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Enterococci

From Commensals to Leading Causes of Drug Resistant Infection

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Over the past 30 years, multidrug resistant enterococci have emerged as leading causes of hospital acquired infection. With the acquisition of resistance to vancomycin in the mid 1980's many of these infections became extremely difficult to treat. To address this problem, researchers with an interest in various aspects of enterococcal biology have come together to consolidate much of what is known about this genus into a single text. *Enterococci: From commensals to leading causes of drug resistant infection* provides state-of-the-art summaries of what is known about 1) Their origins, distribution in nature and gut colonization, 2) Infection – history, incidence, and pathology, 3) Enterococci as indicators of contamination and in public policy, 4) Infection treatment and antibiotic resistance, 5) Pathogenesis and models of enterococcal infection, 6) Comparative enterococcal genomics, 7) Nature, maintenance and transmission of extrachromosomal elements, 8) Enterococcal phage and genome defense, 9) Transcriptional and post transcriptional control of enterococcal gene regulation, 10) Enterococcal cell wall components and structures, 11) Enterococcal biofilm structure and role in colonization and disease, 12) Metabolic and physiologic traits that contribute to the special biology of enterococci, and 13) Enterococcal bacteriocins and other bacterial factors that contribute to niche control.

Editors

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Preface

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The enterococci are an ancient genus of microbes that are highly adapted to living in complex environments and surviving harsh conditions. To the extent that we currently understand their native ecology, they inhabit the gastrointestinal tracts of a wide variety of animals, from insects to man. This suggests that they also inhabited the gastrointestinal tracts of our ancient common ancestors. This would place them among the earliest members of a symbiotic consortium, a microbiota that we now appreciate serves roles ranging from facilitating digestion to providing the host with development cues and protecting it from colonization by more antagonistic microbes. It appears that most of the evolutionary refinement of these organisms occurred prior to the advent of humans. However, it is becoming clear that human activities—from urbanization and the domestication of animals to the introduction and broad application of antibiotics—have selected for the convergence of novel combinations of traits in some species, mainly *Enterococcus faecalis* and *E. faecium,* which are now leading causes of multidrug-resistant hospital-acquired infections. Moreover, the enterococci serve as reservoirs for antibiotic resistances that they are spreading to other important pathogens, most notably *Staphylococcus aureus*.

With the advent of genomic technologies, the number of enterococcal species has exploded. It is likely that many of the traits that have emerged in highly adapted hospital lineages of *E. faecalis* and *E. faecium*, including traits that exacerbate infection and even antibiotic resistance, either originate or have passed through some of these species. Our knowledge of the genetic content of the genus is in its infancy. While we are rapidly learning about the genus (in large part through the genomes of its various species), our general knowledge about the ecology and evolution of these species is almost nonexistent. Although several enterococci inhabit humans, it is unlikely that any currently known species evolved in humans as the definitive host (except for the highly hospital adapted *E. faecium* CC17 lineage, which by some measures can legitimately be regarded as a new species). Microbes evolve into new species when introduced into a new and isolated habitat. However, there currently is no real understanding of any particular isolated habitat that gave rise to a specific species of *Enterococcus*. The problem is that, as we discover novel genes in various species of enterococci, we are at a loss to know their precise native function in the ecology in which they evolved. By understanding the traits that have contributed to the host/microbe dynamic in an evolved, natural context, we will be much better positioned to understand their roles when they emerge in hospital lineages that confound human infection.

To advance the understanding of enterococci as leading causes of multidrug resistant infection, to increase our knowledge of the genus and its relation to humans and other forms of life, and to build a collaborative research community, we organized the 1st International ASM Conference on Enterococci in Banff, Canada, February 27–March 2, 2000. That conference produced a recognized need for a comprehensive text that would provide a definitive resource for members of the community, as well as a point of entry for those interested in helping to address this important problem. In response to these issues, *The Enterococci: Pathogenesis, molecular biology, and antibiotic resistance*, published by ASM Press in 2002, was produced by the research community, and has ably served these roles for the past decade.

However, because of the rapid rate of knowledge growth, which has been catalyzed by the introduction of powerful new technologies, it has become increasingly important to reassess the landscape of knowledge of the enterococci. Moreover, new technologies and resources became available that have made it possible to do this in a new way—to generate a living document that can be updated on a regular basis, and to disseminate it instantly and freely on a global scale. Thus, the genesis of *Enterococci: From commensals to leading causes of drug resistant infection*, a compilation of peer reviewed content contributed by many of the leaders in the *Enterococcus* research community, and provided as an open access book on the U.S. National Institutes of Health NCBI Bookshelf. The *Enterococcus* research community once again pulled together to collaboratively author and review chapters that comprehensively span our knowledge of the enterococci, and this document would not have been possible without their effort and a shared interest in advancing the field. Our collective hope is that this document will

catalyze progress in solving the important problem of enterococci as leading causes of multidrug-resistant hospital-acquired infections, and will further advance knowledge of this important genus and its place in biology.

Taking this new and democratic approach to publication proved a challenging task, as the path was not clear and there were few examples to draw from. Without the services provided by a commercial publisher, chapters had to be manually parsed for peer review, reviews compiled, chapters revised and edited for scientific content, and content had to be edited for readability. Each of the associate editors contributed much to the process, and this project would not have been possible without their input. After attempting to perform many of the review and revision tasks myself, it became obvious that there was a real need for a professional copy editor. The work of Mary Van Tyne was absolutely instrumental in finally bringing this project to fruition. She worked tirelessly to bring the chapters into a consistent style, to check and automate references, and to interface with NCBI. Finally, this project would not have been possible without the helpful guidance of the leadership and staff at NCBI, including Director David J. Lipman, as well as the NCBI Bookshelf Project Lead, Marilu A. Hoeppner.

Finally, sharing and disseminating this knowledge on a leading public health concern would not have been possible without the support of the National Institutes of Health sponsored Harvard-wide Program on Antibiotic Resistance (AI083214), and support of the Department of Ophthalmology, Harvard Medical School and the Massachusetts Eye and Ear Infirmary. On behalf of all of the editors, we extend our deepest thanks to everyone who contributed in so many ways to production of this volume. As a living document, we also extend our deepest appreciation in advance to everyone who will contribute to its further growth, as it continues to serve the community in the future.

Michael S. Gilmore

Editor in Chief

Enterococcus Diversity, Origins in Nature, and Gut Colonization

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Abstract

Enterococci have evolved over eons as highly adapted members of the gastrointestinal consortia of a wide variety of hosts—humans and other mammals, birds, reptiles and insects—but for reasons that are not entirely clear, they emerged in the 1970s as some of the leading causes of multidrug-resistant, hospital-acquired infections. The taxonomy of enterococci has changed considerably over the past ten years, and the genus now includes over forty distinct species with various habitats, tropisms, and metabolic and phenotypic characteristics. These habitats include animal hosts, as well as plants, soil and water, and man-made products, including fermented foods and dairy products. Antibiotic-resistant strains of enterococci have emerged in many of these habitats, and strains with novel resistance mechanisms are isolated with alarming regularity. As a result, the relationship between the non-therapeutic use of antibiotics and the occurrence of enterococci in various non-human habitats is of substantial interest.

Introduction

The term "enterococcus" has its origin at the end of the 19th century when Thiercelin described a saprophytic coccus, of intestinal origin, capable of causing infection (Thiercelin & Jouhaud, 1899; Thiercelin, 1899). The same year, MacCallum and Hastings characterized a similar organism, now known to be *Enterococcus faecalis*, from a lethal case of endocarditis, thus providing a first detailed description of its pathogenic capabilities (MacCallum & Hastings, 1899). These early descriptions constitute the basis for the paradigm of enterococcal pathogenesis as that of a commensal opportunist. The emergence of multi-drug resistant enterococci over the past 50 years has substantially compounded this problem, and antibiotic resistant enterococci are now leading causes of nosocomial infection worldwide (Prabaker & Weinstein, 2011; Werner, et al., 2008).

Phylogenetically, the genus *Enterococcus* belongs to the low GC branch of Gram-positive bacteria (i.e. 37.5% of GC in the *Enterococcus faecalis* chromosome) (Paulsen, et al., 2003). Enterococci were classified as group D streptococci (Sherman J. M., The streptococci, 1937; Sherman J. M., The enterococci and related streptococci, 1938) until 1984, when *Streptococcus faecalis* and *Streptococcus faecium* were reclassified as *Enterococcus faecalis* and *Enterococcus faecium*, respectively (Schleifer & Kilpper-Balz, 1984).

The *Enterococcus* genus is placed in the Enterococcaceae family (Ludwig, Schleifer, & Whitman, 2009), along with genus *Bavariicoccus*, *Catellicoccus*, *Melissococcus*, *Pilibacter*, *Tetragenococcus* and *Vagococcus*, and consists of species that occur in human and animal gastro-intestinal (GI) tracts (Mundt, 1963; Mundt, 1986), as well as in the guts of insects (Martin & Mundt, 1972), traditional fermented food and dairy products (Mundt, 1986), and in various environments including plants ((Mundt, 1963), soil (Mundt, 1961) and water (Ator & Starzyk, 1976). Undoubtedly, an important key to their broad distribution in nature stems from being remarkably resilient organisms that are capable of enduring a broad range in pH and temperatures, as well as hypotonic and hypertonic conditions (Sherman J. M., The enterococci and related streptococci, 1938). Enterococci are ubiquitous in GI tracts even though they constitute a small proportion of the gut consortium, typically

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comprising less than 1% of the adult microflora (Finegold, Sutter, & Mathisen, 1983; Sghir, Gramet, Suau, Violaine, Pochart, & Dore, 2000). Little is known about the main mechanisms used by enterococci to colonize GI tracts of either healthy individuals or hospitalized patients. Nevertheless, exposure of hospitalized patients to antibiotics results in major modifications of the gut microbiota, which facilitate colonization of the GI tract by drug-resistant enterococci (Donskey, et al., 2000; Ubeda, et al., 2010).

In this chapter, we review the literature on the diversity of species that belong to the *Enterococcus* genus, discuss their origin and distribution in nature, and focus on the gut colonization by the enterococci and their transition from commensal to opportunistic pathogens.

The *Enterococcus* genus: definition and diversity

Historical perspective

In his report to the French Society of Biology in 1899, Thiercelin shared his observations of a saprophytic microbe that pullulates in the GI tract of humans along with the *Bacterium coli* (Thiercelin & Jouhaud, 1899). He described this microbe as a Gram-positive diplococcus and proposed the name "Enterocoque" to emphasize its morphology and its intestinal origin (Thiercelin & Jouhaud, 1899). Among the many initial observations in this paper was the observation that this microbe was pathogenic for mice, and susceptible to becoming pathogenic for humans (Thiercelin & Jouhaud, 1899). Microscopically, Thiercelin observed a halo surrounding some cells, similar to that observed for *Pneumococcus,* especially in the context of an intestinal disease, which suggested the presence of a capsule *in situ* (Hancock & Gilmore, 2002; Thiercelin & Jouhaud, 1899). Two months later, he described the morphological polymorphism of enterococcal cells (Thiercelin, 1899). He noted that since his previous report, this microbe had been found to be predominant in the GI tract of a patient with diarrhea, which then translocated from the intestine to the blood, which led to septicemia (Thiercelin, 1899). At nearly the same time, MacCallum and Hastings provided a detailed report of a case of acute endocarditis in a 37-year-old patient, caused by an organism they called *Micrococcus zymogenes* (163), now recognized as a strain of *Enterococcus faecalis*. This bacterium was continuously isolated from the patient's blood until his death due to cardiac failure (MacCallum & Hastings, 1899). The authors described that this bacterium was "very hardy and tenacious of life" (MacCallum & Hastings, 1899), which are now well-recognized traits of the *Enterococcus* genus. MacCallum and Hastings thoroughly investigated the biochemical capabilities of the enterococci and showed that this particular isolate was hemolytic (MacCallum & Hastings, 1899). The accurate detail in the descriptions of the pathogenic traits of these opportunistic commensal bacteria is particularly striking.

In 1906 the name *Streptococcus faecalis* was used by Andrewes and Horder (Andrewes & Horder, 1906) to characterize an organism of fecal origin that clotted milk, similar to the observations of MacCallum and Hastings, and that also fermented mannitol and lactose, but not raffinose. The name *faecalis* was chosen to emphasize that this *Streptococcus* was characteristic of the human intestine, even though the organism was isolated from a patient with endocarditis in this study (Andrewes & Horder, 1906). Orla-Jensen subsequently reported the first description of *Streptococcus faecium*, which differed from the fermentation patterns described for *S. faecalis* (Orla-Jensen, 1919). About twenty years later, a third species, *Streptococcus durans*, was described by Sherman and Wing (Sherman & Wing, 1935) as being similar to *S. faecium,* but with more restricted fermentation capabilities. Sherman subsequently proposed a classification scheme that separated streptococci into four groups: pyogenic, viridans, lactic, and enterococcus (Sherman, 1937). The term "enterococcal group" included *S. faecalis* (hemolysis negative and proteolysis negative), *S. faecalis* var. *liquefaciens* (hemolysis negative and proteolysis positive), *S. faecalis* var. *hemolyticus* (hemolysis positive and proteolysis negative), *S. faecalis* var. *zymogenes* (hemolysis positive and proteolysis positive), and *S. durans*; and was used to describe streptococci that grew between 10 and 45° C, grew in broth at pH 9.6, grew in 6.5% NaCl, and survived heating to 60°C for 30 min (Sherman, 1937). Noteworthy, *S. faecium*, described by Orla-Jensen earlier, was considered by Sherman to

be the same as *S. faecalis* (Sherman, 1937), and was only officially recognized as a separate species in the mid-1960s (Barnes, 1956; Breed, Murray, & Smith, 1957; Deibel, 1964).

In 1957, motile and yellow-pigmented enterococci were described and the name *S. faecium* var. *casseliflavus* (emphasizing the yellow coloration) was proposed (Graudal, 1957; Mundt, 1986). A decade later, Nowlan and Deibel (Nowlan & Deibel, 1967) added *Streptococcus avium* to the enterococcal group. As early as 1970, Kalina (Kalina, 1970) proposed the creation of the taxon *Enterococcus,* based on cellular arrangement and phenotypic characteristics of species in the enterococcal group. However, this genus was not formally accepted until 1984, when Schleifer and Kilpper-Balz (Schleifer & Kilpper-Balz, 1984) provided genetic evidence that *S. faecalis* and *S. faecium* were sufficiently distant from other members of the *Streptococcus* genus. Collins and collaborators (Collins, Jones, Farrow, Kilpper-Balz, & Schleifer, 1984) used a similar genetic approach to show that strains previously named *Streptococcus avium*, *Streptococcus casseliflavus*, *Streptococcus durans* and *Streptococcus gallinarum,* were distinct species but closely related to those of the genus *Enterococcus*. In 2002, an extensive review of the literature (Facklam, Carvalho, & Teixeira, 2002) reported the existence of 23 distinct *Enterococcus* species.

Considering the ubiquitous distribution in nature of the *Enterococcus* genus, it is noteworthy that the three *Enterococcus* species (*E. faecalis*, *E. faecium* and *E. durans*) that were recognized prior to 1950 (Figure 1), are capable of causing human infection. *E. faecalis* and *E. faecium* are considered to be the most abundant enterococci in human feces (Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994; Finegold, Sutter, & Mathisen, 1983; Noble, 1978; Patel, et al., 1998), and account for most enterococcal disease (Malani, Kauffman, & Zervos, 2002). *E. durans* occurs occasionally among the fecal flora of healthy adults (Ruoff, 1990). Until the mid-1990s, *E. faecalis* accounted for 90–95% of clinical isolates, and *E. faecium* infection was rare (Huycke, Sahm, & Gilmore, 1998). Since then, the proportion of *E. faecium* isolates has increased, largely due to the spread of resistance to antibiotics, particularly vancomycin and ampicillin (Arias & Murray, 2012; Gilmore, Lebreton, & van Schaik, 2013; Huycke M. M., et al., 2002). Other species, including *Enterococcus avium*, *gallinarum*, *casseliflavus*, *hirae*, *mundtii*, and *raffinosus*, also have been isolated from human infection (Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994; Hammerum A. M., 2012; Murray, 1990). *Enterococcus gallinarum* and *Enterococcus casseliflavus* infections are of special interest because of their intrinsic resistance to vancomycin, an antibiotic used to treat the aminoglycoside-resistant enterococcal infections that became problematic in the mid-1980s.

Among the 30 species of *Enterococcus* described between 1992 and 2012 (Fig. 1, Table 1), only four (*E. sanguinicola*, *E. gilvus*, *E. pallens* and *E. canintestini*) have been associated with human infection (Carvalho, et al., 2008; Tan, et al., 2010; Tyrell, et al., 2002). Additionally, *E. caccae* has been isolated from human feces (Figure 2) (Carvalho, et al., 2006). The remaining species described since 1992 were isolated from non-human sources, including animals (De Graef, et al., 2003; de Vaux, Laguerre, Diviès, & Prévost, 1998; Devriese, Pot, & Collins, 1993; Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004; Law-Brown & Meyers, 2003; Švec, et al., 2005), plants (Rahkila, Johansson, Säde, & Björkroth, 2011; Sedláček, et al., 2013; Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007; Švec, et al., 2011), water (Niemi, et al., 2012; Sedláček, et al., 2013; Sistek, et al., 2012; Svec, et al., 2001; Svec, et al., 2005; Svec, et al., 2006) and food (Fortina, Ricci, Mora, & Manachini, 2004; Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004; Morandi, Cremonesi, Povolo, & Brasca, 2012; Tanasupawat, Sukontasing, & Lee, 2008) (Figure 2). Although much of the interest in enterococci historically has been driven by their association with infection and human carriage, it is clear that this is only the tip of the *Enterococcus* genus diversity iceberg, and comparison of these species to enterococci from other environments will reveal much about the basic features and origins of the genus.

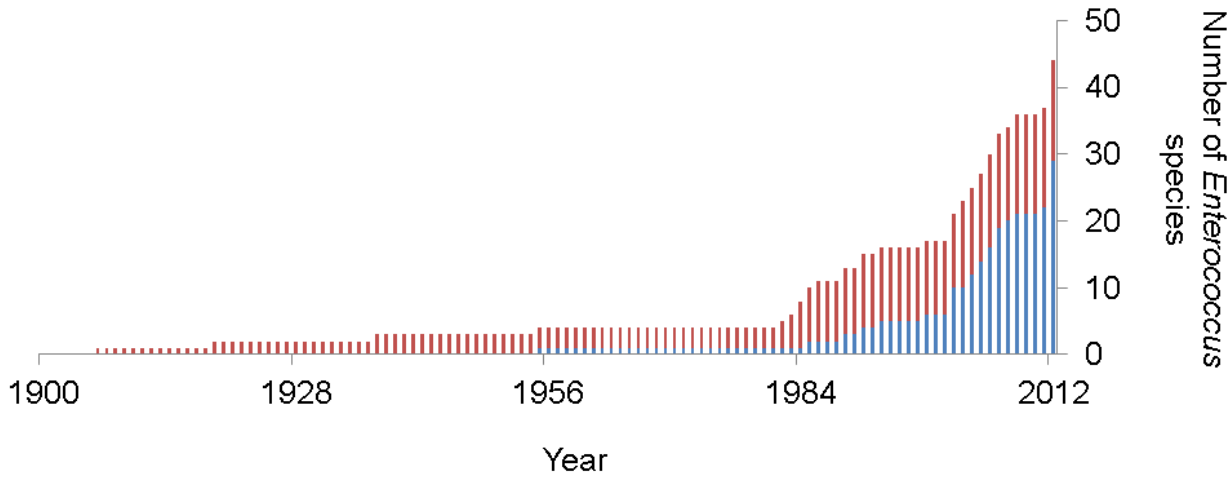

Figure 1. Cumulative *Enterococcus* species identified. Red bars show the number of species associated with human disease.

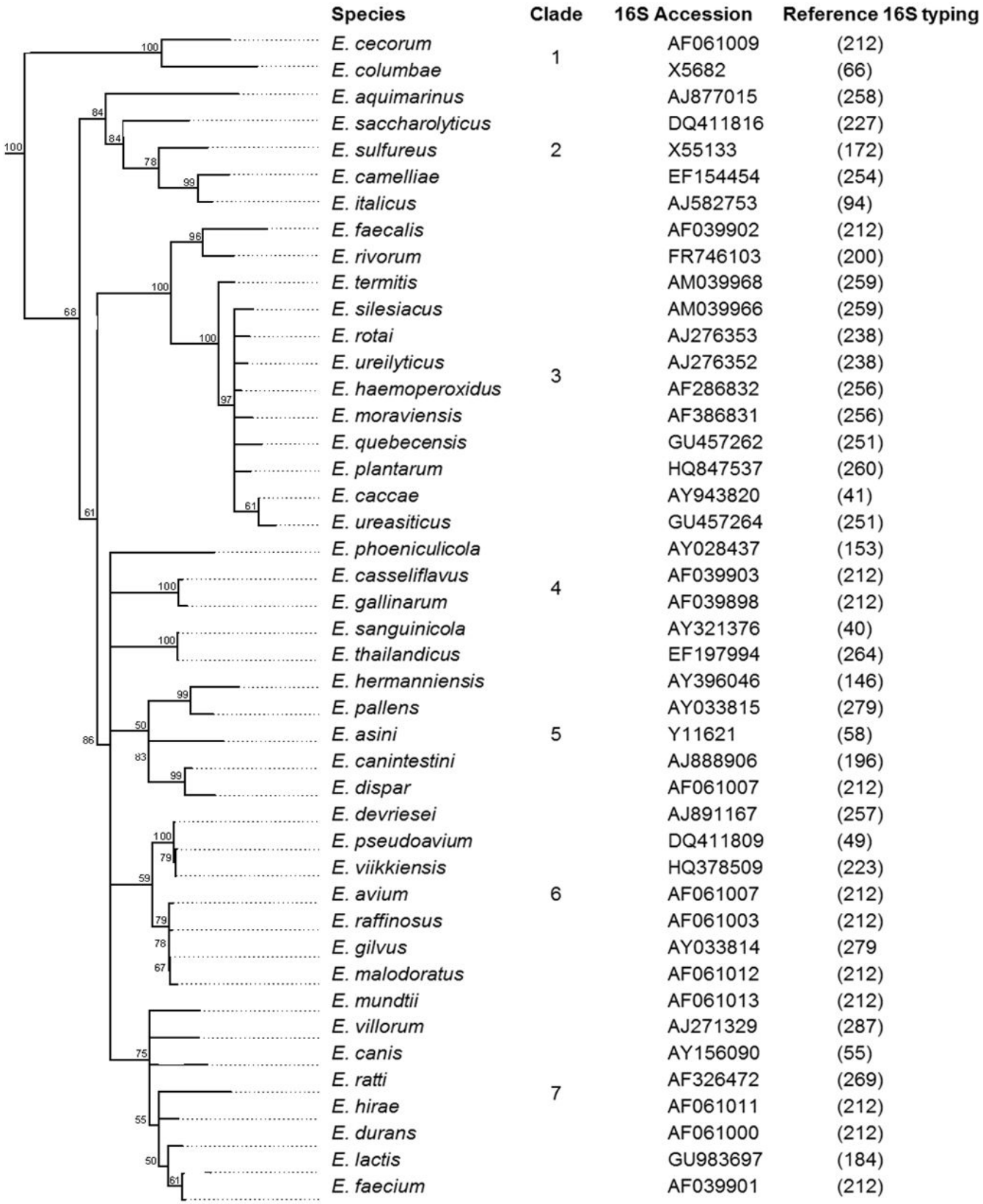

Figure 2. Dendrogram of the genus Enterococcus. A dendrogram of the available 16S rRNA gene sequences for members of the Enterococcus genus was compiled using the Geneious software (Biomatters Ltd) using the neighbour-joining algorithm and the 16S sequence of Tetragenococcus solitarius as an outgroup. Bootstrap values were generated over 1000 iterations. (Adapted from Gilmore et al., 2013)

Table 1. Species part of the *Enterococcus* genus

a.	m.
Enterococcus aquimarinus (Svec, et al., 2005)	Enterococcus malodoratus (Pette, 1955)
Enterococcus asini (de Vaux, Laguerre, Diviès, & Prévost, 1998)	Enterococcus moraviensis (Svec, et al., 2001)
Enterococcus avium (Collins, Jones, Farrow, Kilpper-Balz, & Schleifer, 1984)	Enterococcus mundtii (Collins, Farrow, & Jones, 1986)
c.	p.
Enterococcus caccae (Carvalho, et al., 2006)	Enterococcus pallens (Tyrell, et al., 2002)
Enterococcus camelliae (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007)	Enterococcus phoeniculicola (Law-Brown & Meyers, 2003)
Enterococcus canintestini (Naser, et al., 2005)	Enterococcus plantarum (Švec, et al., 2011)
Enterococcus canis (De Graef, et al., 2003)	Enterococcus pseudoavium (Collins, Facklam, Farrow, & Williamson, 1989)
Enterococcus casseliflavus (Collins, Jones, Farrow, Kilpper-Balz, & Schleifer, 1984)	q.
Enterococcus cecorum (Devriese, Dutta, Farrow, Van De Kerckhove, & Phillips, 1983)	Enterococcus quebecensis (Sistek, et al., 2012)
Enterococcus columbae (Devriese, Ceyssens, Rodrigues, & Collins, 1990)	r.
d.	Enterococcus raffinosus (Collins, Facklam, Farrow, & Williamson, 1989)
Enterococcus devriesei (Švec, et al., 2005)	Enterococcus ratti (Teixeira, et al., 2001)
Enterococcus dispar (Collins, Rodrigues, Pigott, & Facklam, 1991)	Enterococcus rivorum (Niemi, et al., 2012)
Enterococcus durans (Sherman & Wing, 1935)	Enterococcus rotai (Sedláček, et al., 2013)
f.	s.
Enterococcus faecalis (Andrewes & Horder, 1906)	Enterococcus saccharolyticus (Farrow, Kruze, Phillips, Bramley, & Collins, 1984)
Enterococcus faecium (Orla-Jensen, 1919)	Enterococcus sanguinicola (Carvalho, et al., 2008)
g.	Enterococcus silesiacus (Svec, et al., 2006)
Enterococcus gallinarum (Bridge & Sneath, 1982)	Enterococcus sulfureus (Martinez-Murcia & Collins, 1991)
Enterococcus gilvus (Tyrell, et al., 2002)	t.
h.	Enterococcus termitis (Svec, et al., 2006)
Enterococcus haemoperoxidus (Svec, et al., 2001)	Enterococcus thailandicus (Tanasupawat, Sukontasing, & Lee, 2008)
Enterococcus hermanniensis (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004)	u.
Enterococcus hirae (Farrow & Collins, 1985)	Enterococcus ureasiticus (Sistek, et al., 2012)
i.	Enterococcus ureilyticus (Sedláček, et al., 2013)
Enterococcus italicus (Fortina, Ricci, Mora, & Manachini, 2004)	v.
l.	Enterococcus viikkiensis (Rahkila, Johansson, Säde, & Björkroth, 2011)

Table 1. continued from previous page.

Enterococcus lactis (Morandi, Cremonesi, Povolo, & Brasca, 2012)	Enterococcus villorum (Vancanneyt, et al., 2001)

Definition of the genus and criteria for identification

The core morphological and physiological features of all enterococci include being Gram-positive spherical or ovoid cells arranged in pairs or chains (Thiercelin, 1899). They are non-spore-forming facultative anaerobes and obligatory fermentative chemoorganotrophs. They typically have an optimum growth temperature of 35°C and a growth range from 10 to 45°C (Sherman, 1937). They typically grow in broth containing 6.5% NaCl, and hydrolyze esculin in the presence of 40% bile salts (Facklam, 1973). They are catalase reaction negative, and do not express complete cytochromes, although some species produce a catalase and appear catalase positive with weak effervescence. They are usually homofermentative, producing lactic acid as the end product of glucose fermentation, without production of gas (Klein, 2003; Murray, 1990). Some species are motile, such as *E. gallinarum* and *E. casseliflavus* (Graudal, 1957; Mundt, 1986). Pigmentation is a variable trait, with yellow-pigmented species including *E. sulfureus*, *E. casseliflavus*, and *E. mundtii* (Martinez-Murcia & Collins, 1991; Graudal, 1957; Mundt, 1986)), and pigmented species are commonly found among plants (Aarestrup, Butaye, & Witte, 2002). Most enterococci, apart from *E. cecorum*, *E. columbae*, *E. pallens*, and *E. saccharolyticus*, *E. devriesei* (variable among strains), *E. canintestini*, *E. termitis* and *E. viikiensis* are capable of hydrolyzing pyrrolidonyl β naphthylamide and producing leucine aminopeptidase (Facklam, Carvalho, & Teixeira, 2002; Naser, et al., 2005; Rahkila, Johansson, Säde, & Björkroth, 2011; Švec, et al., 2005; Svec, et al., 2006).

Phylogenetic analysis of catalase-negative Gram-positive cocci, based on the comparison of ~1400 bases of the 16s rRNA gene, shows that the *Enterococcus* are more closely related to the *Vagococcus*, *Tetragenococcus*, and *Carnobacterium* than they are to the *Streptococcus* and *Lactococcus* (Facklam, Carvalho, & Teixeira, 2002). The G + C content of DNA ranges from 37 to 45 mol% (237). *Enterococcus faecalis* has long been known to require a number of amino acids (including Val, Leu, Ile, Ser, Met, Glu, Arg, His and Trp) and vitamins (including biotin, nicotinic acid, pantothenate, pyridoxine, riboflavin, and sometimes folic acid) for maximal growth, with other species being similar in their fastidiousness (Facklam, Carvalho, & Teixeira, 2002). As a reflection of their highly evolved role as members of a consortium in an extremely competitive environment, enterococci have reduced genomes that range from 2.7 Mb to 3.6 Mb across species sequenced so far (Gaechter, Wunderlin, Schmidheini, & Solioz, 2012; Qin, et al., 2012; Palmer, et al., 2012; van Schaik & Willems, 2010).

Species of the *Enterococcus* genus identified by DNA-DNA reassociation, 16S rRNA gene sequencing, and/or whole-cell protein analysis to rigorously establish the novel species, and the dates of their initial description are shown in Table 1. DNA-DNA reassociation remains the gold standard for establishing bacterial species (Johnson & Ordal, 1968; Tindall, Rosselló-Móra, Busse, Ludwig, & Kämpfer, 2010), and this analysis has been carried out for most enterococci (Facklam, Carvalho, & Teixeira, 2002). Sequencing of 16S rDNA has become a practical and reliable additional tool (Vandamme, et al., 1996). Additionally, whole-cell protein analysis using standardized techniques correlate well with DNA hybridization, and give unique signatures for each *Enterococcus* species tested (Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994; Merquoir, Netz, Camello, & Texeira, 1997). For practical reasons, 16S rDNA sequencing and whole-cell protein analysis are beginning to replace DNA-DNA reassociation, and are increasingly being used to describe new species. These methodological improvements are important developments, since many enterococcal species vary by only one phenotypic trait (Knudtson & Hartman, 1992; Moore, Zhowandai, Ferguson, McGee, Mott, & Stewart, 2006; Scheidegger, Fracalanzza, Texeira, & Caradarelli-Leite, 2009; Bodnar, Noskin, Suriano, Cooper, Reisberg, & Peterson, 1996) using conventional biochemical tests or commercial test systems (Devriese, Pot, & Collins, 1993; Facklam, Carvalho, & Teixeira, 2002). By itself, 16S rRNA sequence analysis does not appear to provide the resolution needed to distinguish *Enterococcus* species such as *E. casseliflavus* and *E. gallinarum*, which are 99.9%

identical by this criterion (Patel, et al., 1998) (as opposed to 97% identity being the threshold usually used for species identification). As a consequence, additional complementary rapid molecular methods have been developed for routine identification of enterococcal isolates (Facklam, Carvalho, & Teixeira, 2002), including randomly amplified polymorphic DNA (RAPD) (Descheemaeker, Lammens, Pot, Vandamme, & Goossens, 1997; Monstein, Tiveljung, Kraft, Borch, & Jonasson, 2000; Quednau, Ahrné, Petersson, & Molin, 1998), sequencing of the domain V of the 23S rRNA gene (Poyart, Quesnes, & Trieu-Cuot, 2000), amplification of rRNA or tRNA intergenic spacers (Naimi, Beck, Monique, Lefèbvre, & Branlanti, 1999; Tyrell, Bethune, Willey, & Low, 1997), sequencing of the D-ala:D-ala ligase genes (*ddl*) (Ozawa, Courvalin, & Gaiimand, 2000) or the manganese-dependent superoxide dismutase (*sodA*) genes (Poyart, Quesnes, & Trieu-Cuot, 2000), sequencing and hybridization of the chaperonin 60 (*cpn60*) gene (Goh, et al., 2000), and amplification and probing of the *Enterococcus* protein A (*efaA*) gene (Singh, Coque, Weinstock, & Murray, 1998). Several additional molecular tests have been recently introduced to differentiate the *Enterococcus* species, including sequencing genes encoding the RNA polymerase α-subunit (*rpoA*), the phenylalanyl-tRNA synthase (*pheS*) or the elongation factor Tu (*tufA*) (Naser, et al., 2005; Naser, et al., 2005; Picard, et al., 2004), sequencing repetitive extragenic palindromic PCR (REP-PCR) or BOX-PCR (Nayak, Badgley, & Harwood, 2011), pulsed-field gel electrophoresis (PFGE) (Gordillo, Singh, & Murray, 1993; Tenover, Arbeit, & Goering, 1997), and multilocus sequence typing (MLST) (Homan, et al., 2002; Ruiz-Garbajosa, et al., 2006). MLST has proven to be useful in epidemiological studies of *E. faecalis* and *E. faecium* (Leavis, Bonten, & Willems, 2006), and shows similar accuracy to PFGE for the identification of organisms at the subspecies level (Nallapareddy, Duh, Singh, & Murray, 2002).

New species and reclassifications

The taxonomy of the enterococci has changed considerably, as the genus consisted of only 20 species at the end of the 20th century. Numerous new species have been described as the result of improvements in methods for differentiation, combined with growing interest in the enterococci (Figure 1) (Euzéby, 2013). Currently, the genus *Enterococcus* consists of 35 recognized species (Euzéby, 2013), with 8 more species (*Enterococcus lactis*, *plantarum*, *quebecensis*, *rivorum*, *rotai*, *ureasiticus*, *ureilyticus*, and *viikiensis*) likely to be added (Morandi, Cremonesi, Povolo, & Brasca, 2012; Niemi, et al., 2012; Rahkila, Johansson, Säde, & Björkroth, 2011; Sedláček, et al., 2013; Sistek, et al., 2012; Švec, et al., 2011). *E. sanguinicola* (Carvalho, et al., 2008) was recently described as likely to be a junior synonym of *Enterococcus thailandicus* (Shewmaker, et al., 2011), and probably will be removed. The list of *Enterococcus* species (Table 1) will doubtlessly continue to increase and be subject to reevaluation in the rapidly expanding genomic era, which offers new insights into distinctions between bacterial species (Konstantinidis, Ramette, & Tiedje, 2006). Since the diversity of the *Enterococcus* genus was extensively reviewed in 2002 (Facklam, Carvalho, & Teixeira, 2002), this review focuses on new enterococcal species that have been reported since then.

Enterococcus aquimarinus sp. nov. was isolated from sea water and was described by Švec et al. (Svec, et al., 2005) based on whole-cell protein analysis and 16S rDNA sequence. The 16S rDNA sequence shares 96.1–96.9% identity with its closest phylogenetic neighbors, *E. saccharolyticus*, *E. sulfureus*, and *E. italicus* (Svec, et al., 2005). Additional distinguishing features include *pheS* gene sequence polymorphism, (GTG)5-PCR fingerprinting (which identifies short sequence repeats (Lupski & Weinstock, 1992)), and biochemical characteristics. For example, *E. aquimarinus* is not able to produce acid from ribose, which distinguishes it from *E. sulfurous*, *E. saccharolyticus,* and the majority of other enterococcal species; but produces acid from L-Arabinose and other substrates which distinguishes it from *E. italicus* (Svec, et al., 2005). *E. aquimarinus* LMG 16607T was identified as the type strain (Svec, et al., 2005).

Enterococcus caccae sp. nov. (Carvalho, et al., 2006) was recovered from human stool, and was distinguished from other species by DNA–DNA hybridization, whole-cell protein analysis, and sequencing of the 16S rDNA (Carvalho, et al., 2006). Comparative 16S rDNA sequence analysis showed that *E. caccae* shared 99.6% identity with *E. moraviensis* (Carvalho, et al., 2006). However, the level of DNA–DNA relatedness between this novel

taxon and any of the currently recognized species of *Enterococcus* was surprisingly low (32% for *E. moraviensis*), and the differences in the protein pattern supported the novelty of this species (Carvalho, et al., 2006). *E. caccae* can be differentiated from *E. moraviensis* by its ability to grow at 45 °C and a positive Voges-Proskauer test, as well as its inability to produce acid from arabinose, lactose and mannitol (Carvalho, et al., 2006). The type strain of the *E. caccae* species is 2215-02T (Carvalho, et al., 2006).

Enterococcus camelliae sp. nov., isolated from fermented tea leaves in Thailand, was proposed as a new species based on sequence analysis of its 16S rDNA and *rpoA* genes, and global DNA–DNA hybridization analysis (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007). On the basis of the 16S rRNA and the *rpoA* sequence comparison, *E. camelliae* is closely related to *Enterococcus italicus* (99.2% and 93.8% sequence identity, respectively) (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007). However, it shared only 33.8% genome identity based on DNA-DNA hybridization, and possessed phenotypic characteristics, including its inability to produce acid from D-galactose and lactose, which distinguishes it from *E. italicus* and other enterococci (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007). Its type strain is *E. camelliae* FP15-1T (Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007).

The taxonomic position of isolates obtained from healthy dog faeces and originally classified as *Enterococcus dispar* was reinvestigated by Naser and collaborators (Naser, et al., 2005), who determined them to be a different species that they named *Enterococcus canintestini* sp. nov. The 16S rDNA gene sequence showed the highest similarities (between 98–99%) with *E. dispar*, *E. canis* and *E. asini*; however, *E. canintestini* shared only 33.8% genome identity, based on DNA-DNA hybridization with *E. dispar*, and showed a substantially different whole-cell protein profile (Naser, et al., 2005). Multilocus sequence analysis (MLSA) of the genes that encode the alpha subunit of ATP synthase (*atpA*), *rpoA*, and *pheS* was also performed (Naser, et al., 2005). Additionally, biochemical characteristics, including the inability to produce acid from raffinose and D-melibiose, distinguished *E. canintestini* from *E. dispar* and other enterococci (Naser, et al., 2005). The type strain is *E. canintestini* LMG 13590T (Naser, et al., 2005).

Enterococcus canis sp. nov. was proposed by De Graef and collaborators (De Graef, et al., 2003) for strains isolated from rectal swabs and chronic otitis in dogs. While the 16S rDNA sequence shared 98.4% to 99% similarity with members of the *E. faecium* species group, the level of DNA-DNA reassociation with these species was very low (7–13%) (De Graef, et al., 2003). *E. canis* shares a number of phenotypic characteristics with the *E. faecium* species group, but can be distinguished by its inability to hydrolyse arginine or to grow on selective media that contain 0.04% sodium azide (55). The type strain is *E. canis* LMG 12316T (De Graef, et al., 2003).

The species *Enterococcus devriesei* sp. nov. was proposed by Švec *et al.* (Švec, et al., 2005), after having reinvestigated the taxonomic position of two isolates of bovine origin that were originally assigned to the species *Enterococcus raffinosus* based on biochemical reactions. These strains, as well as two additional strains obtained from a charcoal-broiled river lamprey and from the air of a poultry slaughter by-product processing factory, showed key differences from *E. raffinosus* and all other enterococcal species (Švec, et al., 2005). The 16S rDNA sequence indicated a phylogenetic position in the *Enterococcus avium* species group, and showed *Enterococcus pseudoavium* as the closest phylogenetic relative (99.8–99.9% identity) (Švec, et al., 2005). However, whole cell protein electrophoresis, (GTG)5-PCR fingerprinting, ribotyping, and DNA-DNA hybridization (with reassociation values between 25% and 34% with the closest phylogenetic neighbors) confirmed that these strains were a novel species (Švec, et al., 2005). A combination of phenotypic traits, including the ability to produce acid from lactose (but not from melibiose or 2-ketogluconate) is helpful for distinguishing *E. devriesei* from species of the *E. avium* group (Švec, et al., 2005). However, there is variability within the phenotypic traits of this species (Švec, et al., 2005). The type strain is *E. devriesei* LMG 14595T (Švec, et al., 2005).

Enterococcus hermanniensis sp. nov. was proposed by Koort (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004) for strains isolated from modified-atmosphere-packaged broiler meat, and was also detected on canine tonsils. *E. hermanniensis* isolates were placed in the *Enterococcus avium* group, with *E. pallens* as their closest

phylogenetic neighbor, based on 16S rDNA sequence (99% sequence similarity) (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004). Whole-cell protein analysis as well as a low level of DNA-DNA hybridization (12-30%), however, distinguishes this species from *E. pallens* and other known enterococci (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004). Interestingly, *E. hermanniensis* strains fails to produce acid from most common substrates, further distinguishing this species from other enterococci (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004). The type strain is *E. hermanniensis* LMG 12317T (Koort, Coenye, Vandamme, Sukura, & Björkroth, 2004).

Fortina *et al.* (Fortina, Ricci, Mora, & Manachini, 2004) investigated the taxonomic position of atypical *Enterococcus* strains isolated from artisanal Italian cheeses, and proposed the name *Enterococcus italicus* sp. nov. for strains that were found to be unique. The closest relatives are *Enterococcus sulfureus* and *Enterococcus saccharolyticus*, which possess 96% and 97% similarity based on 16S rDNA sequence, respectively (94). *E. italicus* strains can be easily differentiated from the other *Enterococcus* species by DNA–DNA hybridization and phenotypic characteristics (*e.g.*, lack of growth in 6.5% NaCl, and lack of acid production from L-arabinose, melezitose, melibiose, raffinose or ribose). The type strain is *E. italicus* DSM 15952T (Fortina, Ricci, Mora, & Manachini, 2004).

Enterococcus lactis sp. nov. was proposed by Morandi *et al.* (Morandi, Cremonesi, Povolo, & Brasca, 2012) for other atypical *Enterococcus* strains isolated from raw milk Italian cheeses. On the basis of 16S rDNA sequence analysis, the strains were found to be closely related to *E. hirae*, *E. durans*, and *E. faecium*, with a sequence similarity greater that 98.8% (Morandi, Cremonesi, Povolo, & Brasca, 2012). Since no DNA-DNA hybridization or whole-cell protein analysis have been conducted, the authors based the description of this new species on the analysis of the cellular fatty acid composition between *E. lactis* and its phylogenetic neighbors *E. faecium* and *E. durans* (Morandi, Cremonesi, Povolo, & Brasca, 2012). The authors indicated that *E. lactis* strains can be differentiated from other *Enterococcus* species based on the *pheS* gene sequence, RAPD-PCR, 16S–23S rRNA gene intergenic region analysis (Morandi, Cremonesi, Povolo, & Brasca, 2012). The proposed type strain is *E. lactis* DSM 23655T (Morandi, Cremonesi, Povolo, & Brasca, 2012).

Enterococcus phoeniculicola sp. nov. was proposed by Law-Brown and Meyers (Law-Brown & Meyers, 2003) to identify a single strain isolated from the uropygial gland of wild red-billed woodhoopoes (*Phoeniculus purpureus*). Examination of the 16S rDNA sequence showed that its closest relatives are *E. faecium* and *E. avium* (97.3% and 97.5% respectively). However, neither DNA-DNA hybridization nor whole-cell protein analyses were conducted. *E. phoeniculicola* may be differentiated from *E. faecium* and *E. avium* by its inability to form acid from lactose, D-mannitol, D(+)-melezitose or D-sorbitol, as well as its inability to grow in the presence of 40% bile or 6.5% NaCl (153). Its type strain is *E. phoeniculicola* DSM 14726T (Law-Brown & Meyers, 2003).

Enterococcus plantarum sp. nov. was proposed after investigation of the taxonomic position of eight strains isolated during characterization of enterococcal populations on plants (Švec, et al., 2011). Rep-PCR fingerprinting indicated that the isolates constituted a single cluster that was distinct from known enterococcal species (Švec, et al., 2011). In addition, the 16S rDNA sequence showed that these isolates clustered with the *E. faecalis* species group (Švec, et al., 2011). *E. plantarum* was confirmed to be a novel species by whole-cell protein fingerprinting, and can be differentiated from its closest phylogenetic neighbors by *pheS* and *rpoA* gene sequence analysis (Švec, et al., 2011). The authors described the colonies as "yellowish" on agar, but did not describe this species as pigmented (Švec, et al., 2011). The type strain is *E. plantarum* LMG 26214T (Švec, et al., 2011).

Enterococcus quebecensis sp. nov. was recently proposed by Sistek and collaborators (Sistek, et al., 2012) to identify a single isolate that originated from water sampled in the Province of Québec, Canada. Sequence analysis of genes 16S RNA, *rpoA*, *pheS*, *tufA,* and *atpD,* as well as the results of AFLP DNA fingerprinting and DNA–DNA hybridization, confirmed the status of this strain as distinct from other enterococcal species (Sistek, et al., 2012). Based on the 16S rDNA phylogeny, *E. quebecensis* is closely related to *E. moraviensis,* and clusters in the *E. faecalis* group. *E. quebecensis* strains are negative for the Voges-Proskauer test, meaning that they do not

produce acetoin as a result of fermentation (Sistek, et al., 2012). The type strain is *E. quebecensis* DSM 23327T LMG 26306T (Sistek, et al., 2012).

Enterococcus rivorum sp. nov. was proposed by Niemi and collaborators (Niemi, et al., 2012) after taxonomic investigation of strains that originated from pristine waters in Finland. The authors showed by genotypic analysis that *E. rivorum* strains are closely related to the *E. faecalis* group (Niemi, et al., 2012). However, DNA-DNA hybridization confirms that they are a new species (Niemi, et al., 2012). Growth at 45 °C, or in broth containing 6.5% NaCl, is weak or absent; and production of the D antigen is variable (200). Slow growth occurs at 10 °C (Niemi, et al., 2012). The type strain is *E. rivorum* is LMG 25899T = CCM 7986T (Niemi, et al., 2012).

Very recently, Sedláček et al. (Sedláček, et al., 2013), proposed the name *Enterococcus rotai* sp. nov. for urease-producing, yellow-pigmented enterococci isolated from environmental sources, including drinking water, plants, and mosquitoes, and placed them in the *Enterococcus faecalis* species group. These strains show resistance to clindamycin, chloramphenicol, and oxacillin, with most also being resistant to ofloxacin—but they are sensitive to imipenem, and most strains are sensitive to gentamicin as well (Sedláček, et al., 2013). The type strain is *E. rotai* LMG 26678T (Sedláček, et al., 2013).

Two isolates from drinking water in the region of Silesia in Czech Republic were shown to be distinct from other enterococcal species by DNA-DNA hybridization, and were named *Enterococcus silesiacus* sp. nov. (Svec, et al., 2006). The 16S rRNA gene sequence analysis classified these strains in the *E. faecalis* species group. The type strain is *E. silesiacus* LMG 23085T (Svec, et al., 2006). During the same study, Švec and collaborators (Svec, et al., 2006) identified an isolate from the gut of a termite, and proposed the name *Enterococcus termitis* sp. nov. for this species. As for *E. silesiacus*, *E. termitis* is part of the *E. faecalis* species group based on 16S rDNA analysis (Svec, et al., 2006). *E. termitis* strains are pyruvate and Voges-Proskauer test negative, which is unusual among the enterococci (Svec, et al., 2006). The type strain is *E. termitis* LMG 8895T (Svec, et al., 2006).

In 2008, a single strain was isolated from fermented sausage in Thailand, named *Enterococcus thailandicus* sp. nov., was examined and proposed as a new species (Tanasupawat, Sukontasing, & Lee, 2008). By DNA-DNA hybridization, this strain shared less than 70% identity with known species, but was found to be related to *E. hirae*, *E. durans*, and *E. faecium*, based on 16S rDNA and *rpoA* gene sequence analysis (Tanasupawat, Sukontasing, & Lee, 2008). The type strain is *E. thailandicus* FP48-3T (Tanasupawat, Sukontasing, & Lee, 2008).

Enterococcus ureasiticus sp. nov. (which refers to the presence of urease activity) was proposed based on the study of two enterococcal strains isolated from water samples that differed from other *Enterococcus* species by more than 30%, based on DNA-DNA hybridization (Sistek, et al., 2012). 16S rDNA sequence analysis classified them in the *E. faecalis* species group, but this new species harbored divergent *rpoA*, *pheS*, *tufA*, and *atpD* sequences, as well as AFLP-DNA fingerprinting patterns (Sistek, et al., 2012). The type strain is *E. ureasiticus* FP48-3T (Sistek, et al., 2012).

Enterococcus ureilyticus sp. nov. was proposed as a name for a novel *Enterococcus* species identified in a study of strains that inhabit both water and plants (Sedláček, et al., 2013). These urease-producing, yellow-pigmented strains were placed in the *E. faecalis* species group, based on 16S rDNA sequence (Sedláček, et al., 2013). Analysis of *pheS* and *rpoA* genes sequences, as well as as whole-cell protein electrophoresis and DNA-DNA hybridization, indicated significant divergence from other enterococci (Sedláček, et al., 2013). Strains of this species showed resistance to chloramphenicol, oxacillin, and tetracycline, as well as sensitivity to imipenem (Sedláček, et al., 2013). The type strain is *E. ureilyticus* LMG 26676T (Sedláček, et al., 2013).

Finally, Rahkila *et al.* (Rahkila, Johansson, Säde, & Björkroth, 2011) proposed the species *Enterococcus viikkiensis*, with strain LMG 26075T being the type strain. This strain and four others were isolated from air from a broiler processing plant. Based on 16S rDNA sequence analysis, they belong to the *E. avium* group, with *E. devriesei* being their closest neighbor (Rahkila, Johansson, Säde, & Björkroth, 2011). Notably, these strains are also negative for pyruvate utilization (Rahkila, Johansson, Säde, & Björkroth, 2011).

The identification of enterococcal species is dynamic and is continually being revised. The species *Enterococcus seriolicida*, originally proposed by Kusuda (Kusuda, Kawai, Salati, Banner, & Fryer, 1991), has been reclassified as *Lactococcus garvieae*, based on DNA relatedness (Elliott, Collins, Pigott, & Facklam, 1991; Teixeira, et al., 1996)). Additionally, based on biochemical and genetic evidence, *Enterococcus solitarius* (Collins, Facklam, Farrow, & Williamson, 1989), was moved to the *Tetragenococcus* genus, and was named *Tetragenococcus solitarius* (Ennahar & Cai, 2005). Within the *Enterococcus* genus, *E. saccharominimus* (Vancanneyt, et al., 2004) was shown to be a synonym of *Enterococcus italicus* (Fortina, Ricci, Mora, & Manachini, 2004; Naser, Vancanneyt, Hoste, Snauwaert, Vandemuelebroecke, & Swings, 2006), and *E. casseliflavus* was shown to be the senior subjective synonym of *Enterococcus flavescens* (Naser, Vancanneyt, Hoste, Snauwaert, Vandemuelebroecke, & Swings, 2006; Teixeira, et al., 1997). In 2001, *Enterococcus porcinus*, which was isolated and associated with enteric disorders in animals (Teixeira, et al., 2001), was shown to be the same species as *Enterococcus villorum* (Vancanneyt, et al., 2001), as no phenotypic or genotypic differences could be found between these species (De Graef, et al., 2003). Finally, *Enterococcus sanguinicola* sp. nov. was proposed by Carvalho *et al.* (Carvalho, et al., 2008) in 2008, after taxonomic analysis of two isolates recovered from the blood of patients in the United States and in Sweden (Carvalho, et al., 2008), and the proposed type strain was *E. sanguinicola* SS-1729T (Carvalho, et al., 2008). One of the isolates showed a vancomycin-resistant phenotype, due to the presence of the *vanA* operon. However, the authors noted that this species has not been fully validated (Carvalho, et al., 2008), and in fact, a recent study reported that *E. sanguinicola* should be reclassified as *E. thailandicus*, based on DNA-DNA reassociation and sequence of the 16S rDNA and *rpoB* genes (Shewmaker, et al., 2011).

Phenotypic characteristics of the *Enterococcus* genus

The phenotypic tests routinely used to identify members of the *Enterococcus* genus and species have been previously reviewed (Facklam, Carvalho, & Teixeira, 2002). Briefly, Facklam and collaborators (Facklam, Carvalho, & Teixeira, 2002) divided enterococcal species into 5 groups based on acid formation in mannitol and sorbose broth, as well as hydrolysis of arginine. This clustering was applied to the 22 recently identified *Enterococcus* species, based on phenotypic tests performed along species descriptions (Table 2). It is important to note that this phenotypic classification should be distinguished from the 16S rRNA sequence relationship, so although these tests are potentially useful for diagnostics, it does not appear to reflect the evolutionary relationships between *Enterococcus* species.

Group I consists of enterococcal species that form acid in both carbohydrate broths, but do not hydrolyze arginine (Facklam, Carvalho, & Teixeira, 2002). The new species *E. phoeniculicoa*, *E. devriesei*, and *E. canis* (De Graef, et al., 2003; Law-Brown & Meyers, 2003; Švec, et al., 2005) belong to Group I, along with *E. avium*, *E. malodoratus*, *E. raffinosus*, *E. pseudoavium*, *E. saccharolyticus*, *E. pallens*, and *E. gilvus* (Table 2). Group II includes *E. faecalis* and *E. faecium*, and consists of species that form acid in mannitol broth and hydrolyze arginine but fail to form acid in sorbose broth (Facklam, Carvalho, & Teixeira, 2002). New species *E. canintestini*, *E. lactis*, *E. thailandicus*, and *E. sanguinicola* belong to this group (40, 184, 196, 264) (Carvalho, et al., 2008; Morandi, Cremonesi, Povolo, & Brasca, 2012; Naser, et al., 2005; Tanasupawat, Sukontasing, & Lee, 2008). Group III includes species that are not able to form acid in either mannitol or sorbitol broth, but that hydrolyze arginine (Facklam, Carvalho, & Teixeira, 2002). This group now includes *E. villorum*, *E. durans*, *E. dispar*, *E. hirae*, *E. silesiacus*, and *E. rotai* (Sedláček, et al., 2013; Svec, et al., 2006), as well as variants of *E. faecalis* and *E. faecium* (Table 2) (Facklam, Carvalho, & Teixeira, 2002). *E. asini*, *E. sulfureus*, and *E. cecorum* previously formed Group IV, to which we may now add *E. aquamarinus*, *E. plantarum*, *E. caccae*, and *E. termitis* (Carvalho, et al., 2006; Facklam, Carvalho, & Teixeira, 2002; Švec, et al., 2005; Svec, et al., 2006; Švec, et al., 2011). These species are negative for acid formation in mannitol and sorbose broth and do not hydrolyze arginine (Table 2). Finally, Group V consists of the species that form acid on mannitol, but not sorbose broth, and fail to hydrolyse arginine (Facklam, Carvalho, & Teixeira, 2002). This group includes *E. columbae*, *E. rivorum*, *E. hermaniensis*, *E. camelliae*, and *E. viikiensis,* as well as variants of *E. casseliflavus*, *E. gallinarum*, and *E. faecalis* that fail to hydrolyse arginine (Table 2) (Facklam, Carvalho, & Teixeira, 2002; Koort, Coenye, Vandamme, Sukura, &

Björkroth, 2004; Niemi, et al., 2012; Rahkila, Johansson, Säde, & Björkroth, 2011; Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007). The recently described *E. ureilyticus* (Sedláček, et al., 2013) does not appear to fit into any existing group, and we place it as the sole member of Group VI, based on its ability to formate acid from sorbose but not in mannitol broth, as well as its inability to hydrolyze arginine. Because of lack of available information on the fermentation capabilities of *E. quebencensis*, *E. italicus*, and *E. ureasiticus,* they are unable to be classified by this scheme (Table 2) (Fortina, Ricci, Mora, & Manachini, 2004; Sistek, et al., 2012). Zhang and collaborators have identified a gene cluster in the *E. faecium* strain E980 that is responsible for the metabolism of the α-galactoside sugar raffinose (Zhang, Vrijenhoek, Bonten, Willems, & van Schaik, 2011). While (Table 2) *E. faecium* is placed in Group II in the presented table, which is the group unable to use raffinose, these authors showed that raffinose utilization is actually a trait carried by megaplasmids, which indicates that these mobile elements can have important roles in shaping the competitive fitness of the enterococci in the environment; for example, by expanding their metabolic repertoire (Zhang, Vrijenhoek, Bonten, Willems, & van Schaik, 2011).

Table 2. Phenotypic characteristics of recently identified *Enterococcus* species

Species	Phenotypic characteristic[a]										Group
	MAN	SOR	ARG	ARA	SBL	RAF	MOT	PIG	SUC	MGP	
E. phoeniculicola	+	+	-	+	+	-	-	-	+	nd	I
E. devriesei	+	+	-	v	v	v	-	-	+	-	I
E. canis	+	+	-	+	+	-	-	-	v	+	I
E. canintestini	+	-	+	-	-	-	-	-	+	+	II
E. lactis	+	-	+	+	-	-	-	-	-	-	II
E. thailandicus	+	-	+	-	-	-	-	-	+	-	II
E. sanguinicola	+	-	+	-	-	-	-	-	+	-	II
E. silesiacus	-	-	+	-	-	-	-	-	-	-	III
E. rotai	-	-	+	-	-	-	-	+	+	-	III
E. ratti	-	-	+	-	-	-	-	-	-	-	III
E. aquimarinus	-	-	-	+	-	+	-	-	+	-	IV
E. caccae	-	-	-	-	-	-	-	-	+	-	IV
E. plantarum	-	-	-	-	-	-	-	w	+	-	IV
E. termitis	-	-	-	-	-	-	-	-	-	+	IV
E. rivorum	+	-	-	-	+	-	-	-	+	-	V
E. hermaniensis	+	-	-	-	-	-	-	-	-	nd	V
E. camelliae	+	-	-	-	-	-	-	-	+	-	V
E. viikkiensis	+	-	-	-	-	-	-	-	nd	-	V
E. ureilyticus	-	+	-	-	-	-	-	+	+	-	VI
E. quebecensis	+	nd	-	-	-	-	-	-	+	nd	NA
E. italicus	v	-	-	-	v	-	-	-	+	+	NA
E. ureasiticus	v	nd	-	-	-	-	-	-	+	nd	NA

[a] Abbreviations and symbols: MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; MOT, motility; PIG, pigment; SUC, sucrose; MGP, methyl- α-D-glucopyranoside; +, >80% positive; −, <20% positive; v, variable.

Phylogeny of the *Enterococcus* genus

As a reflection of specialization for life in the gut of a host, over millions of years of co-evolution, genome analysis have highlighted the selective advantage for enterococci of their acquisition of many nutrients required for growth and survival from their habitats (usually either from the diet of the host, or through cross-feeding relationships with other microbes in the gut consortium), as opposed to carrying the additional genetic material necessary for their biosynthesis from simpler precursors (Gilmore, Lebreton, & van Schaik, 2013). Figure 2 shows the phylogenetic relationship among the different species of *Enterococcus* based on single-nucleotide polyphormism (SNP) analysis of the 16S rDNA sequences (Fig. 2). This tree can be divided into at least 7 groups, based on deep divides within the genus (Fig. 2). Some patterns emerge with respect to the habitats in which these species were identified and/or reported (Figure 3). Group I members are species that are farthest from other enterococci, which likely means that their speciation from the rest of the genus occurred comparatively early (Fig. 3). This is translated phenotypically by their unusual negative result in the pyruvate utilization test. *E. columbae* was exclusively isolated from pigeon faecal flora (Devriese, Ceyssens, Rodrigues, & Collins, 1990), and *E. cecorum* also occurs mainly in the gut of birds and less frequently in the GI tract of mammals (some mammals like cattle or swine are fed with poultry) (Aarestrup, Butaye, & Witte, 2002). These strains have not yet been isolated from insects that are at the base of the food chain for many birds (Fig. 3), leading to speculation that speciation may have occurred after the evolutionary split among mammals, reptiles, birds, and insects. For strains of Group II, there appears to be a tropism for mammals, as none of these five strains have been isolated from insects, lizards, or birds (Fig. 3). The presence of the strains in the environment may be the consequence of their occurence in the GI tract of humans and animals (Fig. 3), whereas their presence in food products reflects intentional use and not a natural habitat.

E. faecalis in Group III is found ubiquitously along the food chain, in addition to having emerged as an important opportunistic hospital pathogen (Fig. 3). This would suggest that since at least the early Devonian period, ~412 MYA (the time of the last common ancestor of mammals, reptiles, birds, and insects), this species has been a member of gut microbiomes (Gilmore, Lebreton, & van Schaik, 2013). *E. faecalis* is closely related to the recently described *E. rivorum*, which has been isolated from natural water (Niemi, et al., 2012). It would be very interesting to know the extent to which the genome of these two species is conserved. Other species found in Group III have a strong tropism (and almost specificity) to environments like water or plants. In several cases, an attempt was made to exclude contamination from human or animal feces, but it still remains unknown whether these species live freely in the environment (and if so, how their metabolic requirements are satisfied), or whether they are associated with simple eukaryotes like amoeba, as suggested by recent reports (Huws, Morley, Jones, Brown, & Smith, 2008).

Species found in Groups IV, V, and VI show some tropism for humans and animals (especially mammals) (Fig. 3). Enterococcal species that belong to Group V appear to be specific to humans and mammals (Fig. 3). In contrast, species of Groups IV and VI are more ubiquitous, and can be found along the food chain from insects to mammals. Their occurrence in environments such as water or on plants may stem from faecal contamination (Casanovas-Massana & Blanch, 2013; Staley, Reckhow, Lukasik, & Harwood, 2012). It will be of substantial interest to determine precisely how the nutritional requirements of the various species relate to their host or environmental specializations.

Enterococcal species: Natural and man-made habitats

In the 1960's and 1970's Mundt and collaborators explored a variety of environments and found enterococci in the GI tracts and feces of mammals (71.3%), reptiles (85.7%), and birds (31.8%) (Mundt, 1963), as well as insects (53%) (Martin & Mundt, 1972). Culture-positive rates underestimate the presence of microbes (Ward, Weller, & Bateson, 1990), which suggests that the actual rate of colonization may be closer to 100%. This widespread pattern of colonization suggests that since at least the early Devonian period, ~412 MYA (the time of the last

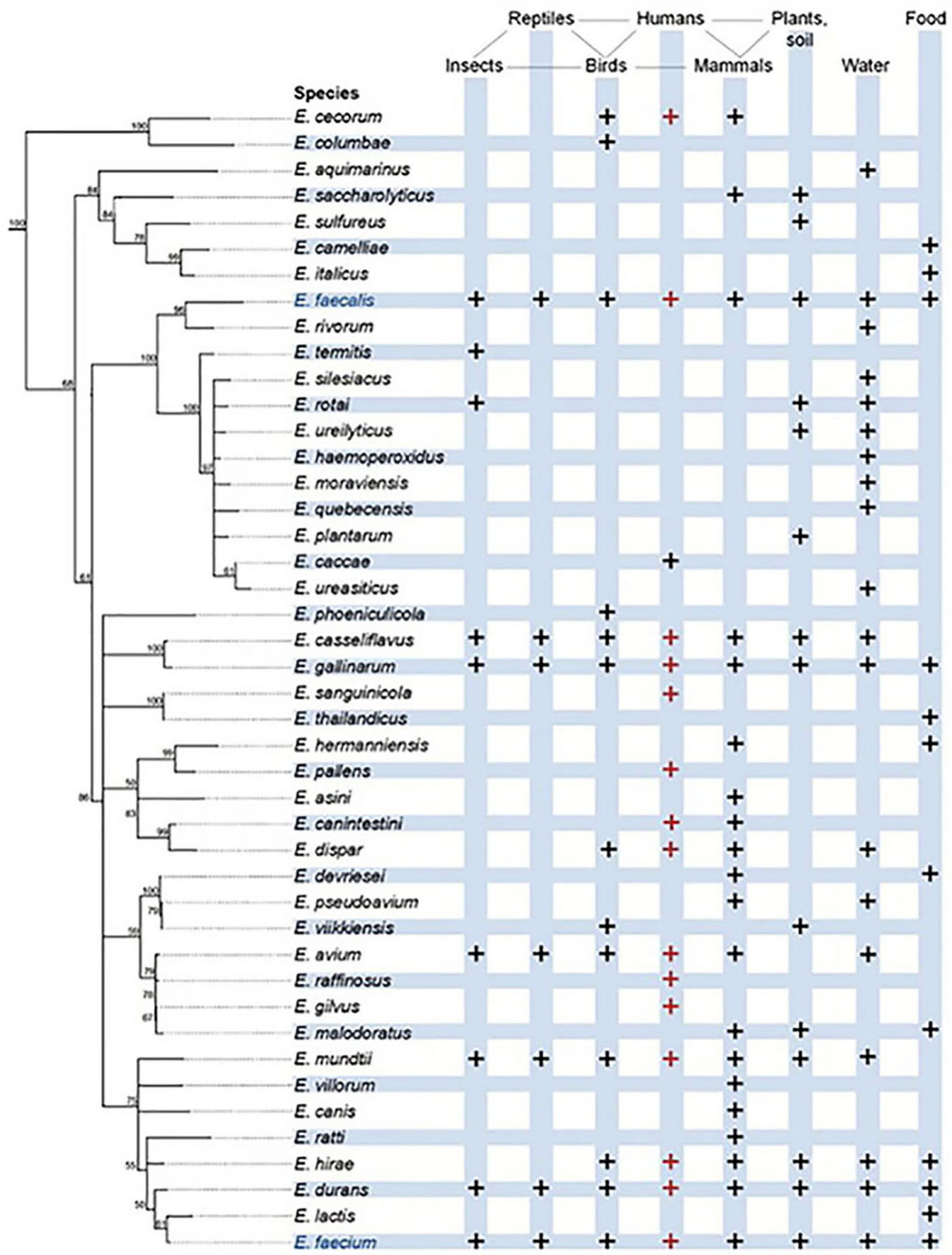

Figure 3. Distribution in nature of species of the genus Enterococcus. The dendrogram shows phylogenic relationships. The sources of isolation are indicated for each species. A simplified food chain is shown. Red and black symbols indicate species that has been described in human infections or colonization, respectively.

common ancestor of mammals, reptiles, birds and insects (Selden, 2007), enterococci have been members of gut microbiomes, which likely place them among the earliest members of GI tract consortia (Gilmore, Lebreton, & van Schaik, 2013).

Enterococci in humans: gut colonization and infection

The human body (especially the GI tract, but also the skin, the upper respiratory tract, the oral cavity, and the vagina) is colonized by microbial communities that, together, constitute the "normal microbiota." Each colonized body site represents an ecosystem that is defined by unique physicochemical and histological characteristics that constitute a competitive environment and select for adapted microbes (Tannock, 1988). The human colon contains about 10^{12} diverse bacteria per gram of contents, with the highest counts in the colon (Sartor, 2008; Vollaard & Clasener, 1994), which contribute to tissue development and immune system homeostasis (Berg, 1996) (Figure 4).

Infections in humans. Enterococcal infection and its treatment are comprehensively covered elsewhere in this volume. Here, we briefly highlight select features of infection that inform the dynamic between colonization and infection by diverse species of enterococci. Enterococci began to emerge as leading causes of multidrug-resistant hospital-acquired infections in the 1970s and 1980s (Huycke, Sahm, & Gilmore, 1998; Jett, Huycke, & Gilmore, 1994), and they now rank among leading causes of hospital-acquired infections of the bloodstream, urinary tract, surgical wounds, and other sites (Hidron, et al., 2008). They are also associated with obligate anaerobes in mixed infections that result in intra-abdominal abscesses (Onderdonk, Bartlett, Louie, & Sullivan-Seigler, 1976). The most recent data available on enterococcal infection from all infection sites and all classes of hospitals in the US, which covers the period 01/01/10–06/30/12, identified 9,309 bloodstream isolates, 54,709 urinary tract isolates, and 20,032 wound isolates (a total of 84,050 isolates) (TSN® Database, Eurofins, Inc., personal communication Daniel F. Sahm, Ph.D.). Of the total, 17,360 are vancomycin-resistant (20.6%) and 64,015 (76%) are *E. faecalis*. Although 24% of isolates are *E. faecium*, they represent 14,998 of the 20,038 (75%) of the vancomycin-resistant isolates. Similar trends have been reported for the European Union (56, 294). *E. durans*, *E. avium*, *E. gallinarum*, *E. casseliflavus*, *E. hirae*, E. *muntdii*, *E. dispar*, and *E. raffinosus* only occasionally cause infection in humans (Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994; Tannock & Cook, 2002). In 2002, Tyrrell and collaborators reported the isolation of *E. gilvus* and *E. pallens* from the bile of a patient with cholecystitis and the peritoneal dialysate of another patient with peritonitis, respectively (Tyrell, et al., 2002). This has been the only description of these species causing human disease thus far. Recently, Ahmed and coworkers (Ahmed, Baig, Gascoyne-Binzi, & Sandoe, 2011) reported a case of human aortic valve infective endocarditis caused by *E. cecorum,* while a study conducted between 2000 and 2008 identified this species as responsible for 0.2% (2 out of 1887) of the bacteremia cases diagnosed at a medical center in Taiwan (Tan, Liu, Li, Huang, Sun, & Qu, 2013). These authors also reported a unique case of bacteremia caused by *E. canintestini* (Tan, Liu, Li, Huang, Sun, & Qu, 2013). While we lack studies that characterize the prevalence of the recently described species in the genus, we can generally assume, based on their recent discovery, that these are not often found as etiological agents of infections in humans.

Gut colonization. Much of what is known about enterococcal colonization of the gut stems from studies of the human GI tract and feces. Enterococci are primarily localized to the human small and large intestine, where enterococci are prominent members of jejunal, ileal, cecal, and recto-sigmoidal consortia (Hayashi, Takahashi, Nishi, Sakamoto, & Benno, 2005). They are also found in human feces, although they constitute a minority population (up to 1%) within the gut microflora (Eckburg, et al., 2005; Sghir, Gramet, Suau, Violaine, Pochart, & Dore, 2000). Enterococci are common in the oral cavity (Smyth, Matthews, Halpenny, Brandis, & Colman, 1987), but occur more rarely in the stomach (Bik, et al., 2005; Monstein, Tiveljung, Kraft, Borch, & Jonasson, 2000). *E. faecalis* and *E. faecium* are most common in human feces, and *E. durans* and *E. avium* are occasionally detected (Finegold, Sutter, & Mathisen, 1983; Tannock & Cook, 2002). *E. caccae* was isolated from human feces, but its prevalence is low (Carvalho, et al., 2006). One study quantified *E. avium* (11%), *E. durans* (33%), *E.*

faecalis (78%), *E. faecium* (100%), *E. gallinarum* (33%), and *E. hirae* (11%) in human fecal specimens (Layton, Walters, Lam, & Boehm, 2010), while *E. casseliflavus* and *E. saccharolyticus* were absent from the nine samples tested (Layton, Walters, Lam, & Boehm, 2010). Other members of the genus *Enterococcus* identified in clinical samples are rarely part of the normal human intestinal microflora, but are present in the gut of other animals or on plants (Fig. 3).

Some members of the microbiota, including indigenous commensal enterococci, can act as opportunistic pathogens and translocate across the mucosal barrier to cause systemic infection in immune-compromised hosts (Berg, 1996; Donskey, 2004). More commonly, however, infection results from the colonization, overgrowth, and translocation of hospital-adapted antibiotic-resistant strains with enhanced pathogenicity. Studies using animal models have shed light on the mechanisms of microflora-mediated colonization resistance to enterococci (Brandl, et al., 2008; Kinnebrew, Ubeda, Zenewicz, Smith, Flavell, & Pamer, 2010). Components of bacterial cells, which include lipoteichoic acid, lipopolysaccharide, and flagellin, are recognized by Toll-like and Nod receptors expressed by intestinal epithelial and Paneth cells. By using a mouse model, Brandl and co-workers (Brandl, et al., 2008) showed that antibiotic reduction of the population of Gram-negative bacteria in the intestine resulted in decreased production of RegIIIγ by Paneth cells, which resulted in intestinal overgrowth of vancomycin-resistant enterococci (VRE) (Brandl, et al., 2008). RegIIIγ is a secreted C-type lectin that selectively kills Gram-positive bacteria, including VRE. RegIIIγ expression was restored in antibiotic treated animals by oral administration of the TLR4 agonist LPS, or systemic administration of the TLR5 agonist flagellin, which reduced VRE counts in the gut of mice (Figure 4B) (Brandl, et al., 2008; Kinnebrew, Ubeda, Zenewicz, Smith, Flavell, & Pamer, 2010). The same mechanisms may also take place in humans, as antibiotic intake by hospitalized patients is associated with an *Enterococcus* outgrowth (Ruiz-Garbajosa, et al., 2012; Ubeda, et al., 2010) that often precedes VRE bloodstream invasion (Ubeda, et al., 2010). Despite increasing knowledge about the interplay between Gram-negative commensals, the host's immune system, and *Enterococcus*, the understanding of synergies and antagonisms between enterococci and other microbes is far from complete. A recent study by Ubeda and coworkers (Ubeda, et al., 2013) observed that the reintroduction of a diverse intestinal microbiota to the GI tracts of mice densely colonized with VRE resulted in a substantial reduction in VRE numbers. They further showed that obligate anaerobic commensal bacteria belonging to the *Barnesiella* genus specifically promoted the clearance of intestinal VRE (Ubeda, et al., 2013). These authors further showed that, in patients undergoing allogeneic hematopoietic stem cell transplantation, intestinal colonization with *Barnesiella* confers resistance to intestinal domination and bloodstream infection with VRE (Ubeda, et al., 2013), which may provide a novel approach for the prevention of infection and the spread of highly antibiotic-resistant bacteria.

Enterococci in animals: gut colonization and infection

Animal GI tracts likely represent the greatest reservoir for enterococci (Gilmore, Lebreton, & van Schaik, 2013). As in humans, enterococci also can infect animals (Aarestrup, Butaye, & Witte, 2002). Ostrolenk and Hunter (Ostrolenk & Hunter, 1946) recovered low levels of enterococci from 49 of 51 fecal samples from various animals, including human, cat, mouse, guinea pig, rabbit, dog, rat, chicken, fly, and monkey. Similarly, Haenel and Mueller-Buethow (Haenel & Muller-Beuthow, 1957) obtained enterococci from human, rat, chicken, and dog, but not in the samples isolated from rabbit, guinea pig, or horse. In 1963, Mundt and colleagues (Mundt, 1963) conducted a thorough analysis of the occurrence of enterococci in animals and natural environments. Enterococci were obtained from the feces of 71% of 216 mammals, 86% of 70 reptiles, and 32% of 22 birds sampled in Great Smoky Mountains National Park (Mundt, 1963). Enterococci occurred only sporadically among the primarily herbivorous mammals, whereas enterococci appear to naturally colonize rodents and larger animals that have varied diets (Mundt, 1963). Enterococci were obtained from most specimens of bats and from the carnivorous mammals, such as fox, bear, raccoon, skunk, and boar (Mundt, 1963). Most probably, microbiota composition in relation to some animals' particular diets determine whether enterococci are able to survive and thrive in their GI tracts (Mieth, 1960).

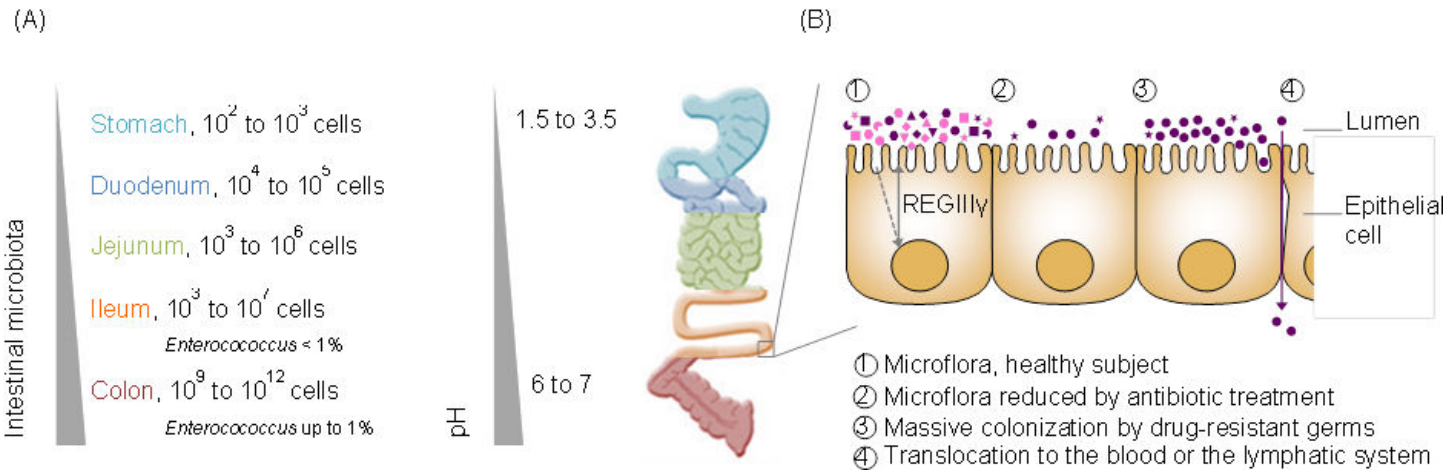

Figure 4. (A) Barriers for gut colonization by the enterococci. The intestinal microbiota, as well as the acidic pH in the stomach, the duodenum, and the jejunum, limit the presence of Enterococcus species. Enterococci are find in the ileum and the colon but represent only a small proportion of the microbiota. (B) The role of antibiotics in the gut colonization by multi-drug resistant enterococci. In response to the Gram-negative bacteria (pink), abundant in the microflora, the intestinal epithelial cells secrete the lectin REGIIIγ that inhibits Gram-positive bacteria (purple) (panel 1). In the course of an antibiotic treatment, the amount of Gram-negative bacteria is reduced and, as a consequence, the production of REGIIIγ decreases (panel 2). Drug-resistant enterococci will benefit of the absence of this natural barrier and massively colonize the gut if their host (panel 3). The novel promiscuity between the bacteria and the epithelial cells may lead to the translocation of enterococcal cells to the blood or the lymphatic system (panel 4).

As new species are described, their associations with hosts are discovered. *E. columbae* is specific to pigeons so far (Devriese, Ceyssens, Rodrigues, & Collins, 1990), and *E. asini* has only been found in donkeys (de Vaux, Laguerre, Diviès, & Prévost, 1998). *E. asini* has not been reported in additional studies since its description in 1998, and as a result, is likely to be rare. *E. columbae* is difficult to isolate, as this species does not grow on selective media that are commonly used for isolation of enterococci and also requires CO_2 for growth, so the prevalence of this species may therefore be underestimated (Devriese, Ceyssens, Rodrigues, & Collins, 1990).

The most commonly encountered enterococcal species in the gut of mammals are *E. faecalis*, *E. faecium*, *E. hirae*, and *E. durans* (Devriese & De Pelsmaecker, 1987). Other species are found only occasionally (Devriese & De Pelsmaecker, 1987), or in particular age groups (such as *E. cecorum* in older poultry) (Aarestrup, Butaye, & Witte, 2002). In fact, an age-dependent succession of enterococcal species colonization appears to occur in chickens (Devriese, Hommez, Wijfels, & Haesebrouck, 1991; Kaukas, Hinton, & Linton, 1987). Chickens are initially colonized by *E. faecalis*, but this population is then displaced, mainly by *E. faecium,* and it was proposed that the use of tylosin (to which *E. faecium* is commonly resistant) as a growth promoter is the reason of this replacement (Kaukas, Hinton, & Linton, 1987). These species then appear to be replaced by *E. cecorum* in the mature chicken. It is of interest that *E. avium* and *E. gallinarum*, originally described from chickens, were rarely found, suggesting that these species may not belong to the normal intestinal flora of poultry (Devriese, Hommez, Wijfels, & Haesebrouck, 1991). *E. avium* was originally described from human feces (Guthof, 1955), but is common in chicken feces (Nowlan & Deibel, 1967). The extent to which the flora of chickens now reflects that native to poultry, or reflects the consequences of intensive production and modern poultry husbandry practices (namely, the frequent use of antibiotics including aminoglycosides), is controversial, but has undoubtedly impacted the representation of various species of enterococci (Aarestrup, Butaye, & Witte, 2002; Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994). Kobayashi and collaborators reported that aminoglycoside modifying enzymes are less prevalent in *E. avium* strains, as compared to *E. faecium* and *E. faecalis* strains isolated from poultry (Kobayashi, Alam, Nishimoto, Urasawa, Uehara, & Watanabe, 2001).

Age-dependent enterococcal colonization also has been described in cattle (Devriese, Laurier, De Herdt, & Haesebrouck, 1992). In preruminant calves, the enterococcal flora mainly consists of *E. faecalis*, *E. faecium,* and

E. avium. This flora is gradually replaced by *E. cecorum* (Devriese, Laurier, De Herdt, & Haesebrouck, 1992). Enterococci occur on the tonsils of preruminating calves; mainly *E. faecalis*, as well as *E. raffinosus*, to a lesser extent (Devriese, Laurier, De Herdt, & Haesebrouck, 1992).

The enterococcal species most frequently isolated from the intestines of swine are *E. faecalis* and *E. faecium*; however, the latter species occurs in low numbers (Devriese & De Pelsmaecker, 1987; Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994). Other enterococcal species found among swine flora include *E. hirae* and *E. cecorum* (Devriese & De Pelsmaecker, 1987; Devriese & Haesebrouck, 1991; Devriese, Pot, Van Damme, Kersters, & Haesebrouck, 1994).

A study on the flora of cats and dogs found that *E. faecalis* was the most frequently encountered enterococcal species in the gut, as well as on the tonsils of these animals, with *E. faecium* and *E. hirae* occurring infrequently (Devriese, Cruz Colque, De Herdt, & Haesebrouck, 1992). Other species, including *E. avium*, *E. raffinosus*, *E. durans*, *E. cecorum*, and *E. gallinarum* were also occasionally isolated (Devriese, Cruz Colque, De Herdt, & Haesebrouck, 1992). As noted above, *E. canis* and *E. canintestini* have more recently been isolated from healthy dogs (De Graef, et al., 2003; Naser, et al., 2005). *E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum*, E. *casseliflavus,* and *E. mundtii* have also been found in horses (Devriese & De Pelsmaecker, 1987; Thal, et al., 1995).

Recent studies in Portugal identified enterococci in a broad range of environments, including wild animals. A total of 144 enterococci (120 *E. faecium*, 14 *E. hirae*, 8 *E. faecalis*, 2 *E. gallinarum*) were recovered in echinoderms collected from Azorean waters (Marinho, et al., 2013). Resistance to erythromycin, ampicillin, tetracycline, and ciprofloxacin in these enterococci was found to be common among them (Marinho, et al., 2013). VRE (*E. faecium*, *E. gallinarum* and *E. casseliflavus*) were recovered in 8 of the 365 analysed fecal samples from Iberian wolf and lynx (Gonçalves, et al., 2013). Similarly, vancomycin-resistant *E. faecalis*, *E. faecium*, *and E. durans* were isolated in 7 of 118 fecal samples (5.9%) of natural gilthead seabream recovered off the coast of Portugal (Barros, et al., 2012).

In other studies, Radhouani and collaborators (Radhouani, Poeta, Gonçalves, Pacheco, Sargo, & Igrejas, 2012) examined 31 enterococcal isolates from 42 common buzzard fecal samples, and found *E. faecium* as the most commonly encountered species (48.4%) recovered. *E. faecium*, *E. durans*, and *E. gallinarum* isolates were detected in fecal samples of other wild birds (Silva, et al., 2011). Enterococci were also isolated from wild boars, partridges and fish (Almeida, et al., 2011), red foxes (Radhouani, et al., 2011), and wild rabbits (Silva, et al., 2010). In addition, enterococci have been found in wild geese (Han, et al., 2011) and from cattle reared with traditional practices in Ethiopia (Bekele & Ashenafi, 2010). In this last study, a total of 298 enterococcal isolates were obtained, which consisted of *E. faecium* (49.6%), *E. durans* (26.9%), *E. hirea* (11.9%), and *E. faecalis* (11.5%).

A large variety of insects, including beetles, flies, bees, termites, and worms have also been found to harbor enterococci. *E. faecalis* and *E. faecium* are predominant but other species occur at lower prevalence. Martin and Mundt (Martin & Mundt, 1972) observed the association of *E. faecalis*, *E. faecium*, and *E. casseliflavus* with a broad range of insect orders. *E. faecalis* (32%), *E. faecium* (22.4%), and *E. casseliflavus* (43.5%) were associated with wild insects from 37 different taxa (Martin & Mundt, 1972). In a survey of laboratory-reared and wild *Drosophila*, Cox and Gilmore (Cox & Gilmore, 2007) found several species of enterococci, including *E. faecalis*, *E. faecium*, *E. gallinaraum*, and *E. durans,* and localized them to the digestive tract. Differences between human and *Drosophila* GI tract anatomy and physiology undoubtedly play a role in determining the composition and physical location of their respective microbial populations.

Anatomically, the GI tract of *Drosophila* (Bodenstein, Cooper, Ferris, Miller, Poulson, & Sonnenblick, 1950) has an overall organization that has parallels to that of humans. With the exception of the *Drosophila* crop, the two systems possess a single alimentary canal that begins at the esophagus, connects to a ventriculus (stomach), extends to the intestine, proceeds to the rectum, and terminates at the anus (Figure 5). In terms of digestive

function, however, they are quite different. *Drosophila* possesses an acidic crop, but maintains an alkaline ventriculus and a neutral to acidic hindgut (Clark, 1999; Dow & Harvey, 1988). Another major difference is the absence of obligate anaerobic phylotypes in Drosophila (Cox & Gilmore, 2007), while the majority of human intestinal microbial phylotypes are obligate or microaerophilic anaerobes (Eckburg, et al., 2005).

Enterococci have been detected on insects in hospitals, and concerns have been raised about this possible reservoir for human contamination (Fotedar, Banerjee, Singh, Shriniwas, & Verma, 1992; Pai, Chen, & Peng, 2004). The importance of insects to the transmission of resistant or infectious enterococci has not been established. Ahmed and collaborators (Ahmed, Baig, Gascoyne-Binzi, & Sandoe, 2011) showed that house flies and cockroaches in the confined swine production environment may act as vectors and/or reservoirs of antibiotic resistant and potentially virulent enterococci. Macovei *et al.* (Macovei, Miles, & Zurek, 2008) showed that houseflies captured in fast-food restaurants commonly carry antibiotic-resistant germs. Among newly characterized *Enterococcus* species, *E. termitis* was isolated from the gut of a termite, while some *E. rotai* strains were isolated from mosquitoes (Sedláček, et al., 2013; Svec, et al., 2006).

Comparison of enterococci isolated from human and animals

In 1993, Bates and collaborators (Bates, Jordens, & Selkon, 1993) described the first vancomycin-resistant enterococci isolated from a non-human reservoir. These authors detected a vancomycin-resistant strain of *E. faecium* (VREF) in farm animals in the UK (Bates, Jordens, & Selkon, 1993). To understand the relationship between enterococci, and more particularly, between antibiotic-resistant strains of agricultural and human origin, *E. faecium* isolates were compared using PFGE, and similar PFGE profiles were found for VRE isolated from stool samples from both humans and animals (Hammerum, et al., 2004; van den Bogaard & Stobberingh, 2000). Besides PFGE, amplified fragment length polymorphism (AFLP) analysis and MLST were used to compare these *E. faecium* isolates (Willems R. J., et al., 2000). VREF strains were largely found to be host-specific, and strains isolated from hospitalized patients were genetically different from the prevailing VREF strains present in the fecal flora of non-hospitalized humans (Willems R. J., et al., 2000). Further, MLST showed that outbreak isolates from hospitalized patients clustered in a subgroup, which was originally termed a clonal complex 17 (CC17), while *E. faecium* isolated from mainly domesticated animals belonged to other types (Willems R. J., et al., 2005; Willems R. J., et al., 2012). Although there is some evidence of host adaptation to *E. faecium* strains, typical hospital-associated clones have been identified in farm animals (Hammerum, 2012), and dogs are frequently colonized by strains similar to those identified in clinical isolates (Damborg, Sørensen, & Guardabassi, 2008; de Regt, et al., 2012). This indicates that sharing a habitat is an important risk factor for the spread of these organisms.

Similarly to *E. faecium*, *E. faecalis* isolates from hospitalized patients tend to derive from select STs and CCs (McBride, Fischetti, LeBlanc, Moellering Jr., & Gilmore, 2007; Ruiz-Garbajosa, et al., 2006), but a diversity of STs can be detected in *E. faecalis* recovered from animals, meat, fecal samples from humans in the community, and patients with bloodstream infections (Kawalec, et al., 2007; Kuch, et al., 2012; McBride, Fischetti, LeBlanc, Moellering Jr., & Gilmore, 2007; Nallapareddy, Wenxiang, Weinstock, & Murray, 2005; Ruiz-Garbajosa, et al., 2006). *E. faecalis* human isolates have been found to be highly related to pig isolates, based on their antimicrobial resistance pattern, virulence gene profile, and MLST/ PFGE types (Larsen, et al., 2011). Similarly, the presence of the pathogenicity island (PAI) of *E. faecalis* can be found in isolates from pigs (Shankar, Baghdayan, Willems, Hammerum, & Jensen, 2006).

Enterococci in the environment (plants, water and soil)

Enterococci are widely used as indicators of fecal contamination from animal or human origin in the environment (see Enterococci as indicators of environmental fecal contamination). However, the extent to which enterococci in the environment (including in water sources) reflects human or animal waste contamination continues to be debated (Harwood, Whitlock, & Withington, 2000). Comparing the genomes of enterococci

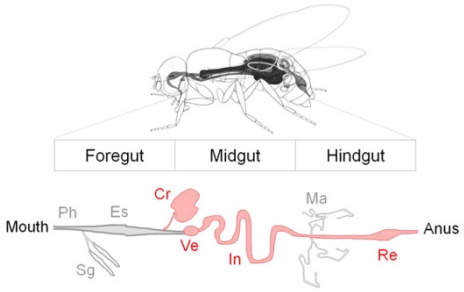

Figure 5. Schematic diagram of the enterococcal colonization of Drosophila GI tract. Red indicates portions of the GI tract naturally colonized by Enterococcus. Ph, pharynx; Sg, salivary gland; Es esophagus; Ve, proventriculus; In, intestine; Mal, Malpighian tubule; Re, rectum. Adapted from Hartenstein, 1993.

obtained from environmental sources to those derived from humans and animals will shed considerable light on this question. Among the extra-enteric niches where enterococci are routinely isolated include soil and sediments, aquatic and terrestrial plants, and ambient waters (Fig. 3). These are heterothermic habitats, in which temperatures are variable—in contrast to the gastrointestinal tract of warm-blooded animals, where the temperature is relatively constant. In addition, a variety of environmental stressors such as UV sunlight, salinity, starvation, and predation also occur (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012). The transition from the animal gastrointestinal tract, an environment rich in many nutrients with key nutrients restricted, to oligotrophic environments that are generally nutrient-poor, exposes enterococci to nutrient starvation. One of the first reports on the survival of enterococci under nutrient-starvation conditions indicated that *E. faecalis* survived for extended periods in sterilized sewage (presumably due to the availability of organic nutrients), but declined rapidly in sterile lake water and in water with a phosphate buffer, indicating that oligotrophic conditions (exemplified by the sterile lake water and phosphate buffer) were deleterious to the survival of enterococci (Sinclair & Alexander, 1984). Indeed, enterococci are auxotrophic bacteria. It is important to note that although the authors observed survival of *E. faecalis* in sterilized sewage, they did not observe growth (Sinclair & Alexander, 1984).

In and on aquatic and terrestrial vegetation. While various enterococcal species can be found on plants (Fig. 3), it is unclear whether or not these strains are contaminants from fecal consortia that are simply surviving, or whether these environments actually constitute a habitat. Ostrolenk and Hunter (Ostrolenk & Hunter, 1946) considered their presence in these locations more an indication of survival, whereas Sherman (Sherman, 1937) considered as evidence against accidental occurrence and survival, given the fact that, of the many cultures isolated from plants, none of the hemolytic types of enterococci had been obtained. Mundt (Mundt, 1961; Mundt, 1963) recovered enterococci from the surface of a variety of plant species, and proposed that enterococci were transient populations, most likely introduced by insects and wind, since their occurrence was seasonal (Mundt, 1961; Mundt, 1963). In another study, however, he demonstrated what appeared to be the ability of *E. faecalis* to grow on plants (Mundt, Coggin, Jr., & Johnson, 1962). Occurrence in seed or soil, movement from the seed to the emergent parts, and ability to reproduce on the growing plant are criteria for bacterial plant epiphytes (Burri, 1903; Dueggeli, 1904; Thaysen & Galloway, 1930; Voznyakovskaya & Khudyakov, 1960). In his work (Mundt, Coggin, Jr., & Johnson, 1962), Mundt suggested that *S. faecalis* is a potential epiphyte on plants, since it was found consistently on most plant parts, it established a cycle on plants in which transmission occurs via the seed, and it reproduced on the growing plant. Although no details are provided, the author concluded that these findings indicate that these bacteria are capable of adaptation to an environment substantially different from that of the intestinal tract in its quantity of nutrients, temperature, and moisture (Mundt, Coggin, Jr., & Johnson, 1962). However, it is important to note that the possibility that the enterococcal cells were commensals of macro or micro-invertebrates, or that they may have benefitted from a more complex bacterial population, were not addressed. Huws and collaborators (Huws, Morley, Jones, Brown, & Smith, 2008) investigated the interactions of

E. faecalis, among other bacteria, with the amoeba *Acanthamoeba polyphaga*. They found evidence of protozoal predation of *E. faecalis*. Interestingly, the extracellular numbers of *E. faecalis* JH2-2 were significantly higher when cultured with amoebae, as compared to growth in the absence of amoebae (Huws, Morley, Jones, Brown, & Smith, 2008). The authors stated that, since not all bacteria are digested by protozoa, outgrowth of select populations occurs, as was observed for the tested enterococcal strain (Huws, Morley, Jones, Brown, & Smith, 2008).

The occurrence of enterococci has been investigated in association with various vegetation, including algae (Whitman, Shively, Pawlik, Nevers, & Byappanahalli, 2003), beach wrack (Anderson, Turner, & Lewis, 1997; Grant, et al., 2001; Inamura, Thompson, Boehm, & Jay, 2011), submerged vegetation (Badgley, Nayak, & Harwood, 2010; Badgley, Thomas, & Harwood, 2010), flowering plants (Mundt, 1963), and forage crops (Müller, Ulrich, Ott, & Müller, 2001; Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012). Whitman and collaborators (Whitman, Shively, Pawlik, Nevers, & Byappanahalli, 2003) showed that the *Cladophora* mats, a green alga found in both fresh and marine waters, were a significant source of *E. coli* and enterococci. In addition to these fecal bacteria, enteric pathogens such as *Shigella*, *Campylobacter*, and *Salmonella* also were isolated (Whitman, Shively, Pawlik, Nevers, & Byappanahalli, 2003). The high densities of enterococci in fresh *Cladophora* have been attributed to *in situ* growth (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003). Indeed, these authors stated that algal leachate readily supported the in-vitro multiplication of enterococci (100-fold in undiluted algal leachate at 35 °C over 24 h), and that such growth was directly related to the concentration of algal leachate. This suggests that leachates contain the necessary growth-promoting substances to sustain these bacteria (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003). However, other adherent bacteria present on these algae may contribute nutrients that enterococci require. Enterococci can persist and survive on submerged vegetations (Badgley, Nayak, & Harwood, 2010; Badgley, Thomas, & Harwood, 2010) or dried algae (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003), and enterococci have also been isolated from plankton and macro-invertebrates (Maugeri, Carbone, Fera, Irrera, & Gugliandolo, 2004; Signoretto, et al., 2004).

Several recently characterized *Enterococcus* species were identified on plants. *E. plantarum* was isolated from plant samples obtained from a meadow in the Czech Republic, not influenced by farm animals and exploited only for hay harvesting; and *E. rotai* and *E. ureilyticus* were isolated from plants, as well as water samples (Švec, et al., 2011).

In water. Large quantities of human and animal wastes are distributed into the environment through sewage or non-sewage systems. Human wastes empty into waterways, usually through a sewage treatment plant, while animal wastes are often used composted (but otherwise untreated) as fertilizers on fields. Enterococci are found in both fresh (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012; Fujioka, Tenno, & Kansako, 198) and marine water (Boehm, et al., 2002; Korajkic, Brownell, & Harwood, 2011). They can survive and persist in fresh (Anderson, Whitlock, & Harwood, 2005) as well as marine water (Litopoulo-Tzanetaki & Tzanetakis, 1992), but it is not clear from the literature whether enterococci can also multiply in these environments. Growth in the absence of essential nutrients obviously will not occur, and studies that report growth in water in the presence of algae (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003) or sediments (Yamahara, Walters, & Boehm, 2009) rarely precisely define their position in more complex food webs.

Shifts in *Enterococcus* species composition generally occur during domestic wastewater treatment. While *E. faecalis* was predominant, different species including *E. pseudoavium*, *E. casseliflavu*s, *E. faecium*, *E. mundtii*, *E. gallinarum*, *E. dispar*, *E. hirae*, *E. durans*, *E. flavescens*, *Enterococcus haemoperoxidus*, and *Enterococcus moraviensis* also can be recovered (Kühn, et al., 2003). This may have implications for the identification of fecal pollution based on the presence of specific bacterial types associated with domestic wastewater (Graves & Weaver, 2010). In water, the species considered as fecal contaminants are mainly *E. faecium* and *E. faecalis*, and the origins of other species are less clear (Aarestrup, Butaye, & Witte, 2002). Data reported by Signoretto *et al.*

(Signoretto, et al., 2004) suggested that the adhesion to plankton of viable but non-culturable cells contribute to the prolonged survival of enterococci in marine waters. Numerous recently-characterized *Enterococcus* species have been isolated from water samples. *E. rotai* and *E. ureilyticus* were isolated from drinking water, *E. acquimarinus* was isolated from sea water, and *E. rivorum* was isolated from a pristine brook (Niemi, et al., 2012; Svec, et al., 2005). Similarly, *E. moraviensis*, *E. silesiacus*, *E. ureasiticus*, and *E. quebecensis* have been isolated from surface water (Sistek, et al., 2012; Svec, et al., 2001; Svec, et al., 2006). Whether they represent flora of microscopic eukaryotes or other larger organisms remains to be definitively determined.

In soil and sediments. Enterococci occur in temperate and tropical soils (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012; Mundt, 1961), fresh or marine water sediments (Ferguson, Moore, Getrich, & Zhowandai, 2005; Obiri-Danso & Jones, 2000), and can be recovered from sand (Halliday & Gast, 2011; Yamahara, Layton, Santoro, & Boehm, 2007). The prolonged survival of enterococci has been observed in freshwater and estuarine sediments (Anderson, Whitlock, & Harwood, 2005). van Donsel *et al.* (Van Donsel, Geldreich, & Clarke, 1967) found that the rates of survival of *S. faecalis* were higher than those of fecal coliforms during spring and winter, but that fecal coliforms survived longer than *S. faecalis* during the summer months. Interestingly, many of the early investigations of the survival and persistence of enterococci in soil environments focused on watersheds impacted by anthropogenic activities, particularly in cattle grazing and field lot operations (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012). All samples taken from urban sewage, and from farmland using pig manure, as well as crops grown on this land, readily yielded *Enterococcus* species (Kühn, et al., 2003). In crops to which animal fertilizer had not been applied, the incidence of *Enterococcus* species was reduced to 33% (Kühn, et al., 2003). *Enterococcus* species can be recovered from sand from freshwater and marine beaches (*E. faecium*, *E. casseliflavus*, *E. durans*), and from marine sediments (*E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, and *E. mundtii*) (Ferguson, Moore, Getrich, & Zhowandai, 2005). As for other environments, whether enterococci grow as free living organisms in sediments, sands, or soils remains highly speculative. While the growth requirements of many bacteria, including *E. coli*, are relatively simple (Andrews, 1991), enterococci require complex nutrients.

Enterococci in food

It is likely that because of their natural occurrence as contaminants of raw meat and dairy products (Franz, Holzapfel, & Stiles, 1999), thermotolerance, low toxicity, and their ability to acidify an environment, enterococci have become an important ingredient in fermented foods. Enterococci are often used for the preparation of traditional cheeses manufactured in Mediterranean countries, including Greece, Italy, Spain and Portugal, from raw or pasteurized goat, sheep, or bovine milk (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Levels of enterococci in different cheeses range from 10^4 to 10^6 CFU/g, with *E. faecium* and *E. faecalis* being the most common (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Enterococci contribute to the ripening and development of aroma in these products because of their proteolytic and esterolytic properties, as well as the production of diacetyl and other important volatile compounds (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Enterococci play an important role in improving flavor development and overall quality of other traditional fermented foods as well, including vegetables and sausages (Franz, Holzapfel, & Stiles, 1999; Giraffa, 2002), including salami and chorizo (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). In addition to desired fermentation properties, both *E. faecalis* and *E. faecium* have been implicated in the spoilage of cured meat products, including canned hams and chub-packed luncheon meats (Magnus, Ingledew, & McCurdy, 1986; Magnus, McCurdy, & Ingledew, 1988). Finally, enterococci commonly occur in large numbers in vegetables (Ben Omar, et al., 2004; Fernandez-Diaz, 1983; Franz, Holzapfel, & Stiles, 1999; Giraffa, 2002; Mundt, 1963); and occur in Spanish-style green olive fermentations (de Castro, Montaño, Casado, Sánchez, & Rejano, 2002; Fernandez-Diaz, 1983; Floriano, Ruiz-Barba, & Jiménez-Díaz, 1998), in which *E. faecalis* and *E. faecium* are frequent contaminants.

While enterococci are used as an indicator of fecal contamination in the environment, their value as hygiene indicators in the industrial processing of foods has been questioned (Birollo, Reinheimer, & Vinderola, 2001).

The resistance of enterococci to pasteurization and their adaptability to different substrates and growth conditions (extreme pH and salinity) explains their occurrence in food products manufactured from raw materials or in nominally–heat-treated food products. The contribution of enterococci to the organoleptic properties of fermented food products (Coppola, Villani, Coppola, Coppola, Parente, & Parente, 1990; Giraffa & Carminati, 1997; Pisano, Fadda, Deplano, Corda, Casula, & Cosentino, 1994; Litopoulo-Tzanetaki & Tzanetakis, 1992; Manolopoulou, Sarantinopoulos, Zoidou, Aktypis, Moschopoulou, & Kandarakis, 2003), and their ability to produce bacteriocins (enterocins) (Cleveland, Montville, Nes, & Chikindas, 2002; De Vuyst & Vandamme, 1994; Ennahar & Cai, 2005; Klaenhammer, 1993; Moll, Konings, & Driessen, 1999; Nes, Diep, Håvarstein, Brurberg, Eijinsk, & Holo, 1996), are important characteristics in food technology. Despite their use in traditionally fermented products, the continuing use of enterococci in food processes is somewhat controversial, because of their association with human infection (De Vuyst, Foulquié Moreno, & Revets, 2003; Vancanneyt, et al., 2002).

E. faecium and *E. faecalis* are generally the most frequently encountered species of *Enterococcus* in food products (Lopez-Diaz, Santos, Gonzalez, Moreno, & Garcia, 1995; Suzzi, et al., 2000). Martin and collaborators (Martín, Corominas, Garriga, & Aymerich, 2009) investigated the species distribution of enterococci in traditional fermented sausages by species-specific multiplx PCR, and by *atpA* and 16S rDNA gene sequencing. They identified *E. faecalis* (31.4%) and *E. faecium* (30.7%), and more rarely *E. sanguinicola* (14.9%), *E. devriesei* (9.7%), *E. malodoratus* (7.2%), *E. casseliflavus* (3.4%), *E. gallinarum* (1.3%), *E. gilvus* (1.0%), *E. hermanniensis* (0.2%), and *E. durans* (0.2%) (Martín, Corominas, Garriga, & Aymerich, 2009). The species *E. camelliae*, *E. italicus*, *E. thailandicus*, and *E. lactis* were originally isolated from tea leaves, an Italian cheese, fermented sausages, and raw milk cheese, respectively (Fortina, Ricci, Mora, & Manachini, 2004; Morandi, Cremonesi, Povolo, & Brasca, 2012; Sukontasing, Tanasupawat, Moonmangmee, Lee, & Suzuki, 2007; Tanasupawat, Sukontasing, & Lee, 2008). The natural habitat of these *Enterococcus* species is very likely not fermented food, but elsewhere in nature in ecologies yet to be defined.

Enterococci are permitted in some countries as probiotics (Franz, Holzapfel, & Stiles, 1999; Franz, Stiles, Schleifer, & Holzapfel, 2003). One *E. faecium* strain (SF68) used as a probiotic, has been suggested to be clinically effective in the treatment of diarrhea in children (Bellomo, Mangiagle, Nicastro, & Frigerio, 1980) and to prevent diarrhea caused by antibiotic treatments (Wunderlich, et al., 1989). In a comprehensive review on the various enterococcal strains used as probiotics, Franz and collaborators (Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011) concluded that data in support of the use of enterococci as probiotics is controversial. While it is possible to argue for the probiotic benefit of some strains, the emergence of enterococci in human disease and as multidrug-resistant germs raises concern.

Drug resistant enterococci in non-human reservoirs

Besides the treatment of human infection, antimicrobial agents are used in food preservation, to treat and promote the growth of animals and pets, in plant production, and for other non-health-related purposes (Dibner & Richards, 2005; Turnidge, 2004). The contribution of each of these applications to resistance among human pathogens is still debated.

In animals. Enterococcal infections of animals are rarely treated with antimicrobial agents; however, enterococci are exposed to antimicrobial selection as members of the GI tract during the course of treatment of other infections, or when antibiotics are used as growth promoters or prophylaxis (Turnidge, 2004). The virtues of antibiotics as growth promoters have been debated since the 1960s (Swann, 1969). Studies conducted in the 1950s confirmed that the use of tetracycline for treatment or growth promotion selected for tetracycline resistance among group D streptococci (enterococci) in chickens (Barnes, 1958; Elliott & Barnes, 1959). More recently, epidemiological studies and feeding experiments have shown that the use of different antimicrobial growth promoters, including glycopeptides (avoparcin), macrolides (tylosin) and oligosaccharides (avilamycin), select for resistance among enterococci in poultry and pigs (Aarestrup, Bager, Jensen, Madsen, Meyling, &

Wegener, 1998; Aarestrup, Bager, Jensen, Madsen, Meyling, & Wegener, 1998; Aarestrup F. M., 2000; Bager, Madsen, Christensen, & Aarestrup, 1997; Christie, Davidson, Novick, & Dunny, 1983; Hinton, Kaukas, Lim, & Linton, 1986). Most interest has focused on the use of avoparcin and the selection of VRE in animal husbandry (Aarestrup F. M., 1995; Bager, Madsen, Christensen, & Aarestrup, 1997). A multitude of global studies describe the occurrence of VRE in many different non-human reservoirs, including pet cats and dogs, horses, birds, wood frogs, ostriches, pigs, pork, broilers, poultry meat, environmental samples, and sewage, as well as from stool samples from farmers and non-hospitalized humans in the community, mainly in Europe (Hammerum A. M., 2012). The linkage of VRE in non-human reservoirs to the use of avoparcin as a growth promoter is debated. Avoparcin was used in animal husbandry in the EU, Australia, and several other locations, but was not allowed for growth promotion in the USA and Canada. As a likely result, there is a low prevalence of VRE in the US and Canada, but nevertheless, there is a high level of occurrence of VRE in US hospitals (Hammerum A. M., 2012). Feeding animals low doses of antimicrobials, in certain conditions, increases productivity by improving feed conversion and decreasing morbidity and mortality caused by infection (Butaye, Baele, Devriese, & Haesebrouck, 2002). The glycopeptide avoparcin was first introduced for growth promotion in 1975 (Hammerum, Lester, & Heuer, 2010), and was mainly used for broilers and pigs, but also for turkeys, veal calves, and other animals (Bates J. , 1997; Hammerum, Lester, & Heuer, 2010; Hammerum A. M., 2012). At that time, it was used extensively in most parts of Europe and the rest of the world, with the notable exceptions of Canada and the USA (McDonald, Kuehnert, Tenover, & Jarvis, 1997). As avoparcin confers cross-resistance to vancomycin, its use selected for outgrowth of VRE, and as a consequence, VRE was common in the intestinal flora of farm animals in Europe during the 1990s (Bager, Madsen, Christensen, & Aarestrup, 1997). In contrast, until 2008, VRE had never been isolated from farm animals in the US. Because of this connection, the use of avoparcin as a growth promoter was banned and the prevalence of VRE in farm animals in Europe rapidly declined, but VRE did not disappear (Aarestrup F. M., 2000). To explain why VRE persist among farm animals, it has been suggested that the use of the macrolide tylosin in pigs co-selects for vancomycin resistance among enterococci, since both the resistance determinants are often on the same plasmid (Aarestrup F. M., 2000). Besides tylosin, Johnsen and collaborators (Johnsen, et al., 200) hypothesized that plasmid addiction systems may contribute to retention of the resistance.

High-level aminoglycoside resistance has been observed among enterococci isolated from a variety of animals. Highly gentamicin-resistant strains were isolated from bovine mastitis (Jayaro & Oliver, 1992) and pigs (Aarestrup, Bager, Jensen, Madsen, Meyling, & Wegener, 1998; Aarestrup F. M., 2000). These gentamicin-resistant isolates include *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* (Aarestrup, Butaye, & Witte, 2002). Resistance to kanamycin has been observed in 30% of *E. faecalis* and *E. faecium* isolates from bovine mastitis (Jayaro & Oliver, 1992), 62% of 62 isolates from chickens, and 36% of 72 isolates from pigs (Rollins, Lee, & LeBlanc, 1985). Streptomycin resistance has been observed among enterococcal isolates from bovine mastitis (80%) (Jayaro & Oliver, 1992), chickens (64%), beef cattle (6%), and pigs (57%) (Rollins, Lee, & LeBlanc, 1985). Thal and collaborators (Thal, et al., 1995) also isolated streptomycin-resistant enterococci from horses and birds. Chloramphenicol use has been limited in food animals for the last several decades. There are few reports on the occurrence of resistance to chloramphenicol among enterococci isolated from animals. Saikia *et al.* (Saikia, Dutta, Devriese, & Kalita, 1995) found that one-third of *E. faecalis* from ducks in India were resistant to chloramphenicol. A low frequency (1–7%) of chloramphenicol resistance occurs in *E. faecium* and *E. faecalis* isolates from broilers and pigs in Denmark (Aarestrup F. M., 2000).

The first finding of quinupristin-dalfopristin–resistant *E. faecium* in non-hospitalized humans was reported in the United Kingdom (UK) by Woodford and collaborators (Woodford, Adebiyi, Palepou, & Cookson, 1998) in the late 1990s. Interestingly, quinupristin-dalfopristin and other streptogramins used for human therapy were not available at that time in the UK for use in humans. Similarly to vancomycin and avoparcin, the finding of quinupristin-dalfopristin–resistant *E. faecium* isolates outside hospitals was assumed to be associated with the use of virginiamycin in animals (McDonald, et al., 2001). The genetic determinant that encodes streptogramin A resistance has been detected in *E. faecium* isolates from poultry, pigs, pork, and sewage worldwide. Enterococci

from animals are also frequently resistant to penicillin or ampicillin. Frequencies of resistance comparable to those observed among isolates from humans have also been reported (Aarestrup F. M., 2000; Yoshimura, Ishimaru, Endoh, & Kojima, 2000).

In food. Antibiotic-resistant enterococci occur in meat products, dairy products, and even within strains used as probiotics (Giraffa, 2002). The contamination of meat with antibiotic resistant enterococci during slaughter of animals to which antibiotics were administered has been reported (Bager, Madsen, Christensen, & Aarestrup, 1997; Del Grosso, et al., 2000; Klare, Badstübner, Konstabel, Böhme, Claus, & Witte, 1999; Kruse, Johansen, Rørvik, & Schaller, 1999). In 2002, Giraffa and collaborators (Giraffa, 2002) highlighted the role played by food reservoirs of VRE in the dissemination of antibiotic resistance traits in the environment. VRE and tetracycline-resistant enterococci have been found among food animals in several countries in Europe (Aarestrup, Butaye, & Witte, 2002). Recently, Ronconi and collaborators (Ronconi, Merino, & Fernández, 2002) assessed the presence of highly aminoglycosides and glycopeptide-resistant enterococci in uncooked foods, and more particularly, in lettuce. The most frequently detected species was *E. faecium* (32.61%), followed by *E. faecalis* (21.74%), *E. gallinarum* (13.04%), *E. casseliflavus* and *E. mundtii* (7.60%), *E. hirae*, (6.52%), *E. durans* (4.35%), *E. raffinossus* and *E. saccharolyticus* (2.17%), and *E. avium* and *E. malodoratus* (1.10%) (Ronconi, Merino, & Fernández, 2002). Susceptibility profiles were found to be similar to those of strains occurring in hospitals (Ronconi, Merino, & Fernández, 2002).

High-level aminoglycoside resistance has been observed among enterococci isolated from food of animal origin (Aarestrup, Butaye, & Witte, 2002). In a US study, gentamicin resistance was found in 7 of 18 enterococci isolates from chicken meat (Thal, et al., 1995). Streptomycin resistance occurred at a similar rate (Thal, et al., 1995). Streptomycin resistance was found at a lower rate (1% of enterococcal isolates) from food in Germany; and has been detected among *E. faecalis* (10%) and *E. faecium* isolates from broilers (2-3%) in Denmark (Aarestrup F. M., 2000). The use of chloramphenicol has been banned in food animal production for more than 30 years, and is largely restricted in human medicine to limited use for systemic treatment or for the treatment of eye infections. As a consequence, a low frequency (1-7%) of chloramphenical resistance, encoded by the gene cat_{pIP501}, has been observed among *E. faecium* and *E. faecalis* isolates from broilers (Aarestrup F. M., 2000). However, Pavia *et al.* (Pavia, Nobile, Salpietro, & Angelillo, 2000) found more than 40% of enterococci from raw meat in Italy to be resistant to chloramphenicol, whereas Klein *et al.* (Klein, Pack, & Reuter, 1998) found 1% of vancomycin susceptible and 29% of vancomycin-resistant enterococci from raw meat in Germany to also be chloramphenicol-resistant.

In the environment. Sewage is an important reservoir of resistant enterococci. As early as the 1970s, tetracycline-resistant enterococci were isolated from sewage (van Embden, Engel, & van Klingeren, 1977). Glycopeptide resistance in *E. faecium* was first detected in 1986 in clinical isolates from hospitals in France (Leclercq, Derlot, Duval, & Courvalin, 1988) and the United Kingdom (Uttley, et al., 1989). The first indication of a reservoir outside hospitals was the detection of VRE in wastewater treatment plants of small towns in Germany that had no hospital (Klare, Heier, Claus, & Witte, 1993); and in sewage in the United Kingdom (Bates, Jordens, & Selkon, 1993). High-level aminoglycoside resistance has been observed worldwide among enterococci isolated in the environment (Aarestrup, Butaye, & Witte, 2002). More specifically, high-level resistance to kanamycin has been observed among 34% of 248 enterococcal isolates from sewage and water in the US (Rice, Messer, Johnson, & Reasoner, 1995). Streptomycin resistance was found in 5% of sewage isolates in the US. Highly gentamicin-resistant enterococci have been isolated from sewage and water in the US (Rice, Messer, Johnson, & Reasoner, 1995) and Germany (Klare, Heier, Claus, & Witte, 1993) as well.

Conclusions

It is clear that the few *Enterococcus* strains and species regularly associated with human infection represent just the tip of the iceberg, as microbes of the genus *Enterococcus* are mainly ancient and highly evolved members of

GI tract consortia of various hosts. More information is sorely needed on the natural habitats of many of the species, including new species that have recently come to our attention, due to of their rare occurrence in clinical specimens. The extent to which enterococcal species naturally inhabit and proliferate in and on plants, soil, and water (as opposed to contaminating them through animal excretion) remains controversial. Genes mirror the requirements of life. As our understanding of enterococcal genomics grows, bacterial genomics will become an important tool for providing new insights into the nature, biology, and habitats of the enterococci.

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Enterococcal Disease, Epidemiology, and Implications for Treatment

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Introduction

During the past few decades, enterococci have emerged as important healthcare-associated pathogens (Arias & Murray, 2012; Austin, Bonten, Weinstein, Slaughter, & Anderson, 1999; Boyce, et al., 1994; Benenson, et al., 2009; Goossens, 1998; Handwerger, et al., 1993). The continuing progress of modern medical care toward more intensive and invasive medical therapies for human disease has undoubtedly contributed to the increased prevalence of these remarkable opportunistic pathogens. This trend has also been attributed to the increasing antibiotic resistance among clinical isolates of enterococci. The rapid spread of enterococci with resistance to vancomycin (VRE) has been of particular concern. Many healthcare-associated strains that are resistant to vancomycin also show resistance to penicillin, as well as high-level resistance (HLR) to aminoglycosides. Finally, as has historically been the case with enterococci, resistance is emerging to newer agents used to treat VRE infections, such as linezolid, quinupristin/dalfopristin, and daptomycin (Chow, Donahedian, & Zervos, 1997; Herrero, Issa, & Patel, 2002; Sabol, Patterson, Lewis II, Aaron, Cadena, & Jorgensen, 2005).

Over the past two decades, *Enterococcus faecium* has emerged as a leading cause of multidrug-resistant enterococcal infection in the United States (Hidron, et al., 2008). *E. faecium* is intrinsically more antibiotic-resistant than *E. faecalis,* with more than half of its pathogenic isolates expressing resistance to vancomycin, ampicillin, and high-levels of aminoglycosides. Treating infections caused by this species can be difficult, and the magnitude of the problem is vast. Approximately 40% of medical intensive care units in a recent National Healthcare Safety Network report found that the majority of device-associated infections (namely, infections due to central lines, urinary drainage catheters, and ventilators) were due to vancomycin- and ampicillin-resistant *E. faecium* (80% and 90.4%, respectively) (Hidron, et al., 2008). Although they were often resistant to high-level aminoglycosides and some macrolides, healthcare-associated infections in these units due to *E. faecalis* remained largely susceptible to vancomycin and ampicillin (93.1% and 96.2%, respectively) for reasons that are not entirely known. Other enterococcal species are rarer causes of human infection, including *E. durans, E. avium, E. casseliflavus, E. hirae, E. gallinarum, E. raffinosus*, and *E. muntdii* (Gordon, et al., 1992).

In the following sections, the common human infections caused by enterococci are briefly described. The epidemiology of antibiotic-resistant enterococci in healthcare settings is summarized, including the role of colonization pressure and host factors on the emergence of VRE in clinical settings. Finally, the current challenges facing clinicians who treat antibiotic-resistant enterococcal infections are reviewed.

Enterococcal Disease

Enterococci can cause a variety of infections. For some of these, other microorganisms are also frequently isolated from the same site. In those situations, it is often not clear whether the manifestations of infection are the result of enterococci, or whether these relatively avirulent and opportunistic organisms are merely bystanders or are playing a minor role in the infection. However, for other types of infections, most notably

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endocarditis and bacteremia, enterococci can clearly cause serious and often life-threatening disease, and specific therapies are associated with improved outcomes (Hoge, Adams, Buchanan, & Sears, 1991).

Urinary Tract Infections

The most common type of enterococcal infection occurs in the urinary tract. Lower urinary tract infections (such as cystitis, prostatitis, and epididymitis) are frequently seen in older men. However, enterococci are exceedingly uncommon as a cause of uncomplicated cystitis in young women. Upper urinary tract infections that can lead to bacteremia occur, not unexpectedly, most often in older men (Graninger & Ragette, 1992). Enterococcal urinary tract infections are more likely to be acquired in hospital or long-term care settings, and thus, are more likely to be resistant to many antibiotics. In the ICU setting, enterococci cause almost 15% of healthcare-associated urinary tract infections. Not unexpectedly, VRE have become major healthcare-associated urinary tract pathogens among ICU patients (Hidron, et al., 2008).

Intra-Abdominal, Pelvic, and Soft Tissue Infections

Enterococci are often recovered from cultures of intra-abdominal, pelvic, and soft tissue infections. They are almost always isolated as only one component of mixed microbial flora and rarely cause monomicrobial infection at these sites. The importance of enterococci in wounds and abscesses has been debated at length. However, with enterococcal bacteremia commonly associated with intra-abdominal and pelvic abscesses and wounds (Graninger & Ragette, 1992; Maki & Agger, 1988; Noskin, Peterson, & Warren, 1995; Patterson, et al., 1995), most clinicians routinely use antibiotic regimens that treat enterococci when confronted with infections at these sites. Drainage of abscesses and debridement of wounds are often essential adjuncts to antibiotic therapy for these infections.

Peritonitis, an infection of the abdominal lining, should be considered separately from intra-abdominal or pelvic mixed aerobic-anaerobic infections. This infection occurs most often in conjunction with liver cirrhosis or in patients who receive chronic peritoneal dialysis. Enterococci can cause monomicrobial infection in these situations—although they occur far less commonly than *Escherichia coli* for spontaneous bacterial peritonitis or coagulase-negative staphylococci, and *Staphylococcus aureus* for dialysis-associated peritonitis. Finally, enterococci are frequently found in cultures from decubiti and foot ulcers, as well as in association with osteomyelitis in diabetics, but their role in infections at these sites is not clearly defined.

Bacteremia

Bacteremia and endocarditis are the more common manifestations of infections due to enterococci. Enterococci are currently the second leading cause of healthcare-associated bacteremia (Hidron, et al., 2008), an increase from the sixth most common cause in the 1980s. In the last few years, the source of a bacteremia is usually the genitourinary tract, although a bacteremia also often arises from intra-abdominal or biliary sources, indwelling central lines, or soft tissue infections. Enterococci are found as a component of polymicrobial bacteremia more often than other organisms (Maki & Agger, 1988; Patterson, et al., 1995).

Enterococcal bacteremias, in contrast to bacteremias with *S. aureus,* rarely seed distant organs or cause metastatic abscesses. The major issue when dealing with enterococcal bacteremia is the presence of endocarditis. The treatment of endocarditis can be more problematic than the treatment of bacteremia due to a noncardiac source. However, even when a specific source is found, the overall mortality rate from enterococcal bacteremia is between 26% and 46% (Maki & Agger, 1988; Malone, Wagner, Myers, & Watanakunakorn, 1986; Patterson, et al., 1995; Shlaes, Bouvet, Devine, Shlaes, al-Obeid, & Williamson, 1989). A large retrospective review of bloodstream infections reported enterococci as the only Gram-positive pathogen associated with a high risk of death (Weinstein, Murphy, Reller, & Lichtenstein, 1983). In some studies, *E. faecium* bacteremia is associated with a higher mortality rate than *E. faecalis* (Noskin, Peterson, & Warren, 1995), and patients with rapidly fatal

underlying diseases can have mortality rates as high as 75%. These high rates likely reflect patients who are at risk for developing enterococcal bacteremia—older adults with multiple underlying diseases, which may include diabetes mellitus, malignancy, heart disease, transplantation, and prior surgery.

Endocarditis

Endocarditis is one of the most serious enterococcal infections. Because of the enterococci's intrinsic resistance to the bactericidal activity of most antibiotics, treatment is difficult, even when relatively susceptible enterococci are involved. Two drugs that exhibit synergistic killing are required for effective therapy. In the situations of VRE or high-level aminoglycoside-resistant enterococcal endocarditis, antibiotic treatment often fails, and surgery to remove the infected valve is essential.

Overall, enterococci cause between 5 to 15% of cases of infectious endocarditis, and this rate has not changed substantially over several decades (Murdoch, et al., 2009). *E. faecalis* remains the more common cause of enterococcal endocarditis than E. *faecium*. These heart valve infections typically occur in older persons (Anderson, Murdoch, Sexton, & Reller, 2004; McDonald, et al., 2005; Wilson, Wikowske, Wright, Sande, & Geraci, 1984). The initial source of bacteremia leading to endocarditis is usually the genitourinary or gastrointestinal (GI) tract. Left-sided involvement is much more common than right-sided involvement. Prosthetic valve enterococcal endocarditis has been increasingly noted, which is perhaps related to the increasing use of these prostheses in older adults who are at an inherently higher risk for enterococcal bacteremia (Anderson, Murdoch, Sexton, & Reller, 2004; Rice, Calderwood, Eliopoulos, Farber, & Karchmer, 1991). In one retrospective analysis of a large endocarditis database (Anderson, Murdoch, Sexton, & Reller, 2004), an equal number of women and men had enterococcal endocarditis, although enterococcal endocarditis is typically reported more often in men than women (McDonald, et al., 2005). Unlike a previous small study (Murdoch, et al., 2009), recent large-case series of enterococcal endocarditis report that between 15% and 39% are healthcare-associated (Anderson, Murdoch, Sexton, & Reller, 2004; McDonald, et al., 2005). The clinical picture of enterococcal endocarditis is usually one of subacute infection characterized by heart failure, rather than embolic events (McDonald, et al., 2005); however, rapidly progressive disease can also occur. Enterococcal endocarditis has a lower mortality rate than other forms of infective endocarditis (odds ratio = 0.49 with 95% confidence interval of 0.24–0.97) (McDonald, et al., 2005), although death rates are still significant at 9% to 15% (McDonald, et al., 2005; Rice, Calderwood, Eliopoulos, Farber, & Karchmer, 1991; Wilson, Wikowske, Wright, Sande, & Geraci, 1984). The most problematic current issue in the management of enterococcal endocarditis is the selection of effective therapy for multidrug-resistant isolates (Stevens & Edmond, 2005).

Uncommon Infections

Other infections less commonly or rarely seen due to enterococci include meningitis, hematogenous osteomyelitis, septic arthritis, and pneumonia. The latter is quite rare, even in association with ventilators, and has only been documented in severely debilitated or immunocompromised patients who receive broad-spectrum antibiotics. There is no evidence that antibiotic-resistant isolates of enterococci, such as VRE, are more or less likely to cause these infections than antibiotic-susceptible isolates of enterococci.

Epidemiology

A large number of studies on enterococcal ecology and epidemiology have been conducted over the past two decades, especially in clinical settings (Arias & Murray, 2012). Non-healthcare–associated investigations show that enterococci are commonplace colonizers over wide swaths of the planet (Byappanahalli, Nevers, Korajkic, Staley, & Harwood, 2012). In addition to being well-recognized colonizers of the GI tract of most animals and insects, these hardy bacteria are routinely recovered from beach sands, freshwater and marine water sediments, soil, and aquatic and terrestrial vegetation. (For more information, see Enterococcus Diversity, Origins in Nature, and Gut Colonization.) Many studies correlate increasing concentrations of environmental enterococci

with GI and dermatological illnesses. As a result, the Environmental Protection Agency recommends enterococci as indicator bacteria for fecal contamination for brackish and marine waters. (For more information, see Enterococci as indicators of environmental fecal contamination.) It must be remembered, however, that enterococci also naturally fill ecological niches, independent of contamination from outside sources. The development of molecular identification and typing methods allows for the facile detection and tracking of enterococci at the strain level. Despite this progress, it remains urgent to more thoroughly define ecological reservoirs, understand host and bacterial traits that promote colonization, and clarify mechanisms for transmission that enhance the spread of multi-drug resistant enterococci.

Enterococcal Reservoirs and Colonization Resistance

Enterococci are normal flora in the GI tract of humans, along with most other animals and insects. *E. faecalis* and *E. faecium* each commonly colonize humans with quantitative stool cultures indicating *E. faecalis* with a higher colonization density than *E. faecium* (Chenoweth & Schaberg, 1990; Noble, 1978; Winters, Schlinke, Joyce, Glore, & Huycke, 1998). The density of enterococci in the colon average 10^7 colony-forming units μg^{-1} (Chenoweth & Schaberg, 1990), although enterococci are found throughout the GI tract and in the oral cavity at lower concentrations. Enterococci are also normal inhabitants of the genital tract, with *E. faecalis* as the predominant species.

The emergence of VRE as leading causes of hospital infection has led to studies that better define characteristics of colonization with this organism. GI colonization, once established, may persist for months to years (Bonten, Hayden, Nathan, Rice, & Weinstein, 1998; Lai, Fontecchio, Kelley, Melvin, & Baker, 1997; Montecalvo, et al., 1995; Noskin, Cooper, & Peterson, 1995; Roghmann, Qaiyumi, Johnson, & Morris, Jr., 1997). Patients with VRE in the GI tract often have the same organism colonizing their skin (Beezhold, et al., 1997). The quantity of VRE increases in healthy volunteers who were given oral glycopeptides (Van Der Auwera, Pensart, Korten, Murray, & Leclerq, 1996). Subsequent studies in both experimental animals and colonized patients have shown that the quantity of VRE found in stool increases several logs when antibiotics with activity against GI anaerobes are administered (Donskey, et al., 2000; Donskey, Hanrahan, Hutton, & Rice, 1999; Ubeda, et al., 2010).

Colonization resistance describes the active exclusion of exogenous pathogens like multi-drug resistant enterococci from the intestine (Vollaard & Clasener, 1994). This trait is primarily provided by "limiting actions" of the normal microbiota, although these mechanisms remain ill-defined. This phenomenon is believed to be predominantly conferred by the anaerobic intestinal microbiota (for humans, this includes *Clostridium* cluster *XIVa*, *Clostridium* cluster *IV*, and *Bacteroides* spp.) (Eckburg, et al., 2005). In the small intestine, one mechanism for colonization resistance arises, in part, by the induction of defensins, cryptdins, and lectins by Paneth cells. In turn, these antimicrobial peptides serve to restrict potentially pathogenic exogenous microorganisms (Cash, Whitham, Behrendt, & Hooper, 2006). An example in mice involves RegIIIγ, a lectin with activity against Gram-positive bacteria that is produced by Paneth cells via the stimulation of toll-like receptors and confers resistance to VRE colonization (Brandl, et al., 2008). Finally, an intact epithelial barrier, coupled with physiological functions that include salivation, immunoglobulin A, peristalsis, and gastric acidity, also contribute to colonization resistance. Breakdown in these ordinary activities, especially when coupled with the administration of broad-spectrum antibiotics, increases the risk for colonization and transmission of antibiotic-resistant enterococci, and thereby promotes infection by these opportunists (Donskey C. J., 2004).

Sources of Infection

In previous years, the source of enterococcal infection for most patients was thought to be their own endogenous flora. However, the marked rise in healthcare-associated enterococcal infections in the 1980s and 1990s led to studies that clearly demonstrated the transmission of pathogenic enterococci among patients in hospital settings (Boyce, et al., 1994; Huycke, Spiegel, & Gilmore, 1991). The primary mode of spread from patient-to-patient occurs through the hands of healthcare workers (Hayden, 2000). Transient carriage of enterococci on the hands

of healthcare workers has been documented in several studies (Antony, Ladner, Stratton, Raudales, & Dummer, 1997; Noble, 1978; Patterson, et al., 1995), although not in all studies (Climo, et al., 2009; Moreno, et al., 1995). Enterococci can persist for as long as 60 minutes after inoculation onto hands (Noskin, Stosor, Cooper, & Peterson, 1995), and as long as 4 months on inanimate surfaces, where they can serve as a reservoir for ongoing transmission in the absence of regular decontamination (Kramer, Schwebke, & Kampf, 2006).

Transmission of enterococci from a healthcare worker's hands to a patient may involve direct inoculation onto intravenous or urinary catheters. A more likely mechanism, however, is that healthcare-associated strains colonize the GI tract of patients with reduced colonization resistance (Donskey C. J., 2004; Vollaard & Clasener, 1994), and then increase in numbers. In this fashion, new strains become part of the patient's endogenous flora, which then serves as a springboard for infection. Acquired enterococcal strains carrying genes that encode antibiotic resistance can persist in the GI tract via selective pressure from broad-spectrum antibiotics frequently used in hospitalized patients (Donskey, et al., 2000; Ubeda, et al., 2010).

Transmission of enterococcal strains has been documented within medical units (D'Agata, Green, Schulman, Li, Tang, & Schaffner, 2001; Handwerger, et al., 1993; Karanfil, et al., 1992), between hospitals (Donskey, et al., 1999; Moreno, et al., 1995), and even from state to state (Chow, Kuritza, Shlaes, Green, Sahm, & Zervos, 1993). The spread of VRE has been noted between acute and long-term care settings and, although uncommon, into the community (Moreno, et al., 1995; Trick, et al., 1999). Frequent contact with healthcare providers and movement of colonized patients among different healthcare settings is undoubtedly responsible for these patterns of transmission.

Role of the Hospital Environment

The hospital environment appears to play an important role in the transmission of multidrug-resistant enterococci (Hota, 2004). The dramatic rise of VRE in the 1990s led to investigations that highlighted the role of the environment in healthcare-associated infections. However, environmental reservoirs for antibiotic-susceptible enterococci are not likely to be different from those for VRE.

Thermometers and thermometer handles appear to be common surfaces involved in the transmission of VRE (Livornese, Jr., et al., 1992; Porwancher, Sheth, Remphrey, Taylor, Hinkle, & Zervos, 1997). A high concordance between strains occurring in the hospital environment and those colonizing patients has been reported (Bonilla, et al., 1997). The healthcare environment is readily contaminated with VRE, with the highest densities found on medical devices (such as blood pressure cuffs, intravenous fluid pumps, or stethoscopes), gowns, bed rails, bedside tables, bed linens, urinals, and bedpans (Bonilla, et al., 1997; Bonten, Hayden, Nathan, Rice, & Weinstein, 1998; Hota, 2004). Not surprisingly, increased environmental contamination has been noted when colonized patients have diarrhea, and there is an increased density of VRE in stool following anti-anaerobic antibiotic use (Donskey, et al., 2000; Roghmann, Qaiyumi, Johnson, & Morris, Jr., 1997; Ubeda, et al., 2010). Several studies have emphasized the tenacity with which enterococci remain viable on environmental surfaces (Hota, 2004), and its subsequent transmission to the hands of healthcare workers. Finally, in one controlled prospective study, environmental contamination with VRE was shown to be highly predictive of VRE acquisition (Drees, et al., 2008).

Host Factors for Antibiotic-Resistant Enterococcal Colonization

Many investigators have defined specific risk factors for GI colonization with antibiotic-resistant enterococci. In the acute care setting, colonization with aminoglycoside-resistant enterococci was shown to be associated with intravenous catheters, bladder catheters, prior surgical procedures, and prior antibiotic therapy (Zervos, Terpenning, Schaberg, Therasse, Medendorp, & Kaufmman, 1987). Additional studies have defined risk factors for colonization with VRE, and have consistently shown that prior antibiotic therapy with vancomycin, third-

generation cephalosporins, and/or agents with anti-anaerobic activity are important to this process (Donskey, et al., 2000).

Other risk factors for VRE colonization include the patient's length of stay in an ICU or hospital (Tornieporth, Roberts, John, Hafner, & Riley, 1996), exposure to other patients with VRE either by close proximity to a VRE-colonized patient, or by care from a nurse providing who is care to another VRE-colonized patient (Austin, Bonten, Weinstein, Slaughter, & Anderson, 1999; Boyce, et al., 1994). Drees et al. (Drees, et al., 2008) showed that "colonization pressure," defined as the percentage of patients in a unit who are colonized with VRE, increased the hazard ratio for acquisition by 1.4 per 10% increase in colonization. When VRE colonization rates exceed 50%, this becomes the dominant risk factor for spread of VRE within a unit (Bonten, et al., 1998).

Certain patient populations, notably those on chronic hemodialysis (D'Agata, Green, Schulman, Li, Tang, & Schaffner, 2001), with hematological malignancies (Ubeda, et al., 2010), or undergoing liver transplantation (Orloff, et al., 1999), are at increased risk for the acquisition of VRE. Many of these patients are cared for in specialized units, and acquisition of GI colonization can be traced back to care within these units and other factors, as noted above. Finally, increasing exposure to patients with VRE has been associated with healthcare workers also being colonized by VRE (Baran, Jr., Ramanathan, Riederer, & Khatib, 2002).

Host Factors Related to Antibiotic-Resistant Enterococcal Infection

The vast majority of VRE-colonized patients do not develop symptomatic infections. The ratio of colonization to infection with VRE is estimated to be approximately 10:1 (Hayden, 2000; Slaughter, et al., 1996). Although unproven, a similar ratio likely exists for enterococcal infections caused by healthcare-associated strains that have a high-level resistance to ampicillin or aminoglycosides (Huycke, Spiegel, & Gilmore, 1991; Willems, et al., 2005). The risk for VRE infection, however, increases among certain patient groups. Patients with neutropenia and those undergoing transplantation are at a particularly increased risk for VRE bacteremia (Lautenbach, Bilker, & Brennan, 1999; Orloff, et al., 1999). In neutropenic patients, the severity of mucositis (Kuehnert, Jermigan, Pullen, Rimland, & Jarvis, 1999) and concomitant infection with *Clostridium difficile* (Roghmann, McCarter, Jr., Brewrink, Cross, & Morris, Jr., 1997) have both been independently associated with an increased risk for VRE bacteremia.

In ICU populations and for those who are immunosuppressed, infection with VRE has been associated with vancomycin, third-generation cephalosporins, and/or antibiotics with activity against anaerobes (Donskey, et al., 2000; Handwerger, et al., 1993; Hayden, 2000; Lautenbach, Bilker, & Brennan, 1999; Montecalvo, et al., 1994; Roghmann, McCarter, Jr., Brewrink, Cross, & Morris, Jr., 1997), increased length of hospital stay (Handwerger, et al., 1993), and the severity of underlying illness (Shay, et al., 1995). A number of risk factors for infection with VRE have also been reported as risk factors for bacteremia with high-level aminoglycoside-resistant enterococci, including chronic renal failure, ICU stay, prior antibiotic use (including cephalosporins), bladder catheterization, expression of cytolysin as an enterococcal virulence determinant, and prolonged hospitalization (Caballero-Granado, et al., 1998; Huycke, Spiegel, & Gilmore, 1991; Noskin, Till, Patterson, Clarke, & Warren, 1991).

Infection Control

The majority of healthcare-acquired infections are caused by microorganisms that are resistant to at least one of the antibiotics most commonly used to treat these infections. This is especially true for infections due to VRE, where treatment options are particularly limited (see below and Enterococcal infection). Therefore, measures that minimize the spread of these resistant organisms are essential. Each healthcare facility needs a comprehensive infection control program that can decrease the transmission of VRE among patients. Specific policies should be based on the rates of resistance within the facility, and should be appropriate for the specific

healthcare setting. For example, specific control measures within an acute care hospital setting may differ somewhat from those applicable to a long-term care setting.

The consensus opinion of experts highlight four interventions as being most important for controlling the spread of VRE in healthcare settings: i) active periodic surveillance cultures (or molecular testing) of patients at highest risk for carriage; ii) decontaminating the hands of healthcare workers using an antiseptic-containing preparation before and after all patient contact; iii) adherence to barrier precautions (*i.e.*, gloves and gowns) and cohorting colonized and/or infected patients; and iv) thorough terminal cleaning for rooms occupied by patient with VRE (Cookson, et al., 2006; Muto, et al., 2003). Although evidence for other control strategies for VRE—antibiotic stewardship to limit inappropriate or excessive antibiotic use, decolonizing patients and/or healthcare workers, and educational initiatives—are potentially useful in selected circumstances, these methods currently find less compelling support in the present literature.

Infection Control Measures

Specific infection control considerations should be based on the type of healthcare facility, the prevalence of VRE in that facility, and the patients' risk for infection. Not unexpectedly, acute care settings warrant strict adherence to isolation precautions, more so than outpatient or long-term care settings. The presence of serious infections in many patients may require additional investigation, including molecular typing of VRE strains, in order to fully understand and break the modes of transmission.

In acute care settings, barrier precautions are the cornerstone of infection control for VRE (Cookson, et al., 2006; Muto, et al., 2003). Assiduous hand antisepsis and use of gloves are the most important features of these precautions. This point is emphasized in studies where VRE has been shown to be transferred from contaminated hands to clean sites on patients or environmental surfaces at an average rate of 10% (Duckro, Blom, Lyle, Weinstein, & Hayden, 2005). Gloves decrease the contamination of hands of healthcare workers by VRE, although contamination is still possible as gloves are removed (Tenorio, et al., 2001). Therefore, hand antisepsis after glove removal is mandatory. When hands are not visibly contaminated with blood, body fluids, or body substances, an alcohol hand rub containing an emollient should be encouraged. Hand washing with soap and water is required when hands are visibly dirty or contaminated with blood, body fluids, or body substances. Monitoring hand hygiene compliance, with appropriate feedback given to healthcare workers, is essential, and is required by several accreditation agencies. Clean single-use gowns should be worn by healthcare workers when entering the rooms of patients with VRE. Medical devices that are required for routine patient care (such as blood pressure cuffs, thermometers, stethoscopes, etc.) should remain in isolation rooms and not be shared among patients. Non-dedicated equipment should be disinfected between uses.

Environmental contamination by VRE is common, can vary in different units, and plays a substantial role in transmission (Hayden, 2000; Muto, et al., 2003). The common occurrence of environmental contamination with VRE has led to recommendations that environmental cleaning be performed with standard disinfecting agents on a daily basis, as well as ensuring that high-touch items such as bedside rails, tables, toilets, and handles are cleaned. Although the efficacy of environmental hygiene on colonization or infection with VRE is unclear, one investigation of a medical intensive care unit with a high prevalence of VRE observed a significant decrease in VRE transmission after the implementation of enhanced environmental cleaning (Hayden, Bonten, Blom, Lyle, van de Vijver, & Weinstein, 2006). Should the skin of patients be considered part of the healthcare environment, interventions that involve daily chlorhexidine bathing have been shown to reduce VRE acquisition by 50%, and decrease the relative risk for VRE bacteremia by three-fold (Climo, et al., 2009). Cohorting colonized or infected patients is an additional targeted intervention of value when single rooms are not available, during outbreaks, or when colonization is hyperendemic within medical units. The efficacy of these control measures has been demonstrated in numerous VRE outbreaks, where the implementation of multifaceted programs has led to successful control (Cookson, et al., 2006; Henard, Lozniewski, Aissa, Jouzeau, & Rabaud, 2011; Lin & Hayden, 2010; Muto, et al., 2003).

Surveillance for VRE

Active surveillance of asymptomatic patients for VRE colonization is a mainstay of targeted control efforts (Muto, et al., 2003). Targeted interventions can help decrease VRE transmission in settings where colonization or infection with VRE is unstable, epidemic, or hyperendemic (Lin & Hayden, 2010). The goal is to identify every colonized patient, so that all colonized patients remain in contact isolation to minimize the spread of VRE to other patients. Surveillance cultures are indicated at the time of hospital admission for patients at high risk for the carriage of VRE. Periodic (*e.g.*, weekly) surveillance cultures are indicated for patients at high risk for VRE because of ward location, antibiotic therapy, underlying disease, and/or the duration of their stay. In facilities with a high prevalence of VRE on initial sampling, a facility-wide culture survey can identify all colonized patients and allow for the implementation of contact precautions.

Colonization with VRE is typically prolonged (Byers, Anglim, Anneski, & Farr, 2002). In hospital settings, removing a patient from contact precautions involves showing that patients are no longer colonized with VRE. The Hospital Infection Control Practices Advisory Committee defines clearance of colonization with VRE as three consecutive negative rectal swabs at least one week apart (Hospital Infection Control Practices Advisory Committee (HICPAC), 1995). However, colonization with VRE can persist despite three consecutive negative weekly surveillance stool cultures (Huckabee, Huskins, & Murray, 2009). Others have proposed defining VRE clearance as a negative rectal swab obtained two to seven days after cessation of a treatment regimen with drugs known to be selective for VRE (such as third-generation cephalosporins, fluoroquinolones, carbapenems, imidazoles, or glycopeptides) implemented for at least five days (Henard, Lozniewski, Aissa, Jouzeau, & Rabaud, 2011). The issue remains unsettled.

Antimicrobial Stewardship

Appropriate use of antibiotics is not only good practice, but is important for controlling the spread of healthcare-associated VRE. The increase in vancomycin resistance among healthcare-associated *E. faecium* isolates in the United States is partially linked to a tremendous increase in vancomycin use during the 1980s and 1990s (Hayden, 2000). The 2003 Society for Healthcare Epidemiology of America published guidelines that stress the avoidance of inappropriate or excessive antibiotic prophylaxis and therapy as a means to control VRE (Muto, et al., 2003). In addition, it was recommended that the correct antibiotic dose and appropriate duration of therapy be used. Vancomycin use should be limited, when possible, to decrease selective pressures that favor vancomycin resistance. An obvious circumstance in which vancomycin restriction should be aggressively pursued is in the isolation of vancomycin-dependent enterococci (Kirkpatrick, et al., 1999). To prevent the establishment of VRE intestinal colonization, considerations should be made to decrease the use of antibiotics with little or no activity against enterococci, such as third-generation and fourth-generation cephalosporins. Finally, when clinically feasible, agents with anti-anaerobic activity should be limited in patients who are colonized with VRE, to prevent persistent high-density colonization.

Education of Healthcare Workers

It is imperative to implement institutional efforts to educate healthcare workers who have direct patient-care responsibilities on infection control policies for the containment of VRE and other multi-drug resistant microorganisms. These efforts must be frequently repeated and reinforced because new workers are constantly being hired, and adherence to the daily tasks required for isolation practices tends to fade over time. This requirement is most important on units or in facilities with high rates of VRE colonization and infection (Bonten, et al., 1998).

Role of the Clinical Microbiology Laboratory

The prompt and accurate identification of antibiotic-susceptible and antibiotic-resistant enterococci is essential to establishing diagnoses, selecting effective therapy, and instituting infection control measures. The clinical microbiology laboratory must employ techniques to identify enterococci to the species level and perform accurate susceptibility testing. In addition to routine testing, laboratories should evaluate all isolates from blood and sterile body sites for high-level streptomycin and gentamicin resistance, and isolates from all sites for vancomycin resistance (Cetinkaya, Falk, & Mayhall, 2000). Routine susceptibility testing for linezolid, daptomycin, and quinupristin/dalfopristin may be necessary at some facilities.

For VRE, the Clinical and Laboratory Standards Institute guidelines recommend standard broth macrodilution or disk diffusion methods for vancomycin-susceptibility testing (Clinical and Laboratory Standards Institute, 2013; Jenkins & Schuetz, 2012). Disk diffusion and E tests should be held for 24 h to obtain accurate readings. Isolates with intermediate zones on disk testing should be tested by an MIC method and further evaluated to the species level, so that non-*E. faecalis* and non-*E. faecium* isolates are identified, and this information should be used to guide infection control measures. Finally, the laboratory must notify the physician and nursing staff and/or infection control personnel when VRE isolates are found, so that appropriate isolation precautions can be promptly instituted.

Culture-based and/or molecular methods are used to perform active surveillance for VRE (Malhotra-Kumar, et al., 2008). Although culture-based methods are slower than molecular-based screening techniques, isolates from cultures have the advantage of being available for further study. However, the time to complete conventional cultures is two to three days, which allows for the potential spread of VRE prior to instituting barrier precautions. Several rapid diagnostic tests for VRE that decrease the time to detection have been approved and may help reduce the risk for transmission (Malhotra-Kumar, et al., 2008). Culture still remains the most commonly used method for screening stool for VRE, although new molecular screening methods are increasing in popularity.

Selective agars that identify VRE in stool samples include *Campylobacter* medium with vancomycin at 10 μg ml^{-1} and *Campylobacter* medium prepared in bile esculin azide agar with vancomycin at 6 μg ml^{-1} (Shigei, Tan, Shiao, de la Maza, & Peterson, 2002). Most VRE screening agars require 24 to 48h of incubation prior to the preliminary identification of colonies, and confirmatory identification and susceptibility testing can take up to five additional days. Chromogenic media for the direct detection of VRE (such as CHROM-agar, chromID, and Spectra VRE media) can reduce turnaround times through early visual identification of colonies (Jenkins, Raskoshina, & Schuetz, 2011; Peltroche-Llacsahuanga, Top, Weber-Heynemann, Lütticken, & Haase, 2009). However, properly assigning differential colony color can be difficult at times, and may require additional biochemical testing. These media all have adequate sensitivity and specificity for VRE screening, although performance generally improves when overnight broth enrichment in liquid media is used prior to plating.

PCR is a sensitive and rapid molecular approach for identifying VRE isolates. Although collecting stool as specimens for these assays is convenient, stool can contain PCR inhibitors that interfere with test results. Therefore, perirectal or perianal swabs are often recommended. Recently, the BD GeneOhm VanR (BD Diagnostics, Spark, MD) and Xpert *vanA/vanB* (Cepheid, Sunnydale, CA) assays were approved for the detection of isolates containing *vanA* and *vanB* genes. These tests can provide results in two to four hours. Any increase in diagnostic speed, however, comes at a greater financial cost than that of culture methods.

Eradication of Colonization

The overall elimination of GI tract colonization with VRE is an attractive prospect for decreasing the spread of these pathogens and lessening the incidence of infection among at-risk patients. Attempts to eliminate VRE from the GI tract, however, have proven to be ineffective with a variety of oral antimicrobials, including

bacitracin, gentamicin, tetracycline, novobiocin, rifampicin, and ramoplanin (Kauffman, 2003). In addition, decolonization regimens have not always been well tolerated. Although some patients have been successfully decolonized, the duration of decolonization has typically been transitory, with VRE often reappearing within several days or weeks. Recolonization most often occurs in patients who are also receiving anti-anaerobic antibiotics (Baden, et al., 2002). Clearly, novel approaches will be needed to achieve the goal of long-term VRE decolonization.

Hemodialysis Centers

Dialysis patients have high rates of VRE colonization (D'Agata, Green, Schulman, Li, Tang, & Schaffner, 2001; Roghmann, et al., 1998), and patients who have been hospitalized and those who have been treated with vancomycin are more likely to be colonized. Restricting the use of vancomycin is an important measure in a specific setting that could help decrease the selective pressure for growth of VRE. Earlier removal of vascular access lines, when feasible, helps decrease the incidence of infection of these catheters and lessen the need for prolonged courses of vancomycin. For dialysis patients who are VRE-colonized but continent, there is no need for additional infection control measures beyond the standard precautions.

Long-Term Care Facilities

The epidemiology of VRE in long-term care facilities differs from that in the acute care settings. Bonilla et al. (Bonilla, et al., 1997) observed VRE rectal colonization rates that varied from 9–22% during a 21-month period. However, transmission of VRE to roommates appeared to be uncommon, as did VRE infections, in this setting. Indeed, VRE infections were not noted until colonized patients were transferred back to an acute care facility for an underlying medical condition (Bonilla, et al., 1997).

Recommendations for infection control for VRE in the long-term care setting have been provided by the Long-Term Care Committee of the Society for Healthcare Epidemiology of America (Benenson, et al., 2009). These recommendations carefully consider the unique mission of long-term care facilities, which become homes for many residents. Because long-term care residents who are colonized with one resistant organism are often colonized with other resistant organisms (Terpenning, Bradley, Wan, Chenoweth, Jorgensen, & Kauffman, 1994), and because strict contact precautions are often impractical in these settings, recommendations for colonized residents with any antibiotic-resistant organism simply consist of standard precautions. Specific recommendations include:

i. A private room for colonized patients, when possible, although it is acceptable to allow a patient colonized with VRE and continent of stool to share a room with another patient, as long as that patient is not severely immunocompromised or has open wounds.
ii. As long as VRE-colonized patients are continent of stool, they may leave their room and participate in group events within and outside the facility.
iii. The appropriate use of gloves and careful hand washing play a primary role in the prevention of VRE transmission to other residents.
iv. Surveillance cultures are not useful unless an outbreak occurs.
v. Knowledge of VRE status should be given when a resident is transferred, but VRE colonization should not preclude transfer to or from a long-term care facility or an acute care hospital.
vi. Suggestions regarding healthcare worker education about VRE and prudent use of vancomycin are the same as in an acute-care facility.

Outpatient Settings

Healthcare continues to shift toward the greater use of outpatient settings, which include surgical centers, infusion centers, dialysis units, and ambulatory care clinics. Patients colonized by VRE in acute care facilities can

become a reservoir for VRE in outpatient settings. However, isolation precautions similar to those carried out in hospitals are neither possible nor practical in most of these settings, and no current data show that they would have an impact on the spread of VRE. This is not to understate risks for VRE infection that undoubtedly exist in outpatient clinics, as posed by the devices, protocols, and therapies used in these settings (Maki & Crnich, 2005). At a minimum, some experts (Herwaldt, Smith, & Carter, 1998) recommend an alert to healthcare workers when VRE-positive patients are scheduled for clinic visits, so that VRE precautions can be instituted where appropriate. Such a strategy is perhaps best justified in outpatient clinics for high-risk patients, such as stem cell transplant recipients, but would be impractical in many outpatient settings.

Home Care

Transmission of VRE to caregivers within a home setting has rarely been reported (McDonald, Kuehnert, Tenover, & Jarvis, 1997). Although VRE colonization of the GI tract has been reported for healthcare workers and healthy adults in the United States and Europe (D'Agata, Jirjis, Gouldin, & Tang, 2001; Goossens, 1998), transmission to healthy caregivers with normal colonization resistance should be low, with colonization posing virtually no risk for VRE infection. Standard precautions (namely, consistent hand hygiene and use of gloves for potential exposure to bodily fluids) should be sufficient.

Treatment

The treatment of enterococcal infections can be difficult. *Enterococcus* species are intrinsically resistant to many antimicrobial agents, including cephalosporins, clindamycin, semisynthetic penicillinase-stable penicillins, and aminoglycosides among others, and have the capacity to acquire resistance genes and mutations (see Enterococcal infection) (Arias & Murray, 2012). In addition, compounds that inhibit the cell wall synthesis—and are considered bactericidal against other Gram-positive cocci—are usually only bacteriostatic against enterococci (Krogstad & Pargwette, 1980). This issue is important when treating life-threatening infections, such as endocarditis, that require bactericidal agents to effect a cure. For enterococci, this involves a combination of agents that can synergistically confer bactericidal activity. *In vitro* synergism is defined as a 100-fold or greater increase in killing at 24h by a combination of agents compared to either agent used alone (Arias & Murray, 2008).

Treatments of enterococcal infections vary, depending on several factors:

i. Is the causative organism susceptible to β-lactams, aminoglycosides, and glycopeptides, or is it resistant to various combinations of these antimicrobial classes?
ii. Is the infection monomicrobial or polymicrobial?
iii. Does the infection involve heart valves or other endovascular structures?

Antibiotic-Susceptible Nonendocarditis Enterococcal Infections

For susceptible isolates, ampicillin and penicillin remain the drugs of choice for enterococcal infections, other than endocarditis, in nonallergic patients. Monomicrobial enterococcal infections, such as urinary tract infections or non-endocarditis bacteremia, can be treated with penicillin or ampicillin alone. Skin and subcutaneous infections and intra-abdominal or pelvic infections rarely yield only enterococci upon culture. Treatment of these polymicrobial infections can be accomplished with a combination of ampicillin and other antibiotics that are effective against a wide range of anaerobic and aerobic Gram-negative bacilli and staphylococci. A simpler alternative in those situations is to use a single agent, such as ampicillin-clavulanic acid or piperacillin-tazobactam, that combines a β-lactamase inhibitor with a β-lactam agent. A glycopeptide, either vancomycin or teicoplanin, can be used as a single agent to treat simple enterococcal infections when the patient has a serious allergy to penicillins. Nitrofurantoin has activity against enterococci, but should only be used to treat lower-tract urinary infections. Although *in vitro* susceptibility studies often show susceptibility to

trimethoprim-sulfamethoxazole, this drug is not effective *in vivo* because enterococci circumvent the mechanism of drug inhibition by utilizing host folates (Zervos & Schaberg, 1985). Finally, quinolones are not particularly effective against enterococci and should not be used for serious infections (Zervos, Bacon 3rd, Patterson, Schaberg, & Kauffman, 1988).

Endocarditis Caused by *Enterococcus faecalis*

Most *E. faecalis* isolates remain susceptible to penicillin and aminopenicillins (Murray B. E., 1992). The combination of a cell wall-active agent and an aminoglycoside remains the standard of care (Baddour, et al., 2005; Habib, et al., 2009). Aminopenicillins are considered the β-lactams of choice as the concentrations required to inhibit enterococci are about half of those of penicillin (Murray B. E., 2000). It is important to note that in cases of serious infection, tests for β-lactamase production should be performed using a higher bacterial inoculum or a penicillinase-detection method (Clinical and Laboratory Standards Institute, 2013). An aminopenicillin combined with a β-lactamase inhibitor (*e.g.*, sulbactam) should be used if a β-lactamase–producing *E. faecalis* is encountered.

Of the available aminoglycosides, gentamicin is generally preferred over streptomycin, as the synergistic agent used with either an aminopenicillin or a glycopeptide. Gentamicin had been recommended because of its greater synergistic effect with cell-wall active agents (Harwick, Kalmanson, & Guze, 1973; Watanakunakorn & Bakie, 1973), although some have reported streptomycin as being more effective than gentamicin (Wilson, Wikowske, Wright, Sande, & Geraci, 1984). Compared to gentamicin, streptomycin is more difficult to obtain and serum concentrations for pharmacokinetic monitoring are not readily available. In penicillin-allergic patients, vancomycin or teicoplanin can be combined with an aminoglycoside. This combination should be reserved only for patients with serious allergies, and the duration of therapy should be 6 weeks (Baddour, et al., 2005).

The dosing of aminoglycosides is somewhat controversial (Falagas, Matthaiou, & Bliziotis, 2006; Graham & Gould, 2002), and until controlled clinical trials are conducted to address this issue, once-daily dosing should not be used in the treatment of enterococcal endocarditis (Baddour, et al., 2005). Gentamicin should be administered every 8 hours, with dosing adjusted to reach a peak serum level of approximately 3 μg ml^{-1} and a trough of <1 μg ml^{-1}. Streptomycin should be administered every 12 hours, with a target peak of 20 to 35 μg ml^{-1} and a trough <10 μg ml^{-1} (Baddour, et al., 2005). The duration of therapy for native valve endocarditis is at least 4 weeks, with 6 weeks favored for those with symptoms for greater than 3 months, or for those with relapse or mitral valve involvement (Wilson, Wikowske, Wright, Sande, & Geraci, 1984). Prosthetic valve endocarditis should be treated for 6 weeks (Rice, Calderwood, Eliopoulos, Farber, & Karchmer, 1991). The prolonged duration of therapy with aminoglycosides for enterococcal endocarditis comes with a significant drawback of increased toxicity in the older populations at risk for this infection. One study suggested a shorter course of aminoglycoside for patients who might be limited by toxicity (Olaison & Schadewitz, 2002).

High-Level Aminoglycoside-Resistant (HLR) Enterococcal Infections

For most simple enterococcal infections, the presence of HLR to aminoglycosides does not influence a treatment regimen, since β-lactam monotherapy is adequate and aminoglycosides are not indicated. For bacteremia, there is no benefit to adding an aminoglycoside. Outcomes are not significantly different for patients who are bacteremic, with enterococci exhibiting HLR to aminoglycosides compared to those with bacteremia with fully susceptible strains (Caballero-Granado, et al., 1998; Patterson, et al., 1995; Watanakunakorn & Patel, 1993).

The development in E. faecalis isolates of HLR to gentamicin (MIC ≥500 μg ml-1 on brain-heart agar) and to streptomycin (MIC ≥2000 μg ml-1 on brain-heart agar or ≥1000 μg ml-1 in brain-heart infusion), eliminates synergism of aminoglycosides with β-lactams, and hence a bactericidal regimen. It is noteworthy that HLR resistance to gentamicin precludes the use of all clinically useful aminoglycosides, except streptomycin (Chow, 2000). E. faecium strains express an aminoglycoside-modifying enzyme that eliminates synergism between cell-

wall inhibitors and aminoglycosides, including kanamycin, netilmycin, and tobramycin. Gentamicin, however, is not affected by this enzyme (Costa, Galimand, Leclercq, Duval, & Courvalin, 1993).

A bactericidal regimen for endocarditis caused by enterococci with HLR to both streptomycin and gentamicin has not yet been established, and as a result, treatment in this situation can be difficult (Chow, 2000). Continuous infusion, high-dose ampicillin monotherapy has been attempted based on animal experiments, but failures of this regimen have been reported (Landman & Quale, 1997). Although the optimal duration of therapy is unknown, given the risk of relapse, therapy beyond 6 weeks and early surgical intervention should both be considered (Eliopoulos, 1993).

In vitro and *in vivo* data shows synergism between amoxicillin or ampicillin and ceftriaxone against *E. faecalis* (Gavaldà, et al., 2007; Gavaldà, et al., 1999; Mainardi, Gutmann, Acar, & Goldstein, 1995). *In vivo* data indicate that for endocarditis due to *E. faecalis* without high-level aminoglycoside resistance, the combination of ampicillin and ceftriaxone is comparable in efficacy to that of ampicillin and gentamicin. The triple combination of ampicillin, ceftriaxone, and gentamicin is not superior to these regimens (Gavaldà, et al., 2003). A recent open-label trial showed that patients with endocarditis due to *E. faecalis* with HLR to aminoglycosides, treated with ampicillin and ceftriaxone, had similar mortality compared to historical controls (Gavaldà, et al., 2007). Of note, the observed synergism between β-lactams against *E. faecalis* does not apply to *E. faecium* (Mainardi, Gutmann, Acar, & Goldstein, 1995). Other therapeutic options for treating *E. faecalis* endocarditis due to strains with HLR to aminoglycosides remain anecdotal, and include combinations of imipenem, vancomycin, and ampicillin (Antony, Ladner, Stratton, Raudales, & Dummer, 1997); a fluoroquinolone and ampicillin (Tripodi, Locatelli, Adinolfi, Andreana, & Utili, 1998); and ciprofloxacin, ampicillin, and gentamicin (Sacher, Miller, Landau, Sacher, Dixon, & Dietrich, 1991).

Vancomycin-Resistant Enterococcal Infections

Infections due to enterococcal strains that express glycopeptide resistance pose a significant challenge, as therapeutic options are limited and somewhat empirical. Given the limitations of antimicrobial therapy, removal of infected foci, such as intravenous catheters, and drainage of abscesses remain important adjunctive measures.

For infections due to penicillin-susceptible VRE, ampicillin remains the drug of choice. Nitrofurantoin, fosfomycin, and doxycycline have intrinsic activity against enterococci, including VRE, and are potential oral options for treating simple VRE infections, such as cystitis (Heintz, Halilovic, & Christensen, 2010). Linezolid and daptomycin are reserved for serious VRE infections that are resistant to penicillins. Other antimicrobials, such as quinupristin/dalfopristin and tigecycline, should be evaluated on a case-by-case basis, due to toxicity concerns . Infections of the urinary tract, skin, or soft tissues due to VRE may respond to drugs such as doxycycline or fluoroquinolones, although susceptibility patterns vary (Landman & Quale, 1997). The use of fluoroquinolones as monotherapy for serious infections, although a possible option for uncomplicated urinary tract infection, is usually not recommended (Arias & Murray, 2008; Zervos, Bacon 3rd, Patterson, Schaberg, & Kauffman, 1988). Finally, trimethoprim-sulfamethoxazole should not be used to treat enterococcal infections, regardless of their susceptibility testing.

Endocarditis caused by VRE poses a great challenge, since there are no reliable bactericidal combinations of antibiotics available. Combinations of agents have been studied in animal models of VRE endocarditis, but results typically depend on the susceptibilities of the strains that are studied, and may not necessarily translate into effective therapy for human infections. In general, clinical experience in treating VRE endocarditis remains limited (Forrest, Arnold, Gammie, & Gilliam, 2011; Stevens & Edmond, 2005). A consultation with a cardiac surgeon for early valve replacement is highly recommended. Some of the varied antimicrobial approaches to the management of these infections are described below.

While most *E. faecalis* isolates expressing vancomycin resistance remain susceptible to ampicillin, the majority of *E. faecium* isolates are resistant to both. For enterococci, the Clinical and Laboratory Standards Institute defines ampicillin resistance as growth at <16 μg ml^{-1} (Clinical and Laboratory Standards Institute, 2013). Endocarditis due to VRE isolates with ampicillin MICs ≤64 μg ml^{-1}, however, have been successfully treated using higher-than-approved doses of ampicillin (*e.g.*, 18-30 gm day^{-1}), usually in combination with an aminoglycoside (Forrest, Arnold, Gammie, & Gilliam, 2011; Murray B. E., 2000). The toxicity of these doses remains unclear, and treatment failures do occur.

Daptomycin

Daptomycin is a bactericidal lipopeptide used to treat skin and soft tissue infections caused by susceptible Gram-positive bacteria, including vancomycin-susceptible *E. faecalis*. An additional indication is for the treatment of *S. aureus* bacteremia and right-sided endocarditis (Enoch, Bygott, Daly, & Karas, 2007). Although daptomycin is not FDA approved for infections caused by *E. faecium* or vancomycin-resistant *E. faecalis*, the bactericidal activity of this agent at doses of 8-10 mg kg^{-1} suggests it could be useful in multi-drug resistant enterococcal endocarditis (Arias, Torres, Singh, Panesso, Moore, & Murray, 2007; Dandekar, Tessier, Williams, Nightingale, & Nicolau, 2003). To date, available data are limited to case reports, which suggest that daptomycin can be effective at higher-than-approved doses of 6 mg kg^{-1} day^{-1} and in combination with other agents (Arias, Torres, Singh, Panesso, Moore, & Murray, 2007; Jenkins I. , 2007; Stevens & Edmond, 2005).

Non-susceptibility of enterococci to daptomycin (MIC >4 μg ml^{-1} by broth dilution, E-test, or zones of inhibition <11 mm by disk diffusion) remains infrequent (Sabol, Patterson, Lewis II, Aaron, Cadena, & Jorgensen, 2005), with an overall prevalence of 0.6% among clinical isolates in a recent series (Kelesidis, Humphries, Uslan, & Peques, 2011). Of these isolates, most were VRE (93.3%) and *E. faecium* (88%). All were from bloodstream infections, with 15% causing endocarditis. Daptomycin resistance can be selected for both *in vitro* and *in vivo* and arises from mutations in diverse genes with putative roles in the biogenesis, permeability, and potential of cell membranes (Arias, et al., 2011; Palmer, Daniel, Hardy, Silverman, & Gilmore, 2011). Limiting the development of resistance to daptomycin may be attempted by using higher than approved doses or combining this lipopeptide with other agents, as described above.

Linezolid

Linezolid is an oxazolidinone used to treat Gram-positive infections, including VRE bacteremia and urinary tract infection. The mechanism of action involves inhibiting the 30S ribosome initiation complex, which renders the drug bacteriostatic against enterococci. Because of this unique mechanism, no cross-resistance with other available agents has been described. Linezolid is active against both E. faecium and E. faecalis (Arias & Murray, 2008). A clinical advantage of linezolid involves an oral and formulation with oral bioavailability approaching 100%. However, myelosuppression, especially thrombocytopenia, is a serious complication that occurs on occasion after prolonged use (Green, Maddox, & Huttenbach, 2001).

Based on anecdotal case reports, and despite its bacteriostatic nature, linezolid has been recommended as a treatment option for VRE endocarditis (Baddour, et al., 2005). Experience using linezolid for VRE bacteremia shows microbiological cure rates of 85.3%, with clinical successes at 78% (Birmingham, Rayner, Meagher, Flavin, Batts, & Schentag, 2003). The efficacy of linezolid in treating endocarditis due to vancomycin-susceptible and vancomycin-resistant *E. faecalis* and *E. faecium,* showed 7 of 8 cases either responded to or were cured by this agent (Falagas, Manta, Ntizora, & Vardakas, 2006). However, treatment failures have also been reported (Tsigrelis, Singh, Coutinho, Murray, & Baddour, 2006). Enterococcal resistance to linezolid remains uncommon (Biedenbach, Farrell, Mendes, Ross, & Jones, 2010). The majority of these bacteria have four to six copies of the 23S rRNA gene—nearly all of which must be mutated in order for resistance to develop (Ntokou, et al., 2012). The development of linezolid resistance has been linked to prolonged and/or inappropriate use of this antibiotic, with the subsequent spread of resistant clones. Of note, linezolid-resistant enterococci have been isolated from

patients without previous exposure to the antibiotic (Ntokou, et al., 2012). To minimize the emergence of resistance, linezolid should be restricted to appropriate indications only and used in courses of therapy as short as feasible, and resistance testing should be performed based on local epidemiology, host risk factors, and/or when treatment failures occur.

Streptogramins

Quinupristin/dalfopristin is a combination agent that consists of streptogramin A (70% dalfopristin) and B (30% quinupristin), with proven efficacy for VRE infection due to *E. faecium* (Linden, et al., 2001). The efficacy of quinupristin/dalfopristin in treating VRE infections in several prospective multicenter studies showed overall showed success rates of 66% (Linden, et al., 2001; Moellering, Linden, Reinhardt, Blumberg, Bompart, & Talbot, 1999). All strains of *E. faecalis* are intrinsically resistant to quinupristin/dalfopristin. These agents work to synergistically inhibit protein synthesis through the 50S ribosomal subunit, and are bacteriostatic as a result. Quinupristin/dalfopristin is poorly tolerated in a minority of patients, due to arthralgias and myalgias. Phlebitis is another common problem that can be avoided by administering the drug through a central venous catheter. Resistance to quinupristin/dalfopristin can occur by target modification, drug inactivation, or active efflux. Clinical isolates of *E. faecium* with resistance to quinupristin/dalfopristin are rare (MIC ≥4 μg ml-1), perhaps because multiple mechanisms are needed to achieve this level of resistance (Thal & Zervos, 1999). Despite this, a high percentage (28.9%) of unrelated E. faecium isolates from Greece was recently noted to have a reduced susceptibility to quinupristin/dalfopristin. These isolates were from patients without exposure to the antibiotic, and were not associated with the veterinary use of virginiamycin, a feed additive used in food animals that promotes streptogramin resistance (Karanika, et al., 2008). Both the acquisition of resistance by E. faecium and superinfection with E. faecalis have been described during treatment with quinupristin/dalfopristin (Chow, Davidson, Sanford 3rd, & Zervos, 1997; Chow, Donahedian, & Zervos, 1997).

The data for using quinupristin/dalfopristin in the treatment of endocarditis due to VRE is limited to anecdotal reports (Bethea, Walko, & Targos, 2004; Furlong & Rakowski, 1997; Mastumura & Simor, 1998). The combination of quinupristin/dalfopristin, doxycycline, and rifampin appears synergistic in vitro and was successfully used to treat a patient with aortic valve endocarditis (Mastumura & Simor, 1998). In another case, a neutropenic patient with persistent bacteremia due to ampicillin-resistant VRE was successfully treated with high-dose ampicillin (24 gm day-1) and quinupristin/dalfopristin (Bethea, Walko, & Targos, 2004). Recently, the package insert for quinupristin/dalfopristin was revised to exclude VRE, with interpretive breakpoints for *E. faecium* deleted.

Lipoglycopeptides

Lipoglycopeptides are a new class of antibiotics that inhibit the bacterial cell wall synthesis like glycopeptides and also disrupt the cell membrane integrity (Zhanel, et al., 2010). Oritavancin, telavancin, and dalvabancin are currently available lipoglycopeptides. They exhibit activity against vancomycin-susceptible enterococci species, and VanB-containing enterococci, although telavancin has marginal activity against VanB isolates. Only oritavancin is active against VanA-containing enterococci, as it can bind to the D-Ala-D-Lac peptidoglycan precursor. These agents are not inferior to comparator agents in clinical trials (Zhanel, et al., 2010). Pending further data, lipoglycopeptides are not routinely recommended for enterococcal infections.

Other antibiotics

Tigecycline is a bacteriostatic agent that binds the 30S ribosomal subunit, and inhibits protein synthesis. It is a broad-spectrum antibiotic that is approved for the treatment of skin and soft tissue infections caused by susceptible organisms, including *E. faecalis.* It is not approved for infections caused by *E. faecium* regardless of susceptibilities (Rubinstein & Vaughan, 2005). Although tigecycline has been successfully used in combination with daptomycin to treat endocarditis due to VRE (Jenkins I. , 2007), its use for serious infections is considered

contraindicated because of excess deaths and noncures in multiple noninferiority studies (Prasad, Sun, Danner, & Natanson, 2012). Teicoplanin, a glycopeptide with a mechanism of action similar to vancomycin, is effective against some VanB-resistant VRE. However, resistance to teicoplanin has developed in some VanB isolates during therapy (Hayden, Trenholme, Schultz, & Sahm, 1993). This agent, which is not commercially available in the United States but widely used in Europe, is not often prescribed for the treatment of enterococcal endocarditis.

Enterococci as probiotics

Probiotics are naturally occurring microorganisms that confer health benefits by supplementing host commensal microbiota, modulating immunity, enhancing intestinal barrier function, or altering pain perception (Forchielli & Walker, 2005). *E. faecalis* and *E. faecium* are human intestinal commensals that also have been used as probiotics, as well as in food production (see Enterococcus Diversity, Origins in Nature, and Gut Colonization). However, no large, randomized, placebo-controlled clinical trials have been conducted to assess their safety or efficacy. As a result, no enterococcal probiotic has been approved by the FDA for the treatment, cure, or amelioration of any human disease. In 2007, the European Food Safety Authority determined that enterococci do not meet the standard for the "Qualified Presumption of Safety" (EFSA Scientific Committee, 2007). Many virulence traits that generally suggest enterococci as poor choices for probiotic therapy support these concerns. In addition, many enterococci have acquired resistance to clinically important antibiotics encoded on a wide variety of conjugative plasmids, transposons, and bacteriophages (see Enterococcal infection). Strains of *E. faecalis* or *E. faecium* should only be considered as potential probiotics when they are shown to lack virulence traits (such as cytolysin, gelatinase, serine protease, aggregation substance, capsular polysaccharide, biofilm production, extracellular superoxide production, and enterococcal surface protein, among others), are unable to translocate the intestinal mucosa, and remain susceptible to phagocytic killing. In addition, any such putative probiotic strain should have limited ability to exchange DNA *in vivo*. No such strain has yet been identified and, until then, alternatives should be explored as probiotics.

Summary and Conclusions

Enterococci are associated with a variety of different clinical syndromes, including bacteremias, endocarditis, and skin or soft tissue and urinary tract infections. The emergence of resistance has made clinicians keenly aware of these opportunistic pathogens. Molecular methods have delineated the epidemiology of VRE and have conclusively demonstrated healthcare-associated acquisition and transmission.

Colonization with VRE occurs approximately 10 times more frequently than actual infection, and occurs in patients with severe underlying illness or who are receiving antibiotics with broad-spectrum anti-anaerobic activity. Infection control efforts have been established to limit the spread of this pathogen. Treatment of serious enterococcal disease requires a synergistic combination of a cell-wall active agent and an aminoglycoside. The relatively few antimicrobial agents available to treat serious VRE infections make therapeutic decision-making for these cases quite challenging. Although enterococci are generally considered safe for use in food production, their role as probiotics is not established, and alternatives should be sought, due to their involvement in therapeutically challenging diseases.

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Enterococci as Indicators of Environmental Fecal Contamination

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Introduction

Enterococci are found in high concentrations in human feces, usually between 10^4 and 10^6 bacteria per gram wet weight (Layton, Walters, Lam, & Boehm, 2010; Slanetz & Bartley, 1957; Zubrzycki & Spaulding, 1962); see also Enterococcus Diversity, Origins in Nature, and Gut Colonization). Although enterococci usually represent less than 1% of the flora (Tendolkar, Baghdayan, & Shankar, 2003), they are usually present in the fecal consortium, but are outnumbered by other bacteria, including *Escherichia coli*, clostridia, and the *Bacteroidales* (Zubrzycki & Spaulding, 1962). Due to their ubiquity in human feces and persistence in the environment, enterococci have been adopted as indicators of human fecal pollution in water. More recently, their densities on human hands have been used as indicators of hand hygiene. The use of enterococci as indicators of human fecal pollution or contamination can be problematic, however, because enterococci are also found in animal feces (Harwood, Whitlock, & Withington, 2000; Layton, Walters, Lam, & Boehm, 2010), in soils (Byappanahalli & Fujioka, 2004; Goto & Yan, 2011), and on plants (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003; Imamura, Thompson, Boehm, & Jay, 2011; Müller, Ulrich, Ott, & Müller, 2001). Although there is debate about the extent to which this happens in nature, there is evidence that enterococci are capable of replicating in extra-enteric environments, such as on beach sands (Bahirathan, Puente, & Seyfried, 1998; Zubrzycki & Spaulding, 1962) and in water containing kelp (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003; Imamura, Thompson, Boehm, & Jay, 2011) and plankton (Mote, Turner, & Lipp, 2012). Identification of human-specific enterococcal species or genotypes could aid in the discrimination of human fecal contamination from other environmental sources of the organisms. Some data suggest that *Enterococcus faecium* and *Enterococcus faecalis* may be more prevalent in human feces than other enterococcal species, while *Enterococcus casseliflavus* and *Enterococcus mundtii* may be more abundant in environmental reservoirs (such as on plants) than other species (Bahirathan, Puente, & Seyfried, 1998; Ferguson, Moore, Getrich, & Zhowandai, 2005; Wheeler, Hartel, Godfrey, Hill, & Segars, 2002). However, a number of species of *Enterococcus* have been isolated from human feces (Layton, Walters, Lam, & Boehm, 2010); Enterococcus Diversity, Origins in Nature, and Gut Colonization), so it will be difficult to derive a single host-specific indicator. It has been suggested that *E. faecium* that contains the enterococcal surface protein (*esp*) gene may be human-specific (Scott, Jenkins, Lukasik, & Rose, 2005), but *esp*-containing *E. faecium* can also be found in select animal hosts (Layton, Walters, & Boehm, 2009; Whitman, Przybyla-Kelly, Shively, & Byappanahalli, 2007).

Fecal enterococci from the GI tract consortia of healthy humans are generally not virulent. Nevertheless, multidrug-resistant *Enterococcus* strains have emerged as leading causes of hospital-acquired infections (Tendolkar, Baghdayan, & Shankar, 2003). Vancomycin-resistant enterococci are particularly important pathogens (Willems, et al., 2005), as are *esp*-containing *E. faecalis* (Shankar, Baghdayan, Huycke, Lindahl, & Gilmore, 1999) and *E. faecium* (Willems, et al., 2001), as well as other types of *E. faecalis* and *E. faecium* (Wisplinghoff, Bischoff, Tallent, Seifert, Wenzel, & Edmond, 2004). It is estimated that there are 800,000 cases of enterococcal infection in the US each year, adding $500,000,000 to annual healthcare costs (Tendolkar,

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Baghdayan, & Shankar, 2003). Therefore, the presence of enterococci in the environment and on hands may have important direct health implications.

Detection of Enterococci

Because of their importance as indicators of fecal pollution, a great deal of effort has gone into developing methods for detection of enterococci in the environment. Selective solid and liquid media are available for one-step detection and isolation of the organisms. Quantitative PCR (QPCR) assays that target the 23s rRNA operon have also been developed (Haugland, Siefring, Wymer, Brenner, & Dufour, 2005; Hou, et al., 2006; Ludwig & Schleifer, 2000). Standardized methods have been approved for the detection of enterococci in water, including the United States Environmental Protection Agency (USEPA) Method 1600 (United States Envinronmental Protection Agency, 2006), the USEPA Method A (United States Environmental Protection Agency, 2010), and the International Organization for Standardization (ISO) methods 7899-2 (International Organization for Standardization, 2000) and ISO 7899-1 (International Organization for Standardization, 1998). IDEXX (Westbrook, ME) defined-substrate assays Enterolert and Enterolert-E are also approved for detection of enterococci in water in the United States (US) and the European Union (EU), respectively. Readers are directed to Edge and Boehm (Edge & Boehm, 2010) for a full description of environmental enterococcal enumeration methods.

Enterococci as Indicators of Fecal Contamination in Recreational Water

Both drinking and recreational waters are monitored for microbial quality. In drinking water, coliforms, including total and fecal coliforms (and *Escherichia coli* in particular) are the primary method of assessing contamination. In the European Union (EU), enterococci are used as indicators of drinking water contamination (The Council of the European Union, 1998). In the EU, enterococci are not permitted in a 100 mL sample of tested drinking water that flows from a tap, and they are not permitted in a 250 mL sample of bottled water.

Enterococci are also used as indicators of fecal contamination of recreational waters throughout the world. In the US, the fecal pollution standard for recreational bathing waters was originally set using concentrations of total coliforms, based on the results of a US Public Health Service study of swimmer health on Lake Michigan in Chicago, IL in 1948 (Stevenson, 1953). In recognition of the fact that related Gram-negative bacteria are naturally present in water, that standard was subsequently revised to a "fecal" coliform standard, which assumes that only a fraction of total coliforms were of fecal origin. In the late 1970s and early 1980s, swimmer health studies were carried out to aid in the identification of new fecal indicator organisms that may be more reliable than fecal coliforms (Cabelli, 1983; Dufour, 1984). Researchers determined that concentrations of enterococci concentrations measured in recreational marine waters polluted by treated wastewater were strongly correlated to the number of swimmers becoming sick with gastrointestinal illness (Cabelli, 1983). Similar results were obtained in other studies around the world (Boehm & Soller, 2011). A meta-analysis of these results (Wade, Pai, Eisenberg, & Colford Jr., 2003) found evidence for a positive association between enterococcal concentrations and swimmer gastrointestinal illnesses (Figure 1). The associations were further confirmed in a suite of epidemiological studies carried out in the EU (Wiedenmann, Krüger, Dietz, López-Pila, Szewzyk, & Botzenhart, 2006) and the US (Wade, et al., 2006) in the 2000s, which supported an association between enterococcal concentrations and swimmer health in recreational freshwaters, as well as marine waters. Given this evidence, the US, EU, and the World Health Organization (WHO) recommend that enterococci be adopted as an indicator of recreational water quality and risk of swimmer illness.

Recreational water quality standards now vary by country, but they generally relate bacterial counts to a geometric mean and a statistical threshold value (also referred to as a single-sample standard). The WHO

compiled recreational water quality standards for countries around the globe in 1999 (World Health Organization, 1999). The policy for each country requires the use of a specific enterococcal enumeration method. In all cases, the method is culture-based, and involves the use of selective and differential media in solid or liquid form (Edge & Boehm, 2010). In the US, a new standard method for measuring enterococci in water has been developed by the USEPA, which uses quantitative polymerase chain reaction (QPCR) in conjunction with a hydrolysis probe (United States Environmental Protection Agency, 2010). However, inter- and intra-laboratory variation of this method, as well as the relationship between QPCR results and those of culture-based assays, are still being debated (Shanks, et al., 2012; Whitman, et al., 2010).

In the US, EPA marine recreational water quality criterion for enterococci in water is not more than 104 colony forming units (CFU) / 100 mL (single-sample standard) and 35 colony forming units / 100 mL (geometric mean standard) (United States Environmental Protection Agency, 2011). These values may soon be revised to extend to fresh waters and to include a standard for enterococcal detection by quantitative PCR. In the EU, bathing water standards for enterococci range from limits of 100-400 CFU/100 mL, depending on whether the beach is marine or fresh, and whether the beach is rated as excellent or sufficient (The Council of the European Union, 2006).

In epidemiological studies used to establish enterococcal standards, the etiologies of swimmer illness were not confirmed, but are not believed to have been directly caused by enterococci. Rather, it is thought that the main etiologies of recreational waterborne illness are viral. One study suggests norovirus-like agents as the main cause of disease (Soller, Bartrand, Ashbolt, Ravenscroft, & Wade, 2010). Thus, the correlative relationship between enterococci concentrations and health might suggest that enterococci in recreational waters are indicative of human viruses. There is a striking lack of data to support an association between enterococcal and virus concentrations, or the concentrations of pathogens in general, in recreational waters (Hellein, Battie, Tauchman, Lund, Oyarzabal, & Lepo, 2011; Jiang & Chu, 2004; Noble & Fuhrman, 2001; Pusch, et al., 2005; Viau, Lee, & Boehm, 2011). A few reports have found positive associations between enterococci and the bacterial enteric pathogens *Campylobacter* and *Salmonella* in surface waters (Viau, et al., 2011; Walters, Thebo, & Boehm, 2011), but the causative link between enterococci and swimmer illness remains unknown. As pathogen detection techniques advance to allow for the more sensitive and specific detection of human viruses, as well as enteric bacterial and protozoan pathogens in water, the basis for associations between waterborne enterococci and pathogens may be discovered.

Sources of enterococci in recreational waters include sewage, agricultural and urban runoff, stormwater, direct input by animals via defecation, bather shedding, boats, plant debris (for example, wrack), polluted groundwater, soils, sediments, and sands (Figure 2). In developed countries, sewage is typically well-treated prior to discharge through an outfall that is usually located far from recreational waters. Direct inputs of untreated sewage, however, can impact recreational waters during storm events in regions that have combined sewer overflows and in regions with leaking sewer lines (Sercu, Van De Werfhorst, Murray, & Holden, 2009). Runoff, generated by storms, urban activities like car washing and irrigation, and agricultural activities can contain extremely high concentrations of enterococci, sometimes that surpass concentrations measured in raw sewage (Olivieri, Boehm, Sommers, Soller, Eisenberg, & Danielson, 2007; Reeves, Grant, Mrse, Copil Oancea, & Boehm, 2004). The source of enterococci in runoff can include soil, animal feces, exfiltrated raw sewage, and decaying plant material. Recent research indicates that decaying vegetation on both fresh and marine beaches can contain enterococci (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003; Imamura, Thompson, Boehm, & Jay, 2011), while sediments and soils may also harbor enterococci (Byappanahalli & Fujioka, 2004; Mote, Turner, & Lipp, 2012). Additionally, beach sands have been shown to harbor enterococci which can grow (Zubrzycki & Spaulding, 1962) and be transported into the adjacent waters (Yamahara, Layton, Santoro, & Boehm, 2007). Table 1 shows example concentrations of enterococci in various sources mentioned here; however, it should be noted that concentrations in these sources can be quite variable in time and space.

When the source of enterococci to surface waters is not fecal, their presence may not indicate a health risk. Epidemiology studies have investigated the correlation between enterococci and swimmer illness in recreational waters not impacted by wastewater, and the results are equivocal (Boehm & Soller, 2011). For example, in Mission Bay, California (CA), a site where enterococci are believed to be from birds and runoff, swimmer illness did not correlate to enterococci (Colford Jr., et al., 2007). At Doheny Beach, CA, a site polluted with urban runoff, swimmer illness correlated to enterococci when runoff was discharging into the ocean, but the correlation did not persist when runoff was not discharging (Colford Jr., et al., 2012).

Surface waters throughout the world are plagued by high concentrations of fecal bacteria. In the US, 24% of surface water bodies are listed as impaired, due to elevated levels of fecal indicator bacteria, and a subset of these are impaired because of high concentrations of enterococci (United States Environmental Protection Agency, 2012). When a surface water is known to contain concentrations of enterococci that exceed regulatory standards, actions must be taken to reduce their concentrations. Microbial source tracking (MST) using animal host-specific gene markers in *Bacteroidales* has become an increasingly popular tool for identifying sources of enterococcal contamination in water. There are *Bacteroidales* assays that identify the presence of dog, horse, human, cow, and ruminant feces (Dick, et al., 2005; Kildare, Leutenegger, McSwain, Bambic, Rajal, & Wuertz, 2007; Shanks, et al., 2008; Shanks, Kelty, Sivaganesan, Varma, & Haugland, 2009). Unfortunately, it may be difficult, if not impossible, to allocate enterococci sources using *Bacteroidales* genetic markers, due to the differential fate and transport of bacterial DNA and culturable enterococci in the environment (Jeanneau, et al., 2012; Walters, Yamahara, & Boehm, 2009). Ongoing work is assessing the feasibility of this approach and investigating the possibility of source tracking with enterococcal genetic markers.

Another approach to reducing enterococcal concentrations in water is to identify inputs based on an understanding of the fate and transport of enterococci. Figure 3 shows the various processes that control the concentrations of enterococci in surface waters. Upon entering a surface water, enterococci concentrations vary due to dispersion and advection, which are controlled by concentration gradients and fluid velocities, respectively. Enterococci concentrations are further influenced by sedimentation/deposition, resuspension, particle interactions, growth, predation, and light and dark inactivation due to environmental stresses, such as sunlight and oligotrophy, respectively.

Enterococci are subject to light and dark inactivation and potentially to growth in the environment. Of these processes, the photoinactivation of enterococci has perhaps been the most extensively studied. Sunlight can cause direct damage to nucleic acids or other cellular components, or catalyze the formation of reactive oxygen species, which can cause photooxidative damage to enterococci. Enterococcal sunlight decay rates range between 0.1 and 6 h^{-1} (Fisher, Iriarte, & Nelson, 2012; Marracini, Ferguson, & Boehm, 2012). Enterococci species that contain a yellow carotenoid pigment decay at slower rates when subjected to photostress, due to the ability of the carotenoid to quench reactive oxygen species within the cell (Marracini, Ferguson, & Boehm, 2012). Recent work (Sassoubre, Nelson, & Boehm, 2012) suggests that enterococci are inactivated primarily by endogenously-produced reactive oxygen species generated by cellular chromophores, when illuminated by sunlight in seawater that does not contain colored dissolved organic matter. Dark decay rates are reported between 0.005 and 0.03 h^{-1} (Boehm, Keymer, & Shellenbarger, 2005); dark decay may be caused by stress from exposure to variable salinities, non-ideal temperatures, or a lack of carbon or essential vitamins. While there is no evidence showing that enterococci can grow in ambient oligotrophic waters, experiments showed enterococci can grow in sands (Yamahara, Walters, & Boehm, 2009) as well as in water augmented with decaying kelp (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003; Imamura, Thompson, Boehm, & Jay, 2011). Growth in oligotropic water has been observed for *E. coli* O157:H7, which is also an enteric bacterium (Vital, Hammes, & Egli, 2008), so this might be possible for enterococci as well. Finally, some researchers have reported that enterococci can enter a viable but non-culturable (VBNC) state in water from which they may be resuscitated (Heim, Del Mar Lleo, Bonato, Guzman, & Canepari, 2002; Lleò, Bonato, Benedetti, & Canepari, 2005). Most studies of enterococcal decay and growth have used culture-based assays for enterococci enumeration; these assays would not be able to

detect the VBNC population. A more thorough understanding of the VBNC population would be useful to more fully understand the extra-enteric lifestyle of enterococci (Lleò, Bonato, Benedetti, & Canepari, 2005).

Enterococcal predation can occur by bacterivorous protozoa (such as amoebas, forams, nanoflagellates, and ciliates) and various other zooplankton (hereafter collectively referred to as grazers). An additional possible biological removal mechanism for enterococci is through infection by lytic bacteriophages (Purnell, Ebdon, & Taylor, 2011); however, to our knowledge, this removal mechanism has not been thoroughly evaluated in natural waters. Two studies have documented grazing rates of enterococci in natural waters. Boehm et al. (Boehm, Keymer, & Shellenbarger, 2005) used a dilution method that is typically used in oceanography to measure grazing rates on phytoplankton, and measured a grazing rate of 0.02 h^{-1} for enterococci. Menon et al. (Menon, Billen, & Servais, 2003) reported enterococci mortality rates due to grazing of 0.01 to 0.03 h^{-1} by observing the disappearance of radioactivity from the enterococcal DNA labeled with tritiated thymidine.

A comparatively small number of studies have considered the interaction between particles and enterococci in natural waters. It has been assumed that the bacteria-particle interactions are in equilibrium, and an isotherm model has been applied. Liu et al. (Liu, et al., 2006) assumed that 10% of the total enterococci in Lake Michigan waters were associated with a particle. Jeng et al. (Jeng, England, & Bradford, 2005) assumed that 9% of enterococci were associated with particles in stormwater. Characklis et al. (Characklis, Dilts, Simmons 3rd, Likirdopulos, Krometis, & Sobsey, 2005) showed that 20%–55% of enterococci were associated with settlable particles in stormwater and background water samples. Mote et al. (Mote, Turner, & Lipp, 2012) found that enterococci in an estuary were associated with particles greater than 30 μm. More research is needed to understand if kinetic models of bacterial attachment to particles in surface waters are needed, and to understand the mechanisms by which the attachment of enterococci to particles occurs. A single study has characterized the surface properties of *E. faecalis* and found the bacterium is negatively charged at all pHs, even in the presence of ions (Schinner, Letzner, Liedtke, Castro, Eydelnant, & Tufenkji, 2010). A mechanistic understanding of how electrostatic, hydrophopic, and other surface-surface interactions control enterococci adhesion to particles would be useful.

The deposition of enterococci to sediments at the base of the water column can occur if planktonic enterococci settle, or if they are attached to larger particles that settle to the base of the water column. Settling velocity is a function of particle (in this case, bacterial) size, shape and density, and fluid density and viscosity. Schinner et al. (Schinner, Letzner, Liedtke, Castro, Eydelnant, & Tufenkji, 2010) determined that *E. faecalis* has an equivalent spherical diameter of about 0.8 μm, but assumptions about the exact shape and density of enterococci must be made in order to infer a settling rate. Liu et al. (Liu, et al., 2006) estimate that enterococci settle at a rate of 0.023 m/d in Lake Michigan when they are not associated with particles. Enterococcal association with particles of different sizes, shapes, or densities will affect the settling rate. Resuspension of enterococci that have previously been deposited in the sediments can occur when the sediment is disturbed and experiences shear stresses greater than the critical shear stress. Readers are directed to Nevers and Boehm (Nevers & Boehm, 2010) for more discussion on these processes. Deposition and resuspension of *E. coli* have been studied in a stream using an antibiotic resistant strain not typically found in the environment (Jamieson R. , Joy, Lee, Kostaschuk, & Gordon, 2005; Jamieson R. C., Joy, Lee, Kostaschuk, & Gordon, 2005), but no similar study with enterococci has been carried out. Rather, mathematical formulations for deposition and resuspension of enterococci have primarily been used in models of enterococci in water (Sanders, Arega, & Sutula, 2005; Steets & Holden, 2003).

Long-term spatially and temporally intense studies of enterococci concentrations in marine waters indicates that they vary at predictable time scales, due to various fate and transport processes mentioned above (Figure 4). Rainfall, which is heavier in some areas during El Niño events, leads to higher concentrations of enterococci in ambient waters, due to inputs of stormwater (Boehm, et al., 2002). Enterococci concentrations also vary due to the tides—fortnightly and semi-diurnal signals in enterococci concentrations can be found that correspond to the spring-neap and ebb-flood tidal cycles (Boehm, et al., 2002; Yamahara, Layton, Santoro, & Boehm, 2007).

The tides control transport of enterococci in marine waters through tidal currents, and tides also can modulate enterococci inputs (Boehm & Weisberg, 2005). For example, only during falling ebb tides will tidal lagoons that contain high concentrations of enterococci from bird feces discharge to coastal waters (Grant, et al., 2001). Sunlight suppresses enterococci concentrations near high noon, due to photoinactivation (Boehm, Yamahara, Love, Peterson, McNeill, & Nelson, 2009). Finally, enterococci concentrations vary at high frequencies in marine waters, due to mixing processes that generate patches and ligaments of enterococci in waters free of enterococci (Boehm A. B., 2007).

Knowledge of enterococci sources, fate, and transport can inform the creation of models that predict enterococcal concentrations in surface waters. Process-based models of enterococci in surface waters (Boehm, Keymer, & Shellenbarger, 2005; Cho, et al., 2010; Liu, et al., 2006) have been used to better understand sources of contamination and implement pollution control strategies through total daily maximum loads (TMDLs). Relatively simple statistical models of enterococci concentrations are used for beach management in some regions of the US and EU (Francy, 2009; Hou, Rabinovici, & Boehm, 2006; Stidson, Gray, & McPhail, 2012); the main goal of these models is to identify conditions when health risks are high and conditions are unsafe for swimmers. Predictors like rainfall, tide, time of day, and wave height are used as independent variables in statistical models to predict concentrations of enterococci. If models predict concentrations over a specific threshold, a beach warning is issued so that the public knows that swimming conditions may not be safe. The USEPA has recently developed software for creating statistical models of enterococci concentrations in surface waters, called Virtual Beach (Frick, Ge, & Zepp, 2008).

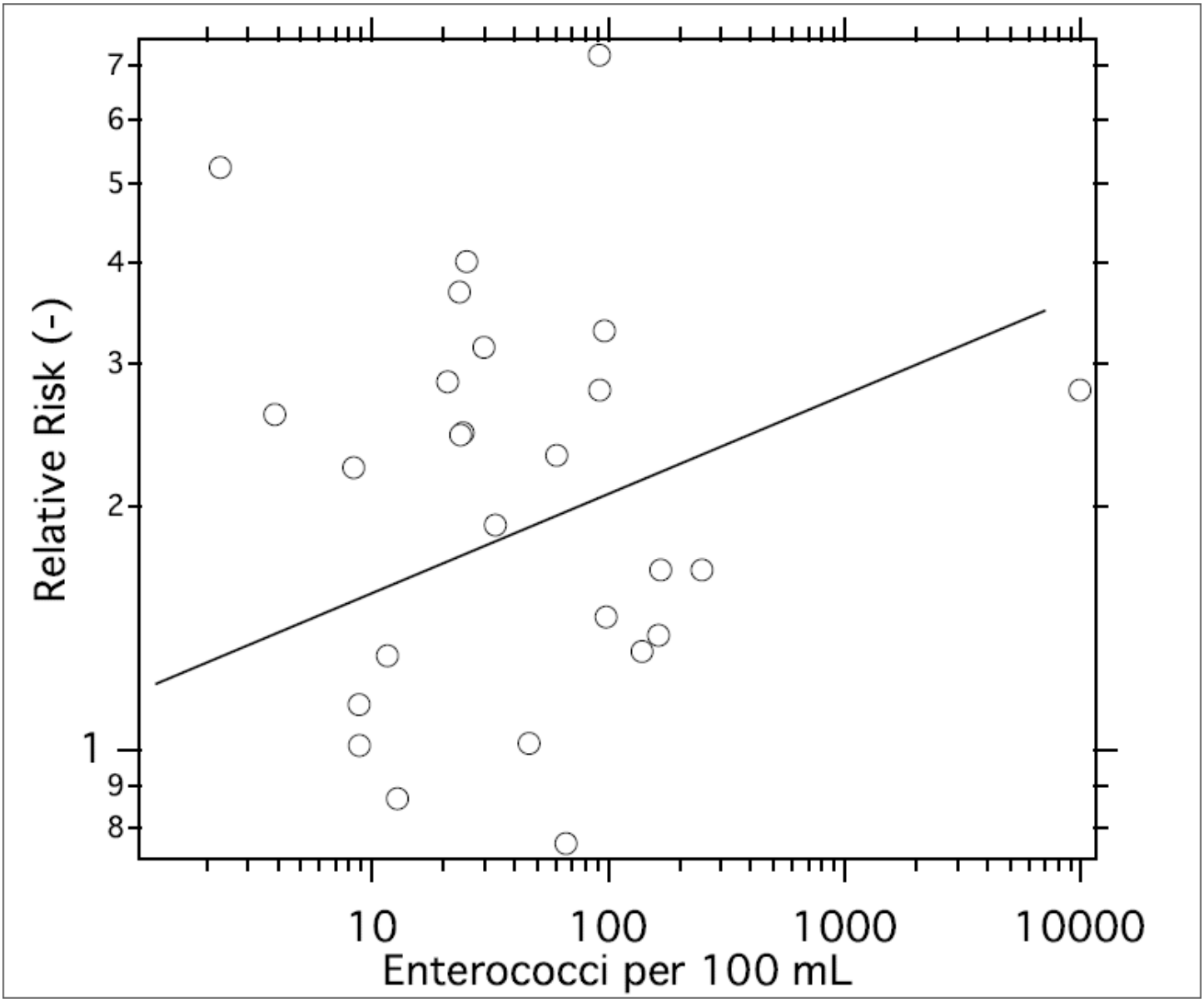

Figure 1. Enterococccci concentrations in marine waters versus relative risk of acquiring gastrointenstinal illness, as reported in the meta-analysis of Wade et al. (Wade, Pai, Eisenberg, & Colford Jr., 2003). The line shown is the weighted best-fit line (r=0.37, p=0.051) and has a slope of 0.3, as reported by the authors. The figure is created using data extracted from Figure 1 of Wade et al. (Wade, Pai, Eisenberg, & Colford Jr., 2003).

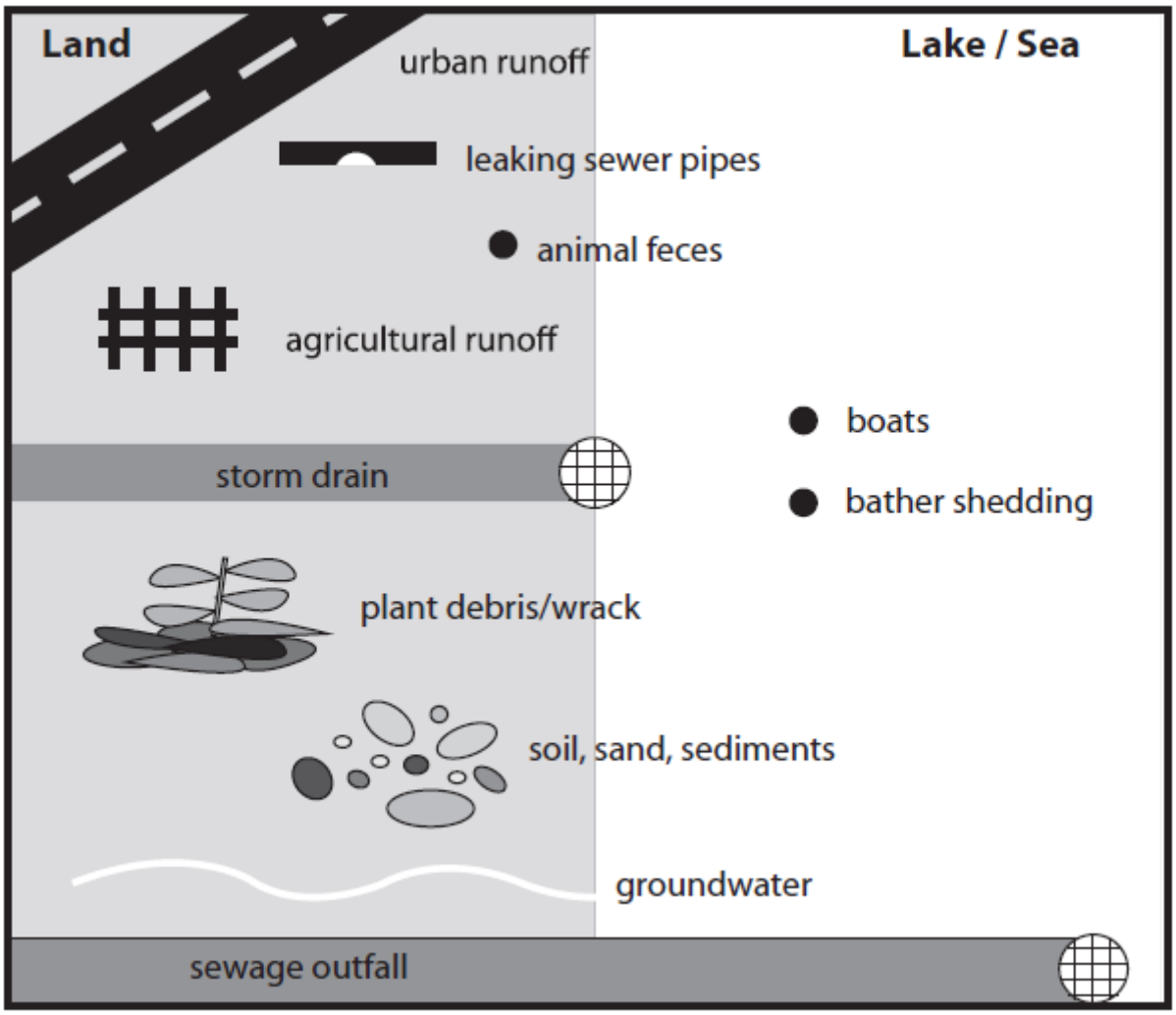

Figure 2. Sources of enterococci in recreational waters. See Table 1 for example concentrations associated with the sources.

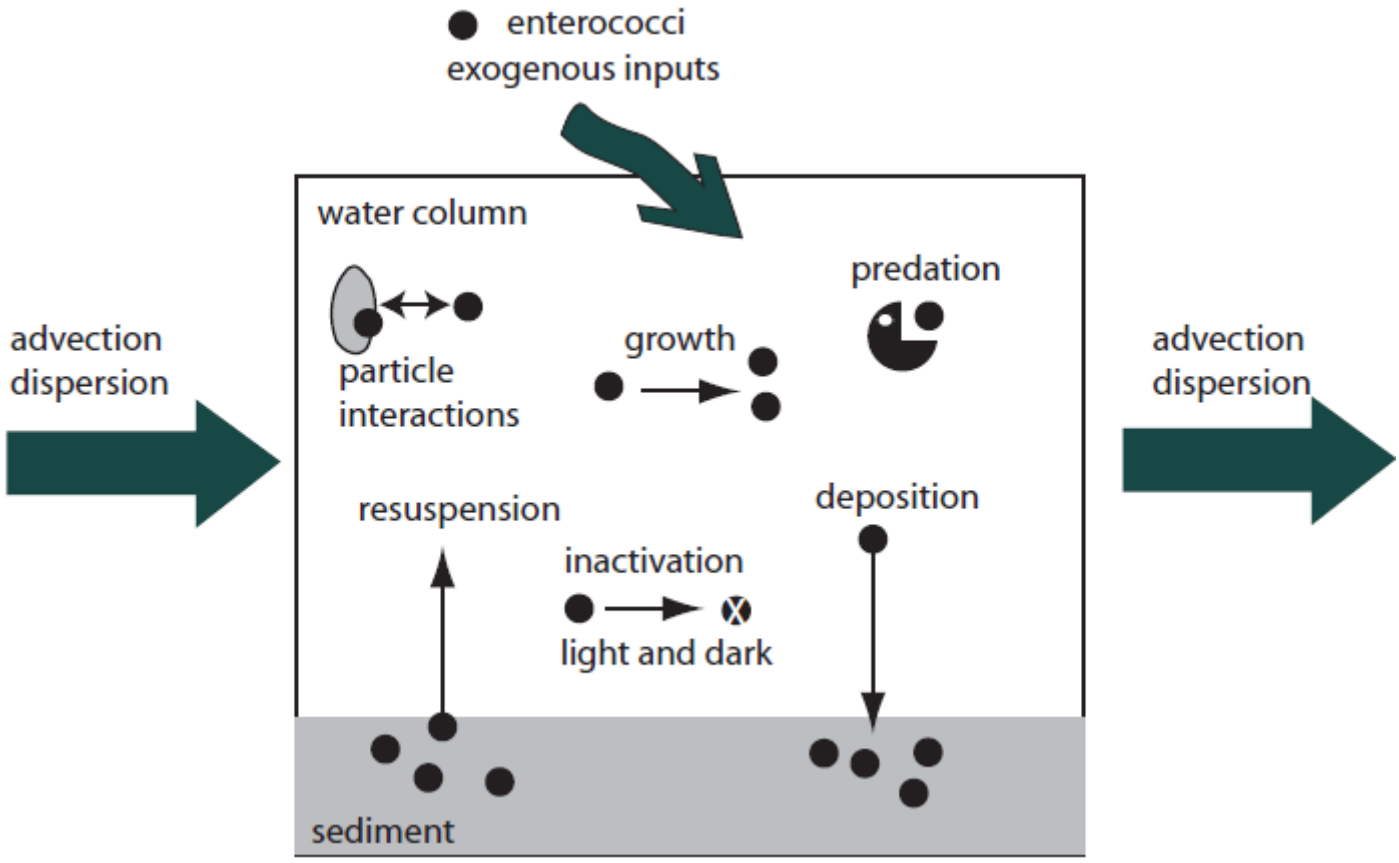

Figure 3. Processes that affect concentrations of enterococci in surface waters.

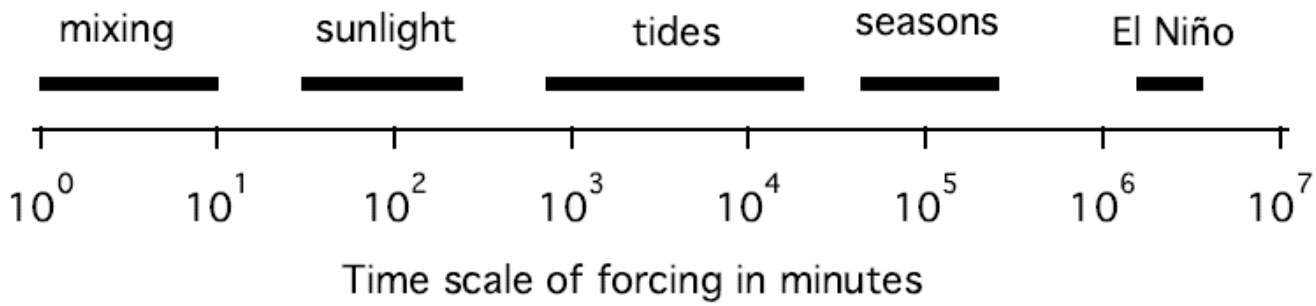

Figure 4. Time scales over which enterococci vary in marine waters, due to natural forcing mechanisms. Figure adapted from Boehm et al. (Boehm, et al., 2002).

Table 1. Concentrations of enterococci measured in common sources to recreational waters.

Source	Concentration	Reference
Kelp wrack	10^1-10^4 CFU/ dry g	(Imamura, Thompson, Boehm, & Jay, 2011)

Table 1. continued from previous page.

Source	Concentration	Reference
Sand	1-10^4 CFU/g	(Halliday & Gast, 2011; Yamahara, Layton, Santoro, & Boehm, 2007)
Bather shedding	10^6 CFU/person	(Elmir, et al., 2007)
Urban runoff	10^3 MPN/100 ml	(Reeves, Grant, Mrse, Copil Oancea, & Boehm, 2004)
Stormwater	0-10^6 MPN/100 ml	(Olivieri, Boehm, Sommers, Soller, Eisenberg, & Danielson, 2007)
Dog feces	10^4-10^8 CFU/g feces	(Wright, Solo-Gabriele, Elmir, & Fleming, 2009)
Bird feces	10^2-10^6 CFU/g	(Wright, Solo-Gabriele, Elmir, & Fleming, 2009)
Groundwater	10^2 MPN/100 ml	(Boehm, Shellenbarger, & Paytan, 2004)
Raw sewage	10^5 MPN/100 ml	(Ahmed, Stewart, Gardner, & Powell, 2008)
Agricultural runoff	10^3 MPN/100 ml	(Díaz, O'Geen, & Dahlgren, 2010; Reeves, Grant, Mrse, Copil Oancea, & Boehm, 2004)

Note that these are representative concentrations only, as they are quite variable in most sources, particularly in secondary sources, like runoff and sewage. CFU is colony forming units and MPN is the most probable number.

Enterococci as Indicators of Fecal Contamination on Hands

Contaminated hands are believed to be a vector for infectious diseases, particularly enteric and respiratory illnesses. For this reason, hand washing has been promoted as a way to save millions of lives (Curtis, 2003). Studies to document the prevalence of hand washing in hospitals, child care centers, and in developing countries where sanitation infrastructure is poor and hand washing facilities are rare, have sought to identify good, unbiased hand washing indicators. The presence of enterococci on hands has been investigated as such an indicator.

When used as an indicator of hand washing and hygiene, the source of enterococci is primarily believed to be fecal in origin. Enterococci can also be found in the mouth (Gold, Jordan, & van Houte, 1975), so oral secretions may also be a source. Soil represents an additional enterococcal source to hands. Limited work has been done to confirm the sources of enterococci found on hands.

Several studies have used both enterococci (or fecal streptococci) and *E. coli* (or fecal coliforms) as indicators of hand hygiene in developing and developed countries. These studies found that enterococci are superior indicators (Kaltenthaler, Elsworth, Schweiger, Mara, & Braunholtz, 1995; Kaltenthaler & Pinfold, 1995; Pickering, Julian, Mamuya, Boehm, & Davis, 2011; Pinfold & Horan, 1996) because they strongly correlated to other hygiene indicators (such as good hygiene knowledge), or that their accumulation on hands could be traced to specific activities (like defecating) during structured observations. The superior performance of enterococci over coliforms has been attributed to the prolonged survival of enterococci on inoculated clean hands (Pinfold, 1990) and inanimate surfaces (Kramer, Schwebke, & Kampf, 2006).

Most studies of enterococci on hands have reported the presence or absence of the organism in hand rinse, fingertip rinse, or fingertip impression samples (Judah, Donachie, Cobb, Schmidt, Holland, & Curtis, 2010; Kaltenthaler, Elsworth, Schweiger, Mara, & Braunholtz, 1995; Kaltenthaler & Pinfold, 1995; Pinfold, 1990; Pinfold & Horan, 1996). Only a few studies have reported concentrations. Pickering et al. (Pickering, et al., 2010; Pickering, Julian, Mamuya, Boehm, & Davis, 2011) found between 1000 and 10000 CFU enterococci per 2 hands on women and children under 5 years old in peri-urban Dar es Salaam, Tanzania; every person tested had measurable enterococci on their hands. A survey in the United Kingdom (UK) detected enterococci on the hands of 28% of commuters who used public transit (Judah, Donachie, Cobb, Schmidt, Holland, & Curtis, 2010).

While these results suggest a difference in enterococcal prevalence on the hands of individuals in regions with good (UK) and poor sanitation infrastructure (Tanzania), enterococcal enumeration was carried out using different sampling and cultivation methods, so care should be taken in comparing the two studies.

Measuring enterococci on hands may be useful for understanding post-collection stored water contamination in developing countries, as well as the spread of infectious disease in both developing and developed countries. Pickering et al. (Pickering, et al., 2010) reported a positive correlation between enterococci on hands and enterococci in stored drinking water in households in peri-urban Dar es Salaam, suggesting that post-collection contamination of stored waters in areas with low levels of sanitation could be facilitated by hands contaminated during defecation or other activities (Pickering, Julian, Mamuya, Boehm, & Davis, 2011). The authors also found a correlation between enterococci on hands and gastrointestinal and respiratory symptoms. A similar correlation was found between enterococci on hands of children in child care centers in California, US and respiratory illness (Julian, Pickering, Leckie, & Boehm, 2013).

There are no current regulations or standard methods for measuring enterococci on hands. Based on research conducted over the last 20 years, it appears that the presence and concentration of enterococci may be good indicators for hygiene and health. However, further work will need to be done to confirm this.

Future Research Needs

In water, enterococci are used as indicators of environmental contamination, because they are found in high concentrations in feces, and exposure to enterococci is linked to adverse health effects in swimmers. Recreational water quality standards are based on enterococci concentrations, so understanding their sources, fate, and transport in the environment is central to assessing and maintaining good water quality. A method to allocate sources of enterococci found in a surface waters would be beneficial to the community of water quality managers. This could be in the form of a molecular microbial source tracking tool, analogous to the tools used to track sources of *Bacteroidales*, or perhaps it could be in the form of a process-based model that links concentrations to particular sources, given spatial-temporal variation in the enterococci concentration signal.

Researchers have just recently started to use enterococci on hands as indicators of hand hygiene. Investigation into sources of enterococci on hands, the time scale of their survival, and their potential to grow on skin will add to the understanding of the strengths and limitations of this hand hygiene indicator. Additional studies that link enterococci density on hands to hand hygiene practices (like hand washing) and health outcomes such as respiratory disease and gastrointestinal illness will further lend credence to their use as hygiene indicators.

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Enterococcal Infection—Treatment and Antibiotic Resistance

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Introduction

The clinical importance of the genus *Enterococcus* is directly related to its antibiotic resistance, which contributes to the risk of colonization and infection. The species of the greatest clinical importance are *Enterococcus faecalis* and *Enterococcus faecium*. Although the resistance characteristics of these two species differ in important ways, they can generally be categorized as intrinsic resistance, acquired resistance, and tolerance.

Relative to the streptococci, enterococci are intrinsically resistant to many commonly used antimicrobial agents. All enterococci exhibit decreased susceptibility to penicillin and ampicillin, as well as high-level resistance to most cephalosporins and all semi-synthetic penicillins, as the result of expression of low-affinity penicillin-binding proteins. For many strains, their level of resistance to ampicillin does not preclude the clinical use of this agent. In fact, ampicillin remains the treatment of choice for enterococcal infections that lack other mechanisms for high-level resistance. Enterococci are also intrinsically resistant to clindamycin, which is mediated by the product of the *lsa* gene, although the mechanism remains poorly defined. Trimethoprim-sulfamethoxazole appears to be active against enterococci when tested *in vitro* on folate-deficient media, but fails in animal models, presumably because enterococci can absorb folate from the environment (Zervos & Schaberg, 1985). Enterococci also have a native resistance to clinically achievable concentrations of aminoglycosides, which precludes their use as single agents. Although *E. faecalis* is naturally resistant to quinupristin-dalfopristin, this combination is highly active against *E. faecium* strains that lack specific resistance determinants.

Enterococci are tolerant to the (normally) bactericidal activity of cell-wall active agents, such as β–lactam antibiotics and vancomycin. Tolerance implies that the bacteria can be inhibited by clinically achievable concentrations of the antibiotic, but will only be killed by concentrations far in excess of the inhibitory concentration. Enterococcal tolerance can be overcome by combining cell-wall active agents with an aminoglycoside. The mechanism by which β–lactam-aminoglycoside combinations yield synergistic bactericidal activity remains a mystery, but *in vitro* data indicate that a higher concentration of aminoglycoside enters cells that are also treated with agents that inhibit cell wall synthesis, which suggests that the cell wall active agents promote uptake of the aminoglycoside (Mohr, Friedrich, Yankelev, & Lamp, 2009).

Tolerance is normally detected *in vitro* by plotting survival in kill curves, and can be observed for a number of antibiotic-bacteria combinations. I*n vitro* tolerance has an important impact on therapy for treating enterococcal infections. The treatment of endocarditis requires bactericidal therapy, due to the inaccessibility of the bacteria within the cardiac vegetations to the mammalian immune system. Recognition of synergism between penicillin-streptomycin led to an improvement in cure rates for enterococcal endocarditis, from approximately 40% to greater than 80% (Jensen, Frimodt-Møller, & Aarestrup, 1999; Rice & Carias, 1998). Despite considerable effort, investigators have yet to find other combinations of antibiotics that are synergistically bactericidal against enterococci.

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In addition to intrinsic resistance and tolerance, enterococci have been extraordinarily successful at rapidly acquiring resistance to virtually any antimicrobial agent put into clinical use. Introduction of chloramphenicol, erythromycin and tetracyclines was quickly followed by the emergence of resistance, in some cases reaching a prevalence that precluded their empirical use. While the occurrence of ampicillin resistance in *E. faecalis* has been quite rare, there is now widespread, high-level resistance to ampicillin among clinical *E. faecium* isolates. High-level aminoglycoside resistance, which negates the synergism between cell-wall active agents and aminoglycosides, has been recognized for several decades. Vancomycin resistance is widely prevalent in *E. faecium*, although it remains relatively rare in *E. faecalis*. In response to the growing problem of vancomycin resistance in enterococci, the pharmaceutical industry has developed a number of newer agents that have activity against vancomycin-resistant enterococci (VRE). However, none of these newly licensed agents (quinupristin-dalfopristin, linezolid, daptomycin, tigecycline) has been entirely free of resistance. Thus, the widespread resistance of enterococci has had a substantial impact on our use of both empirical and definitive antibiotics for the treatment of enterococcal infections, a situation that is likely to persist for the foreseeable future.

Molecular Mechanisms of Antibiotic Resistance in Enterococci

As previously noted, enterococci exhibit significant resistance to a wide variety of antimicrobial agents. This resistance is almost certainly relevant in most natural ecological settings in which enterococci dwell. As normal commensals of the human gastrointestinal tract, enterococci are routinely exposed to a myriad of antibiotics in the course of contemporary medical treatment, and enterococcal resistance plays a key role in the ecological dynamics that occur during and after antibiotic therapy. In addition, their resistance has confounded the best efforts of contemporary medicine to cope with infections caused by enterococci.

Intrinsic resistance—that which is encoded within the core genome of all members of the species—differs from acquired resistance, in that the latter is present in only some members of the species and is obtained via the horizontal exchange of mobile genetic elements (or via selection upon antibiotic exposure). A great deal of effort has been devoted to understanding the molecular mechanisms of resistance in enterococci. This has resulted in identification of determinants that specify resistance for many antibiotics, including those that are (or once were) clinically useful as therapeutics to treat enterococcal infections, as well as those to which enterococci, as commensals of humans, are incidentally exposed in the course of therapy for infections caused by other bacteria. In many cases, this research has led to the development of an understanding of the regulation and biochemical activities of the resistance determinants, and, in selected cases, has provided insight into the consequences of antibiotic resistance on the biological fitness of enterococci. This section will provide an overview of mechanisms of resistance that have been examined in the past 10 years.

Glycopeptide resistance

The glycopeptides vancomycin, teicoplanin, and newer derivatives, are used to treat serious infections due to resistant Gram-positive bacteria. Most Gram-negative bacteria are not susceptible to glycopeptides because their outer membrane prevents access to the peptidoglycan targets located in the periplasmic space. Glycopeptides inhibit bacterial growth by interfering with peptidoglycan biosynthesis. The antibiotics form complexes with the D-Ala-D-Ala peptide termini of peptidoglycan precursors on the outer surface of the cell, which prevents the cell wall biosynthetic enzymes (*i.e.*, the PBPs) from using them as substrates for transglycosylation and transpeptidation and, hence, impairment of cell wall integrity.

Glycopeptide resistance has been extensively reviewed (Arthur & Courvalin, 1993; Arthur & Quintiliani, Jr., 2001; Courvalin, 2005; Courvalin, 2006; Depardieu, Podglajen, Leclercq, Collatz, & Courvalin, 2007; Jaspan, et al., 2010). The biochemical basis for resistance derives from modification of the antibiotic target. Glycopeptide-resistant enterococci produce altered peptidoglycan precursors in which the D-Ala-D-Ala termini have been modified such that they terminate in either D-Ala-D-lactate or D-Ala-D-Ser. These substitutions reduce the

binding affinity of the antibiotics for the peptidoglycan precursors (~1000 fold reduction for D-Ala-D-lac; ~7 fold for D-Ala-D-Ser). The altered precursors can still serve as substrates for the cell wall biosynthetic enzymes to enable the construction of functional peptidoglycan, but the reduced affinity of glycopeptides renders the drugs unable to inhibit cell wall biosynthesis. The capacity to produce the alternative glycopeptide-resistant peptidoglycan precursors is encoded by resistance operons usually encoded on mobile genetic elements (and thus transferable to otherwise susceptible hosts). Specific types of glycopeptide resistance are encoded in the chromosome as part of the core genome of certain enterococcal species.

Overview of genetic mechanisms of glycopeptide resistance

Nine distinct gene clusters conferring glycopeptide resistance have been described in enterococci. These determinants differ from each other both genetically and phenotypically, based on their physical location (encoded on a mobile genetic element or in the core genome); the specific glycopeptides to which they confer resistance (often distinguished operationally as providing resistance to both vancomycin and teicoplanin, or providing resistance to vancomycin but not teicoplanin); the level of resistance they confer; whether resistance is inducible or constitutively expressed; and the type of peptidoglycan precursor that is produced by their gene products. The Van gene clusters encode several functions: (i) a regulatory module, namely a two-component signal transduction system that is responsible for sensing the presence of glycopeptides and activating expression of the resistance genes in inducible Van types; (ii) enzymes that produce the modified peptidoglycan precursors, including enzymatic machinery that is required to produce the appropriate substitute (D-Lac or D-Ser), and a ligase that fuses D-Ala to either D-Lac or D-Ser to make the corresponding dipeptide that can be incorporated into peptidoglycan precursors via the normal biosynthetic machinery of the cell; and (iii) D,D-carboxypeptidases that eliminate any of the normal (unmodified) peptidoglycan precursor synthesized by the natural biosynthetic machinery of the cell, thereby ensuring that nearly all precursors reaching the cell surface are of the modified variety. The Van gene clusters are typically referred to by the names given to the ligases they encode (VanA, VanB, VanC, and so on). The VanA and VanB types are the most common among clinical isolates and have been studied in the greatest detail.

The VanA determinant (Figure 1) confers a high level of resistance to vancomycin and teicoplanin. VanA is typically encoded on Tn*1546* or related transposons, and includes seven open reading frames transcribed from two separate promoters. The regulatory apparatus is encoded by the VanR (response regulator) and VanS (sensor kinase) two-component system, which are transcribed from a common promoter, while the remaining genes are transcribed from a second promoter. Gene products that specify the production of modified peptidoglycan precursors include VanH (dehydrogenase that converts pyruvate to lactate) and VanA (ligase that forms D-Ala-D-Llac dipeptide). The VanX (dipeptidase that cleaves D-Ala-D-Ala) and VanY (D,D-carboxypeptidase) peptidases serve to eliminate the natural peptidoglycan precursors from the cell. The 7th gene, VanZ, is often referred to as an "accessory" function, but its role in resistance is not fully understood.

The VanB locus (Figure 1) confers moderate to high-level resistance to vancomycin, but is not induced by teicoplanin. VanB is usually acquired onTn*5382*/Tn*1549* type transposons, which occur on plasmids or in the chromosome of the host. The genetic organization of VanB is similar to that of VanA, in that it contains two distinct promoters transcribing seven open reading frames, but there are some significant differences. For example, although VanB encodes a two-component system (named $VanR_B$ and $VanS_B$), this signaling system is considerably different from that encoded in VanA. VanB encodes homologs of VanH and the D-Ala-D-Ala ligase (encoded by VanB), as well as the peptidases (VanX and VanY). However VanB lacks a homolog of VanZ, and instead encodes a protein named VanW, whose role in resistance is not fully understood.

Regulation of glycopeptide resistance

Expression of vancomycin resistance is controlled via the VanR/VanS two-component signal transduction system, shown in Figure 1. The VanS sensor kinase is thought to recognize a (poorly defined) stimulus that

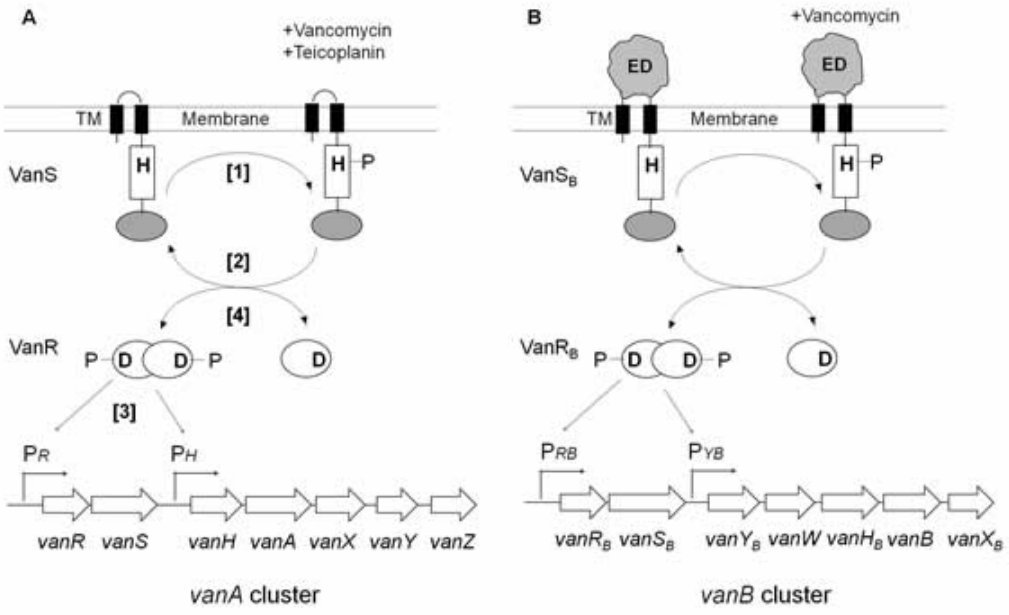

Figure 1. Regulation of vancomycin resistance gene clusters. Comparison of VanA regulation (panel A) and VanB regulation (panel B). The VanS (or VanSB) sensor kinases are anchored in the cytoplasmic membrane by two transmembrane segments (TM) that flank the predicted sensory input domain. VanS (which is inducible by both vancomycin and teicoplanin) contains only a short extracellular loop, and may receive activating signals in, or immediately adjacent to, the membrane. VanSB (which is inducible by vancomycin, but not teicoplanin) contains a large extracellular domain (ED) that likely serves as the ligand-recognition domain. In both cases, the presence of the appropriate antibiotic stimulus leads to the activation of kinase activity and ATP-dependent autophosphorylation on a highly conserved His residue [1]. This phosphoryl group is transferred to the VanR (or VanRB) response regulator [2], which leads to dimerization, enhanced binding to DNA, and activation of transcription from the two promoters found in the Van gene cluster [3]. In the absence of inducing stimuli, VanS (VanSB) serves as a phosphatase to ensure that VanR (VanRB) remains in the inactive state [4] and Van expression is off. Figure is adapted from (Arthur & Quintiliani, Jr., 2001) with modifications.

signals the presence of vancomycin in the environment. VanS thereby becomes activated and autophosphorylates a conserved histidine residue on the cytoplasmic side of the protein. That phosphoryl group can be transferred to a conserved aspartate residue on the VanR response regulator, which leads to VanR dimerization, enhanced VanR binding to the 2 promoters located in the Van locus, and consequently, an increased transcription of both the Van resistance genes as well as the regulatory genes (Depardieu, Courvalin, & Kolb, 2005). Additionally, there is substantial evidence that VanS serves as a phosphatase for VanR under non-inducing conditions, to prevent activation of Van expression via spurious phosphorylation from other sensor kinases in the cell (cross-talk), or via autophosphorylation of VanR from acetyl-phosphate (Depardieu, Courvalin, & Kolb, 2005). As a result, the phosphatase activity of VanS is critical to maintain the signaling pathway in the off state in the absence of an inducing antibiotic. Mutations that impair the phosphatase activity

of VanS (or remove VanS completely) lead to constitutive expression of the resistance genes (Arthur & Quintiliani, Jr., 2001). This is now known to be true for many of the Van clusters (Depardieu, Kolbert, Pruul, Bell, & Courvalin, 2004; Panesso, et al., 2010). In fact, constitutively resistant mutants that carry lesions in VanS can be isolated from patients during glycopeptide therapy. For example, examination of successive isolates of *E. faecium* obtained from a patient suffering from an infection with a VanB strain revealed that a short deletion in the $VanS_B$ kinase led to the loss of phosphatase activity and constitutive glycopeptide resistance (Depardieu, Courvalin, & Msadek, 2003).

Although both VanA and VanB rely on two-component signaling systems to control Van expression, it is clear that there are important differences between these regulatory systems. For example, the VanS and $VanS_B$ sensor kinases exhibit relatively little sequence identity in the N-terminal portion that serves as the site of stimulus recognition. In fact, the amino acid sequence of the predicted extracellular ligand-binding domain of VanS is short, and is likely to comprise only a short loop that connects the two transmembrane helices outside the membrane, which suggests that VanS belongs to the intramembrane-sensing family of sensor kinases (Mascio, Alder, & Silverman, 2007), whereas the predicted extracellular domain of $VanS_B$ is substantially larger and likely constitutes an independently-folded extracellular domain that serves to recognize cognate signals. Given the distinct architecture of these two sensor kinases, it seems plausible that they recognize and respond to different molecular signals to trigger kinase activation and expression of the resistance genes. In fact, this predicted difference in ligand binding—and consequently, in the inducibility of the signaling system—underlies the difference in teicoplanin susceptibility of enterococci that contain VanA vs. those that contain VanB. Although the molecular identity of the actual inducing signal(s) remain unclear, the VanA resistance genes are induced by the presence of both vancomycin and teicoplanin (thereby conferring resistance to both), but the VanB resistance genes are only induced by vancomycin—hence, VanB strains remain susceptible to teicoplanin. Of note, $VanS_B$ can acquire mutations of various types that lead to constitutive expression of the resistance genes or to inducibility by teicoplanin, thereby altering the phenotype of such mutants carrying the VanB locus.

Regulation by host factors?

Some evidence suggests that one or more sensor kinases encoded in the genome of the enterococcal host can contribute to the regulation of the Van resistance genes. For example, $VanR_B$-dependent gene expression remains inducible even in the absence of $VanS_B$ (Baptista, Rodrigues, Depardieu, Courvalin, & Arthur, 1999), suggesting that another sensor kinase can phosphorylate and activate $VanR_B$. Similarly, the VanE cluster in *E. faecalis* encodes a $VanS_E$ kinase that is predicted to be nonfunctional due to a premature stop codon (Abadía Patiño, Courvalin, & Perichon, 2002), but such resistance is nevertheless inducible by vancomycin (Foucault, Depardieu, Courvalin, & Grillot-Courvalin, 2010). Such findings suggest that enterococci encode endogenous two-component signaling systems whose natural function is to monitor the integrity of the cell wall for perturbations, and activate appropriate adaptive responses to ensure cell wall maintenance; and further, that the glycopeptide resistance gene cassettes have managed to exploit these endogenous systems to assist in the regulation of glycopeptide resistance. Other host factors may also play a role in regulation of Van expression. For example, expression of the VanE vancomycin resistance genes may be influenced by the alteration of DNA supercoiling in *E. faecalis* (Paulsen, et al., 2003).

The expanding Van alphabet

While the VanA- and VanB-type vancomycin resistance clusters continue to be the predominant forms that account for vancomycin resistance in hospitals, new Van resistance gene clusters have been recently described, which brings the number of known gene clusters capable of conferring Van resistance to nine. Lebreton and colleagues (Lee, Huda, Kuroda, Mizushima, & Tsuchiya, 2003) recently described such a new gene cluster, named VanN, that specifies incorporation of D-Ala-D-Ser at the terminus of the peptidoglycan precursors. VanN joins other recently described Van clusters (Boyd, Willey, Fawcett, Gillani, & Mulvey, 2008; Xu, et al.,

2010) known to specify incorporation of either D-Ala-D-Ser (VanC, VanE, VanG, and VanL types) or D-Ala-D-Lac (VanA, VanB, VanD, and VanM types) into peptidoglycan precursors.

Fitness cost of vancomycin resistance

Despite the complex mechanism that underlies glycopeptide resistance, resistant enterococci have disseminated worldwide, suggesting that resistance imposes little or no biological cost to the bacteria. This hypothesis was carefully examined by using pairs of isogenic enterococcal strains with specific mutations, to evaluate the fitness cost of vancomycin resistance during growth *in vitro* and *in vivo* (Franke & Clewell, 1981). The investigators found that expression of vancomycin resistance imposed a significant fitness cost, both when expression is induced by the antibiotic and when expression is constitutive due to mutation of the regulatory apparatus. However, uninduced vancomycin resistance did not impose a measurable fitness cost. Thus, these results offer a strong evolutionary rationale for the tight regulation of vancomycin resistance by the VanS/VanR two-component signaling system found in Van gene clusters.

Alternative mechanism of glycopeptide resistance

A novel mechanism of glycopeptide resistance has been described in laboratory-selected vancomycin-resistant mutants of *E. faecium* (Cremniter, et al., 2006). This mechanism is unrelated to that encoded by the Van gene clusters (namely, those with production of peptidoglycan precursors containing D-Lac or D-Ser substitutions). The investigators selected highly resistant mutants *in vitro* and performed extensive analysis of peptidoglycan structure in the mutants. Their analysis revealed that the beta-lactam insensitive L,D-transpeptidase pathway (discussed in more detail below, under *Ampicillin resistance*) was activated. This alternative transpeptidase (named Ldt_{fm}) is capable of crosslinking enterococcal peptidoglycan using the L-Lys found at the 3rd position of the peptide stem (rather than the D-Ala found at position 4, as is typical of most PBPs). The investigators found that a cryptic D,D-carboxypeptidase was activated in the glycopeptide-resistant mutants, whose activity resulted in production of peptidoglycan peptide stem precursors that are tetrapeptides (lacking the terminal D-Ala), rather than pentapeptides. Such precursors are not substrates for binding by glycopeptide antibiotics, but can be cross-linked by the Ldt_{fm} transpeptidase. However, it remains unknown whether this mechanism of glycopeptide resistance is relevant in clinical isolates.

Daptomycin resistance

Daptomycin is a lipopeptide antibiotic with potent *in vitro* bactericidal activity against Gram-positive bacteria. The mechanism of antimicrobial action for daptomycin has not been unequivocally established, but is thought to involve calcium-dependent insertion into the cytoplasmic membrane followed by membrane depolarization, release of intracellular potassium ions, and rapid cell death (Alborn, Jr., Allen, & Preston, 1991; Matsumura & Simor, 1998; Silverman, Perlmutter, & Shapiro, 2003). Because its mechanism of action is distinct from those of other antibiotics, daptomycin is useful for treatment of infections that are caused by multidrug-resistant Gram-positive strains. Daptomycin resistance has been observed in clinical isolates following daptomycin therapy, typically as a result of mutations in chromosomal genes. In *Staphylococcus aureus*, resistance is associated with mutations in genes encoding proteins such as MprF, a lysylphosphatidylglycerol synthetase; YycG, a sensor histidine kinase; and RpoB and RpoC, the β and β′ subunits of RNA polymerase (Galimand, et al., 2011).

Daptomycin-nonsusceptible clinical *E. faecium* strains have been described (Muller, Le Breton, Morin, Benachour, Auffray, & Rincé, 2006). The investigators determined that these strains did not carry mutations in homologs of genes known to confer nonsusceptibility to daptomycin in *S. aureus* (*yycG*, *mprF*, *rpoB*, *rpoC* were evaluated in this study), which suggests the existence of one or more novel mechanisms of daptomycin resistance in enterococci. However, the genes responsible for resistance were not identified.

Recent studies have begun to explore the genetic basis of daptomycin resistance in enterococci (Arias, et al., 2011). The genomes of a pair of *E. faecalis* strains isolated from the same patient before and after daptomycin

therapy were sequenced to identify polymorphisms contributing to resistance (Arias, et al., 2011). In that study, unique-sequence polymorphisms were found in *cls*, *gdpD* (both thought to be involved in phospholipid metabolism) and *liaF*, but no polymorphisms were found in homologs of the genes identified in daptomycin-resistant *S. aureus* isolates. Follow up analysis with *in vitro* selection for daptomycin-resistant variants of the original susceptible strain led to the identification of mutations in *liaF* and *gdpD*. Importantly, the daptomycin-resistant phenotype was determined to be a consequence of the identified mutations, as site-directed mutagenesis to recapitulate these mutations in an otherwise daptomycin-susceptible host conferred enhanced daptomycin resistance, demonstrating that mutations in these genes confer resistance. Similarly, in a clinical strain pair of *E. faecium* recovered from a patient both before and after daptomycin therapy, a polymorphism in *cls* was identified (but not in *liaFSR* or *gdpD*) in the daptomycin-resistant derivative. Changes in *cls*, *liaF*, *liaS*, or *liaR* were also identified in other daptomycin-resistant clinical isolates of enterococci, which suggests that such mutations play a key role in the development of daptomycin resistance *in vivo*. Development of daptomycin resistance appeared to be associated with profound ultrastructural changes in the cell envelope and septal apparatus, although it remains unclear if these changes are functionally important for resistance or merely an incidental consequence of the mutations. LiaF is part of the three-component LiaFSR regulatory system, which is known to coordinate the response of the cell envelope to antibiotics and antimicrobial peptides in some Gram-positive bacteria, which suggests that perturbations in the activity of this signaling system may alter envelope properties in a such a way that daptomycin can no longer interact with, or insert into, the membrane efficiently.

In parallel studies, Palmer and coworkers systematically selected daptomycin-resistant variants of *E. faecalis in vitro* (Palmer, Kos, & Gilmore, 2010) in a stepwise manner, and characterized the order of appearance of mutations that correspond to increased resistance to daptomycin. Whole-genome sequence comparison identified mutations in seven genes, including *cls*, *rpoN*, and additional genes whose cellular functions in other contexts have not been established. Transfer of the *cls* mutant allele to a susceptible *E. faecalis* strain conferred enhanced resistance to daptomycin, which unambiguously proved that the *cls* mutation is sufficient for resistance. Time-resolved analysis of the emergence of mutations during daptomycin exposure revealed that *cls* mutations appeared early in multiple independent selections, which highlights its importance. Additional daptomycin-resistant mutants were obtained that lacked such *cls* mutation, which shows that alternative paths to daptomycin resistance also exist. Collectively, the results from these two studies suggest that, while the underlying mechanisms are genetically distinct from those identified in *S. aureus*, a role for altered membrane phospholipid composition and/or surface properties in both staphylococcal and enterococcal daptomycin resistance is likely.

Aminoglycoside resistance

Aminoglycosides act by binding to the 16S rRNA of the 30S ribosomal subunit and interfering with protein synthesis. Enterococci generally exhibit a moderate level of intrinsic aminoglycoside resistance that has been attributed to poor uptake of antibiotics. For example, analysis of selected mutants that exhibited enhanced gentamicin resistance *in vitro* suggested that impaired uptake of gentamicin can contribute directly to enhanced resistance (Aslangul, et al., 2006), although the mutations or genes responsible for the alteration in uptake were not unambiguously identified. However, some evidence suggests that other mechanisms may contribute to, or even be primarily responsible for, intrinsic resistance of some enterococci to aminoglycosides.

Moderate species-specific intrinsic resistance to aminoglycosides in *E. faecium* is enhanced by a chromosomally encoded rRNA methyltransferase, EfmM (Galloway-Peña, Roh, Latorre, Qin, & Murray, 2012) that uses S-adenosyl methionine as a methyl donor to methylate a specific residue on 16S rRNA, in the context of the 30S ribosomal subunit. The inactivation of *efmM* in *E. faecium* increases susceptibility to the aminoglycosides kanamycin and tobramycin, and conversely, expression of a recombinant version of *efmM* in *Escherichia coli* confers enhanced resistance to these drugs. Addition of the 5-methyl to C1404 sterically hinders aminoglycoside binding. In addition, a chromosomally encoded 6′-N-aminoglycoside acetyltransferase (*aac(6′)-Ii)* confers low-

level intrinsic resistance (Costa, Galimand, Leclercq, Duval, & Courvalin, 1993). The physiological effects of these two factors seems to be additive, in the sense that mutation of both genes led to the largest reduction in resistance. However, it remains unclear if aminoglycoside resistance is the primary function of EfmM. The position of m5C1404 at the junction between the ribosomal A-site and the P-site in 16S rRNA suggests that this modification might also play a more basic role in protein synthesis by influencing codon-anticodon interactions.

High-level resistance to aminoglycosides (HLGR) is conferred by a mechanism distinct from those described above, and importantly, abolishes the synergistic bactericidal activity of aminoglycosides in combination with cell-wall–active agents that are important in the treatment of severe enterococcal infections, such as endocarditis. HLGR is usually acquired on a mobile element that encodes an aminoglycoside-modifying enzyme. Such enzymes can be phosphotransferases (APHs) that use ATP to phosphorylate a hydroxyl group on the antibiotic, acetyltransferases (AACs) that use acetyl-CoA to acetylate an amino group on the antibiotic, or nucleotidyltransferases (ANTs) that use ATP to adenylylate a hydroxyl group on the antibiotic. HLGR is most often associated with members of the APH(2′′)-I phosphotransferase family or the bifunctional AAC(6′)-Ie-APH(2′′)-Ia family that are encoded on various transposons or conjugative plasmids (reviewed in (Kak, Donabedian, Zervos, Kariyama, Kumon, & Chow, 2000)).

Rifampicin resistance

Rifampicin inhibits bacterial growth by binding to the beta subunit of RNA polymerase (RpoB) and preventing initiation of transcription (Wehrli, Knüsel, Schmid, & Staehelin, 1968). Rifampicin has been used for decades as part of an antibiotic cocktail to treat infections caused by *Mycobacterium tuberculosis*, and has recently found increasing use in the treatment of staphylococcal infections associated with indwelling medical devices, such as artificial joints. Most resistance to rifampicin results from mutations of specific sites in the gene encoding the beta subunit of the RNA polymerase, which reduces the affinity of rifampicin for the polymerase. Mutations in RpoB responsible for rifampicin resistance have been identified in numerous and diverse species of bacteria. Additionally, enzymatic inactivation of rifampicin has been observed in a handful of cases.

Although rifampicin has not been used extensively to treat enterococcal infections, acquired resistance to rifampicin is nonetheless common in enterococci—nearly 79% of 71 clinical isolates were found to exhibit rifampicin resistance (Andrews, Ashby, Jevons, Marshall, Lines, & Wise, 2000), as well as >57% of a diverse collection of isolates from six countries in Europe (Lautenbach, Schuster, Bilker, & Brennan, 1998). Presumably this is at least partially a consequence of commensal enterococci being exposed to rifampicin during treatment for non-enterococcal infections, but other as-yet-unknown factors may contribute to the occurrence of resistant enterococcal isolates as well. In *E. faecium*, substitutions in RpoB are associated with rifampicin resistance, and most of the identified RpoB polymorphisms have been previously implicated in conferring resistance in other species of bacteria. The biological cost of rifampicin resistance is variable, depending on the particular mutation in RpoB, as well as other potential compensatory mutations that may occur elsewhere in the genome (Enne, Delsol, Roe, & Bennett, 2004).

Spontaneous rifampicin-resistant mutants of *E. faecalis* and *E. faecium* are readily isolated *in vitro* (Kristich, Little, Hall, & Hoff, 2011). Mutations were identified in the *rpoB* gene of all such mutants at sites known to be associated with resistance to rifampicin in other species of bacteria. For two particular mutants, confirmation that the *rpoB* point mutations are indeed responsible for rifampicin resistance was obtained by expressing the mutant alleles in an otherwise rifampicin-susceptible host. One unexpected observation was that some RpoB mutations led to an alteration in cephalosporin resistance in an allele-specific manner. In particular, the *rpoB* H486Y mutation conferred a substantially enhanced resistance to cephalosporins in multiple lineages of *E. faecalis,* as well as in *E. faecium*, whereas other Rif-resistant substitutions in *rpoB* did not affect cephalosporin resistance. The mechanistic basis for this observation is not known, but the *rpoB* H486Y substitution may alter rates of transcription of genes that contribute to intrinsic cephalosporin resistance in enterococci.

Rifampicin resistance in some isolates of *E. faecium* can be reversed by inclusion of subinhibitory concentrations of daptomycin. This phenomenon could not be explained by 1) alteration of rifampicin transport into, or efflux from, the cell by daptomycin; 2) the direct inactivation of rifampicin; nor 3) mutations at the rifampicin-binding site in *rpoB* (Reynolds & Courvalin, 2005). The molecular explanation for this effect remains unknown and authors speculate that there may be another mechanism for rifampicin resistance besides the two known mechanisms (namely, mutation in the *rpoB* gene and rifampicin inactivation). This third mechanism has been dubbed "daptomycin-reversible resistance," and further investigation will be needed to define its basis.

Quinolone resistance

Quinolones generally exhibit only moderate activity against enterococci. Quinolones inhibit the growth of bacteria by interfering with DNA replication, specifically by binding to the type II topisomerases that control DNA supercoiling (DNA gyrase and DNA topoisomerase IV) and inhibiting their function, leading to lethal double-strand breaks in the DNA. Quinolone resistance in many species of bacteria occurs via mutations in the "quinolone resistance determining regions" of the genes that encode gyrase and topoisomerase IV. These mutations prevent efficient binding of the antibiotic to the enzyme, which enables DNA replication to continue despite the presence of the antibiotic. Such mutations have been observed in clinical and lab-derived quinolone-resistant isolates of enterococci (Kak, Donabedian, Zervos, Kariyama, Kumon, & Chow, 2000; Oyamada Y. , Ito, Inoue, & Yamagishi, 2006; Palmer, Daniel, Hardy, Silverman, & Gilmore, 2011; Werner, Fleige, Ewert, Laverde-Gomez, Klare, & Witte, 2010), and presumably act to confer enhanced quinolone resistance through a similar mechanism.

A second mechanism known to contribute to quinolone resistance in other species of bacteria is efflux of the antibiotic out of the cell. Such efflux is often a function of pumps with relatively broad or nonspecific substrate specificities, which are sometimes referred to as multidrug-resistance efflux pumps (MDRs). Genes encoding MDRs are usually found on the bacterial chromosome. Although the primary physiological functions for most MDRs remain unclear, these proteins are known to actively transport toxic compounds out of the cell. The genome of *E. faecalis* V583 is predicted to encode 34 MDRs (Davis, et al., 2001), which suggests that drug efflux may play an important role in antibiotic resistance. Two of these pumps have been experimentally implicated in promoting quinolone resistance. The first is EmeA, a homolog of *Staphylococcus aureus* MDR NorA. Mutation of EmeA resulted in modest increases in susceptibility to several compounds, including quinolones (Jung, et al., 2010), and treatment of wild-type *E. faecalis* OG1RF with known MDR inhibitors (reserpine, lansoprazole, and verapamil) inhibits the efflux of the quinolone norfloxacin, as well as the toxic compound ethidium bromide. The second pump is EfrAB, an ABC-type transporter that enhances resistance to a variety of structurally unrelated compounds, including quinolones, when expressed in *E. coli* (Lefort, Saleh-Mghir, Garry, Carbon, & Fantin, 2000). Its function in enterococci was not investigated.

A new mechanism of quinolone resistance has been identified—protection of DNA gyrase and topoisomerase IV from inhibition by quinolones. The activity is provided by members of the Qnr protein family that were originally identified in enterobacteria as a transmissible type of quinolone resistance (Mascher, Helmann, & Unden, 2006). The natural function of Qnr proteins remains unclear. These proteins are characterized by tandem pentapeptide repeats, and homologs of Qnr appear to be encoded in the genomes of various bacteria. Inactivation of a homolog of Qnr identified in the genome of *E. faecalis*, comprised of 42 predicted pentapeptide repeats, resulted in a modest decrease in resistance to fluoroquinolones. Overexpression of the corresponding gene yielded an increase in resistance. Furthermore, expression of the *E. faecalis* gene in heterologous organisms, including *S. aureus* and *Lactococcus lactis*, increased the level of quinolone resistance in those hosts (Arsène & Leclercq, 2007). Purified EfsQnr inhibited the ATP-dependent DNA supercoiling activity of *E. coli* gyrase (Hellinger, Rouse, Rabadan, Henry, Steckelberg, & Wilson, 1992), suggesting that EfsQnr may protect *E. faecalis* from the effects of quinolones by modulating the action of gyrase in cells.

Macrolide, lincosamide, and streptogramin resistance

Macrolides, lincosamides, and streptogramin antibiotics inhibit protein synthesis by binding to the 50S subunit of the ribosome. Macrolides and lincosamides are not used to treat enterococcal infections, but resistance to them is nonetheless widespread (Jonas, Murray, & Weinstock, 2001). The most common form of acquired resistance to macrolides is production of an enzyme that methylates a specific adenine in the 23S rRNA of the 50S ribosomal subunit, which reduces the binding affinity of the macrolide for the ribosome. This modification also reduces the binding of lincosamide and streptogramin B antibiotics to the ribosome. The responsible enzyme is typically encoded by the *ermB* gene, and the phenotype is often referred to as MLS_B. An efflux pump, encoded by the transferrable *mefA* gene, is also known to pump macrolides out of the cell, but confers a lower level of resistance than *ermB* (Clancy, et al., 1996).

E. faecalis and *E. faecium* are known to exhibit different intrinsic susceptibilities to quinupristin-dalfopristin (Q-D), members of the streptogramin family that act synergistically. *E. faecalis* is sufficiently intrinsically resistant that these antibiotics cannot be used therapeutically, whereas *E. faecium* is usually susceptible. The molecular basis for this difference appears to stem from the existence of a chromosomally encoded putative ABC transporter in the *E. faecalis* genome, named Lsa (198). Lsa is encoded in the genome of all isolates of *E. faecalis* evaluated (n=180), but in none of the genomes of other enterococci (n=189). Disruption the *lsa* gene in *E. faecalis* OG1RF resulted in a >40-fold decrease in MIC to Q-D. In addition, expression of the *E. faecalis* V583 *lsa* gene in otherwise susceptible *E. faecium* led to a 6-fold increase in MIC. Expression of *lsa* in the heterologous host *Lactococcus lactis* also moderately increased Q-D resistance (Singh & Murray, 2005). Further support for a role of Lsa in Q-D resistance stems from the observation that clinical isolates of *E. faecalis* that are susceptible to lincosamides and dalfopristin harbor mutations in *lsa* that result in premature stop codons (Dina, Malbruny, & Leclercq, 2003). However, the influence of Lsa on efflux or transport of the antibiotics has not been directly evaluated. Such studies will be helpful in determining the mechanism by which Lsa provides resistance, as the protein itself contains ATP-binding domains characteristic of ABC transporters, but lacks identifiable transmembrane sequences that would be expected of an authentic efflux pump. As such, the molecular mechanism of Lsa action remains unknown. Acquired resistance to Q-D has emerged in *E. faecium*, and is mediated by members of the streptogramin acetyltransferase family of enzymes that acetylate streptogramin A (such as VatH), and by the Vga genes, which encode an ABC transporter that presumably function to export the antibiotic from the cell (Kak & Chow, 2002).

Beta-lactam resistance

Antibiotics in the beta-lactam family inhibit bacterial growth by serving as suicide substrates for the D,D-transpeptidases (also known as penicillin-binding proteins, or PBPs) that catalyze cross-linking of peptidoglycan peptide side chains during the synthesis of mature peptidoglycan. Once modified by a beta-lactam antibiotic, PBPs are inactivated, thereby preventing continued cell wall synthesis. Enterococci exhibit intrinsic nonsusceptibility to beta-lactam antibiotics, but the extent of nonsusceptibility varies among the different classes of beta-lactams and between enterococcal species: penicillins have the most activity against enterococci (and *E. faecium* is inherently a bit more resistant than *E. faecalis*), carbapenems slightly less, and cephalosporins exhibit the least activity. This spectrum of activity is reflected in the utility of these drugs for treatment, insofar as ampicillin remains an effective therapy for susceptible enterococcal infections, but cephalosporins are completely ineffective against enterococci. In fact, prior use of cephalosporins is a major risk factor for the acquisition of an enterococcal infection (reviewed in (Shepard & Gilmore, 2002)).

The intrinsic nonsuceptibility of enterococci to beta-lactams involves the production of the low-affinity class B penicillin-binding protein 5 (Pbp5), an ortholog of the low-affinity Pbp2a expressed by methicillin-resistant isolates of *Staphylococcus aureus* (Gonzales, Schrekenberger, Graham, Kelkar, DenBesten, & Quinn, 2001). Due to its relatively low affinity for beta-lactams, the chromosomally encoded Pbp5 is capable of carrying out

peptidoglycan synthesis at concentrations of beta-lactam antibiotics that saturate all of the other enterococcal PBPs (Canepari, Lleò, Cornaglia, Fontana, & Satta, 1986), and therefore is required for beta-lactam resistance (Arbeloa, et al., 2004; Rice L. B., Carias, Rudin, Lakticová, Wood, & Hutton-Thomas, 2005). Pbp5 is required for both the intrinsic beta-lactam resistance traits of enterococci, such as their intrinsic cephalosporin resistance, as well as for the acquired (enhanced) resistance to members of the beta-lactam family (such as ampicillin), to which enterococci are otherwise clinically susceptible. Below, we discuss resistance to two different families of beta-lactam antibiotics (cephalosporins and ampicillin) in more detail.

Intrinsic cephalosporin resistance

E. faecalis and *E. faecium* are naturally (intrinsically) resistant to cephalosporins. This trait has been known for decades and is encoded by chromosomal determinants in the core genome of these organisms, but its molecular basis remains incompletely understood. Thus far, in *E. faecalis*, a handful of genetic determinants have been shown to be required for intrinsic cephalosporin resistance: the low-affinity penicillin-binding protein Pbp5; a two-component signal transduction system, CroRS; a transmembrane Ser/Thr kinase, IreK; and one of the early enzymes involved in synthesis of peptidoglycan precursors, MurAA. The role of these determinants has been best studied in *E. faecalis*, although it appears likely that similar mechanisms are present in in *E. faecium*.

pbp5

Genetic studies on isogenic mutants of *E. faecalis* and *E. faecium* provide clear evidence of a requirement for Pbp5 in intrinsic cephalosporin resistance (Arbeloa, et al., 2004; Rice L. B., Carias, Rudin, Lakticová, Wood, & Hutton-Thomas, 2005). Deletion mutants that lack *pbp5* exhibit large reductions in the level of cephalosporin resistance. The deletion mutants also exhibit a reduction in resistance to the non-cephalosporin beta-lactam, ampicillin, although the magnitude of the reduction is more modest (especially for *E. faecalis*). High-molecular-weight PBPs are often categorized as Class A (bifunctional, exhibiting both transglycosylase and transpeptidase activities) and Class B (monofunctional, exhibiting transpeptidase activity but lacking transglycosylase activity). As a Class B PBP, Pbp5 contains a transpeptidase domain, but lacks a transglycosylase domain, which is necessary for the initial polymerization of the disaccharide moiety of peptidoglycan precursors. Therefore, even though Pbp5 is capable of synthesizing crosslinks between the peptide side chains of peptidoglycan, Pbp5 must cooperate with one or more transglycosylases for cell wall biosynthesis. Candidate transglycosylase partners include the three bifunctional Class A enterococcal PBPs (or in principle, monofunctional transglycosylases analogous to Mgt of *Staphylococcus aureus*, although no examples have yet been identified in enterococci). Analysis of isogenic deletion mutants lacking one or more Class A PBPs in both *E. faecalis* and *E. faecium* revealed that Pbp5 cooperates with one or both Class A PBPs, either PbpF or PonA, to permit growth in the presence of cephalosporins. The third Class A PBP (PbpZ) encoded by these organisms is unable to provide transglycosylase activity in the presence of cephalosporins. Triple mutants of either *E. faecalis* or *E. faecium* that lack all three class A PBPs are viable (although susceptible to cephalosporins), which indicates that as-yet-unidentified additional transglycosylases capable of peptidoglycan polymerization must exist. Given that double mutants lacking *pbpF* and *ponA* are susceptible to cephalosporins, it appears that these unidentified transglycosylases do not have the capacity to participate in functional interactions with Pbp5. Of note, while much of the PBP deletion analysis indicates substantial similarities in the underlying mechanisms of Pbp5 function in *E. faecalis* and *E. faecium*, deletion of the Class A PBPs in *E. faecium* revealed an unexpected dissociation between the expression of resistance to ceftriaxone and ampicillin that was not observed in *E. faecalis* (Rice L. B., Carias, Rudin, Lakticová, Wood, & Hutton-Thomas, 2005), suggesting that Pbp5-mediated crosslinking of peptidoglycan in *E. faecium* was differentially susceptible to beta-lactams, depending upon partner glycosyltransferase.

croRS

The genome of *E. faecalis* V583 encodes 17 two-component signal transduction systems (TCSs) (Hancock & Perego, 2004). Systematic inactivation of these TCSs and phenotypic characterization of the mutants revealed that the inactivation of CroRS rendered *E. faecalis* susceptible to extended spectrum cephalosporins, but not to a panel of antibiotics that perturb other cellular processes (Hartmann, et al., 2010). These results are consistent with studies in another lineage of *E. faecalis*, in which deletion of the genes encoding the CroRS TCS resulted in a loss of resistance to ceftriaxone (Comenge, et al., 2003). Further analysis of the deletion mutant revealed that the loss of CroRS function did not alter the expression of Pbp5, peptidoglycan precursor production, or peptidoglycan crosslinking. The CroRS TCS appeared to function according to conventional models for TCSs, in that the CroS kinase could autophosphorylate itself in an ATP-dependent manner, followed by the transfer of the phosphoryl group to the CroR response regulator. The CroR response regulator contains a functional DNA binding domain, which suggests that transcriptional remodeling is necessary for adaptation to the stress imposed by cephalosporins. However, only a few genes controlled directly by CroR have been identified thus far. These include *salB*, encoding a secreted protein that does not contribute to cephalosporin resistance (Murray, 1990), *croRS* itself (Murray, 1990), and genes that encode a putative glutamine transporter (Lebreton, et al., 2011) with no obvious connection to cephalosporin resistance. Treatment of *E. faecalis* cells with a panel of cell-wall-active antibiotics resulted in induction of a CroR-dependent promoter, but nothing more is known about the nature of the physiological signal(s) that influences CroS kinase regulation of cephalosporin resistance.

ireK

In addition to the CroRS TCS, a second signal transduction protein (IreK) is required for cephalosporin resistance in *E. faecalis*. IreK exhibits a characteristic bipartite domain architecture that includes a "eukaryotic-type" Ser/Thr kinase coupled, through a putative transmembrane segment, to a series of five repeats of the PASTA domain. Homologs of IreK are nearly universal among the genomes of Gram-positive bacteria. The function of the extracellular PASTA domains is not well understood, but it has been proposed that they bind to peptidoglycan or fragments thereof (Moellering, Jr. & Weinberg, 1971; Squeglia, et al., 2011; Yeats, Finn, & Bateman, 2002), which suggests that IreK could serve as a transmembrane receptor kinase that senses damage or perturbation of the peptidoglycan and initiates a signaling circuit to restore cell wall integrity. Consistent with that view, a homolog of IreK in *Bacillus subtilis* (PrkC) responds to fragments of peptidoglycan released by growing cells, which signals exit from dormancy by *B. subtilis* spores (Shah, Laaberki, Popham, & Dworkin, 2008). Analysis of an *E. faecalis* deletion mutant revealed that IreK is required for intrinsic cephalosporin resistance and for resistance to certain other cell-envelope stresses, such as detergents that are present in bile salts (Kuch, et al., 2012). IreK exhibits protein kinase activity *in vitro*, and its kinase activity is required to promote cephalosporin resistance in *E. faecalis* cells (Kristich, Wells, & Dunny, 2007). As with other members of this kinase family, IreK can catalyze autophosphorylation of threonine residues contained with a specific segment of the kinase domain known as the "activation loop". Phosphorylation at these sites is usually thought to lead to a conformational change, which results in enhanced activity of the kinase (i.e., "activation"). Analysis of site-directed mutants bearing phosphomimetic substitutions at these sites in IreK support the hypothesis that phosphorylation of the IreK activation loop enhances kinase activity *in vivo* and leads to increased cephalosporin resistance (Kristich, Wells, & Dunny, 2007). Other than itself, physiological substrates of IreK in *E. faecalis* cells that are important for cephalosporin resistance have not yet been described.

E. faecalis IreK and its homologs in other low-GC Gram-positive bacteria are encoded immediately adjacent to a gene that encodes a PP2C-type protein phosphatase (called IreP in *E. faecalis*). IreP can dephosphorylate both IreK and substrates of IreK *in vitro*, and analysis of deletion mutants lacking IreP indicate that this activity is important *in vivo*. IreP mutants exhibit substantial hyperresistance to cephalosporins, a finding which is consistent with hyperactivation of the IreK kinase (Kristich, Wells, & Dunny, 2007). Furthermore, mutants that lack IreP exhibit a large reduction in fitness in the absence of cephalosporins, as compared to wild-type *E.*

faecalis, which indicates that uncontrolled activation of cephalosporin resistance mechanisms imparts a significant fitness cost to the cell. The complex regulatory circuitry controlling intrinsic cephalosporin resistance in *E. faecalis* may therefore stem from the fitness cost that is associated with expression of this phenotype.

MurAA

A recent transposon mutagenesis screen in *E. faecalis* revealed a new determinant of intrinsic cephalosporin resistance in enterococci (Vesić & Kristich, 2012). As with most low-GC Gram-positive bacteria, the genome of *E. faecalis* encodes two homologs (annotated as MurAA and MurAB) of the enzyme that catalyzes the first committed step in the synthesis of the peptidoglycan precursor UDP-N-acetylglucosamine 1-carboxyvinyl transferase, which performs PEP-dependent conversion of UDP-N-acetylglucosamine to UDP-N-acetylglucosamine-enolpyruvate. Deletion of *murAA*, but not *murAB*, led to an increased susceptibility of *E. faecalis* to cephalosporins. This enhanced cephalosporin susceptibility does not reflect a general growth or cell-wall synthesis defect of the mutant, because the deletion mutant is not sensitized to antibiotics in general—or even to all antibiotics that inhibit cell wall biosynthesis—but exhibits a loss of resistance specifically for extended spectrum cephalosporins and for fosfomycin (an antibiotic known to target MurA homologs). Chemical genetic analysis revealed synergistic action of ceftriaxone with fosfomycin that was also observed with two strains of *E. faecium*, suggesting that MurAA of *E. faecium* functions in a similar manner to promote cephalosporin resistance. In addition, expression of *murAA*-enhanced cephalosporin resistance in an *E. faecalis* mutant that lacked IreK, which suggests that MurAA may function downstream of IreK in a pathway that leads to cephalosporin resistance. Further genetic analysis revealed that MurAA catalytic activity is necessary, but not sufficient, for this role.

Ampicillin resistance

Modifications in Pbp5 are associated with increased resistance to beta-lactams, such as ampicillin. For example, the Pbp5-encoding gene found in hospital-associated, ampicillin-resistant strains of *E. faecium* differs by ~5% from the corresponding gene in community-associated, ampicillin-susceptible strains (Garnier, Taourit, Glaser, Courvalin, & Galimand, 2000). Most studies that report an association between mutations in Pbp5 and enhanced ampicillin resistance have been performed on non-isogenic clinical isolates, in which unknown factors other than Pbp5 may influence resistance. To circumvent this limitation, Rice and colleagues (Rice, Calderwood, Eliopoulos, Farber, & Karchmer, 1991) used a plasmid-based *pbp5* expression system to explore the impact of specific amino acid substitutions in Pbp5 on ampicillin resistance.

Substitutions that had previously been implicated as contributing to ampicillin resistance in clinical strains conferred modest levels of resistance when expressed from the plasmid-borne *pbp5* in an otherwise-susceptible *E. faecium* host, thereby providing direct evidence of their influence. Combinations of point mutations, especially Pbp5 M485A with a Ser insertion at position 466, yielded substantially enhanced levels of resistance. Furthermore, a correlation was established between the affinity of purified, recombinant Pbp5 mutants for antibiotic binding, with resistance levels provided by these alleles. Further analysis revealed that the chromosomally-encoded *pbp5* determinant could be transferred between strains of *E. faecium* (Rice L. B., 2005) by conjugation, which suggests a mechanism by which high-level ampicillin resistance conferred by mutant *pbp5* alleles could be disseminated among clinical isolates. Although the mechanism of conjugative transfer was not established in that study, a plausible mechanism could involve mobilization mediated by co-integrated enterococcal conjugative plasmids, as recently described in *E. faecalis* (Marshall, Donskey, Hutton-Thomas, Salata, & Rice, 2002). Similar to *E. faecium*, mutations in Pbp5 of clinical isolates of *E. faecalis* may also lead to enhanced resistance to beta-lactam antibiotics, such as imipenem and ampicillin (Oyamada Y. , et al., 2006).

L,D transpeptidation

A multi-step *in vitro* selection process was used to generate a highly ampicillin-resistant mutant of *E. faecium* (Mainardi, et al., 2002). The mutant was found to contain exclusively L-Lys-3-D-Asx-L-Lys cross-links in its peptidoglycan (a product of L,D-transpeptidation, rather than the typical D,D-transpeptidation carried out by PBPs), and peptidoglycan composition was unaffected by the presence of ampicillin. Analysis of peptidoglycan composition of strains with intermediate levels of resistance obtained during the selection process revealed that the balance between D,D-transpeptidation and L,D-transpeptidation influences ampicillin resistance (Manson, Hancock, & Gilmore, 2010). High-level resistance requires elevated activity of a beta-lactam insensitive D,D-carboxypeptidase, which cleaves the peptide stem termini of normal peptidoglycan precursors to generate the substrate for the L,D transpeptidase. The metallo-D,D-carboxypeptidase (DdcY) responsible for cleavage of the peptidoglycan precursors has been identified (183), and its enhanced expression appears to be mediated by mutations that result in activation of a putative cryptic two-component signal transduction system (DdcSR). The enzyme responsible for the formation of the L,D- crosslinks (called Ldt_{fm}) has been identified (Mainardi, Gutmann, Acar, & Goldstein, 1995) and appears to be constitutively expressed in *E. faecium*. Ldt_{fm} is evolutionarily unrelated to the D,D-transpeptidases, in that it uses an active-site Cys nucleophile, rather than a Ser nucleophile. In addition, homologs of Ldt_{fm} are encoded in the genomes of a variety of Gram-positive bacteria (Mainardi, et al., 2005; Mainardi, Gutmann, Acar, & Goldstein, 1995). The normal physiological function of the L,D-transpeptidase is unclear, but it has been proposed to have a role in the maintenance of peptidoglycan structure in stationary-phase cells. Surprisingly, although beta-lactams are usually thought to specifically inhibit the D,D-transpeptidase activity of PBPs, a particular sub-class of the beta-lactam antibiotic family was found to inhibit Ldt_{fm} via covalent modification of the active site Cys (Mainardi, Legrand, Arthur, Schoot, van Heijenoort, & Gutmann, 2000). As noted above, activation of the L,D transpeptidase cross-linking pathway can result in emergence of cross-resistance to beta-lactams and glycopeptides.

Linezolid resistance

Linezolid is a member of the oxazolidinone family of antibiotics developed for use against multidrug-resistant Gram-positive bacteria. Linezolid interferes with bacterial growth by inhibiting protein synthesis through interaction with the translational initiation complex. Resistance to linezolid can be selected *in vitro*, and has also been observed in clinical settings (89, 164). Mutations within the central loop of domain V of 23S rRNA, including a G2576U mutation, are associated with resistance to linezolid, and presumably prevent or reduce binding of the antibiotic to the ribosomal subunit. Analysis of linezolid-resistant *E. faecalis* and *E. faecium* isolates selected during therapy, or obtained from patients following linezolid treatment failure, revealed a direct correlation between the percentage of rRNA genes that carry a G2576U mutation (each genome encodes several copies of the rRNA genes—four in *E. faecalis* and six in *E. faecium*) and the phenotypic level of linezolid resistance, which suggests that the percentage of ribosomes that carry rRNA with the G2576U substitution is the primary determinant for the level of linezolid resistance (Martínez-Martínez, Pascual, & Jacoby, 1998). A similar correlation was observed for linezolid-resistant mutants of *E. faecalis* selected *in vitro* in a recombination-proficient genetic background (Lu, Chang, Perng, & Lee, 2005). Attempts to derive linezolid resistant mutants in a recombination-deficient mutant genetic background did not yield resistant mutants at comparable frequencies. Collectively, these results suggest that recombination between rRNA genes after the emergence of the G2576U mutation may enable the amplification of the level of linezolid resistance in enterococci under the selective pressure imposed by antibiotic treatment.

Enterococci are Generous with Their Genes: Mobile Genetic Elements Enable Facile Horizontal Transfer and Spread of Antibiotic Resistance Determinants Among Enterococci

Gene mobility and exchange have figured prominently in the rise of the enterococcus as a nosocomial pathogen. It is interesting to note that *E. faecalis* OG1X, a fully susceptible strain studied for decades by Don Clewell and others, was found to have no mobile elements and no acquired DNA when its genome was completely sequenced (Bourgogne, et al., 2008). This contrasts sharply with the genome of *E. faecalis* V583, one of the first vancomycin-resistant clinical isolates, in which approximately 25% of its genome consists of acquired DNA (Polidori, et al., 2011). Clearly, existence in clinical settings and the selective pressure of antibiotics in the environment have yielded clinical isolates that have an array of mechanisms to acquire and exchange DNA.

Plasmids

Enterococci were among the first Gram-positive bacteria to have extensively characterized plasmids (Table 1). While a range of different types of plasmids are undoubtedly present in clinical enterococcal strains, the most frequently studied plasmids in the area of antimicrobial resistance are the pheromone-responsive plasmids and the broad host range plasmids (Panesso, Abadía-Patiño, Vanegas, Reynolds, Courvalin, & Arias, 2005). (For more details on plasmid structure and movement, see Extrachromosomal and Mobile Elements in Enteroocci.) The most thoroughly studied pheromone responsive plasmids include pAD1 (Ehrenfeld & Clewell, 1987) and pCF10 (Christie, Korman, Zahler, Adsit, & Dunny, 1987). These plasmids encode responses to small peptide fragments known as pheromones, which leads to the coating of the donor cells with aggregation substance, a sticky protein that promotes clumping of donor and recipient cells and that facilitates the efficient transfer of plasmids (with frequencies as high as 10^{-2}-10^{-1}/recipient CFU). Mating pair aggregation can be observed as the macroscopic clumping of cells in liquid media, and high-frequency transfer is observed both in liquid and on solid media. pAD1 does not encode resistance to any antibiotics, but does encode the production of cytolysin (Segarra, Booth, Morales, Huycke, & Gilmore, 1991), which serves as a virulence factor in all models tested (Clewell, 2007). pCF10 harbors the tet(M) conjugative transposon Tn*925,* which encodes resistance to tetracycline and minocycline Pheromone-responsive plasmids have also been implicated in the transfer of VanB-type vancomycin resistance (Zheng, Tomita, Inoue, & Ike, 2009).

The most completely studied of the pheromone-responsive plasmids have had a host range that appears to be restricted to *E. faecalis*. However, early in the vancomycin resistance era, the transfer of VanA-type vancomycin resistance from *E. faecium* to *E. faecalis* was reported to result from either the transfer of a VanA-encoding pheromone responsive plasmid or through the cointegration of a VanA plasmid and a pheromone-responsive plasmid (Heaton, Discotto, Pucci, & Handwerger, 1996; Hegde, Vetting, Mitchenall, Maxwell, & Blanchard, 2011). Regardless of their origin, pheromone-responsive plasmids described to date do not appear to be able to replicate in non-enterococcal species.

The other major class of enterococcal plasmids associated with the transfer of antimicrobial resistance genes are the so-called "broad host-range," or Inc18 plasmids (Bruand, Chatelier, Ehrlich, & Janniere, 1993). The prototypes for these plasmids are pAMβ1, which encodes resistance to macrolides, and pIP501, which confers resistance to macrolides and chloramphenicol. These plasmids have been shown to transfer to other species (including staphylococci and streptococci), and then to transfer back into enterococci *in vitro*. The frequency of transfer is considerably lower than that seen with pheromone-responsive plasmids, and requires that matings be performed on solid surfaces, as a means of providing cell-cell contact.

Inc18 broad host-range plasmids, or their remnants, have been found in association with a range of different resistance determinants. Tn*5385*, a large, composite, transferable, multi-resistant (streptomycin, tetracycline, gentamicin, erythromycin, β-lactamase) element found in the chromosome of *E. faecalis* CH116 (173), includes

sequences identical to the replication region of pAM β1. Other Inc18 plasmids have been implicated in the transfer of the VanA resistance determinant from *E. faecalis* to *S. aureus* (Zhu, et al., 2010). Inc18 plasmids that mediate vancomycin resistance do not appear to be stable in *S. aureus*, but vancomycin resistance can be stabilized by transposition from the broad host range plasmid to one of the staphylococcal plasmids or to the staphylococcal chromosome.

Table 1. Prototype plasmids and transposons of Enterococci

Plasmid type	Examples	References
Pheromone-responsive	pAD1, pCF10	(Christie, Hammond, Reising, & Evans-Patterson, 1994; Ehrenfeld & Clewell, 1987)
Broad host range (Inc18)	pAMβ1, pIP501	(Qu, Yang, Shen, Wei, & Yu, 2012)
Large, virulence encoding	pLRM23, pLG1	(Le Breton, Muller, Auffray, & Rincé, 2007; Rice & Marshall, 1992)
Transposon type	Examples	References
Composite	Tn*5281*, Tn*5384*, Tn*5385*	(Hong, Hutchings, & Buttner, 2008; Rice L. B., Carias, Hutton-Thomas, & Rudin, 2007; Rice L. B., et al., 2009)
Tn3-family	Tn*917*, Tn*552*, Tn*1546*	(Arthur, Molinas, Depardieu, & Courvalin, 1993; Rowland & Dyke, 1990; Tomich, An, & Clewell, 1980)
Conjugative	Tn*916*, Tn*5382*	(Carias, Rudin, Donskey, & Rice, 1998; Clewell, et al., 1991)

Transposons

Many varieties of transposons have played important roles in the emergence and spread of antimicrobial resistance in enterococci. As more strains become sequenced, it is clear that the enterococcal genome is quite plastic, with mobile elements liberally sprinkled throughout and implicated in a variety of genome rearrangements and transfers. Much of the work to define specific transposons has involved elements that encode resistance to antimicrobial agents, for their obvious clinical importance and for the relative ease of following the movement of such elements. It is now clear, however, that enterococcal transposons confer mobility to a variety of determinants that impact various phenotypes, such as virulence, colonization ability and cell-to-cell communication.

Enterococcal transposons can be broadly classified into three basic categories: composite transposons;, Tn*3*-family transposons, and conjugative transposons (Table 1). Composite transposons are those elements whose mobility is conferred by flanking insertion sequences, or IS elements. In general, the IS elements that form the ends of a composite transposon are identical, and may be oriented as direct or as inverted repeats. Several composite transposons have been described in enterococci, and perhaps the most commonly identified composite transposon are those of the Tn*5281* type (Hong, Hutchings, & Buttner, 2008). Tn*5281* encodes resistance to all aminoglycosides except streptomycin. It consists of the *aac*-6′/*aph*-2′′ bifunctional aminoglycoside-modifying enzyme gene flanked by inverted copies of the insertion sequence IS*256*. As originally described, Tn*5281* has two IS*256* copies on one end and one on the other. In most respects, it is identical to Tn*4001*, a staphylococcal composite transposon in which the bifunctional gene is flanked by single inverted copies of IS*256* (Magnet, et al., 2007). The bifunctional enzyme is responsible for the vast majority of gentamicin resistance observed in enterococci, and it is found almost exclusively in the context of Tn*5281*-like structures.

Tn*1547* is a composite element conferring mobility to the VanB glycopeptide resistance determinant in *E. faecalis* (Rand, Houck, & Silverman, 2007). This 64kb element owes its mobility to flanking copies of IS*16* and an IS*256*-like element. Although Tn*1547* was the first of the VanB glycopeptide resistance elements in enterococci to be

described, subsequent work has shown that the VanB operon is far more frequently associated with Tn*5382*-like elements (Bjørkeng, Rasmussen, Sundsfjord, Sjöberg, Hegstad, & Söderquist, 2011; Dahl, Lundblad, Rokenes, Olsvik, & Sundsfjord, 2000; Lyon, May, & Skurray, 1984).

An interesting example of how IS elements can yield a variety of different mobile elements can be observed with Tn*5385*, a multi-resistance composite element identified in *E. faecalis* (Figure 2). Tn*5385* is a 65kb mobile element whose ends are composed of directly-repeated copies of IS*1216*. Several other putative transposons lie within the larger element, including Tn*4001*, which confers gentamicin resistance, and Tn*5384*, a 26kb element formed by one IS*256* terminus of Tn*4001* and a second IS*256* located 26kb away (Rice L. B., et al., 2009). Between these two IS elements lay determinants for gentamicin, macrolide, and mercury resistance, which makes Tn*5384* a multi-resistance mobile element. Although not conjugative by itself, Tn*5384* is capable of transposing from the *E. faecalis* chromosome to a transferable plasmid. Also included within the larger Tn*5385* are a Tn*916*-like conjugative transposon designated Tn*5381* and an element identical to the staphylococcal β–lactamase transposon Tn*552* (Rice L. B., Carias, Hutton-Thomas, & Rudin, 2007). The mechanisms by which Tn*5385* transfers between enterococcal strains has not been well delineated, but there are data to suggest that its insertion into the recipient chromosome can occur either through homologous recombination across flanking sequences, or through recombination across internal mobile elements and similar structures in the recipient chromosome.

The first Tn*3*-family element described in enterococci was Tn*917*, a 5kb element that confers resistance to macrolides, lincosamides, and streptogramin B through expression of the *ermB* gene (Shaw & Clewell, 1985). Sequence analysis showed that Tn*917* was identical to the macrolide-resistance transposon Tn*551* described in *S. aureus* (Wu, de Lencastre, & Tomasz, 1999). Tn*917* transposes through a replicative mechanism, and resistance expression is repressed by translational attenuation inducible by exposure to erythromycin (but not clindamycin) (Hall, Steed, Arias, Murray, & Rybak, 2012). Neither Tn*917* nor any other Tn*3*-family element encodes transfer functions.

A second Tn*3*-family element that has been identified, albeit rarely, in enterococci is the staphylococcal β–lactamase transposon Tn*552* (Sacco, et al., 2010). Tn*552* expression of β–lactamase is inducible in staphylococci by exposure to penicillin and other related drugs. It is interesting that in most of the β–lactamase-producing *E. faecalis* isolates that have been described, the regulation genes found upstream of the structural β–lactamase gene have been deleted or inactivated, which suggests that the expression of the regulation genes may reduce the fitness of those enterococcal isolates (Ono, Muratani, & Mastumoto, 2005; Rowland & Dyke, 1990; Tomayko, Zscheck, Singh, & Murray, 1996). The consequence of loss of the induction method is that β–lactamase in these strains is expressed constitutively, but at very low levels, resulting in only a modest impact on susceptibility of the isolates. Perhaps the fact that little resistance advantage is achieved explains the rarity with which such strains are isolated. A recent report identified an identical β–lactamase, including the regulatory gene cluster, in eight strains of *E. faecium* (Sarti, Campanile, Sabia, Santagati, Gargiulo, & Stefani, 2012). The impact of this β–lactamase expression on ampicillin susceptibility was marginal, given the higher levels of intrinsic resistance to β–lactams that is characteristic of this species.

The Tn*3*-family transposon that has had the greatest impact on the management of enterococcal infections over the past two decades is Tn*1546*, an 11kb element that encodes the VanA-type glycopeptide resistance operon (Arthur, Molinas, Depardieu, & Courvalin, 1993). As with other resistance genes in Tn*3*-family transposons found in enterococci, the expression of the VanA operon is inducible by exposure; in this case, exposure to glycopeptide antibiotics (Arthur, Molinas, & Courvalin, 1992). The VanA operon is always found in the context of Tn*1546*, although as more clinical isolates are sequenced, increasing numbers of variants (generally created by the insertion of IS elements within parts of the transposon, sometimes with deletion of non-essential segments) are reported. In one recent report, Tn*1546* was modified by insertion of the *fosB* gene, which resulted in an element that confers resistance to both vancomycin and fosfomycin (Quintiliani, Jr. & Courvalin, 1996). Tn*1546*

can be found on both the chromosome and transferable plasmids. Interspecies transfer of these Inc18-type VanA-encoding plasmids has been examined *in vitro*, and the rate of transfer is considerably lower than that seen between enterococcal strains (Werner, et al., 2011). Transfer of vancomycin resistance to *S. aureus* has involved exclusively Tn*1546*-like elements, and in most cases is associated with Inc18-type plasmids (Zhu, et al., 2010).

Conjugative transposons were described at roughly the same time in enterococci and *Streptococcus pneumoniae*. The first enterococcal conjugative transposon described was Tn*916*, which is 18kb in length and confers resistance to tetracycline and minocycline through the *tet(M)* gene (Friedman, Alder, & Silverman, 2006). Its pneumococcal counterpart is Tn*1545* (Courvalin & Carlier, 1987)—and although it was several kb larger than Tn*916* and conferred resistance to macrolides and chloramphenicol in addition to tetracycline and minocycline, Tn*1545* had identical ends and genes that conferred excision and integration capabilities (Clewell, Flannagan, & Jaworski, 1995). Conjugative transposons excise from their point of origin and transfer to their target sites without replication; as a result, their transposition is considered to be conservative. The circular intermediate that forms by excision appears to have heteroduplex DNA at its "joint" region, and the formation of this circle allows transcription to proceed through to the putative conjugation genes (Celli & Trieu-Cuot, 1998). As a result, excision stimulates intercellular transfer. Their host ranges appear to be broad and insertion specificity lax, although they generally appear to target regions that are rich in adenines and thymidines.

Tn*916*-like elements appear to be the primary mechanism by which *tet(M)* genes spread among Gram-positive bacteria. They can transfer to Gram-negative bacteria *in vitro* and their remnants have been found around *tet(M)* genes present in tetracycline-resistant strains of *Neisseria gonorrhoeae* (Swartley, McAllister, Hajjeh, Heinrich, & Stephens, 1993). They encode their own conjugation genes, although they may also transfer between strains incorporated into transferable plasmids. To date, no compelling data exist that conjugative transposons can mobilize other replicons.

The first expansion of the Tn*916*-family transposons came with the description of Tn*5382*, which is an approximately30 kb element that encodes the VanB glycopeptide-resistance operon and has substantial homology with the Tn*916* integrase and excisase genes (Carias, Rudin, Donskey, & Rice, 1998). It also transposes through a circular intermediate. It is able to transfer between enterococcal strains, but in most cases, this transfer appears to occur within larger segments of transferred chromosomal DNA. A likely identical transposon was fully sequenced after the discovery of Tn*5382*, was designated Tn*1549* (Gavaldà, et al., 2007), and is frequently referred to as Tn*5382*/*1549*. Molecular studies of diverse enterococcal strains suggest that Tn*5382*/*1549* is the main structure within which the VanB glycopeptide resistance operon is located (Bjørkeng, Rasmussen, Sundsfjord, Sjöberg, Hegstad, & Söderquist, 2011; Dahl, Lundblad, Rokenes, Olsvik, & Sundsfjord, 2000; Lyon, May, & Skurray, 1984).

With the availability of increasing numbers of fully sequenced genomes, it has become clear that Tn*916*-family transposons are present in many strains and incorporate a variety of different genetic structures. One such transposon, Tn*5386*, is located in the genome of *E. faecium* strain D344R and was shown to interact with a second Tn*916*-like element to yield the deletion of a large segment of chromosome that includes the *pbp5* ampicillin resistance determinant, thereby rendering this *E. faecium* mutant susceptible to ampicillin (Rice L. B., Carias, Marshall, Rudin, & Hutton-Thomas, 2005; Rice, Carias, & Marshall, 1995). Tn*916* itself has been shown to mediate significant changes in the pulsed field patterns of bacterial strains, which suggests that these elements may be major drivers of genomic evolution (Thal, Silverman, Donabedian, & Zervos, 1997). Tn*5386* does not encode tetracycline/minocycline resistance. Instead, what appears to be a bacteriocin immunity operon is located in the place of *tet(M)* . As more genomes sequences are made available for analysis, there is little doubt that the variety of these elements will increase.

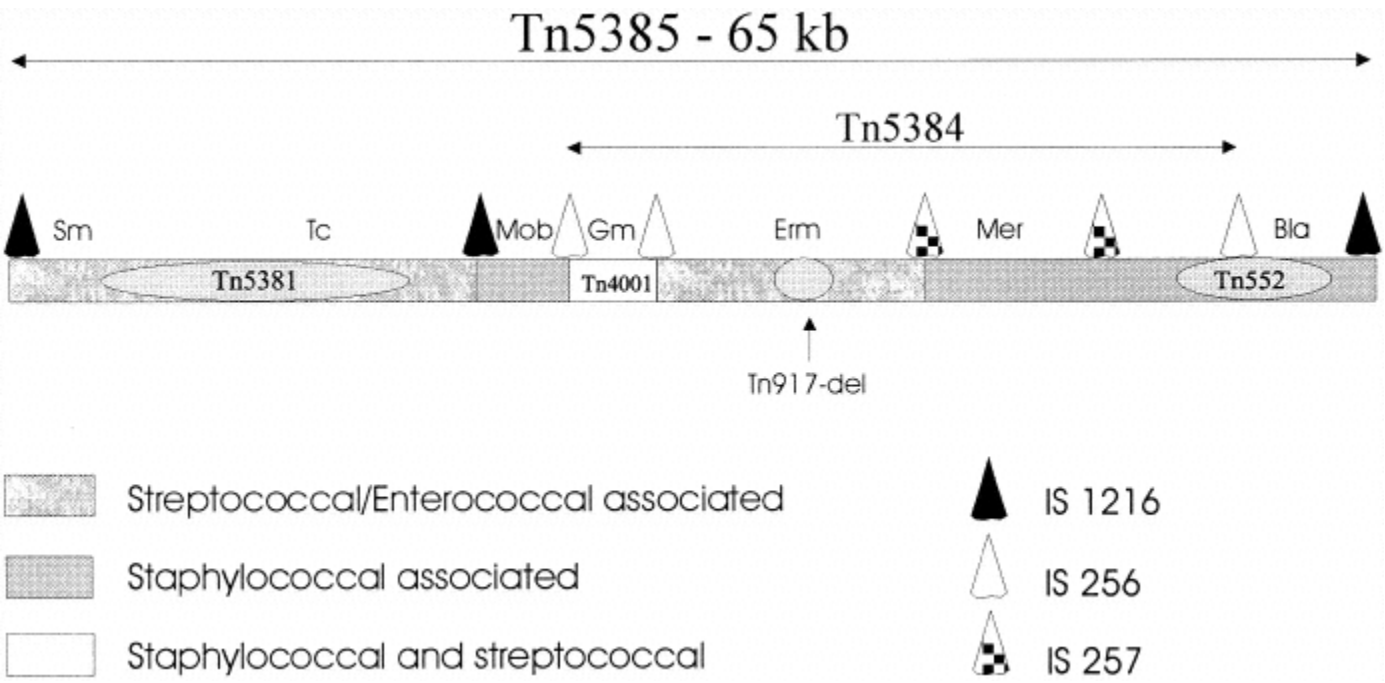

Figure 2. Graphic depiction of the structure of Tn*5385*. The key indicates the presumed origins (based on sequence data reported elsewhere) of the different regions of Tn*5385*. The figures marking the placement of insertion sequences are also indicated in the key. Transposons within the larger element are indicated in various ways, which depend on their degree of overlap. Resistance genes and other important loci are noted above the element. Bla, the β-lactamase gene; Erm, the erythromycin-resistance determinant; Gm, the *aac*-6′-*aph*-2″ bifunctional aminoglycoside-resistance gene; IS, the insertion sequence; Mer, the mercuric chloride-resistance determinant; Mob, the mobilization region, similar to that found on small staphylococcal plasmids; Sm, the *aadE* streptomycin-resistance gene; Tc, the tet(M) tetracycline-minocycline-resistance determinant within Tn*5381*; Tn*917*-del, the region that contains a version of Tn*917* in which the transposition genes have been deleted.

Genomic exchange in *E. faecalis* and *E. faecium*

The emergence and spread of glycopeptide-resistant *E. faecium* has prompted extensive molecular investigations of the strains that both colonize and infect hospitalized patients. Early phenotypic studies suggested differences between strains that colonized patients in Europe but rarely caused infections, and those that caused infections in hospitalized patients in the United States. While both types of strains carried similar determinants that conferred glycopeptide resistance, it was quickly apparent that the infecting strains in the U.S. were far more likely to express high levels of resistance to ampicillin than were the colonizing strains from Europe (Descheemaeker, Chapelle, Devriese, Butaye, Vandamme, & Goossens, 1999). These distinctions led to hypotheses that the strains on the two continents represented two different lineages. The U.S. strains appeared to be a hospital-adapted lineage with higher degrees of resistance to antibiotics, whereas the European strains appeared to be of community origin that were perhaps transferred from the feces of animals that had been given the growth-promoting glycopeptide antibiotic avoparcin. Further work has also demonstrated that the hospital-adapted strains were more likely to contain genes that encode putative virulence determinants such as enterococcal surface protein (*esp*Efm) and hyaluronidase (*hyl*Efm), and that some of these virulence determinants can be encoded on transferable plasmids (Le Breton, Muller, Auffray, & Rincé, 2007; Rice & Marshall, 1992).

It was not especially surprising when transfer of vancomycin-resistance determinants between *E. faecium* strains was demonstrated *in vitro*, since these determinants were known to be encoded on transposons—some of which may be conjugative, but all of which may be incorporated into conjugative plasmids. What was surprising was the demonstration that resistance to ampicillin, which is encoded on the chromosome by the intrinsic *pbp5* gene,

was also transferable at a readily detectable frequency *in vitro* ((Rice L. B., 2005). Exquisite and painstaking work by Manson and colleagues in *E. faecalis* showed that transfers of large regions of the *E. faecalis* V583 genome could be transferred to *E. faecalis* recipients, in the presence of conjugative plasmids (Marshall, Donskey, Hutton-Thomas, Salata, & Rice, 2002). These plasmids contain IS elements identical to those within the enterococcal genome. Cross-over between the IS elements in the plasmids and the chromosome create cointegrates that then use the transfer mechanism of the plasmid to transfer variable-length segments of the donor chromosome. Once these segments are in the recipient cell, these regions can then integrate into the recipient genome, either through homologous recombination or the activity of the transferred IS element. These investigators placed a selectable marker at regions throughout the donor genome and showed that virtually any region could be transferred to the recipient, as long as the pheromone-responsive plasmid was present in the donor. They concluded that these chromosomal mobilizations were the result of recombination between plasmid and chromosomal copies of IS*256*. Similar evidence for IS element-mediated transfer of large genomic resistance segments in *E. faecium* has been observed in one of our laboratories (L.B.R., unpublished).

It now appears that such genomic exchange is common among enterococci isolated in clinical settings. As noted above, it is estimated that as much as 25% of the *E. faecalis* V583 genome has been acquired (Polidori, et al., 2011). Moreover, careful work performed by R. Willems and colleagues has identified a specific loose lineage of *E. faecium*, designated clonal complex 17 (CC17), that is responsible for the vast majority of infection-causing *E. faecium* worldwide (220). This clonal complex first became prevalent in the U.S., while the less resistant and pathogenic strains were becoming common in Europe (67). Prohibition of the use of avoparcin to promote growth in food animals led to reductions in colonization by non-virulent and non-resistant strains, and since that time, CC17 strains have become the predominant disease-causing *E. faecium* strains worldwide (de Regt, et al., 2008). Analysis of *pbp5* genes from CC17 and other strains does not suggest that the entire ampicillin-resistant *E. faecium* outbreak is due to the transfer of single *pbp5* genes among strains, but rather that several different lineages of mutated *pbp5* genes have contributed to the outbreak of ampicillin-resistant, glycopeptide-resistant *E. faecium* (Garnier, Taourit, Glaser, Courvalin, & Galimand, 2000). Recent work also suggests that the CC17 lineage predates the clinical use of antibiotics, suggesting that it is an opportunist that is ideally suited to the antibiotic era, rather than a strain created by selective antibiotic pressure (Galloway-Peña, Rice, & Murray, 2011).

Antibiotics and colonization by multi-resistant *E. faecium*

As noted above, CC17 *E. faecium* strains are ideally suited to proliferate in a world occupied by immunocompromised patients who are given large and prolonged doses of antimicrobial agents. Although they are intrinsically more resistant to ampicillin than their *E. faecalis* counterparts, the emergence of *E. faecium* strains that were highly resistant to ampicillin occurred during the 1980s (Gryczan, Grandi, Hahn, Grandi, & Dubnau, 1980). The scattered reports of the emergence of these strains merited little attention at the time, mostly because *E. faecium* was a relatively minor cause of infection (relative to *E. faecalis*, for instance), and because vancomycin was always available for the treatment of resistant strains. The emergence of glycopeptide resistance in enterococci caught everyone's attention, primarily because there was significant concern that this resistance could transfer to methicillin-resistant *S. aureus*, for which vancomycin was the only reliable therapy at the time. Over time it became apparent that the vast majority of glycopeptide-resistant enterococci were *E. faecium*, both in Europe and in the United States.

With the exception of *E. faecalis* V583, the early glycopeptide-resistant enterococcal strains were isolated and characterized in Europe (Shlaes, Bouvet, Devine, Shales, al-Obeid, & Williamson, 1989; Williamson, Al-Obeid, Shlaes, Goldstein, & Shlaes, 1989). The use of the glycopeptide antibiotic avoparcin as a growth promoter in European food animals suggested that these strains may have arisen in the gastrointestinal tracts of animals, and were transmitted to humans. Early data suggested that a significant percentage of individuals in the European community were colonized with GRE, and that GRE could be isolated from food purchased in supermarkets

(Wegener, Madsen, Nielsen, & Aarestrup, 1997). However, very few GRE infections were reported in European hospitals.

The emergence of GRE in the United States occurred later than its emergence in Europe and had a distinctly different character. U.S. GRE strains became significant causes of infection in hospitalized patients, especially in those that were significantly immunocompromised (Vergis, et al., 2001). Moreover, these strains were far more likely to express high levels of resistance to ampicillin than the strains isolated in Europe. Avoparcin had never been used in U.S. farms and GRE were essentially absent from the feces of U.S. farm animals. Similarly, attempts to isolate GRE from the feces of community dwellers were unsuccessful (Coque, Tomayko, Ricke, Okhyusen, & Murray, 1996). In essence, the European GRE outbreak was a community phenomenon that did not extend significantly into the hospital, and the U.S. outbreak was a hospital phenomenon that did not extend significantly into the community.

Both outbreaks were the results of the application of significant concentrations of glycopeptide antibiotics to the gastrointestinal tract. In Europe, this occurred on farms; in the U.S., it occured in the gastrointestinal tracts of hospitalized patients who were being treated with oral vancomycin for antibiotic-associated diarrhea due to *Clostridium difficile*. These concentrations of glycopeptides eliminated much of the Gram-positive flora of the colon, but left the enterococci, which are tolerant to the bactericidal activity of these agents. Naturally glycopeptide-resistant bacteria then colonized the gut, passing their glycopeptide resistance genes to the resident enterococci.

In animals, this transmission yielded vancomycin-resistant *E. faecium* that otherwise reflected the routine enterococci of the animals, which would be generally susceptible to ampicillin and not well suited for pathogenicity. In humans, transmission occurred into strains that were already hospital-adapted; in other words, strains that were more resistant to antibiotics and those that were more likely to express virulence determinants that promote infection. Recent data suggest that CC17 strains, which are well suited for survival and pathogenicity in the hospital, have been present for a long period of time (Galloway-Peña, Rice, & Murray, 2011). Prior to the emergence of GRE, these strains became the predominant hospital flora when extended-spectrum cephalosporin use became widespread, because extended-spectrum cephalosporins are potent selectors of ampicillin-resistant *E. faecium* colonization of the gastrointestinal tract (Chirugi, Oster, Goldber, & McCabe, 1992). When the vancomycin-resistance determinants became available, they were transferred into these strains. In Europe, it appears that the prevalence of the non-pathogenic strains from animals forestalled the emergence of glycopeptides-resistant CC17 strains. Political pressure led to the discontinuation of glycopeptide use in animals in the 1990s. Since that time, the prevalence of GRE in farms animals and in the community has decreased. Unfortunately, many European countries now have increasing reports of nosocomial outbreaks of infection and colonization, caused by CC17 strains (Bourdon, et al., 2011).

An understanding of the impact of antibiotic administration on colonization by GRE is important when evaluating strategies to try to reduce GRE gastrointestinal colonization in the clinical setting. For example, shortly after the likely association between oral vancomycin therapy and GRE colonization was recognized, recommendations were proposed that *C. difficile*-associated diarrhea should be treated with metronidazole rather than vancomycin (Cohen, et al., 2010). Before long, however, it was recognized that exposure to metronidazole was also associated with an increased risk of GRE colonization (Cervera, et al., 2011). Animal studies confirmed that while anti-anaerobic antibiotics, such as metronidazole, promoted the persistence of high levels of GRE gastrointestinal colonization, antibiotics that lacked anaerobic activity did not (Donskey, Hanrahan, Hutton, & Rice, 1999). Donskey and colleagues (Donskey, et al., 2000) extended these studies to humans, and showed that fecal output of VRE significantly increased after colonized patients were exposed to anti-anaerobic antibiotics.

It was also surprising to many that association of GRE colonization with intravenous vancomycin exposure was only inconsistently observed in clinical studies, and that association with exposure to extended-spectrum

cephalosporins was much more consistently found (Carmeli, Eliopoulos, & Samore, 2002). However, intravenous vancomycin is not secreted into the gastrointestinal tract in detectable concentration, at least in the first five days of therapy (Currie & Lemos-Filho, 2004). On the other hand, some extended-spectrum cephalosporins are secreted into the gastrointestinal tract in large concentrations (especially ceftriaxone). A series of animal studies convincingly documented that the selection of GRE colonization by ceftriaxone is dependent on the expression by the GRE of high levels of ampicillin resistance, which translate to very high levels (>5,000 µg/ml) of ceftriaxone resistance (Rice, Lakticová, Carias, Rudin, Hutton, & Marshall, 2009). Since within a short period of time more than 90% of GRE isolated in the U.S. were ampicillin-resistant *E. faecium*, the connection between extended-spectrum cephalosporins and GRE is understandable. It is interesting to note that these animal studies also suggested that piperacillin-tazobactam can promote the persistence of high levels of GRE colonization by virtue of being a potent anti-anaerobic agent, but can prevent the establishment of GRE colonization, through its inhibitory effect on growth of GRE in the upper gastrointestinal tract (Donskey, Hanrahan, Hutton, & Rice, 1999). Consistent with these findings, piperacillin-tazobactam has been only inconsistently associated with GRE colonization in clinical studies.

Enterococcal antimicrobial resistance and the use of antibiotics in food animals

As previously noted, there are convincing data to suggest that the emergence of VRE in the European population is tied to the use of the glycopeptide antibiotic avoparcin in food animals. The use of growth-promoting or therapeutic antibiotics in animals has been associated with a variety of resistance determinants found in human isolates of the same species. Specifically, enterococcal resistance to quinupristin-dalfopristin has been tied to the use of virginiamycin in food animals (Welton, et al., 1998). Whether the specific strains that emanate from animals are likely to cause disease in humans can be argued, but should not be used in an argument to promote the continued use of antibiotics in animals for the purpose of increased growth. Recent data are clear that the intra-species transfer of enterococcal resistance determinants is far more common than inter-species transfer (Dahl, et al., 2007), and that the rates of transfer *in vivo* exceed those that we are able to measure *in vitro*. As such, any practices that encourage the acquisition of important resistance determinants by any enterococcal strains should be avoided.

Enterococcal Infection: Strategies for, and Consequences of, Antibiotic Treatment

Treatment of enterococcal infections has become one of the most challenging issues facing clinicians in the 21st century. The increased prevalence of strains that are resistant to almost all antibiotics with *in vitro* bactericidal activity against enterococci is a worrisome trend. Indeed, among the most common enterococcal species isolated from critically ill patients in the USA, *Enterococcus faecium* has become one of the most predominant, becoming almost as often isolated from clinical samples at some hospitals as *Enterococcus faecalis* (Hodel-Christian & Murray, 1991; Mir, Asong, Li, Cardot, Boons, & Husson, 2011). This trend in the epidemiology of enterococcal infections has important clinical consequences, since resistance to antibiotics such as ampicillin and vancomycin (which used to be the cornerstone antibiotics for the treatment of enterococcal infections) is the "norm" in modern-day hospital-associated isolates of *E. faecium*. Conversely, resistance to ampicillin is rarely seen in *E. faecalis*, and vancomycin resistance is much less frequent in this species. Furthermore, the infectious Disease Society of America (IDSA) has included *E. faecium* among the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *Pseudomonas aeruginosa*, and *Enterobacter* spp.) for which new therapies are urgently needed (Arias & Murray, 2012; Rice, et al., 2004).

One of the main challenges in the treatment of severe enterococcal infections is that these organisms are either tolerant or intrinsically resistant to a variety of antimicrobial agents. For example, compared to streptococci,

higher concentrations of penicillin (10 to >100 times) than those used against streptococci are needed to kill enterococci *in vitro* (Murray, 2000). Moreover, enterococci are often tolerant to β-lactam antibiotics with minimal bactericidal concentrations (MBC) of penicillin and ampicillin that exceed ≥32 times the minimal inhibitory concentration (MIC) of the corresponding β-lactam compound. Also, enterococci often possess genes that make them intrinsically resistant to compounds used in clinical practice. For example, *E. faecium* carries an acetylase enzyme (AAC(6′)) that produces higher MICs to several aminoglycosides (such as tobramycin and amikacin), which results in a loss of synergistic effect with cell-wall agents (Arias, Contreras, & Murray, 2008). Similarly, most *E. faecalis* contain a gene (designated *lsa*) that encodes an ATP-binding protein which confers intrinsic resistance to quinupristin/dalforistin (Q/D) (Singh, Weinstock, & Murray, 2002). These therapeutic issues have had an important impact since the early days of the antibiotic era (1940s); indeed, cure rates in the treatment of enterococcal endocarditis with penicillin monotherapy were disappointing (~60%) when compared to those for streptococcal endocarditis (Arias, Contreras, & Murray, 2010; Murray, 2000), and only the addition of an aminoglycoside increased the number of favorable outcomes.

We will focus the discussion in this section on issues related to the treatment of severe enterococcal infections (namely, bacteremia and endocarditis) in both susceptible and multidrug-resistant (MDR) enterococci. Additionally, we will include comments related to the influence of antibiotic therapy in the selection of drug resistance, and will discuss the changes in the gastrointestinal (GI) tract caused by the use of antibiotics that promote the growth and dissemination of MDR enterococci.

Treatment of enterococcal infections: susceptible vs. resistant enterococci

The majority of enterococcal infections are described in critically ill patients for whom treatment is usually required. The clinical approach to the treatment of susceptible versus resistant enterococal infections differs significantly. For practical purposes, the majority of modern-day "susceptible" enterococcal infections are caused by isolates of *E. faecalis* that lack resistance to ampicillin (or penicillin) and vancomycin, and do not exhibit high-level resistance (HLR) to aminoglycosides. Nonetheless, although ampicillin resistance continues to be uncommon in clinical isolates of *E. faecalis*, high-level resistance (HLR) to aminoglycosides has become more common, and vancomycin resistance appears to be increasing. On the other hand, most MDR enterococcal infections are caused by *E. faecium*, in which both ampicillin and vancomycin have become obsolete, and the isolates often exhibit HLR to aminoglycosides. Thus, we will divide this section in three parts: *i*) treatment of *Enterococus faecalis* infections that are susceptible to ampicillin and vancomycin, and do not exhibit HLR to aminoglycosides; *ii*) treatment of *E. faecalis* infections that are susceptible to ampicillin and vancomycin but exhibit HLR to aminoglycosides; and *iii*) treatment of MDR *E. faecium*.

Treatment of ampicillin and vancomycin-susceptible *Enterococus faecalis* without HLR to aminoglycosides

The cornerstone for treating susceptible enterococcal infections is the β-lactam antibiotics, and vancomycin is usually reserved for cases in which ampicillin cannot be used, due to a β-lactam allergy. Among the β-lactams, the compounds with the best *in vitro* activity include the amino-penicillins (such as ampicillin) and ureidopenicillins (such as piperacillin), followed by penicillin G and carbapenems (imipenem). However, as previously mentioned, the use of β-lactam monotherapy is often non-bactericidal for enterococci, and was associated with poor outcomes in endovascular infections. Of note, a bactericidal regimen is of paramount importance to the treatment of deep-seated infections such as endocarditis. Pioneer studies performed in the 1970s (Moellering, Jr. & Weinberg, 1971; Zimmermann, Moellering, Jr., & Weinberg, 1971) provided compelling evidence that the association of a cell-wall agent, such as a β-lactam (or a glycopeptide, such as vancomycin) plus an aminoglycoside produced a synergistic bactericidal effect against enterococci. Synergism is defined *in vitro* as a decrease of >2 log10 in bacterial counts (CFU/ml) by 24 hours, as compared to the cell wall agent alone (Moellering, Jr., Linden, Reinhardt, Blumberg, Bompart, & Talbot, 1999), or a 99.9% reduction in colony counts

from the starting inoculum when the combination of a β-lactam (or glycopeptide) and an aminoglycoside is used. Additionally, the concentration of the aminoglycoside should not have any effect on the growth curve of the tested microorganism (Murray, 2000). The basis for such a synergistic effect appears to be related to the increase in the uptake of the aminoglycoside molecule by the bacterium when the cell wall is altered (Arias, Contreras, & Murray, 2008).

Gentamicin and streptomycin are the two main aminoglycosides used in clinical practice to achieve bactericidal therapy, and clinical experience supports the use of the combination of ampicillin (or penicillin) and either of these two aminoglycosides as the first line of therapy for severe enterococcal infections that are susceptible to both classes of antibiotics. It is of paramount importance that the clinical microbiology laboratory tests isolates for the presence of HLR to aminoglycosides, since the presence of HLR abolishes the synergistic effect. HLR is defined in the clinical laboratory by the presence of at least one colony-forming unit at concentrations of 2000 mg/L and 500 mg/L of streptomycin and gentamicin, respectively, on brain heart infusion agar (BHI), or evidence of growth at a concentration of 1000 mg/L of streptomycin when using BHI broth (Mir, Asong, Li, Cardot, Boons, & Husson, 2011). It is also important to note that the use of aminoglycosides other than gentamicin or streptomycin is not recommended for the treatment of severe *E. faecalis* infections where synergistic bactericidal therapy is necessary, since many enterococcal isolates may harbor the *aph(3′)-IIIa* gene, which confers HLR to kanamycin and resistance to the synergistic effect of amikacin (Mir, Asong, Li, Cardot, Boons, & Husson, 2011). Moreover, testing for the presence of resistance or a lack of synergism to aminoglycosides other than gentamicin or streptomycin has not been standardized for routine use in the clinical laboratory.

Some clinicians have advocated the use of continuous infusions of penicillin for the treatment of enterococcal endocarditis (Vogler, Dorney, & Bridges, 1962) in order to improve the time above MIC (*T*>MIC) parameter that is crucial for β-lactam-related killing. However, animal models of enterococcal endocarditis have shown conflicting results. Using a rat endocarditis model, Thauvin et al. (Thauvin, Eliopoulos, Willey, Wennersten, & Zervos, 1987) showed that continuous infusion of ampicillin was superior than intermittent intramuscular injection in reducing bacterial titers in cardiac vegetations. Conversely, Hellinger et al. (Hellinger, Rouse, Rabadan, Henry, Steckelberg, & Wilson, 1992) did not find any difference in efficacy between the administration of ampicillin by continuous infusion or intermittent administration (both by intravenous route and in combination with gentamicin) in an experimental model of rabbit endocarditis. Prospective clinical data comparing the two regimens in humans are lacking, and as a result, it is difficult to make any recommendations for the use of one therapeutic strategy above the other.

In rare cases of *E. faecalis* infections, the presence of β–lactamase in these isolates may compromise the clinical effectiveness of ampicillin or penicillin (Arias, Singh, Panesso, & Murray, 2007; Norris, Reilly, Edelstein, Brennan, & Schuster, 1995). The Clinical Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2012) recommends testing for penicillinase in specific clinical settings, such as endocarditis. These isolates should not cause major therapeutic dilemmas, since the ampicillin-sulbactam combination should be effective against these penicillinase-producing strains (Table 2).

Table 2. Options for the treatment of severe *E. faecalis* infections

Ampicillin and vancomycin-susceptible *E. faecalis* without HLR to aminoglycosides
Ampicillin (or penicillin) plus aminoglycosides (gentamicin or streptomycin) In the case of a β-lactam allergy, vancomycin is the drug of choice

Ampicillin and vancomycin-susceptible *E. faecalis* with HLR to aminoglycosides
Ampicillin plus ceftriaxone Ampicillin plus imipenem plus vancomycin Ampicillin plus fluoroquinolones[1] Ampicillin plus high-dose daptomycin[2]
Penicillinase-producing *E. faecalis* (rare)
Ampicillin-sulbactam plus aminoglycosides (gentamicin or streptomycin)
Vancomycin-resistant *E. faecalis*
They are usually susceptible to ampicillin, and ampicillin-based regimens are preferred

Treatment of ampicillin and vancomycin-susceptible *E. faecalis* that exhibit HLR to aminoglycosides

One of the important clinical challenges in the treatment of E. faecalis endovascular infections is the emergence of HLR to gentamicin, which is usually mediated by the bifunctional aminoglycoside modifying enzyme AAC(6′)-Ie-APH (2′′)-Ia (Chow, 2000), as well as HLR to streptomycin, which is associated with ribosomal mutations and/or the presence of an streptomycin-nucleotidyltransferase enzyme (9). In some regions of the world, the prevalence of E. faecalis with HLR to aminoglycoside appears to be increasing, and represents up to 60% of the isolates (Patel, et al., 2001). HLR to gentamicin or to streptomycin abolish the synergistic bactericidal effect of the cell-wall agent/aminoglycoside combination in most E. faecalis isolates and represent a serious issue in the treatment of IE. Another important consideration is that, due to the toxicity profile of aminoglycosides, clinicians are sometimes reluctant to use the aminoglycoside class of antibiotics in critically ill patients who may have compromised renal function. Therefore, alternatives to the aminoglycosides are an important clinical necessity in patients in whom these compounds cannot be used due to resistance or toxicity.

Clinical and animal studies have shown that the combination of cephalosporins (such as ceftriaxone or cefotaxime) with an amino-penicillin (such as ampicillin or amoxicillin) is synergistic and bactericidal against strains of E. faecalis that exhibit HLR but are fully susceptible to ampicillin (Mainardi, et al., 2007; Tascini, Doria, Leonildi, Martinelli, & Menichetti, 2004). The basis for this effect is thought to be the result of the differential saturation of the E. faecalis penicillin-binding proteins (PBPs) by ampicillin and cefotaxime (or ceftriaxone). The amino-penicillin may partially saturate PBPs 4 and 5, but not PBPs 2 and 3; the latter PBPs may still participate in cell-wall synthesis. In the presence of the cephalosporin/amino-penicillin combination, complete saturation of PBPs 2 and 3 may be achieved, which results in a bactericidal synergistic effect (Mainardi, et al., 2007). Clinical support for the use of the combination of ampicillin (2 g every 12 h) plus ceftriaxone (2 g every 4 h) was provided by a prospective, open-label, non-randomized trial in 13 Spanish hospitals. The study included 43 patients (21 and 22 patients with E. faecalis isolates that exhibited HLR to aminoglycoside and non-HLR, respectively). The rate of clinical cure for isolates with HLR to aminoglycosides and non-HLR was 71.4% and 72.7%, respectively, at the end of treatment (six weeks), and 71.4% and 63.6% at three months, respectively. However, only 13 patients completed the protocol, with an overall clinical cure rate at three months of 67.4%, although microbiological cure was 100%. The data are promising and the combination may be an interesting option for E. faecalis infective endocarditis (IE) in which aminoglycosides cannot be used (Goffin & Ghuysen, 1998) (Table 2). More recently, an observational, nonrandomized, comparative multicenter cohort study at 17 Spanish and 1 Italian hospitals provided robust clinical evidence indicating that the combination of ceftriaxone plus ampicillin was as effective as the ampicillin-aminoglycoside combination for the treatment of E. faecalis with less toxicity (Fines, Perichon, Reynolds, Sahm, & Courvalin, 1999).

In Japan, the aminoglycoside arbekacin, which is more stable than gentamicin to the action of the AAC(6′)-Ie-APH (2′′)-Ia enzyme, is available for the treatment of enterococcal infections. Interestingly, in vitro studies have

found that arbekacin produced synergism when combined with ampicillin in 40% of enterococci that possessed the AAC(6′)-Ie-APH (2′′)-Ia enzyme (Kinnebrew, Ubeda, Zenewicz, Smith, Flavell, & Pamer, 2010). Moreover, in vivo studies in experimental endocarditis (rabbits) showed that the combination of ampicillin plus arbekacin was able to achieve a more statistically significant decrease in bacterial counts from vegetations, as compared to ampicillin plus gentamicin (Kak, You, Zervos, Kariyama, Kumon, & Chow, 2000), which suggests a possible role of arbekacin in the treatment of certain isolates that exhibit HLR to gentamicin, although prospective, comparative clinical data in humans are not available.

Other regimens that have been used successfully in sporadic cases of infective endocarditis or bacteremia caused by E. faecalis with HLR to aminoglycosides include the combination of ampicillin plus imipenem plus vancomycin (Antony, Ladner, Stratton, Raudales, & Dummer, 1997), and ampicillin plus ofloxacin (Tripodi, Locatelli, Adinolfi, Andreana, & Utili, 1998), and daptomycin plus ampicillin. Additionally, a new generation of cephalosporins with good in vitro activity against some strains of E. faecalis (but not E. faecium) has recently been made available. Ceftobiprole, which is only available in Canada and Switzerland, and ceftaroline (available in the USA) are members of this new class of compounds. The available data suggest that ceftobiprole may have some potential in the treatment of E. faecalis infections. Indeed, ceftobiprole has been shown to exhibit potent in vitro synergistic bactericidal activity against E. faecalis, including against strains that produce β-lactamase and carry a vancomycin resistance gene cluster (Arias, Singh, Panesso, & Murray, 2007). Moreover, ceftobiprole showed potent in vivo activity against E. faecalis in a mouse model of peritonitis (Arias, Singh, Panesso, & Murray, 2007) and urinary tract infection (Singh & Murray, 2012). Further clinical data are needed in order to evaluate the specific role of ceftobiprole in the treatment of E. faecalis infections.

Treatment of multidrug-resistant *E. faecium*

As previously mentioned, the relatively recent rise in frequency of nosocomial infections caused by multidrug-resistant *E. faecium* is one of the most important challenges in clinical settings, since reliable therapies for severe, deep-seated infections caused by these MDR strains are not available. The majority of current *E. faecium* isolates recovered from hospitals across the USA are resistant to ampicillin. Moreover, the ampicillin MICs of nosocomial isolates of *E. faecium* that are currently isolated from hospitals in the USA are much higher than those that were isolated 20 years ago (Garnier, Taourit, Glaser, Courvalin, & Galimand, 2000). This observation has important clinical consequences, since it has been postulated that *E. faecium* isolates with ampicillin MICs $\leq$ 64 μg/ml could potentially be treated with high doses of ampicillin (up to 30 g/day) combined with an aminoglycoside (if HLR is not present) (Murray, Mederski-Samoraj, Foster, Brunton, & Harford, 1986) (Table 3), since concentrations of ampicillin in serum between 100 to 150 mg/L can be readily obtained with a high-dose regimen. Moreover, for an *E. faecium* isolate with an ampicillin MIC of 16 μg/mL, the combination of ampicillin with imipenem (MIC of 32 μg/ml) was superior in reducing bacterial counts from vegetations, as compared with the most active single agent in experimental endocarditis (Brandt, Rouse, Laue, Stratton, Wilson, & Steckelberg, 1996). Unfortunately, *E. faecium* isolates with ampicillin MICs $\leq$ 64 μg/ml are now rare in clinical settings. Furthermore, vancomycin, which used to be the most reliable alternative for the treatment of ampicillin-resistant *E. faecium* isolates, has now become obsolete in a high proportion of infections, particularly in the USA, where more than 80% of isolates are resistant to glycopeptides. Similarly, a high percentage of these isolates also exhibit HLR to both gentamicin and streptomycin, reducing even further the possibilities of obtaining synergistic bactericidal therapy.

Only two compounds are currently approved by the Food and Drug administration for the treatment of vancomycin-resistant *E. faecium* infections: linezolid and quinupristin-dalfopristin (Q/D), and both compounds are recommended by the American Heart Association (AHA) for the treatment of MDR *E. faecium* IE. The clinical experience with both compounds is limited since controlled, prospective, randomized clinical trials are not available. Q/D is a mixture of the streptogramin antibiotics quinupristin (streptogramin B) and dalfopristin (streptogramin A) that inhibit protein synthesis after their interaction with the 50S ribosomal subunit. As

mentioned above, most *E. faecalis* are intrinsically resistant to Q/D—but the antibiotic does have potent *in vitro* bactericidal activity against *E. faecium*. The clinical efficacy of Q/D for the treatment of severe infections caused by MDR *E. faecium* was assessed in two multicentric, prospective, non-comparative studies (Lobritz, Hutton-Thomas, Marshall, & Rice, 2003; Moellering, Jr. & Weinberg, 1971). The overall success rates (both clinical and bacteriological) in both studies were ca. 65%. Of note, Q/D has some important side effects, such as phlebitis and myalgias/arthralgias, which have led to treatment interruptions (Arias, Contreras, & Murray, 2008).

A retrospective analysis of 113 patients with VRE bacteremia treated with Q/D found an attributable mortality rate (related to the infection) of 50%, which was significantly higher than the rate observed in patients treated with linezolid (p=0.002) (Erlandson, Sun, Iwen, & Rupp, 2008). Moreover, the use of Q/D monotherapy has been associated with several reports of clinical failure, which has led to the suggestion that Q/D may be used as part of a combination regimen against isolates of MDR *E. faecium* (Table 3). Indeed, the association of Q/D plus doxycycline and rifampin cleared the bloodstream of a patient with VRE endocarditis who had failed Q/D monotherapy (Mave, Garcia-Diaz, Islam, & Hasbun, 2009). Similarly, the association of Q/D plus ampicillin was successfully used in the treatment of a patient with recurrent IE endocarditis due to *E. faecium* (ampicillin MIC >16 μg/ml) after clinical failure with linezolid monotherapy (Bethea, Walko, & Targos, 2004). Thus, the limited clinical data suggests that Q/D may be used as part of a combination therapy when treating severe *E. faecium* infections. (Table 3)

Linezolid is a synthetic oxazolidinone with bacteriostatic activity against enterococci (all species) whose mechanism of action is thought to involve interactions with the A site of bacterial ribosomes that inhibit protein synthesis. As with Q/D, this compound is also FDA-approved for the treatment of VRE infections (both *E. faecalis* and *E. faecium*) and is also recommended by the AHA for IE caused by vancomycin-resistant *E. faecium* (Baddour, et al., 2005). Clinical support for the use of linezolid for severe VRE infections is derived mostly from retrospective studies, case reports, and a small meta-analysis. In a comparative study using linezolid for compassionate indications in VRE infections, favorable clinical and microbiological outcomes were 78% and 85% in the intention to treat population, respectively. However, in the subgroup of patients with VRE IE, microbiological eradication was obtained only in 63% of the patients, whereas 76% achieved a clinical cure (Birmingham, Rayner, Meagher, Flavin, Batts, & Schentag, 2003). Another study which included patients with solid organ transplants and VRE bacteremia reported a clinical success rate with linezolid of only 67% (El-Khoury & Fishman, 2003).

Three retrospective studies have attempted to compare the clinical outcomes of linezolid vs daptomycin in the treatment of bacteremia caused by VRE. The first one by Mave et al. (Mave, Garcia-Diaz, Islam, & Hasbun, 2009) analyzed the clinical outcomes of 68 patients treated with linezolid. Overall mortality was ca. 21% with a median duration of bacteremia of two days and a relapse rate of 2.9%. The overall microbiological eradication rate was 88.2%. The second study included 34 patients treated with linezolid, in which the mortality rate was 29.4%. IE treated with linezolid was associated with a prolonged duration of blood cultures (Crank, et al., 2010). A logistic regression analysis performed in this study suggested that previous linezolid treatment was associated with increased mortality (Odds ratio [OR] 6.63 p=0.031). The third and largest study included 138 patients with VRE bacteremia who were treated with linezolid (Twilla, Finch, Usery, Gelfand, Hudson, & Broyles, 2012). The rates of clinical and microbiological cures were 74% and 94%, respectively, with a rate of recurrence of 3%. The overall mortality in the linezolid arm was ca. 18%. Additionally, a meta-analysis attempted to examine the efficacy of linezolid in the treatment of VRE endocarditis. From six cases reported in this meta-analysis, four were *E. faecium*, and clinical cure with linezolid was achieved in all of them (Falagas, Manta, Ntziora, & Vardakas, 2006). Conversely, clinical failures when using linezolid monotherapy have been reported in several cases of VRE infections (Berdal & Eskesen, 2008; Webster, Griffiths, & Bowler, 2009), including some cases of endocarditis and central nervous system infections. Therefore, with the available clinical data, linezolid appears to be a reasonable option for the treatment of VRE infections; however, its bacteriostatic activity may preclude the routine use of linezolid in endovascular infections. If this is the case, linezolid should be reserved as an alternate

option when other bactericidal therapies are unavailable. Furthermore, a worrisome recent report of transferable plasmid-mediated linezolid resistance in enterococci mediated by the methyl-transferase Cfr (Diaz, Kiratisin, Mendes, Panesso, Singh, & Arias, 2012) raises concern about the possible emergence and rapid dissemination of linezolid resistance in enterococci. Linezolid may be an attractive option for the treatment of central nervous system infections caused by MDR *E. faecium,* due to the favorable pharmacokinetics of the drug in the cerebrospinal fluid and CNS tissues.

Daptomycin is a lipopeptide antibiotic with bactericidal activity against enterococci, including VRE (both *E. faecium* and *E. faecalis*), which makes it an attractive compound for the treatment of MDR *E. faecium* infections. Daptomycin is FDA-approved for the treatment of vancomycin-susceptible enterococcal skin and soft tissue infections, but has no indication for any VRE infection or for *E. faecium*. However, clinicians often use this compound in the treatment of severe VRE infections, due to a lack of other bactericidal options. As we have seen with other compounds, the clinical data that supports the use of daptomcyin for VRE infections are composed mostly of retrospective studies and case reports.

Mohr et al. (Mohr, Friedrich, Yankelev, & Lamp, 2009) retrospectively analyzed 159 patients with enterococcal bacteremia (120 of those patients with *E. faecium*) who received treatment with daptomycin. Clinical success was observed in 104 patients (87%), with adverse events possibly related to daptomycin observed in 9.5%, eight of which were considered serious. Apart from bacteremia, daptomycin has been used in the treatment of several other enterococcal infections, including endocarditis, skin and soft tissue infections, bone and joint infections and urinary tract infections (Cantón, Ruiz-Garbajosa, Chaves, & Johnson, 2010). In IE, which is an infection where bactericidal therapy is necessary for optimal outcomes, the European Cubicin Outcomes Registry and Experience (EU-CORE) reported 22 cases of enterococcal endocarditis (18 of these isolates were *E. faecalis* and 4 were *E. faecium*) in which the overall clinical cure was 73% (Cervera, et al., 2011) but robust clinical data to support the use of daptomycin in enterococcal IE are still lacking. As mentioned above, three studies have retrospectively compared the clinical outcomes of patients with enterococcal bacteremia using linezolid vs daptomycin (Crank, et al., 2010; Diaz, Kiratisin, Mendes, Panesso, Singh, & Arias, 2012; Murray, Mederski-Samoraj, Foster, Brunton, & Harford, 1986). In all three studies, no statistical significant difference in mortality was found between the two compounds (mortality in patients treated with daptomycin ranged from 24% to 46%), although a trend towards increased mortality was observed in one of these comparative studies. Patients in the daptomycin arm appeared to be sicker, making it difficult to extrapolate the data from this study to clinical practice.

An important issue in the treatment of deep-seated enterococcal infections during daptomycin monotherapy is the report of clinical failures and emergence of resistance during therapy (mostly reported in bacteremia and endocarditis) (Arias, et al., 2011). In the majority of these cases, a lack of a clinical cure has been correlated with the use of dose schemes that were approved for the treatment of soft tissue infections (only with vancomycin-susceptible enterococci) and *S. aureus* bacteremia (4 and 6 mg/kg, respectively). Indeed, the MIC breakpoint for enterococci is four times higher than that of staphylococci (4 ug/ml versus 1 ug/ml) (Clinical and Laboratory Standards Institute, 2012), which leads to the suggestion that higher doses are needed for the treatment of enterococcal infections. Thus, doses that range from 8 to 14 mg/kg have been used in several case reports of difficult-to-treat and recurrent enterococcal infections (Mir, Asong, Li, Cardot, Boons, & Husson, 2011). A recent work, which uses an *in vitro* model of simulated endocardial vegetations, suggested that higher doses of this compound achieved the best bactericidal activity and prevented the emergence of resistance (Hancock & Perego, 2002). As previously mentioned for other compounds, prospective clinical trials that have evaluated the efficacy and safety of higher doses of daptomycin are lacking, but the available clinical evidence suggest that doses of 8-10 mg/kg or higher should be strongly considered in the treatment of severe enterococcal bacteremia and endocarditis.

Some of the disappointing outcomes observed with daptomycin monotherapy in the treatment of *E. faecium* infections have been overcome by the addition of compounds with *in vitro* activity against the infecting strains. For example, failure of daptomycin in the treatment of two patients with *E. faecium* endocarditis was overcome by the addition of high-dose ampicillin plus gentamicin, and gentamicin plus rifampin, respectively (Arias, et al., 2007; Stevens & Edmond, 2005). Moreover, three additional patients with recurrent *E. faecium* IE were successfully treated with a combination of daptomycin plus tigecycline (Jawetz & Sonne, 1966; Prystowsky, et al., 2001; Schutt & Bohm, 2009). Tigecycline is a bacteriostatic compound derived from minocycline (tetracycline class of antibiotics) that is approved by the FDA for the treatment of soft tissue infections and community-associated pneumonia caused by susceptible organisms. This antibiotic has good *in vitro* activity against enterococci, concentrates well in intra-abdominal tissues, and readily penetrates vegetations. However, the blood levels achieved by tigecycline are low, making the use of this antibiotic as monotherapy less attractive for enterococcal bloodstream infections. Nonetheless, the combination of tigecycline with daptomycin may be an interesting regimen for the treatment of MDR *E. faecium* IE and difficult-to-treat bacteremias, although more clinical data are needed in order to clarify the role of this combination in the treatment of these infections.

Another intriguing combination used for the treatment of MDR *E. faecium* IE is daptomycin plus ampicillin in an *E. faecium* isolate that exhibited high MICs to ampicillin. Indeed, a recent report by Sakoulas et al. (Sakoulas, et al., 2012) describes a patient with *E. faecium* endocarditis who failed daptomycin monotherapy, but whose bloodstream infection was cleared by adding ampicillin (the isolate had an ampicillin MIC of > 128 μg/ml). Ampicillin appears to enhance the killing of daptomycin in these isolates by mechanisms that are unclear, but seem to be related to the enhanced binding of the lipopetide to the cell membrane. Further clinical data on the use of this combination are needed.

Chloramphenicol is an antibiotic that has been used in the treatment of severe infections caused by VRE (mostly *E. faecium*), since this compound retains *in vitro* activity against most isolates. Chloramphenicol was used in 14 patients with VRE bloodstream infections, achieving 73% of microbiological eradication, but only 53% of favorable clinical response (Okamoto, Okubo, & Inoue, 1996). Another study retrospectively evaluated the clinical response of chloramphenicol treatment for VRE bacteremia in 51 patients (Laverde Gomez, et al., 2011) with rates of microbiological eradication and clinical cure of 79% and 61%, respectively. Moreover, chloramphenicol has been used as an anti-enterococcal agent in cases of CNS infections caused by enterococci (ventriculitis and meningitis) (Heaton & Handwerger, 1995; Scapellato, Ormazabal, Scapellato, & Bottaro, 2005), and also in IE (in combination) (Safdar, Bryan, Stinson, & Saunders, 2002). One of the main concerns regarding the use of chloramphenicol is its inherent bone marrow toxicity, and as a result, the limited clinical data suggest that this compound may be used in "desperate" cases when other therapies or combinations have failed.

Finally, oritavancin is an investigational glycopeptide antibiotic with *in vitro* activity against vancomycin-susceptible enterococci and VRE, including *E. faecium* with the VanA phenotype. *In vivo* studies using animal models of endocarditis (Linden, et al., 2001; Saleh-Mghir, et al., 1999) have shown that oritavancin was able to reduce the number of bacterial colony-forming units of VRE infecting vegetations, as compared to untreated controls and vancomycin, although it did not sterilize the vegetations. Moreover, combination of oritavancin plus gentamicin produced a significant reduction in bacterial counts from vegetations, as compared to untreated controls and prevented emergence of oritavancin-resistant mutants which were readily selected with the use of oritavancin monotherapy. Thus, this compound may offer promise for the treatment of VRE, particularly when used in combination with other agents in the treatment of severe VRE infections (Arias, Mendes, Stilwell, Jones, & Murray, 2012).

Table 3: Possible alternatives for the treatment of severely vancomycin-resistant *E. faecium* infections

E. faecium with ampicillin MIC ≤ 64 µg/ml
No HLR to aminoglycosides
High-dose ampicillin plus aminoglycosides (gentamicin or streptomycin)
HLR to aminoglycosides
HD ampicillin[1] plus daptomycin[2]
HD ampicillin plus Q/D
HD ampicillin plus imipenem[3]
E. faecium with ampicillin MIC > 64 µg/ml
No HLR to aminoglycosides
Daptomycin[2] plus an aminoglycoside + another active agent[4]
Q/D plus + another active agent[4]
Linezolid + another active agent[4]
HLR to aminoglycosides
Daptomycin plus another active agent[4,5]
Q/D plus another active agent[4,6]
Linezolid + another active agent

Q/D: Quinupristin/dalfopristin
[1] Doses up to 30 g/day may be considered
[2] Doses of 8-12 mg/kg are preferred
[3] If imipenem MIC < 32 µg/ml
[4] Agents with potential activity include ampicillin (even if resistant), tigecycline, doxycycline with rifampin, ampicillin, or fluoroquinolones (if susceptible to each agent)
[5] Agents that have been successfully used in case reports include tigecycline (three cases) and ampicillin (one case)
[6] The combination of Q/D plus doxycycline plus rifampin was successfully used in one patient

Treatment of less severe enterococcal infections

As previously mentioned, there are some cases in which enterococci are isolated from clinical samples, but may not need to be treated. This situation usually occurs in patients who have intravascular or urinary catheters, since these devices are usually the source of the infection. Although prospective studies have not specifically identified the subset of patients who have enterococci in the bloodstream or urine and may do well in the absence of specific antimicrobial therapy, clinical experience suggests that this group of patients may include: ***i***) immunocompetent patients without signs of infection (such as those with asymptomatic bacteriuria or bacteremia) in whom positive cultures harbor low bacterial burdens (for example, only one positive bottle in a set of many other blood cultures); ***ii***) cultures usually clear upon removal of the catheter; and ***iii***) patients do not have any risk for the development of endocarditis (that is, patients with prosthetic valves or those in whom prosthetic material was used for valve replacement, history of previous IE, congenital heart disease or patient with history of cardiac transplantation) (Baddour, et al., 2005).

In some individuals with true uncomplicated urinary tract infections (UTIs), the use of systemic agents may not be necessary, and antibiotics that concentrate in the urine may be useful in these settings. Fosfomycin and nitrofurantoin are two drugs that concentrate in the urine and still retain good *in vitro* activity against enterococci, and should be considered for the treatment of uncomplicated UTIs caused by enterococci. Fosfomycin has an FDA approval for the treatment of UTIs caused by vancomycin-susceptible enterococci, but not for VRE or any *E. faecium*. Furthermore, due to high concentrations of the amino-penicillins in the urine, ampicillin or amoxicilin may be useful in cases in which the ampicillin MICs of the enterococcal isolate is <128 µg/ml. It is important to emphasize that removal of the urinary catheter is of paramount importance for the successful eradication of the infective microorganisms in these cases (Arias & Murray, 2012).

Emergence of resistance during therapy

The malleability of enterococcal genomes, the intrinsic resistance of enterococci to several antimicrobial agents, and their abilities to recruit and disseminate antibiotic resistance determinants make these organisms a real challenge in clinical practice. One the main concerns that physicians face during the treatment of enterococcal disease is the possibility of developing resistance during therapy, which may eventually lead to therapeutic failures and, in several instances, contribute to the increased mortality of patients infected with these microorganisms. This issue is more relevant when the mechanism of resistance involves mutations of existing genes rather than the acquisition of new resistance determinants. Development of *in vivo* resistance to linezolid and daptomycin are two of the best examples of this phenomenon.

As previously mentioned, the level of linezolid resistance directly correlates with the number of rRNA genes mutated—the more mutated alleles, the higher the linezolid MIC. The link between mutations in the rRNA genes and the dynamics of emergence of linezolid resistance in *E. faecalis* during therapy was evaluated in a study using an artificial intestinal ecosystem in mono-associated gnotobiotic mice (Bourgeois-Nicolaos, Massias, Couson, Butel, Andremont, & Doucet-Populaire, 2007). This study provided compelling evidence that the selection of linezolid resistance in gnotobiotic mice was highly associated with concentrations of linezolid in the gut, which were directly correlated with the dose regimen used.

For example, in mice exposed to a regimen of 0.5 g/L of linezolid via oral gavage (which mimics the human dosing scheme), linezolid-resistant mutants were selected as early as two days after starting therapy and were identified up to 21 days after the initiation of treatment. However, mutations in only two out of the four rRNA alleles were identified in the *E. faecalis* derivatives with decreased linezolid susceptibility after 21 days of treatment with this regimen. Conversely, a lower-dose regimen of 0.05 g/L selected mutants more readily *in vivo* and mutants harbored mutations in all four copies of the 23S rRNA genes with higher MICs to linezolid than those identified in the higher-dose regimen (Bourgeois-Nicolaos, Massias, Couson, Butel, Andremont, & Doucet-Populaire, 2007).

The study supports the strong correlation between pharmacokinetic/pharmacodynamic parameters of linezolid and the emergence of *in vivo* resistance during therapy. Similarly, emergence of daptomycin resistance during therapy is an important cause of concern, as noted above. These data further emphasize the fact that the success of antimicrobial therapy against enterococci depends on many factors, including the optimization of the *in vitro* and *in vivo* activity of the antimicrobials, since enterococci have a remarkable ability to adapt to environmental stresses and respond to the "attack" of antibiotics.

Impact of antibiotic use on emergence of enterococcal nosocomial infections

Enterococci are commensals of the gastrointestinal (GI) tract of humans and animals, and, in the absence of antibiotics, establish a symbiotic relationship with the human microbiota. In normal circumstances, enterococci are found in low numbers in the colon, as compared with other members of the gut flora (such as anaerobes) (Murray, 2000). The "natural" balance in the microbiota becomes altered by the presence of antibiotics; the administration of cephalosporins and metronidazole, which are often used as part of an empiric regimen in critically ill patients, kills many Gram-negative and anaerobic bacteria in the intestine, but enterococci tend to survive, since these compounds have no potent activity against them. Moreover, some of the cephalosporins (such as ceftriaxone) are excreted in bile, a pharmacokinetic property that increases concentrations of this class of antibiotics in the gut, which maximizes the effect against the gut microbiota and promotes the expansion of enterococci (particularly VRE) in the GI tract (Donskey, et al., 2000).

The establishment of enterococci as the predominant flora in the gut of hospitalized patients has important clinical consequences, since it is from this biological niche that these microorganisms are capable of reaching the

bloodstream or persisting as colonizers that invade other tissues, such as the skin of hospitalized patients (Ubeda, et al., 2010). Indeed, once colonization is established, it may persist for prolonged periods. In a study that included 53 liver and kidney transplant recipients colonized by VRE, persistent colonization for more than three weeks was demonstrated in 66% of patients (Patiño, Chippaux, Courvalin, & Périchon, 2005). Moreover, VRE colonization with isolates that have similar pulsed field gel electrophoresis patterns to those of the initial colonizing strain has been shown to persist for more than a year (Baden, et al., 2001). Colonization of a patient in a medical unit is usually the main factor that triggers the dissemination of enterococcal strains (VRE) to other patients. Epidemiological factors that enhance this dissemination in hospital settings include prolonged hospitalization, physical proximity to patients infected or colonized by VRE, hospitalization in critical care units, and hospitalization in a room with a previous patient known to have had an infection or colonization with VRE, among others (Arias & Murray, 2012). The antibiotics that have been strongly associated with VRE colonization (*E. faecium*) in the GI tract include clindamycin, metronidazole, piperacillin-tazobactam, and second- or third-generation cephalosporins (Sullivan, Edlund, & Nord, 2001).

The actual molecular mediators that promote the colonization of VRE in the presence of antibiotics are unknown, but recent work using murine models of GI colonization has yielded important information to understand the dynamics of enterococcal colonization in the GI tract. Two recent pioneer studies (Brandl, et al., 2008; Kristich & Little, 2012) have been able to demonstrate that surface components of Gram-negative flora play an important role in maintaining the equilibrium between enterococci and their counterparts in the GI tract. Lipopolysaccharide (LPS) and flagellin of Gram-negative bacteria are capable of stimulating (via Toll-like receptors) the production of the lectin RegIIIγ (a C-type lectin) by intestinal epithelial cells. RegIIIγ is capable of killing a variety of Gram-positive organisms, including VRE. Therefore, upon the reduction in Gram-negative flora produced by the presence of antibiotics in the gut, the synthesis of REgIIIγ by the intestinal epithelium is drastically decreased. This effect directly promotes the overgrowth of VRE in the gut.

In mice, the predominance of VRE after the administration of metronidazole, neomycin, and vancomycin was observed for up to two months after discontinuation of the regimen (Ubeda, et al., 2010). Moreover, the high MICs of piperacillin and cephalosporins displayed by ampicillin-resistant *E. faecium* strongly contribute to maintaining VRE as the dominant flora of hospitalized patients in the presence of antibiotics. Ubeda et al. (Ubeda, et al., 2010) elegantly showed that critically ill patients (those undergoing bone marrow transplant due to hematological malignancy) in whom VRE had become the predominant intestinal flora after several courses of antimicrobials, were more likely to develop a bloodstream infection with these organisms, which supports the role of the GI tract as the source of infecting VRE strains in patients receiving antibiotics.

For the foreseeable future, MDR enterococci will continue to pose immense clinical challenges in hospitalized patients. The trends in the epidemiology of enterococcal infections suggest that MDR *E. faecium* may become the most common species isolated from hospitalized patients in the near future. As a result, novel therapeutic strategies focused on treating infections with these organisms are urgently needed.

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Pathogenesis and Models of Enterococcal Infection

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Introduction

The vast majority of enterococci, including species that are major agents of nosocomial infection, are peaceful inhabitants of the gastrointestinal (GI) tracts of animals that range from insects to humans. However, situations arise in which enterococci can cause serious disease. The era of modern medicine has created circumstances that facilitate the pathogenic behavior of these microbes in the following ways. First, the use, and arguable overuse, of antibiotics has selected for bacteria that are resistant to these medicines. Even though enterococci may not be as inherently virulent as some other bacterial pathogens, they are facile at collecting and exchanging antibiotic resistance determinants, in addition to being naturally resistant to many antibitoics. Such properties give enterococci a selective advantage in environments with heavy antibiotic usage, such as the hospital, and may allow them to out-compete other species that would normally keep them in check. Another factor is the increasing susceptible population of immunocompromised individuals, which includes the elderly, solid organ and bone marrow transplant patients, and cancer patients. The action of the immune system likely contributes to the commensal balance, and a severely weakened defense system may be unable to keep these species in check. Finally, there are genetic factors that contribute to the ability of enterococci to survive and cause infection in a host environment, which are defined as virulence determinants for the purpose of this discussion. Some of these factors are part of the core genome, while others are traits that can be acquired and shared. Much work has been done over the last 20 years to identify these virulence determinants, and to characterize their mechanisms of action, which is is the main focus of this chapter.

Animal models have made critical contributions to our understanding of the pathogenesis of enterococcal infection. A number of advances recently have been made that allow for new and more facile models to be employed in these studies. However, since the motivation behind a given study usually relates to some aspect of human infection, it is important to understand the parallels and limitations of each model, in order to know the extent to which the results can be extrapolated to humans.

Models for Studying Enterococcal Infection

Models of infection have been used to test the infectivity of enterococci since 1899, when MacCallum and Hastings (MacCallum & Hastings, 1899) infected mice and rabbits with enterococci in an attempt to test Koch's postulates. Both mice and rabbits succumbed when injected intravenously and intraperitoneally with large inocula of human-derived enterococci (MacCallum & Hastings, 1899). However, their study also illustrated some of the challenges inherent in all investigations of bacterial pathogenesis, i.e., the varying infectivity of

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different strains, and the varying susceptibility of different hosts. Since this first study, mice and rabbits have served as the most common infection models for studies of enterococcal pathogenesis.

In the last decade, invertebrate models of enterococcal infection have been developed as a complement to mammalian models. The use of invertebrates, such as the nematode *Caenorhabditis elegans* and the Greater Wax Moth caterpillar *Galleria mellonella,* has provided new options for pathogenesis studies, in that experimenting with these organisms can be inexpensive and practical. In addition, the large-scale early-phase screens needed for the identification of virulence factors or new therapeutics is prohibitively difficult in mammalian models due to the large numbers of animals required, but can easily be carried out in these small invertebrates (Garsin, et al., 2001; Maadani, Fox, Mylonakis, & Garsin, 2007). In the invertebrates that are amenable to genetic manipulation, it is also possible to examine the host response, as has been done by transcriptional profiling of *C. elegans* infected with *E. faecalis* (Englemann, et al., 2011; Wong, Bazopoulou, Pujol, Tavernarakis, & Ewbank, 2007). However, a crucial requirement for interpreting the results from any animal model is a good understanding of the model's limitations.

Invertebrates

C. elegans

C. elegans is a well-characterized model organism that has been used to elucidate important biological processes in development, gerontology, and, more recently, host-pathogen interactions. It is a small, soil-dwelling nematode that ingests microbes found on rotting fruit as a food source. Presumably because this lifestyle exposes the animal to a variety of potentially pathogenic organisms, *C. elegans* has evolved an intricate innate immune system that shares many features with higher animals (Ewbank & Zugasti, 2011; Irazoqui, Urbach, & Ausubel, 2010; Tan & Shapira, 2011). Structural barriers, such as a tough collagenous cuticle, protect the epidermal surface from direct penetration by all but a few highly specialized pathogens. A variety of antimicrobial peptides including lysozymes, caenopores, lectins, and ABFs (antibacterial factors) are secreted by the intestinal epithelial cells, while NLPs (neuropeptide-like peptide) and caenacins are secreted by the epidermis (Ewbank & Zugasti, 2011). The intestine (and possibly the epidermis) also releases reactive oxygen species (ROS) as part of the defense response (Chávez, Mohri-Shiomi, & Garsin, 2009; Chávez, Mohri-Shiomo, Maadani, Vega, & Garsin, 2007). Several highly conserved metazoan signaling pathways orchestrate the *C. elegans* innate immune response including components of the insulin signaling, p38 MAPK, and β-catenin signaling pathways (Irazoqui, Urbach, & Ausubel, 2010). When comparing the innate immune response of *C. elegans* to the mammalian immune response, it is important to keep in mind that *C. elegans* does not have an adaptive immune system, nor does it have a cellular aspect to its innate immune response. These differences limit the types of comparisons that can be made between microbial interactions with *C. elegans*, on one hand, and microbial interactions with mammals, on the other (Ewbank & Zugasti, 2011; Irazoqui, Urbach, & Ausubel, 2010; Tan & Shapira, 2011).

In addition to the fact that *C. elegans* can be infected by a large array of human pathogens (Powell & Ausubel, 2008), the animal has many characteristics that facilitate the study of such infections. *C. elegans* are easy to maintain in the laboratory due to their small size (the adult is 1 mM long) and short generation time of three days. They possess a hermaphroditic reproductive style that allows one adult animal to produce up to 300 genetically identical progeny, but also a male sex, generated at a low frequency, that allows for the crossing of strains. The animal has a fully sequenced genome, is easily subjected to RNAi-mediated knockdown of genes, and many mutants and genetic tools exist (Hope, 1999). Because of these features, large-scale screens of both host and pathogen factors that contribute to infection, as well as screens for novel antimicrobials, have been possible (Kurz & Ewbank, 2007).

Host-pathogen interactions with various human infectious agents, including *E. faecalis*, can be studied easily in *C. elegans* by exploiting its propensity to feed on a wide range of microbes including bacteria and yeasts (Powell & Ausubel, 2008). The consumption of *E. faecalis* results in an infection in the lumen of the animal's intestine

that eventually causes its demise. To inoculate *C. elegans* with *E. faecalis* by feeding, L4 stage larvae are transferred from a lawn of their normal laboratory food, *E. coli* strain OP50, to plates of solid agar medium that contain a lawn of *E. faecalis* (Garsin, et al., 2001). Brain heart infusion (BHI) is the medium most often used, but for certain expression profiling studies in *C. elegans*, *E. faecalis* has also been placed on nematode growth medium (NGM), which is a minimal medium that causes slower killing kinetics (Englemann, et al., 2011; Wong, Bazopoulou, Pujol, Tavernarakis, & Ewbank, 2007). Adding appropriate antibiotics prevents contamination by OP50, which is carried over when *C. elegans* are transferred to *E. faecalis*. After transfer, the animals die with an LT_{50} of approximately 4–5 days, depending on the particular *E. faecalis* strain, medium, and temperature (Garsin, et al., 2001). Usually *C. elegans* are examined daily and any dead animals are removed from the plates. The animals can be followed on the agar plates, or alternately, they can be moved into liquid culture after an initial inoculation period of 8–12 hours. A liquid medium that was empirically determined to be efficacious for *E. faecalis*-mediated killing is 10–20% BHI diluted in M9 medium (Moy, et al., 2006). Liquid medium is most commonly used in studies that examine the effects of different drugs on the infectious process — for example, screens for new antimicrobials (Moy, et al., 2006; Moy, et al., 2009).

Experiments that examined the features of an *E. faecalis* infection of *C. elegans* showed that there is both colonization and growth inside the intestinal lumen, such that each *C. elegans* eventually contains 10^4 to 10^5 CFU. Invasion of the intestinal cells or the rest of the animal has not been reported. The infection is persistent in that transferring *E. faecalis*-exposed *C. elegans* to a lawn of non-pathogenic bacteria does not displace the *E. faecalis*, and the animals continue to die (Garsin, et al., 2001; Moy, et al., 2006). Infecting *C. elegans* with a small inoculum of *E. faecalis* (by mixing it with a bacterium that does not kill *C. elegans*, such as particular strains of *Enterococcus faecium,* as described below) still results in the eventual engorgement of the intestine and the demise of the animals. Infection by *E. faecalis* requires the ability to divide, and *E. faecalis* exposed to bacteriostatic antibiotics will not kill *C. elegans* (Garsin, et al., 2001). Electron microscopy allows for the observation of actively dividing *E. faecalis* cells in the *C. elegans* intestine (Figure 1). Importantly, *C. elegans* does not reproduce on *E. faecalis*. The animals lay eggs, but few hatch, and these few hatchlings almost never grow beyond the initial L1 larval stage (Garsin, et al., 2001). It is not known whether *C. elegans* larvae are unable to extract nutrients from *E. faecalis* or whether *E. faecalis* kills them.

In contrast to *E. faecalis*, many strains of *E. faecium* grown aerobically colonize, but do not grow or persist within the *C. elegans* gut, and do not kill the animal (Garsin, et al., 2001). However, when *E. faecium* is cultured anaerobically and then restored to aerobic conditions, *E. faecium* kills the nematodes with an LT_{50} of 4–6 hours, due to hydrogen peroxide production by the bacterium (Moy, Mylonakis, Calderwood, & Ausubel, 2004). The mechanism by which *E. faecium* produces H_2O_2 is related to its exclusive use of glycolysis and fermentation, which unlike *E. faecalis* metabolism, can involve a partial electron transport chain (see The physiology and metabolism of enterococci). These metabolic features under aerobic conditions cause *E. faecium* to regenerate NAD^+ by water- and peroxide-forming NADH oxidases (Huycke M. M., 2002). Moy *et al.* (Moy, Mylonakis, Calderwood, & Ausubel, 2004) provided evidence that these oxidases generate the H_2O_2 observed. Normally, an NADH peroxidase degrades the H_2O_2, which prevents its accumulation. Moy *et al.* speculated that the gene for this enzyme is expressed only under aerobic conditions, such that NADH peroxidase levels are low when transitioning the plates from anaerobic to aerobic conditions, which allows for significant amounts of H_2O_2 to accumulate (Moy, Mylonakis, Calderwood, & Ausubel, 2004).

The *C. elegans* model has been used to study and discover factors with a possible role in *E. faecalis* virulence. Cytolysin and the Fsr system affect virulence in a wide variety of hosts, and *C. elegans* is no exception. The presence of cytolysin significantly increases the rate of killing, both on solid and in liquid medium (Garsin, et al., 2001; Moy, et al., 2006). The Fsr system and two of the proteases it regulates, gelatinase and serine protease, also affect virulence in the *C. elegans* model (Garsin, et al., 2001; Moy, et al., 2006; Sifri, et al., 2002). Response to nutritional stress regulated by the stringent response is another important virulence-related factor for optimal *E. faecalis* infection in *C. elegans*, as the loss of both ppGpp synthases that control the stringent response, RelA and

RelQ, limits colonization and infection (Abranches, et al., 2009). Somewhat surprisingly, many surface proteins with possible functions in attachment or adhesion, such as Esp, Ace, Acm, and EbpA, do not play essential roles in the *C. elegans* infection model, perhaps as a consequence of functional redundancy. Mutations in the corresponding genes have not been identified in genetic screens and no attenuated phenotypes have been observed in direct testing ((Maadani, Fox, Mylonakis, & Garsin, 2007), Garsin lab, unpublished data). However, the loss of *ef3314*, which encodes a surface protein with some structural similarity to Esp, does significantly attenuate virulence and suggests that some surface factors do play essential roles in *C. elegans* (Creti, et al., 2009). A forward genetic screen of *E. faecalis* transposon mutants for defects in killing *C. elegans* uncovered potential virulence determinants with predicted functions in transcription, metabolism, and damage control. Five of nine mutants tested were attenuated in a mouse peritonitis model, which demonstrates the utility of using this invertebrate model to identify *E. faecalis* genes that are involved in mammalian infection (Maadani, Fox, Mylonakis, & Garsin, 2007).

Another exciting use of *C. elegans* is as a tool to identify low molecular-weight compounds that are protective against *E. faecalis* infection. To develop this model, it was first demonstrated that traditional antibiotics such as tetracycline and vancomycin at clinically relevant concentrations cure *C. elegans* of an *E. faecalis* infection (Moy, et al., 2006). Then various libraries of compounds were tested for the ability to protect *C. elegans* from *E. faecalis* by screening for increased survival in a liquid infection model. As a result, two compound screens have been described. A small scale screen of about 7,000 compounds, in which curing was scored manually by visual inspection, provided proof of principal for the approach (Moy, et al., 2006). The second screen of approximately 37,000 compounds used robotic liquid-handling to set up the assay, including distribution of *C. elegans* into 384 well assay plates, and automated microscopy and image analysis to score the animals for mortality (Moy, et al., 2009). An unexpected and potentially important outcome of these screens is that many of the compounds identified did not prevent *in vitro E. faecalis* growth at the concentrations that were found to be protective of *C. elegans*. Many compounds were protective at 10- to 100-fold lower concentrations than their corresponding *in vitro* MICs for *E. faecalis*. This is in contrast to traditional antimicrobials, in which the concentration that exerts an inhibitory effect on *in vitro* bacterial growth is significantly lower than the concentration required at the site of infection to achieve clearance in animal models and human patients.

How these drugs are acting to protect *C. elegans* from *E. faecalis*-mediated killing, and whether or not the same effects will be observed in vertebrate models of infection, are areas of active study. Some hypotheses include that these compounds exert their effects on the host innate immune system, block bacterial virulence gene expression, and/or are modified into a more active form within the host (Moy, et al., 2006; Moy, et al., 2009). Recent work has shown that one compound (referred to as RPW-24) activates the *C. elegans* p38 MAPK pathway, thereby conferring resistance not only to *E. faecalis* but also to *P. aeruginosa* infection (Pukkila-Worley, et al., 2012). The ability to use *C. elegans* infected with *E. faecalis* as a model to identify compounds that protect against infection could lead to new classes of anti-infectives of clinical importance, and the continuation of this work will be followed with much interest.

Insects

Insects, particularly the larva of the Greater Wax Moth Caterpillar, *Galleria mellonella*, have recently emerged as important models for testing the roles of potential virulence factors in infectious human pathogens, including enterococci. Insects have surprisingly complex innate immune systems, based on both cellular and humoral mechanisms with parallels in mammals. The insect blood equivalent, called the hemolymph, contains innate immune cells called haemocytes, which can phagocytose invading pathogens in a manner similar to human macrophages and neutrophils. Though insects do not generate specific antibodies, they do produce a wide range of substances that constitute the humoral response. The factors include components of the clotting and melanization cascades and various anti-microbial peptides, some of which are shared with vertebrates (lysozymes, metalloproteinases, defensins) (Kavanagh & Reeves, 2004).

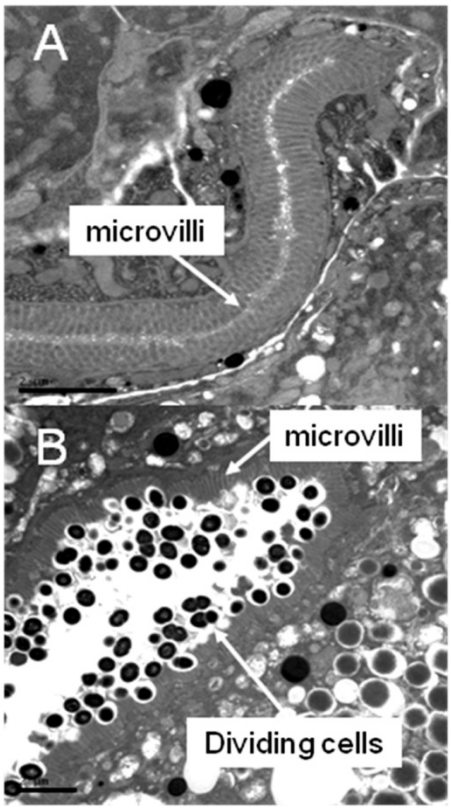

Figure 1. Electron micrographs of *C. elegans* intestine from animals infected with *E. faecalis* for 48 hours (B), compared to uninfected animals (A). Note that the infected animals display a distended intestinal lumen, erosion of the microvilli, and colonization with actively dividing bacteria. Picture courtesy of S. Kolodziej, P. Navarro R. van der Hoeven and D. A. Garsin, University of Texas Health Science Center at Houston.

G. mellonella is an attractive model, because larvae can easily be reared at low cost or cheaply obtained from commercial sources. The animals are infected by injection into the hemolymph and survival is monitored over a few days, with death usually measured as the endpoint. Staff can also be quickly and easily trained in the procedures. Limitations to the model include the lack of a sequenced genome, the inability to genetically manipulate the organism because no molecular tools are currently available, and sometimes, an imperfect recapitulation of pathogen-human interactions (Mylonakis E. , 2008; Olsen, Watkins, Cantu, Beres, & Musser, 2011).

G. mellonella has only recently been used as a model for *E. faecalis* infection. *E. faecalis, E. faecium*, and other species of enterococci are naturally found to be associated with insects (Cox & Gilmore, 2007; Martin & Mundt, 1972). The use of *G. mellonella* as an infection model began when a group isolated a strain of *E. faecalis* from larvae that died of a bacterial infection. Injection of this strain back into hemolymph of the animal resulted in death (Park, Kim, Lee, Seo, & Lee, 2007). In other work, a dose-dependent sensitivity to infection by *E. faecalis* was discovered (Yan, et al., 2009). Depending on the strain of *E. faecalis* being used, investigators observed death within one to two days of inoculating with anywhere between 1×10^5 to 5×10^8 CFUs (Michaux, et al., 2011; Yan, et al., 2009; Zhao, et al., 2010). *E. faecium* and *E. durans* are significantly less infective in this model (Gaspar, et al., 2009). Only large inocula (1×10^8) of some strains of *E. faecium* resulted in caterpillar mortality. However, at lower inoculums (1×10^6), better colonization was observed among some hospital-adapted isolates of *E. faecium* (Lebreton, et al., 2011). Infection by oral feeding of *E. faecalis* was shown to be ineffective, which is perhaps not surprising when considering *E. faecalis*'s common role as a gut commensal (Fedhila, et al., 2010).

Several virulence factors affect time to death in *G. mellonella* larvae when mutants that carry lesions in the genes-of-interest are compared to their isogenic, wild type controls. Not surprisingly, the Fsr quorum-sensing

system and specifically one of the genes it regulates, *gelE*, were discovered to be important. Park *et al.* (Park, Kim, Lee, Seo, & Lee, 2007) observed that a secreted substance had insecticidal activity, and purification identified it as GelE. They then demonstrated that GelE could digest cecropin and other antimicrobial peptides produced by *G. mellonella* (Park, Kim, Lee, Seo, & Lee, 2007). Gaspar *et al.* (Gaspar, et al., 2009) showed that strains that lacked *gelE* were less virulent, as compared to isogenic controls. The Fsr quorum-sensing system is generally necessary for *gelE* expression, and loss of *fsrB* caused attenuated phenotypes in two *E. faecalis* strains, but not in the OG1RF background—perhaps because it has residual gelatinase activity (Gaspar, et al., 2009; Singh K. V., Nallapareddy, Nannini, & Murray, 2005). Loss of the ppGpp synthase, *relA*, which is critical for stress adaption, was shown to attenuate virulence in *G. mellonella*. A more stress-resistant mutant that consisted of a C-terminal truncation was more virulent (Yan, et al., 2009). Loss of another stress resistance factor, the heat-shock protein ClpB, also attenuated virulence (de Olivera, et al., 2011). The collagen- and laminin-binding protein Ace resulted in attenuated infection in *G. mellonella* when the gene was deleted (Lebreton, et al., 2009). Other *E. faecalis* factors that affect virulence in this model include methionine sulfoxide reductases (encoded by *msrA* and *msrB*) and the transcription factor SlyA, whose loss actually increases virulence. The methionine sulfoxide reductases and SlyA have also been shown to affect persistence within mouse organs following IV injection and survival within peritoneal macrophages, which demonstrates that their importance during infection extends beyond *G. mellonella* (Michaux, et al., 2011; Zhao, et al., 2010).

In addition to examining specific potential virulence determinants, *G. mellonella* is being used to identify new ones. One group looked at the ways in which lyosogeny with different phages affects virulence. The authors observed changes in *G. mellonella* survival, both increased and decreased, that were dependent upon the phage employed. Though the genetic determinants have not yet been identified, these experiments suggest that the phages are introducing or disrupting factors that affect virulence (Yasmin, et al., 2010). Additionally, *G. mellonella* was employed in a screen for *in vivo* activated genes using recombination-based in vivo expression technology (R-IVET). Among the genes found, the inactivation of a two-component system, *ef_3196* and *ef_3197*, resulted in a virulence defect, whereas deletion of a gene encoding an ankyrin repeat protein caused a hypervirulent phenotype (Hanin, et al., 2010). The *G. mellonella* model has also been demonstrated to be a powerful tool for large-scale investigations, such as the screening of a targeted *E. faecalis* mutant library for new fitness factors (Rigottier-Gois, et al., 2011).

E. faecalis was first reported to infect *Drosophila melanogaster* in 2001. Infection of the fly was achieved by pricking the animal with a needle previously dipped into a concentrated culture of bacteria. The goal of this investigation, and most of the *Drosophila* studies afterward, was to elucidate host immune responses to a Gram-positive agent, rather than to understand *E. faecalis* virulence mechanisms (Michel, Reichhart, Hoffmann, & Royet, 2001).

Cox and colleagues (Cox & Gilmore, 2007) characterized the gut microbiota of wild-caught and laboratory reared *Drosophila*, and localized enterococci within that consortium. Enterococci were among the most abundant gut microbes found using sequence-based metagenomics. Feeding *Drosophila* strains that were cytolytic resulted in increased mortality (40% mortality vs. 12%), which demonstrated that this virulence factor could compromise this host by a natural infection route (Cox & Gilmore, 2007).

Vertebrates

In contrast to infection studies carried out in invertebrates, vertebrate animal infection models afford researchers the ability to study enterococcal pathogenesis in the context of the innate and adaptive immune responses, and to evaluate bacterial effects on organs that closely resemble those found in humans. An important challenge in attempting to approximate human infection in a model is that enterococcal strains of interest are either commensals or are recently derived from commensals, and they infrequently infect humans or animals with intact immune systems. Therefore, to achieve a progressive infection using healthy vertebrate animals, large inocula, either with or without foreign bodies (such as intravascular catheters for the endocarditis model) are

needed. Some models have been developed that induce neutropenia in animals prior to infection (Griffith, Rodriguez, Corcoran, & Dudley, 2008; Leendertse M. , et al., 2009; Onyeji, Nicolau, Nightingale, & Bow, 2000) or that take advantage of naturally immune limited tissues (such as the endophthalmitis model (Callegan, Booth, Jett, & Gilmore, 1999; Jett, Atkuri, & Gilmore, 1998; Jett, Jensen, Nordquist, & Gilmore, 1992; Stevens, Jensen, Jett, & Gilmore, 1992) further discussed below), which enables the production of disease manifestations using fewer organisms. Although these models are generally more technically demanding, the use of animal models of enterococcal infection has greatly enhanced our understanding of the chromosomal- and plasmid-encoded determinants that enable enterococci to cause disease, as well as the pathogenesis of those infections in mammalian tissues.

Endocarditis

Enterococci are the third most common cause of endocarditis (Murdoch, et al., 2009), which is one of the most life-threatening infections caused by *E. faecalis* and *E. faecium*, and as a result, using a mammailian model to elucidate the role of enterococcal virulence traits is critical to derive new approaches for treatment and prevention. Enterococcal endocarditis involves the formation of a biofilm on heart valves at sites of damage that become integrated into masses called vegetations. A number of enterococcal adhesins or proteins known to function in biofilm formation have been tested in endocarditis models. Among those that contribute to *E. faecalis* endocarditis virulence are gelatinase (Thurlow L. R., et al., 2010), the protease Eep (Frank, et al., 2012), aggregation substance (Chow, et al., 1993; Chuang, et al., 2009), Ebp pili (Nallapareddy S. R., et al., 2006), and Ace (Singh K. V., Nallapareddy, Sillanpää, & Murray, 2010). Endocarditis was observed to be lethal in a rabbit model in the presence of aggregation substance-expressing cells that also expressed the toxin cytolysin, and vegetations were larger when caused by an isogenic strain that expressed aggregation substance alone (Chow, et al., 1993). The severity of endocarditis was reduced in rats that were injected with a recombinant Ace protein or passively immunized with anti-Ace antibodies (Singh K. V., Nallapareddy, Sillanpää, & Murray, 2010). Besides the cytolysin toxin, adhesins, and biofilm determinants, the general stress response protein Gls24 was also shown to contribute to *E. faecalis* endocarditis in rats (Nannini, Teng, Singh, & Murray, 2005). In *E. faecium*, the adhesin Esp was found to be necessary for full virulence in the rat endocarditis model (Heikens, et al., 2011).

Enterococcal endocarditis models have generally used either Sprague-Dawley rats or New Zealand white rabbits, with a number of procedural variations. In both models, damage to the aortic valve is induced by advancing a catheter into the right carotid artery to the point where it crosses the valve. Catheters are then either left in place for the duration of the infection (Thurlow L. R., et al., 2010), or removed after two hours (Chuang, et al., 2009; Schlievert, et al., 1998). A small study aimed at examining the effect of leaving the catheter in place during infection found that the number and size of vegetations formed by strains was increased, as compared to rabbits from which the catheters had been removed (Schlievert, et al., 1998). This observation led the study authors to propose that vegetation formation in the presence of a transaortic catheter may be artificially enhanced (Schlievert, et al., 1998).

In the rat model, inocula of 10^7-10^8 CFU are often administered through the catheter twenty minutes after its placement, the catheter is then heat-sealed, and the end into which microbes were added is embedded in subcutaneous tissue. The animals are typically euthanized at 24 hours post-inoculation (Nallapareddy S. R., et al., 2006). Bacteria may also be injected through the rat tail vein. In rabbits with the catheter remaining throughout infection, bacteria are injected through an ear vein 24 hours after surgery, and the experiment is terminated at 48 hours post-inoculation (Thurlow L. R., et al., 2010). Alternatively, bacteria are injected through an ear vein within two hours following catheter removal, and rabbits are euthanized at 96 hours post-inoculation (Chuang, et al., 2009; Frank, et al., 2012). The reported inoculum used for rabbit experiments was ~10^7 CFU for strain *E. faecalis* V583 in animals with retained catheters, and ~10^9 of OG1SSp or OG1RF in animals with the catheters removed (Chuang, et al., 2009; Frank, et al., 2012; Thurlow L. R., et al., 2010). Following euthanasia, hearts are dissected to expose the aortic valve in order to harvest vegetations for bacterial enumeration by

quantitative culture. Bacterial loads in the blood, kidneys, spleen, and liver can also be determined (Nallapareddy S. R., et al., 2006; Thurlow L. R., et al., 2010).

The number of variables that differ between endocarditis models requires that caution be exercised in interpreting the results of these experiments. The differences that should be considered include vegetation formation in the presence or absence of a foreign body, whether bacteria encounter the damaged heart valve by circulation or by direct delivery to the infection site, and how long after valve damage bacteremia is induced. The latter is important because sterile vegetations, or nonbacterial thrombotic lesions, can form on damaged valves over time, such that the delayed onset of enterococcal bacteremia (such as in (Thurlow L. R., et al., 2010)) will result in bacterial colonization of an extant mass on the damaged valve. By comparison, the induction of bacteremia shortly after valve damage likely results in the bacteria being present at the valve concurrent with the onset of vegetation formation. Therefore, it is possible that different genetic determinants may be involved in enterococcal colonization of damaged heart valves, and that discrepancies may arise as to whether a gene contributes to endocarditis virulence, based on the model in which it was tested.

Urinary tract infection

Enterococcal species are the second most common cause of catheter-associated urinary tract infection (CAUTI) reported to the National Healthcare Safety Network over a 22-month period beginning in January 2006 (Hidron, et al., 2008). Several murine models of *E. faecalis* urinary tract infection (UTI) have been used to study the pathogenesis of infection over the past two decades. Most are variations of ascending unobstructed urinary tract infections and are adapted from models used to study Gram-negative uropathogens (Johnson, Clabots, Hirt, Waters, & Dunny, 2004; Kau, et al., 2005; Shankar, et al., 2001). Typically, 10^6-10^8 organisms are administered to anesthetized mice via a transurethral catheter, and animals are sacrificed at intervals ranging from one to fourteen days, at which time urine, the bladder, and the kidneys are harvested for quantitative bacterial culture and histopathology (Kau, et al., 2005; Shankar, et al., 2001; Singh, Nallapareddy, & Murray, 2007). *E. faecalis* shows a tropism for the kidneys in this type of model, making it useful for studies focused on factors that contribute to enterococcal pyelonephritis (Johnson, Clabots, Hirt, Waters, & Dunny, 2004; Kau, et al., 2005; Shankar, et al., 2001; Singh, Nallapareddy, & Murray, 2007). Infection of C57BL/6J mice with an *E. faecalis* urinary tract isolate caused an inflammatory response in the kidneys, but not the bladder (Kau, et al., 2005). The inflammatory response was different than that induced by uropathogenic *E. coli* and was found to be independent of receptor TLR2 (Kau, et al., 2005). Deletion or disruption of the coding sequences for a number of *E. faecalis* adhesins, including Ace, Esp, Epa, and Ebp pili, resulted in reduced virulence in mouse models of ascending UTI infection (Lebreton, et al., 2009; Shankar, et al., 2001; Singh, Lewis, & Murray, 2009; Singh, Nallapareddy, & Murray, 2007). The *E. faecium* pilus also contributes to UTI virulence (Sillanpää J. , et al., 2010).

A model that more closely parallels *E. faecalis* CAUTI has been developed (Guiton, Hung, Hancock, Caparon, & Hultgren, 2010), which involves implantation of silicone catheter segments into the bladders of C57BL/6Ncr mice immediately before inoculation with 10^7 organisms. Biofilms formed *in vivo* by strain OG1RF on implanted catheter segments contained 10^7 CFU/implant, from two to seven days post-infection. The presence of the implants increased bladder colonization by three orders of magnitude at 24 hours post-infection, and increased kidney colonization by more than one order of magnitude between 24 hours and 7 days post-inoculation, as compared to control infections. Implantation of silicone pieces resulted in marked histological damage to the uroepithelium, and caused increased expression of several cytokines, including IL-6, G-CSF, and keratinocyte-derived cytokines. Genetic studies indicate that a sortase A-dependent substrate is necessary for virulence in this model (Guiton, Hung, Hancock, Caparon, & Hultgren, 2010).

Endophthalmitis

Because of the challenges presented in handling immune-compromised animals, and because of the difficulty studying the progression of infection in a closed tissue, such as the heart or peritoneal cavity, an endophthalmitis

model was developed (Callegan, Booth, Jett, & Gilmore, 1999; Jett, Atkuri, & Gilmore, 1998; Jett, Jensen, Nordquist, & Gilmore, 1992; Stevens, Jensen, Jett, & Gilmore, 1992) that capitalizes on 1) the immune privileged nature of the interior of the eye and its natural susceptibility to infection; 2) the fact that the infection remains largely localized, and the presence of an uninfected control in the same animal in the contralateral eye; 3) the ability to visually follow the infection using inexpensive and readily available instrumentation (an ophthalmoscope); and 4) the ability to objectively and sensitively measure changes in organ function by using electroretinography. In the rabbit model, which is less technically demanding than a similar mouse model, as few as 1000 enterococci are injected typically (although as few as 10 organisms can seed an infection (Stevens, Jensen, Jett, & Gilmore, 1992)) in a 10 µl volume of PBS through an avascular point in the sclera, called the pars plana, to limit intraocular bleeding. The placement of the inoculum into the vitreous immediately behind the lens allows the process to be followed using a surgical microscope. Because of the gel-like nature of the vitreous, the inoculum forms a visible bubble of PBS that refracts light slightly differently than does vitreous. After about 6 hours, fibrin contained within the vitreous begins to coalesce around the 10 µl bubble, and this can be seen with an ophthalmoscope. The clearest direct view of the microvasculature of the body can be found in the retina. Examination of the retina with an ophthalmoscope after about 12 hours post-infection reveals capillaries in the infected eye that begin to dilate because of the microbe-induced inflammation. As early as 16 hours post-infection, a whitening of the microvessels in the retina can be observed using an ophthalmoscope, as neutrophils begin adhering to the vessel walls. Shortly after that, streams of neutrophils that emanate from the optic nerve head can be seen (Callegan, Booth, Jett, & Gilmore, 1999; Jett, Jensen, Nordquist, & Gilmore, 1992; Stevens, Jensen, Jett, & Gilmore, 1992).

In addition to direct observation, measurements routinely performed during the course of infection include assessment of retinal function by electroretinography (ERG). This involves specialized equipment for assessing electrophysiological behavior of neural cells of the retina. In brief, a fine gold electrode is placed on the surface of the eye and a grounding electrode is placed elsewhere on the anaesthetized animal. It is then exposed to a series of xenon strobe flashes. As the photoreceptors and interconnecting neurons fire, an electrical potential is generated, which is detected by the electrodes. The magnitude of the potential is proportional to the number of cells firing. Using this tool, a decline in the function of the photoreceptor layer can be measured, from 100% functionality to 0%, typically over the course of a 48–72 hour experiment. Following termination of the study and dissection of the eye, other parameters can be measured, including enumeration of inflammatory cells in the cornea, anterior chamber, vitreous and retina, as well as histopathological examination of changes in the vitreous and the architecture of the retina and surrounding structures (Callegan, Booth, Jett, & Gilmore, 1999; Jett, Jensen, Nordquist, & Gilmore, 1992; Stevens, Jensen, Jett, & Gilmore, 1992). Inoculation with approximately 10^2 organisms leads to a vitreous bacterial load of 10^8–10^9 organisms within 24 hours (Callegan, Booth, Jett, & Gilmore, 1999; Jett, Jensen, Nordquist, & Gilmore, 1992; Stevens, Jensen, Jett, & Gilmore, 1992). Changes in these parameters have been used to examine the role of the cytolysin of *E. faecalis* in the pathogenesis of enterococcal infection, as well as its susceptibility to treatment (Callegan, Booth, Jett, & Gilmore, 1999; Choi, Hahn, Osterhout, & O'Brien, 1996; Jett, Jensen, Atkuri, & Gilmore, 1995; Jett, Jensen, Nordquist, & Gilmore, 1992; Stevens, Jensen, Jett, & Gilmore, 1992; Wada, et al., 2008). Additionally, the contributions to the pathogenesis of infection of the *E. faecalis* quorum-sensing *fsr* regulatory locus (Mylonakis, et al., 2002), and its regulated proteases gelatinase and serine proteases (Engelbert, Mylonakis, Ausubel, Calderwood, & Gilmore, 2004) have been studied.

Peritonitis and lethality experiments

One of the most widely used infection models is the intraperitoneal infection/peritonitis model. This typically involves mice or rats and consists of intraperitoneal injection of enterococci, followed by daily monitoring for signs of morbidity or death. Parameters measured, often at pre-determined time points, include bacterial counts and assessment of the host response in peritoneal lavage fluid and organs. Without an adjuvant, high concentrations of organisms, ranging from 10^7–10^{11} CFU/ml, are required to achieve LD_{50}. This approach was

used in initial studies that compared the relative lethality of strains that expressed cytolysin or aggregation substance (Dupont, Montravers, Mohler, & Carbon, 1998; Ike, Hashimoto, & Clewell, 1984). Singh *et al.* (Singh, Lewis, & Murray, 2009) found that incorporation of sterile rat fecal extracts into the inoculum lowered the LD_{50} of strain OG1RF, and this approach was used to evaluate the relative virulence of several strains with mutations in putative virulence determinants (Singh, Qin, Weinstock, & Murray, 1998). The cytolysin was observed to enhance toxicity upon intraperitoneal challenge approximately 100-fold (Singh, Qin, Weinstock, & Murray, 1998). Other *E. faecalis* traits that lower LD_{50} values, or reduce times to death upon intraperitoneal challenge include *gelE* (Singh, Qin, Weinstock, & Murray, 1998), the *epa* locus (Xu, Singh, Murray, & Weinstock, 2000), *fsr* genes and *sprE* (Qin X. , Singh, Weinstock, & Murray, 2000), *etaRS,* which encodes a two component system (Teng, Wang, Singh, Murray, & Weinstock, 2002), *gls24* (Teng, Nannini, & Murray, 2005), and the gene that encodes the transcriptional regulator Ers (Riboulet-Bisson, et al., 2008). A mouse peritonitis model was recently used to screen a recombination-based *in vivo* expression technology library in order to identify genes that are upregulated during growth in infection (Hanin, et al., 2010).

In addition to studying entrococcal virulence, an *E. faecium* peritonitis model was established to study the way in which the mouse innate immune system responds to bacterial infection (Leendertse M. , et al., 2008). Peritoneal infection with 10^8 CFU in healthy mice led to gradual clearing of enterococci over 48 hours, which was accompanied by an initial increase in neutrophils (6 hours) and a later upsurge in macrophages (24–48 hours). By using mice deficient for TLR2 and the TLR common adaptor protein MyD88, the authors showed that bacterial clearance and neutrophil influx were diminished during the first several hours post-inoculation. In a follow-up study (Leendertse M. , et al., 2009), the same group demonstrated that bacterial loads were increased for longer periods of time in mice with induced neutropenia, and that this was associated with increased levels of TNF-alpha and IL-6 and decreased recruitment of macrophages to the peritoneal fluid. Opsonization by complement and the presence of peritoneal macrophages appeared to be necessary for optimal clearance of *E. faecium* by 48 hours post-infection (Leendertse M. , et al., 2010; Leendertse M. , et al., 2009). This series of experiments suggests that many aspects of the innate immune system are required in a host for maximal protection against *E. faecium* intra-abdominal infection.

Subcutaneously implanted foreign bodies

Models employing subcutaneously implanted foreign bodies, or tissue cages, have served as a means to study both subdermal abscess formation and implanted device biofilm infections caused by *E. faecalis* (Frank, et al., 2012; Furustrand Tafin, et al., 2011). Hollow, perforated plastic or Teflon devices are subcutaneously placed in the flank of rabbits (Frank, et al., 2012) or guinea pigs (Furustrand Tafin, et al., 2011) under aseptic conditions, and wounds are allowed to heal for 2–6 weeks. During healing, the implanted chambers are encapsulated by host tissues and fill with fluid. Cages are inoculated by injecting organisms through the cage perforations, and infected cage fluid can be withdrawn at any time during the course of infection. In the rabbit model, it is known that bacteria remain localized within the chamber, rather than escaping to the bloodstream to cause bacteremia, as the animals do not exhibit overt signs of illness in experiments carried out over as many as seven days. The rabbit model has been used to characterize *E. faecalis* gene expression during growth in a mammalian host through transcriptional microarrays and recombinase-based *in vivo* expression technology (Frank, et al., 2012; Frank, Lemos, Schlievert, & Dunny, 2012). Since the rabbits do not become outwardly sick, the rabbit subdermal abscess model is not particularly useful for assessing the systemic impact of enterococcal infection, but conclusions can be drawn about persistence *in vivo* in the context of an activated host immune response (Frank, et al., 2012). The guinea pig model has been shown to be useful for testing antibiotic activity against *E. faecalis* in both the planktonic and biofilm states (Furustrand Tafin, et al., 2011).

Analysis of Virulence of *E. faecalis*

Because most enterococcal infections have historically been caused by strains of *E. faecalis,* and because the virulence of *E. faecium* strains is more subtle and the strains are often more difficult to manipulate, most virulence studies of enterococci have examined *E. faecalis* and its factors. Both secreted factors, as well as surface localized properties, have been found to contribute to the severity of infection.

Secreted Factors

The cytolysin

The cytolysin contributes to the virulence of *E. faecalis* in humans and all animal models tested (268a), and is a structurally novel toxin and member of the lantibiotic class of bacteriocins expressed by many strains of *E. faecalis* (Haas & Gilmore, 1999; Van Tyne, Martin, & Gilmore, 2013). It was first characterized in the 1930s and was studied for the hemolytic activity that it conferred to group D streptococcal isolates (Todd, 1934). Later, it was noted that hemolytic isolates of *S. faecalis* (now *E. faecalis*) also inhibited a broad range of Gram-positive organisms (Brock, Peacher, & Pierson, 1963; Stark, 1960). The simultaneous loss of both bactericidal and hemolytic activities by these strains upon UV exposure, with subsequent reversion, suggested a single determinant that encodes both activities (Brock & Davie, 1963).

The bacteriocin activity of enterococcal strains was examined by Sherwood *et al.* (Sherwood, Russell, Jay, & Bowman, 1949) who found that of 61 hemolytic streptococci, 17 were found to produce an "antibiotic substance" active against other streptococci. Five of the eight hemolytic group D (enterococcal) strains in this group were also found to be capable of bacteriocin production. Later, Brock *et al.* (Brock, Peacher, & Pierson, 1963) found that over 50% of enterococcal isolates examined produced some type of bacteriocin. These authors defined five different types of bacteriocins, based on activity spectrum and biochemical characteristics, noting that type 1 bacteriocin was produced by all strains of *Streptococcus zymogenes* (hemolytic *E. faecalis*), and that this bacteriocin had a particularly wide spectrum of activity against Gram-positive bacteria.

Epidemiological data supports a role for the cytolysin as a toxin in human infection. Infection-derived isolates of *E. faecalis*, particularly those that cause multiple infections in a hospital ward, showed enrichment for the cytolysin (Huycke & Gilmore, 1995; Huycke, Spiegel, & Gilmore, 1991; Ike, Hashimoto, & Clewell, 1987). Another study found that cytolysin was as prevalent in hospital-derived fecal samples as clinical samples, but was significantly less common in community fecal samples, which suggests that the hospital environment selects for cytolysin (Coque, Patterson, Steckelberg, & Murray, 1995). The cytolysin has been associated with lethality to humans following analysis of an outbreak of multiple antibiotic-resistant *E. faecalis* (Huycke, Spiegel, & Gilmore, 1991). Patients infected with cytolytic, gentamicin/kanamycin-resistant strains were found to be at a five-fold increased risk of an acutely terminal outcome. Another large prospective study of 398 patients with enterococcal bacteremia, however, did not find a statistically significant correlation between 14-day mortality and gelatinase, cytolysin or Esp, either singly or in combination (Vergis, et al., 2002).

In well controlled animal models, ranging from mammals to invertebrates, the cytolysin makes a readily demonstrable contribution to the severity of infection (Chow, et al., 1993; Garsin, et al., 2001; Huycke, Spiegel, & Gilmore, 1991; Ike, Hashimoto, & Clewell, 1984; Ike, Hashimoto, & Clewell, 1987; Jett, Jensen, Nordquist, & Gilmore, 1992). In most of these studies, the role of the cytolysin was evaluated using isogenic mutants of the cytolysin operon. The first demonstration of the toxigenic activity of the cytolysin was presented by Ike *et al.* (Ike, Hashimoto, & Clewell, 1984), who employed murine lethality tests to show that *E. faecalis* strains that express the cytolysin are an order of magnitude more toxic than isogenic, non-cytolytic strains. Also, a hypercytolytic strain derived by a Tn*917* insertion in the cytolysin operon resulted in a further two-fold increase in toxicity. Using different strains of enterococci, others have confirmed that the cytolysin contributes to the 50%

lethal dose (LD50), increasing lethality by one or more orders of magnitude (Dupont, Montravers, Mohler, & Carbon, 1998; Singh, Qin, Weinstock, & Murray, 1998).

The role of cytolysin in the pathogenesis of *E. faecalis* infectious endocarditis was assessed in a rabbit model (Chow, et al., 1993). By examining *E. faecalis* strains that expressed either cytolysin and/or aggregation substance, it was determined that these factors had a synergistic effect on rabbit mortality. Mortality was about 15% in rabbits infected with strains that expressed aggregation substance alone, whereas mortality jumped to 55% when both aggregation substance and cytolysin were expressed. In contrast, the lethal effect of the cytolysin in this model was essentially abrogated in the absence of aggregation substance. In retrospect, these results are not altogether surprising, given that we now know that the expression of cytolysin is quorum-dependent, and aggregation substance most likely facilitates *E. faecalis* adherence at the infection site, which promotes the manifestation of the toxic effects of the cytolysin (Haas, Shepard, & Gilmore, 2002).

E. faecalis is frequently implicated in surgical site infections, including post-operative endophthalmitis, which is often associated with poor visual outcomes (Callegan, Engelbert, Parke III, Jett, & Gilmore, 2002). In a rabbit model of experimental endophthalmitis, the *E. faecalis* cytolysin was found to contribute significantly to ocular virulence. Infection with a cytolytic *E. faecalis* strain, using as few as 100 organisms, was associated with a rapid loss of vision and severe retinal tissue damage, both of which were not observed upon infection with a mutant strain that was defective in cytolysin production (Jett, Jensen, Nordquist, & Gilmore, 1992). Furthermore, combined antimicrobial and anti-inflammatory therapy that allowed for recovery in rabbits infected with a non-cytolytic mutant proved completely ineffective in the treatment of rabbits infected with an isogenic, cytolytic strain (Jett, Jensen, Atkuri, & Gilmore, 1995).

Given that one natural habitat of the enterococci is the gastrointestinal tract, it has been of interest to see which enterococcal factors might promote translocation across the intestinal epithelium. With regard to the cytolysin, although a direct comparison between isogenic strains differing in cytolysin production was not made, Wells *et al.* (Wells, Jechorek, & Erlandsen, 1990) demonstrated high levels of translocation by a cytolytic *E. faecalis* strain. Concentrations of *E. faecalis* in this study ranged from 10^9 to 10^{10}, which suggests conditions that would enable the production of cytolysin. Huycke *et al.* (Huycke, Joyce, & Gilmore, 1995) observed that during *in vitro* growth of a 1:1 mixture of cytolytic and isogenic non-cytolytic strains of *E. faecalis*, the cytolytic strain outgrew the mutant. However, in a murine colonization model achieved by orogastric administration to antibiotic-treated mice, a 1:1 mixture of cytolytic and non-cytolytic *E. faecalis* strains resulted in equal colonization, as detected by measuring the CFUs from the stools of mice after one or seven days. As with negative results from all models, however, it is difficult to know in this case whether these results mean that the bacteriocin activity of the cytolysin does not contribute to colonization of hospitalized patients, or whether the test as conducted does not fully model the ecological conditions and competing flora that exist in the gastrointestinal tracts of hospitalized patients.

The contribution of cytolysin to proliferation in the bloodstream has been evaluated. In a peritonitis model evaluating isogenic mutants, about four orders of magnitude greater numbers of cytolytic *E. faecalis* were quantified in the bloodstream at 48 hours of infection, as compared to the non-cytolytic mutant (Huycke, Sahm, & Gilmore, 1998). Although a mechanism for this observation is not readily apparent, the cytolysin is known to possess activity against macrophages and polymorphonuclear leukocytes (PMNs), which implies a potential role in immune evasion (Miyazaki, et al., 1993). An *in vitro* study that evaluated PMN-mediated killing of *E. faecalis* failed to observe differences between cytolytic *E. faecalis* and isogenic mutants, and it was not clear if cytolysin was indeed expressed under the experimental conditions used for the assay (Arduino, Murray, & Rakita, 1994). Along these lines, the survival of a cytolytic strain of *E. faecalis* within peritoneal macrophages *in vitro* was not different from that of a non-cytolytic strain as tested (Gentry-Weeks, Karkhoff-Schweizer, Pikis, Estay, & Keith, 1999).

As mentioned, expression of the cytolysin by *E. faecalis* resulted in a significant increase in lethality when *C. elegans* nematodes were fed on a lawn of bacteria (Garsin, et al., 2001). More recently, a study examining the native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis found that high-level colonization with a cytolytic strain of *E. faecalis* increased killing of *Drosophila*, as compared to an isogenic non-cytolytic strain (Cox & Gilmore, 2007).

In summary, a wide variety of infection models have shown a role for the cytolysin in *E. faecalis* virulence. The broad activity of the cytolysin against both prokaryotic and eukaryotic cell types suggests that cytolytic activity might provide access to key nutrients that are not accessible to non-cytolytic strains. Supporting this hypothesis, it has been shown that *E. faecalis* is capable of assembling an electron transport chain through the synthesis of *b*-, *d*- and *o*-type cytochromes (Pritchard & Wimpenny, 1978; Ritchey & Seeley Jun, 1974). Assembly of these cytochromes requires the uptake of exogenous hemin, which could presumably become available through lysis of erythrocytes or other target cells. The net effect of this process is an increase in energy yield through aerobic respiration.

Although it is clear that cytolysin contributes to virulence in animal models, the conflicting data on its effect on host cells precludes a clear understanding of the mechanisms involved. The synergism exhibited with a surface adhesin–like aggregation substance may be most relevant during biofilm-type infections, where bacterial densities reach a quorum beyond the threshold required for the production and toxic activity of cytolysin. The inclusion of the genes that encode for the cytolysin along with those encoding adhesions, such as aggregation substance and Esp, on the *E. faecalis* pathogenicity island, may thus be a selected outcome that favors fitness (Shankar, Baghdayan, & Gilmore, 2002).

The molecular mechanism of cytolysin action on erythrocytes has been well studied. Initial studies of the hemolytic activity of the cytolysin revealed that when *E. faecalis* strains that were hemolytic on blood agar were cultured in standard liquid laboratory media, no such activity could be detected in the culture fluid (Todd, 1934), as noted as a caveat in the above studies with immune cells in culture. These observations prompted the author to term it a "pseudohaemolysin." Kobayashi (Kobayashi, 1940) later observed that some, but not all, erythrocytes were susceptible to cytolysin-mediated hemolysis; specifically, erythrocytes from human, horse, cow, and rabbit, but not sheep or goat, were susceptible. Later, it was suggested that phosphatidylcholine may serve as the preferred target on the erythrocyte membrane, due to the relatively higher levels of phosphatidylcholine in the outer leaflet of human, horse, rabbit, and mouse erythrocytes (Roelofsen, de Gier, & van Deenen, 1964). Miyazaki *et al.* (Miyazaki, et al., 1993) provided further support for this contention by demonstrating that phosphatidylcholine was able to inhibit the lysis of horse erythrocytes by hemolytic *E. faecalis*. By producing non-cytolytic mutants of *E. faecalis* through repeated exposure to the mutagen nitrosoguanidine, and examining zones of hemolysis on blood agar between pairs of defined mutants, it was determined that the cytolysin consisted of an activator component and a lytic component (Granato & Jackson, 1969).

The cytolysin determinant was first identified on large conjugative plasmids (Clewell, et al., 1982; Dunny & Clewell, 1975; Granato & Jackson, 1969; LeBlanc & Lee, 1982). Localization to conjugative plasmids, which were also found to be responsive to pheromones, was achieved through broth mating experiments (Dunny, Brown, & Clewell, 1978; Dunny, Craig, Carron, & Clewell, 1979). Through transposon mutagenesis and restriction analysis of a prototype pheromone-responsive transmissible plasmid, pAD1, the cytolysin determinant was mapped to a region that spanned 8 kb. The generation of a mutant in this locus allowed investigation into the role of the cytolysin in the pathogenesis of enterococcal infection (Clewell, et al., 1982). Further characterization of cytolysin determinants in different strains revealed its location on either pheromone-responsive plasmids (LeBlanc, Lee, Clewell, & Behnke, 1983), or the chromosome (Ike & Clewell, 1992) within a pathogenicity island (Shankar, Baghdayan, & Gilmore, 2002).

The organization, expression, and regulation of the cytolysin operon have been described in a number of reviews (Coburn & Gilmore, 2003; Cox, Coburn, & Gilmore, 2005; Shankar, Coburn, Pillar, Haas, & Gilmore, 2004; Van Tyne, Martin, & Gilmore, 2013) and are depicted in Figure 2. Briefly, the cytolysin is encoded by a complex operon that consists of eight genes, with expression driven by a pair of divergent, overlapping promoters. Structural genes for the cytolysin subunits (*cylLL* and *cylLS*), post-translational modification and secretion functions (*cylM*, *cylB* and *cylA*) and cell immunity (*cylI*) are transcribed as a single unit. The regulatory genes, *cylR1* and *cylR2*, are transcribed in the opposite direction as a second transcriptional unit. The cytolysin subunits CylLL and CylLS are synthesized as 68- and 63-residue precursors, respectively, and then are post-translationally modified by the product of the third gene (*cylM*) in the operon (Booth, et al., 1996; Gilmore, et al., 1994). CylM, a 993-residue polypeptide, introduces modifications that are characteristic of the lantibiotic class of bacteriocins. The CylB protein functions as both an ATP-binding cassette transporter (Gilmore, Segarra, & Booth, 1990; Gilmore, et al., 1994) and a signal peptidase (Håvarstein, Diep, & Nes, 1995), and these dual functions serve to export the cytolysin subunits and to proteolytically remove a leader peptide from each subunit. The secreted subunits, which are still inactive at this point, require further protelolytic processing by the CylA protease to acquire activity (CylLS'' and CylLL'' that is capable of lysing target cells (Booth, et al., 1996). CylI provides a self-immunity function by protecting the producer from the bactericidal activity of the cytolysin.

CylR1 and CylR2, both of which reduce expression from the cytolysin promoter by about 40-fold, regulate cytolysin production. Purified CylR2 specifically binds to an inverted repeat sequence overlapping the -35 sequence of the cytolysin structural gene promoter (Rumpel, et al., 2004). The crystal structure of CylR2 has revealed dimerization and the presence of a helix-turn-helix DNA binding domain. A model for the action of cytolysin that links sensing of the inducer molecule CylLS'' to the presence or absence of a suitable target cell has been proposed (Coburn, Pillar, Jett, Haas, & Gilmore, 2004). In the absence of a target cell, CylLS'' and CylLL'' are maintained only at basal levels and can further interact to form an insoluble oligomeric complex that effectively diminishes the concentration of free inducer, CylLS''. Thus, in the absence of a target cell, CylLL'' acts to titrate the level of free CylLS'' in solution to below the threshold level necessary to trigger high-level cytolysin production.

However, in the presence of a target cell, CylLL'' binds preferentially to the target membrane, which reduces the concentration of CylLL'' in solution, and allows the accumulation of free CylLS'' to the level necessary to activate high-level cytolysin expression. Thus, this provides a mechanism by which the bacterium produces high levels of cytolysin only when required by simultaneously detecting both the accumulating inducer CylLS'' molecule and a target cell through the membrane sensor CylLL''. Furthermore, this would provide an additional fail-safe mechanism to protect the producer from the bactericidal effects of the cytolysin.

Gelatinase, serine protease, and their regulation by Fsr

While bacterial proteases are generally considered to be factors that enhance an organism's ability to acquire nutrients by proteolysis, they are frequently implicated in damage to host tissues during infections. A broad range of host functions are impacted by bacterial proteases, and include direct or indirect degradation of host connective tissues and proteins (Burns, Jr., Marciel, & Musser, 1996; Lantz, Allen, Duck, Switalski, & Hook, 1991), interference with host cell signaling pathways to facilitate microbial survival (Duesbery, et al., 1998; Maeda & Yamamoto, 1996) and degradation of key components of the host immune system (Plaut, 1983), to name a few. In many cases, they can act as virulence determinants in animal models of infection (Maeda & Yamamoto, 1996).

In *E. faecalis*, the gelatinase (GelE, a matrix metalloprotease) and a serine protease (SprE) comprise the two secreted proteases that have been well studied (Qin X. , Singh, Weinstock, & Murray, 2001). The term gelatinase was coined for the protease activity based on its observed ability to hydrolyze gelatin (MacCallum & Hastings, 1899). The proteolytic activity, combined with the presence or absence of a hemolytic phenotype, remained the basis for the classification of *Streptococcus faecalis* into the subspecies *S. faecalis*, *S. faecalis var. liquefaciens*, *S.*

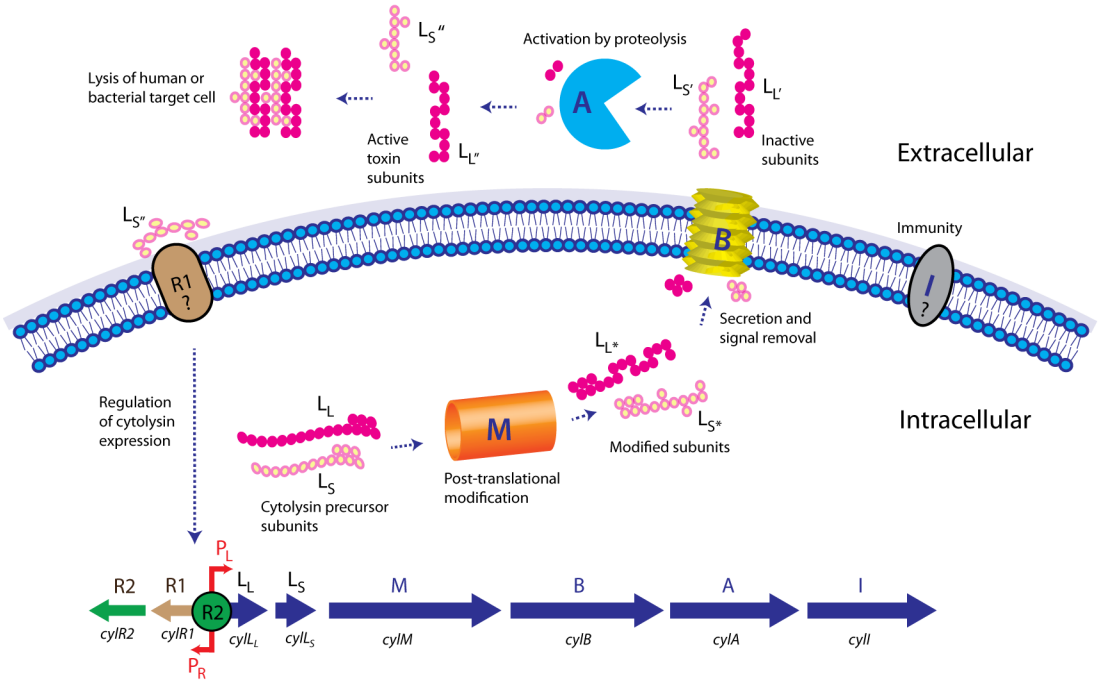

Figure 2. Model for the expression of the cytolysin in E. faecalis. The toxin subunits L_L and L_S are ribosomally synthesized in precursor form, then are post-translationally modified by CylM, which introduces modifications characteristic of the lantibiotic class of bacteriocins. The modified peptides L_L* and L_S* are then further processed and secreted by CylB, which serves as both an ATP-binding cassette transporter and a signal peptidase. The mature externalized peptide subunits, L_L' and L_S', undergo activation by CylA through proteolytic cleavage, which removes identical 6-amino acid residues from the N-terminus of each subunit and generates the active toxin subunits, L_L'' and L_S''. CylI confers self-immunity from the bactericidal action of cytolysin. Cytolysin expression is regulated by R1 and R2. The toxin subunit L_S'' has been shown to possess signaling activity, which results in the autoinduction of the cytolysin operon by a quorum-sensing mechanism.

faecalis var. hemolyticus, and *S. faecalis var. zymogenes*—until biochemical and DNA hybridization studies established that *S. faecalis* and its subspecies *liquefaciens* and *zymogenes* were, in fact, the same species, and were later regrouped under the present genus *Enterococcus* (Farrow, Jones, Phillips, & Collins, 1983). Early reports on the characterization of the gelatinase from *E. faecalis* suggested that it was a zinc-metalloprotease (Bleiweis & Zimmermann, 1964), and later purification by Makinen *et al.* (Mäkinen, Clewell, An, & Mäkinen, 1989) from a human oral *E faecalis* isolate (OG1-10) classified it as a metalloprotease II (EC 3.4.24.4) with broad substrate specificity. The ability of the enzyme to inactivate human endothelin prompted an attempt to rename it as coccolysin, but the term gelatinase has prevailed in the literature (Mäkinen & Mäkinen, 1994). Sequencing of the *gelE* gene by Su *et al.* (Su, et al., 1991) showed that it encodes a 509-residue protein predicted to contain a 29 amino-acid signal sequence followed by 162 residues of pro-sequence and a 318 amino-acid mature protein with a mass of 34.5kDa.

Early studies that examined the prevalence of gelatinase production among *E. faecalis* clinical isolates reported that 72% of hospital isolates were positive (Kühnen, Richter, Richter, & Andries, 1988), while a later study that examined both hospital (clinical and fecal) and community (fecal) isolates noted a higher frequency among hospital isolates (both clinical and fecal), as compared to community fecal isolates. The data suggest a possible enrichment for a Gel+ phenotype in the hospital environment (Coque, Patterson, Steckelberg, & Murray, 1995). The latter study also observed the absence of the Gel+ phenotype in 46% of endocarditis isolates, which suggests no role for this enzyme in this particular disease. Subsequent studies also reported the identification of *E. faecalis* isolates that carried the *gelE* gene, but which were phenotypically negative (Eaton & Gasson, 2001; Qin X. ,

Singh, Weinstock, & Murray, 2000). A 23.9 kb deletion in the region that encodes the *fsr* genes, which drive *gelE* expression, was reported among clinical urine isolates of *E. faecalis* (Nakayama, Kariyama, & Kumon, 2002). A survey of 215 *E. faecalis* isolates concluded that neither the *fsr* locus or gelatinase production was enriched in disease-associated isolates, as compared to isolates from healthy individuals (Roberts, Singh, Okhuysen, & Murray, 2004). The majority of gelatinase-negative isolates in this study also exhibited the 23.9 kb deletion previously mentioned. And as noted above, a large prospective study of enterococcal bacteremia patients failed to find a statistically significant correlation between 14-day mortality and gelatinase, cytolysin, or Esp, either singly or in combination (Vergis, et al., 2002).

In well-controlled animal studies that used similar or isogenic strains of *E. faecalis* and inbred animals, a contribution of gelatinase to the severity of infection is usually shown. Using germ-free rats, Gold *et al.* (Gold, Jordan, & van Houte, 1975) showed that the protease-positive *E. faecalis* isolate (OG1) was cariogenic when compared to three other non-proteolytic strains. Further, Gutschik *et al.* (Gutschik, Møller, & Christensen, 1979) evaluated 10 strains of *E. faecalis* with varying gelatinase activity in a rabbit endocarditis model, and concluded that the clinical severity of the disease correlated with proteolytic activity. In more controlled studies using isogenic strains, the role of gelatinase has been evaluated in mouse and rat peritonitis models (Dupont, Montravers, Mohler, & Carbon, 1998; Singh, Qin, Weinstock, & Murray, 1998). In both studies using the mouse model, it was noted that a gelatinase-positive strain, OG1RF, exhibited a lower LD50 and an earlier time to death, as compared to a gelatinase-negative strain, OG1X, which was derived from a common parent by nitrosoguanidine mutagenesis. In the rat peritonitis model, however, the OG1X strain induced no mortality and was deemed less pathogenic. Due to the residual gelatinase activity still exhibited by OG1X and the likelihood of other unknown mutations caused by the nitrosoguanidine treatment, a defined insertion mutation in the *gelE* gene of OG1RF was created (Singh, Qin, Weinstock, & Murray, 1998). The resulting strain had no detectable gelatinase activity, and corroborated earlier observations in the mouse peritonitis model. The *gelE* disruption mutant was, however, found to abrogate expression of the downstream *sprE* gene that expressed the second serine protease, which limited the conclusions drawn from the earlier work (Qin X. , Singh, Weinstock, & Murray, 2000). More recent work indicated a potential role for GelE in infection, as well as housekeeping functions (Waters, Antiporta, Murray, & Dunny, 2003). In this study, GelE was shown to limit chain length, clear the bacterial surface of misfolded aggregation substance mutant proteins, enhance autolysis, and reduce the titer of the cCF10 pheromone that induces conjugation of pCF10. While the importance of proteases in biofilm formation through control of extracellular DNA release has been reported (Thomas, Thurlow, Boyle, & Hancock, 2008), others have also shown a role for gelatinase and the Fsr system in facilitating *E. faecalis* translocation *in vitro* across polarized human enterocyte-like T84 cells (Zeng, Teng, & Murray, 2005).

Identification of the determinant that encodes the second protease (named SprE, due to its similarity to the *S. aureus* V8 protease) came about after sequencing the region immediately downstream of *gelE* in strain OG1-10 (Su, et al., 1991). Qin *et al.* (Qin X. , Singh, Weinstock, & Murray, 2000) used nucleic acid hybridization with *gelE* and *sprE* probes and RT-PCR to demonstrate co-localization and co-transcription of *gelE* with *sprE* and the loss of the *sprE* transcript in the *gelE* disruption mutant. Also, an OG1RF *sprE* disruption mutant exhibited gelatinase activity, but not serine protease activity. Further, the *sprE* deletion mutant resulted in significantly prolonged survival in a mouse peritonitis model, as compared to the wild type OG1RF strain, which implies an independent role for *sprE* in this model. The attenuated virulence of *sprE* mutants has also been demonstrated in the *C. elegans* model (Garsin, et al., 2001; Sifri, et al., 2002) and in the rabbit endophthalmitis model (Engelbert, Mylonakis, Ausubel, Calderwood, & Gilmore, 2004; Suzuki, et al., 2008).

The regulatory aspects of gelatinase and serine protease expression in *E. faecalis,* as detailed in Figure 3, were unraveled following the identification of the *fsr* (*E. faecalis* regulator) locus, a set of three genes (*fsrA*, *fsrB*, and *fsrC)* upstream of *gelE* (Qin X. , Singh, Weinstock, & Murray, 2000). This locus bears significant similarity to the *agr* (accessory gene regulator) locus in *S. aureus*, a quorum-sensing system that positively regulates the expression of several secreted proteins and toxins, and down-regulates the expression of surface proteins (Novick

& Geisinger, 2008). Based on sequence homology, FsrA (247 amino acids) and FsrC (447 amino acids) likely constitute a two-component histidine kinase sensor (FsrC) and response regulator (FsrA) system. The FsrB (242 amino acids) protein is similar to AgrB, but has a 50 amino acid carboxy terminal extension, which is processed into an 11-residue peptide pheromone termed the gelatinase biosynthesis-activating pheromone (Nakayama, et al., 2001; Qin X. , Singh, Weinstock, & Murray, 2001).

Isogenic *E. faecalis* OG1RF mutants, with disruptions in the *fsrA*, *fsrB*, and *fsrC* genes, were tested for virulence in a mouse peritonitis model, and all were shown to significantly prolong the time course of survival when compared to the parental strain OG1RF (Qin X. , Singh, Weinstock, & Murray, 2000). In experiments conducted with an *fsrB* deletion mutant and *fsrA*, *fsrB*, and *gelE* disruption mutants using a *C. elegans* model, results similar to that in the mouse peritonitis model were seen (Garsin, et al., 2001; Shu & Zhulin, 2002). However, since none of the *fsr* mutants showed detectable gelatinase or serine protease activity, it was difficult to assign independent role(s) to each, which implies that the observations could have resulted from either polar or regulatory effects of the *fsr* genes on *gelE* and *sprE* expression. Transcriptional and Northern blot analyses provided further insights into the organization of the *fsr* locus and showed that: (i) disruption of each of the *fsr* genes abolished a *gelE/sprE* transcript; (ii) complementation of the *fsr* locus in trans restored gelatinase activity to each of the three *fsr* mutants, but not to a *gelE* disruption mutant; (iii) *fsrB* and *fsrC* are co-transcribed; (iv) both *fsrB* and *fsrC* are required for *fsr* locus function; and (v) the Fsr system is autoregulated and functions as a two-component regulatory system (Qin X. , Singh, Weinstock, & Murray, 2001; Qin X. , Singh, Weinstock, & Murray, 2000). Furthermore, primer extension analysis identified promoter sequences upstream of *fsrA, fsrB*, and *gelE*, but not *fsrC* or *sprE,* and deletions in conserved repeats within these regions abolished promoter activity (Qin X. , Singh, Weinstock, & Murray, 2000).

Experiments analyzing induction of *fsrC* expression by supernatants from post-exponential and stationary phases of OG1RF and an *fsrB* deletion mutant suggested that an inducer molecule regulates *fsrC* expression (Qin X. , Singh, Weinstock, & Murray, 2000). Consistent with these observations, Nakayama *et al.* (Nakayama, et al., 2001) described the isolation of an 11-aa inducer (pheromone) from late exponential phase cultures and named it gelatinase-biosynthesis activating pheromone (GBAP). The peptide appeared to be derived from residues 220-230 at the carboxy terminus of *fsrB*. A similar autoinducing peptide lactone (AgrD) of the *agr* system drives expression of the *agr* locus in *S. aureus* (Nakayama, et al., 2001). More recently, a revised model for GBAP synthesis has been proposed, based on the demonstration that GBAP is encoded by a fourth gene, *fsrD,* within the *fsr* locus that is separate from *fsrB.* Even though *fsrD* is in-frame with *fsrB*, it is translated independently (Nakayama, et al., 2006). A recent study demonstrated a high degree of conservation of the *fsrD* gene sequence among multiple MLSTs (multilocus sequence types) in the genome of 22 unrelated *E. faecalis* strains, although there was considerable variation in the *fsr* locus outside of *fsrD* (Galloway-Peña, Bourgogne, Qin, & Murray, 2011).

The effects of mutations in the *fsr* locus have been examined in a rabbit model of endophthalmitis (Mylonakis, et al., 2002; Engelbert, Mylonakis, Ausubel, Calderwood, & Gilmore, 2004). While an *fsrB* mutant shown to be defective in the production of both gelatinase and serine protease was attenuated in virulence (Mylonakis, et al., 2002), a later study revealed that the greater degree of attenuation in the *fsrB* mutant might be due either to synergy or to effects outside of gelatinase and serine protease (Engelbert, Mylonakis, Ausubel, Calderwood, & Gilmore, 2004). Another recent study examined the protease-positive strain OG1S and its isogenic protease-deficient strain OG1X in an aphakic (lacking the eye lens) rabbit model of postoperative endophthalmitis (Suzuki, et al., 2008). The protease-positive bacteria or culture supernatants derived from their *in vitro* growth in BHI broth decreased ERG *b*-wave amplitude and caused morphological changes to the posterior capsule and retina (Suzuki, et al., 2008). In a recent study, Singh *et al.* found that an *E. faecalis* OG1RF mutant with a nonpolar deletion in *fsrB*, which produced low levels of gelatinase upon prolonged *in vitro* incubation, was similar to the wild-type strain in induction of endocarditis in a rat model. In contrast, a *gelE* insertion mutant was attenuated (Singh K. V., Nallapareddy, Nannini, & Murray, 2005). To further explore if additional genes

beyond *gelE* and *sprE* are influenced by the *fsr* locus, Bourgogne *et al.* (Bourgogne, Hilsenbeck, Dunny, & Murray, 2006) compared the transcription profiles of OG1RF and an isogenic *fsrB* deletion mutant by microarray analysis. The results revealed that a number of other genes, including those related to biofilms, surface proteins, and metabolic pathways, may be impacted by the *fsr* locus, and that it may also act as a negative regulator.

Based on the observation that gelatinase destroys a defense system in insect hemolymph (Park, Kim, Lee, Seo, & Lee, 2007), the virulence of *fsrB* and *gelE* deletion mutants were recently evaluated in the *Galleria mellonella* insect model (Gaspar, et al., 2009). When inoculated with wild-type *E. faecalis* OG1RF or an isogenic *fsrB* deletion mutant (TX5266), it was observed that 80% of the larvae were dead at 48 hours, as compared to 50% mortality in larvae infected with an isogenic *gelE* deletion mutant (TX5264), which suggests that only *gelE* played a role in OG1RF virulence in this model. However, when similar isogenic mutants prepared from two unrelated *E. faecalis* food isolates were compared in this model, the *fsrB* deletion mutants were more significantly attenuated than the *gelE* deletion mutants (Gaspar, et al., 2009).

The Fsr system regulates some of the other virulence determinants discussed in this chapter. For example, regulation of the expression of Ace, an adhesion molecule on the surface of *E. faecalis* that mediates binding to collagen (see below), was recently described (200). Both *fsr* and *gelE* mutants were shown to express significantly higher levels of Ace at the cell surface, and GelE-dependent cleavage of Ace from the surface of *E. faecalis* was also correlated with improved adhesion to collagen. In a microarray analysis of the Fsr system's regulon, Bourgogne *et al.* (Bourgogne, Hilsenbeck, Dunny, & Murray, 2006) described the positive regulation of EbpR, a transcription factor necessary for the production of pili, in addition to other regulators. Negative regulation of the *eut* locus, which encodes the proteins necessary for the catabolism of ethanolamine, was also noted (Bourgogne, Hilsenbeck, Dunny, & Murray, 2006). As a result of these contributions to the microbe/host dynamic, the Fsr quorum-sensing system has been suggested as a potential target for antimicrobial development. Recent studies that examined culture filtrates of *Streptomyces* sp. strain Y33-1 identified the known peptide antibiotic siamycin as having a potent inhibitory effect on the production of both gelatinase and GBAP, possibly through a direct interaction with FsrC (Nakayama, et al., 2007).

Adhesins

Aggregation substance

Aggregation substance (AS) proteins are a group of closely related, multifunctional, surface-anchored polypeptides encoded by pheromone-responsive, conjugative plasmids with roles in *E. faecalis* plasmid transfer and virulence. The AS proteins Asa1, Asc10, and Asp1, encoded by the *asa1*, *prgB*, and *asp1* genes of conjugative plasmids pAD1, pCF10, and pAD1, respectively, are greater than 90% identical at the amino acid level, outside of a variable region in the N-terminal portion of the protein (Galli, Friesenegger, & Wirth, 1992; Galli, Lottspeich, & Wirth, 1990; Kao, Olmsted, Viksnins, Gallo, & Dunny, 1991). The AS proteins have an N-terminal signal sequence, a C-terminal LPxTG cell wall anchor domain, two Arg-Gly-Asp (RGD) motifs, and a number of experimentally determined domains that were found to mediate aggregation and lipoteichoic acid (LTA)-binding (Hendrickx, Willems, Bonten, & van Schaik, 2009). Expression of AS on the surface of pheromone-induced donor cells leads to strong binding of donors to plasmid-free recipient cells to enhance the efficiency of plasmid transfer by conjugation (194). In addition to its function in bacterial cell aggregation, AS is associated with increased virulence and mortality in rabbit models of infective endocarditis (Chow, et al., 1993; Hirt, Schlievert, & Dunny, 2002; Schlievert, Chuang-Smith, Peterson, Cook, & Dunny, 2010). AS may enhance *E. faecalis* virulence by serving as an adhesin in bacterial-host interactions, promoting host cell internalization of bacteria, and by contributing to the evasion of killing by phagocytic cells. AS was found to be more prevalent in both clinical and fecal samples in the hospital environment than in samples from the community environment (Coque, Patterson, Steckelberg, & Murray, 1995).

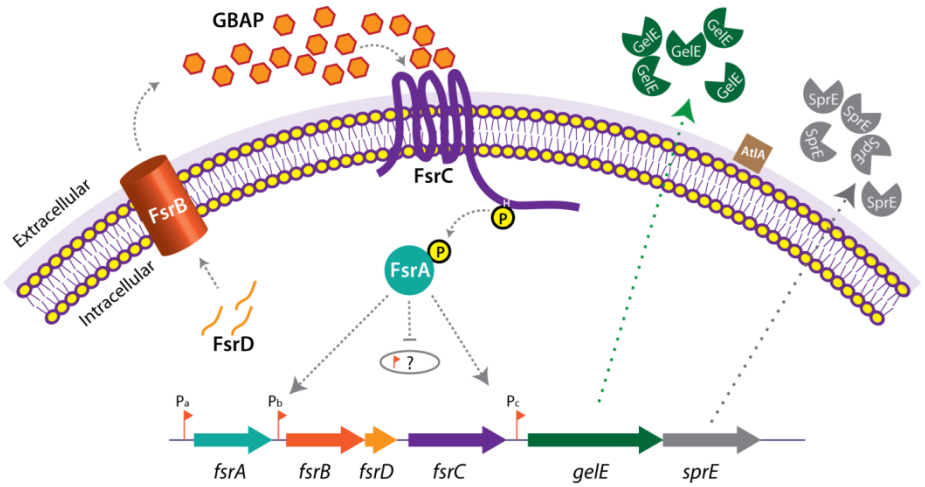

Figure 3. Model for the mechanism of *fsr* activation in *E. faecalis* and its effect on the synthesis of gelatinase and serine protease. The *E. faecalis fsr* system bears global similarity to the staphylococcal *agr* quorum-sensing system. The secreted gelatinase biosynthesis-activating pheromone (GBAP) is an 11-amino-acid autoinducing peptide, which is produced by the proteolytic activity of FsrB on the GBAP propeptide encoded by *fsrD*. The gene, *fsrD,* which resides in the 3′-end of *fsrB*, is translated independently of *fsrB*. Extracellular GBAP interacts with the two-component histidine kinase sensor, FsrC, which induces the phosphorylation of the response regulator, FsrA. Activated FsrA positively regulates the expression of the *fsr* operon and the co-transcribed downstream genes, *gelE* and *sprE*, which encode secreted gelatinase (GelE) and serine protease (SprE), respectively, in a cell-density–dependent manner. SprE acts as an immunity protein in concert with autolysin AtlA to control GelE-mediated autolysis and release of eDNA. Recent studies have also shown that the *fsr* system can function as a negative regulator of genes that are predicted to encode surface proteins and metabolic functions.

Since many *E. faecalis* infections appear to originate from bacteria that translocate through the intestinal or genitourinary epithelium (77), *E. faecalis* must have mechanisms for interaction with host cells and/or extracellular matrix (ECM) proteins. AS has been investigated for this capacity due to its known adherence properties, coupled with the observation that it has dual RGD motifs, which are found in eukaryotic proteins that interact with integrins. AS was first shown to increase the ability of a pAD1-positive strain, relative to a plasmid-free strain, to bind to cultured renal tubular cells (Kreft, Marre, Schramm, & Wirth, 1992). Adherence was disrupted by pre-incubation of the renal cells with synthetic Arg-Gly-Asp-Ser peptides (Kreft, Marre, Schramm, & Wirth, 1992). The N-terminal RGD motif of Asa1 was found to interact with integrin CD11b/CD18 (complement receptor type 3), which led to augmented binding of AS+ cells to macrophages (Süßmuth, et al., 2000); similar results were obtained with polymorphonuclear leukocytes (Vanek, et al., 1999). Enhanced internalization of AS-expressing *E. faecalis* cells has been demonstrated in HT-29 enterocytes (Olmsted, Dunny, Erlandsen, & Wells, 1994; Sartingen, Rozdzinski, Muscholl-Silberhorn, & Marre, 2000), other intestinal epithelial cell lines (Sartingen, Rozdzinski, Muscholl-Silberhorn, & Marre, 2000), and in colonic mucosa (Isenmann, Schwarz, Rozdzinski, Marre, & Beger, 2000). The presence of surface-expressed AS substantially increased macrophage phagocytosis of *E. faecalis* cells. The bacteria subsequently suppressed the macrophage respiratory burst and were resistant to intracellular killing (Süßmuth, et al., 2000). Similarly, AS augmented opsonization-independent phagocytosis, yet the bacteria were resistant to intracellular killing in activated human PMNs (Rakita, et al., 1999). Asa1 was also shown to significantly enhance binding to the ECM components fibronectin, thrombospondin, vitronectin, and type I collagen, but not to laminin or type IV collagen (Rozdzinski, Marre, Susa, Wirth, & Muscholl-Silberhorn, 2001). The particularly strong interaction between Asa1 and fibronectin was dependent on an intact variable region within the N-terminal half of the protein (Rozdzinski, Marre, Susa, Wirth, & Muscholl-Silberhorn, 2001).

A significant effort has been undertaken to dissect the functional domains of pCF10's Asc10, with respect to aggregation, host cell binding, and endocarditis virulence. Using a library of insertional mutants distributed throughout *prgB*, Waters and Dunny (Waters & Dunny, 2001) defined an aggregation domain in the N-terminal

half of the protein (amino acids 473-683) that included the first RGD motif and overlapped part of the variable region. They further showed that an intact aggregation domain was required for the efficient uptake by HT-29 cells through an unknown mechanism that was independent of the RGD motifs. Aggregation itself was not a prerequisite for uptake (Waters, Wells, & Dunny, 2003). Site-specific mutations in the RGD motifs also had no effect on aggregation (Waters, Wells, & Dunny, 2003). In experiments that studied the direct interaction of purified Asc10 variants with LTA, Waters *et al.* (Waters, et al., 2004) discovered that the LTA-binding domain is a separate domain situated upstream of the Asc10 (473-684) aggregation domain, at amino acids 150-358, and that this domain was also required for aggregation and internalization by HT-29 enterocytes. Recent advances in enterococcal genetic manipulation techniques (Kristich, Chandler, & Dunny, 2007) have facilitated the construction of a markerless in-frame *prgB* deletion in pCF10, as well as the generation of pCF10 variants carrying mutant *prgB* alleles that have deletions or point mutations in the LTA-binding aggregation domain and the RGD motifs (Chuang, et al., 2009). When evaluated in a rabbit experimental endocarditis model, the pCF10Δ*prgB* strain and a strain expressing a full-length *prgB* variant with glycine-to-alanine point mutations in both RGD motifs (RGD double mutant) showed the most pronounced decreases in virulence (Chuang, et al., 2009). Mutation of either RGD motif alone revealed that the N-terminal RGD motif was more important in virulence. In fact, a strain with a 450-amino acid C-terminal region deletion, which encompassed the second RGD domain, remained fully virulent. Disruption of either aggregation domain resulted in intermediate levels of attenuation. In an *ex vivo* porcine heart valve model that evaluated bacterial adherence at early time points, the pCF10Δ*prgB* strain was found to colonize valve tissues at only 40% of the level of the Asc10+ strain after four hours (Chuang-Smith, Wells, Henry-Stanley, & Dunny, 2010). Mutations in either or both aggregation domains, or both RGD motifs, caused a slight impairment in valve colonization, whereas deletion of the C-terminal subdomain did not cause any significant defects (Chuang-Smith, Wells, Henry-Stanley, & Dunny, 2010). The authors propose separate virulence functions for the multiple domains—the aggregation domains likely mediate binding at the heart valve surface, whereas it was speculated that the RGD motifs may contribute to immune evasion (Chuang, et al., 2009; Chuang-Smith, Wells, Henry-Stanley, & Dunny, 2010). In heart valve infections caused by AS+ enterococci, heart valve colonization may be accelerated by the ability of interbacterial aggregates to attach to the host surface as a single event (Chuang-Smith, Wells, Henry-Stanley, & Dunny, 2010).

As noted above, the combination of AS and cytolysin resulted in increased virulence in experimental endocarditis and a mouse intraperitoneal injection model (Chow, et al., 1993; Dupont, Montravers, Mohler, & Carbon, 1998). However, when tested in other infection models, the presence of AS did not enhance the killing of *C. elegans* (Garsin, et al., 2001) or contribute to pathogenesis in endophthalmitis or ascending unobstructed urinary tract infections (Jett, Atkuri, & Gilmore, 1998; Johnson, Clabots, Hirt, Waters, & Dunny, 2004). This may be partially due to the failure of AS induction *in vivo* (Johnson, Clabots, Hirt, Waters, & Dunny, 2004).

In the course of infection, AS can be induced in the absence of recipient cells or the addition of an exogenous pheromone. This was first observed in tissue culture experiments and was found to be caused by a component found in serum and plasma (Hirt, Schlievert, & Dunny, 2002; Kreft, Marre, Schramm, & Wirth, 1992). The prevention of autocrine induction of Asc10 on pCF10 (by self-produced pheromone) is a tightly regulated process that involves an 80:1 molar ratio of the inhibitor peptide, iCF10, to the activating peptide, cCF10 (Nakayama, Dunny, Clewell, & Suzuki, 1995). Genetic and functional evidence supports a model for Asc10 self-activation *in vivo* that occurs in response to disruption of the inhibitor:activator peptide ratio, which follows the inactivation of iCF10 by a component in plasma. The component is possibly albumin or albumin/lipid complexes (Chandler, Hirt, & Dunny, 2005). Induction of Asc10 in endocarditis (Hirt, Schlievert, & Dunny, 2002) is supported by data that demonstrate that pCF10Δ*prgB* leads to significant attenuation in this model (Chuang, et al., 2009). Despite a demonstrable role for aggregation substance in enterococcal endocarditis, the vaccination of rabbits with a purified surface-exposed region of Asc10 failed to provide protection (McCormick, et al., 2001). Asc10 antibodies were excluded from vegetations in passively immunized animals, and opsonized bacteria in animals pre-immunized with Asc10 were still found in vegetations (McCormick, Tripp, Dunny, & Schlievert, 2002). Unexpectedly, immunization with heat- and gentamicin-killed AS+ organisms increased

severity of infection when immunized animals were challenged with the AS+ organism, as compared to animals vaccinated with an AS- strain (Schlievert, Chuang-Smith, Peterson, Cook, & Dunny, 2010). However, passive immunization with anti-AS IgG Fab significantly reduced endocarditis disease that was caused by an AS+ strain of *E. faecalis* (Schlievert, Chuang-Smith, Peterson, Cook, & Dunny, 2010).

Enterococcal surface protein, Esp

Enterococcal surface protein, or Esp, was identified initially in a highly virulent, gentamicin-resistant, *E. faecalis* isolate from a bacteremia (Shankar V. , Baghdayan, Huycke, Lindahl, & Gilmore, 1999). This ~202 kDa protein possesses structural features that are characteristic of Gram-positive surface proteins, such as a transport signal sequence and a cell wall-anchor sequence that is slightly divergent from the consensus LPxTG motif. The core region consists of repeat units that make up about 50% of the protein, and has a unique architecture made up of distinct tandem repeating units. The first repeating unit located downstream of the 694 amino acid N-terminal domain consists of three 84-residue repeats that are specified by nearly identical 252-nucleotide tandem repeats (A repeats). Seven, nearly identical, 246-nucleotide tandem repeating units (C repeats), which encode reiterations of an 82-amino acid sequence, are flanked by the B repeats, which share 74% sequence identity at the amino acid level. These highly conserved, multiple repeat structures allow for expression of alternate forms that differ in the number of repeat units as a result of recombination mediated addition or deletion of units (Shankar V. , Baghdayan, Huycke, Lindahl, & Gilmore, 1999). Esp exhibits global structural similarity to the *Streptococcus pyogenes* protein R28 (Stålhammar-Carlemalm, Areschoug, Larsson, & Lindahl, 1999), *Streptococcus agalactiae* Rib and C-alpha proteins (Michel, Madoff, Kling, Kasper, & Ausubel, 1991) and the *Staphylococcus aureus* biofilm-associated protein, Bap (Cucarella, et al., 2001). This similarity is restricted to a highly conserved region within the C repeat units of the Esp protein, corresponding to regions within the group A and B streptococcal proteins, while the similarity with Bap is limited to the non-repeat N-terminal region.

The *esp* gene was found to be enriched in infection-derived *E. faecalis* isolates (Shankar V. , Baghdayan, Huycke, Lindahl, & Gilmore, 1999), and an *esp* homolog was reported in *E. faecium* isolates (Baldassarri, et al., 2001; Willems, et al., 2001). The variant *esp* gene was significantly enriched ($P < 0.0001$) among epidemic vancomycin-resistant *E. faecium* isolates (VREF) that are genetically distinct from non-epidemic VREF obtained from hospitals on three continents (Willems, et al., 2001). A comparison of 22 vancomycin-resistant *E. faecium* isolates from catheter-related, bloodstream infections (VREF-CRB) to 30 VREF isolates from the gastrointestinal tract of control patients resulted in no correlation between *esp* in VREF and bacteremia, or with more biofilm formation (Raad, et al., 2005). A study in the United Kingdom detected *esp* in over 60% of vancomycin-resistant and vancomycin-sensitive clinical isolates, but not in environmental isolates (Woodford, Soltani, & Hardy, 2001). Although no correlation with vancomycin resistance was observed, another survey of 201 clinical bloodstream isolates found *esp* to be present in 52% of *E. faecium*, 40% of *E. faecalis*, and one *E. raffinosus* strain (88). A screening study of enterococcal virulence factors also identified the *esp* homolog to be enriched among clinical *E. faecium* isolates, as compared with food or starter isolates (Eaton & Gasson, 2001). The *esp* gene was found to be significantly associated with ampicillin-resistant strains, as compared to ampicillin-sensitive strains of *E. faecium* ($P < 0.001$), regardless of the isolation site (Coque, Willems, Cantón, Del Campo, & Baquero, 2002). However, as noted above, one enterococcal bacteremia study did not find a statistically significant correlation between 14-day mortality and gelatinase, cytolysin, or Esp, either singly or in combination (Vergis, et al., 2002).

Genotyping of endodontic enterococcal isolates showed that 20 of 31 *E. faecalis* and 2 of 2 *E. faecium* isolates carried the *esp* gene (Sedgley, et al., 2005). Seno *et al.* (Seno, Kariyama, Mistuhata, Monden, & Kumon, 2005) examined 352 *E. faecalis* isolates from patients with complicated urinary tract infections and found that 75% of the strains carried the *esp* gene. Isolates carrying both *esp* and *asa1* genes (315/352) were found to be better biofilm formers ($P = 0.038$) than isolates that carried neither gene. In a study of *E. faecium* clinical isolates from Germany, the *esp* gene was found among multiple MLST types belonging to clonal complex 17 (CC-17) (Klare, et al., 2005), in which Esp was later described as being an important biofilm determinant (Heikens, Bonten, &

Willems, 2007). A recent study in Australia of 41 VREF *vanB* isolates from immunocompromised patients (n= 41; 14 infected and 27 colonized) found the *esp* gene was highly prevalent, but not associated with 30-day mortality (Worth, et al., 2008). Another study in India found the *esp* gene to be significantly enriched among 200 infection-derived isolates of *E. faecalis* from patients at a tertiary care center, as compared to 100 commensal isolates. The presence of the gene was highly correlated with biofilm formation *in vitro* (Upadhyaya, Lingadevaru, & Lingegowda, 2011).

Toledo-Arana *et al.* (Toledo-Arana, et al., 2001) found a significant correlation between the presence of *esp* and the ability of *E. faecalis* to form biofilms on polystyrene. Ninety-three percent of the tested *esp*-positive isolates formed a biofilm, as compared to none of the *esp*-negative isolates. The presence of *esp* did not, however, promote the adhesion of *E. faecalis* to other medically relevant substrates, such as silicone rubber, fluoroethylene-propylene, or polyethylene (Waar, van der Mei, Harmsen, Degener, & Busscher, 2002). In evaluating the adhesion of enterococcal strains to two types of urinary catheter materials, Joyanes *et al.* (Joyanes, Pascual, Martínez-Martínez, Hevia, & Perea, 2000) concluded that *E. faecalis* showed greater adherence than *E. faecium*. Furthermore, adherence was not related to either bacterial surface hydrophobicity or hemolysin or gelatinase production. An investigation of adhesive properties of *E. faecalis* strains to intestinal Int-407 and Girardi heart cell lines revealed no role for Esp in adhesion (Archimbaud, et al., 2002). Infection-derived *E. faecium* isolates that were positive for *esp* were reported to adhere better to Caco-2 cells than *esp*-negative isolates ($P < 0.05$), but these studies were done with non-isogenic strains (Lund & Edlund, 2003). In a later study, these authors also showed that *esp*-positive, infection-derived *E. faecium* exhibited higher conjugation frequencies ($P < 0.01$) with respect to acquisition of *vanA,* as compared to *esp*-negative isolates (Lund, Billström, & Edlund, 2006). The *esp* gene was also found to be present in a large number of antibiotic resistant, cross-transmitted, *E. faecium* isolates prevalent in Swedish hospitals (Billström, Sullivan, & Lund, 2008).

The role of Esp in colonization and persistence of *E. faecalis* in an animal model of ascending urinary tract infection was evaluated by comparing an Esp-positive strain of *E. faecalis* to its isogenic Esp-deficient mutant (Shankar, et al., 2001). Groups of CBA/J mice were challenged transurethrally with 10^8 CFU of either the parent or the mutant strain, and bacteria were enumerated in the urine, bladder, and kidneys five days post-infection. Significantly higher numbers of bacteria were recovered from the bladder and urine of mice challenged with the Esp-bearing parent strain than from mice challenged with the Esp-deficient mutant, which points to a role for Esp as a virulence factor in this infection model. The study suggested that Esp may serve to promote bacterial adhesion to the bladder epithelium through specific components of the bladder wall, such as mucin or uroplakin (Shankar, et al., 2001).

The role of *esp* in experimental peritonitis and urinary tract infection has been recently evaluated using *E. faecium* E1162 and an isogenic *esp*-deficient mutant (Leendertse, et al., 2009). Results from this study corroborated observations made for *E. faecalis* and revealed enhanced binding of the wild type strain to bladder and kidney epithelial cells *in vitro*, as well as higher numbers in both organs among infected mice. No difference was observed in the peritonitis model. The same isogenic pair was recently tested in a mouse bacteremia model (Sava, et al., 2010). In this study, passive immunization with antibodies to the N-terminal portion of Esp did not protect mice from bacteremia ($P > 0.05$), while in comparison, antibodies to LTA from both *E. faecalis* and *E. faecium* resulted in fewer numbers of *E. faecium* in the blood.

The role of *E. faecium* Esp in a rat model of infectious endocarditis was recently reported (Heikens, et al., 2011). In this study, higher numbers of the Esp-positive strain (E1162) were recovered at 24 hours from heart vegetations, as compared to an isogenic, Esp-deficient mutant. Further, anti-Esp antibodies were detected in sera of infected mice and from infected patients. In similar studies, the *esp*-positive strain MMH594 was recovered in significantly higher numbers ($P < 0.01$) from 48-hour vegetations on infected heart valves in a rabbit endocarditis model, as compared to an isogenic, *esp*-deficient mutant (unpublished). Although the presence of

antibodies to Esp was not evaluated in this study, the presence of Esp appeared to significantly enhance vegetation weight.

Ace, an adhesin to collagen of *E. faecalis*

Attachment of bacteria to host tissue components, such as the extracellular matrix (ECM), is an important early step of the infection process. Several studies have reported the ability of some *E. faecalis* isolates to adhere to a number of extracellular matrix (ECM) proteins, such as collagen, laminin, fibrinogen, fibronectin, lactoferrin, vitronectin, and thrombospondin, but most of them agree that adherence to these proteins is exhibited by relatively few isolates after growth in standard laboratory media (233, 291, 298). For example, Xiao *et al.* (Xiao, Höök, & Weinstock, 1998) found that only 2 of 44 *E. faecalis* isolates adhered to collagen and/or laminin after growth in brainheart infusion broth (BHI) at 37°C; but growth at an elevated temperature of 46°C led to a significant increase in the adherence of most clinical *E. faecalis* isolates to collagen and laminin, but not to fibronectin, fibrinogen, or albumin. Similar conditional adherence to collagen, fibrinogen, and fibronectin was later found after growth of *E. faecalis* in the presence of serum, versus its growth in BHI (180). Of note, a brief exposure (<5 min) to serum caused an immediate increase in adherence to fibronectin and collagen, to a lesser extent, while growth in the presence of serum was required for fibrinogen adherence (Nallapareddy & Murray, 2008). Thus, these observations suggest that adherence phenotypes to ECM proteins are not constitutively expressed by most clinical *E. faecalis* isolates, but are instead elicited by a stress condition or a host-derived cue, or are mediated by a potential factor(s) in serum-forming bridges between bacterial adhesins and host ECM proteins.

Subsequent searches for genes that encode potential adhesins in the first available genome sequence of *E. faecalis* (strain V583) led to the discovery of Ace (adhesin to collagen of *E. faecalis*), an MSCRAMM (microbial surface components recognizing adhesive matrix molecules) protein with similarity to the ligand-binding region of the *S. aureus* collagen adhesin Cna. A recombinant version of Ace was shown to bind to collagen type I, an important fibrillar collagen type with a wide tissue distribution, although with different kinetics than Cna (Rich, et al., 1999). Unlike Cna, however, Ace was also found to bind to collagen type IV and laminin, both of which are major network-forming components of mammalian basement membranes (Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000). Using an isogenic *ace* mutant of *E. faecalis* strain OG1RF, Nallapareddy *et al.* (Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000) further demonstrated that native Ace displayed on the cell surface mediates the majority of the 46°C-elicited collagen and laminin adherence of OG1RF. Direct binding of native Ace to collagen was confirmed by a far-western analysis of mutanolysin extracts from 46°C-grown OG1RF cells. Rabbit antibodies specific to the collagen-binding A-domain of Ace almost completely inhibited the conditional collagen and laminin adherence of OG1RF, as well as two other clinical *E. faecalis* strains, which further corroborates the specificity of the Ace-ligand interaction (Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000). Similar inhibition was also seen with human-derived antibodies purified from serum of an endocarditis patient (Nallapareddy S. R., Singh, Duh, Weinstock, & Murray, 2000). Ace has also been shown to be important for attachment of *E. faecalis* to dental roots (Hubble, Hatton, Nallapareddy, Murray, & Gillespie, 2003). Further studies identified the collagen-rich dentin as the target for this Ace-mediated adherence, which suggests that Ace may have a role in the common isolation of *E. faecalis* from periapical periodontitis, as well as its ability to persist in the root canal in the presence of antibacterial medications (Kowalski, et al., 2006). In addition, Hall *et al.* (Hall, et al., 2007) reported that Ace binds to collagen type VI as a recombinant protein, and that fluorescent beads coated with rAce A-domain bind to cultured human epithelial and endothelial cells. However, the moiety(ies) interacting with Ace on these cells remains uncharacterized.

Studies of *ace* expression revealed much higher levels of *ace*-specific mRNA after growth in the presence of serum or collagen type IV than in a routine laboratory medium (BHI) (Nallapareddy & Murray, 2006; Shepard & Gilmore, 2002). The increased mRNA was correlated with abundant surface display of Ace on *E. faecalis* OG1RF and its increased collagen and laminin adherence (Nallapareddy & Murray, 2006). Similar upregulation

of *ace* expression after exposure to collagen was also seen with other *E. faecalis* strains of different origins, which suggests that this may be a common programmed response of this species to a host-derived signal (Nallapareddy & Murray, 2006). The detection of anti-Ace antibodies in 90% of serum samples from patients with *E. faecalis* endocarditis suggested that most, if not all, strains produce Ace during infection and that Ace is antigenic in humans (Nallapareddy S. R., Singh, Duh, Weinstock, & Murray, 2000). A recent flow cytometry analysis of bacteria harvested from endocarditis vegetations infected with *E. faecalis* found Ace on 40-45% of bacterium-size particles, which confirmed the production of Ace *in vivo* (Singh K. V., Nallapareddy, Sillanpää, & Murray, 2010). While details of regulatory pathways that control *ace* expression are largely unknown, the transcriptional regulator Ers has been shown to negatively regulate *ace* by binding to its promoter region (Lebreton, et al., 2009). This study also indicated bile salts as another stress/factor that increases production of *ace* mRNA. Although the molecular details remain uncharacterized, this bile salts effect was proposed to be mediated by the deregulation of *ers* (Lebreton, et al., 2009). More recently, Pinkston *et al.* (Pinkston, et al., 2011) reported that gelatinase specifically cleaves Ace from the cell surface in later stages of *in vitro* growth, which provides at least one explanation for the previous detection of low levels of surface Ace and collagen adherence under these conditions. Using an *fsrB* mutant, this study further showed that surface display of Ace is modulated by the Fsr system through the activity of gelatinase, which demonstrates a link between Ace-mediated collagen adherence of *E. faecalis* and the multi-target Fsr quorum-sensing regulatory network (Bourgogne, Hilsenbeck, Dunny, & Murray, 2006; Pinkston, et al., 2011).

Several studies have implicated an important role for Ace in virulence. Using a mouse model of septic arthritis, Xu *et al.* (Xu, Rivas, Brown, Liang, & Höök, 2004) showed that exogenous expression of the collagen-binding A-domain of Ace in *S. aureus* increased the virulence of this organism to levels similar to an isogenic *S. aureus* strain that expressed Cna. Evidence for the role of Ace in virulence of *E. faecalis* has been shown by attenuation of an *ace* deletion mutant in a mouse UTI model, a *G. mellonella* insect model, and in survival within murine peritoneal macrophages (Lebreton, et al., 2009). Singh *et al.* (Singh K. V., Nallapareddy, Sillanpää, & Murray, 2010) recently demonstrated the importance of Ace for *E. faecalis* pathogenesis in endocarditis by showing that the deletion of *ace* leads to significant attenuation in experimental rat endocarditis, while no difference was observed in a peritonitis model. Furthermore, Ace was shown to be important in the early attachment stage of endocarditis, in addition to being expressed on the surface of *E. faecalis* cells *in vivo* within vegetations—a finding that is consistent with the concept that Ace mediates attachment of *E. faecalis* to exposed collagen and/or laminin at sites of valvular damage. Finally, vaccination with a recombinant collagen-binding A-domain of Ace, as well as passive immunization with anti-Ace A-domain antibodies, both conferred protection against endocarditis and reduced *E. faecalis* colonization of vegetations in the rat model (Singh K. V., Nallapareddy, Sillanpää, & Murray, 2010). This is in agreement with the previously shown efficient inhibition of collagen and/or laminin adherence by both polyclonal and monoclonal anti-Ace A-domain antibodies *in vitro* (Hall, et al., 2007; Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000; Nallapareddy S. R., Singh, Duh, Weinstock, & Murray, 2000). These results suggest that Ace, which was also found to be immunogenic in humans (Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000), could be a useful target for immunoprophylactic or therapeutic strategies (Singh K. V., Nallapareddy, Sillanpää, & Murray, 2010).

EfaA, *E. faecalis* antigen A

EfaA is a major surface antigen of *E. faecalis* and was identified using sera from patients with known *E. faecalis* endocarditis (Lowe, Lambert, & Smith, 1995). The *efaA* gene is the third gene of a three-gene operon, *efaBCA*, which is predicted to encode components of an ABC-type transporter, with EfaA as its putative substrate-binding lipoprotein component (Low, Jakubovics, Flatman, Jenkinson, & Smith, 2003). EfaA has sequence identity with a group of streptococcal ABC transporter proteins, some of which have also been identified as prominent surface adhesins and/or as factors associated with adherence or pathogenesis (Andersen, Ganeshkumar, & Kolenbrander, 1993; Kolenbrander, Andersen, Baker, & Jenkinson, 1998; Viscount, Munro, Burnette-Curley, Peterson, & Macrina, 1997). Evidence for the previously implied importance of *efaA* in *E.*

faecalis infection was provided by Singh *et al.* (Singh, Coque, Weinstock, & Murray, 1998), who showed that mice infected with an isogenic *efaA* disruption mutant of *E. faecalis* OG1RF had significantly prolonged survival, as compared to those infected with the wild-type parent strain, in an experimental peritonitis model. Screening for the prevalence of the *efaA* gene found it to be ubiquitously present in nearly all *E. faecalis* isolates and identified an equally prevalent homolog in *E. faecium* (Eaton & Gasson, 2001; Singh, Coque, Weinstock, & Murray, 1998). Low *et al.* (Low, Jakubovics, Flatman, Jenkinson, & Smith, 2003) reported that EfaA production is up-regulated *in vitro* when environmental Mn^{2+} concentrations are low. They proposed that the *efaBCA* operon encodes a high-affinity manganese permease that is expressed in tissues or serum where Mn^{2+}, an important micronutrient for *E. faecalis*, is not freely available, which explains the importance of EfaA for the infection of human host tissues (Low, Jakubovics, Flatman, Jenkinson, & Smith, 2003).

Ebp, endocarditis, and biofilm-associated pili

Although filamentous structures resembling pili or fimbriae were seen in electron microscopy studies of enterococci by Handley and Jacob in the 1980s (Handley & Jacob, 1981), the genetic and structural basis of enterococcal pili remained unknown until the discovery of a three-gene *ebpABC* (endocarditis and biofilm-associated pili) locus and an adjacent downstream sortase-encoding gene, *bps* (biofilm and pilus-associated sortase), that are necessary for the assembly of pili on the surface of *E. faecalis* strain OG1RF (Nallapareddy S. R., et al., 2006). The first clue to the importance of the Ebp pili for enterococcal virulence came from the finding of high titers of antibodies against recombinant proteins corresponding to the three structural pilus subunits, EbpA, EbpB, and EbpC, in sera from patients with *E. faecalis* endocarditis, a finding that implies that they are expressed *in vivo* and are immunogenic in the human host (Sillanpää, Xu, Nallapareddy, Murray, & Höök, 2004). Nallapareddy *et al.* (Nallapareddy S. R., et al., 2006) then showed that a non-piliated *ebp* allelic replacement mutant was significantly attenuated in a rat endocarditis model, and that this mutant was also impaired in its ability to attach to plastic surfaces and form biofilm. In addition, further studies found the *ebp* locus to be important for the colonization of kidneys in a murine model of ascending UTI (Singh, Nallapareddy, & Murray, 2007). A *bps* sortase deletion mutant of OG1RF that is unable to polymerize Ebp pilins on the cell surface was similarly attenuated in experimental UTI, and had a decreased ability to form biofilm (Kemp, Singh, Nallapareddy, & Murray, 2007). These studies have identified a role for Ebp pili of *E. faecalis* in two clinically important infections, in which the ability of *E. faecalis* to form biofilm is considered to play a major role.

As discussed above, the adherence of most *E. faecalis* isolates to several host ECM proteins is enhanced by growth in the presence of serum. Recently, deletion of the *ebpABC* genes was shown to nearly eliminate serum-elicited adherence of *E. faecalis* OG1RF to fibrinogen, a major serum and ECM component (Nallapareddy S. R., Singh, Sillanpää, Zhao, & Murray, 2011), while no effect on fibronectin adherence was observed. The involvement of Ebp pili in fibrinogen adherence was confirmed by complementation and inhibition of fibrinogen adherence of OG1RF by antibodies against the three Ebp pilins; the latter results also pointed to the possibility of using anti-Ebp antibodies for immunization against *E. faecalis* infections. Comparison of single and double deletion mutants of *ebpABC* and *ace* and their complemented derivatives showed that Ebp pili are also involved in serum-elicited adherence of *E. faecalis* OG1RF to collagen, although to a lesser degree than Ace. These mutants also showed reduced adherence to a collagen-secreting fibroblast cell line 3T6 (Nallapareddy S. R., Singh, Sillanpää, Zhao, & Murray, 2011). Furthermore, native pili extracted from OG1RF cells were found to bind to collagen, but not fibronectin, which gives further support for a role of Ebp pili in collagen adherence (Nallapareddy S. R., Singh, Sillanpää, Zhao, & Murray, 2011). In contrast, the *ebpABC* deletion had no significant effect on adherence to two other cell lines—human intestinal (Caco-2) and urinary bladder (T24) epithelial cells—nor on translocation across a human intestinal epithelial (T84) monolayer. This suggests that Ebp pili, unlike pili from streptococci, either do not have a major role in adherence to host cells/translocation or that the cell lines studied do not express the necessary surface receptors/factors.

Bacterial interactions with platelets are known to contribute to colonization of vegetations on heart valves during endocarditis (Fitzgerald, Foster, & Cox, 2006; Moreillon, Que, & Bayer, 2002). Studies with enterococci have shown variable platelet adherence and aggregation properties among isolates (Rasmussen, Johansson, Söbirk, Mörgelin, & Shannon, 2010; Scheld, Zak, Vosbeck, & Sande, 1981; Usui, Ichiman, Suganuma, & Yoshida, 1991). Nallapareddy *et al.* (Nallapareddy S. R., et al., 2011) recently reported that growth in the presence of serum increases adherence of *E. faecalis* OG1RF to human platelets. In the same study, using the pilus-deficient *ebpABC* deletion mutant and its complemented derivative, Ebp pili were shown to mediate the majority of this serum-elicited platelet adherence, while *ace* and *fss2*, a gene that encodes a fibrinogen-binding MSCRAMM protein (Sillanpää J. , et al., 2009), had no effect (Nallapareddy S. R., et al., 2011). Consistent with these findings, a pilus negative *bps* deletion mutant, as well as a housekeeping sortase (*srtA*) mutant and a sortase double mutant, were also found to be impaired in adherence to platelets (Nallapareddy S. R., et al., 2011).

Studies on regulation of the *ebp* locus identified a gene designated *ebpR* (endocarditis- and biofilm-associated pilus regulator), that is immediately upstream of the *ebpABC* genes and oriented in the opposite direction (Bourgogne, et al., 2007). The EbpR protein was found to have similarity to the AtxA/Mga family of regulator proteins and contains two predicted helix-turn-helix DNA-binding domains (Bourgogne, et al., 2007). Deletion of *ebpR* led to a 100-fold reduction in the expression of the *ebpABC* genes (Bourgogne, et al., 2007), which were previously shown to be co-transcribed as an operon (Nallapareddy S. R., et al., 2006). Only a slight effect was seen on *bps*, which is likely explained by its independent transcription from a second promoter, in addition to a four-gene *ebpABC–bps* transcript.

Although the regulatory pathway(s) involved in Ebp pilus production largely remain to be elucidated, several studies have shown that production of Ebp pili is affected by environmental factors. These include increased pilus expression when *E. faecalis* OG1RF is grown in tryptic soy broth + glucose (TSBG; standard biofilm medium) versus BHI, and even greater expression with growth in the presence of serum or bicarbonate (see below) (Bourgogne, et al., 2007; Bourgogne, Thomson, & Murray, 2010; Nallapareddy S. R., et al., 2006). Analysis of a large collection of diverse *E. faecalis* isolates demonstrated that serum-elicited Ebp pilus production is a general property exhibited by all 91 isolates tested (Nallapareddy S. R., et al., 2011). Further studies by flow cytometry revealed two distinct populations in terms of Ebp surface expression, which suggests a bistable mode of expression similar to recent reports with *S. pneumoniae* (Basset, et al., 2011; De Angelis, et al., 2011). Even more enhanced Ebp pilus expression was seen when OG1RF cells were harvested directly from endocarditis vegetations; ~70% of bacterial size particles that reacted with anti-*E. faecalis* whole-cell antibodies were found to produce EbpC compared to ~30% of serum-grown OG1RF, which demonstrates that Ebp pili are actively generated within host vegetations during endocarditis. These results corroborate the previous studies that indicate *in vivo* Ebp pilus expression by the common finding of anti-Ebp antibodies in sera from endocarditis patients infected with *E. faecalis* (Sillanpää, Xu, Nallapareddy, Murray, & Höök, 2004).

As mentioned above, bicarbonate,which is another environmental factor that may mimic physiologic conditions in the host, was recently demonstrated to induce Ebp pilus production via EbpR, a positive regulator of the *ebpABC* operon (Bourgogne, Thomson, & Murray, 2010). Although many other regulators of the AtxA/Mga family and virulence factors of other bacteria are known to be activated by the presence of CO_2 or CO_2/HCO_3^-, the increase in *ebpR* and *ebpABC* transcripts was found to be caused by the addition of HCO_3^- and not CO_2 (Bourgogne, Thomson, & Murray, 2010). Apart from the increased expression of cytolysin when *E. faecalis* cells are grown in an atmosphere of 80% H_2/20% CO_2 (41), Ebp pili were described as the first virulence-associated factor of *E. faecalis* whose expression is activated in response to elevated levels of bicarbonate. It was postulated that the release of HCO_3^- in the upper intestinal tract in response to the acidic discharges from the stomach may be sensed by *E. faecalis* and consequently lead to increased Ebp pilus expression to favor intestinal colonization. The same study also showed that the previously identified role of the Fsr quorum-sensing system as a weak repressor of the *ebp* locus (Bourgogne, Hilsenbeck, Dunny, & Murray, 2006) is independent of the *ebpR*-mediated bicarbonate effect. A recent report by Gao *et al.* (Gao, et al., 2010) identified a previously

uncharacterized gene, *rnjB*, which encodes a putative RNase J2, as another activator of the *ebp* operon. The *ebpABC* mRNA levels were significantly lower in an *rnjB* deletion mutant compared to the wild-type parent strain OG1RF, which suggests that *rnjB* regulates Ebp pilus expression at the mRNA level. Of note, both the *ebpR* and *rnjB* mutants showed lower biofilm formation versus the parent strain, which is consistent with the reduced Ebp pilus production. Considering the findings with *ebpR*, *rnjB* and *fsr*, it appears that several pathways with multiple target genes control the production of Ebp pili. However, the molecular details of these potential regulatory networks are still emerging.

Taken together, the above studies have revealed a multifunctional role for Ebp pili of *E. faecalis* in host-pathogen interactions. It could be argued that these properties enable circulating *E. faecalis* cells to adhere to exposed ECM proteins, such as collagen, on damaged vascular endocardial surfaces and to platelets and fibrinogen in vegetations on heart valves, which potentially explains the importance of Ebp pili in experimental endocarditis. Further studies are needed to determine which of the individual Ebp pilins is/are involved in these processes. Similar virulence-associated properties, such as adherence to various host tissue components, biofilm formation, and a role in animal infection models have also been assigned to pili or pilins of an increasing number of other Gram-positive bacteria, including *E. faecium* (Sillanpää J. , et al., 2010), *S. pyogenes* (Abbott, et al., 2007), *S. agalactiae* (Maisey, Hensler, Nizet, & Doran, 2007; Maisey, et al., 2008), *S. pneumoniae* (Barocchi, et al., 2006; Gianfaldoni, et al., 2007), and *C. diphtheriae* (Mandlik, Swierczynski, Das, & Ton-That, 2007), which points to a widespread role of these structures in infections caused by Gram-positive bacteria.

Enterococcal Capsule, Lipoteichoic Acid and Cell Wall Polysaccharide

Detailed information about the structure, chemistry, and genetics that underlie the carbohydrate and teichoic acid components of the enterococcal cell wall are presented in Enterococcal cell wall components and structures. The focus of this section will be on the role that some of these components play in enterococcal pathogenesis. Generally, these non-protein components on the outer surface of the bacterium can influence how well the host recognizes the invading pathogen, which affects the efficiency of the mechanisms of clearance, including phagocytosis and/or cytokine production (Hancock & Gilmore, 2002; Huebner, Quaas, Krueger, Goldmann, & Pier, 2000; Theilacker, et al., 2011; Thurlow R. L., Thomas, Fleming, & Hancock, 2009). The cell wall components that enhance the ability of the bacterium to overcome various host defenses are considered to be virulence factors.

The polysaccharide antigenic components of the Gram-positive cell wall can be divided into two categories: those that are common to all members of a given species and those that vary within a species in terms of their composition and/or structure. Antibodies can be raised against components that are surface-exposed, and typing schemes to differentiate enterococcal strains have been developed, based on whole-cell agglutination with particular antibodies (Hufnagel, et al., 2004; Maekawa, Yoshioka, & Kumamoto, 1992; Sharpe, 1964). These antibodies do not simply indicate differences between polysaccharides, which have been shown to vary, but can also indicate whether a static component of the cell wall is exposed or not, due to the variable presence of a capsule in *E. faecalis* strains (Hufnagel, Carey, Baldassarri, Reinert, & Huebner, 2006; Thurlow, Thomas, & Hancock, 2009). As detailed below, the presence of a capsule generally suggests a more virulent strain because of the immune evasion capabilities that the capsule imparts.

Variable capsular carbohydrate

Several studies have demonstrated that an important method of immune clearance of enterococci is neutrophil-mediated opsonization, with or without the involvement of complement. However, some strains were reported to be resistant to opsonization, and a polysaccharide, possibly a capsular component, was identified as the mediator of resistance by several indirect observations (Arduino, Murray, & Rakita, 1994; Huebner, et al., 1999; Rakita, et al., 2000; Rakita, et al., 1999). For example, exposure of *E. faecium* strain DO (also known as TEX16) to sodium periodate, but not proteases or phospholipases, eliminated resistance to opsonization (Arduino,

Murray, & Rakita, 1994). However, antibodies raised against this strain that targeted the carbohydrate fraction, also eliminated opsonic resistance, which restored the neutrophil killing of this strain (Rakita, et al., 2000).

Identification of an *E. faecalis* capsule was achieved by the purification of a carbohydrate that was localized to the surface of the bacterium when using specific antibodies. A genetic locus, *cps* for capsule polysaccharide, which encodes the genes necessary to generate this carbohydrate, was also identified (Hancock & Gilmore, 2002). A strain with an isogenic mutation in one of the genes (*cpsI*) was more susceptible to opsonophagocytotic killing by neutrophils and was compromised in its ability to persist within the lymph nodes of a mouse, which shows that the capsule contributes to virulence in strain OG1RF. Moreover, when capsule-specific antibodies were included in the phagocytic assay, the capsule-producing strains of *E. faecalis* were more effectively targeted for killing, which suggests that such antibodies could have therapeutic indications (Hancock & Gilmore, 2002). Further work (Thurlow R. L., Thomas, Fleming, & Hancock, 2009) showed that a capsule was protective against C3 complement, opsonophagocytic killing by macrophages. C3 was shown to deposit equally well on encapsulated versus non-encapsulated cells by Western blot analysis, but the capsule appeared to mask C3 epitopes on encapsulated whole cells, as there was less C3-antibody binding, as determined by flow cytometry (Thurlow R. L., Thomas, Fleming, & Hancock, 2009). Additionally, a capsule was shown to mask epitopes associated with LTA, which is a common target of anti-enterococcal antibody production (see below) (Thurlow, Thomas, & Hancock, 2009). A significant decrease in TNFα by the macrophages was associated with encapsulated strains, likely due to the decrease in LTA-exposed epitopes (Thurlow R. L., Thomas, Fleming, & Hancock, 2009). Further work on the structure of the capsular material revealed it to be a diheteroglycan, and antibodies raised against this material protected mice from bacteremia caused by encapsulated strains (Theilacker, et al., 2011).

Lipoteichoic acid

Various typing schemes for distinguishing strains of differing properties have been developed, based on differences in cell surface structures. These typing methods have been confounded by changes to the taxonomic classification of enterococci (Sharpe, 1964), and by variation in non-polysaccharide epitopes (Maekawa, Yoshioka, & Kumamoto, 1992). Hufnagel *et al.* (Hufnagel, et al., 2004) proposed a new typing scheme in 2004 that classified a majority of the tested *E. faecalis* strains into one of four types: CPS-A, B, C, or D. Strains of serotypes A and B only contained the *cpsAB* genes in their capsule locus, while serotypes C and D strains possess an additional eight to nine genes. Further work defined CPS-C serotype strains as containing the *cpsF* gene, which encodes an enzyme that carries out a glucosylation only found in the CPS-C capsule (Thurlow, Thomas, & Hancock, 2009). It was believed that serotypes A and B possessed a capsular polysaccharide of a different chemical nature than serotypes C and D, based on a purification of capsular material from a serotype A strain (Wang, et al., 1999). However, further investigation revealed that the material was actually LTA that had been inadvertently modified during the purification procedure (Theilacker, et al., 2006). Antiserum to serotypes A and B contain antibodies against LTA, which is accessible due to a *lack* of a capsule in these strains (Huebner, et al., 1999; Theilacker, et al., 2006; Theilacker, et al., 2011; Thurlow, Thomas, & Hancock, 2009). *E. faecalis* LTA is an important cell-wall component that influences the way in which the immune system recognizes infection. Antibodies raised specifically against LTA resulted in opsonic killing (Huebner, et al., 1999; Theilacker, et al., 2011), and are protective in a mouse model of enterococcal infection (Huebner, Quaas, Krueger, Goldmann, & Pier, 2000). All sequenced strains of *E. faecium* contain *cpsAB* homologues, but not the other capsule genes, which suggests that its ability to make this type of a capsule is rare or non-existent.

Common cell wall polysaccharide: Epa, enterococcal polysaccharide antigen

The existence of a cell wall polysaccharide that is common to all strains of *E. faecalis* was first detected by a study that screened for common antigens produced during *E. faecalis* human infections. DNA fragments from *E. faecalis* strain OG1RF were expressed in *E. coli,* and the production of antigenic material was detected by using serum from infected humans (Xu, Jiang, Murray, & Weinstock, 1997). One immunopositive clone produced

proteinase K-resistant antigenic material, and subsequent study confirmed that the material was a carbohydrate (Xu, Jiang, Murray, & Weinstock, 1997; Xu, Murray, & Weinstock, 1998). Sequencing of the clone revealed a locus in which many of the genes encoded proteins with similarity to bacterial polysaccharide biosynthesis enzymes (Xu, Murray, & Weinstock, 1998). A later characterization defined the genes involved in generating the Epa polysaccharide as *epaA–epaR*, which corresponded to EF2198–EF2177 in the V583 genome (Teng, Singh, Bourgogne, Zeng, & Murray, 2009). Overall, by both predicted gene function and by compositional analysis of the antigenic material, the *epa* locus appears to be involved in generating a rhamnose-containing polysaccharide (Teng, Singh, Bourgogne, Zeng, & Murray, 2009; Xu, Murray, & Weinstock, 1998). The polysaccharide is hypothesized to be present in all strains of *E. faecalis*, as well as relatively invariant, based on sequence analysis of 12 strains (Teng, Jacques-Palaz, Weinstock, & Murray, 2002). A similar *epa* locus is found in all sequenced strains of *E. faecium*, except that three genes are missing (*epaI, epaJ, epaK*). Epa is postulated to reside deep in the cell wall due to difficulty in detecting it on the surface with antibodies, at least *in vitro* (Hancock & Gilmore, 2002; Xu, Murray, & Weinstock, 1998; Xu, Singh, Murray, & Weinstock, 2000).

Loss of the Epa polysaccharide resulted in attenuated virulence phenotypes in a variety of models and assays, which suggests that this polysaccharide contributes to the pathogenic properties of *E. faecalis*. Insertion mutants in *epaB* and *epaE* resulted in attenuated killing in a mouse peritonitis model with a two- to three-fold increase in the LD_{50} (Xu, Singh, Murray, & Weinstock, 2000). These two mutants were also more susceptible to neutrophil-mediated phagocytosis and killing, which suggests that this cell wall polysaccharide contributes to *E. faecalis* evasion of the immune system (Teng, Jacques-Palaz, Weinstock, & Murray, 2002). The polysaccharide may also contribute to colonization and invasion of tissue, as the *epa* mutants were additionally deficient in biofilm formation on a polystyrene surface (Mohamed, Huang, Nallapareddy, Teng, & Murray, 2004) and translocation across a polarized monolayer of colon epithelial cells (Zeng, Teng, Weinstock, & Murray, 2004). Consistent with a role in colonization and biofilm formation, the *epaB* mutant was also defective in a mouse model of ascending UTI (Singh, Lewis, & Murray, 2009).

Metabolic Characteristics

Bacterial adaptation to growth in a host as a pathogen inevitably results in metabolic changes, as the organism acclimates to niche-specific nutritional availabilities and arms itself to thrive in the face of the host immune response. Transcriptional microarray analysis of *E. faecalis* OG1RF harvested after eight hours of incubation in a rabbit subdermal abscess infection model revealed the differential regulation of nearly 300 genes, nearly 90% of which were down-regulated (58). Analysis of the down-regulated genes, which included those that coded for ribosomal proteins, aminoacyl tRNA synthetases, DNA replication enzymes, RNA polymerase subunits, ATP synthase machinery subunits, cell division-related proteins, and acyl carrier proteins, suggested activation of the stringent response among bacteria during infection in the subdermal abscess environment—although the stressor that triggered such a reaction remains unidentified. The stringent response has been shown to be necessary for *E. faecalis* infection in the *C. elegans* killing model (Abranches, et al., 2009). In *E. faecalis*, (p)ppGpp, the alarmone whose accumulation activates the stringent response during nutrient deprivation or environmental stress is synthesized by the enzymes RelA and RelQ. RelQ maintains (p)ppGpp levels during homeostatic growth, whereas RelA produces (p)ppGpp during stress conditions (Abranches, et al., 2009). Analysis of OG1RF Δ*relA*, Δ*relQ*, and Δ*relA*Δ*relQ* in-frame deletion mutants in the subdermal abscess model demonstrated that both *relA* and *relQ* affect survival in this environment (Frank, Lemos, Schlievert, & Dunny, 2012). The Δ*rel*A strain, which displays higher basal levels of (p)ppGpp, exhibited increased survival during the early hours of infection, which suggests a protective role for the alarmone in the initial stages of infection. In contrast, Δ*relA*Δ*relQ* and Δ*relQ* showed reduced abilities to persist in the abscesses for several days, which suggests that production or maintenance of basal (p)ppGpp is a key factor for persistence. Further analysis of the same mutants in rabbit endocarditis showed that Δ*relA* is severely attenuated, but Δ*relA*Δ*relQ* , which has no (p)ppGpp, is not attenuated (Frank, Lemos, Schlievert, & Dunny, 2012). This phenotype indicates (p)ppGpp

levels affect virulence and that the ability to hydrolyze (p)ppGpp is indispensible for *E. faecalis* to infect heart valves.

The ability to produce extracellular superoxide is a common trait among *E. faecalis* isolates, particularly those that cause bacteremia, and is infrequently found in *E. faecium* isolates (Huycke, Joyce, & Wack, 1996). It has been hypothesized that extracellular superoxide produced by intestinal commensal *E. faecalis* cells may be a potential source of chromosomal instability (CIN) in colonocytes, which may result in colorectal cancer (Huycke, Abrams, & Moore, 2002). An experiment that measured increased DNA damage in the colon cells of rats colonized with wild-type *E. faecalis,* as compared to those colonized with an isogenic superoxide non-producing strain, provided data in support of this hypothesis (Huycke, Abrams, & Moore, 2002). After further study, a detailed mechanism has emerged that states that *E. faecalis* extracellular superoxide production activates the release of DNA-damaging agents from activated macrophages, which results in CIN and cell cycle arrest in nearby epithelial cells (Wang, et al., 2008; Wang & Huycke, 2007; Wang, et al., 2012). In effect, this line of research, which is described in more detail elsewhere in this volume, suggests that superoxide-producing *E. faecalis* commensals induce bystander effects, which may ultimately contribute to the pathogenesis of colorectal carcinogenesis (Wang, et al., 2012).

Another metabolite associated with virulence in *E. faecalis*, at least in the *C. elegans* model, is ethanolamine (Maadani, Fox, Mylonakis, & Garsin, 2007). Ethanolamine can serve as a source of both carbon and nitrogen for those microbes that are equipped to catabolize it. Ethanolamine is present in large quantities in the intestine, and the ability to utilize this compound has been associated with intestinal pathogens, such as *Salmonella* species and *Listeria monocytogenes* (Garsin D. A., 2010). Though loss of one of the *E. faecalis* genes necessary to metabolize ethanolamine caused an attenuated phenotype in the worm model (Maadani, Fox, Mylonakis, & Garsin, 2007), it remains unclear whether the ability to metabolize ethanolamine affects *E. faecalis* intestinal commensalism or pathogenesis in mammals.

Other Factors Affecting Virulence of *E. faecalis*

Despite extensive research on enterococcal pathogenicity, few virulence factors present in all clinical isolates have been identified. This fact illustrates that infection is the result of complex interactions between factors derived from the host, as well as from the microbe. Enterococcal virulence depends on strain-variable combinations of factors belonging to the pan and core genomes that result in infection when expressed together. This includes factors that confer fitness inside the infected host.

Pathogens that invade a host are exposed to numerous stresses, and stress resistance has been linked to virulence. An interesting protein implicated in stress resistance identified in recent years is the general stress protein Gls24, of unknown function. This 20 kDa protein was initially identified to be strongly induced in stationary phase cells, and also in cultures exposed to bile salts or the heavy metal cadmium in *E. faecalis* strain JH2-2 (Giard, Rince, Capiaux, Auffray, & Hartke, 2000). The *gls24* gene forms an operon with *glsB*, which encodes a small polypeptide of unknown function. This genetic organization is highly conserved among sequenced *E. faecalis* strains. The *gls24* and *glsB* genes can also be expressed as a large mRNA that includes 4 other cistrons (*orf1–orf4*) upstream of *gls24* (Giard, Rince, Capiaux, Auffray, & Hartke, 2000; Teng, Nannini, & Murray, 2005). The ORF that precedes *gls24* (*orf4*) is a close paralog of *gls24*. Of note, *E. faecalis* strain V583 harbors a second, albeit incomplete *orf1–orf4* operon (*orf1* is missing in this copy) situated in the pathogenicity island. The induction of *gls24* expression in `stationary phase is due to promoter P2 situated immediately upstream of *gls24,* whereas bile salts treatment causes induction of the remote promoter P1, which is located in front of *orf4*. Stationary phase cultures of *gls24* mutants constructed in two different strains are more sensitive to bile salts exposure than the parental strains (Giard, Rince, Capiaux, Auffray, & Hartke, 2000; Teng, Nannini, & Murray, 2005). Of greater importance, the *gls24* disruption mutant (but not a *glsB* mutant) constructed in strain OG1RF was highly attenuated in a mouse peritonitis virulence model (Teng, Nannini, & Murray, 2005) and a rat endocarditis model (Nannini, Teng, Singh, & Murray, 2005). In this last study, a separate experiment also

showed also that when administrated as a mixture with the parental strain, the *gls24* mutant, but not the *glsB* mutant, was out-populated at the end of the experiment in vegetations, organs, and blood, despite being inoculated in greater numbers.

Phagocytes form the front line of defense used to fight invading pathogens. These cells have specific enzymes that generate antimicrobial reactive oxygen species (ROS). As a consequence, antioxidant defense activities of pathogens play an important role in colonization and persistence at the site of infection. *E. faecalis* is well equipped to survive oxidative stress *in vitro*, and is also highly resistant to intracellular killing by phagocytic cells (La Carbona, et al., 2007; Verneuil, et al., 2006). Part of this resistance is due to a manganese superoxide dismutase (Mn-SOD) (Verneuil, et al., 2006), a heme-dependent catalase (Frankenberg, Brugna, & Hederstedt, 2002) and three NADH-dependent peroxidases (alkylhydroperoxide reductase, thiol peroxidase, and NADH-peroxidase) (La Carbona, et al., 2007). Mutants with an inactivated *sodA* gene (that encodes the Mn-SOD), and with inactivated genes that encode the peroxidases (*ahpCF, tpx*, and *npr*), have been constructed and tested for attenuation in infection (La Carbona, et al., 2007; Verneuil, et al., 2006). These studies showed that the *sodA* and *tpx* mutants were highly compromised in survival inside mouse peritoneal macrophages. In addition, reduced survival inside microglial cells was recently shown for the *sodA* mutant (Peppoloni, et al., 2011), and attenuated virulence in a mouse peritonitis model was found for the Tpx-deficient strain (La Carbona, et al., 2007).

When antioxidant defense systems fail to protect the cell against ROS, damage repair pathways are activated. Methionine (Met) side chains of proteins are particularly vulnerable to oxidation, as they form methionine sulfoxide (MetSO). Methionine sulfoxide reductases A and B are antioxidant-repair enzymes that reduce methionine sulfoxides back to methionine (Zhao, et al., 2010). Deficiency of either Msr-enzyme in *E. faecalis* reduced survival inside mouse peritoneal macrophages stimulated with recombinant gamma interferon plus lipopolysaccharide (but not in naïve phagocytes), and reduced virulence in a systemic and urinary tract infection (UTI) model (Zhao, et al., 2010).

Several proteins implicated in host-pathogen interactions are characterized by a leucine-rich repeat (LRR) domain. The best characterized of these LRR proteins are *IlnA* and *IlnB* from *Listeria monocytogenes*, which trigger internalization into cells (Hamon, Bierne, & Cossart, 2006). Two internalin-like proteins were identified in *E. faecalis* V583 (EF2250 and EF2686), both of which are characterized by an additional C-terminal WxL domain involved in peptidoglycan binding (Brinster, et al., 2007). The polypeptide encoded by *ef2686*, named ElrA for *Enterococcus* leucine-rich protein A, was recently shown to be important for *E. faecalis* virulence, since a disruption mutant was significantly attenuated in a mouse peritonitis model, demonstrated reduced survival in macrophages, and displayed a decreased interleukin-6 response *in vivo* (Brinster, et al., 2007).

Several transcriptional regulators that are likely to control determinants important for virulence or fitness have also been identified in *E. faecalis*. The disruption of *hypR*, which encodes a regulator that shows some sequence homology to the main oxidative stress regulator OxyR of *E. coli*, reduced resistance to oxidative stress, survival in *in vivo* infected peritoneal macrophages, and virulence in a mouse peritonitis model (Verneuil N. , et al., 2005; Verneuil, et al., 2004). Additionally, inactivation of a homologue of PerR, the most important transcriptional regulator of the oxidative stress response of *Bacillus subtilis*, decreased virulence of the mutant in the peritonitis model (Verneuil N. , et al., 2005). Using transposon mutagenesis, a new gene locus implicated in biofilm formation has been identified (Hufnagel, Koch, Creti, Baldassarri, & Huebner, 2004). This locus is composed of four genes that are likely expressed as an operon, designated *bop* (biofilm on plastic surfaces). The transposon was inserted into the second gene of this operon, and the corresponding mutant showed reduced biofilm production and decreased persistence in a mouse bacteremia model. The last gene of the operon encodes a transcriptional regulator belonging to the LacI/GalR family, which was less expressed in the transposon mutant and was responsible for the observed phenotypes (Hufnagel, Koch, Creti, Baldassarri, & Huebner, 2004). An AraC-type transcriptional regulator, designated PerA and encoded by a gene located within the pathogenicity island, was also implicated in biofilm formation and virulence. A corresponding mutant constructed in the

clinical isolate E99 produced more biofilm, but was less pathogenic in a mouse peritoneal infection model and was attenuated for survival within macrophages *in vitro* (Coburn, Baghdayan, Dolan, & Shankar, 2008). The enterococcal regulator of survival (Ers), a member of the Crp/Fnr family of transcriptional regulators, is a close homologue of PrfA, which is the main regulator of virulence genes in *Listeria monocytogenes*. Survival of a mutant with a disrupted *ers* gene within peritoneal macrophages was highly reduced and the strain was also less virulent in a mouse peritonitis model (Giard, et al., 2006; Riboulet-Bisson, et al., 2008). In contrast, the mutation of *slyA*, which encodes a transcriptional regulator of the MarR/SlyA family, led to increased virulence of the mutant strain in the *Galleria mellonella* model, better survival inside *in vivo* infected mouse macrophages, and longer persistence in mouse kidneys and livers (Michaux, et al., 2011). A strain with a deleted *sigV* gene encoding an extracytoplasmic function (ECF) sigma factor was shown to have decreased persistence in both the kidneys and liver, and in a UTI model, showed reduced colonization of the kidneys and bladders (Le Jeune, et al., 2010).

Factors Contributing to Virulence in *E. faecium*

Over the past three decades, there has been a gradual shift towards a higher proportion of enterococcal infections being caused by *E. faecium* (from ~5% to more than 35%) in the USA (94) and in many European hospitals (Top, Willems, van der Velden, Asbroek, & Bonten, 2008; Werner, et al., 2008). Besides resistance to antibiotics, including ampicillin and vancomycin, it appears that other properties have enhanced the hospital adaptation and infectivity of *E. faecium*, such as traits that contribute to colonization of the intestinal tract, translocation through the intestinal wall, and/or attachment to internal organs and other tissue sites. A significant challenge to studying *E. faecium* pathogenesis has been difficulty in engineering specific mutations, which stems in part from the scarcity of selection markers for use in antibiotic-resistant clinical strains, as well as their poor transformability. Some of these obstacles have been overcome by the introduction of conjugative, temperature-sensitive plasmids and positive counterselection systems (Kristich, Chandler, & Dunny, 2007; Nallapareddy, Singh, & Murray, 2006). Thus far, only three genes/loci, *acm* (Nallapareddy, Singh, & Murray, 2008), the *ebpfm* (Sillanpää J. , et al., 2010) operon, and *esp* (Heikens, Bonten, & Willems, 2007; Heikens, et al., 2011), have been shown to play a role in *E. faecium* virulence when tested in animal models.

Acm

Adherence studies with *E. faecium* have shown that, like *E. faecalis*, many isolates can adhere to collagen, but typically do so after growth in a routine laboratory medium (BHI) (Nallapareddy, Weinstock, & Murray, 2003), in contrast to the conditional adherence exhibited by most *E. faecalis* isolates (Xiao, Höök, & Weinstock, 1998). A highly significant association was found in collagen adherence of *E. faecium* isolates of clinical origin, versus community fecal or animal origin, which suggests that such adherence to collagen may contribute to the ability of *E. faecium* to persist, colonize, and cause infection in the hospital environment (Nallapareddy S. R., Singh, Okhuysen, & Murray, 2008; Nallapareddy, Weinstock, & Murray, 2003). Analysis of an early draft version of the first *E. faecium* genome (strain TX16, also known as DO) (Fox, 2000) identified *acm* (adhesin of collagen from *E. faecium*), a gene that encodes an MSCRAMM family protein with higher similarity to the *S. aureus* MSCRAMM Cna than to Ace of *E. faecalis* (Nallapareddy, Weinstock, & Murray, 2003). Recombinant Acm was subsequently shown to bind to collagen type I and, to a lesser degree, type IV, but not to laminin, fibrinogen, or fibronectin (Nallapareddy, Weinstock, & Murray, 2003). The introduction of a functional *acm* gene into two collagen adherence-negative natural *E. faecium acm* mutants, on a low-copy-number shuttle vector, led to a collagen adherence phenotype for both strains (Nallapareddy, Weinstock, & Murray, 2003). Acm was later confirmed to mediate collagen adherence of *E. faecium* using an *acm* deletion mutant, which nearly eliminated the collagen binding of strain TX82 (Nallapareddy, Singh, & Murray, 2006). Although the *acm* gene is carried by almost all *E. faecium* isolates, it occurs as a functional gene almost exclusively in multi-drug resistant *E. faecium* isolates of clinical origin, while it is present as an inactive pseudogene, often interrupted by a transposon, in approximately one quarter of isolates from non-clinical sources (Nallapareddy, Singh, & Murray, 2006; Nallapareddy S. R.,

Singh, Okhuysen, & Murray, 2008). Moreover, the level of collagen adherence correlates strongly with the amount of Acm produced on the cell surface of diverse *E. faecium* isolates, which supports the role of Acm as the primary collagen adhesin of *E. faecium,* at least *in vitro.*

A recent study by Nallapareddy *et al.* (Nallapareddy, Singh, & Murray, 2008) reported that the *acm* deletion mutant of strain TX82 was highly attenuated in experimental endocarditis using a rat model, both in early adherence/colonization of the heart valve, and in established vegetations, while no differences in mortality were seen in a mouse peritonitis model. Further examination of 17 endocarditis isolates revealed that, although 5 of them did not produce detectable surface Acm nor adhere to collagen, all had an intact *acm* sequence that was highly identical to those of Acm-producing isolates. Not surprisingly, mRNA levels of *acm* were reduced in some of these Acm non-producers (*e.g.*, TX16) under standard *in vitro* growth conditions, explaining the lack of surface Acm and collagen adherence (Nallapareddy, Singh, & Murray, 2008). However, flow cytometry analysis of extracts processed directly from rat vegetations infected with TX16, detected Acm on ~40% of TX16 cells, which demonstrates the active production of Acm during infection. This finding is consistent with the presence of antibodies against Acm in the serum of the endocarditis patient infected with TX16 (Nallapareddy, Singh, & Murray, 2008). A larger survey of patient sera found anti-Acm antibodies in all sera from *E. faecium* endocarditis patients, and in most sera from patients with other *E. faecium* infections, despite the lack of Acm expression or collagen adherence by some of the infecting strains, which suggests that Acm expression *in vivo* is common even by those *acm*+ clinical isolates that do not express it *in vitro* (Nallapareddy S. R., Singh, Okhuysen, & Murray, 2008). Anti-Acm antibodies purified from an endocarditis patient serum were able to significantly inhibit the adherence of *E. faecium* to collagen (Nallapareddy, Singh, & Murray, 2008), similar to rabbit antibodies raised against the collagen-binding subdomains of Acm (Nallapareddy S. R., Sillanpää, Ganesh, Hook, & Murray, 2007). These studies support the concept of Acm as a potential target for developing antibody-based strategies for immunoprophylaxis, or in combination with antibiotics to treat *E. faecium* infections. A further possibility could be active or passive immunization with of a mixture of two or more *E. faecium* surface adhesins, perhaps in combination with the ubiquitous and highly conserved *E. faecalis* Ace and/or Ebp pili, for broader coverage of both major enterococcal pathogens.

Ebpfm pili

Recent analyses of the first available draft *E. faecium* genome (endocarditis strain TX16/DO) identified 15 genes (including *acm*) that encode LPXTG-motif, cell-wall anchored proteins with MSCRAMM-like characteristics (see Enterococcal cell wall components and structures). These include a cluster of three pilin-encoding genes, initially named *ebpAfm, ebpBfm*, and *ebpCfm*. Although the gene organization of the *ebpfm* locus, including a downstream sortase gene, is nearly identical to the *ebp-bps* locus of *E. faecalis*, these encoded proteins show relatively large amino acid sequence divergence between these two species, with similarities ranging from 66% (EbpBfm vs. EbpB) to 85% (EbpCfm vs. EbpC) (Nallapareddy S. R., et al., 2011). Also, transcriptional organization of the two *ebp* operons were shown to be different, with separate *ebpABCfm* and *bpsfm* transcripts produced by *E. faecium* compared to a four-gene *ebpABC-bps* transcript, plus an additional *bps* transcript, produced by *E. faecalis* (Nallapareddy S. R., et al., 2006; Sillanpää J. , et al., 2008). Using an *ebpABCfm* deletion mutant, this operon was recently shown to be important for the ability of *E. faecium* TX82 to cause infection in an ascending UTI model, as well as to form biofilm (Sillanpää J. , et al., 2010), which indicates functional similarity between the Ebp type pili of *E. faecium* and *E. faecalis*. However, it is currently unclear whether the Ebpfm pili have a similar role in endocarditis and adherence to host collagen, fibrinogen, and platelets, as shown by their counterpart in *E. faecalis*. Although *E. faecium* TX16 has been shown/predicted to produce three additional pili, as well as individual MSCRAMMs (see Enterococcal cell wall components and structures)—with several of these genes reported to be enriched in isolates of the hospital-associated clade versus those in the community clade—direct evidence for their involvement in virulence is lacking.

Esp

Similar to *E. faecalis*, *E. faecium* clinical isolates are enriched in an *esp* homolog that shares >90% amino acid identity (Leavis, et al., 2004) and is encoded by a pathogenicity island (Leavis, et al., 2004; Willems, et al., 2001). The *esp* gene encodes a very large LPXTG-motif cell-wall anchored protein that represents one of the few identities within the pathogenicity islands of these two species. Studies with an *esp* deletion mutant showed that as for *E. faecalis*, *esp* of *E. faecium* contributes to biofilm formation (Heikens, Bonten, & Willems, 2007), virulence in UTI in a mouse model (Leendertse, et al., 2009), and virulence in endocarditis (Heikens, et al., 2011), similar to previous reports on *esp* of *E. faecalis*. No contribution for *esp* was detected in mouse peritonitis (Leendertse, et al., 2009), intestinal colonization (Heikens, et al., 2009), or adherence to the intestinal epithelial cell line Caco-2 (Heikens, et al., 2009).

Other factors

Increased virulence of *E. faecium* transconjugants that harbor conjugative megaplasmids has been reported in mouse peritonitis (Arias, Panesso, Singh, Rice, & Murray, 2009) and colonization models (Rice, et al., 2009). In both models, these plasmids were found to contain the hyaluronidase *hyl* gene, which was previously associated with a higher prevalence in clinical isolates, as compared to community isolates. However, the subsequent deletion of *hyl* caused no effect on peritonitis, which suggests that this model bypasses a step where it contributes, or that other uncharacterized virulence factors are encoded by the *hyl*-containing plasmid, and this gene is a passive marker in virulent strains (Panesso, et al., 2011).

Two homologs of the *E. faecalis* general stress protein, Gls24 (Giard, Rince, Capiaux, Auffray, & Hartke, 2000; Nannini, Teng, Singh, & Murray, 2005; Teng, Nannini, & Murray, 2005), have recently been characterized in *E. faecium* (Choudhury, Singh, Sillanpää, Nallapareddy, & Murray, 2011). The genes that encode these proteins, *gls33* and *gls20* (named according to the calculated molecular weights of their encoded proteins), are both followed by homologs of *E. faecalis glsB* (designated as *glsB* and *glsB1* in *E. faecium*), and are part of larger operons that are only partially similar to the *gls* operon of *E. faecalis*. Comparisons among sequenced Efm genomes revealed clade-specific differences between the *gls33* and *gls20* operons, corresponding to ~7% nucleotide sequence difference between hospital-associated (HA) and community-associated (CA) *gls33* operons, and ~3.5% difference between HA and CA *gls20* operons (Choudhury, Singh, Sillanpää, Nallapareddy, & Murray, 2011; Galloway-Peña, Rice, & Murray, 2011). This genetic divergence between the HA and CA lineages is in agreement with that recently reported for most of the MSCRAMM- and pilus-encoding genes of *E. faecium*, as well as the clade-specific differences seen at the core genome level (see Enterococcal Genomics). A double deletion mutant that lacked both *gls33-glsB* and *gls20-glsB1*, but neither single deletion mutant, was shown to be highly attenuated in a mouse peritonitis model, while mutants that lack either *gls33-glsB* or *gls20-glsB1* or both were all more sensitive to bile salts than the wild-type parent strain (Choudhury, Singh, Sillanpää, Nallapareddy, & Murray, 2011). Similar attenuation has previously been demonstrated with a *gls24* mutant of *E. faecalis* in experimental peritonitis (Teng, Nannini, & Murray, 2005) and endocarditis (Nannini, Teng, Singh, & Murray, 2005), in addition to a decrease in bile salts tolerance (Giard, Rince, Capiaux, Auffray, & Hartke, 2000; Nannini, Teng, Singh, & Murray, 2005), which demonstrates that the *gls* loci of both species are important for virulence, and suggests their involvement in adaptation to the intestinal environment. Finally, protection studies using anti-Gls24 serum against experimental peritonitis by *E. faecalis* suggest Gls proteins as useful targets for immunotherapy.

Conclusions

In the strictest definition, "virulence factor" is defined as a substance that is necessary for causing disease in the host, but not necessary for survival in other contexts. Toxins, like the one produced by *Bacillus anthracis* and other toxin-producing pathogens, mostly fit this criterion. While a significant number of genetic determinants contribute to the ability of a given enterococcal strain to cause infection, these determinants are not necessarily

found in every clinical isolate, which highlights the point that enterococcal infection is multifactorial and involves contributions by the microbe, as well as the host. Many enterococcal factors that contribute to fitness in the host also contribute to the overall fitness of the bacterium in other ecologies, including its normal habitat, the GI tract. The virulence of enterococci is more complex than the simple presence of some main players, and appears to be dependent on strain-variable combinations of factors that lead to improved infection and colonization when expressed together in the right background. Virtually all studies on enterococci as pathogens have focused on the genetic factors that contribute to their pathogenic potential, with fewer studies focusing on the role of the host or on factors that contribute to the commensal lifestyle of enterococci. Promoting such noninfectious behavior could be a novel strategy for potentially preventing and treating infections. Understanding how enterococci contribute to the human microbiome as well as infection would help to illuminate exactly where they occur on the commensal-pathogen continuum.

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Enterococcal Genomics

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Introduction

Enterococcal genomics is a rapidly growing area of study. The first enterococcal genome sequence—that of *Enterococcus faecalis* V583—was published ten years ago (McShan & Shankar, 2002; Paulsen, et al., 2003), and complete or draft genome sequences of various enterococcal strains and species now number in the hundreds (http://www.ncbi.nlm.nih.gov/genome). Concurrent with rapid advances in genome sequencing, the sequencing-based classification scheme of multilocus sequence typing (MLST) has been used to interrogate population structures of the two enterococcal species that are most associated with human health and disease, *E. faecalis* and *Enterococcus faecium*. These two species also constitute the bulk of enterococcal genome sequence data that has been generated to date. This wealth of genomic data has allowed for an investigation of enterococcal diversity at a depth not previously achievable. Genomic studies in enterococci have been driven by overarching questions, such as: Why do multiple species of enterococci exist that inhabit seemingly identical niches, such as *E. faecalis* and *E. faecium* in the human gut, and what ecological factors have contributed to their divergence from a common ancestor? Within an enterococcal species such as *E. faecalis* or *E. faecium*, what qualities distinguish one strain from another? Are infection- or hospital-derived strains evolutionarily distinct from strains that benignly co-exist in the complex microbial consortium of the healthy human intestine? Related to this, have antibiotic use and the nosocomial environment led to changes in the enterococcal genome and/or its population structure?

This chapter highlights major advances in enterococcal genomics, including the development of MLST schemes to study the population structures of *E. faecalis* and *E. faecium;* comparative genome hybridization (CGH) studies to catalog the genomic contents of hundreds of *E. faecalis* and *E. faecium* strains; and significant findings from genome sequencing of multiple enterococcal species, beginning with the discovery and sequencing of the *E. faecalis* pathogenicity island (PAI). Additionally, we review the use of genome resequencing as a tool to study the short-term evolution of *E. faecalis* and the use of metagenomics to assemble *in situ* enterococcal genomes. In concluding the chapter, we discuss future perspectives in enterococcal genomics, including pressing questions that should drive future research in this field. While comparative genomics in enterococci has rapidly advanced over the last ten years, the number of genomes discussed here pales in comparison to what has been emerging—136 enterococcal genomes have been sequenced as part of the Human Microbiome Project (http://www.hmpdacc.org/), and 406 more were sequenced in a large-scale enterococcal genome sequencing endeavor performed in a multi-national collaboration with the Broad Institute (Cambridge, MA). Clearly, our foray into enterococcal genomics has only just begun.

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Population structures of *E. faecalis* and *E. faecium* defined by MLST

MLST is a sequencing-based typing method that was designed to infer the global and long-term epidemiology of a particular bacterial species. In addition, MLST has been widely used in both population and evolutionary biology (Urwin & Maiden, 2003). The technique relies upon the amplification and sequencing of internal fragments of multiple housekeeping genes that are present at different locations on a chromosome. MLST is similar in concept to 16S rRNA sequencing, in that both techniques rely upon the sequencing of genomic loci for which diversifying selection is low, but is distinguished from 16S rRNA sequencing in its ability to resolve relationships at a subspecies level. Although MLST is able to infer bacterial population structure using hundreds or thousands of strains, one of its drawbacks is the limited number of alleles that are analyzed—only seven for *E. faecium* and *E. faecalis*—which are presumed to represent the evolutionary history of the entire genome. This limitation can be overcome by comparing full genome sequences, as has been shown by studies of the pathogens *Staphylococcus aureus* and *Streptococcus pneumoniae*, in which the recent evolution of pathogenic lineages was described by the analysis of several dozen genome sequences (Croucher, et al., 2011; Harris, et al., 2010). However, as a facile classification method, MLST has shown that certain lineages within *E. faecalis* and *E. faecium* are consistently associated with infections, multidrug resistance, and hospital persistence, and this information has helped guide the selection of strains for genome sequencing projects.

E. *faecium* MLST

The utility of MLST in evaluating enterococcal population structures was first explored in *E. faecium*. The need to understand epidemiology and population structure has been an important driver for research into this organism. In particular, the recognition that hospital-acquired infections are not caused by *E. faecium* clones that normally reside in the gut, but rather are caused by specific *E. faecium* clones that are acquired during hospitalization and are apparently well adapted to thrive in the perturbed microbiota of hospitalized patients, prompted research into potential virulence genes that were significantly enriched in these hospital clones. The first molecular epidemiological studies that showed host specificity of *E. faecium* and the existence of a distinct genogroup of hospital associated isolates used techniques such as amplified fragment length polymorphism (AFLP) and ribotyping (Borgen, Wasteson, Kruse, & Willems, 2002; Brisse, Fussing, Ridwan, Verhoef, & Willems, 2002; Coque, et al., 2005; Jureen, et al., 2003; Vancanneyt, et al., 2002; Willems, et al., 2000). However, these electrophoresis-band migration-based techniques are difficult to standardize and bands of different sequences may migrate similar distances in a gel, which makes these methods less than ideal for studying long term or global epidemiology. MLST overcomes these limitations (Maiden, et al., 1998).

In *E. faecium* MLST, the sequences of internal gene fragments (between 395 and 583 nucleotides [nt] in size) of seven housekeeping genes (*atpA*, *ddl*, *gdh*, *purK*, *gyd*, *pstS*, *adk*) are determined (Homan, et al., 2002). An allele number is assigned to the sequence of each MLST gene, which results in a "barcode" of seven numbers for each strain. This barcode is represented by a sequence type (ST). One of the major advantages of MLST is its portable nature: the presence of a large and publicly accessible database of *E. faecium* MLST profiles (http://efaecium.mlst.net/) enables researchers from around the world to add allelic profiles for strains that they have collected and typed by using MLST. The use of a uniform typing method and the establishment of databases that can be consulted through the Internet also leads to a unambiguous nomenclature for clones, which is pivotal for studying the global spread and long-term transmission of particular strains. This has resulted in a database that consists of thousands of strains that comprise nearly a thousand distinct STs. This dataset, which for all strains combined consists of more than 6 Mbp of sequence, also allows for the study of evolutionary relationships between STs within the species *E. faecium*.

Initially, analyses of the genetic relatedness of *E. faecium* MLST data were performed using eBurst (Feil, Li, Aanensen, Hanage, & Spratt, 2004), an algorithm widely used to study relatedness of isolates and to generate

hypotheses about patterns of recent evolutionary descent in bacteria. This resulted in the identification of a large cluster of clinical *E. faecium* isolates that was first termed lineage C1 and later renamed clonal complex 17 (CC17), after ST17, from which these clinical isolates presumably evolved (Willems, et al., 2005). Practically all strains from CC17 were found to be resistant to ampicillin and ciprofloxacin (Leavis H. L., Willems, Top, & Bonten, 2006; Willems, et al., 2005). Strains from CC17 were enriched for several genes with putative roles in virulence, such as the large surface protein *esp*, and carbohydrate metabolism (Heikens, van Schaik, Leavis, Bonten, & Willems, 2008; Leavis H. L., et al., 2007). The most prominent marker for such CC17 strains was found to be the presence of IS*16* (Leavis H. L., et al., 2007; Werner, et al., 2011). However, the assumption that CC17 isolates have emerged from a single "founder" ST appears to be erroneous. This was first suggested by Turner *et al*. (Turner, Hanage, Fraser, Connor, & Spratt, 2007), who showed that eBurst performs unreliably in species in which recombination is a more prominent driver of genetic diversity than mutation. *E. faecium* has a high ratio of recombination over mutation (Willems, et al., 2005) which indicates that algorithms other than eBURST would better reflect *E. faecium*'s population structure. Indeed, analysis of MLST data by algorithms that account for homologous recombination events indicate that clinical *E. faecium* isolates are not closely linked in purely evolutionary terms (Willems & van Schaik, 2009).

An alternate approach is to perform an analysis of population structure that has the power to identify deep-branching lineages as well as recombination between them. This can be implemented by using Bayesian analysis of genetic population structure (BAPS) software (Corander, Marttinen, Sirén, & Tang, 2008; Corander & Tang, 2007; Tang, Hanage, Fraser, & Corander, 2009). BAPS uses a statistical model to partition molecular variation in a population into different groups based on both clonal ancestry and recombination patterns, as identified from their DNA sequence. BAPS analysis of 491 distinct STs found among 1720 *E. faecium* isolates identified 13 groups of related *E. faecium* strains (Willems, et al., 2012). Phylogenetic analysis based on concatenated MLST gene sequences of isolates contained in the two largest BAPS groups (BAPS 2-1 and BAPS 3-3) showed that currently circulating clinical isolates belong almost exclusively to three different lineages (lineage-17, lineage-18, and lineage-78) (Willems, et al., 2012). BAPS analysis indicated that lineage-78, which belongs to BAPS 2-1 and which has emerged in the first decade of the 21st century as a major cause for nosocomial infections, seems to have an evolutionary history which is distinct from lineages-17 and -18 that are both contained in BAPS 3-3.

This means that these three major hospital lineages stem from at least three different ancestral strains, and have independently acquired genes that characterize clinical isolates through convergent evolution (Willems, et al., 2012). This observation raises questions as to exactly how monophyletic strains belonging to the group previously termed CC17 are, and precisely how long ago they may have emerged. BAPS analysis also indicated that clinical isolates are unrelated to *E. faecium* strains that commonly colonize healthy humans as commensals, which belong to BAPS group 1, but instead that the genetic evolution of hospital clones possibly involved animals (Willems, et al., 2012). BAPS analysis showed that hospital-associated *E. faecium* have undergone lower levels of admixture (cases in which sequence types contain sequence characteristics of more than one subpopulation) as compared to animal isolates, which is indicative of either fairly restricted recombination or recent emergence. This suggests that although the evolutionary development of hospital-associated *E. faecium* from animal isolates most probably included horizontal gene transfer with successive acquisition of adaptive elements like *esp*, once adapted to a distinct pathogenic niche, hospital-associated *E. faecium* may have become isolated (Willems, et al., 2012).

The availability of large amounts of MLST gene sequences has also allowed for a detailed analysis of the origin of genetic diversity in the species. Observations that the majority (60%) of gene tree topologies of individual MLST genes are not congruent and that a high average number of nucleotide differences occur in single-locus variants indicate that genetic diversity in *E. faecium* has mainly been driven by recombination and not by mutation (Willems, 2010).

E. *faecalis* MLST

An MLST scheme has also been developed to study *E. faecalis* population structure (http://efaecalis.mlst.net) (Ruiz-Garbajosa, et al., 2006). Similar to *E. faecium*, this scheme relies on the amplification and sequencing of internal regions of seven housekeeping genes (*gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt*, and *yqiL*) for which diversifying selection signatures are low (Ruiz-Garbajosa, et al., 2006). Using this system, clonal complexes CC2 and CC9 were found to be enriched among hospital-derived strains (Ruiz-Garbajosa, et al., 2006). (Note that there is no relationship between *E. faecium* and *E. faecalis* ST and CC number designations.) Earlier studies that relied on different MLST schemes indicated that several infection-derived, β-lactamase-producing, or vancomycin-resistant *E. faecalis* strains were clonally related (Nallapareddy, Duh, Singh, & Murray, 2002; Nallapareddy, Wenxiang, Weinstock, & Murray, 2005). This lineage was designated the BVE (Bla^+-Van^R-endocarditis) clonal complex (Nallapareddy, Wenxiang, Weinstock, & Murray, 2005), which overlaps *E. faecalis* CC2 in the more commonly used MLST scheme (Ruiz-Garbajosa, et al., 2006). Analysis of 106 *E. faecalis* strains isolated worldwide from the early 1900s to 2006 revealed that acquired antibiotic resistance is enriched in the CC2, CC8, and CC9 lineages (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007). Analysis of more recently isolated strains from Europe (primarily isolated from 2006-2009) found that multidrug resistance is enriched in the CC2, CC16, and CC87 lineages, while CC2 and CC87 lineages were found nearly exclusively in hospitals (Kuch, et al., 2012). Collectively, these studies and others (Freitas, Novais, Ruiz-Garbajosa, Coque, & Peixe, 2009; Kawalec, et al., 2007; Willems, Hanage, Bessen, & Feil, 2011) highlight the success of certain MLST lineages—in particular, CC2—in acquiring multidrug resistance, as well as in hospital persistence and infection. CC2 and CC9 have been identified as potential "high-risk" *E. faecalis* lineages (Leavis, Bonten, & Willems, 2006), although CC87 may displace CC9 as a potential high-risk lineage in Europe (Kuch, et al., 2012).

MLST analyses have revealed a potentially important difference between *E. faecalis* and *E. faecium*. For both species, particular CCs appear to be significantly associated with hospital-derived isolates. However, most *E. faecalis* CCs contain both clinical and commensal isolates, while the same is not true for *E. faecium*. This suggests that human commensal and clinical *E. faecalis* isolates are not as evolutionarily distinct as those in *E. faecium*.

CGH, PCR, and blot-based screening methods have shown that horizontally acquired traits other than antibiotic resistance are enriched in certain *E. faecalis* MLST lineages. PCR and blot screens found that certain *E. faecalis* PAI genes are enriched in infection-derived *E. faecalis* isolates (Nallapareddy, Duh, Singh, & Murray, 2002; Shankar, Baghdayan, & Gilmore, 2002), and in the CC2, CC8, and CC9 lineages (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007). The genome sequence of *E. faecalis* V583, a bloodstream infection-derived, vancomycin-resistant ST6/CC2 strain and the first *Enterococcus* strain to be sequenced (Paulsen, et al., 2003), allowed for the development of microarrays that could be used for CGH studies (Aakra, Nyquist, Snipen, Reiersen, & Nes, 2007; Lepage, et al., 2006; Solheim, Aakra, Snipen, Brede, & Nes, 2009; Solheim, et al., 2011). CGH studies of *E. faecalis* strains of diverse provenance (Aakra, Nyquist, Snipen, Reiersen, & Nes, 2007; McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007), as well as more focused studies on food (Lepage, et al., 2006) and infant fecal isolates (Solheim, Aakra, Snipen, Brede, & Nes, 2009), have found that most variation between these strains and a V583 reference occurs in regions of the V583 genome that were thought to be horizontally acquired (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007; Paulsen, et al., 2003). One CGH study identified a prophage (V583 prophage 3), a putative genomic island (V583 ORFs EF1847-EF1897) sometimes referred to as *efaB5* (Lepage, et al., 2006), and a putative genomic island flanking the V583 *vanB* transposon as being among genes enriched in CC2 strains of hospital and non-hospital origins, as compared to non-CC2 strains (Solheim, et al., 2011). In this study, none of the well-characterized *E. faecalis* virulence factors were among the CC2-enriched genes (Solheim, et al., 2011), which highlights the nature and occurrence of *E. faecalis* strains related to hospital isolates in other ecologies. Collectively, CGH studies appear to show that the acquisition and exchange of mobile genetic elements are major contributors to the genomic

diversity of *E. faecalis*. Further, the extent of horizontally acquired material in *faecalis* genomes varies among different MLST lineages, with certain elements being enriched in CC2.

Similar to *E. faecium*, population genetic analyses of MLST alleles indicate that the *faecalis* species is highly recombinogenic, which may contribute to its genome diversity. Alleles at different MLST loci are significantly associated with each other when considering both distantly related STs and STs within CCs that may have recently diversified, which is suggestive of a clonal population that evolves primarily by mutation (Ruiz-Garbajosa, et al., 2006). However, this association disappears if only distantly related STs are considered (Ruiz-Garbajosa, et al., 2006). Additionally, gene trees for individual MLST loci are incongruent, and individual MLST alleles are distributed throughout the concatenated MLST phylogeny (Ruiz-Garbajosa, et al., 2006). These observations are consistent with an epidemic population structure, where recombination occurs frequently among all members of the population—yet signatures of recombination are masked by the emergence of a highly successful lineage (or perhaps multiple lineages) that rapidly increase in frequency (Smith, Smith, O'Rourke, & Spratt, 1993). The CC2 lineage has not been detected in isolates collected prior to the 1980s (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007), suggesting that it has recently emerged and rapidly disseminated. Notably, because of the recombinogenic nature of *E. faecalis*, eBurst analysis may oversimplify the population structure of the species, as has occurred for *E. faecium* MLST analyses. It remains to be determined whether BAPS analysis will alter our current view of *E. faecalis* population structure.

Early Comparative Genome Analysis Reveals the Existence of a Pathogenicity Island in *E. faecalis*

In a retrospective study of an outbreak of 206 enterococcal bacteremias at the University of Wisconsin Hospitals and Clinics over a 17-month period, 190 were found to be caused by *E. faecalis*, most of which were resistant to high levels of aminoglycosides and macrolides (Huycke, Spiegel, & Gilmore, 1991). Moreover, nearly half of the 190 infections were caused by a single *E. faecalis* lineage, as defined by pulsed-field electrophoresis (PFGE) patterns (now known to be ST6/CC2 (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007)), which was multidrug resistant as well as hemolytic, whereas the remainder were caused by largely non-hemolytic idiosyncratic strains with few identities by PFGE (Huycke, Spiegel, & Gilmore, 1991). This indicated that one strain, termed MMH594, had become highly hospital-adapted, antibiotic resistant, and unusually pathogenic. Nearly simultaneously, the first vancomycin-resistant *Enterococcus* in the United States was isolated at Barnes-Jewish Hospital at Washington University (Sahm, et al., 1989). This strain, V583 (now also known to be ST6/CC2 (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007)) was identified as *E. faecalis*, and represented a novel vancomycin resistance phenotype, termed VanB. Identical VanB strains were isolated over a span of three months from the bloodstream, urine, and feces of a chronically infected patient who had received vancomycin therapy prior to the first isolation (Sahm, et al., 1989). An identical isolate was later obtained from another patient in the same ICU, but who had not received vancomycin therapy, and identical isolates were obtained from urine and blood over the course of two weeks (Sahm, et al., 1989).

In examining the genomes of the multidrug-resistant hospital outbreak strain from Wisconsin and the vancomycin-resistant strains from St. Louis by sequence analysis, a number of new traits for enterococci were discovered and found to be common to the hospital strains from both locations. These included a capsule (Hancock & Gilmore, 2002; Hancock, Shepard, & Gilmore, 2003), a novel adhesin termed enterococcal surface protein (Esp) that was enriched in clinical isolates (Shankar, et al., 2001; Shankar V. , Baghdayan, Huycke, Lindahl, & Gilmore, 1999), and a bile acid hydrolase. An operon for the enterococcal cytolysin was also found, which is a factor that confers lethality to enterococcal infection in humans (Huycke, Spiegel, & Gilmore, 1991) and animals (Chow, et al., 1993; Garsin, et al., 2001; Ike, Hashimoto, & Clewell, 1984; Ike, Hashimoto, & Clewell, 1987; Jett, Jensen, Nordquist, & Gilmore, 1992; Singh, Qin, Weinstock, & Murray, 1998); is enriched in bloodstream *E. faecalis* isolate collections (Huycke & Gilmore, 1995; Huycke, Spiegel, & Gilmore, 1991; Ike, Hashimoto, & Clewell, 1987); is novel in structure (Bogie, Hancock, & Gilmore, 1995; Booth, et al., 1996;

Coburn & Gilmore, 2003; Coburn, Hancock, Booth, & Gilmore, 1999; Cox, Coburn, & Gilmore, 2005; Gilmore, Segarra, & Booth, An HlyB-type function is required for expression of the Enterococcus faecalis hemolysin/bacteriocin, 1990); and is regulated by a novel quorum-sensing mechanism (Haas, Shepard, & Gilmore, 2002) that enables enterococci to detect target cells at a distance (Coburn, Pillar, Jett, Haas, & Gilmore, 2004). Aggregation substance, an enterococcal surface factor that causes clumping, was first described in the Clewell lab (Yagi, et al., 1983), and was shown to be lethally synergistic with the cytolysin in endocarditis (Chow, et al., 1993), was also present in the genomes of these clinical isolates. Intriguingly, all of these factors, except the capsule, occurred on a single macrorestriction fragment (Shankar, Baghdayan, & Gilmore, 2002). Further sequence analysis revealed that these traits were organized into an island of over 150 kb in size that included a phage-like integrase and excisionase, differed in G+C content from the rest of the chromosome, possessed flanking 10 bp direct repeats, and was inserted at a lysyl-tRNA locus (Shankar, Baghdayan, & Gilmore, 2002), which was reminiscent of the organization of PAIs in Gram-negative bacteria (Langille, Hsiao, & Brinkman, 2010). The *E. faecalis* PAI was the first canonical PAI to be identified in a Gram-positive bacterium.

Compared to those occurring in V583 and V586, the 153,571 bp PAI of strain MMH594 was found to be closest to the prototype, in that the islands from the vancomycin-resistant St. Louis strains V583 and V586 from the chronically infected patient possessed additional IS elements (IS*256* and IS*905*) that disrupted the cytolysin operon (Shankar, Baghdayan, & Gilmore, 2002) (Figure 1). Moreover, these IS elements volatilized this region of the island, which resulted in a 17 kb deletion that distinguishes V583 from V586 and occurs at a very high frequency of 1 in 10^3 V586 cells (Shankar, Baghdayan, & Gilmore, 2002). This indicates that the chronically infected St. Louis HIV/AIDS patient was infected with a mixed population of V583/V586 cells. Adaptation of the microbe in chronic infection, as seen with V583/V586, is similar to that seen with *P. aeruginosa* in chronically infected cystic fibrosis lungs (Hoboth, et al., 2009). Thus, from comparative sequence analysis and *in vitro* experiments, it is clear that the PAI is a dynamic component of *E. faecalis* genomes, and rapid changes in its structure are likely to impact *E. faecalis* virulence (Figure 1).

Further studies have found that the PAI has a modular structure and likely evolves by accretion of modules obtained through horizontal gene transfer (McBride, et al., 2009). A collection of 53 esp^+ *E. faecalis* strains were interrogated for the presence of MMH594 PAI genes using dot blot hybridization, PCR, and a novel pathoarray with probes that corresponded to each of the 129 open reading frames of the MMH594 PAI. Presence of *esp* was chosen as a marker for PAI presence in the 53 strains, as *esp* in *E. faecalis* had only been detected within the PAI. Incongruence between phylogenies built from MLST alleles and from PAI gene content in the 53 strains suggested that the PAI and the core *E. faecalis* genome evolve through different mechanisms. Transversal clustering of PAI hybridization data revealed that the canonical PAI contains six modules (named A through F, moving from 5′′ to 3′ PAI ends) of consecutive genes (with the exception of module E) that appear to be acquired or inherited as discrete units. Module A is composed of the pAM373-like plasmid remnant that is present at the 5′ end of the MMH594 PAI (PAI ORFs EF0005-EF0033; (Shankar, Baghdayan, & Gilmore, 2002)). That this module has high identity to a pheromone-responsive plasmid provides a straightforward mechanism for its horizontal transfer. It is flanked on one end by a putative phage integrase and excionase and by transposases on the other end, which could also contribute to the mobility of this module. Module B (EF0042-EF0049) contains the cytolysin operon and occurs 3′ to a conjugated bile salt hydrolase gene that is flanked by transposases (EF0039-EF0041). Module C (EF0051-EF0074) possesses *esp* and the *gls24*-like gene, among others, as well as an internal recombinase gene and flanking transposase (EF0075). This transposase also flanks Module D (EF0076-EF0092), which is composed of sugar uptake and metabolism genes. Module E is non-contiguous (EF0093-EF0108 and EF0124-EF0128), and encodes various genes that are likely involved in metal transport and cofactor biosynthesis. Module F (EF0108-EF0122) is internal to Module E and possesses several transposases that may provide mobility to this module.

Collectively, these data suggest that the PAI evolves by the accretion of discrete modules bounded by transposases, and is transmitted by horizontal gene transfer, as opposed to a model where a single progenitor

PAI entered an ancestral *E. faecalis* strain and was diversified by differential gene loss in diverging MLST lineages. If an evolution-by-accretion model is correct, we could expect to discover novel PAI modules in comparative genome analyses. Consistent with this proposal, a novel PAI module has been identified in genomes of the CC2 strains HH22 and TX0104 (Solheim, et al., 2011). This module encodes several hypothetical proteins and a predicted mucin-binding domain protein (Solheim, et al., 2011), and occurs between MMH594 PAI ORFs EF0040 and EF0065, which correspond to Module B and part of Module C as defined above. Despite its absence in MMH594 and V583 genomes, the presence of this module was found to be enriched among CC2 strains, compared to non-CC2 strains, in a PCR-based screening analysis (Solheim, et al., 2011). This result suggests that comparative genomics will reveal much about the module diversity and evolutionary history of the PAI. Indeed, one *E. faecalis* strain for which genome sequence is available, T1, possesses a ~60 kb PAI located at the same PAI *att* site, which is composed entirely of novel modules not present in the prototypical MMH594 PAI (Palmer and Gilmore, unpublished).

The First Complete Enterococcal Genome, *E. faecalis* V583, and Comparison With OG1RF

E. faecalis V583 was provided to TIGR in 1998 and its genome sequence was published in 2003 (Paulsen, et al., 2003). The release of this genome was significant for many reasons, including its provision of a complete, high-quality reference for subsequent genome projects. V583 was selected because it was the first vancomycin-resistant *Enterococcus* isolated in the United States, and as noted above, was found to be similar to MMH594 and representative of the ST6/CC2 lineage (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007; Sahm, et al., 1989). A significant and striking finding of the V583 genome is the amount of mobile DNA contained within it—calculated to be over 25% of the genomic content (Paulsen, et al., 2003). This includes seven predicted prophages, multiple integrated plasmids, IS elements and genomic islands (including the PAI), a *vanB*-type transposon that confers vancomycin resistance, and three extrachromosomal plasmids (pTEF1, pTEF2, and pTEF3), including one that encodes multiple antibiotic resistance genes (pTEF1) (Lepage, et al., 2006; McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007; Paulsen, et al., 2003) (Figure 2). Two of the pTEF plasmids, pTEF1 (66.3 kb) and pTEF2 (57.7 kb), are predicted pheromone-responsive plasmids similar to the extensively studied pheromone-responsive plasmids pAD1 (Clewell, 2007) and pCF10 (Dunny, 2007), respectively (see Extrachromosomal and Mobile Elements in Enteroocci). These plasmids detect small peptides produced by plasmid-free cells to induce high-efficiency transfer (transfer frequencies of up to one transconjugant per 10–100 donors; (Hirt, Schlievert, & Dunny, 2002; Huycke, Gilmore, Jett, & Booth, 1992; Licht, Laugesen, Jensen, & Jacobsen, 2002)), and their replication mechanisms appear to be functional only in the *faecalis* species (Palmer, Kos, & Gilmore, 2010). pTEF3 (18.0 kb) possesses the broad host range Inc18 replicon of pAMβ1 (Paulsen, et al., 2003). The remarkable amount of horizontally acquired material in the V583 genome leads to the proposition that enterococci have a special propensity to acquire and disseminate mobile elements, such as those that encode antibiotic resistance genes (Paulsen, et al., 2003).

A second complete *E. faecalis* genome was published in 2008 (Bourgogne, et al., 2008), which provides a comparator to V583. *E. faecalis* OG1RF is a laboratory-generated rifampicin and fusidic acid-resistant derivative of the non-antibiotic resistant human oral isolate OG1 (Gold, Jordan, & van Houte, 1975), which was originally isolated prior to 1975. OG1RF is an ST1 strain, and is not a member of any of the high-risk *E. faecalis* MLST lineages. Compared to V583, the OG1RF genome has little mobile content, lacks plasmids and the PAI, and possesses only one prophage that is core to the *faecalis* species (prophage 2; (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007)), as well as a putative transposable element that lacks antibiotic resistance genes (Bourgogne, et al., 2008). The abundance of horizontally acquired DNA in V583, as compared to OG1RF, results in a large size discrepancy between their genomes: 3.36 Mb for V583, compared to 2.74 Mb for OG1RF. Interestingly, despite the 620 kb of additional coding potential in the V583 genome, including the PAI, OG1RF can cause disease in animal models of infection (Bourgogne, et al., 2008), which highlights the point that even

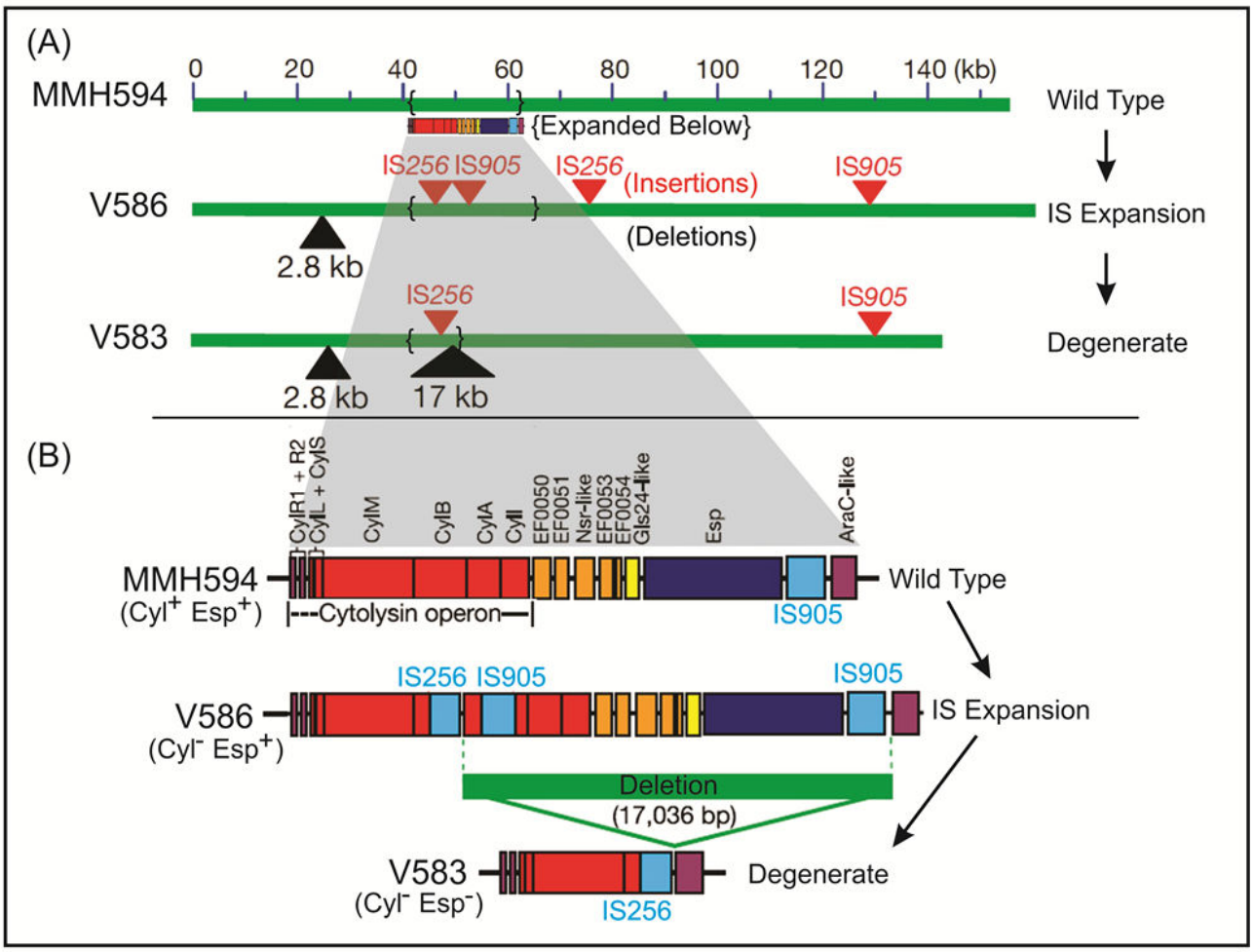

Figure 1. A dynamic PAI in hospital-adapted *E. faecalis*. Alignment of PAI sequences from hospital infection isolates E. faecalis MMH594, V586, and V583. (A) Overall comparison of PAIs reveals expansion of the prototypical MMH594 PAI by IS acquisition (indicated by inverted red triangles) in strain V586, followed by ~17 kb deletion of an IS-flanked region (indicated by a black triangle) in strain V583. (B) Expanded view of PAI showing genes affected by PAI structural variation. The prototypical PAI of MMH594 encodes cytolysin and Esp, among other factors important for virulence. The cytolysin operon of V586 is disrupted as a result of IS expansion; this strain does not produce cytolysin. V583 does not produce cytolysin or Esp as a result of IS expansion in the V586 progenitor and a subsequent ~17 kb internal deletion.

commensal strains of *E. faecalis* can be opportunists, and that basic fitness functions are important to the ability of members of this species (and probably genus) to cause disease.

Dynamic *E. faecalis* Genomes and CRISPR-Cas Defense

Further genome sequencing has illuminated the extent of mobile content and range of genome sizes in the *faecalis* species. A third complete *E. faecalis* genome has been announced (Brede, Snipen, Ussery, Nederbragt, & Nes, 2011), although a detailed comparative analysis has not yet been published. *E. faecalis* 62 was isolated from the feces of a healthy human infant and is a ST66 strain, which is a lineage that has not been associated with either infection or hospitals (Solheim, Aakra, Snipen, Brede, & Nes, 2009; Solheim, et al., 2011). The extrachromosomal elements of this strain, and to some extent the PAI, have been described (Brede, Snipen, Ussery, Nederbragt, & Nes, 2011), and their abundance and presence, respectively, indicate that the *E. faecalis* 62 genome is more similar to that of V583 than OG1RF in its mobile genome content. *E. faecalis* 62 possesses three predicted plasmids—one similar to the cryptic plasmid pS86 (EF62pA; 5.1 kb) and two putative pheromone-

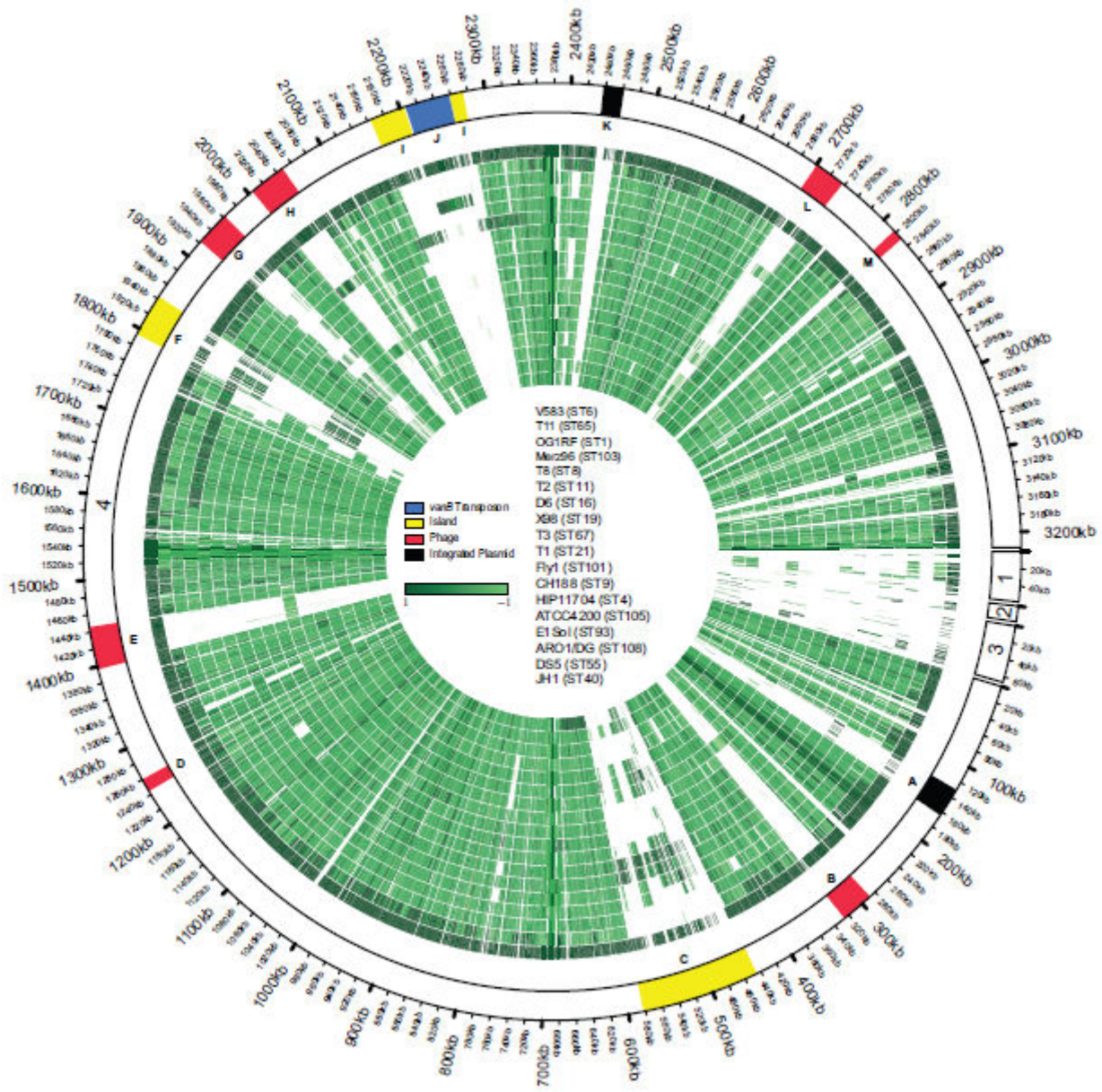

Figure 2. *E. faecalis* genome plot. The outermost ring shows E. faecalis V583 chromosomal (scaffold 4) and plasmid scaffolds (scaffold 1, pTEF2; scaffold 2, pTEF3; scaffold 3, pTEF1), with each V583 gene represented as a radial position along the ring. The locations of V583 variable regions are shown (98): A, integrated plasmid; B, prophage 1; C, PAI; D, prophage 2; E, prophage 3; F, putative island efaB5; G, prophage 4; H, prophage 5; I, putative island; J, vancomycin resistance (vanB) transposon; K, integrated plasmid; L, prophage 6; M, prophage 7. Seventeen additional E. faecalis genomes are represented by the rings below V583. Genes are colored by phylogenetic distance from V583 (from dark to light green with increasing phylogenetic distance). Strains shown, from outermost to innermost rings, are V583, T11, OG1RF, Merz96, T8, T2, D6, X98, T3, T1, Fly1, CH188, HIP11704, ATCC4200, E1Sol, AR01/DG, DS5, and JH1. Patterns suggestive of recombination (darker colored genes indicate close phylogenetic relatedness to V583) occur in Merz96, T2, and JH1, which flank genomic islands.

responsive plasmids that are similar to pCF10 (EF62pB; 51.1 kb) and pAM373 (EF62pC; 55.4 kb) (Brede, Snipen, Ussery, Nederbragt, & Nes, 2011). A pseudotemperate linear phage (EF62φ; 30.5 kb) is also present, as is a chromosomally encoded Tn*916* and a variant PAI encoding *esp* (Brede, Snipen, Ussery, Nederbragt, & Nes, 2011). Collectively, this results in a genome size of 3.13 Mb, which is intermediate between those of V583 and OG1RF.

Sixteen additional *E. faecalis* genomes were sequenced and assembled to high-quality draft status as part of a enterococcal genome sequencing project that increased the amount of available sequencing data for the genus by approximately 10-fold (Palmer, et al., 2010). The 16 *E. faecalis* strains were selected to represent the deepest nodes in the *faecalis* MLST phylogeny, and were isolated over an approximately 80-year period from clinical and non-clinical sites around the world (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007). Analysis of the 16 draft *E. faecalis* genomes has further supported the proposition that mobile element acquisition is a major contributor to genome diversity in the *faecalis* species. The genome sizes of all 16 strains are intermediate between the extremes of V583 and OG1RF, with multidrug-resistant strains generally possessing larger genomes (Palmer, et al., 2012). Strains with genomes larger than 3 Mb encode significantly more protein domains associated with mobile elements, including plasmid replication initiation and mobilization domains, a plasmid addiction toxin domain, a transposase domain, and the anti-restriction protein ArdA domain, which is encoded by the self-mobilizable transposon Tn*916* (Palmer, et al., 2012). This is consistent with these strains acquiring foreign genetic elements conferring new traits, which results in larger genomes. The observed range of genome sizes suggests that the propensity to acquire mobile elements *in situ* varies within the *faecalis* species.

Genome size and the extent of acquired antibiotic resistance appear to be related to the CRISPR-*cas* status of *E. faecalis* strains. Clustered, regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (*cas*) genes provide a type of acquired immune defense in prokaryotes (Wiedenheft, Sternberg, & Doudna, 2012). CRISPR-Cas systems have been shown to block phage infections and plasmid uptake, and thus are endogenous barriers to horizontal gene transfer (see Enterococcal bacteriophages and genome defense). In the comparative analysis of V583 and OG1RF, it was noted that OG1RF possesses CRISPR-*cas* while V583 lacks it, which suggests that CRISPR-Cas defense prevented the accumulation of mobile genetic elements in OG1 prior to its isolation from the human mouth (Bourgogne, et al., 2008). Supporting a role for CRISPR-Cas systems in *E. faecalis* genome defense, genome size distributions significantly differ between *E. faecalis* strains that possess or lack CRISPR-*cas*, with strains that lack CRISPR-*cas* having larger genomes (with an average size of 3.1 Mb, compared to 2.9 Mb for strains that possess CRISPR-*cas*) (Palmer, et al., 2012). Similarly, strains that lack CRISPR-*cas* in their genomes encode significantly more conserved protein domains associated with mobile elements (Palmer, et al., unpublished). Analysis of 48 *E. faecalis* strains using comparative genomics and a PCR-based screening approach found a significant relationship between acquired antibiotic resistance and CRISPR-*cas*, with strains that lacked CRISPR-*cas* possessing more acquired antibiotic-resistance genes (Palmer & Gilmore, 2010). Strains deficient for CRISPR-*cas* are additionally enriched for cytolysin and aggregation-substance genes (Lindenstrauss, et al., 2011), both of which are encoded on pheromone-responsive plasmids and the PAI. Finally, all CC2 strains characterized thus far lack CRISPR-*cas* (Palmer & Gilmore, 2010), which suggests that the absence of this line of defense is a core property of the lineage that perhaps contributes to its success in acquiring novel genomic content. The role of CRISPR-Cas defense in regulating the influx of mobile elements into *E. faecalis* genomes is of high interest.

The Recombinogenic Nature of *E. faecalis*

Genomics has illuminated the extent of recombination in *E. faecalis*. As might be expected from MLST analyses, the inter-relationships of 18 *E. faecalis* strains (16 draft genomes discussed above, along with V583 and OG1RF) could not be confidently resolved by a phylogeny built from 847 core gene sequences (Palmer, et al., 2012). In pairwise comparisons of the same 18 strains, the average nucleotide identity in shared genes varied little (97.8-99.5% average identity). However, total shared gene content varied widely, which is likely a function of the varying genome sizes of the strains (Palmer, et al., 2012). This is consistent with *E. faecalis* strains generally being closely related in their core genes, with most variation introduced through the acquisition of horizontally transferred material. That said, signatures of recombination are apparent in some *E. faecalis* genomes (Fig. 2). Three *E. faecalis* strains, JH1 (ST40), Merz96 (ST103), and T2 (ST11), appear to have acquired genomic islands (the PAI, *efaB5*, and a putative genomic island that flanks the V583 *vanB* transposon, respectively), as well as a

sequence adjacent to the islands, from strains closely related to V583 (Palmer, et al., 2012). The recombinant region of Merz96 overlaps the *fsr* locus, of interest because variation in the *fsr* region influences production of gelatinase (42). The Merz96 *fsr* region was previously shown to be similar to that present in V583, MMH594, and HH22—all ST6 strains (Galloway-Peña, Bourgogne, Qin, & Murray, 2011). It is likely that Merz96 acquired this region, as well as *efaB5,* through recombination with a ST6 strain.

A mechanism for recombinatorial transfer of large chromosomal regions between *E. faecalis* strains has been elucidated, and provides an explanation for decades of observations that implicate chromosomal DNA exchange in enterococci (Manson, Hancock, & Gilmore, 2010). In laboratory mating experiments with V583 as donor and OG1RF as recipient, hybrid V583-OG1RF transconjugants were observed that possessed up to 857 kb of the V583 genomic sequence. Depending on the location of the selectable marker in the V583 donor genome, transconjugants were observed that possessed the entire PAI, the V583 capsule biosynthesis operon, the V583 vancomycin resistance operon, and/or V583 *gdh* and *yqiL* MLST markers, which generated a double locus MLST variant of OG1RF in one mating experiment (Manson, Hancock, & Gilmore, 2010). Displacement of the OG1RF CRISPR-*cas* locus by V583 sequence was also observed (Palmer & Gilmore, 2010). Transfer of large chromosomal regions was dependent upon the presence and transfer functions of either of the pheromone-responsive plasmids pTEF1 or pTEF2 (Manson, Hancock, & Gilmore, 2010). Both pTEF1 and pTEF2 were found to integrate into the chromosome at shared IS*256* sequences, and from there, likely initiated transfer induced by OG1RF pheromones, which resulted in mobilization of the V583 chromosome. The PAI and *efaB5* appear to be hotspots for IS element accumulation, and IS elements occur in the genomic island that surrounds *vanB* (Paulsen, et al., 2003), which suggests that pheromone-responsive plasmids that possess similar IS elements could readily integrate into these regions. It is likely that Merz96, JH1, and T2 have acquired genomic islands, along with surrounding sequences, by a similar mechanism. This pheromone-responsive plasmid-dependent chromosome transfer mechanism likely contributes to the recombinogenic population structure of the *faecalis* species.

E. faecium Genome Sequencing

Table 1 provides an overview of several important characteristics of the first 31 publicly available *E. faecium* genomes. Most (23/31) of these isolates originated from human infection or from hospitalized patients that were colonized by antibiotic resistant strains. Six genomes are from strains that were isolated from feces of healthy humans (and likely represent human commensal isolates). *E. faecium* strains from non-human sources are poorly represented in this first set of *E. faecium* genomes, with genomes of only two representative strains sequenced. Both strains had been isolated from dog feces (de Regt, et al., 2012). There is a large sample bias toward clinical isolates in *E. faecium* strains that have been selected for genome sequencing studies. However, clinical isolates form only a minute fraction of the total *E. faecium* population, and it is important that genomes of *E. faecium* strains from non-human sources be included in sequencing projects. Another bias that is evident from Table 1 is the overrepresentation of strains from Europe and North America. In fact, only three strains (a clinical isolate from Brazil and a commensal and a clinical isolate from Australia) do not originate from these two continents. More balance is needed to fully understand the global diversity of *E. faecium*.

The first completely finished *E. faecium* genome sequence became available in March 2012 and was determined for strain Aus0004. Aus0004 is a vancomycin-resistant strain that was isolated from the bloodstream of a patient in Melbourne, Australia, in 1998 (Lam, et al., 2012). The Aus0004 genome sequence was determined by a combination of 454 and Illumina sequencing and was finished by primer walking. A bacterial artificial chromosome (BAC) library was prepared to resolve chromosomal duplications. A second complete genome sequence was determined for strain TX16 (also named TX0016 or DO), which was isolated from the blood of a patient with endocarditis in 1992. Unassembled fragmentary sequences were first sequenced by Sanger sequencing in 1999, and were completed with additional 454 sequencing and PCR to close gaps (Qin, et al., 2012).

Other draft *E. faecium* genomes have been generated by either 454 or Illumina sequencing. Most fragmentary assemblies are a consequence of the large number of repetitive elements in the genomes of *E. faecium*, which are challenging to resolve bioinformatically. These elements include IS elements (for example, there are 95 IS elements in Aus0004 [77]), but also repeats within protein coding sequences, such as those that are present in the gene that encodes the Esp surface protein (Leavis, et al., 2004). Practically all *E. faecium* strains with available genome sequences were isolated in the last 20 years. The oldest strain is E1636, which was isolated in 1961 from a bloodstream infection in the Netherlands. Interestingly, this strain encodes the tetracycline resistance gene *tetM* (van Schaik, et al., 2010), though this antibiotic had only been on the market since 1952. It would be of particular interest to sequence the genomes of *E. faecium* strains from the pre-antibiotic era, as this would definitively answer the question if *E. faecium* strains are a natural source of antibiotic-resistance genes or have acquired all their resistances through lateral gene transfer in the last 70 years. While limitations exist for the first available set of *E. faecium* genome sequences, these data have provided us with important new insights into the evolution and basic biology of this organism.

Table 1. Initial 31 publicly available *E. faecium* genome sequences

Strain	Source[a]	Country	Year	Sequencing Center[b]	Assembly size (nt)[c]	No. contigs	Resistances, virulence genes and remarks[d]	Ref
Aus0004	Clinical isolate from blood	AUS	1998	UM	2.96 Mbp	1	VAN; *esp*+	(Johnson, et al., 2010; Lam, et al., 2012)
TX16	Clinical isolate from blood	USA	1992	WU	2.70 Mbp	1	Also named DO or TX0016. AMP; hyl+	(Qin, et al., 2012)
1,141,733	Clinical isolate from blood	USA	2005	BI	2.87 Mbp	101		(Palmer, et al., 2010; Palmer, et al., 2012)
1,230,933	Clinical isolate from wound	USA	2005	BI	2.95 Mbp	304	AMP, CIP and VAN; *esp*+ and *hyl*+	(Palmer, et al., 2010)
1,231,408	Clinical isolate from blood	USA	2005	BI	2.89 Mbp	379	AMP and CIP	(Palmer, et al., 2010; Palmer, et al., 2012)
1,231,410	Clinical isolate from skin and soft tissue	USA	2005	BI	2.94 Mbp	230	AMP, CIP and VAN; *esp*+ and *hyl*+	(Palmer, et al., 2010; Palmer, et al., 2012)
1,231,501	Clinical isolate from blood	USA	2005	BI	2.80 Mbp	140		(Palmer, et al., 2010; Palmer, et al., 2012)
1,231,502	Clinical isolate from blood	USA	2005	BI	2.92 Mbp	220	AMP, CIP and VAN;*esp*+ and *hyl*+	(Palmer, et al., 2010; Palmer, et al., 2012)
C68	Isolated from feces of hospitalized individual	USA	1996	BI	2.94 Mbp	182	VAN, AMP; *esp*+ and *hyl*+	(Carias, Rudin, Donskey, & Rice, 1998)
Com12	Isolated from feces of healthy individual	USA	2006	BI	2.69 Mbp	67		(Palmer, et al., 2010; Palmer, et al., 2012)

Table 1. continued from previous page.

Strain	Source[a]	Country	Year	Sequencing Center[b]	Assembly size (nt)[c]	No. contigs	Resistances, virulence genes and remarks[d]	Ref
Com15	Isolated from feces of healthy individual	USA	2007	BI	2.77 Mbp	70		(Palmer, et al., 2010; Palmer, et al., 2012)
D344SRF	Spontaneous mutant of strain D344R	USA	?	BI	2.75 Mbp	215	ERY, CLI, FUS and RIF.	(Rice, et al., 2009)
E1039	Isolated from feces of healthy individual	NLD	1998	UMCU	2.50 Mbp	124		(van Schaik, et al., 2010)
E1071	Isolated from feces of hospitalized individual	NLD	2000	UMCU	2.70 Mbp	96	VAN, CHL, ERY, SPC, STR and TET	(van Schaik, et al., 2010)
E1162	Isolated from a bloodstream infection	FRA	1997	UMCU	2.71 Mbp	139	AMP and TET, *esp*+	(van Schaik, et al., 2010)
E1636	Isolated from a bloodstream infection	NLD	1961	UMCU	2.84 Mbp	223	AMP and TET	(van Schaik, et al., 2010)
E1679	Isolated from the tip of a vascular catheter	BRA	1998	UMCU	2.93 Mbp	340	AMP, CIP, ERY, GEN, SPC and VAN *esp*+	(van Schaik, et al., 2010)
E4452	Isolated from dog feces	NLD	2008	UMCU	2.77 Mbp	268	AMP	(de Regt, et al., 2012)
E4453	Isolated from dog feces	NLD	2008	UMCU	2.82 Mbp	374	AMP	(de Regt, et al., 2012)
E980	Isolated from feces of healthy individual	NLD	1998	UMCU	2.79 Mbp	131	ERY	(van Schaik, et al., 2010)
LCT-EF90	Derived from *E. faecium* type strain[e]	SWI	1940s?	BGI	2.77 Mbp	118		(Chang, et al., 2012)
PC4.1	Isolated from feces of healthy individual	AUS	2008	JCVI	2.81 Mbp	78		
TC 6	Transconjugant of mating between C68 and D344SRF	USA	?	BI	2.88 Mbp	278	Resistant to TET. *hyl*+	(Rice, et al., 2009)
TX0082	Isolated from bloodstream of patient with infective endocarditis	USA	1999	WU	2.69 Mbp	151	AMP, ERY and VAN	(Nallapareddy, Singh, & Murray, 2006)

Table 1. continued from previous page.

Strain	Source[a]	Country	Year	Sequencing Center[b]	Assembly size (nt)[c]	No. contigs	Resistances, virulence genes and remarks[d]	Ref
TX0133A[f]	Isolated from bloodstream of patient with infective endocarditis	USA	2006	WU	2.93 Mbp	223	VAN; *esp*+	(Arias, et al., 2007)
TX0133a01[f]	Isolated from bloodstream of patient with infective endocarditis	USA	2006	WU	3.07 Mbp	252	VAN; *esp*+; *hyl*+	(Arias, et al., 2007)
TX0133a04[f]	Isolated from bloodstream of patient with infective endocarditis	USA	2006	WU	2.92 Mbp	211	*esp*+	(Arias, et al., 2007)
TX0133B[f]	Isolated from bloodstream of patient with infective endocarditis	USA	2006	WU	2.93 Mbp	221	*esp*+	(Arias, et al., 2007)
TX0133C[e]	Isolated from bloodstream of patient with infective endocarditis	USA	2006	WU	2.91 Mbp	215	VAN; *esp*+	(Arias, et al., 2007)
TX1330	Isolated from feces of healthy individual	USA	1994	BCM	2.72 Mbp	156		(Singh, Weinstock, & Murray, 2002)
U0317	Isolated from urine of patient with a urinary tract infection	NLD	2005	UMCU	2.89 Mbp	227	AMP, CIP, ERY, GEN and STR. *esp*+ *hyl*+	(van Schaik, et al., 2010)

[a] All isolates are from human subjects, unless mentioned otherwise.
[b] Abbreviations used: UM: University of Melbourne; BI: Broad Institute; JCVI: J. Craig Venter Institute; UMCU: University Medical Center Utrecht; BGI: Beijing Genomics Institute; BCM: Baylor College of Medicine; WU: Washington University School of Medicine.
[c] The size of the assembly does not include the estimated sizes of gaps between contigs.
[d] The abbreviations used for antibiotic resistances are AMP: ampicillin; CIP: ciprofloxacin; VAN: vancomycin; ERY: erythromycin; CLI: clindamycin; RIF: rifampicin; FUS: fusidic acid; CHL: chloramphenicol, SPC: spectinomycin, STR: streptomycin; GEN: gentamicin.
[e] This strain was derived from the *E. faecium* type strain. The type strain was cultured at 15°C and 37°C for more than four weeks, after which DNA was isolated and sequenced. The *E. faecium* type strain was first deposited to culture collections in 1946. We have been unable to determine the isolation date and the exact source of this isolate.
[f] These strains originate from a study in which the evolution of an *E. faecium* strain was followed during antibiotic therapy and during *in vitro* culture. See (Arias, et al., 2007) for further details.

Toward a Genome-Based Reconstruction of *E. faecium* Evolution

In the first genome-based study of *E. faecium*, seven isolates were sequenced (148). Based on a phylogenetic analysis of the concatenated alignments of 649 protein sequences, it was shown that the human commensal

strain E980 was relatively distantly related to the other six *E. faecium* isolates, which suggested the presence of a deeply branching division within the species. Interestingly, a similar division between two major clades within *E. faecium* had previously been suggested by non-sequence based studies, in which random amplified polymorphic DNA (RAPD)-PCR, AFLP, and PFGE were used to characterize a collection of *E. faecium* strains (Vancanneyt, et al., 2002; Vankerckhoven, et al., 2008).

A divide between two major clades in *E. faecium* was also found by analyses of MLST data and additional genome sequence-based studies (Galloway-Peña, Roh, Latorre, Qin, & Murray, 2012; Galloway-Peña, Rice, & Murray, 2011; Palmer, et al., 2012). BAPS analysis of MLST data (Willems, et al., 2012), combined with genome sequence data, confirmed a major split in the *E. faecium* population (Figure 3). To further analyze the differences between the two clades, termed A and B, Palmer *et al.* (Palmer, et al., 2012) performed pairwise comparisons of 8 *E. faecium* genomes to determine the average nucleotide identity (ANI) in genes shared between the genomes. Pairwise genome comparisons between clade A and clade B strains revealed ANI values that ranged from 93.9 to 95.6%. In examining other species, ANI values of 95 ± 0.5% have been found to correlate with 70% DNA-DNA hybridization values, which is the threshold usually used to distinguish different species (50). By this criterion, clade A and clade B would represent separate species-level subdivisions of *E. faecium.* A recent study including 51 newly sequenced *E. faecium* genomes (Lebreton, et al., 2013) provided evidence for a second split in the *E. faecium* population, which distinguishes modern clinical isolates (clade A1) from most animal-derived strains (clade A2) in the dataset. After removing recombined regions in the genomes, mutation rates were calculated using the BEAST algorithm (Drummond, Suchard, Xie, & Rambaut, 2012) for the three clades (A1, A2 and B) of the *E. faecium* population. Interestingly, a significantly higher mutation rate was found for clade A1 strains ($4.9 \times 10^{-5} \pm 0.3 \times 10^{-5}$ substitutions per nucleotide per year) than for both clade A2 ($3.6 \times 10^{-6} \pm 0.6 \times 10^{-6}$ substitutions per nucleotide per year) and clade B ($1.3 \times 10^{-5} \pm 0.2 \times 10^{-5}$ substitutions per nucleotide per year). The inferred mutation rates for each clade were used to estimate the time of divergence between the different *E. faecium* clades A1, A2, and B, placing the time of the split between clade A and B at 2,776 ± 818 years ago and the split between clade A1 and clade A2 at 74 ± 30 years ago (Lebreton, et al., 2013).

Human commensal isolates overwhelmingly belong to clade B (which corresponds to BAPS-group 1 defined by Willems *et al.* (Willems, et al., 2012)), while strains from other sources, including hospital-acquired infections and animal isolates, cluster in clade A1 and A2, respectively (Lebreton, et al., 2013). However, this ecological distinction between two clades is not absolute: of the strains with sequenced genomes, strain 1,141,733 was isolated from a bloodstream infection but clusters in clade B, while strain E1039, which was isolated from the feces of a healthy volunteer, clusters in clade A. Notably, the presence of evolutionarily distinct clades within what we currently characterize as *E. faecium* is very different from what genomics has revealed for *E. faecalis*, where isolates can be so closely related in core genes as to be virtually indistinguishable from one another.

Recently, a lack of CRISPR-Cas defense has been implicated in the evolution of multidrug-resistant *E. faecalis* (Palmer & Gilmore, 2010). CRISPR-Cas systems appear to be relatively rare in *E. faecium*. Among the first 31 publicly available *E. faecium* genomes, a CRISPR-Cas system can be identified in only five isolates (strains TX1330, Com12, 1,141,733, PC4.1, and 1,231,408) (Palmer & Gilmore, 2010) and unpublished observations). Except for strain 1,231,408, each of these strains belongs to clade B; strain 1,231,408 is a hybrid clade A-clade B strain that appears to have acquired CRISPR-*cas* through recombination with a clade B strain (Palmer, et al., 2012). It has been hypothesized that the absence of CRISPR-Cas in *E. faecium* would allow for the facile acquisition of foreign DNA, such as plasmids that encode genes for antibiotic resistance (Palmer & Gilmore, 2010). However, even commensal *E. faecium* isolates with very few resistances (such as E1039 and E980) lack a CRISPR-Cas system, which indicates that the absence of CRISPR-Cas does not necessarily lead to the rapid accumulation of antibiotic resistance genes (van Schaik, et al., 2010). Further genome sequencing of antibiotic-resistant and antibiotic-sensitive isolates is required to determine if CRISPR-Cas defense contributes significantly to the evolution of multidrug resistance in *E. faecium*. Nevertheless, based on available genome

data, it appears that CRISPR-Cas defense is an ancestral aspect of the clade B *E. faecium* lineage and not of clade A.

Intraspecies Diversity of *E. faecium*

Bacterial genomes differ as the result of drift in the sequences of shared genes (with some mutations becoming fixed by selection), as well as from the acquisition of new genes by horizontal gene transfer. A first insight into variation in gene content of different *E. faecium* isolates was provided by a study in which comparative genomic hybridization was used to characterize the accessory gene pool of *E. faecium* (Leavis H. L., et al., 2007). This led to the identification of 175 genes found more frequently in clinical isolates than in non-clinical isolates. This approach identified the transposon IS*16* as most specifically enriched in clinical isolates (Leavis H. L., et al., 2007). This study also showed that, based on gene content, hospital isolates were more similar to each other than to isolates from other sources, such as healthy humans and animals.

Clinical *E. faecium* isolates are predominantly found within clade A1, and these appear to have emerged from a background of animal isolates, while human commensal isolates are more distantly related (Lebreton, et al., 2013). Genome sequence analysis of two ampicillin-resistant strains (E4452 and E4453) that were isolated from dogs (de Regt, et al., 2012) appear to corroborate the hypothesis that strains from lineage-78 in clade A1 have evolved from animal isolates, since strains E4452 (ST266) and E4453 (ST192) both belong to lineage-78, as defined in the previously described BAPS analysis of *E. faecium* MLST data (159). Phylogenomic analyses underscore the relatively close relationship of E4453 with strains that belong to either lineage-78 or lineage-18 (de Regt, et al., 2012). Both these canine *E. faecium* strains carry IS*16*, which was previously shown to be highly enriched among clinical isolates; but lack virulence genes, such as *hyl* or *esp*, which are commonly found in clinical isolates. Strain E4453, but not strain E4452, carries a genomic island that is putatively involved in the breakdown of complex sugars (Heikens, van Schaik, Leavis, Bonten, & Willems, 2008). However, the two canine *E. faecium* strains share more genes with each other than with the clinical strains E1162 and U0317, which indicates that niche adaptation is occurring on the level of the accessory genome of these isolates. For instance, both strains carry an element that is putatively involved in the breakdown and metabolism of xylopolysaccharides, which is absent from all other isolates for which the genome sequences are publicly available, and appears to be relatively rare in clinical isolates (de Regt, et al., 2012). A PCR-based analysis of the presence of this element in ampicillin-resistant strains isolated from the community showed that this element is relatively common among ampicillin-resistant *E. faecium* strains originating from dogs (18 positives from 25 tested strains) and cats (8 positives from 11 tested strains). This element was also found in two of only three ampicillin-resistant strains that were isolated from a total of 40 healthy volunteers who were given a week-long course of amoxicillin. All ampicillin-resistant isolates from the community were typed with MLST and found to be related to ampicillin-resistant canine *E. faecium* strains. The low number of ampicillin-resistant enterococci that were isolated from healthy humans underscores the relatively low colonization rate of this group by ampicillin-resistant enterococci, and indicates that ampicillin-resistant *E. faecium* are more frequently present in pets than in healthy humans. The finding that ampicillin-resistant isolates from pets are phylogenetically related to ampicillin-resistant clinical isolates of lineage-78 may indicate that ampicillin-resistant isolates have first emerged in pets or other animals and have acquired additional elements (such as virulence determinants, like the *esp* gene and transposons that confer vancomycin resistance) during their adaptation to the lifestyle of nosocomial pathogens. Further genome sequencing of ampicillin-resistant and ampicillin-sensitive strains from non-clinical and non-human reservoirs is required to clarify the evolutionary pathways that have led to the emergence of multidrug-resistant *E. faecium* strains that can successfully colonize and infect hospitalized patients.

To further quantify intraspecies genomic diversity and to estimate the size of the total gene pool that is accessible to a single species, pan-genome analyses are required (reviewed in (Tettelin, Riley, Cattuto, & Medini, 2008)). In these analyses, pairwise comparisons are made between all possible combinations of genome sequences of a

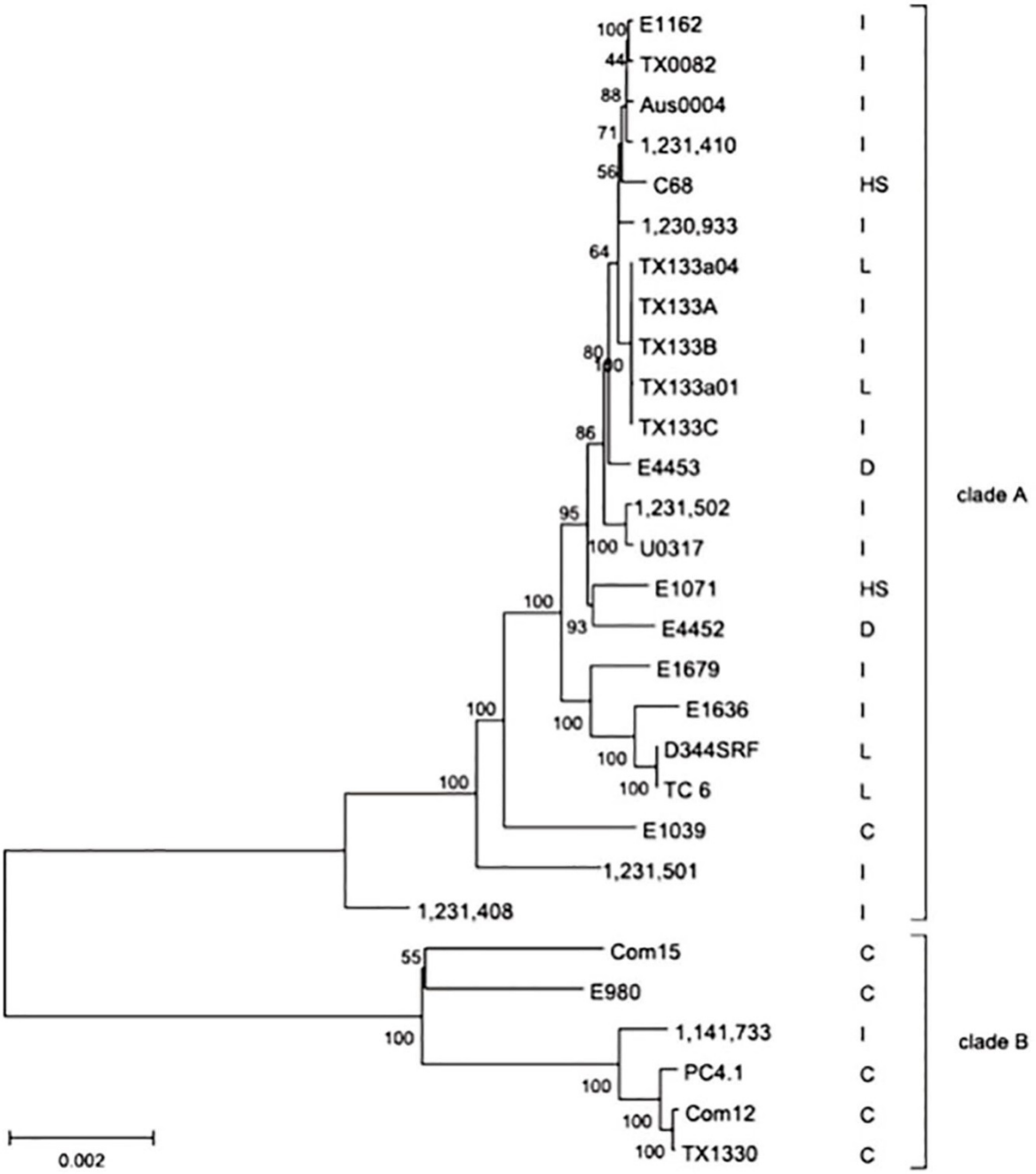

Figure 3. Phylogenomic analysis of *E. faecium*. Minimum evolution tree based on the concatenated alignments of 274 orthologous proteins conserved in draft genome sequences of 30 *E. faecium* isolates. Bootstrap values are indicated and are based on 1000 permutations. The two *E. faecium* clades termed A and B by Palmer *et al.* (110) are indicated. The origins of the strains are also provided (I: isolated from site of infection; HS: hospital surveillance isolate; C: human commensal isolate; D: isolated from dog feces; L: strain was experimentally generated in the laboratory; see Table 1 for further details).

single species, leading to estimates of the total gene pool that is accessible to the organism, the size of the core genome (namely, the number of genes that are conserved in all strains of the same species), and the number of new genes that might be expected to be discovered when additional genomes are sequenced. By this approach and by using 73 *E. faecium* genomes, the size of the *E. faecium* core genome is estimated to consist of 1597

coding sequences (Lebreton, et al., unpublished data). The total number of coding sequences in an *E. faecium* genome can range from 2272 (for strain EnGen0014) to 3318 (for strain TX0133a01), which indicates that between 29% and 52% of the DNA in any *E. faecium* isolate is non-core and is thus variably present between strains. For strain Aus0004, 38% of the genome is accessory (Lam, et al., 2012). These analyses have also found that the *E. faecium* pan-genome is comparatively open, which signifies that *E. faecium* can efficiently acquire and incorporate novel DNA into the collective gene pool of the species. This indicates that *E. faecium* can rapidly acquire genes that contribute to fitness, which enables it to inhabit a wide variety of environmental niches.

Diversity in the *E. faecium* Accessory Genome: Plasmids, Phage and Genomic Islands

Genome sequencing has shown that plasmids, prophages, and genomic islands are important drivers of diversity in the accessory genome of *E. faecium*.

Plasmids

As in *E. faecalis*, most *E. faecium* isolates carry plasmids that significantly contribute to the genome. Plasmids in *E. faecium* are mostly studied because of their conveyance of antibiotic resistance genes (Palmer, Kos, & Gilmore, 2010), but plasmids can also confer traits that contribute to gastrointestinal colonization (Arias C. A., Panesso, Singh, Rice, & Murray, 2009; Rice, et al., 2009) and the metabolism of carbohydrates (Zhang, Vrijenhoek, Bonten, Willems, & van Schaik, 2011). A broad discussion of enterococcal plasmids is presented in Extrachromosomal and Mobile Elements in Enteroocci.

Megaplasmids occur in some *E. faecium* isolates, including those that encode the hyl_{Efm} gene. hyl_{Efm} is more frequently present in clinical than in non-clinical *E. faecium* isolates, and was initially erroneously classified as a hyaluronidase (Rice, et al., 2003). However, hyaluronidase activity has not been detected in strains that carry this gene (Laverde Gomez, et al., 2011). More recent sequence analyses have shown that the protein encoded by hyl_{Efm} is likely to encode a family 84 glycosyl hydrolase, which may function as a β-N-acetylglucosaminidase (Freitas, et al., 2010; Sheldon, et al., 2006). Transfer of a plasmid that encodes hyl_{Efm} increases the virulence of *E. faecium* in a mouse peritonitis model (Arias C. A., Panesso, Singh, Rice, & Murray, 2009), as well as in a mouse model of gastrointestinal colonization (Rice, et al., 2009). However, specific deletion of the hyl_{Efm} gene did not lead to lower virulence in a mouse peritonitis model (Rice, et al., 2009), which indicates that other genes encoded by the plasmid contribute to virulence. Plasmids that encode hyl_{Efm} range in size from 150 to 375 kb (Arias C. A., Panesso, Singh, Rice, & Murray, 2009; Freitas, et al., 2010; Rice, et al., 2009). Sequencing of the 280 kb plasmid pLG1 revealed that hyl_{Efm} is encoded within a conserved 18-kb gene cluster that is flanked by incomplete inverted copies of the IS*1476* putative transposase gene. Additional pLG1 genes have functions in plasmid maintenance, replication, and conjugation. Genes involved in carbon uptake and metabolism, as well as those that confer resistance to heavy metals and antibiotics, were also identified (Laverde Gomez, et al., 2011). The *pilA*/*fms21-fms20* gene cluster that encodes a pilus structure in *E. faecium* is also present on pLG1 (Kim, et al., 2010; Laverde Gomez, et al., 2011). Large multifunctional plasmids in *E. faecium* appear to play an important role in the adaptation of *E. faecium* to new environments, as in a single event (such as the acquisition of a plasmid), many genes are acquired with diverse roles in gastrointestinal colonization, virulence, and antibiotic survival. Sequencing of additional antibiotic resistance plasmids has shed new light on plasmid maintenance systems and the genetic context of the vancomycin-resistance transposon Tn*1546* (Li, Alvarez, Harper, & Wang, 2011; Sletvold, et al., 2008; Sletvold, et al., 2007; Sletvold, et al., 2010).

Bacteriophages

Enterococcal bacteriophages are comprehensively discussed in Enterococcal bacteriophages and genome defense. In the genomes of *E. faecium* strains, integrated prophages appear to be important drivers of diversity.

Genes of phage origin make up 2.3% to 5.1% of seven sequenced *E. faecium* genomes, and in most of those strains, integrated prophages can be activated by the addition of the DNA cross-linking agent mitomycin, which leads to the release of phage particles that can be visualized by electron microscopy (van Schaik, et al., 2010). In this study, all bacteriophages activated by mitomycin had the typical morphology of *Siphoviridae*, with an isometric head and a long non-contractile segmented tail. This family of bacteriophages is common in lactic acid bacteria, and a study aimed at identifying temperate bacteriophages in *E. faecalis* also exclusively found *Siphoviridae* (Yasmin, et al., 2010). However, lysogenic phages from other families have also been detected in *E. faecium* and other enterococci (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010). In strain Aus0004, three distinct prophages can be identified, of which one is present as a 50-kb inverted repeat (Lam, et al., 2012). These two identical prophages appear to have facilitated a 683 kb chromosomal inversion, which leads to a shift in the predicted replication terminus, which is normally positioned 180° from the origin of replication, but is at 115° in Aus0004 instead.

The presence of integrated prophages poses a potential threat to survival of the *E. faecium* cell, as the activation of the phage may lead to cell death. Even the presence of phage DNA may come at a cost, in terms of the increased metabolic burden required for the replication of phage DNA during cell division. For these reasons, bacteria have evolved mechanisms by which they can withstand attack by bacteriophages, such as the CRISPR-Cas system previously discussed. Alternately, prophages can be retained in the population if the phage confers a fitness benefit to the host, in a process termed lysogenic conversion (Brüssow, Canchaya, & Hardt, 2004). There are currently no data available that would indicate that phages carry genes that contribute significantly to the fitness of *E. faecium*, but systematic studies aimed at characterizing phage genes have not been performed. Phages may also be involved in the spread of antibiotic resistance genes among enterococci, as they can transfer antibiotic resistance genes between different enterococcal species, which expands the mobility of resistance genes in this genus (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2011).

Genomic islands

Similar to what was found in *E. faecalis* (Schwarz, Perreten, & Teuber, 2001), genome sequencing of *E. faecium* led to the identification of two genomic islands that are enriched in clinical *E. faecium* isolates. The most thoroughly studied genomic island in *E. faecium* carries the *esp* gene, which encodes a large (~200 kDa) peptidoglycan-anchored surface protein that contributes to biofilm formation (Heikens, Bonten, & Willems, 2007). In animal models, it has been shown to contribute to urinary tract infection (Leendertse, et al., 2009) and endocarditis (Heikens, et al., 2011), but not to gastrointestinal colonization in the model tested (Heikens, et al., 2009). In *E. faecium*, the *esp* gene is part of a large genomic island that ranges from 64 to 125 kbp in size (Figure 4), and which has been sequenced to completion in the strains E1162, E1679, U0317, and Aus0004 (Lam, et al., 2012; van Schaik, et al., 2010). As in *E. faecalis* (124), the *esp* genomic island in *E. faecium* carries all the hallmarks of a prototypical genomic island, as its %G+C content is 1.1% to 2.3% lower than the entire genome of this strain, and it is flanked by two 54-bp direct repeats. In all strains, the *esp* genomic island has been integrated between a small gene that encodes a hypothetical protein and the *rpsI* gene, which encodes a small ribosomal protein. In strain U0317, a genomic rearrangement event appears to have occurred after the initial integration of the *esp* genomic island, which resulted in the nearly complete island reintegrating 5′ of the *tuf* gene (van Schaik, et al., 2010). The locus in which the *esp* island integrates appears to be a hotspot for the integration of foreign elements; in several strains that lack the *esp* island, smaller elements of possible phage or plasmid origin have integrated at this position.

In *E. faecium* strains E1162 and UW3308, the *esp* island can be mobilized and horizontally transferred to *esp*$^-$ acceptor strains by conjugation (Oancea, Klare, Witte, & Werner, 2004; van Schaik, et al., 2010). The observation that the *esp* island is mobilizable and self-transmissible has led to the renaming of this island to the integrative conjugative element (ICE) ICE*Efm1* (Top, et al., 2011). Sequencing of ICE*Efm1* in different *E. faecium* strains has revealed that this element encodes many functions that are potentially beneficial for the bacterial cell during

infection of the human host. Therefore, the conjugative spread of ICE*Efm1* may significantly contribute to the recent emergence of *E. faecium* as a nosocomial pathogen of major importance.

The overall structure of ICE*Efm1* is similar in strains E1162, E1679, U0317 and Aus0004, but several insertions and rearrangements have occurred. The 5′ end of ICE*Efm1* consists of an element that resembles the conjugation module of the widely spread conjugative transposon Tn*916*. At the extreme 5′ end of ICE*Efm1* in strains E1162, E1679, and U0317, a predicted peptidoglycan-anchored surface protein is encoded. This protein is 91% identical on the amino-acid level to the collagen adhesin EcbA (Hendrickx, et al., 2009). This gene is also present in strain Aus0004, but an additional 23.8 kb fragment is present between the gene that encodes the *ecbA* homolog and the 54 bp sequence that forms the outer boundary of ICE*Efm1*. The central part of ICE*Efm1* consists of the *esp* gene and several other genes that have predicted roles in regulation of transcription, transport, and other functions. There is considerable heterogeneity in the *esp* gene among different strains, particularly in the 3′ end of the gene where repeats are encoded (Leavis, et al., 2004). In the 3′ end of ICE*Efm1*, a 10-kb region can be identified that is essentially identical (98% nucleotide sequence identity) to a region of the *E. faecalis* PAI, which also encodes *esp* (Shankar, Baghdayan, & Gilmore, 2002). Several of the genes in this region have a predicted role in the uptake of manganese, which is an important element for virulence in both Gram-negative and Gram-positive bacteria (Papp-Wallace & Maguire, 2006). On the extreme 3′ end of ICE*Efm1*, an integrase gene is present, which is indispensable for excision of ICE*Efm1* (Top, et al., 2011). Multiple transposases are scattered throughout ICE*Efm1*, and the presence of other elements (such as a group II intron in strain E1162) can vary from strain to strain. The most striking difference among the currently sequenced ICE*Efm1* elements is the presence of a Tn*1549*-like transposon (Garnier, Taourit, Glaser, Courvalin, & Galimand, 2000) in the ICE*Efm1* of Aus0004. This transposon carries the *vanB* gene cluster, which confers resistance to vancomycin. Another similar integration in ICE*Efm1* has occurred in strain E1679, where a gene cluster putatively encoding a pathway for the metabolism of inositol has been inserted. This element appears to be functional, as strain E1679 can grow on inositol as carbon source, while *E. faecium* strains that lack these genes cannot (van Schaik *et al.*, unpublished data). Genome sequencing of strain 1,231,502 also revealed the presence of this inositol utilization gene cluster as part of ICE*Efm1* in this strain.

A second genomic island was first identified in the draft TX16 genome, due to its aberrant dinucleotide frequency and %G+C content. The island is an 8.4 kb element that is more commonly found in clinical isolates than in strains isolated from non-clinical settings (Heikens, van Schaik, Leavis, Bonten, & Willems, 2008). This genomic island is flanked by inverted and direct repeats, contains an integrase, has an anomalous %G+C; and in addition to DO, is present in the genomes of the clinical isolates 1,230,933, 1,231,502, 1,231,410, Aus0004, C68, E1162, E1679, TX0133, TX0082, and U0317, all of which are clade A strains. The only non-clinical isolate in which this element is also found in the genome sequence is the canine *E. faecium* strain E4453. The functional role of this element remains to be experimentally determined, but sequence analysis points towards its role in the uptake and metabolism of complex carbohydrate substrates.

Genomics of Other Enterococci

Although most sequencing efforts have has focused on genomes of *faecalis* and *faecium* species, data are increasingly available for other enterococcal species as well. These genomes, in addition to being interesting in their own right, are important for understanding the diversity of the genus *Enterococcus,* and to understand the evolution of the species within this genus. Annotated and unannotated draft genome sequences are available for strains of *E. casseliflavus, E. gallinarum*, *E. italicus*, *E. saccharolyticus,* and *E. mundtii* (Magni, et al., 2012; Palmer, et al., 2010; Palmer, et al., 2012) and unpublished data in GenBank).

E. casseliflavus and *E. gallinarum*, historically thought to be primarily associated with plants (Mundt & Graham, 1968) and birds (Collins, Jones, Farrow, Kilpper-Balz, & Schleifer, 1984), respectively, have also been associated with life-threatening infections, such as bacteremia and meningitis (Choi, et al., 2004; de Perio, Yarnold, Warren,

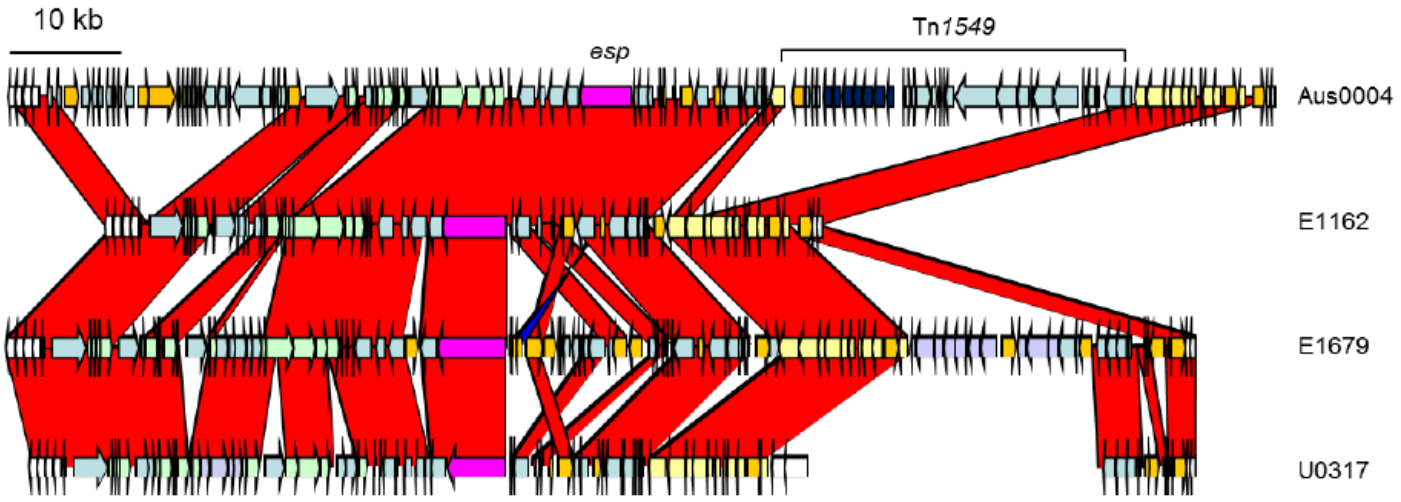

Figure 4. ICE*Efm1* in *E. faecium* strains Aus0004, E1162, E1679 and U0317. The ICE*Efm1* elements of strains Aus0004, E1162, E1679, and U0317 were aligned using the Artemis Comparison Tool (15). Arrows indicate coding sequences (CDS) and the direction of transcription. Red-colored bands indicate matching regions between ICE*Efm1* of different strains. A small blue band represents an inversion of a transposase fragment between E. faecium E1162 and E1679. The yellow arrows indicate a region that is identical to a fragment of the E. faecalis MMH594 PAI (126). The green arrows represent CDS that are homologous to the conjugation module of Tn*916*. The purple arrows in E1679 represent CDS putatively encoding a functional pathway for inositol uptake and metabolism. Orange arrows represent transposases and integrases. Dark blue arrows represent the genes of the *vanB* cluster on Tn*1549*. The *esp* gene is colored pink. White arrows indicate flanking genes that are not part of ICE*Efm1*.

& Noskin, 2006; Khan & Elshafi, 2011; Prakash, Rao, & Parija, 2005). A hospital outbreak of *E. gallinarum* has been reported (Contreras, et al., 2008). The endogenous low-level vancomycin resistance of *E. casseliflavus* and *E. gallinarum*, combined with the intrinsic resistance of all enterococci to antibiotics such as cephalosporins (Tannock & Cook, 2002), may contribute to a rise in opportunistic infections caused by these species. Three of the sequenced *E. casseliflavus* genomes (EC10, EC20, and EC30) and the *E. gallinarum* EG2 genome derive from clinical isolates obtained from patients in the United States (Palmer, et al., 2012). *E. casseliflavus* ATCC 12755 (Genbank accession AEWT00000000) is a reference genome for the Human Microbiome Project. Of the remaining sequenced species, to our knowledge, only *E. mundtii* has been associated with human infection, and reports of these types of infection are few (de Perio, Yarnold, Warren, & Noskin, 2006; Prakash, Rao, & Parija, 2005). *E. mundtii* ERL1656, for which a genome sequence is available, was isolated from cow′s milk and is a bacteriocin-producing strain that is thought to have probiotic properties (Magni, et al., 2012). *E. saccharolyticus* 30_1 (Genbank accession ADLY00000000) was isolated from inflamed ileum tissue taken from a patient with Crohn's disease (http://www.broadinstitute.org/annotation/genome/Enterococcus_group), and is a reference genome for the Human Microbiome Project. Finally, *E. italicus* DSM 15952 (Genbank accession AEPV00000000), which is also a reference genome for the Human Microbiome Project, was isolated from an Italian cheese and is the type strain for the *italicus* species (Fortina, Ricci, Mora, & Manachini, 2004).

Two phenotypes typically absent in *E. faecalis* and *E. faecium* have been of particular interest for clinical discrimination of enterococcal species. *E. casseliflavus* and *E. gallinarum* are distinguished from *E. faecalis* and *E. faecium* by their motility, while *E. casseliflavus* and *E. mundtii* are further distinguished by their production of a yellow pigment (Facklam, Carvalho, & Teixeira, 2002). However, variability in these phenotypes occurs, which complicates species designations when phenotypic characteristics are used (Devriese, Pot, & Collins, 1993; Tannock & Cook, 2002; Vincent, Knight, Green, Sahm, & Shlaes, 1991). The genetic basis for these traits, as well as whether they are truly species-specific, are of interest. Genomic analysis has revealed genetic bases for motility and pigment production in *E. casseliflavus* and *E. gallinarum* (Palmer, et al., 2012), among other traits.

Flagellar motility

A set of 48 predicted flagellar biosynthesis and chemotaxis genes are co-localized on one contig of the draft *E. casseliflavus* EC10 genome (contig 22; ORFs ECAG_01613-ECAG_01660) (Palmer, et al., 2012). Orthologs of all or most of these genes are present in *E. casseliflavus* EC20, EC30, and *E. gallinarum* EG2 (Palmer, et al., 2012). The *E. casseliflavus* ATCC 12755 genome possesses a highly conserved homologue of the putative flagellin protein (ECAG_01617) encoded by *E. casseliflavus* EC10 (Palmer, unpublished), which suggests that a similar gene cluster is present in that strain. Most top BLASTP hits to the *E. casseliflavus* EC10 motility cluster proteins are from *Lactobacillus ruminis* strains, and a few are from *Carnobacterium* sp. strains (Palmer, et al., 2012); both genera are within families that are closely related to the *Enterococcaceae*. The distribution of flagellar motility genes among available enterococcal genomes suggest either that motility was once a core property of the *Enterococcus* genus, and was subsequently lost by the progenitors of the *faecalis* and *faecium* lineages; or that flagellar motility was laterally acquired by the progenitor of the *casseliflavus*/*gallinarum* lineage from closely related bacteria. Because motility is a rare phenotype in the *Enterococcus* genus (Facklam, Carvalho, & Teixeira, 2002), the most parsimonious explanation would appear to be lateral transfer, although we note that motility is also present in some species of *Vagococcus*, another genus within the *Enterococcaceae* family (Lawson, Foster, Falsen, Ohlén, & Collins, 1999; Shewmaker, et al., 2004; Teixeira, et al., 1997).

Surprisingly, a homolog of *E. casseliflavus* EC10 flagellin is present in the draft *E. saccharolyticus* 30_1 genome (HMPREF9478_02662; 94% amino acid identity to ECAG_01617), and is flanked by additional genes with likely roles in flagellar biosynthesis and chemotaxis (Palmer, unpublished). The presence of a flagellin homologue in this bacterium is surprising, because motility has not been noted as a characteristic of the *saccharolyticus* species (Facklam, Carvalho, & Teixeira, 2002). Analysis of the 16S rRNA sequence of the *E. saccharolyticus* 30_1 genome (HMPREF9478_03015) reveals that it is 100% identical to the 16S rRNA sequence of *E. gallinarum* EG2 and 99% identical to several partial *E. gallinarum* 16S rRNA sequences in GenBank. Further, preliminary analyses indicate that the housekeeping genes of *E. saccharolyticus* 30_1 are >99% identical to those of *E. gallinarum*, which suggests that strain 30_1 may have been misclassified (Palmer and van Schaik, unpublished). Whether the *E. saccharolyticus* 30_1 strain is a motile variant of the *saccharolyticus* species or is in fact an *E. gallinarum* strain should be investigated further.

Carotenoid production

Pigment production is a property of only a few described *Enterococcus* species, including *E. casseliflavus* and *E. mundtii* (Facklam, Carvalho, & Teixeira, 2002), although pigment production appears to be an unreliable phenotypic marker for species designations (Tannock & Cook, 2002). The yellow pigment elaborated by *E. casseliflavus* is a carotenoid pigment that likely protects it, as well as other species that produce the pigment, from photo-oxidation (Maraccini, Ferguson, & Boehm, 2012; Taylor, Ikawa, & Chesbro, 1971). *Staphylococcus aureus* also produces a carotenoid pigment, staphyloxanthin, which has been more extensively studied. Staphyloxanthin biosynthesis is directed by the *crtOPQMN* operon, and *crtOPQMN* expression *in trans* is sufficient to confer staphyloxanthin production on the natively non-pigmented *Staphylococcus carnosus* (Pelz, et al., 2005). Biochemical characterization of the staphyloxanthin biosynthesis pathway expressed in *S. carnosus* revealed that *crtMN* expression alone is sufficient for production of the yellow pigment and pathway intermediate 4,4-diaponeurosporene (Pelz, et al., 2005). The additional expression of *crtOPQ* is required to complete the biosynthetic pathway to staphyloxanthin (Pelz, et al., 2005).

Homologues of all five *crt* genes are present in the *E. casseliflavus* EC10, EC20, and EC30 genomes, while only *crtM* and *crtN* are present in *E. gallinarum* EG2 (Palmer, et al., 2012). For the enterococci, *crt* gene organization differs from that of *S. aureus*. In *E. gallinarum* and *E. casseliflavus*, *crtNM* are putatively co-transcribed, while *crtPQO* are putatively transcribed and are encoded elsewhere on the *E. casseliflavus* genome. The significance of this is unknown, but is suggestive of two "modules" of pigment biosynthesis genes, one composed of *crtNM* and

one composed of *crtPQO*—although note that CrtM and CrtN activities should be required for the later steps to be functional. *Lactobacillus plantarum* also possesses the *crtNM* module, and a collection of 18 *L. plantarum* strains that possessed *crtNM* were found to be variable for production of the yellow pigment 4,4-diaponeurosporene, with some strains forming yellow cell pellets with high amounts of pigment detectable by chromatographic analysis, and others forming white cell pellets with low or trace amounts of pigment detected (Garrido-Fernández, Maldonado-Barragán, Caballero-Guerrero, Hornero-Méndez, & Ruiz-Barba, 2010). For *L. plantarum*, 4,4-diaponeurosporene production varies with the growth medium used (Garrido-Fernández, Maldonado-Barragán, Caballero-Guerrero, Hornero-Méndez, & Ruiz-Barba, 2010). The presence of *crtNM* in *E. gallinarum* EG2 is surprising, as it suggests that this strain could also produce the yellow pigment 4,4-diaponeurosporene, which has not been observed in routine laboratory culture. Whether these genes are functional and expressed, and perhaps induced under conditions so far not encountered in laboratory culture, remains to be determined. Regardless, it appears that yellow pigment production by certain enterococcal strains is due to the growth medium-dependent production of one or both of two structurally distinct carotenoids, which likely explains the inconsistencies that have been observed in this phenotype.

BLAST analysis reveals the distribution of the *crt* genes among other sequenced enterococci and closely related bacteria (Palmer, unpublished). Consistent with a conserved pigment biosynthesis pathway in *E. casseliflavus*, putative *crtNM* and *crtPQO* genes are present in *E. casseliflavus* ATCC12755. Nucleotide sequences with high identity to *crtN* and *crtPQO* are also present in the unannotated *E. mundtii* ERL1656 genome, although some sequences are partial and operon structures are unclear. This suggests that a similar pathway is present in *E. mundtii*, although further bioinformatic analysis and perhaps additional sequencing will be required for confirmation. Interestingly, *crt* genes are also present in *Carnobacterium* sp. strains 17-4 and AT7, arranged in a cluster as *crtNMP-ywdH-crtQO* (*ywdH* encodes a putative aldehyde dehydrogenase). This further highlights the similarities between *Carnobacterium* sp. and motile, pigmented enterococci.

Differences Between Enterococcal Species Revealed by Genomics

Beyond the phylogenetic classification, what does it mean to be an *E. faecalis*, an *E. faecium*, an *E. casseliflavus*, or another enterococcal species? Are there certain traits that define these species relative to one another? A great deal of work in the clinical microbiology of enterococci has focused on biochemical or phenotypic tests that are capable of discriminating one species from another (Facklam, Carvalho, & Teixeira, 2002). However, phenotypic variability within a species and atypical isolates complicate those analyses. Comparative genomics, through the interrogation of tens or hundreds of genomes, offers a new way to search for traits that are characteristic of a species.

Species-specific metabolism

Comparative analysis of metabolic pathways encoded by enterococcal genomes has been limited, although some insights in this area have emerged. A COG (clusters of orthologous genes)-based analysis of seven *E. faecium* and six *E. faecalis* genomes identified 70 COGs specific to *E. faecium* and 140 COGs specific to *E. faecalis* (van Schaik, et al., 2010). Several of these belong to pathways known to differ between the two species, including that of arabinose catabolism (present in *faecium*, but not *faecalis*) and of heme-dependent cytochrome biosynthesis (present in *faecalis*, but not *faecium*). Other species-specific differences identified by COG analysis were the presence of putative pectin and lignocellulose catabolism genes in *faecium* but not *faecalis*, and ethanolamine utilization genes in *faecalis* but not *faecium*. Ethanolamine catabolism genes were confirmed to be specific to the *faecalis* species in a separate study evaluating 30 *faecalis*, *faecium*, *casseliflavus* and *gallinarum* genomes (Palmer, et al., 2012). It is interesting that genes for the catabolism of complex plant polysaccharides and their degradation products are specific to *E. faecium*, while genes for catabolism of the membrane phospholipid head group ethanolamine are specific to *E. faecalis*. This suggests that these two species can occupy fundamentally different nutrient niches in the human intestine.

A combination of Biolog carbon utilization analysis and comparative genomics identified additional metabolic reactions and pathways that discriminate different enterococcal species (Palmer, et al., 2012). Genes that putatively direct synthesis of the bacterial second messenger signal cyclic-di-GMP, and genes for an acetoin dehydrogenase were identified in *E. casseliflavus,* and not *E. faecalis*, *E. faecium*, or *E. gallinarum*. Inulin catabolism was additionally found to be specific to *E. casseliflavus* using Biolog analysis; however, no species-specific genes conferring this phenotype have been identified. As mentioned above, ethanolamine catabolism genes were found to be specific to *E. faecalis*, as are the *bkdDABC* genes encoding a branched-chain α-keto acid dehydrogenase complex, and a putative formate dehydrogenase gene (*fdhA*). Consistent with the distribution of the *bkdDABC* genes, catabolism of α-ketovalerate in Biolog assays is specific to *E. faecalis*. Finally, a gene that encodes a putative glutaminase was found to be *E. faecium*-specific. Each of these genes and metabolic reactions present opportunities for the development of novel biochemical assays or genetic screens capable of rapidly discriminating enterococcal species. Biolog analysis has also provided information on *E. faecium* intraspecies metabolic diversity by aiding in the identification of genetic determinants that confer raffinose catabolism (Zhang, Vrijenhoek, Bonten, Willems, & van Schaik, 2011).

Extracellular polysaccharide biosynthesis

Extracellular polymer biosynthesis has been most studied for *E. faecalis*, which in addition to lipoteichoic and wall teichoic acids, produces a rhamnopolysaccharide called the enterococcal polysaccharide antigen (Epa), and a capsule (Hancock & Gilmore, 2002). Biosynthesis of the Epa polymer is directed by the *epa* locus, a cluster of genes that encompasses *epaA* to *epaR,* which encode functions that are likely to be important for synthesis and export of the rhamnopolysaccharide polymer (Teng, Singh, Bourgogne, Zeng, & Murray, 2009; Xu, Murray, & Weinstock, 1998). Based on PCR-based screening and dot blot hybridization studies, it was reported that the *epa* locus is core to the *faecalis* species (Teng, Singh, Bourgogne, Zeng, & Murray, 2009). Conversely, the presence of the *cps* genes that direct capsule biosynthesis varies among *E. faecalis* strains (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007). Little is known about extracellular polymer synthesis by enterococcal species other than *faecalis*. A comparative analysis of 30 *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* genomes confirmed that the *epa* locus is core to the *faecalis* species, and also identified a similar *epa* locus as being core to *E. faecium* (Palmer, et al., 2012). The presence of *epa* loci in both *E. faecalis* and *E. faecium*, but not in *E. casseliflavus* or *E. gallinarum*, suggests that the Epa polymer might contribute in some significant way to the colonization of mammalian hosts, with which these two species are most closely associated. Surprisingly, the *E. faecalis cps* locus was not detected outside of that species (Palmer, et al., 2012). Instead, a second putative capsule biosynthesis mechanism was identified, being present in the genomes of *E. faecium*, *E. casseliflavus*, and *E. gallinarum*, as well as the *E. saccharolyticus* 30_1 and *E. italicus* DSM 15952 genomes (Palmer, et al., 2012). This putative capsule biosynthesis mechanism appears to be similar to that of *Streptococcus pneumoniae*, and consists of a core phosphoregulatory system encoded by *wzg-wzd-wze-wzh* and a variable set of genes that occur downstream of *wzg-wzd-wze-wzh,* which appear to direct biosynthesis of a polysaccharide polymer. Other than *E. faecalis*, *wzg-wzd-wze-wzh* is core to all enterococcal species for which a genome sequence is available, which highlights a significant evolutionary deviation of *faecalis* from other enterococci.

Additional Genomic Applications: Metagenomics and Genome Resequencing

To this point, we have discussed comparative genomic analyses of closed and draft enterococcal genomes, each obtained by *de novo* sequencing of DNA obtained from an isolate grown in pure culture. Here, we discuss two additional genomic techniques that have been applied to *E. faecalis*: metagenomics and genome resequencing.

Metagenomic assembly of *E. faecalis* UC1ENC from a complex microbial community

Enterococci are abundant in the feces of formula-fed infants and are generally more prevalent in infant intestinal communities compared to adult communities, which suggests that their presence contributes to the conditioning of the infant immune system (Tannock & Cook, 2002). A molecular phylogenetic analysis of the fecal community of a premature human infant revealed diet-dependent fluctuations in community structure, with enterococcal 16S rRNA sequences being most abundant after a dietary switch from enteral feedings with maternal breast milk to commercial infant formula (Morowitz, et al., 2011). A complete enterococcal genome, designated UC1ENC, was assembled from metagenomic sequence generated from DNA extracted from fecal samples obtained from this infant. The UC1ENC 16S rRNA gene sequence is 100% identical to that of multiple *E. faecalis* strains, which indicates that the UC1ENC genome originated from an *E. faecalis* strain abundant in the infant gut. *In silico* MLST analysis of UC1ENC assigned the genome to ST179, an ST previously recovered from hospitalized patients in Spain and the Netherlands (http://efaecalis.mlst.net), and non-hospitalized individuals in Spain (Kuch, et al., 2012). Two putative enterococcal plasmids (plasmid 1 and plasmid 2) and two putative enterococcal phages (phage 1 and phage 2) were also assembled from the infant metagenomic data (Morowitz, et al., 2011).

Comparative analysis of the UC1ENC genome with the V583 genome revealed substantial relatedness in core functions as well as shared mobile genetic elements. Predicted protein sequences encoded by the two genomes possess an average amino acid identity of 98.7% (Morowitz, et al., 2011). UC1ENC shares two prophage (prophages 2 and 4) with V583, as well as portions of the PAI (Morowitz, et al., 2011) that lack the module F and the 3′ region of module D, as defined by previous studies of the PAI (McBride, et al., 2009). BLAST analysis of predicted enterococcal plasmid and phage sequences additionally assembled from infant fecal metagenomic data reveal substantial identity to previously characterized *E. faecalis* mobile elements (Palmer, unpublished analysis). Plasmid 1 (68.7 kb) is similar to the pheromone-responsive plasmid pMG2200 (Zheng, Tomita, Inoue, & Ike, 2009), although it lacks the Tn*1549 vanB*-type vancomycin resistance transposon that is present in pMG2200. Plasmid 2 (8.3 kb) appears to be derived from the conjugative plasmid pRE25 (124) and possesses a similar zeta-epsilon-delta toxin-antitoxin system. Phage 1 (8.3 kb) is similar to chromosomal sequence of the infant fecal E. faecalis strain 62, while phage 2 (28.9 kb) is similar to the pseudotemperate linear phage present in E. faecalis 62, EF62ϕ (Brede, Snipen, Ussery, Nederbragt, & Nes, 2011). In situ copy numbers of the putative UC1ENC plasmids and phages were calculated using metagenomic reads from the premature infant fecal analysis, using samples extracted at 16, 18, and 21 days post-birth (Morowitz, et al., 2011). Plasmid 1 copy numbers were calculated to be approximately 2 at each day of sampling, which is consistent with the known copy numbers of pheromone-responsive plasmids (Weaver, Rice, & Churchward, 2002). Copy numbers of plasmid 2 and phage 1 were around 1, perhaps indicating that these elements are integrated into the UC1ENC genome. Copy numbers of phage 2 fluctuated from 5 to 12, which is consistent with this element being a pseudotemperate phage that replicates independently from the chromosome, or perhaps with a virulent phage being liberated from UC1ENC cells. To our knowledge, this study is the first instance in which copy numbers of enterococcal extrachromosomal elements have been calculated for an in vivo environment.

In vitro evolution and resequencing identifies a genetic basis for daptomycin resistance

In prokaryotic genome resequencing, derivatives or populations of a reference strain are sequenced to high coverage, and reads are aligned to the reference genome to identify polymorphisms that occur within. A notable use of this technique was the identification of mutations that occurred over a long-term evolution experiment with *Escherichia coli* (Barrick, et al., 2009). This technique has also proven useful for identifying a genetic basis for enterococcal resistance to the last-line antibiotic daptomycin (Palmer, Daniel, Hardy, Silverman, & Gilmore, 2011). Daptomycin is a lipopeptide antibiotic with a unique mechanism of action that causes cell death in the

absence of lysis (Cotroneo, Harris, Perlmutter, Beveridge, & Silverman, 2008). Daptomycin was approved by the US Food and Drug Administration in 2003 for the treatment of certain staphylococcal infections and is used off-label to treat enterococcal infections, such as bacteremia and endocarditis (Cantón, Ruiz-Garbajosa, Chaves, & Johnson, 2010). Enterococcal resistance to daptomycin has emerged in the clinic, but occurs sporadically and does not appear to be conferred by the acquisition of mobile elements, such as plasmids and transposons (Kelesidis, Humphries, Uslan, & Pegues, 2011; Munoz-Price, Lolans, & Quinn, 2005).

In triplicate *in vitro* experiments, *E. faecalis* V583 was passaged through increasing concentrations of daptomycin, which resulted in the emergence of three high-level daptomycin resistant strains (Palmer, Daniel, Hardy, Silverman, & Gilmore, 2011). Illumina resequencing of these strains identified mutations that were putatively responsible for this phenotype, including single nucleotide polymorphisms, deletions, duplications, and the transposition of an IS*256* element. Mutations in one gene, a putative cardiolipin synthase (*cls*; V583 ORF EF0631), were among the first to occur in each of the three evolution experiments. Strikingly, EF0631 mutations were observed in daptomycin-resistant *E. faecalis* clinical isolates, as well as in a homolog of EF0631 in daptomycin-resistant *E. faecium* clinical isolates, but not their susceptible parent strains isolated prior to daptomycin therapy. Expression in OG1RF of EF0631 alleles from daptomycin-resistant strains conferred resistance to OG1RF, which conclusively demonstrated that the EF0631 mutations identified by resequencing analysis conferred resistance. Collectively, *in vitro* evolution experiments and genome resequencing identified mutations that confer resistance to a last line antibiotic, with those mutations also occurring in resistant clinical isolates. This study illustrates the utility of genomic approaches to study rapid adaptation in enterococci.

Not all daptomycin-resistant *E. faecalis* clinical isolates possess EF0631 mutations, which indicates that mutational pathways to resistance exist that are independent from this gene (Palmer, Daniel, Hardy, Silverman, & Gilmore, 2011). *De novo* genome sequencing of the daptomycin-resistant *E. faecalis* human bloodstream infection isolate R712 and its daptomycin-susceptible parent strain S613, isolated from the same patient prior to daptomycin therapy, revealed mutations in the R712 EF0631 *cls* and in two additional genes, *liaF* (EF2913) and *gdpD* (EF1904) (Arias C. A., et al., 2011). *liaF* and *gdpD* mutations were not observed in the resequencing study (Palmer, Daniel, Hardy, Silverman, & Gilmore, 2011). Experimental interrogation of the *liaF* and *gdpD* mutant alleles revealed that the *liaF* mutation alone, or *liaF* and *gdpD* mutations together, were sufficient to confer daptomycin resistance to S613 (Arias C. A., et al., 2011), which demonstrates that a *cls*-independent pathway to resistance exists. In the case of enterococcal daptomycin resistance, genomic approaches have significantly contributed to an understanding of how resistance emerges.

Conclusion and Perspectives

Genome sequencing and comparative genomics has significantly advanced our understanding of enterococcal virulence, biology, and evolution. Insights discussed here include the identification of adaptive elements that likely contribute to the diverse ecology of the enterococci, analyses illuminating the fluidity of the *E. faecalis* and *E. faecium* genomes, and the discovery that the species *E. faecium* is actually composed of distinct phylogenetic clades between which recombination occurs. Despite rapid and significant advances in this area, much remains to be learned from existing genome data.

For example, for currently available *E. faecalis* genomes, most comparative analysis has focused on recombination and horizontal gene transfer, which is precipitated by multiple lines of evidence that indicate that horizontal gene transfer is rampant in the species. However, a detailed analysis of the mobile elements that populate these genomes remains to be performed. What novel traits do these plasmids, phage, and genomic islands encode? What impact does the insertion of mobile elements have on the genome; namely, are core functions disrupted? What are the core functions of the *faecalis* genome, and what roles might these genes play in infection, niche colonization, and persistence? Beyond *faecalis*, what functions are core to the other enterococcal species for which multiple genome sequences are available—*faecium* and *casseliflavus*—and what

do they tell us about what it means to be classified as one of these species? Further, what can comparative genomics tell us about phylogenetic relationships and gene exchange among genera closely related to *Enterococcus*, such as *Melissococcus* and *Tetragenococcus*, each of which already have sequenced representatives available in GenBank? These are a handful of questions that are meant to illustrate that we have only scratched the surface of this field.

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Extrachromosomal and Mobile Elements in Enterococci: Transmission, Maintenance, and Epidemiology

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Introduction

Extrachromosomal elements are ubiquitous in the prokaryotic world and play important roles in the adaptation and survival of cell populations, especially in changing environments. Plasmids are readily found in enterococci, and it is not unusual for clinical and commensal strains (e.g. *Enterococcus faecalis* and *Enterococcus faecium*) to harbor a number of such elements. Indeed, plasmid-free isolates are only infrequently identified. Enterococcal plasmids commonly encode: i) resistance to one or more antibiotics; ii) elevated resistance to ultraviolet light; iii) virulence factors, such as cytolysin and aggregation substance; and iv) bacteriocins. In addition, intercellular transmissibility is frequently a plasmid-determined trait. As in many bacterial species, plasmids generally range in size from 3–4 kb to well over 100 kb and may be present at relatively low copy number (1–2 copies) or up to 20 or more per cell. Table 1 presents a list of enterococcal plasmids recently compiled by one of the authors (Teresa M. Coque).

Conjugation is a primary means for intercellular DNA mobility in enterococci—natural transformation has never been reported, and information is only beginning to be reported with regard to transduction involving a bacteriophage (see Enterococcal bacteriophages and genome defense). Some conjugative plasmids transfer efficiently from donor to recipient in broth, whereas others transfer well only on solid surfaces. In the case of *E. faecalis*, peptide sex pheromones secreted by recipient cells induce conjugation-related mating functions, determined by certain plasmids (*e.g.* pAD1, pCF10, and a host of others). Another group of plasmids, such as pMG1 and related elements identified mainly in *E. faecium*, are also able to transfer efficiently in broth, but do not appear to make use of sex pheromones. A group of plasmids exemplified by pAMβ1 do not transfer well in broth, but are able to move if the cells are on a solid surface. Nonconjugative plasmids are also commonly present in enterococci, and some are readily mobilized by conjugative elements *in trans* or move *via* co-integration in some cases. Representatives of some of the above-noted elements have been sequenced, and studies relating to their transfer mechanisms have been published. In addition, reports relating to replication and partitioning provide significant information on the ways in which certain transmissible elements are maintained in their host.

Other types of transmissible elements common in enterococci are the so-called conjugative transposons, which are exemplified by the Tn*916* family. Usually found integrated in the chromosome, their movement involves an excision event that results in a non-replicative circular intermediate that is able to transfer conjugatively, followed by insertion into the genome of a recipient cell. Originally identified in *E. faecalis*, these elements, which commonly encode antibiotic resistance traits, have a broad host range and are widespread among

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numerous bacterial genera. In a similar vein and as found to be the case for many species of bacteria in recent years, enterococci have been shown to carry a plethora of "genomic islands," some of which are mobile and called "integrative conjugative elements" (ICEs). Some of these represent "pathogenicity islands" that confer significant virulence traits and even antibiotic resistance.

Rapidly accumulating genomic sequencing data are facilitating identification of the enterococcal "mobilome," which includes not only transmissible elements, but also insertion sequences, transposons, and integrons that move intracellularly. Studies based on functionality, including replication and maintenance, complement this rapidly expanding picture, and the significant extent to which enterococci have participated in horizontal transfer within the bacterial world is becoming readily apparent. Below we attempt to summarize recent developments in various aspects of mobile genetic elements (MGEs) in enterococci and try to provide a perspective that is relevant to bacterial-human interaction.

Plasmid Transmission

From a general perspective, the pheromone-responding plasmids and the pMG1-related plasmids, which are usually of sizes greater than 60 kb, are able to transfer at frequencies on the order of 10^{-3} per donor (or higher) within a few hours in broth suspensions (Clewell & Dunny, 2002; Clewell & Francia, 2004); they transfer at much higher frequencies—approaching 100%—on solid surfaces (such as nutrient agar). Thus far, their ability to replicate in a particular host appears limited to the genus *Enterococcus*; although in some cases they are able to transfer to (but do not establish autonomously in) other genera (Francia & Clewell, 2002). pAMβ1, and related elements (commonly 27-50 kb) that do not transfer well in broth, move at frequencies on the order of 10^{-4} or more per donor on solid surfaces. Significantly, these elements often exhibit a broad host range that enables them to move into and establish in a variety of Gram-positive species (Clewell & Francia, 2004; Grohmann, Muth, & Espinosa, 2003).

Sex pheromone systems

E. faecalis was the first bacterial species reported to utilize sex pheromones in the transfer of plasmid DNA—well over 30 years ago—when it was found that recipient bacteria secreted short peptides that were able to induce a mating response by donors that carried certain conjugative plasmids (Dunny, Brown, & Clewell, 1978; Dunny, Craig, Carron, & Clewell, 1979) (*E. faecalis* was designated *Streptococcus faecalis* at that time). Studies showed that such plasmids encode a response to a specific peptide, which leads to expression of a number of mating functions, including a protein "aggregation substance" (AS) that appears on the cell surface. AS facilitates mating-pair formation in liquid cultures, a phenomenon that visibly manifests as a "clumping together" of donors and recipients. AS binds to lipoteichoic acid, which is present on both recipients and donors (Ehrenfeld, Kessler, & Clewell, 1986). Exposure of donors to a culture filtrate of recipients for a few hours results in a self-clumping response facilitated by the synthesized AS; indeed, transfer of plasmid DNA even occurs between donors, although at a significantly reduced rate, as compared to transfer that involves plasmid-free recipients (Clewell & Brown, 1980). The clumping response proved to be useful in quantifying pheromone activity using a microtiter dilution assay (Dunny, Craig, Carron, & Clewell, 1979). AS was found not to be necessary for transfer if matings were conducted on solid surfaces; thus, its primary role in mating would appear to be the initiation of donor-recipient contact upon random collision of cells in liquid suspensions. AS was later found to also be a virulence factor in various pathogenicity models (see Enterococcal Disease, Epidemiology, and Implications for Treatment and Pathogenesis and models of enterococcal infection).

Plasmid-free strains of *E. faecalis* were found to secrete a number of different pheromones, each specific to a different family of conjugative plasmids. Some of the best-studied plasmid representatives are pAD1, pCF10, pPD1, and pAM373, with corresponding sex pheromones referred to as cAD1, cCF10, cPD1, and cAM373, respectively. Such plasmids are common among clinical isolates, and as many as three different pheromone-

responding plasmids have been identified in a single isolate (Clewell, Yagi, Ike, Craig, Brown, & An, 1982; Murray, An, & Clewell, 1988). When a given recipient acquires a copy of a pheromone-responding plasmid, the related pheromone is no longer detectable in culture supernatants; however, the transconjugants (now donors) secrete a plasmid-encoded peptide that acts as a competitive inhibitor of the pheromone. Inhibitors encoded by pAD1, pCF10, pPD1, and pAM373 are referred to as iAD1, iCF10, iPD1, and iAM373, respectively. These pheromones and inhibitors have all been characterized and represent hydrophobic, linear, octa- or hepta-peptides, and synthetic forms of the peptides are active at sub-micromolar concentrations (Clewell & Dunny, 2002). Figure 1 shows their amino acid sequences.

The pheromones derive from a segment of the signal sequences of lipoprotein precursors (Clewell, An, Flannagan, Antiporta, & Dunny, 2000) and result from processing by a lipoprotein signal peptidase and a metallopeptidase that is referred to as Eep (An, Sluvaik, & Clewell, 1999). The latter is a member of a large family of intramembrane proteases known as RIP (regulated intramembrane proteolysis), which are found in both prokaryotic and eukaryotic organisms (Brown, Ye, Rawson, & Goldstein, 2000). As for the plasmid-encoded inhibitors, the precursors correspond to a pre-peptide that resembles an unattached signal sequence, with only Eep being required for processing. There is an interesting exception with regard to processing in that the precursors of cAM373 and iAM373 appear to use a mechanism that does not require Eep; and an alternate Eep-like protein that might serve such a function has not been detected in *E. faecalis* genomes, based on amino acid homology with Eep. Chandler and Dunny (Chandler & Dunny, 2008) have reported evidence that the amino acid residues that precede the cAM373 moiety are likely responsible for the insensitivity of the cAM373 precursor to Eep. This finding was based on the construction of a hybrid pre-peptide that contains cCF10 and the upstream region of the cAM373 precursor; while Eep normally processes the precursor of cCF10, it was not active on the hybrid.

There is no current evidence that the lipoprotein moiety of the various pheromone precursors plays a role in the conjugation or related regulatory processes. In the cases of cAD1 and cAM373, mutations affecting only the lipoprotein component do not affect secretion of the pheromone; and these derivatives behave normally as recipients and donors (An & Clewell, 2002; Flannagan & Clewell, 2002). It is noteworthy, however, that a deletion of the *eep* determinant has been observed by Frank *et al.* (Frank, Barnes, Grindle, Manias, Schlievert, & Dunny, 2012) to severely impair pathogenicity of *E. faecalis* in a rabbit endocarditis model in a manner unrelated to plasmid content. Thus, Eep might be involved in the processing necessary for expression of one or more lipoproteins, if not for precursor-linked peptides that contribute to virulence.

Secretion of pheromones is reduced by a plasmid-encoded membrane protein, which is designated TraB in the case of pAD1 or homologue PrgY in the case of pCF10. Mutations in these related determinants result in increased secretion (An & Clewell, 1994; Chandler & Dunny, 2008; Ike & Clewell, 1984; Weaver & Clewell, 1990); TraB/PrgY, as well as Eep, are possibly associated with a general secretion complex of the host [see Rosch & Caparon, 2004]. Recent studies by Chandler and Dunny (Chandler & Dunny, 2008) have provided some insights into the activity of PrgY, as well as its interaction with cCF10. Interestingly, and perhaps related to the fact that pre-cAM373 does not get processed by Eep, pAM373 does not encode a TraB/PrgY homologue (De Boever, Clewell, & Fraser, 2000).

With regard to the uptake of pheromones, a specific plasmid-encoded surface-bound sensor/receptor protein (TraC encoded by pAD1 and PrgZ encoded by pCF10) specifically binds and "passes" the peptide to a host ABC oligopeptide transporter complex for internalization (Leonard, Podbielski, Hedberg, & Dunny, 1996; Tanimoto, An, & Clewell, 1993). The TraC/PrgZ protein appears to discriminate between the pheromone and the cognate inhibitor peptides, which results in a competitive inhibition that allows selective passage of the pheromone to the more generalized peptide uptake system (Leonard, Podbielski, Hedberg, & Dunny, 1996). However, there is evidence in the pCF10 system that iCF10 may enter the cell and bind competitively with cCF10 to the negatively regulating PrgX protein, which binds to a key promoter involved in regulation of the pheromone response (see

below). For a more detailed and illustrative discussions of pheromone biosynthesis, secretion, and uptake, see the references (Chandler & Dunny, 2008; Clewell, 2007).

cAM373 activity produced by different bacterial genera

It has been known for a number of years that sex pheromone cAM373 activity is also produced by the human pathogen *Staphylococcus aureus* and oral commensal *Streptococcus gordonii*, a component of dental plaque (Clewell, An, White, & Gawron-Burke, 1985). That is, culture filtrates of these species induce clumping by *E. faecalis* strains that specifically harbor pAM373. The related peptides are not identical to cAM373 but are similar (Fig. 1); and similar to the case in enterococci, the related precursors correspond to hydrophobic segments of signal sequences of pre-lipoproteins. The corresponding lipoproteins, however, are very different, and while there is no direct evidence that the peptides serve as pheromones within these non-enterococcal species, it has been shown that they are able to induce intergeneric plasmid transfer from *E. faecalis* (Francia & Clewell, 2002; Vickerman, Flannagan, Jesionowski, Brossard, Clewell, & Sedgley, 2010). While pAM373 is unable to replicate in *S. aureus* or *S. gordonii*, a vector that carries the origin of transfer from pAM373 can be readily mobilized by pAM373 and established as a replicating plasmid in a heterologous host.

Genetic analyses conducted in *S. gordonii* showed that an Eep homologue and a signal peptidase II used in processing of lipoprotein precursors are necessary for the production of detectable gordonii-cAM373 (Vickerman, Flannagan, Jesionowski, Brossard, Clewell, & Sedgley, 2010). As noted above, and contrary to the case in *S. gordonii*, the Eep identified in *E. faecalis* is not necessary for processing the enterococcal cAM373 precursor; this probably relates to differences in the amino acid residues upstream in the two precursors (Chandler & Dunny, 2008).

A potentially significant factor in the production of cAM373 activity by non-enterococcal pathogens, such as *S. aureus,* is the possibility of its involvement in the ever-feared acquisition of vancomycin-resistance, which is now frequently carried by *E. faecalis*. In this regard, at least one plasmid, pAM368, which carries a vancomycin-resistance determinant and encodes a cAM373-response, has been identified in an enterococcal isolate (Showsh, De Boever, & Clewell, 2001) and could be "poised" for transfer in a clinical setting. In a similar vein, *S. aureus* strains that harbor plasmids of the pSK41/pGO1 family secrete a cAD1 activity (in addition to cAM373) that is encoded as part of the signal sequence of the plasmid-encoded lipoprotein TraH precursor (Firth, Fink, Johnson, & Skurray, 1994; Firth, Fink, Johnson, & Skurray, 1994), which could facilitate the uptake of vancomycin-resistance determinants from *E. faecalis* through mobilization or transfer by a pAD1-like element (Garnier, Taourit, Glaser, Courvalin, & Galimand, 2000). While there has been evidence of a limited number of clinical cases of *S. aureus* acquiring high-level vancomycin resistance (VanA) from enterococci in recent years, a direct involvement of pheromone-responding plasmids has not yet been demonstrated (Flannagan, et al., 2003; Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008).

It is noteworthy that the pheromone-responding plasmid pCF10 has been reported to mobilize a small vector plasmid that carries an inserted pCF10-*oriT* site into two different genera, *Lactococcus lactis* and *Streptococcus agalactiae,* when matings were conducted on nutrient agar plates (Staddon J. H., Bryan, Manias, Chen, & Dunny, 2006). Since the related recipients are not known to produce cCF10 activity**,** the extent to which the conjugation system was induced is not clear. The fact that pCF10 itself did not establish in these hosts is consistent with the view that while transfer of pheromone-responding plasmids to a variety of other Gram-positive organisms may readily occur, their ability to replicate appears to be limited to enterococci.

A unique regulatory theme involved in the pheromone response

There is a basic similarity in the organization of pheromone-responding plasmids, in that determinants involved in replication and partitioning are located adjacent to a pheromone-response sensing and regulatory region that is, in turn, upstream of an extensive set of structural genes that manifest the overall mating process (see Figure 2). The latter stretch of conjugation genes can involve up to ~30 kb of DNA. Regulation involves both negatively

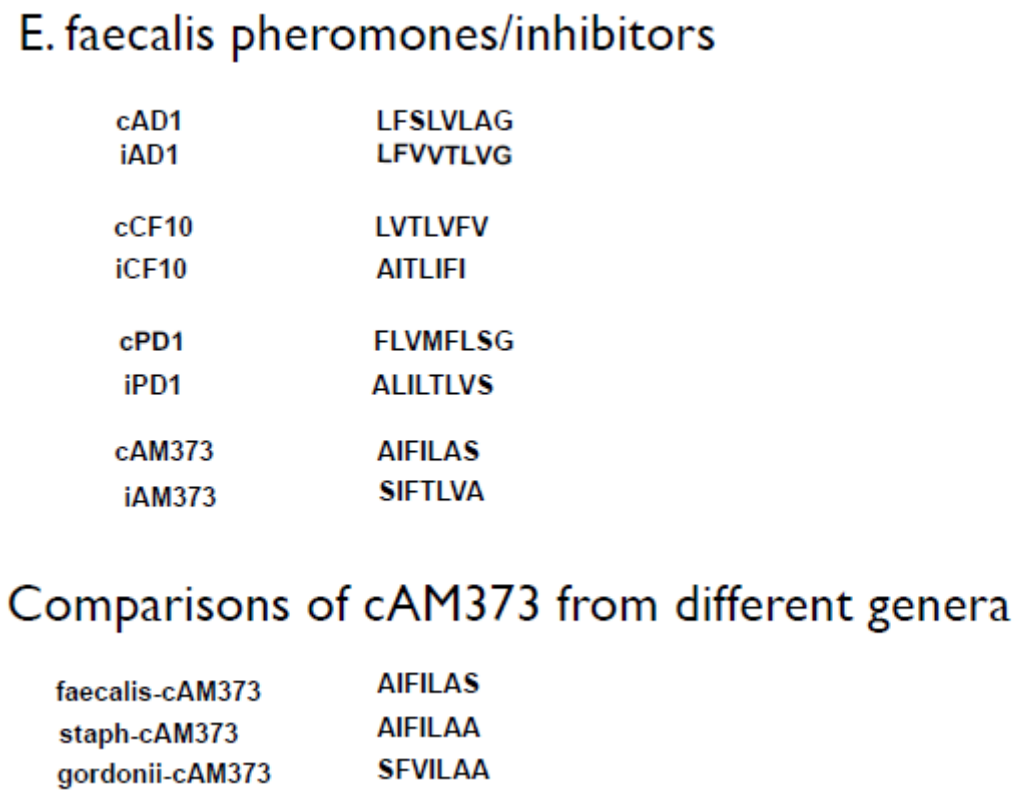

Figure 1. The structure of sex pheromones and cognate inhibitor peptides secreted by *Enterococcus faecalis*, as well as a comparison of the *E. faecalis* cAM373 sequence to those with similar activities secreted by *S. aureus* and *Streptococcus gordonii*.

and positively acting features, and structural genes that encode significant surface proteins, such as AS and the product associated with entry/surface exclusion (such as those involved in reduction of uptake of plasmid DNA from other donors), are usually among determinants that are close to the regulatory center. Based on hybridization analyses and sequence comparisons, significant homology exists between most pheromone-responding plasmids, particularly with regard to AS and the determinant for surface exclusion (Clewell & Francia, 2004; Hirt, Wirth, & Muscholl, 1996). An exception is pAM373, which encodes a significantly different AS and no surface exclusion determinant (De Boever, Clewell, & Fraser, 2000). A transfer origin (*oriT*) is generally located well downstream and close to a relaxase determinant. The plasmids pAD1 and pCF10 have been the most heavily studied over the years and have been comprehensively reviewed (Clewell, 2007; Clewell & Dunny, 2002; Dunny G. M., 2007; Dunny & Johnson, 2011). Based on analyses of these systems as well as significant studies of pPD1 (Folli, et al., 2008; Fujimoto, Tomita, Wakamatsu, Tanimoto, & Ike, 1995; Horii, Nagasawa, & Nakayama, 2002) and more limited analyses of pAM373 (De Boever, Clewell, & Fraser, 2000; Ozawa, De Boever, & Clewell, 2005), a consistent and somewhat unique regulatory theme has become evident.

As illustrated in Fig. 2, a key control feature relates to the expression and interplay (counter transcription) from two opposing promoters P_i and P_n, which ultimately controls transcription termination at a factor independent terminator, t. Because different systems utilize different terminology, the model here is presented in generic terms. P_i governs transcription of the inhibitor peptide precursor determinant, designated as *inh* (*iad* for pAD1 and *icf/prgQ* for pCF10). In the uninduced state, transcription from P_i occurs at a basal level that facilitates production of appropriate amounts of the inhibitor peptide (Inh) with termination occurring at t. The other promoter P_n is located downstream of *inh*, about 150–450 nt from the opposing P_i, depending on the particular plasmid. This promoter relates to the expression of a key regulatory protein Neg (TraA for pAD1, pPD1 and

pAM373, and PrgX for pCF10), whose determinant *neg* is located upstream of, and in the opposite orientation to, *inh*. Neg negatively regulates transcription from P_i through its interaction with at least two binding sites. In the presence of a pheromone, however, which binds to the C-terminal region of Neg (Fujimoto & Clewell, 1998; Shi, et al., 2005), the significant up-regulation of transcription from P_i occurs. In at least one system (pAM373), there is evidence that Neg remains associated with the transcription complex during induction (Ozawa, De Boever, & Clewell, 2005). In the uninduced state, transcription from P_n occurs at a high level; however, only a fraction of the transcription extends all the way through *neg*. A significant degree of termination of this transcript occurs relatively early and is apparently related to an as yet unrecognized terminator, which gives rise to a short (102 nt) RNA designated Anti-Inh that can assume an extensive secondary (cloverleaf) structure (Shokeen, Johnson, Greenfield, Manias, Dunny, & Weaver, 2010). An important function of Anti-Inh is its ability to enhance termination of the P_i transcript at t (Bae, Kozlowicz, & Dunny, 2004; Johnson, et al., 2010; Tomita & Clewell, 2000) through regional complementarity and consequent conformational alteration of the *inh* transcript.

During pheromone induction, transcription from P_n becomes greatly reduced, while up-regulated transcription from P_i extends beyond t, which ultimately results in the expression of the conjugation system. An important aspect of regulation concerns the need to make termination at t very "tight" when cells are not exposed to exogenous pheromone. This is because once transcription goes beyond t, it not only facilitates expression of down-stream conjugation functions, but it also results in significant up-regulation. For example, in the case of pAD1, the first determinant beyond t encodes a positively acting regulator, TraE1, that up-regulates itself, as well as certain down-stream genes. While Anti-inh contributes to termination at t, there are suggestions that Neg can also bind near t in some systems, which probably further enhances termination in the absence of pheromone (Clewell, 2007; Folli, et al., 2008). Some plasmids (*e.g.* pCF10 and pAD1) even have a second terminator (t2) closely behind t that probably helps decrease transcriptional leakage in the uninduced state.

Adding to the complexity of the overall process, there is also evidence that in the uninduced state, the P_i transcript interacts with the P_n transcript in such a way as to facilitate cleavage by RNAse III (cleaves double stranded RNA), which possibly facilitates some processing, as well as subsequent degradation (Johnson, Haemig, Chatterjee, Wei-Shou, Weaver, & Dunny, 2011). Finally, mathematical analyses of the pCF10 system has supported the view that transcriptional interference between RNA polymerase molecules that initiate at P_i and P_n is also a component of regulation (Chatterjee, et al., 2011).

With regard to the above regulatory pattern, it is important to note a report by Ibrahim *et al.* (Ibrahim, Nicolas, Bessières, Bolotin, Monnet, & Gardan, 2007) who, in a search of the data base for short, non-annotated ORFs within numerous bacterial genomes, were able to identify a new family of coding sequences for hydrophobic peptides (20-23 amino acids) located just upstream of determinants for positive transcriptional regulators of the Rgg family. Rgg was first identified as a regulator of glycosyl transferase synthesis in *S. gordonii* (Sulavik & Clewell, 1996; Sulavik, Tardif, & Clewell, 1992), but homologues have been subsequently identified in numerous low G+C Gram-positive species, and include proteins involved in the regulation of virulence and biofilm formation in *Streptococcocus pyogenes* (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011; Declerck, et al., 2007; Lyon, Gibson, & Caparon, 1998; Rocha-Estrada, Aceves-Diez, Guarneros, & de la Torre, 2010). Rgg relates to what now corresponds to a superfamily of cytoplasmic regulatory proteins that directly bind to peptide ligands (Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011; Mashburn-Warren, Morrison, & Federle, 2010), many of which relate to signaling cell density (namely, quorum sensing). Additionally, Neg proteins (e.g. PrgX and TraA) are included in this group. Members of this family do not necessarily exhibit significant amino acid homology; however, secondary and tertiary structure prediction algorithms reveal a high degree of similarity [see Chang, LaSarre, Jimenez, Aggarwal, & Federle, 2011]. Notably, high-resolution structures determined for the pCF10 Neg protein PrgX as an apo-protein, and in complex with its cognate pheromone and inhibitor peptides, represented the first successful structure determinations for any receptor for a bacterial intercellular-signaling peptide (Kozlowicz, Dworkin, & Dunny, 2006; Shi, et al., 2005). Thus, the Neg-Inh peptide aspect of

the pheromone response expression system described above bears a resemblance to what may be a widespread pattern of intercellular peptide communication control in Gram-positive bacteria.

Non-pheromone-responding plasmids that transfer efficiently in broth. The pMG1 family.

In 1998, Ike *et al.* (Ike, Tanimoto, Tomita, Takeuchi, & Fujimoto, 1998) reported the identification of a 65 kb plasmid, pMG1, that confers resistance to gentamicin (a Tn*4001*-like transposon) in *E. faecium* that would transfer relatively well to other *E. faecium* strains in broth, as well as to *E. faecalis* and *E. hirae*. Transfer frequencies were on the order of 10^{-4} per donor in 3–4 hour matings. Studies showed that a sex pheromone was not involved.

Donor-recipient aggregates were not generally visible to the naked eye, in contrast to systems that involved a pheromone response, although microscopic examination revealed the formation of aggregates (Ike, Tanimoto, Tomita, Takeuchi, & Fujimoto, 1998). In addition, unlike the case for pheromone-responding plasmids like pPD1 and pAD1, the aggregates were not dispersed upon EDTA exposure. It was then found that closely related plasmids, including those that carry high-level vancomycin-resistance (VanA) determinants associated with Tn*1546*–like elements, were common among antibiotic-resistant strains of *E. faecium* and *E. avium* (Tomita, Pierson, Lim, Clewell, & Ike, 2002; Tomita, et al., 2003). These plasmids exhibited incompatibility (namely, an inability to co-exist), and members of the group now constitute the pMG1 family. DNA hybridization studies showed no detectable homology with pheromone-responding plasmids or the broad host-range pAMβ1 and pIP501 plasmids (Ike, Tanimoto, Tomita, Takeuchi, & Fujimoto, 1998). It would appear that pMG1 family elements have contributed significantly to the spread of vancomycin- and gentamicin-resistance among enterococci, particularly within *E. faecium*. However, while they exhibit transfer between various species of enterococci, the extent to which they might transfer to different genera is not yet known.

pMG1 and the related pHTβ carrying a vancomycin-resistance transposon (Tn*1546*-like) have been sequenced and found to closely resemble each other (Tanimoto & Ike, 2008; Tomita & Ike, 2005). Conjugation-related determinants identified by BLAST analysis appear to span well over 30 kb, and there are similarities at the amino acid level to proteins encoded by pXO2 (10-50% identity with regard to 22 different proteins), a virulence-related plasmid carrying capsule determinants in *Bacillus anthracis*. In the *E. faecalis* host FA2-2, the presence of pHTβ is associated with a self-aggregation of cells, which are visible when allowed to grow overnight in broth. Genetic analyses suggested that this is related to the aggregation that occurs between donors and recipients during mating. The phenomenon was narrowed down to a region that spans 6 kb (Tomita & Ike, 2005), which contained 5 ORFs (an operon) with one (ORF10) being relatively large (1209 amino acids) and possibly encoding an "aggregation substance." There was no similarity of this protein to the AS of pAD1, pCF10, pPD1, *etc.*; however, it has exhibited amino acid homology with a surface protein encoded by pXO2. Analyses revealed additional regions that are important in the mating process, as well as a key positive regulator, *traB*. Based on BLAST analyses, there was evidence for the presence of a coupling protein (namely, a protein utilized in DNA transfer), a VirB11-family sec protein, a transfer origin, and a relaxase (Tomita & Ike, 2005).

Plasmids that transfer well on solid surfaces but not in broth (the Inc18 elements)

Broad host range conjugative enterococcal plasmids, which are transferable mainly on solid surfaces and not responsive to pheromones, and are typically between 26 and 50 kb, are usually members of what has been referred to as the Inc18 family, based on their incompatibility with each other (Horaud, Le Bouguenec, & Pepper, 1985; Jannière, Gruss, & Ehrlich, 1993) (see Table 1). Homologs can be found in a wide variety of bacterial genera [see Clewell & Francia, 2004; Grohmann, Muth, & Espinosa, 2003]. In enterococci, they typically encode resistance to Em (MLS), and often encode resistance to additional antibiotics. Representative enterococcal

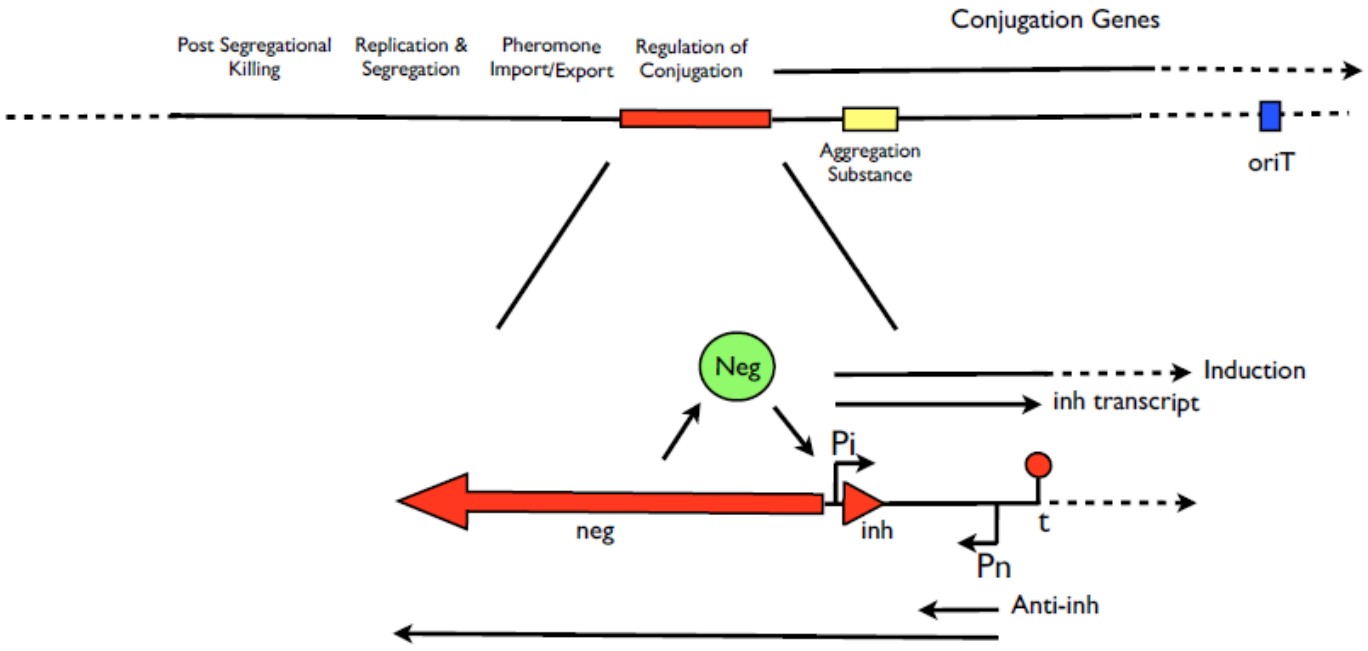

Figure 2. A generic model that focuses on key aspects of regulation of the plasmid-encoded sex pheromone response. The primary promoters are P_i and P_n, which govern expression of oppositely oriented transcripts. Neg (which corresponds to TraA or PrgX, depending on the particular plasmid system) acts on P_i to influence expression of *inh*, whose product corresponds to the inhibitor peptide. In the uninduced state, P_i expresses at a basal level, which allows the expression of Inh. Exposure of cells to sex pheromone results in the internalized peptide binding to Neg and up-regulating expression from P_i. This results in expression beyond the transcription terminator t and into the structural genes related to conjugation. P_n transcription is elevated in the absence of sex pheromone. It gives rise to a short transcript that enhances termination of the P_i transcript at t and a lower amount of an extended product that serves as the *neg* transcript. During induction, transcription from P_n ceases.

plasmids of this class are the *E. faecalis* plasmids pAMβ1 (28 kb) and pRE25 (50 kb), and related conjugation determinants closely resemble those of the *S. agalactiae* plasmid pIP501 (30 kb) (Grohmann, Muth, & Espinosa, 2003; Horodniceanu, Bouanchaud, Bieth, & Chabbert, 1976; Schwarz, PerretenV., & Teuber, 2001), whose transfer functions have been analyzed in greater detail. The host-range of the latter includes numerous Gram-positive genera, and appears to extend to the multicellular *Streptomyces lividans,* as well as the Gram-negative *Escherichia coli,* where both replication and conjugation genes appear functional (Kurenbach, Bohn, Prabhu, Abudukerim, Szewzyk, & Grohmann, 2003). Sequence comparisons show that there is significant similarity with the tra regions of the *S. aureus* plasmids pGO1 and pSK41, as well as the *Lactococcus lactis* plasmid, pMRC01 (Grohmann, Muth, & Espinosa, 2003).

pAMβ1 and pIP501 have been observed to transfer in the environment as well as in animal intestines (Byzov, et al., 1999; Igimi, Ryu, Park, Sasaki, Sasaki, & Kumagai, 1996; McConnell, Mercer, & Tannock, 1991; Morelli, Sarra, & Bottazzi, 1988). In mixed infections in humans, closely related plasmids appear to have been involved in the delivery of a Tn*1546*-like vancomycin-resistance transposon from *E. faecalis* to *S. aureus* (Flannagan, et al., 2003; Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008; Zhu, et al., 2010). It is noteworthy that the above-mentioned pRE25, which was originally identified in raw-fermented sausage, confers resistance to 12 different antibiotics (Schwarz, PerretenV., & Teuber, 2001; Teuber, Schwarz, & Perreten, 2003)—which supports

the view that Inc18 elements are likely involved in human acquisition of antibiotic-resistant bacteria from many sources.

Origins of transfer, relaxases and mobilization

As previously mentioned, conjugative plasmids generally have a specific origin of transfer (*oriT*) that corresponds to a site at which a plasmid-encoded relaxase binds, with the potential to introduce a strand-specific nick that initiates, and also terminates, the process of intercellular DNA transfer. A complex that consists of relaxase and accessory proteins associated with *oriT* is referred to as the relaxosome. In the case of enterococci, examples of plasmids where *oriT*s and relaxases have been identified include: i) pheromone-responding plasmids pAD1, pAM373 and pCF10; ii) a member of the pMG1 family; iii) the Inc18-type pIP501; and iv) the mobilizable plasmid pAMα1. A number of pheromone-responding plasmids are able to mobilize otherwise non-conjugative elements (Clewell, Yagi, Ike, Craig, Brown, & An, 1982; Murray, An, & Clewell, 1988; Oliver, Brown, & Clewell, 1977) as well as various cryptic elements (404), and there is strong evidence for the mobilization of non-conjugative elements facilitated by pAMβ1, pIP501, and related plasmids (Burdett, 1980). In addition, it is likely that the relaxase of certain plasmids might act on *oriT* sites that happen to be located on the bacterial chromosome (see the subsequent section on conjugative transposons and integrative conjugative elements). Interestingly, and for reasons not yet known, the pheromone responding plasmid pAD1 strongly inhibits the transfer of pAMβ1 if both are present in the same host (Clewell, Yagi, Ike, Craig, Brown, & An, 1982).

In the case of pAD1, there are two transfer origins, *oriT1* and *oriT2*; the latter of which is utilized about 1000-fold more efficiently (Francia & Clewell, 2002; Francia, et al., 2001). The *oriT1* site is located within the *repA* determinant (which is required for replication; see below) and exhibits strong similarity to the IncP-type of *oriT*s (8). The *oriT2* site closely resembles the single known *oriT* site of pAM373, both of which are located within a ~285 bp segment upstream and are relatively close to a relaxase determinant (designated *traX* for pAD1). When a fragment of DNA that contains the *oriT2* segment of pAD1 is cloned in an otherwise non-mobilizable vector, the chimera can be readily mobilized by the pAD1, but not by the pAM373 system, and vice versa. These *oriT* loci are unique compared to those of other characterized transfer systems. The *oriT nic* sites are located within large inverted repeat structures spanning about 140 bp, and specificity is based primarily on a series of adjacent 5–6 bp direct repeats that are separated by similarly-sized spacer sequences (Figure 3). The inverted repeats of pAD1 and pAM373 exhibit strong homology; however, the adjacent direct repeats are non-homologous, and there is strong evidence that they are involved in the binding of the specific relaxases (Francia & Clewell, 2002). The relaxases differ significantly from others that have been characterized, in that they are lacking the relatively common "3-histidine motif" (Francia, Varsaki, Garcillán-Barcia, Latorre, Drainas, & de la Cruz, 2004; Garcillán-Barcia, Francia, & de la Cruz, 2009). However, highly related pAD1/pAM373 relaxase-like sequences are readily found in databases that contain recently reported enterococcal genomes.

The pheromone-responding pCF10 bears *oriT* and relaxase determinants that are similar to those of a large superfamily of conjugation systems, which includes the staphylococcal pC221 and pC223 (53) and is completely different from those present in pAD1 and pAM373. The relaxase (PcfG) contains the more typical 3-histidine domain, and studies by Chen *et al.* (53) showed that the interaction of PcfG with *oriT* includes an accessory protein PcfF. Analysis at the protein and DNA levels of the specificity determinants for recognition of *oriT* by the PcfG relaxase and PcfF accessory protein allowed for a detailed model for coordinated DNA binding, unwinding, and site-specific nicking within the pCF10 *oriT* region. The DNA sequence that includes *oriT* (Fig. 3) and *pcfG* is very similar to that of the *Lactococcus lactis* plasmid pRS01. The relaxase determinant of pRS01 contains a group II intron within a segment identical to a region of *pcfG*, and studies showed that the intron could insert in the related pCF10 site through a homing mechanism (339). The data suggest a mechanism whereby group II introns may spread horizontally. A group II intron also has been identified on the pMG1-like plasmid pHTγ, although not within a relaxase determinant (365).

The *oriT* sites of the non–pheromone-responsive pMG1 and the closely related pHTβ are located in a noncoding region that is close, but not immediately adjacent, to a relaxase determinant (351, 362). In this case, the relaxase contains the three domains typical of many relaxases, including the three-histidine motif (135). The *oriT* region contains no significant similarity to those of other known classes of plasmids. It contains three direct repeats and two inverted repeats, one of which probably contains the *nic* site.

The pIP501 conjugation system uses an *oriT* site that resembles that of IncQ plasmids, and a *nic* site that is identical to that of RSF1010. Genetic studies on conjugation were initially conducted by Macrina and coworkers (197, 372), who identified the *oriT* site and showed that it could be specifically nicked by the product of the adjacent relaxase determinant *traA*. Grohmann and colleagues (199, 200) subsequently analyzed the system in some detail using an *E. faecalis* host background. They showed that *traA* is the first of 15 open reading frames that constituted an operon that was expressed at a similar level during various stages of growth (200). The relaxase was found to negatively regulate the operon, and the *oriT* nicking target overlapped the promoter of the operon (200). The relaxase, TraA, contains characteristic motifs and was shown to exist as a dimer in solution. With a full length of 654 amino acids, a 246-amino acid truncated (N-terminal) version, which also exhibits dimer formation, maintained nicking activity on DNA that contained the specific *oriT*. pIP501 and pAMβ1 have highly homologous relaxases (96% identity) and only slightly different *oriT* sequences (Figure 4). (See below for comments on the identification and association of the pIP501 conjugation with a Type IV secretion system.)

The interesting case of pAMα1 and the role of relaxase

E. faecalis strain DS5 harbors a nonconjugative multicopy plasmid pAMα1 (9.75 kb) that encodes resistance to tetracycline. It was originally of interest because of the generation of multiple tandem copies of its resistance determinant *tet*(L), when cells were grown protractedly in the presence of tetracycline (Clewell, Yagi, & Bauer, 1975; Yagi, Clewell, & Bauer, 1976). The phenomenon was shown to involve recombination between two directly repeated homologous, but not identical, ~380 bp recombination sequences, RS1 and RS2, which flanked *tet*(L) (Yagi & Clewell, 1977). It was subsequently reported that pAMα1 corresponded to a structure that resembled a cointegrate of plasmids similar to pBC16 (from *Bacillus cereus*) and pS86 (from *E. faecalis*), and that the two RS sequences probably arose as a result of a site-specific recombination between homologous sites on the original plasmids (Perkins & Youngman, 1983). The pBC16-like element, referred to as pAMα1Δ1, carries the *tet*(L) determinant and appears to be unable to replicate alone in the *E. faecalis* host. The pS86-like element was dubbed pAMα1Δ2. Amplification in pAMα1-containing cells during growth in the presence of tetracycline corresponds to the generation of tandem repeats of the pBC16-like component, separated by RS sequences. The RS sequences were eventually found to represent *oriT* sites. pAMα1 encodes two relaxases (MobB and MobE) that are encoded by the two components of the cointegrate (Francia & Clewell, 2002). Each is able to facilitate mobilization through its interaction with one or both *oriT* sites, when present with a conjugative plasmid, such as pAD1. Both MobB and MobE are members of the pMV158 family of relaxases, and both RS1 and RS2 contain regions that resemble the pMV158 *oriT* sites (Fig. 4).

The pAMα1 amplification was shown to begin with a site-specific recombination between RS1 and RS2 that is catalyzed by either MobB or MobE (Francia & Clewell, 2002). However, after the initial generation of two copies of the *tet*(L)-containing segment, additional amplification is accomplished by homologous (RecA-dependent) recombination between the larger redundant DNA segments (Yagi & Clewell, 1980). Site-specific recombination activity of relaxases has been previously reported (Broome-Smith, 1980; Llosa, Bolland, Grandoso, & de la Cruz, 1994); the TraX relaxase of pAD1 was found to exhibit such an activity (Francia & Clewell, 2002).

The above system illuminates multiple functions that a site-specific recombination system, which involves a relaxase in this case, can serve: 1) Initiation of plasmid transfer; 2) Facilitation of plasmid cointegration, which could subsequently enable transfer and establishment of a component plasmid that is otherwise not able to replicate in a particular host; and 3) Initiating the amplification of tandem copies of a gene.

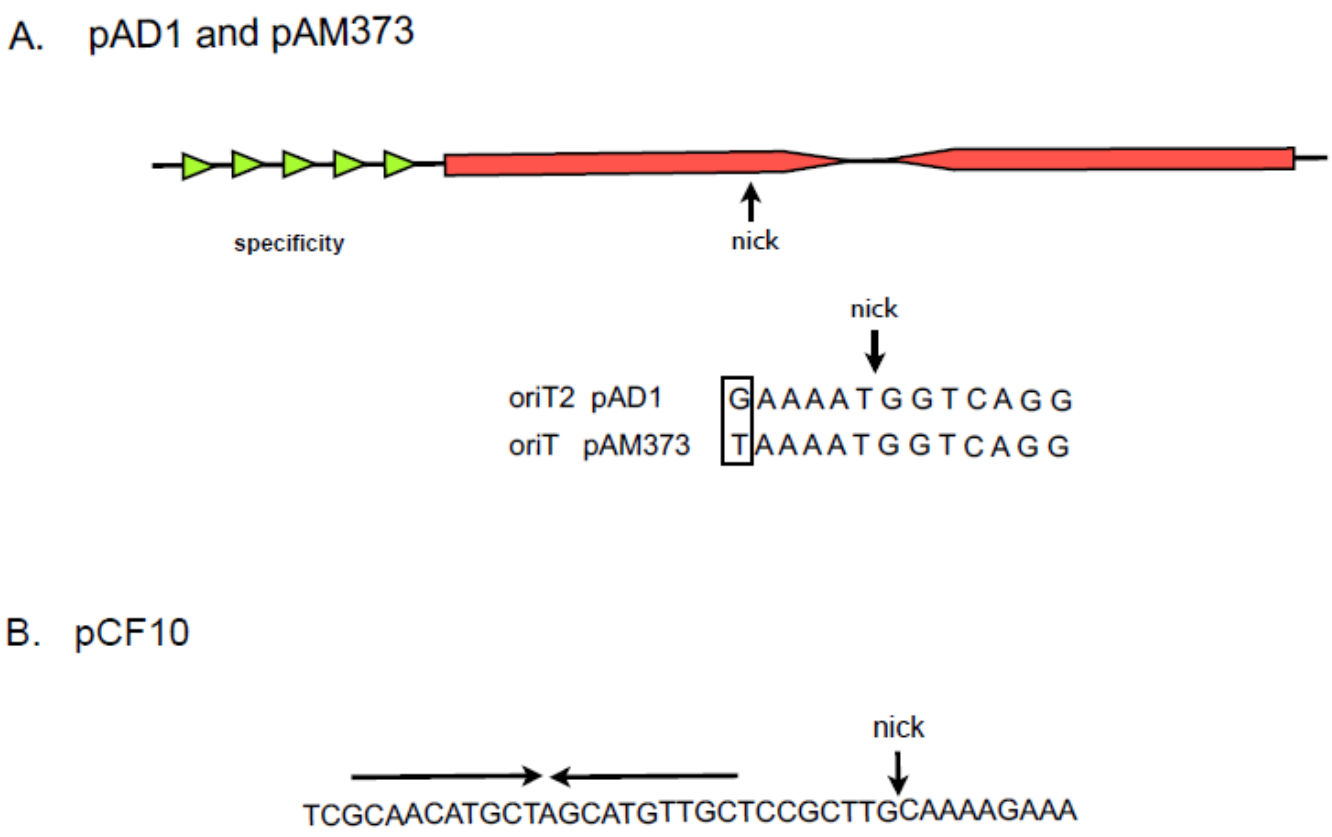

Figure 3. The *oriT* sites of three sex pheromone plasmids with the corresponding nick sites indicated by the arrows. A. The red indicates a large inverted repeat structure that is similar for both pAD1 and pAM373. The green represents short direct repeats that differ for pAD1 and pAM373 and relate to the specificity of binding of the corresponding relaxases. B. The *oriT* for pCF10 is very different from pAD1 and pAM373, as indicated by the nick site located outside of a much shorter and very different inverted repeat.

On the presence of Type IV secretion systems

The structures involved in DNA transfer constitute a divergent Type IV secretion system (T4SS). A key feature of T4SS is the intercellular transfer of a protein substrate (a DNA-protein in the case of conjugation). What is known about Type IV-mediated DNA transfer comes primarily from studies of plasmids in Gram-negative bacteria (Cascales & Christie, 2003; Zechner, et al., 2000), but it has become evident in the past ten years that Gram-positive systems, including enterococci, make use of analogous systems. This was first recognized based on comparative analysis of DNA sequence data (Grohmann, Muth, & Espinosa, 2003), whereas an understanding of protein organization and function for the Gram-positive systems has emerged more slowly. A combined genetic (yeast 2-hybrid analysis) and biochemical (pull-down) analysis of the pIP501 conjugation system established that the protein products of the *tra* operon interact to form a T4SS machine (Abajy, et al., 2007). However, the proteins involved in the Gram-positive T4SS are surprisingly divergent from counterparts of Gram-negatives, which is likely the result of major differences in the cell envelopes that are traversed by the DNA.

A highly conserved T4SS component, termed a coupling protein, interfaces with the relaxosome and a complex involved in mating pair formation (MPF) (Cascales & Christie, 2003). In the case of enterococcal plasmids, homologs of coupling proteins include: Orf10 of pIP501 (Abajy, et al., 2007); TraW (Orf53) of pAD1 (Francia &

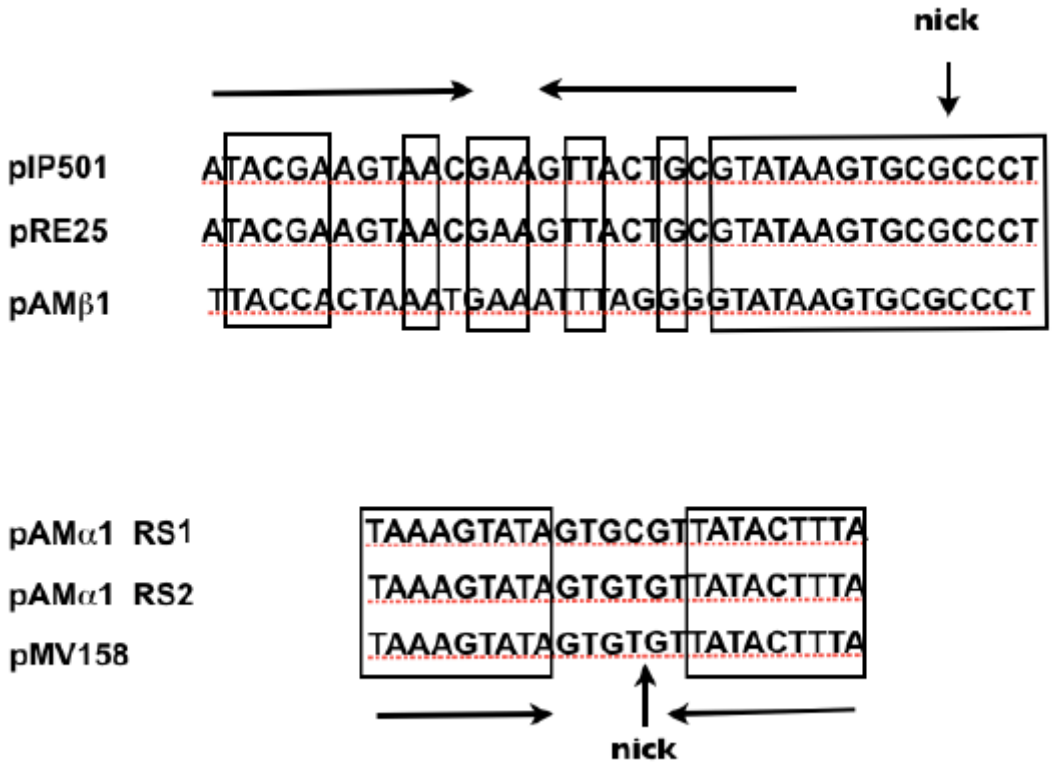

Figure 4. The *oriT* sites of several enterococcal plasmids. The horizontal arrows represent inverted repeats and the vertical arrows indicate the nick sites.

Clewell, 2002); PcfC of pCF10 (53); and Orf22 of pHTβ (a pMG1-type plasmid) (Tomita & Ike, 2008). Studies of pCF10 demonstrated that PcfC is indeed the functional coupling protein that physically links the relaxosome with the T4SS machine and leads to ATP-dependent transfer through the mating channel (Chen, Zhang, Manias, Yeo, Dunny, & Christie, 2008). Common and unique features of T4SSs from Gram-positive and Gram-negative bacteria have recently been detailed (Alvarez-Martinez & Christie, 2009).

Conjugative transposons and integrative conjugative elements

Conjugative transposons are widespread in bacteria and have been found in numerous genera, including in both Gram-negative and Gram-positive bacteria. The prototype, Tn*916*, was first identified in *E. faecalis* DS16, and consists of an 18 kb element that encodes resistance to tetracycline/minocycline [Tet(M)]. It was originally recognized as a transposon because of its ability to insert at multiple sites on the co-resident plasmid pAD1 (Franke & Clewell, 1981; Gawron-Burke & Clewell, 1982). Conjugative transposons have a remarkably broad host range (including Gram-negatives), due to the presence of comparatively few restriction sites, an anti-restriction system, and proteins that function in multiple host backgrounds. The properties of Tn*916* and its extended family of related elements have been reviewed in detail (Clewell, Flannagan, & Jaworski, 1995; Clewell & Gawron-Burke, 1986; Rice L. B., 1998; Roberts & Mullany, 2010; Roberts & Mullany, 2011; Scott & Churchward, 1995).

Tn*916*-like elements are usually found to be integrated in the bacterial chromosome, although they can occur on low-copy plasmids (Clewell & Francia, 2004). They are characterized by their ability to excise, which generates a circular intermediate that is non-replicative but is able to transfer conjugatively to a recipient by a plasmid-like mechanism. Excision of Tn*916* has been shown to require the activity of a specific integrase (Int_{Tn916}), a

member of the lambda family, and excisionase, Xis (Su & Clewell, 1993), and is accomplished by a mechanism that has been well characterized (Hinerfeld & Churchward, 2001; Jia & Churchward, 1999; Rocco & Churchward, 2006). The transposon is generally flanked by 6-bp coupling sequences (usually not identical) that may influence the rate of excision (Caparon & Scott, 1989; Jaworski & Clewell, 1994). Int_{Tn916} brings the ends of the transposon together by binding to direct repeats (DR2) located within each terminus. It then generates a staggered cut, which gives rise to a 6-bp "core" (heteroduplexed if the flanking coupling sequences differ), that enables the ends to join to form the excised circular intermediate.

Intercellular transfer is initiated by a transposon-encoded relaxase (Orf20) that acts at an *oriT* site (adjacent to the relaxase determinant) on the circular intermediate (Jaworski & Clewell, 1995; Rocco & Churchward, 2006). BLAST searching has not resulted in any hits against known relaxases; however, similarity to members of the Rep_trans superfamily of replication initiators has been noted (Garcillán-Barcia, Francia, & de la Cruz, 2009). Int_{Tn916} also binds within *oriT* and facilitates recognition of the specific *nic* site by the relaxase (Rocco & Churchward, 2006). Orf21, encoded on the other side of the *oriT* from the relaxase determinant, is a member of the FtsK/SpoIIIE family, and therefore may correspond to a coupling protein, as such proteins are distantly related [see (228)]. In any case, support for the involvement of a T4SS system in Tn*916* conjugation is also provided by Orfs 14-16, which exhibit similar sequences to products that participate in such a process.

Upon entering the recipient with a single strand transferring and the complementary strand being synthesized in the recipient, a double-stranded circular form then inserts into the genome through a mechanism that resembles the reverse of excision. Insertion of the transposon occurs with relatively low target specificity at sites that are generally AT-rich. Transfer frequencies can range from 10^{-9} or less to as high as 10^{-4} per donor, depending on the particular insertion in the donor. Tn*916* contains 26-bp terminal inverted repeats (identity at 20 of 26 bp), which provide an AT-richness that may facilitate the alignment of intermediates with the AT-rich target sites. Transfer studies in the laboratory are generally conducted on solid surfaces, where frequencies are 1-2 orders of magnitude higher than in broth (Jaworski & Clewell, 1994).

Transfer of Tn*916* often gives rise to multiple insertions; indeed as many as half of the transconjugants in a given mating experiment may exhibit more than one and as many as six insertions in different sites (Gawron-Burke & Clewell, 1982). When more than one copy is present in a donor, the transfer of one may activate in *trans* the transfer of another (Flannagan & Clewell, 1991). The basis of multiple insertions is not known, although at least two possibilities can be considered: 1) during intercellular DNA transfer, replacement replication from the 3′ end within the donor might result in multiple rounds that, in the recipient, give rise to dimeric/oligomeric forms that subsequently resolve with monomers inserting at different sites; and 2) an initial insertion may take time to stabilize, during which it might, after the chromosomal replication fork has passed through it, undergo an intracellular transposition into a new target.

Rice et al. (Rice L. B., Carias, Rudin, Hutton, & Marshall, 2010) have identified a clinical isolate of *E. faecium* (C68) that contains three closely-related functional Tn*916*-like elements, which suggests that the acquisition of multiple copies can occur naturally. In a different clinical isolate of *E. faecium* (D344R), the same group (Rice L. B., Carias, Marshall, Rudin, & Hutton-Thomas, 2005) reported the presence of an 18 kb Tn*916*-like element, together with a heterologous, functional 29 kb Tn*916*-like element (Tn*5386*). In this case, they showed that the integrase from either could act on both transposons, giving rise to a deletion of the entire 178 kb of DNA between the two elements (Rice L. B., Carias, Hutton-Thomas, & Rudin, 2007)—a segment that contained a determinant, *pbp5*, that is associated with increased resistance to ampicillin. One could envision a case whereby the entire 178 kb segment along with one (or even both) of the flanking transposons could transfer as a unit, which enables the movement of the ampicillin-resistance trait. The reader is referred to a later section of this chapter for a broader and more epidemiologically-related description of conjugative transposons in enterococci.

As noted earlier, analyses of complete genome sequences, which are continuing to be reported at ever-increasing rates, are revealing the great extent to which horizontal gene transfer occurs in the bacterial world. Large

segments of DNA that are being identified as components of the bacterial chromosome appear to have been "acquired" from an unrelated source;, and in some cases they appear to be able to conjugate. Such segments, which can correspond to well over 100 kb, are turning out to be ubiquitous and quite diverse (Guglielmini, Quintais, Garcillán-Barcia, de la Cruz, & Rocha, 2011; Wozniak & Waldor, 2010); and they frequently bear multiple determinants for integrases and insertion sequences, as well as genes that resemble those involved in conjugative transfer. Putative *oriT* sites, relaxase determinants and plasmid-like conjugation genes—sometimes even similar to those found in Tn*916*—have also been identified. The presence of such determinants has given rise to the term "integrative conjugative elements" (ICEs), although direct demonstration of such transfer has not always been possible. The additional presence in ICEs of genes that facilitate survival or the ability to take advantage of a new environment is common, with determinants that encode antibiotic resistance and virulence being good examples. Conjugation frequencies can be quite low (such as 10^{-10} to 10^{-9}); and insertion into the recipient genome is often into a specific site, such as a tRNA determinant. Secondary transfer events observed in the laboratory sometimes occur at much higher frequencies, which suggests that the initial transfer event may have been associated with an alteration/rearrangement of the element or help from a resident conjugative plasmid. "Pathogenicity island" (PAI) and "genomic island" are commonly used terms to describe these entities, which may or may not be capable of conjugation without a "helper" system (like a mobilizing plasmid). Many of these "islands" include modules derived from plasmids, transposons and/or bacteriophages. Some excellent reviews of ICEs can be found in the literature (Burrus, Pavlovic, Decaris, & Guédon, 2002; Guglielmini, Quintais, Garcillán-Barcia, de la Cruz, & Rocha, 2011; Osborn & Böltner, 2002; Wozniak & Waldor, 2010).

In enterococci, a PAI was initially identified in E. faecalis strain MMH594; it corresponded to about 150 kb, contained numerous putative virulence determinants and 11 transposases/IS elements, and was flanked by 10-bp direct repeats (Shankar, Baghdayan, & Gilmore, 2002). Similar islands have been identified in E. faecium (Leavis H. L., et al., 2003; Oancea, Klare, Witte, & Werner, 2004). Many strains of enterococci associated with nosocomial infections have since been shown to contain PAIs, although with significant variability; and genomic analyses have suggested the involvement of a modular accretion in their evolution (McBride, et al., 2009; McBride, Fischetti, Moellering, Jr., & Gilmore, 2007). In the case of the MMH594 strain, a ~28 kb segment that contains virulence determinants from within the PAI was shown to excise, circularize, and transfer at a very low frequency (10-10 per donor) to the plasmid-free E. faecalis strain OG1RF; however, secondary transfer occurred at a high frequency (10-1 per donor) and was shown to involve a pheromone-like plasmid into which the segment integrated (Coburn, Baghdayan, Dolan, & Shankar, 2007). The transfer of PAI components between E. faecalis and E. faecium has also been reported (Coburn, Baghdayan, Dolan, & Shankar, 2007; Oancea, Klare, Witte, & Werner, 2004).

Although most reports appear to demonstrate the movement of only internal segments of PAIs, Laverde-Gomez et al. (Laverde-Gomez, et al., 2011) have recently shown that an entire PAI (~200 kb) in *E. faecalis* UW3114 was able to precisely excise, transfer, and integrate site-specifically into the chromosome of *E. faecalis*, as well as *E. faecium*. (It is noteworthy that along with precise excisions, imprecise excisions of internal segments of the PAI were also detected in the donor.) A 66-kb pheromone-responsive plasmid pLG2 (which encoded resistance to erythromycin) was transferred in parallel and was probably involved in promoting transfer of the PAI (Laverde-Gomez, et al., 2011). Manson et al (Manson, Hancock, & Gilmore, 2010) reported that in the case of *E. faecalis* V583 transfer of PAI sequences was accompanied by a resident plasmid (pTEF1 or pTEF2). They also observed plasmid-promoted transfer of DNA outside the PAI that involved determinants widely scattered around the chromosome, which is a finding that is consistent with earlier reports in enterococci, where pheromone-responding plasmids were found to mobilize certain chromosome-borne mutational markers (Franke & Clewell, 1981; Franke, et al., 1978).

Inasmuch as PAIs in enterococci represent a variety of segments that carry different clusters of genes, including conjugation-related determinants, integrases, transposons, and IS sequences, it is likely that there are multiple ways by which a given "island," part of it, or even segments that include a region outside of the island, could be

excised and mobilized. Multiple IS sequences, as well as resident plasmids (for example, strain V583 carries at least ten *IS256* sequences, ten *IS1216*s, and six *ISEf1*s, as well as three plasmids) might easily participate in the incorporation, rearrangement, or deletion of segments of DNA in the ever-changing PAI mosaic.

Plasmid Maintenance

Replication

Bacterial plasmids require three basic components for replication within their host cells: 1) a replication initiator, which serves to recruit the cellular DNA replication machinery; 2) an origin of replication, at which the initiator acts; 3) a negative regulatory circuit that serves to limit the frequency of replication. These three components comprise the minimal replicon and will be the focus of this section of the chapter.

A recent survey of plasmid replicons in enterococci identified eleven classes of replicons, based on sequence homology and phylogeny of the initiator proteins (185). These plasmids can be further grouped into four families, based on sequence homology and the presence of conserved domains. The RepA_N plasmid family encodes initiators with the RepA_N domain in their N-terminus, usually within the first 80 amino acids. This family includes the pheromone responsive conjugative plasmids of *E. faecalis*, as well as the pRUM-related plasmids of *E. faecium* (Weaver, Kwong, Firth, & Francia, 2009).

The Inc18 family (Brantl, Behnke, & Alonso, 1990) includes the well-studied broad host range plasmids pAMβ1, pIP501, and pSM19035, and provides the backbone of many cloning vectors used in the enterococci. The initiators of this plasmid family include the PriCT_1 domain, which is associated with primases, although these initiators have not been shown to have primase activity. The RCR family (Khan, 2005) is a large group of ubiquitous plasmids that replicate through a rolling circle mechanism. Several classes of RCR initiators have been detected in the enterococci. The Rep_3 family of replicons includes several plasmids detected in both *E. faecalis* and *E. faecium*, but these plasmids have not yet been well studied in these species. However, the Rep_3 domain is conserved in the initiators of many well-studied enterobacterial plasmids, and may provide a model for studies of those identified in enterococci. The details of what is known about the enterococcal members of these plasmid families, as well as what we may infer about their function from relatives in other species, will be discussed below.

RepA_N plasmids

The RepA_N plasmid family is broadly distributed in the low G+C Gram-positive bacteria, including both conjugative and non-conjugative plasmids that range in size from the 3.3 kb *Lactobacillus helveticus* plasmid pLJ1 (Takiguchi, Hashiba, Aoyama, & Ishii, 1989) to the 281 kb *E. faecium* plasmid pLG1 (Laverde-Gomez, et al., 2011). Proteins that contain the RepA_N conserved domain have also been associated with streptococcal bacteriophages and ICE elements, although their precise function on these elements is unclear (Weaver, Kwong, Firth, & Francia, 2009). While the replicon family appears to be widespread, individual plasmids have a relatively narrow host range that is frequently restricted to their species of origin. Phylogenetic analysis further suggests that they have not been recently disseminated by horizontal transfer (Weaver, Kwong, Firth, & Francia, 2009).

The RepA_N initiator proteins have a tripartite domain organization (Weaver, Kwong, Firth, & Francia, 2009). The N-terminal domain is the most highly conserved among family members and contains the eponymous conserved domain. The C-terminal domain is relatively poorly conserved across phylogenetic boundaries, but is better conserved among plasmids present in the same genus. This pattern of conservation suggests that the N-terminal domain performs some essential function common to all plasmid family members, such as DNA binding and/or strand separation, while the C-terminal domain performs some host-specific function. In plasmids of Gram-negative bacteria, such host specificity has been associated with interaction with host

replication proteins, particularly the loading of the host replicative helicase, DnaB (195). The C-terminal domain of the RepA_N plasmids could perform a similar function.

The central domain of the RepA_N initiator proteins is highly variable and the corresponding genes contain multiple complex nucleotide repeats, which usually consist of short 5-12 nucleotide (nt) inverted repeats that are embedded in longer direct repeats. In some cases, the repeats are translated into amino acid repeats, but in others they are not, which suggests that it is the DNA sequence and not the protein sequence that is important. This region has been shown to function as an origin of replication *in vivo* in several plasmids, including the prototype pheromone-responsive plasmid pAD1 (Francia, Fujimoto, Tille, Weaver, & Clewell, 2004). *In vitro* studies demonstrated that pAD1 RepA_N protein RepA binds to the inverted repeats within the origin region and that the conserved N-terminal domain is sufficient for binding. The C-terminal domain, including two of the repeats, did not bind the origin (Francia, Fujimoto, Tille, Weaver, & Clewell, 2004). Interestingly, in several RepA_N family plasmids, including the pheromone-responsive plasmids pAD1 and pCF10 and the non-conjugative *E. faecium* plasmid pRUM, the inverted repeats converge on a poly-A tract of 3-5 nt. In the *S. aureus* RepA_N plasmid pSK41, RepA has been shown to leave a single A unprotected in binding studies (Kwong, Skurray, & Firth, 2004). This suggests that the structure of the repeats is important for function, but precisely what this function is remains unclear. The origin repeats have been demonstrated to function as incompatibility determinants in pAD1 (Francia, Fujimoto, Tille, Weaver, & Clewell, 2004), the *Bacillus subtilis* plasmid pLS32 (Tanaka & Ogura, 1998) and the *S. aureus* plasmid pSK1 (Kwong, Lim, Lebard, Skurray, & Firth, 2008), which suggests that the high sequence variability within this region may be required to prevent competition between related plasmids in the same host.

The molecular events that allow RepA_N proteins to facilitate replication initiation at their origins of replication are as yet unknown. It has been demonstrated that pAD1 RepA has non-sequence specific single stranded DNA binding activity, in addition to its sequence specific double stranded DNA binding activity (Francia, Fujimoto, Tille, Weaver, & Clewell, 2004). Similar activities have been observed in the unrelated initiator proteins of the Inc18 family plasmids, where single-stranded DNA binding facilitates strand separation (Le Chatelier, Jannière, Ehrlich, & Canceill, 2001). Origin function apparently requires multiple *rep*-associated repeats, since recombination between the two large repeats in pAD1 eliminates origin function (Francia, Fujimoto, Tille, Weaver, & Clewell, 2004). Note that the RepA protein retains initiator function, despite an in-frame 35-codon deletion.

The mechanism of copy number control in the enterococcal RepA_N plasmids is also unknown**.** In pSK41, an antisense RNA has been implicated in regulating translation of the RepA protein (Kwong, Lim, Lebard, Skurray, & Firth, 2008; Kwong, Skurray, & Firth, 2006; Kwong, Skurray, & Firth, 2004) but an examination of the pAD1 sequence in the analogous region showed no evidence of a similar regulatory RNA on this plasmid (Weaver, Kwong, Firth, & Francia, 2009) In pLS32, mutations in some of the repeats increase the copy number of the plasmid. Similar mutations have not yet been made in the enterococcal RepA_N origin regions (Tanaka, Ishida, & Maehara, 2005). Interestingly, there are several potential binding sites for the RepC partition protein, similar to the iterons involved in partition [see the following section and Francia et al. (Francia, Weaver, Goicoechea, Tille, & Clewell, 2007)], in the putative promoter for pAD1 RepA, but it remains unknown whether these binding sites are actually bound by RepC or are important for regulation.

Inc18 plasmids

The term "incompatibility group" was traditionally applied to plasmids whose replication functions were so closely related that they could not be maintained together in the same cell culture, due to competition for limiting replication and/or stability components (Novick, 1987). In keeping with this traditional definition, the Inc18 incompatibility group originally included plasmids pIP501 from *Streptococcus agalactiae*, pSM19035 from *Streptococcus pyogenes*, and pAMβ1 from *E. faecalis* (Brantl, Behnke, & Alonso, 1990). Since that time, the terms Inc18-like or Inc18 family have been used, perhaps inappropriately, to refer to plasmids that encode replication

initiators with sequence homology to the initiators of the original three plasmids of the group. While this homology probably indicates a similar replication initiation mechanism, it does not imply that the replicons are closely related enough to be truly considered incompatible. With this caveat in mind, the term Inc18 plasmid family will be used below to refer to plasmids with related initiator proteins, without regard for their actual incompatibility properties.

The Inc18 plasmid family is comprised of relatively low copy number (<10 copies/cell) plasmids that range in size from 25 to 50 kb and frequently carry multiple antibiotic-resistance genes. They have a broad host range and many are self-transmissible. As noted in an earlier section, they appear to be particularly important in the spread of vancomycin resistance both within the enterococci and to other species (Flannagan, et al., 2003; Freitas, et al., 2013; Freitas, et al., 2012; Freitas, et al., 2010; Rosvoll, et al., 2010; Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008). A recent PCR-based screening of enterococcal isolates indicated that Inc18 family plasmids were the most common plasmids in both *E. faecalis* and *E. faecium* (Jensen, Garcia-Migura, Valenzuela, Løhr, Hasman, & Aarestrup, 2010). In addition, their replicons form the backbone of some of the most broadly used cloning vectors in the enterococci.

Another class of plasmids that may be related to the Inc18 family is the pMG1/pHT plasmids. While these plasmids have not been reported to have a broad host range, the only replication initiator protein homolog identified in the pMG1 sequence is approximately 30% identical to Inc18 initiators (Tanimoto & Ike, 2008) and includes the PriCT_1 domain. This protein was shown to be essential in supporting the replication of a mini-plasmid in *E. faecalis*, perhaps due to its function at an essential inverted repeat sequence. Sequence differences or alterations in origin function may limit the host range of these replicons, relative to the better-studied Inc18 replicons.

Studies on the replication of Inc18 family plasmids have focused primarily on the original three members of the group. Most of this work was performed in the model Gram-positive bacterium *B. subtilis*, because of its amenability to genetic manipulation, but the plasmids in enterococci most likely replicate by a similar mechanism. Nomenclature is somewhat confusing, since homologous proteins on the three plasmids have different letter designations, despite the fact that the replicons share an overall >92% DNA sequence homology (Lioy, Pratto, de la Hoz, Ayora, & Alonso, 2010). For simplicity, initiator and copy control proteins for all three plasmids will be designated Rep and Cop, respectively, and the regulatory RNA will be referred to as *ct* for countertranscript. It will be assumed that the molecular mechanisms of replication and copy control described for one plasmid apply to all three.

Replication initiation of Inc18 plasmids requires the Rep protein, a small origin region located immediately downstream of the *rep* gene, a transcription fork that passes through the replication origin, and the host DNA Pol I (Bruand, Le Chatelier, Ehrlich, & Jannière, 1993). The replication primer is provided by a transcript that initiates from the *repE* promoter and passes through the origin region (Bruand & Ehrlich, 1998). Rep has both sequence-specific dsDNA binding and non-specific ssDNA binding capabilities. It uses the former to bind to the origin region, which causes bending and supercoiling-dependent melting, while it uses the latter to extend and stabilize the single-stranded bubble within the origin. The *rep* transcript then passes through the replication bubble and is processed in a poorly defined Rep-dependent manner to provide the primer for DNA Pol I-dependent replication (Le Chatelier, Jannière, Ehrlich, & Canceill, 2001). Replication proceeds for approximately 150 bp until an *ssiA* site for *priA*-dependent priming of lagging strand synthesis is exposed. At this point, DNA Pol I is replaced by DNA Pol III, which directs simultaneous unidirectional replication of both strands away from the *rep* gene.

The Rep proteins are rate-limiting for replication and as a result, their production must be tightly regulated. Regulation of Rep synthesis occurs by the concerted action of two regulators, an antisense RNA, *ct*, and a transcriptional repressor, Cop. Binding of *ct* to the 5′ untranslated region of the *rep* message causes a refolding of the nascent transcript that results in the premature termination of transcription (Brantl, Birch-Mirschfeld, &

Behnke, 1993; Heidrich & Brantl, 2007; Le Chatelier, Ehrlich, & Jannière, 1996). Cop acts as a transcriptional repressor at the *rep* promoter by competing with RNA polymerase binding (Brantl, Birch-Mirschfeld, & Behnke, 1993; Le Chatelier, Ehrlich, & Jannière, 1996; Licht, Freede, & Brantl, 2011). In addition, Cop-mediated repression of the *rep* promoter reduces transcriptional interference with the convergent *ct* promoter, which indirectly increases the production of *ct* and tightens the repression of replication (Brantl & Wagner, 1997). When the copy number decreases, decreased Cop levels result in an increase in *rep* transcription and a concommitant decrease in the production of *ct*. This extra layer of control is essential because, unlike the regulatory RNAs of most other plasmids, *ct* is relatively stable and therefore does not directly reflect the plasmid copy number (Brantl & Wagner, 1996). The cooperativity of this system is indicated by the fact that interference with either component alone results in a 10-20 fold increase in copy number, but simultaneously disabling both Cop and *ct* has no further effect (Brantl & Behnke, 1991). Another layer of regulation is provided by the plasmid-encoded ω protein, which represses the synthesis of Cop (de la Hoz, et al., 2000). ω also binds to the promoters for genes involved in active partition and post-segregational killing, and presumably coordinates replication and stable inheritance functions.

Because plasmids are commonly present in multiple copies in bacterial cells and those copies are 100% identical, they are frequently subjected to recombination that results in multimerization. Because copy control systems count numbers of origins rather than the number of plasmid molecules, and plasmids with multiple origins have a replication advantage, systems to resolve multimers are an important adjunct to copy control mechanisms (Summers, Beton, & Withers, 1993). In Inc18 plasmids, this function is accomplished by β recombinase, which acts at a resolution site that is located immediately upstream of its gene (Alonso, Ayora, Canosa, Weise, & Rojo, 1996). In addition to its role in multimer resolution, β also has been implicated in the switch from DNA Pol I to DNA Pol III-directed DNA replication at *ssiA* (Jannière, Bidnenko, McGovern, Ehrlich, & Petit, 1997). Binding of β to its target presents a roadblock to continued DNA Pol I-directed replication, and the resolvase site is appropriately positioned to facilitate primosome assembly at *ssiA*. The product of the *top* gene has also been implicated in the polymerase switch (Bidnenko, Ehrlich, & Jannière, 1998). The Top protein displays significant sequence homology to *E. coli* topoisomerase I, and has been shown to possess DNA relaxation activity, but only on plasmids that use DNA Pol I for initiating replication. Top-mediated relaxation of negative supercoils has been proposed to interfere with the ability of DNA Pol I to separate the DNA strands for replication, thereby causing its arrest.

RCR plasmids

RCR plasmids are a ubiquitous family of plasmids that were originally described in *S. aureus*, are widespread in Gram-positive bacteria, and include representatives in Gram-negative bacteria and Archaea. Replication initiator proteins from three classes of RCR replicons have been detected in enterococci; the pT181-family that contains the Rep_trans conserved domain, the pMV158 family that contains the Rep_2 conserved domain, and the pUB110 family that contains the Rep_1 conserved domain (see the section below on categorizing plasmids). Only two natural isolates have been described in any detail in the enterococci—the *E. faecium* plasmids pRI1 (Rep_trans) (Garcia-Migura, Hasman, & Jensen, 2009) and pJB01 (Rep_2) (Kim, et al., 2006)—but related initiators have been detected in sequencing projects and PCR-based screens (Jensen, Garcia-Migura, Valenzuela, Løhr, Hasman, & Aarestrup, 2010). While Rep_1 initiators have been identified in the enterococci, they are commonly associated with alternative replicons and may not be functional. Indeed, the pAMα1 Rep_1 initiator is known not to function in *E. faecalis*, and replication of this plasmid depends on the Rep_3 initiator. (See previous section on pAMα1.) Several commonly used enterococcal cloning vectors have been derived from RCR plasmids that were isolated from other organisms. RCR plasmids have been the subject of numerous extensive reviews (del Solar, Giraldo, Ruiz-Echevarría, Espinosa, & Díaz-Orejas, 1998; del Solar, Hernández-Arriaga, Gomis-Rüth, Coll, & Espinosa, 2002; Espinosa, del Solar, Rojo, & Alonso, 1995; Khan, 2005), and the reader is referred to these for more extensive information and detailed citations on this subject.

Rolling circle replication was originally described in single-stranded DNA (ssDNA) coliphages, such as φX174, and the basic mechanisms of ssDNA phage and RCR plasmid replication are similar, except for several modifications to the plasmid systems that serve to regulate replication frequency and limit copy number. Replication in all RCR replicons is initiated by the introduction of a single-strand nick within a specific origin: the double-stranded origin, or *dso*, by the replicon-encoded initiator protein. Nicking is accomplished through a conserved active site tyrosine that is present in all RCR initiators. The plasmid *dso* contains two separate sequence elements, one that is required for sequence-specific binding of the initiator and a second that contains the nick site. The initiator protein consists of two domains, a DNA-binding domain that binds specifically to its cognate origin and a nicking domain that contains the active site tyrosine. Binding of the initiator to supercoiled plasmid DNA causes a conformational change in the *dso* that exposes the nick site as ssDNA. This is essential, since the initiator-nicking domain cannot recognize the nick site in double-stranded DNA (dsDNA). The active site tyrosine becomes covalently attached to the 5′ nucleotide at the nick site and remains attached until the replication cycle is complete. A DNA helicase (PcrA in Gram-positive bacteria) and DNA Pol III are then attracted to the origin and replication is primed by the free 3′-OH at the nick site. After a single round of replication, synthesis through the origin regenerates a copy of the *dso*. A nicking/religation reaction catalyzed by the bound initiator terminates replication, which results in the release of a completely replicated duplex plasmid copy and the displaced ssDNA strand. Replication of the displaced strand is accomplished from an origin distinct from the *dso*, which is referred to as the single-strand origin, or *sso*. These are imperfect palindromes that are recognized by the host RNA polymerase, which synthesizes a short RNA primer. Host DNA Pol I initiates DNA synthesis from the primer and eventually is displaced by DNA Pol III, which completes the synthesis of the complementary strand.

In a bacteriophage, RCR initiators function as monomers and contain two closely-spaced active site tyrosines. Following completion of the first round of replication, a second round of replication can be immediately initiated by using the second tyrosine. By alternating between the two tyrosines, multiple replication rounds can be completed after a single initiation event. Some RCR plasmid initiators also function as monomers, but contain only a single tyrosine. Termination is accomplished by an acidic residue close to the active site tyrosine, thereby preventing reinitiation. In other plasmids, the initiator also contains a single tyrosine, but functions as a dimer. In these plasmids, the noninitiating tyrosine of the dimer could, in principle, terminate and reinitiate replication as in a bacteriophage. However, while this second tyrosine is used for termination, the termination reaction results in the attachment of an oligonucleotide that corresponds to approximately ten nucleotides of newly synthesized DNA beyond the *dso*. Oligonucleotide attachment inactivates the initiator, which prevents reuse and requires *de novo* initiator synthesis prior to a second round of replication.

Initiator synthesis is also regulated in RCR plasmids by a regulatory RNA that is transcribed from the opposite strand at the 5′ end of the initiator gene. This antisense RNA or ctRNA binds to the initiator message within the complementary region, which either causes a refolding of the message that results in premature transcription termination, or interferes directly with ribosome binding. Because the antisense RNA is unstable, its level in the cell always reflects the number of plasmid copies, which makes it an effective control element. RCR plasmids of the pMV158-family (Rep_2) also encode a small transcriptional repressor protein, CopG, that acts synergistically with the antisense RNA to repress initiator synthesis.

Replication at an RCR plasmid *dso* initiates with a nick, which provides the free 3′-OH required for the priming of DNA Pol III. Therefore, RCR plasmids have no need for interaction with host proteins, like DnaA or primase. This fact is probably responsible for the broad host range of RCR replicons, some of which are capable of replicating in both Gram-positive and Gram-negative bacteria. However, inefficient use of *sso*s, either by the host RNA polymerase or DNA Pol I, may result in the accumulation of ssDNA in non-native hosts. Some plasmids have partially solved this problem by evolving *ssos* that are capable of functioning in more than one host. Others may contain two or three *sso*s and presumably use the best one in different hosts.

The production of ssDNA as an intermediate of rolling circle replication imposes certain limits on the biology of RCR plasmids, and on their utility as cloning vectors. Most naturally-occurring RCR plasmids are less than 12 kb in size, presumably because larger sizes would increase the probability of the presence of partially homologous sequences that would lead to recombination and deletion during the ssDNA phase of replication. This propensity for recombination also limits the usefulness of RCR plasmids as cloning vectors. Such vectors are notoriously "structurally" unstable, often deleting all or portions of cloned genes over time. Cloning of large DNA fragments, fragments with repeat sequences, or fragments carrying genes toxic to the host is particularly problematic.

Rep_3 plasmids

Plasmid replication initiator proteins that contain the Rep_3 conserved domain are ubiquitous in bacteria, and include some of the best-studied bacterial plasmids, including the enterobacterial plasmids F, P1, pSC101, and R6K (del Solar, Giraldo, Ruiz-Echevarría, Espinosa, & Díaz-Orejas, 1998). Several plasmids that contain initiators with the Rep_3 domain have been identified in the enterococci, including *E. faecium* plasmids pMBB1 (399), pDT1 (Todokoro, Tomita, Inoue, & Ike, 2006) and pCIZ2 (Criado, et al., 2008), and *E. faecalis* plasmids pS86 (Martínez-Bueno, Valdivia, Gálvez, & Maqueda, 2000), pAMα1 (Francia & Clewell, 2002), and pEF1071 (Balla & Dicks, 2005). Of these plasmids, the pCIZ2 replicon is the best characterized. The minimal replicon consists of the initiator protein gene, *repE*, and two sets of direct repeats, DR1 and DR2, immediately upstream of the *repE* gene. The DR1 repeats are distal to the *repE* promoter and consist of 3.5 copies of a 12 bp sequence. The DR2 repeats are between the DR1 repeats and the *repE* promoter and consist of 4.5 copies of a 22 bp sequence. A fragment that contains both sets of repeats is capable of supporting replication if the *repE* gene is supplied *in trans*, which indicates that the repeats function as an origin of replication. 2D-gel analysis confirmed that the plasmid replicates through a theta mode rather than by rolling circle. Attempted transformation into several species indicated that the plasmid has a relatively narrow host range. Other enterococcal plasmids within this group have a similar organization of iterons upstream of the Rep_3-containing gene.

While the mechanism of copy control is not known for the enterococcal plasmids, it has been intensively studied in their Gram-negative counterparts. In these plasmids, replication initiator monomers represent the active initiator form, while dimers are inhibitory. As plasmid copy number increases, so does initiator concentration leading to increased dimer formation. Initiator dimers cross-link the excess plasmid copies at their initiator repeats, or iterons, "handcuffing" replication and simultaneously inhibiting initiator transcription. As copy number falls, handcuffing is released, initiators are monomerized by chaperones, and plasmid replication can proceed (Chattoraj, 2000). Whether a similar cycle occurs in the Gram-positive representatives remains to be determined.

Several Rep_3 replicons from Gram-positive plasmids have been used for the construction of cloning vectors [see Benachour, Auffray, & Hartke, 2007; Criado, et al., 2008]. Theoretically, these plasmids should make good vectors, because they are generally smaller than the RepA_N plasmids and their theta mode of replication makes them structurally more stable than rolling circle replicons. Further studies on copy control mechanisms should help determine if high copy derivatives can be constructed.

Plasmid partition cassettes: organization and activity

Replication control systems maintain plasmid copy numbers within defined windows that are characteristic for different plasmids, as outlined above. Low copy number plasmids, including those that mediate antibiotic resistance and virulence in enterococci, cannot rely on passive diffusion through the cytoplasm to ensure their stable maintenance. Instead, these plasmids possess accessory mechanisms to promote their persistence in the population. As discussed below, plasmids frequently encode post-segregational cell-killing mechanisms that act specifically in plasmid-free progeny (Hayes & Van Melderen, 2011). Low–copy-number plasmids also specify active partition mechanisms that guarantee their precise distribution during bacterial cytokinesis. Recent studies

focused on biochemical, structural and subcellular localization analyses have revealed crucial aspects of the partitioning process, especially in *E. coli* and in certain *Bacillus* spp. In particular, understanding of assembly of the segrosome, the nucleoprotein complex that drives plasmid segregation, and of the action of motor protein components of the complex have provided fascinating glimpses into plasmid trafficking and positioning during partition (Hayes & Barillà, 2006; Schumacher M. A., 2012).

The loci that mediate plasmid segregation are of four currently known types, based on genetic organization and evolutionary relationships of the encoded proteins (Schumacher M. A., 2008). Types I to III have been most well characterized, whereas type IV has been investigated less thoroughly. Types I to III are each comprised of two autoregulated genes and a centromere-like sequence. In each case, one of the genes specifies an NTPase, either an ATPase of the ParA (type I) or actin (type II) superfamilies, or a GTPase related to tubulin (type III). ParA proteins vary in size from ~200 to ~400 amino acids. The larger proteins typically possess a DNA binding domain that is required for transcriptional regulation of the locus and which is absent from smaller homologs. Type II actin-like proteins are similarly evolutionarily diverse (Derman, et al., 2009), whereas type III tubulin-like proteins are apparently confined to *Bacillus thuringiensis* and related bacilli (Larsen, Cusumano, Fujioka, Lim-Fong, Patterson, & Pogliano, 2007).

The second gene in type I to III partition cassettes invariably encodes a centromere-binding factor (CBF). The proteins that accompany large ParA homologs in the type I class are members of the ParB family of dimeric DNA-binding proteins. ParB proteins possess two DNA binding motifs: a helix-turn-helix (HTH) motif and a six-stranded β-sheet coiled-coil that contact distinct repeat motifs in the centromere site (Schumacher & Funnell, 2005). By contrast, the primary sequences of the CBFs that associate with small ParA proteins are more variable (Fothergill, Barillà, & Hayes, 2005). Nevertheless, in the few cases for which tertiary structures of these CBFs have been solved, they have proven to possess a dimeric ribbon-helix-helix (RHH) fold. This fold is characteristic of a family of DNA-binding proteins that are widely disseminated in both bacteria and archaea (Schreiter & Drennan, 2007). Similarly, the only CBF of type II segregation complexes for which structural information is available is a RHH protein. Here, multiple RHH dimers assemble on the cognate centromere to form an extended, higher order nucleoprotein structure (Møller-Jensen, Ringgard, Mercogliano, Gerdes, & Löwe, 2007; Schumacher, et al., 2007). The crystal structure of one type III class CBF has been determined, and reveals a dimeric winged HTH motif. However, instead of canonical HTH-DNA interactions, the protein inserts the N termini of the recognition helices into a single DNA groove and the wings into adjacent grooves (Ni, Xu, Kumaraswami, & Schumacher, 2010). Thus, there is considerable diversity in the modes of centromere recognition by CBFs in the three major classes of partition complexes.

In accord with the variations in CBF sequences and tertiary structures, plasmid centromeres are remarkably heterogeneous (Hayes & Barillà, 2006). The sites typically comprise multiple direct and/or inverted repeat motifs that may be arrayed over ≈100-bp. However, the sequences, lengths, and numbers of repeats differ from centromere to centromere (Wu, Zampini, Bussiek, Hoischen, Diekmann, & Hayes, 2011). Recognition of centromeres by the cognate CBFs is specific, with little cross-talk with non-cognate centromeres (Fothergill, Barillà, & Hayes, 2005). Thus, different plasmids that co-exist in the same cell may ensure their independent segregation by using distinct CBF-centromere interactions.

The centromere is not contacted directly by the partition NTPase that instead is recruited to the mature segrosome by interactions with the CBF. Plasmid pairing through the segrosomes is considered to be an early step in the partitioning process. Subsequently, nucleotide-induced polymerization of the NTPase into filamentous structures directs the attached plasmids to opposite halves of the dividing bacterial cell. However, the mechanisms by which the polymerizing NTPases in type I-III partition complexes achieve partitioning differ. For the type II system, ATP-mediated symmetrical growth of the actin-like filament propels bound plasmids towards opposite cell poles (Campbell & Mullins, 2007). By contrast, the tubulin-like polymers in type III complexes undergo treadmilling, in which protein subunits are added to the plus end and disassembled from the

minus end of the filament. The attached CBF-plasmid can be transferred from the minus end to the subunits in the elongating plus end, which induces plasmid movement towards the cell pole (Barillà, Rosenberg, Nobbmann, & Hayes, 2005; Larsen, Cusumano, Fujioka, Lim-Fong, Patterson, & Pogliano, 2007; Ni, Xu, Kumaraswami, & Schumacher, 2010). The ParA protein in type I partition complexes also undergoes nucleotide-mediated polymerization that is modulated by the partner CBF (Barillà, Rosenberg, Nobbmann, & Hayes, 2005). Current models suggest that depolymerization of ParA filaments pulls bound plasmids towards the cell poles (Ringgaard, van Zon, Howard, & Gerdes, 2009), but the molecular mechanism by which plasmid transport is achieved in type I systems remains uncertain.

Enterococcal plasmid segregation cassettes

Despite their prevalence and significance in disseminating antibiotic resistance (see the later section on epidemiology), the molecular mechanisms that underpin segregation of enterococcal plasmids are poorly understood. Only the partition cassettes from the prototypical, pheromone-responsive plasmid pAD1 (see above) and the gentamicin-resistance plasmid pGENT have been described to date. The two modules conform to the type I archetype: both cassettes include genes for similarly sized ParA homologs, but encode distinctive CBFs and centromeric sites (Figure 5) (Derome, et al., 2008; Francia, Weaver, Goicoechea, Tille, & Clewell, 2007). For pAD1, the centromere comprises arrays of 13 and 12 8-bp direct repeats with the consensus 5′-TAGTARRR-3′. Provision of the RepB (ParA) and RepC (CBF) proteins *in trans* promoted enhanced segregational stability of a vector bearing either all 25 repeats or 12 motifs, but not a vector that possessed 3 repeats. Thus, more than three repeats are required for effective segrosome assembly (Francia, Weaver, Goicoechea, Tille, & Clewell, 2007). RepC binds the centromere directly and specifically *in vitro* and recruits the RepB protein, which itself does not contact the site. Interestingly, although other enterococcal plasmids carry *repB* genes, the sequences of the accompanying downstream genes and of the putative centromeres are variable which emphasizes how diversity in the CBF-centromere interaction may confer segregation-specificity in multiplasmid strains (Francia, Weaver, Goicoechea, Tille, & Clewell, 2007). Moreover, although RepB homologs are widely distributed in enterococci and other Gram-positive species, there is no linkage between the homologs and any particular plasmid replicon type, which suggests mosacism in enterococcal plasmids, as has been observed in plasmids of other Gram-positives (Weaver, Reddy, Brinkman, Patel, Bayles, & Endres, 2009).

The pGENT plasmid (≈70-kb) was detected in a clinical isolate of *E. faecium*, and confers high-level resistance to gentamicin and a range of other aminoglycosides (Simjee, Fraise, & Gill, 1999). Screening of a library of pGENT fragments cloned in a segregation probe vector identified *cenE-prgP-prgO* as a maintenance locus (Derome, et al., 2008). The *prgP-prgO* genes were previously noted on other enterococcal plasmids, but were of uncertain function (Hedberg, Leonard, Ruhfel, & Dunny, 1996). PrgP is a ParA homolog, whereas the dimeric PrgO protein binds the *cenE* centromere site. Like the pAD1 centromere, the *cenE* site consists of two clusters of repeats, although in this case, seven 5′-TATA-3′ motifs separated by half-helical turns are located in each array (Fig. 5). The *cenE* site shows a modest intrinsic curvature that may be important in assembling a functional segrosome (Derome, et al., 2008). Recent database searches revealed >100 PrgO homologs, principally in *Enterococcus sp*. but occasionally in other Gram-positives. Although annotation of many of these homologs is incomplete and it is frequently unclear whether they are encoded by chromosomal or plasmid genes, *prgP-prgO* loci may prove to be the most common partition cassettes in enterococci.

The descriptions of the pAD1 and pGENT segregation cassettes represent only the first steps in probing the molecular basis of plasmid segregation in enterococci. For example, it remains unclear whether the RepB and PrgP proteins polymerize in response to nucleotide binding, as established for other ParA superfamily members, and whether the cognate CBFs modulate this process. No tertiary structures of enterococcal segregation proteins, nor of these proteins in complex with centromeric DNA are currently available, which precludes a detailed understanding of the mechanics of segrosome assembly in these important bacteria. Moreover, it is unknown whether enterococcal chromosomal factors contribute to plasmid segregation, whether *repB-repC* and

prgP-prgO are the only type I partition modules in enterococci, and whether type II and III partition cassettes also are prevalent.

Enterococcal partitioning as an antibacterial drug target

High-throughput screening (HTS) of small molecule libraries is a powerful approach that is geared at identification of lead compounds that can be developed into new drugs. Moreover, recent progress in compound synthesis and screening now make mechanism-directed small molecule strategies a robust and feasible experimental alternative (Fischbach & Walsh, 2009). The bacterial DNA segregation apparatus has significant potential as a novel target for antibacterial agents that are now urgently required, as existing antibiotics inexorably fail in treating infections caused by enterococci and other pathogens. Thus, small-molecule HTS directed at the enterococcal partition machinery may produce new compounds that could be developed into new antibacterial drugs. Protein-protein interactions involved in CBF dimerization, ParA homolog polymerization, or in CBF-ParA associations might be targeted. Small molecules that disrupt the assembly of enterococcal CBFs on their centromeres or on operator sites that reflect their anticipated roles in partition loci transcriptional regulation might be identified. Compounds isolated from a library screen that suppressed the ATPase activity of a mycobacterial ParA protein were recently described (Nisa, et al., 2010). Nucleotide binding or hydrolysis by enterococcal ParA proteins could be targeted analogously. Thus, as our understanding of plasmid segregation in enterococci develops and as potential HTS approaches evolve, the identification of small molecules that intervene in defined steps of the process may open new avenues towards combating enterococcal infections.

Toxin-Antitoxin Systems

Post-segregational killing (PSK) systems were originally defined as loci that stabilize bacterial plasmids by programming the death of any host cell that fails to inherit a plasmid copy at cell division (Gerdes, Rasmussen, & Molin, 1986; Jaffé, Ogura, & Hiraga, 1985; Ogura & Hiraga, 1983). They accomplish this feat by producing a stable toxin and an unstable antitoxin. As long as the plasmid is retained, the antitoxin is continually replenished and the toxin is inhibited. If the plasmid is lost, the antitoxin is degraded and the toxin kills the cell. PSK systems have also been referred to as addiction modules; the host cell is said to become "addicted" to the presence of the plasmid and suffers the ultimate withdrawal symptom (death) upon plasmid loss (Yarmolinsky, 1995).

PSK systems come in three varieties, designated Types I, II, and III, which differ in the composition and mode of action of their antitoxins (Fineran, Blower, Foulds, Humphreys, Lilley, & Salmond, 2009; Hayes, 2003). In Type I and Type III systems, the antitoxin is a regulatory RNA. In Type III systems, the RNA binds directly to the toxin protein and inhibits its activity (Blower, Fineran, Johnson, Toth, Humphreys, & Salmond, 2009; Blower, et al., 2011). No Type III system has yet been described in enterococci. In Type I systems, the RNA antitoxin binds to complementary sequences in the toxin mRNA, inhibiting translation and/or stimulating degradation. The paradigm for Type I systems is the *hok/sok* system of the *E. coli* plasmid R1 (Gerdes, Rasmussen, & Molin, 1986). The *par* determinant of the *E. faecalis* plasmid pAD1 is the only Type I PSK system characterized so far in Gram-positive bacteria (Weaver, Jensen, Colwell, & Sriram, 1996; Weaver & Tritle, 1994). In Type II systems, the antitoxin is a protein that inhibits toxin activity by direct binding to toxin proteins (Jensen & Gerdes, 1995). These antitoxins typically consist of two domains, a disordered toxin-binding domain and a DNA-binding domain. The toxin-binding domain adopts a more orderly structure upon toxin binding, but remains more susceptible to recognition by proteases than the toxin. The DNA binding domain binds to the promoter responsible for transcription of the bicistronic antitoxin-toxin mRNA, and is responsible for autorepression of the system. In most cases, the toxin facilitates promoter binding and expression may be controlled by the ratio of the toxin and antitoxin proteins. The best-studied Type II system of Gram-positive bacteria is the ωεζ system found on Inc18 plasmids (Ceglowski, Boitsov, Karamyan, Chai, & Alonso, 1993; Lioy, Pratto, de la Hoz, Ayora, & Alonso, 2010). A second enterococcal Type II system, Axe-Txe, was identified on the *E. faecium* plasmid pRUM (Grady & Hayes, 2003). Both the ωεζ and the Axe-Txe systems have been shown to be common in enterococci

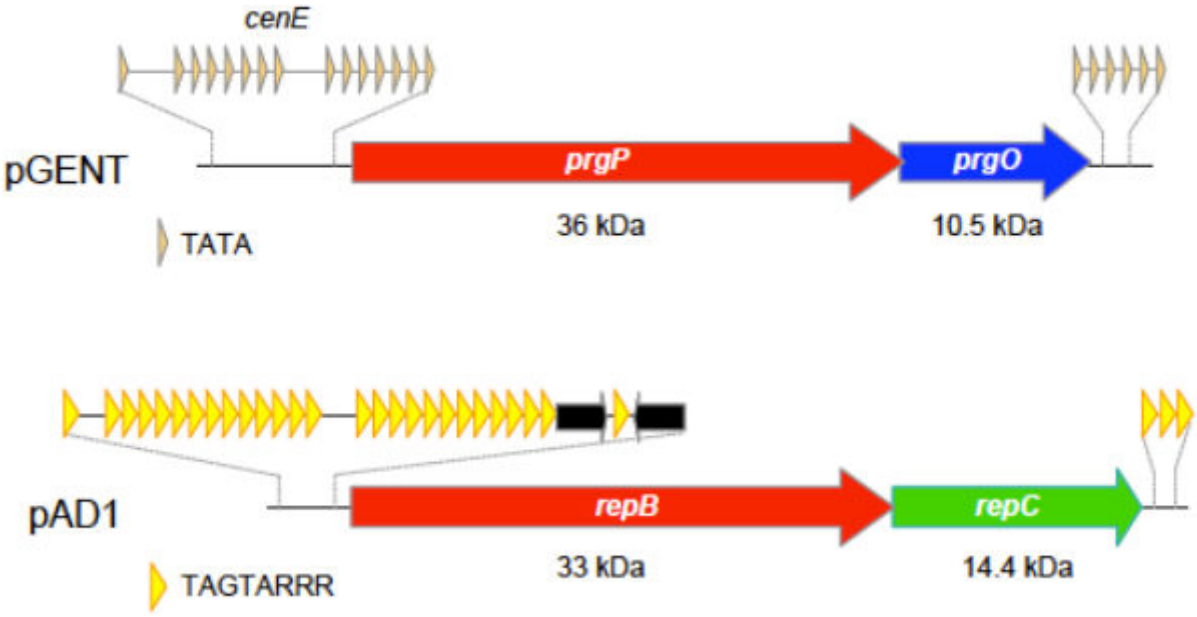

Figure 5. Organization of the pGENT and pAD1 segregation loci (90, 119). ParA homologs are denoted by red arrows and genes for CBFs by blue and green arrows. Molecular masses of protein monomers are indicated. Repeat motifs in the centromere sites located upstream of the genes are shown by arrowheads. Additional repeats of unknown function are situated downstream of the genes.

(Moritz & Hergenrother, 2007; Rosvoll, et al., 2010). Other Type II systems related to those described in *E. coli,* including RelBE and MazEF, have been identified on enterococcal plasmids (Moritz & Hergenrother, 2007), and ribonuclease activity has been demonstrated for an enterococcal MazF toxin (Wang & Hergenrother, 2007). The RelBE and MazEF systems are ubiquitous in both Gram-positive and Gram-negative bacteria and have been extensively reviewed elsewhere (Condon, 2006; Gerdes, Christensen, & Løbner-Olesen, 2005; Hayes & Van Melderen, 2011), and as a result, will not be discussed in detail here.

Shortly after their description, homologs of plasmid-encoded PSK systems were identified on bacterial chromosomes (Masuda, Miyakawa, Nishimura, & Ohtsubo, 1993; Poulsen, Larsen, Molin, & Andersson, 1989). Evaluation of accumulating genomic sequences has revealed that most bacterial chromosomes encode multiple homologs of both Type I and Type II PSK systems (Fozo, Makarova, Shabalina, Yutin, Koonin, & Storz, 2010; Leplae, Geeraerts, Hallez, Guglielmini, Drèze, & Van Melderen, 2011). Since these chromosomal homologs clearly are not involved in post-segregational killing, they have been referred to as toxin-antitoxin or TA modules. This designation is now commonly applied to both plasmid and chromosomal systems. The precise role of many of these systems is still obscure, and in some cases, is controversial (Hayes, 2003). These systems have been implicated in stress response, persistence, programmed cell death, biofilm formation, and maintenance of integrated mobile genetic elements. Numerous extensive reviews have been published on TA systems, of which a few of the most recent are cited here (Fozo, Hemm, & Storz, 2008; Hayes & Van Melderen, 2011; Van Melderen, 2010; Wang & Wood, 2011). This section will focus only on those systems that have been identified in the enterococci.

Type I systems: *par*$_{pAD1}$ and its relatives

The pAD1 *par* determinant was originally identified as a locus required for maximal stability of the plasmid's basic replicon (Weaver, Clewell, & An, 1993). The first indication that *par* might be a PSK/TA module came from the investigation of a serendipitously isolated pAD1 mini-plasmid that triggered host cell death when induced with pheromone (Weaver & Clewell, 1989). Later work showed that this phenomenon resulted from the fortuitous fusion of a pheromone-inducible promoter, P_0 (or the generic P_i described in the section above on conjugation) to RNA I (Weaver, Jensen, Colwell, & Sriram, 1996; Weaver & Tritle, 1994), an mRNA that encodes the 33–amino-acid peptide toxin of the *par* locus Fst. Sequence and RNA analysis identified a short transcript convergently transcribed and partially complementary to RNA I (384), which is designated RNA II. It was later demonstrated that RNA II was capable of counteracting the toxic effects of RNA I both *in cis* and *in trans*, which confirms its role as the antitoxin of the system (Greenfield & Weaver, 2000; Weaver, Jensen, Colwell, & Sriram, 1996). It was further demonstrated that the *par* locus, which is contained on a fragment of 457 bp, stabilized heterologous plasmids at the expense of host cell growth, which confirms its role as a PSK system (Weaver K. E., 1995; Weaver, Jensen, Colwell, & Sriram, 1996; Weaver, Walz, & Heine, 1998).

The genetic organization of *par* and the structure of its transcripts are shown in Figures 6 and 7. The *par* RNAs are convergently transcribed and share a bidirectional intrinsic terminator. The terminator loop provides one region of complementarity at which the two RNAs interact. The RNAs are also transcribed across a pair of direct repeats, DRa and DRb, which provide a second region of complementarity between RNA I and RNA II. Interaction at both the terminator loop and the direct repeats is essential for the proper regulation of RNA I translation, but the function of these interactions differs. The interaction between RNA I and RNA II is initiated at a U-turn motif that is present in the terminator loop of RNA I (Greenfield, Franch, Gerdes, & Weaver, 2001). These motifs, with a consensus sequence of YUNR, have been demonstrated to accelerate the rate of RNA-RNA interaction in a variety of systems (Franch & Gerdes, 2000). Indeed, mutations in the terminator loop reduce the rate of interaction of the two RNAs *in vitro* (Greenfield, Franch, Gerdes, & Weaver, 2001) and abrogate RNA II-mediated protection *in vivo*, which suggests that the rate of interaction is important to translational suppression (Greenfield & Weaver, 2000; Shokeen, Patel, Greenfield, Brinkman, & Weaver, 2008). Following the initial reversible interaction between the terminator loops, binding is rapidly extended to the DRa and DRb repeats that sequester the initiation codon, interfere with ribosomal binding, and inhibit translation of the toxic peptide, Fst (Greenfield & Weaver, 2000; Shokeen, Patel, Greenfield, Brinkman, & Weaver, 2008).

Two RNA I intramolecular structures, 5′-SL and 5′-UH (which are boxed and labeled in Fig. 7), also impact the regulation of *par* function. The 5′-SL is a stem-loop structure that sequesters the ribosome-binding site for the Fst open reading frame, which suppresses translation (Greenfield, Ehli, Kirshenmann, Franch, Gerdes, & Weaver, 2000; Shokeen, Patel, Greenfield, Brinkman, & Weaver, 2008). Translational suppression is not complete, since low levels of translation can be observed *in vitro* and wild-type RNA I is toxic *in vivo* in the absence of RNA II. However, mutations that destabilize the 5′-SL cannot be established in cells that express RNA II (Shokeen, Patel, Greenfield, Brinkman, & Weaver, 2008), in spite of the fact that RNA II is capable of binding to and suppressing translation from such mutants *in vitro* (Greenfield, Ehli, Kirshenmann, Franch, Gerdes, & Weaver, 2000). This discrepancy between *in vivo* and *in vitro* results may relate to the timing of RNA I's interaction with its two competing partners, ribosomes and RNA II. Because the interaction between RNA I and RNA II is initiated at the terminator loop, the ribosome binding site is transcribed and available for ribosome binding before RNA II can initiate binding. The 5′-SL is postulated to temporarily inhibit ribosome binding until the terminator loop can be transcribed. It is also possible that RNA I is processed to remove the 5′-SL *in vivo* before it can be translated, but no such processing product has been observed, despite multiple attempts.

The 5′-UH is an "upstream helix" composed of the extreme 5′ end of the RNA I transcript and a complementary sequence further downstream that folds back to interact with it. This helix sequesters the 5′ nucleotides from digestion by cellular RNases, and is at least partially responsible for the greater stability of RNA I relative to RNA

II (Shokeen, Greenfield, Ehli, Rasmussen, Perrault, & Weaver, 2009). Mutations in the 5′-UH result in a >4-fold drop in RNA I half-life from >40 minutes to around 9 minutes; the half-life of free RNA II is approximately 4 minutes. Mutation of the 5′-UH makes RNA I more susceptible to RNases J1 and J2, which have 5′ to 3′-exonuclease activity (Mathy, Bénard, Pellegrini, Daou, Wen, & Condon, 2007; Shokeen, Greenfield, Ehli, Rasmussen, Perrault, & Weaver, 2009). Whether these are the primary RNases responsible for degradation of RNA I is not clear. It is also possible that other features of RNA I, such as its relatively inaccessible 3′ end and its compact structure, may also contribute to its stability.

In order for TA loci to function as plasmid stabilization systems, the antitoxin must be less stable than the toxin. Indeed, *par* RNA II is significantly less stable than RNA I, with a half-life of 4 minutes, while RNA I shows negligible decay even after 40 minutes. Interestingly, in the presence of RNA I, RNA II basal levels increase more than two-fold, and its half-life increases to 16 minutes (Weaver, Ehli, Nelson, & Patel, 2004). Similarly, the basal level and stability of the RNA I 5′-UH mutant was increased more than two-fold in the presence of RNA II (Shokeen, Greenfield, Ehli, Rasmussen, Perrault, & Weaver, 2009). These results suggest that formation of the RNA I-RNA II complex protects both RNAs from degradation by cellular RNases. While most regulatory RNAs appear to destabilize their targets, target stabilization is not without precedent (Opdyke, Kang, & Storz, 2004). Indeed, target destabilization would not be an effective mechanism of regulation for Type I PSK systems, since a pool of toxin mRNA must persist in plasmid-free segregants in order to kill the cells. In the *hok-sok* PSK system, newly transcribed *hok* mRNA adopts a conformation in which both the translation initiation region and *sok* interacting region are sequestered by intramolecular secondary structures, which allows for the accumulation of a pool of translationally inactive *hok* mRNA. Slow processing from the 3′ end of the *hok* message results in a conformational change that exposes both the *sok* and ribosome interacting sequences (Franch, Gultyaev, & Gerdes, 1997). If the plasmid is still present, *sok* RNA binds to *hok*, and the complex is rapidly degraded by RNase III (Gerdes, Nielsen, Thorsted, & Wagner, 1992). If the plasmid is lost, ribosomes translate the *hok* toxin, which kills the cell. In the case of *par*, the 5′-SL appears to prevent ribosome binding to RNA I until RNA II binding can occur. The translationally inactive complex then accumulates as a pool in the cells, with RNA I to RNA II ratios maintained at about 1:1.1 (Weaver, Ehli, Nelson, & Patel, 2004). It is possible that the discontinuous nature of the interacting sites in the RNA I-RNA II complex prevents efficient degradation by RNase III, which requires at least two helical turns of double-stranded RNA for binding and activity (301). The lower stability of RNA II suggests that it is preferentially removed from the complex and is degraded by means that have yet to be described. If plasmid remains in the cell, sufficient RNA II is produced to replace that which has been removed from the complex. If the plasmid is lost, degraded RNA II cannot be replaced, the Fst ribosome binding site becomes accessible—either through the processing of the 5′-SL or by the utilization of a ribosomal standby site (de Smit & van Duin, 2003)—and sufficient Fst is produced to kill the cell.

The toxin of the *par* TA module is a 33–amino-acid peptide, designated Fst for *faecalis* stabilizing toxin. The peptide has a charged N-terminus, a hydrophobic central region with features of a transmembrane domain, and a highly charged C-terminal tail. Amino-acid substitution experiments revealed that the central hydrophobic domain was critical for toxin function (Weaver, Reddy, Brinkman, Patel, Bayles, & Endres, 2009) and that charged amino acids in the N terminus were also important. In contrast, the charged C-terminal tail appears to have little contribution to toxin function; a nonsense mutation that resulted in truncation of the C-terminal eight amino acids retained its toxicity.

Fst is toxic to *E. faecalis* (Patel & Weaver, 2006; Weaver, Weaver, Wells, Waters, Gardner, & Ehli, 2003), *S. aureus* (Weaver, Reddy, Brinkman, Patel, Bayles, & Endres, 2009), and *B. subtilis* (Patel & Weaver, 2006) when over-expressed from the native RNA I transcript. Toxicity can also be observed in *E. coli* if the 5′-SL structure is disrupted (Shokeen, Patel, Greenfield, Brinkman, & Weaver, 2008). In all four species, the primary effect is condensation of the nucleoid. In *E. coli* and *B. subtilis*, this results in elongation of cells, perhaps because the collapsed nucleoid interferes with formation of the division septum at the cell center through nucleoid occlusion (NO) (Wu & Errington, 2011). In *S. aureus*, the division septum forms and invaginates, but the nucleoid is

frequently trapped at the convergence point, and the completion of cell division is inhibited. In this case, NO appears to be ineffective in stopping invagination of the cell wall, or Fst abrogates its function. Since new cell wall growth occurs only at the septum (Pinho & Errington, 2003) in *S. aureus*, the presence of a condensed nucleoid effectively blocks both division and growth. In *E. faecalis*, cells initially elongate, then produce mis-placed division septae, and finally mis-segregate the nucleoids producing cells that contain little or no DNA. Since cell wall growth in chaining ovococci, like enterococci, occurs both longitudinally and septally (Morlot, Zapun, Dideberg, & Vernet, 2003), elongation apparently occurs even though the nucleoid trapped at the division site blocks septation. In at least some cells, the partition apparatus mobilizes the condensed chromosome, but only into one of the daughter cells. Thus, specific differences in the effects of Fst may relate directly to fundamental differences in cell growth and division.

The specific target of Fst is unknown. The putative transmembrane domain and its importance to toxin function suggest that it is membrane-localized. However, exposure to Fst, unlike Hok (Gerdes, Rasmussen, & Molin, 1986), does not result in the leakage of cell contents and the formation of "ghost cells." An increase in cell permeability is observed following Fst over-expression, but only after the appearance of cell growth and division anomalies, which suggests that membrane defects may be a secondary effect (Patel & Weaver, 2006; Weaver, Weaver, Wells, Waters, Gardner, & Ehli, 2003). Nisin and Fst have a synergistic effect, which suggests that they have different, but complementary targets (Weaver, Weaver, Wells, Waters, Gardner, & Ehli, 2003). Nisin is a pore-forming lantibiotic that docks on lipid II and also affects peptidoglycan synthesis (Wiedemann, et al., 2001). Unlike nisin, synthetic Fst has no effect on cell growth when added externally (Weaver, Weaver, Wells, Waters, Gardner, & Ehli, 2003), which suggests either that it is modified in some way within the cell, or that is targets a component present only on the inner surface of the membrane or in the cytoplasm. Recent microarray data indicates that exposure to Fst results in the induction of a variety of energy-dependent membrane transporters; interference with this induction by RNA polymerase mutation or interference of ABC transporter activity with reserpine leads to Fst resistance (Brinkman, Bumgarner, Kittichotirat, Dunman, Kuechenmeister, & Weaver, 2013). It is possible that hyperactivity of energy-utilizing membrane transporters depletes the cells of energy, which thereby leads to the observed toxic effect.

An atomic resolution structure of Fst has been determined in the membrane mimetic dodecylphosphocholine (DPC) by NMR spectroscopy (Göbl, Kosol, Stockner, Rückert, & Zangger, 2010). These results indicated that Fst forms a transmembrane α-helix with the first two and the last seven amino acids protruding. The charged C-terminal seven amino acids are disordered and were predicted to extend from the cytoplasmic side of the membrane. These authors suggested that the primary function of membrane insertion was to facilitate interactions with a specific target, rather than being directed against the membrane itself. They also predicted that the disordered C–terminus might become structured upon recognition of the target, but this conclusion conflicts with mutagenic studies that indicate that the last eight amino acids are not required for toxicity (Weaver, Reddy, Brinkman, Patel, Bayles, & Endres, 2009).

Work by several groups has revealed that Fst belongs to a large family of RNA-regulated peptide toxins (Fozo, Hemm, & Storz, 2008; Fozo, Makarova, Shabalina, Yutin, Koonin, & Storz, 2010; Kwong, Jensen, & Firth, 2010; Weaver, Reddy, Brinkman, Patel, Bayles, & Endres, 2009). These peptides are smaller than 60 amino acids, are hydrophobic, and are predicted to contain an α-helical transmembrane domain. Indeed, many of the smaller peptides may consist solely of the transmembrane helix. Most are toxic when over-expressed in their native hosts (Fozo, Hemm, & Storz, 2008). An exhaustive bioinformatic search across 774 bacterial genomes identified hundreds of these peptides in the γ-proteobacteria and Firmicutes that were divided into eight families (Fozo, Makarova, Shabalina, Yutin, Koonin, & Storz, 2010). Fst is the founding member of the Fst/Ldr family of peptide toxins, which in this analysis consisted of 161 members. In addition, Kwong, *et al*. reported the identification of more than 200 Fst related peptides in a diversity of Gram-positive bacteria (Kwong, Jensen, & Firth, 2010). While there is likely significant overlap between these two lists, it seems apparent that Fst-related peptides are ubiquitous in Gram-positive bacteria and that the related Ldr peptides are prevalent in the γ-proteobacteria.

Although Fst homologs were frequently present on mobile genetic elements, phylogenetic analysis showing coherence between the phylogeny of the peptides and their hosts of origin suggests that their distribution is not due to recent horizontal gene transfer (Fozo, Makarova, Shabalina, Yutin, Koonin, & Storz, 2010). The gene organization of the Fst/Ldr-homologs further distinguishes the two subgroups. Examination of the DNA sequences that surround the Fst-like peptides revealed the existence of all of the elements originally defined in the pAD1 *par* locus, including the convergent promoters for RNA I and RNA II transcripts, a bi-directional intrinsic terminator, the DRa and DRb interacting sequences, and sequences that provide the 5′-SL and 5′-UH of RNA I, which suggests that they may be regulated in a similar manner to *par* (Kwong, Jensen, & Firth, 2010; Weaver, Kwong, Firth, & Francia, 2009). In contrast, the regulatory RNA of the Ldr message, the Rdl-RNA, is transcribed from the opposite strand of the 5′ end of its target in a manner reminiscent of the *hok/sok* system (Gerdes & Wagner, 2007; Kawano, Oshima, Kasai, & Mori, 2002). Nevertheless, overexpression of Ldr in *E. coli* produces nucleoid condensation effects similar to those seen upon over-expression of Fst, which indicates functional as well as sequence homology (Kawano, Oshima, Kasai, & Mori, 2002). Whether this is a case of variation in regulation of an ancestral locus or convergent evolution is unclear.

It is important to note that, while the general features of the Fst-encoding *par* homologs are conserved, their sequences are not, particularly in the regions that are predicted to provide interactions between the Fst message and its regulatory RNA (Kwong, Jensen, & Firth, 2010; Weaver, Reddy, Brinkman, Patel, Bayles, & Endres, 2009). This feature would allow related *par* systems present on different plasmids to operate in the same cell without interfering with one another. Furthermore, a number of *par* homologs are chromosomally encoded. While many of these are associated with integrated MGE or their remnants, some are not. For example, the Fst homolog EF0409 is located between genes that appear to be associated with mannitol transport and metabolism, and is present in all sequenced *E. faecalis*, but not *E. faecium* strains. Recent work in the Weaver laboratory (unpublished) indicates that it neither interferes with nor is essential for pAD1 *par* function.

Chromosomal homologs of both Type I and II TA systems are common in bacteria, and their function is a matter of ongoing debate (Gerdes, Christensen, & Løbner-Olesen, 2005; Hayes F. , 2003; Van Melderen, 2010). Some chromosomal TA systems are clearly associated with integrated MGE, including plasmids, prophage, pathogenicity islands, and ICE elements, and could serve to facilitate retention of these elements in the absence of overt selection for them. Others are not apparently associated with MGE and have been suggested to perform various functions in response to stress, including suppression of translation, formation of a subpopulation of persister cells, and apoptosis. Others have been suggested to be merely selfish elements that ensure nothing but their own maintenance. It seems likely that no single function will apply to all chromosomally encoded TA systems. Five of the *par* homologs not associated with MGE are intimately linked to genes involved in carbohydrate metabolism (Kwong, Jensen, & Firth, 2010; Weaver, Reddy, Brinkman, Patel, Bayles, & Endres, 2009). This includes the *E. faecalis* EF0409 locus described above, the *Staphylococcus saprophyticus* SSP0870 locus situated between genes for 6-phosphogucono-lactonase and an aldehyde dehydrogenase, the *Lactobacillus caseii* LSEI2682 locus situated between genes for a mannose-6-P isomerase and a two-component signal transduction system, a locus in *S. aureus* MRSA252 located between genes that encode a putative ABC transporter and glycerate kinase, and a *Listeria monocytogenes* locus downstream of a gene that encodes a glycosyl hydrolase. The locations of these *par* homologs, along with the association of Fst effects with ABC transporters, are suggestive of a role in fine-tuning carbohydrate metabolism. This possibility is under active investigation.

Type II (Proteic) systems

α. ωεζ

The ωεζ PSK system was originally identified and characterized on the *S. pyogenes* plasmid pSM19035 (Ceglowski, Boitsov, Karamyan, Chai, & Alonso, 1993; Lioy, Pratto, de la Hoz, Ayora, & Alonso, 2010). An identical locus is present on the related plasmid, pAMβ1 from *E. faecalis* strain DS5 (Clewell, Yagi, Dunny, &

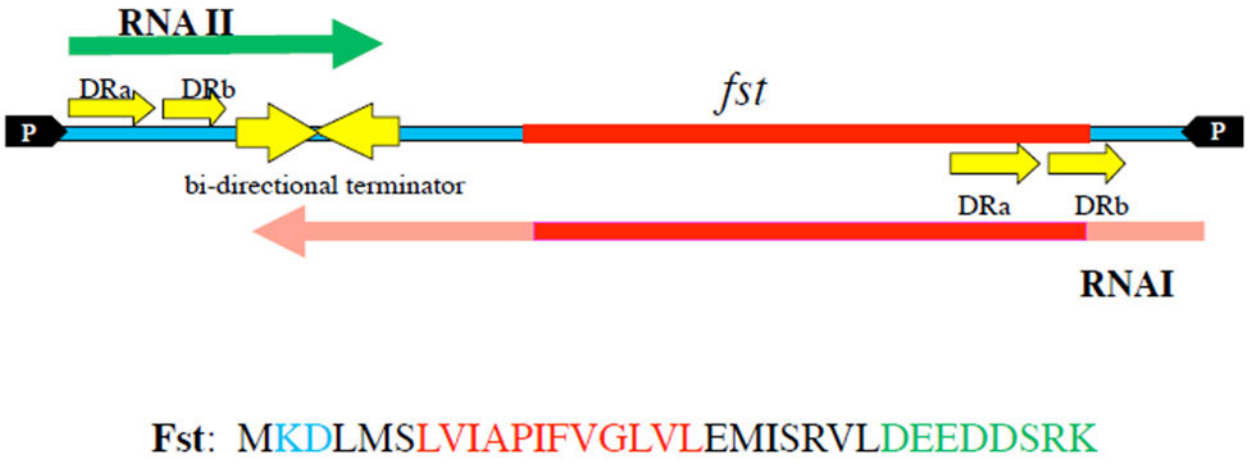

Figure 6. Organization of the pAD1 *par* locus. Converging promoters (black arrowheads labeled P) transcribe the toxin-encoding RNA I (red arrow) and the antitoxin RNA II (green arrow) toward a bi-directional intrinsic transcriptional terminator (converging yellow arrows). The RNAs are transcribed across direct repeats (yellow arrows labeled DRa and DRb) at which interaction occurs, which suppresses translation of the Fst coding sequence (red box on blue line). The protein sequence of the Fst toxin is shown below using standard single-letter amino-acid designations. The essential, conserved hydrophobic domain is shown in red. This forms part of a transmembrane domain in the recently published structure of Fst (142). The two amino acids in blue at the N-terminus must be charged to retain toxin function. The non-essential C-terminal tail is shown in green.

Schultz, 1974), and related loci have been identified on the pheromone plasmid pSL1 from *E. faecalis* (Lim, Tanimoto, Tomita, & Ike, 2006), as well as several vancomycin-resistance plasmids from *E. faecium* (Rosvoll, et al., 2010; Sletvold, Johnsen, Hamre, Simonsen, Sundsfjord, & Nielsen, 2008; Sletvold, Johnsen, Simonsen, Aasnæs, Sundsfjord, & Nielsen, 2007). pSM19035, pAMβ1, and the *E. faecium* plasmids are members of the Inc18 plasmid family (see above).

The ε and ζ proteins are the antitoxin and toxin components, respectively, of the pSM19035 system. As in other TA systems, the ε antitoxin is less stable than the ζ toxin and is specifically targeted by the ATP-dependent protease Lon *in vivo*, although *in vitro* studies have failed to reconstitute degradation of ε from the toxin-antitoxin complex. *In vivo* ε has a half-life of <20 minutes, while ζ has a half-life >60 minutes (Camacho, et al., 2002; Lioy, et al., 2006). The toxin and antitoxin form an extremely stable heterotetramer complex organized as $\zeta\varepsilon_2\zeta$. Structural data and mutagenesis experiments revealed that the ζ toxin contains a P-loop NTPase superfamily domain, and indicated that toxicity is due to an ATP-dependent phosphorylation event. ε antitoxin was inferred to interfere with the binding of ATP at the P-loop of the toxin in the $\zeta\varepsilon_2\zeta$ complex, thereby inhibiting toxin function (Meinhart, Alonso, Sträter, & Saenger, 2003).

Unlike most Type II TA systems, which are autoregulated by their antitoxin components, transcription of the εζ TA pair is regulated by a third component, ω. ω, ε, and ζ are transcribed in order as a tricistronic message from the P_ω promoter, which is repressed by the binding of ω dimers (ω_2) to seven binding sites within the promoter region. This binding inhibits transcription without inhibiting RNA polymerase promoter binding (de la Hoz, et al., 2000). ε and ζ are also expressed from a weak promoter, P_ε, upstream of the ε gene, which provides a low basal level of the two TA components in the presence of ω_2-mediated repression. It is hypothesized that, in the event of a downward fluctuation in copy number, ω_2 levels drop, which leads to increased production of ε and ζ to a threshold level at which the amount of stable ζ protein exceeds the level of the unstable ε protein, and leads

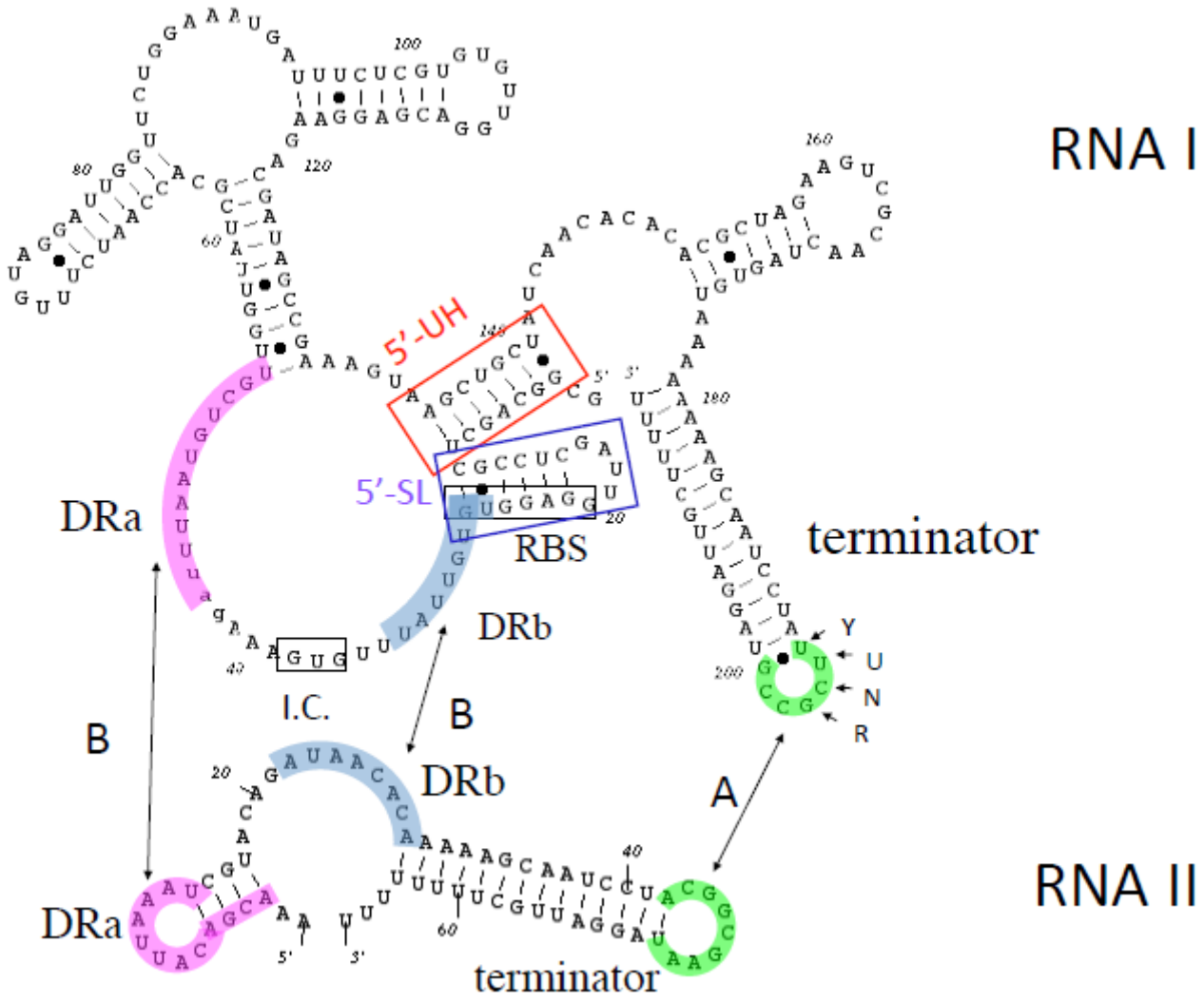

Figure 7. Secondary structure of RNA I and RNA II. The terminator region and the direct repeats (DRa and DRb) are shaded pink and blue, respectively. The two 5′ structures, 5′-SL and 5′-UH, of RNA I are boxed and labeled as are the fst ribosome binding site (SD) and initiation codon (I. C.). The two RNAs have three dispersed complementary segments. Interaction between the two RNAs is initiated at the U-turn motif (labeled YUNR) present in the loop of the terminator of RNA I (green shaded). This interaction is indicated by the arrow labeled A. The interaction then extends to the direct repeat sequences (with interaction indicated by arrows labeled B) and prevents the translation of the toxin, Fst, since the initiation region of the toxin is overlapped by the interacting RNAs.

to toxin expression. ω_2 acts to repress not only the ωεζ operon, but also the copy control function CopS and the active partition protein δ, thereby coordinating the replication and stability functions.

Work with the pSM19035 system suggested that the effects of ζ toxin are bacteriostatic, inducing a viable but non-culturable (VBNC) state in which most major metabolic pathways are inhibited. The effects of ζ over-expression can be partially reversed by expression of the ε antitoxin, but a proportion of cells (~20%) are killed by lysis (Lioy, et al., 2006). Recently, a chromosomal homolog of ζ toxin, PezT of *Streptococcus pneumoniae*, was demonstrated to be a kinase that phosphorylates the nucleotide sugar uridine diphosphate-N-acetylglucosamine (UNAG), a key cell wall precursor (Mutschler, Gebhardt, Shoeman, & Meinhart, 2011). This activity inhibits cell wall synthesis by depleting the pool of UNAG required for peptidoglycan synthesis and by competitive inhibition of MurA by the UNAG-3P product. The difference between the predominantly bacteriostatic effect of ζ toxin over-production and the lytic effect of PezT over-production may simply reflect a difference in levels of

the two toxins produced. Interestingly, ζ toxin has been shown to inhibit yeast cell growth, as well as bacterial cell growth (Zielenkiewicz, Kowalewska, Kaczor, & Cegłowski, 2009). Since yeast do not produce peptidoglycan, inhibition of these cells must occur through a different mechanism.

As observed in most other TA systems, the toxin proteins of ωεζ-like systems are more highly conserved than the antitoxin proteins, which probably reflects the constraints that are required to maintain biological activity. Even though the PezT toxin shares only 42% homology with ζ, their 3D structures are identical (Khoo, et al., 2007; Meinhart, Alonso, Sträter, & Saenger, 2003). ω, on the other hand is perfectly conserved in plasmid systems, but is absent from the chromosomal systems, which indicates that it performs plasmid-specific functions. In the PezAT chromosomal system, the PezA antitoxin functions as an autorepressor with PezT as a corepressor, as in canonical Type II TA systems (Khoo, et al., 2007), which suggests that the εζ TA pair was brought under ω control to more efficiently coordinate its expression with other plasmid-maintenance functions.

b. Axe-Txe

The Axe-Txe locus was originally described on the multi-resistance plasmid pRUM from a clinical isolate of *E. faecium* (Grady & Hayes, 2003). It was demonstrated to stabilize heterologous plasmids in its native host, as well as in *B. thuringiensis* and *E. coli*, and over-expression of Txe was shown to be toxic in *E. coli*. Toxicity was alleviated by coexpression of Axe, which confirmed that Axe-Txe was a TA locus. An identical locus was later identified on a vancomycin-resistance plasmid related to pRUM, designated pS177, in a second *E. faecium* clinical isolate (Halvorsen, Williams, Bhimani, Billings, & Hergenrother, 2011). The Txe toxin was shown to inhibit translation by cleaving mRNA one base downstream of the AUG initiation codon in *E. coli*. These results are consistent with phylogenetic analyses, which suggests that Txe belongs to the RelE superfamily of RNA interferases, a widespread group of proteins found on both bacterial plasmids and chromosomes that possess ribosome-dependent RNase activity (Condon, 2006; Gerdes, Christensen, & Løbner-Olesen, 2005). RelE toxins are commonly encoded in TA systems with RelB antitoxins. However, the Axe antitoxin is more closely related to Doc, the antitoxin for the unrelated Phd toxin, suggesting that recombinational "mixing and matching" has occurred in the evolution of TA systems (Hayes & Van Melderen, 2011). pS177 also encodes a *relBE* TA system, designated $relBE_{Ef}$. It is not known whether this locus is functional.

Work on a chromosomally-located *relBE* locus in *E. coli* suggests that the RelE "toxin" is not bactericidal (Pedersen, Christensen, & Gerdes, 2002). Cells affected by RelE over-expression can be rescued by subsequent expression of the antitoxin. It has been postulated that the function of chromosomally-encoded RNA interferases is to rapidly and temporarily halt translation during periods of nutritional stress, which puts cells into a kind of stasis from which they can awaken when circumstances become more favorable for their growth. Similarly, the effects of ζ toxin were also shown to be reversible by over-expression of the ε antitoxin (Lioy, et al., 2006). The fact that the activities of some toxins have been shown to be reversible begs the question of whether the function of PSK "toxins" in general is to kill plasmid-free segregants. In this regard, it is important to note that most studies of toxin function have relied on regulated over-production of the toxin, and it is unlikely that toxins ever reach such high levels under natural circumstances. It is possible that the real role of plasmid-encoded PSK systems is simply to delay cell division in the case of downward fluctuations in plasmid copy number in order to allow sufficient time for plasmid replication to restore homeostasis. Once plasmid copy numbers are restored and antitoxin levels are increased, the effects of the toxin could be reversed and the plasmid-containing cell would be free to grow. Alternately, in the case of conjugative plasmids (particularly in chained bacteria like enterococci), the division of plasmid-free segregants could be delayed long enough for cells to regain a copy of the plasmid by conjugation. Evaluation of these possibilities awaits methods that will allow the investigation of toxin and antitoxin levels expressed from their native loci in single cells.

On the Categorization of Plasmids

Plasmids have been traditionally classified as RCR or theta based on their mode of replication, and Table 1 lists currently known plasmids in enterococci accordingly, with theta replication being the most prevalent. As described in the earlier section on plasmid replication, plasmids can be further subdivided into six families, based on the presence of conserved domains in their replication initiators: Rep_3, Inc18 and RepA_N families of theta-replicating plasmids and Rep_trans, Rep_1 and Rep_2 classes of RCR plasmids. This classification scheme was initially developed by Jensen *et al.* (Jensen, Garcia-Migura, Valenzuela, Løhr, Hasman, & Aarestrup, 2010), and the related multiplex-PCR approach has recently been applied to different collections of enterococcal isolates (Freitas, et al., 2013; Freitas, et al., 2012; Garcia-Migura, Sanchez-Valenzuela, & Jensen, 2011; Jensen, Garcia-Migura, Valenzuela, Løhr, Hasman, & Aarestrup, 2010; Romo, et al., 2008; Rosvoll, et al., 2010). Table 2 provides an updated list of enterococcal replication initiators that cluster into the above six families. As reflected in the table, a number of plasmids encode more than one replicon (up to three different initiator sequences), which hampers their definitive classification.

While sometimes multiple replication initiators are encoded, plasmids rarely carry multiple determinants for mobilization. Thus, a categorization based on the latter could serve as an alternate classification scheme. Mobile plasmids are characterized by the presence or absence of: i) a MOB region, which is involved in DNA processing and provides the relaxase, accessory proteins, and an origin of transfer (*oriT*); and ii) a MPF (mating pair formation) region that includes construction of the mating channel. Self-transmissible elements carry both MOB and MPF regions, whereas mobilizable elements encode just the MOB module and need the MPF genes from a co-resident conjugative element for mobilization. As relaxases are crucial for initiation of DNA transfer, their amino acid sequences can serve as the basis of a classification criterion. Seven families of relaxases have thus far been described: MOB_P, MOB_F, MOB_H, MOB_Q, MOB_V, MOB_C, and MOB_T (Francia, Varsaki, Garcillán-Barcia, Latorre, Drainas, & de la Cruz, 2004; Garcillán-Barcia, Francia, & de la Cruz, 2009; Smillie, Garcillán-Barcia, Francia, Rocha, & de la Cruz, 2010). It is fitting that this classification extends to the entire MOB region (which also includes the *oriT* and the nicking accessory proteins).

A grouping of the enterococcal mobile elements whose DNA sequences were deposited in GenBank by the end of December 2011 into mobilization families was performed, based on PSI-BLAST searches using the N-terminal 300 amino acids of prototype relaxases, as previously described (Garcillán-Barcia, Francia, & de la Cruz, 2009; Guglielmini, Quintais, Garcillán-Barcia, de la Cruz, & Rocha, 2011). Relaxases encoded in contigs derived from enterococcal genome sequencing projects were not included, unless a VirB4-like and/or a T4SS coupling protein (T4CP) gene were also identified in its proximity. Close co-occurring relaxase-like, T4CP-like and VirB4-like hits were indicative of putative conjugative elements (Guglielmini, Quintais, Garcillán-Barcia, de la Cruz, & Rocha, 2011), and in some cases, the determinants were related to integrative elements (ICEs) rather than plasmids. Relaxases from mobile plasmids, as well as ICEs, in enterococci were found to cluster into five families: MOB_P (17 plasmids and 4 ICEs), MOB_Q (6 plasmids), MOB_V (8 plasmids), MOB_C (6 plasmids and 1 ICE), and MOB_T (12 ICEs and 1 plasmid) (Table 3). MOB_F or MOB_H relaxases were not found in enterococci, while the MOB_P family was clearly the most represented with 21 members. MOB_Q, MOB_C, and MOB_T were found only in conjugative elements. In contrast, MOB_V was present only in mobilizable plasmids. MOB_P is found in both conjugative and mobilizable elements. MOB_P is the most diverse family and can be found associated with RepA_N, Inc18-like and small theta-replicating plasmids, including Rep_3 elements, while MOB_C comprises RepA_N and Inc18-like plasmids. MOB_Q is associated with a group of broad host range Inc18-like plasmids and MOB_V includes RCR and small theta plasmids. MOB_T relaxases are actually members of the Rep_trans superfamily of replication initiation factors.

This classification scheme can be easily applied to any newly sequenced plasmid, with only BLAST alignments needed to assign the relaxase to one of the MOB families. The related multiplex-PCR approach (Goicoechea, et al., 2012) has been used to identify the relaxase determinants in different collections of enterococcal isolates.

Only three examples of more than one relaxase have been identified thus far: pAMα1, pCF10, and pMG2200. As previously described, pAMα1 is a cointegrate of two mobilizable plasmids (Francia & Clewell, 2002) while pCF10 and pMG2200 are pheromone-responding plasmids that contain a copy of a tetracycline resistance Tn*916*-like element and a vancomycin-resistance Tn*1549*-like element, both of which encode relaxases, respectively (Hirt, et al., 2005; Zheng B. , Tomita, Inoue, & Ike, 2009).

The implementation of these typing schemes has revealed a significant degree of mosaicism, which mainly involves the Inc18 and RepA_N families of plasmids (Freitas, et al., 2013). It shows a variable content of replication and relaxase determinants that deserve attention, as these elements carry important adaptive traits such as antibiotic resistance, bacteriocins, or virulence (see Table 1).

Epidemiology and the Flow of Mobile Genetic Elements: Opportunities and Limitations

Enterococci are recovered from a diversity of environments that include dairy products, foodstuffs, biofilms on food-processing and medical equipment, and the gastrointestinal tracts and oral cavities of animals and humans —locations where horizontal gene transfer (HGT) between groups of bacterial species readily occurs (Aarestrup, Butaye, & Witte, 2002; Palmer, Kos, & Gilmore, 2010). The ever-increasing numbers of reports of enterococcal strains that have acquired new adaptive traits that enable them to survive in different conditions (such as resistance to antibiotics, biocides, heavy metals, and different metabolic capabilities, or the ability to colonize different epithelial tissue or cause infection) illustrates the role of HGT in the combinatorial evolution of these microorganisms. Phylogenomic analyses have revealed the influence of HGT in the evolutionary trajectories of some sequence types (ST) within major clonal complexes of *E. faecium* and *E. faecalis*, and also in determining strain-specific properties (de Regt, et al., 2012; van Schaik, et al., 2010). Although other enterococcal species have not been studied in such detail, we might consider each one as constituted by a variety of populations with individual properties, which enables adaptation to particular conditions and therefore likely to have been influenced by HGT. The number of acquired genes that enterococci share with other species and genera from related habitats supports this notion (see Enterococcus Diversity, Origins in Nature, and Gut Colonization and Enterococcal Genomics).

Two main post-transfer barriers protect a given host cell from invasion by foreign DNA: *restriction-modification* (RM) *systems,* and *clustered, regularly interspaced short palindromic repeats* (CRISPR). Little is known about the diversity of RM and anti-RM systems associated with enterococci. To date, the presence of anti-RM systems is confined to analogs of ArdA (alleviation of restriction of DNA) proteins that act against Type I restriction systems (detected in Tn*916* and CTn*6000,*) and other genes predicted to be involved in methylation (e.g. in CTn*6000*) (Brouwer, Mullany, & Roberts, 2010; Serfiotis-Mitsa, et al., 2008). Interestingly, the presence of RM in pathogens such as *S. aureus* has been observed to limit the transfer of enterococcal DNA to this species (Corvaglia, François, Hernandez, Perron, Linder, & Schrenzel, 2010; Sung & Lindsay, 2007; Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008). CRISPRs are defence systems that provide a type of acquired immunity against specific sequences [for a more complete review, see Marraffini & Sontheimer, 2010]. There is evidence that the presence of complete CRISPR loci is inversely related to the presence of MGE (Palmer & Gilmore, 2010), a relationship that, in retrospect, is not surprising.

Whereas plasmids with different mechanisms of replication, conjugation abilities, and host ranges have been described in enterococci, their occurrence varies between species and strain origin. For example, while Inc18 plasmids appear to be widely distributed among different species, other plasmid types seem to be confined to either *E. faecium* (e.g. RCR pRI1-like, small theta pCIZ2-like, and pRUM-like plasmids, as well as a variety of megaplasmids) or *E. faecalis* (e.g. pheromone-responsive plasmids) (Freitas, et al., 2013; Freitas, et al., 2010; Leon-Sampedro, et al., 2012). Antibiotic resistance is frequently associated with Inc18, pRUM-like, and

pheromone-responsive plasmids. In addition, mosaics are often reported, and relevant epidemiological differences are common (Table 1).

The various sources of enterococcal plasmids indicated in Table 1 illustrate only the "tip of the iceberg" when considering the vast populations of organisms with the potential to colonize and adapt to specific environments. The presence of plasmid-borne passenger genes should reflect an epidemiological "history" of sorts. Traits carried on transferable elements can be kept within the host bacterium ("private" traits") or secreted ("public traits"), based on whether they make their hosts "helpers" or "harmers" of neighboring cells, respectively (Rankin, Rocha, & Brown, 2011). "Private" traits would comprise a wide diversity of genes involved in basic and adaptive functions, while "public traits" would comprise secreted proteins that are involved in capturing resources (such as siderophores), metabolic expansion (such as the catabolism of emerging energy resources, such as complex carbohydrates and polycyclic aromatic hydrocarbons), biofilm formation (traits that enhance adhesion), killing of competing lineages (such as bacteriocins), exploitation of hosts (virulence factors), or addictive systems (such as toxin-antitoxin systems). Such characteristics, which are commonly associated with plasmids, can play important roles as enterococci evolve. Indeed, many of the related determinants are able to move between plasmids and between chromosome and plasmid because of their association with site-specific recombination systems. Such elements correspond to transposons that are able to move from one site to another, and, in some cases, that use flanking insertion sequences to facilitate movement. Of course, insertion sequences themselves are an important component of the bacterial mobilome, in that they can "jump" independently or serve as portable regions of homology able to facilitate homologous recombination, which enables integration or excision of plasmids into or from other replicons. As discussed below, all of these elements are ubiquitous in enterococci.

Movement between replicons

Insertion sequences and composite transposons

ISs have been identified in different enterococcal species that belong to 10 IS families that are widespread among *Firmicutes* (Table 4). Some ISs that are multiply represented in the chromosomes, and MGEs of both *E. faecalis* and *E. faecium* [e.g. IS*1216* and IS*257* (IS*6* family), IS*Ef1*/IS*6770* (IS*30* family) and IS*16* and IS*256* (IS*256* family)] have been useful for typing purposes (Leavis H. L., Willems, van Wamel, Schuren, Caspers, & Bonten, 2007; Rice & Thorisdottir, 1994; Thorisdottir, et al., 1994; Werner, Fleige, Geringer, van Schaik, Klare, & Witte, 2011). Additionally, a role in the formation of functional promoters for biofilm production in virulent *E. faecalis* strains has been suggested for IS*1191* (Coburn, Baghdayan, Dolan, & Shankar, 2008).

Composite transposons that are flanked by terminal IS elements and encode antibiotic resistance are common among enterococci, and range in size from 4.7 to 65 kb (Table 5a). Examples of widespread composite transposons are Tn*5405*, which encodes aminoglycoside and streptothricin resistance, Tn*1547,* which confers vancomycin resistance, and elements that encode resistance to high levels of gentamicin (Tn*5281*, which contains a tandem duplication of IS*256* at one terminus of Tn*4001*, a Tn*4001*-IS*257* hybrid, and different Tn*4001*-truncated elements that can also be included in larger platforms such as Tn*5384*, Tn*5385*, or Tn*924*). While Tn*5405* and Tn*4001* derivatives have been identified in commensal enterococci, staphylococci, and streptococci from pets, farm animals, and humans (mainly associated with worldwide-spread elements such as Inc18 plasmids), Tn*1547* has been identified in a few *E. faecium* strains from the USA and Europe. ISs are frequently involved in the genesis of novel IS-based composite transposons in enterococci that yield composite mosaic platforms (Bonafede, Carias, & Rice, 1997; Rice & Carias, 1998; Rice & Marshall, 1994).

Tn*3*- and Tn*7*-related elements

Among enterococci, Tn*3*-family tranposons are represented by Tn*1546* and Tn*917*, which closely resemble Tn*551* in staphylococci (Arthur, Molinas, Depardieu, & Courvalin, 1993; Grindley, 2001; Shaw & Clewell, 1985).

Although they have a highly related transposase (TnpA), they differ in transposon organization, particularly in the relative orientation of the *tnpR*, with Tn*917* resembling the Tn*501* organization and Tn*1546* being similar to that of Tn*3* (Grindley, 2001). Tn*1546* has an atypical *res* site related to the β recombination systems of Inc18 plasmids pSM19035 and pAMβ1 (Arthur, Molinas, Depardieu, & Courvalin, 1993; Grindley, 2001).

Tn*1546* (10.8-kb) contains a cluster of 7 *van* genes (*vanRSHAXYZ*) that code for resistance to vancomycin and teicoplanin (Arthur, Molinas, Depardieu, & Courvalin, 1993; Werner, et al., 2008). A large number of Tn*1546* variants that include deletions, rearrangements and/or insertions have been described, and their differences have been exploited for epidemiological purposes (Jensen, Ahrens, Dons, Jones, Hammerum, & Aarestrup, 1998; López, et al., 2010; Novais, et al., 2008; Willems, et al., 1999; Woodford, Adebiyi, Palepou, & Cookson, 1998). They are often found on members of Inc18 and RepA_N, plasmids, which has probably facilitated their wide distribution among enterococci and non enterococcal species isolated from animals, humans, and soil (Clark, Weigel, Patel, & Tenover, 2005; Flannagan, et al., 2003; Freitas, et al., 2013; Freitas, et al., 2012; Guardabassi & Agersø, 2006; Guardabassi, Perichon, van Heijenoort, Blanot, & Courvalin, 2005).

Tn*917* (5.3-kb) and its highly related variant Tn*3871*, contain the *ermB* gene that encodes macrolides-lincosamide-streptogramin B (MLS_B) resistance (Banai & LeBlanc, 1984; Shaw & Clewell, 1985; Tomich, An, & Clewell, 1980), and its transposition, as well as the expression of resistance, is inducible by erythromycin (Tomich P. K., An, Damle, & Clewell, 1979). These transposons seem to be widely spread among humans and farm animals since the early 1980s (LeBlanc, Inamine, & Lee, 1986; Rollins, Lee, & LeBlanc, 1985).

An example of production of beta-lactamase among enterococci (Murray, 1990; Sarti, Campanille, Sabia, Santagati, Gargiulo, & Stefani, 2012) is due to the *blazZ-blaI-blaR1* operon located on Tn*552*, a transposon that is widely spread among staphylococcal plasmids. Tn*552* is a Tn*7*-like element with an atypical transposition module that includes a TnsB-like transposase homologous with DNA integrases of eukaryotic retroviruses and retrotransposons, a putative ATP-binding protein similar to the B protein of phage Mu, and a co-integrate resolution system homologous with those of Tn*3* family *resL binL* (Rowland & Dyke, 1990). Although Tn*552* has been identified among globally spread CC2 *E. faecalis* and CC17 *E. faecium* clinical isolates that belong to main human lineages ST17, ST18, and ST78 previously considered as CC17, its occurrence remains rare among enterococci (75, 251, 312, 393). A Tn*552* variant that lacks regulatory *blaI* and *blaR1* genes due to an IS*256* insertion has been identified on plasmids of *E. faecalis* isolates that belong to CC2 (ST6) recovered in the mid-1980s from certain American states and Argentina (Murray, 1990). A full copy of the Tn*552* element surrounded by sequences related to the α-family of staphylococcal plasmids was identified on a large composite structure designated Tn*5384*, located on the chromosome of *E. faecalis* isolates of the clonal complex 9, that caused an outbreak in Boston in the early 1980s (Murray, 1990; Rice L. B., Carias, Marshall, & Bonafede, 1996). Only two reports have documented the plasmid location of the *blaZ-blaI-blaR1* operon among *E. faecium* isolates. One describes a single isolate in an American hospital with endemic presence of *E. faecalis* beta-lactamase producers (Coudron, Markowitz, & Wong, 1992); the other documents eight *E. faecium* clinical isolates that belong to the main human lineages ST17, ST18, and ST78, previously considered as CC17, which were recovered in a single Italian hospital during 2010 (Willems, et al., 2012).

Conjugative transposons /integrative conjugative elements

A diversity of conjugative transposons (CTns) have been identified among enterococci since the description of Tn*916* in the early 1980s (see earlier section and Table 5 for details). They display a common synteny, but differ in their integrase/excisionase sequences and the specificity for the insertion site (Roberts & Mullany, 2009; Roberts & Mullany, 2011). Many of them encode resistance to tetracycline (Tn*916-like,* Tn*6000,* Tn*5801,* Tn*5397*), kanamycin and erythromycin (Tn*1545*), or glycopeptides (Tn*1549*/Tn*5382*) (Carias, Rudin, Donskey, & Rice, 1998; Garnier, Taourit, Glaser, Courvalin, & Galimand, 2000; Rice L. B., 1998; Roberts & Mullany, 2011).

A large number of Tn*916*/Tn*1545* family members (*tetM*) have been detected in different enterococcal species recovered from humans, pets, farm animals, wild boars, house flies from food settings, and foods (Hegstad, Mikalsen, Coque, Werner, & Sundsfjord, 2010; Roberts & Mullany, 2011) (see Table 5C). Besides the prototype Tn*916,* characterized enterococcal Tn*916*-like transposons are Tn*918*, Tn*925,* Tn*3702* (Horaud, Delbos, & de Cespédès, 1990), Tn*5031/5032/5033*, Tn*5381* and Tn*5383*, Tn*6084*, Tn*6085*a, and Tn*6085*b (Rice L. B., Carias, Rudin, Hutton, & Marshall, 2010) or Tn*6009*, which is a Tn*916*-like transposon that is directly linked to a functional staphylococcal mercury resistance operon located upstream of the Tn*916* conjugation module. Tn*1549* (*vanB2*) is a Tn*916*-like element that comprises a central part, with the *vanB2* operon replacing the *tet(M)* gene (Garnier, Taourit, Glaser, Courvalin, & Galimand, 2000).

Other CTns that code for resistance to tetracycline (*tetM*) encode integrases that are highly homologous to those of certain *S. aureus* PAIs (Tn*6000*) or the large serine recombinase *tndX* gene of *Clostridium difficile* (Tn*5397*), which is responsible for the excision and insertion of the element (Brouwer, Mullany, & Roberts, 2010; Hegstad, Mikalsen, Coque, Werner, & Sundsfjord, 2010; Novais, Freitas, Sousa, Baquero, Coque, & Peixe, 2008; Roberts & Mullany, 2011). Tn*6000,* formerly EfcTn1 (Brouwer, Mullany, & Roberts, 2010; Roberts, Davis, Seville, Villedieu, & Mullany, 2006), is a mosaic element whose integrase is highly homologous with those of SaPIbov and SaPIbov2, two bovine staphylococcal pathogenicity islands (42% and 41% identity with Int_{Tn916}, respectively). It also encodes: i) a group II intron that is identical to that of Tn*5397* from *C. difficile*; ii) the *vap* and *hel* genes which are homologous to genes coding for a virulence associated protein; iii) a DEAD helicase of the Superfamily 2 within the virulence-related transposon from *Dichelobacter nodosus* (which seems to be transferred horizontally between bacteria and is possibly mediated by bacteriophages); and iv) a putative type I restriction/modification system (Brouwer, Mullany, & Roberts, 2010). Recent studies have detected both $TndX_{5397}$ and $Int_{5801/CW459}$ among enterococcal species of different origins, although $TndX_{5397}$ is enriched among *E. faecium* isolates of poultry origin (Agersø, Pedersen, & Aarestrup, 2006; Novais, et al., 2008). Tn*6000* appears to be spread among enterococci from community based humans, animals, and soil since at least the early 1980s (Novais, et al., 2012).

Enterococcal CTns that lack antibiotic resistance genes have also been described (Burrus, Pavlovic, Decaris, & Guédon, 2002; Fujimoto, Tomita, Wakamatsu, Tanimoto, & Ike, 1995; Rice L. B., Carias, Marshall, Rudin, & Hutton-Thomas, 2005) (Table 5). A Tn*916*-like element, *efaB5*, which consists of EF_1846 to EF_1897, which is ~49.5 kb in *E. faecalis* strain V583, seems to be enriched among CC2 isolates, and plays a role in niche adaptation (Galloway-Peña, Bourgogne, Qin, & Murray, 2011; Solheim, Brekke, Snipen, Willems, Nes, & Brede, 2011). ICE*Efm1,* previously designated as an *esp* pathogenicity island in *E. faecium* (*esp*$PAI_{E.\ faecium}$) is now classified as an ICE element, as it has recently been shown that it is self-transmissible and requires its IntA integrase for excision (Top, Sinnige, Majoor, Bonten, Willems, & van Schaik, 2011). The element is flanked by two 54-bp-direct imperfect repeats, with only the 54-bp DR at the 5′ end being present at the junction site, which suggests that the *esp* PAI is similarly transferred as described for the ICE-like element Tn*916*. The *esp* gene is involved in biofilm formation and infection in a mouse model (Leavis, et al., 2004; van Schaik, et al., 2010).

Enterococcal isolates that contain different CTns and/or multiple copies of a given CTn have been described (see previous section). Besides the emblematic example of the widespread VanB *E. faecium* C68 clone widely disseminated in Cleveland, Ohio (USA) in the early 90s, which harbors three Tn*916*-like transposons (Tn*6084*, Tn*6085*a and Tn*6085*b, and Tn*5382* (*vanB2, pbp5*), enterococcal isolates from humans and animals that contain different CTns (different combinations of Tn*916*, Tn*5397*, Tn*6000* and other ICEs) have been frequently reported (Agersø, Pedersen, & Aarestrup, 2006; Novais, et al., 2012).

Some CTns have an extraordinary ability to undergo genetic exchange processes within a diversity of genomes, which often result in mosaic platforms that carry fragments of transposons, plasmids, RM systems, and self-splicing elements, as demonstrated for the Tn*6000* (Brouwer, Mullany, & Roberts, 2010; Novais, et al., 2012). It has been suggested that they might also contribute to genome evolution by favoring large deletions (Rice L. B.,

Carias, Marshall, Rudin, & Hutton-Thomas, 2005). Mosaic platforms that contain Tn*916-like* transposons, such as Tn*5385* (Tn*5381*-Tn*5384*-Tn*552*), Tn*5382* (*pbp5*-CTn*1549)*, or Tn*6009* (Tn916-mer) have also been described (Carias, Rudin, Donskey, & Rice, 1998; Rice L. B., 1998; Soge, Beck, White, No, & Roberts, 2008).

Self-splicing elements

Group II introns are active mobile elements that relate to a family of self-splicing RNAs. Different introns have been identified in chromosomal DNA, conjugative plasmids, CTns, and pathogenicity islands of *E. faecalis* and *E. faecium* (Coburn, et al., 2010; Rice L. B., Carias, Rudin, Hutton, & Marshall, 2010; Sletvold, Johnsen, Wikmark, Simonsen, Sundsfjord, & Nielsen, 2010) (Table 6). These belong to what has been designated as class B, and thus far have been observed only in enterococci**.** A class A intron Ll.ltrB located on the *L. lactis* plasmid pRS01 has been observed to target conjugative elements of other Gram-positive bacteria, including enterococci (Belhocine, Yam, & Cousineau, 2005).

Integrons

Data related to the spread of integrons in enterococci are scarce. In 1998, the antiseptic resistance gene *qacE* delta 1, frequently encoded in the 3´conserved region of integrons, was identified in *Enterococcus*, and bore a nucleotide sequence identical to that of *qacE* delta 1 from Integron InC from *Pseudomonas* (Kazama, Hamashima, Sasatsu, & Arai, 1998). In 1999, the presence in *E. faecalis* of an integron-related antibiotic-resistance cassette *aadA* (identical to *aadA* genes from integrons in several *E. coli* plasmids) was observed (Clark, Olsvik, Swenson, Spiegel, & Tenover, 1999). While these two reports suggested the appearance of integrons in enterococci, the presence of intact class 1 and class 2 integrons in hospital-associated *E. faecalis* and *E. faecium* isolates has recently been reported in China (Xu, et al., 2010). The appearance of identical cassettes in *Staphylococcus*, *Pseudomonas,* and *E. coli* suggested the intergeneric dissemination of integrons in that hospital.

Bacteriophages

Most of the bacteriophages currently described in *Enterococcus* spp. belong to tailed families of *Podoviridae*, *Siphoviridae*, and *Myoviridae* (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010). Data related to the spread of virulence traits or antibiotic resistance by lysogenic bacteriophages among enterococci are limited (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2011; van Schaik, et al., 2010; Yasmin, et al., 2010). Similar to the case in other MGEs, the incidence of prophage elements is mainly detected in the genomes of *E. faecium* and *E. faecalis* (V583) that are poor in CRISPR systems (van Schaik, et al., 2010) and seem to be more predominant in human isolates than in those from animal origin (Lepage, et al., 2006). Nevertheless, the high incidence of phage-specific genes in *E. faecium* and *E. faecalis* suggests that they play an important role in the genome diversity observed among members of these species (Leavis H. L., Willems, van Wamel, Schuren, Caspers, & Bonten, 2007; McBride, Fischetti, Moellering, Jr., & Gilmore, 2007; van Schaik, et al., 2010). See Enterococcal bacteriophages and genome defense for more extensive coverage of enterococcal bacteriophages.

Concluding Remarks

It is evident that the employment of mobile genetic elements by enterococci in the spread of clinically significant traits, such as antibiotic resistance and virulence, is a greatly used and varied process. The efficiency with which certain conjugation systems can operate is highly evolved, which suggests that features of the enterococcal cell surface may be particularly suitable for this type of DNA transfer. Enterococci are especially hardy organisms, and their widespread distribution in the environment has undoubtedly facilitated the collection of traits important to their eventual establishment as human commensals, as well as made them one of the most common organisms involved in nosocomial infections. As members of the normal intestinal flora, their conjugation proficiency likely makes them a significant reservoir of genetic information for other bacteria, and they likely contribute to the passage of a variety of survival traits to other genera. The recent focus on the molecular biology

and epidemiology of these organisms and their involvement in horizontal DNA transfer has begun to shed significant light on the role these organisms play in human health and disease.

Appendix

Table 1. Enterococcal plasmids

Plasmid family	Plasmid[b]	Size (kb)	Original host (country)	Origin	Host range	Genetic markers (pheromone response)	Reference
ROLLING-CIRCLE REPLICATING PLASMIDS							
Rep_trans	pJS42	4.1	*E. faecium* JH95		*E. faecium*	cryptic	Garcia-Migura, Hasman, & Jensen, 2009
(pRI1-like)	pRI1	6.0	*E. faecium* 9631160-1	F (chicken), A (poultry), H F (Japanese rice bran paste)	*E. faecium, E. hirae*	cryptic	Freitas, et al., 2012; Garcia-Migura, Hasman, & Jensen, 2009; Jensen, Garcia-Migura, Valenzuela, Løhr, Hasman, & Aarestrup, 2010; Ridenhour, Fletcher, Mortensen, & Daneo-Moore, 1996
	pEFNP1	ND	*E. faecium* N15		*E. faecium*	cryptic	
	pKq10	ND	*E. faecium*		*E. faecium*	*tet(U)*	
Rep_2	pMV158	5.5	*S. agalactiae*	H	*S. agalactiae, E. faecalis*	*tet(L)*	Burdett, 1980; van der Lelie, Bron, Venema, & Oskam, 1989
	pJB01	2.2	*E. faecium*	H	*E. faecium*	Cryptic	Kim, et al., 2006
Rep_1	pNJAKD	3.8	*E. faecium*	F (milk)	*E. faecium*	cryptic	Kumar, Ponnaluri, Putarjunan, Ranganathan, Roy, & Das, 2012
THETA REPLICATING PLASMIDS							
Rep_3	pCIZ2	7.4	*E. faecium* L50	F (sausage, chicken), H	*E. faecium*	cryptic	Criado, et al., 2006
	p200B[c]	12.5	*E. faecium*	HH	*E. faecium*	*bac32(also enterocin IT)*	Inoue, Tomita, & Ike, 2006
	pHY	6,037	*E. faecium*	HH	*E. faecium*	*bac51*	Yamashita, Tomita, Inoue, & Ike, 2011
	pB82	6.2	*E. faecium* VR82	H, F (chicken), SW	*E. faecium, E. hirae, E. durans*	*bac43*	Todokoro, Tomita, Inoue, & Ike, 2006
	pEF1071	9.3	*E. faecalis* BFE 1071 (Germany)	A (pig)	*E. faecalis*	bacteriocins (1071A and 1071B)	Balla & Dicks, 2005
	Plasmid	>3.2[b]	*E. faecalis* FAIR-E 309 (Argentina)	F (cheese)	*E. faecalis*	bacteriocin	Unpublished

Table 1. continued from previous page.

	pGL (also pDGL)	8.3	*E. durans* 41D	F (cheese)	*E. durans*	bacteriocin	Du, Somkuti, & Renye, Jr., 2012
	pEFR	3.2	*E. faecium*	NA	*E. faecium*	cryptic	Unpublished
	pAMα1	9.8	*E. faecalis* DS5	H	*E. faecalis*	*tet(L)*	Francia & Clewell, 2002
	pS86	5.1	*E. faecalis* S-86	H	*E. faecalis,*	cryptic	Martínez-Bueno, Valdivia, Gálvez, & Maqueda, 2000
	pEF47	5.5	*E. faecalis* 47	A (cow)	*E. faecalis, other Gram positives*	cryptic	Sprincova, Stovcik, Javorsky, & Pristas, 2005
	p703/5	ND	*E. faecalis* KBL703	F (milk)	*E. faecalis*	NA	Cha, Lim, Jang, Lim, Kim, & Chang, 2007
	pEF418[d]	>15.9[j]	*E. faecalis* 418	HH	*E. faecalis, E. faecium*	*aadE*	GenBank AF408195.1125
	EF62pA	5.1	*E. faecalis* 62	HH	*E. faecalis*	cryptic	Brede, Snipen, Ussery, Nederbragt, & Nes, 2011
	pJS33	3.1	*E. faecium* JH95	NA	*E. faecium*	cryptic	unpublished
	pMBB1	>1.9[j]	*E. faecium*	NI	*E. faecium*	cryptic	Wyckoff, Barnes, Gillies, & Sandine, 1996
Inc18[a]	pIP501	30.6	*S. agalactiae* B96	H, A, SW	*E. faecalis, E. faecium*	*cat, erm(B)*	Thompson & Collins, 2003
	pRE25	50.2	*E. faecalis* RE25	F (sausage, chicken), H, HH, A (poultry, pig), SW	*E. faecalis, E. faecium*	*cat, erm(B), sat4, aph(3′)-III, aadK*	Schwarz, PerretenV., & Teuber, 2001
	pAMβ1	27.8	*E. faecalis* DS5	H	*E. faecalis, E. faecium*	*erm(B)*	Clewell & Francia, 2004; Clewell, Yagi, Dunny, & Schultz, 1974
	pTEF3	17.9	*E. faecalis* V583	H	*E. faecalis*	*UV*	Paulsen, et al., 2003
	pEF-01	35,9	*E. faecalis* EF-01	A (bovine)	*E. faecalis*	*cfr fex(B)*	Liu, et al., 2011
	pEH-1[e]	>18.1	*E. hirae*	A (pig)	*E. hirae*	*fex(B)*	Liu, et al., 2012
	pEFM-1	14.8	*E. faecium*	A (pig)	*E. faecium*	*fex(B)*	Liu, et al., 2012)
	pPPM1000	ND	*E. faecium*	A (monkey)	Unknown	Sm^r, Hg^r	Davis, Roberts, Ready, Richards, Wilson, & Mullany, 2005
	pM7M2	19.5	*E. faecium* (USA)	F (cheese)	*E. faecium, S. mutans*	*tet(M), tet(L)*	Li, Alvarez, Harper, & Wang, 2011

Table 1. continued from previous page.

	pIP816 [f]	34.6	*E. faecium* BM4147	H	*E. faecium*	*vanA, str*	Sletvold, Johnsen, Wikmark, Simonsen, Sundsfjord, & Nielsen, 2010
	pVEF1 [f]	39.6	*E. faecium* 399/F99/H8 (Norway)	HH, F (chicken), A (poultry)	*E. faecium, E. hirae, E. durans*	Tn*1546*(*vanA*)	Sletvold, Johnsen, Simonsen, Aasnæs, Sundsfjord, & Nielsen, 2007
	pVEF2 [f]	39.7	*E. faecium* 399/F99/A9 (Norway)	F (chicken), A (poultry)	*E. faecium, E. hirae, E. durans*	Tn*1546*(*vanA*)	Sletvold, Johnsen, Simonsen, Aasnæs, Sundsfjord, & Nielsen, 2007
	pVEF3 [f]	63.1	*E. faecium* 399/S99/A7 (Norway)	A (poultry)	*E. faecium*	Tn*1546*(*vanA*)	Sletvold, Johnsen, Hamre, Simonsen, Sundsfjord, & Nielsen, 2008
	pVEF4 [f]	>44[j]	*E. faecium* 399/F98/A4 (Norway)	A (poultry)	*E. faecium*	Tn*1546*(*vanA*)	Sletvold, Johnsen, Wikmark, Simonsen, Sundsfjord, & Nielsen, 2010
	pWZ1668 [f]	48.3	*E. faecalis*	HH	*E. faecalis*	Tn*1546*(*vanA*)	Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008
	pWZ909 [f]	42.6	*E. faecalis*	HH	*E. faecalis*	Tn*1546*(*vanA*)	Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008
	pWZ7140 [f]	47.2	*E. faecalis*	HH	*E. faecalis*	Tn*1546*(*vanA*)	Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008
	pEF1[g]	21.3	*E. faecium* 6T1a	F (olive), H, HH, F (chicken), A (pig), SW	*E. faecium*	*uvrA*	Ruiz-Barba, Floriano, Maldonado-Barragán, & Jiménez-Díaz, 2007
	pLG2 [h]	62.6	*E. faecalis* UW3114	H	*E. faecalis*	*ant(6)-Ia, sat, aph(3′)-IIIa, tet(M)*	Laverde-Gomez, et al., 2011
	pHTβ	52.9	*E. faecium* FH	H, HH, F (chicken)	*E. faecium, E. avium, E. durans*	Tn*1546*(*vanA*)	Tomita & Ike, 2008
	pMG1 and closely related plasmids	65.0	*E. faecium*	H, HH, F (chicken)	*E. faecium, E. durans*	Tn*4001,* Tn*1546*	Tanimoto & Ike, 2008

Table 1. continued from previous page.

	pZB18	68	*E. faecium*	H	*E. faecium*	Tn*1546*(*vanA*)	Zheng, Tomita, Xiao, Wang, Li, & Ike, 2007
RepA_N	pAD1	59.3	*E. faecalis* DS16	H, F (chicken), A (pig), SW	*E. faecalis*	*uvrB* (cAD1), Hly/cyl	Colmar & Horaud, 1987; Francia, et al., 2001
	pTEF1	66.3	*E. faecalis* V583	H	*E. faecalis*	Tn*4001, erm(B), qacZ* (cAD1)	Paulsen, et al., 2003
	pTEF2	57.7	*E. faecalis* V583	H	*E. faecalis*	(cCF10)	Paulsen, et al., 2003
	pJH2	59	*E. faecalis* JH1	H	*E. faecalis*	*Hyl/bac*(cAD1)	Clewell & Francia, 2004; Clewell & Weaver, 1989; Colmar & Horaud, 1987; Galli & Wirth, 1991
	pIP964	65	*E. faecalis*	H	*E. faecalis*	*Hyl/bac* (cAD1)	Clewell & Francia, 2004; Colmar & Horaud, 1987
	pAMγ1	60	*E. faecalis* DS5	H	*E. faecalis*	*Hyl/bac , uvr* (cAD1)	Clewell & Francia, 2004; Clewell, Yagi, Dunny, & Schultz, 1974; Colmar & Horaud, 1987
	pAMγ2	~60	*E. faecalis* DS5	H	*E. faecalis*	(cAMγ2)	Clewell & Francia, 2004; Galli & Wirth, 1991
	pAMγ3	~60	*E. faecalis* DS5	H	*E. faecalis*	(cAMγ3)	Clewell & Francia, 2004; Galli & Wirth, 1991
	pBEM10	70	*E. faecalis* HH2	H	*E. faecalis*	*bla,* Tn*4001* (cAD1)	Murray, An, & Clewell, 1988
	pCF10	67.7	*E. faecalis* SF-7	H, F (chicken)	*E. faecalis*	*tet(M)*-CTn*925, uvrA* (cCF10)	Hirt, et al., 2005
	pAMS1	130	*E. faecalis* MC4	HH	*E. faecalis*	(cCF10)	Sedgley, Clewell, & Flannagan, 2009
	pMB1	90	*E. faecalis* S-48	HH	*E. faecalis*	(cCF10)	Clewell & Francia, 2004
	pMB2	56	*E. faecalis* S-48	HH	*E. faecalis*	(cPD1)	Clewell & Francia, 2004
	pPD1	59	*E. faecalis* 39-5	HH	*E. faecalis*	(cPD1)	Fujimoto, Tomita, Wakamatsu, Tanimoto, & Ike, 1995
	pYI14	61	*E. faecalis* 39-5	H	*E. faecalis*	(cPD1)	Tomita, Kamei, & Ike, 2008

Table 1. continued from previous page.

	pEJ97-1	11.3	*E. faecalis* EJ97	SW	*E. faecalis*	(cPD1)	Sánchez-Hidalgo, Magueda, Gálvez, Abriouel, Valdivia, & Martínez-Bueno, 2003
	pAM373	36.8	*E. faecalis* RC73	H, F (chicken)	*E. faecalis*	(cAM373)	De Boever, Clewell, & Fraser, 2000
	pAM368	107	*E. faecalis* 368	H	*E. faecalis*	(cAM373); Tn*1546* (*vanA*)	Showsh, De Boever, & Clewell, 2001
	pBEE99	80.6	*E. faecalis* E99	H	*E. faecalis*	*bee, uvr* (UK pheromone)	Coburn, et al., 2010
	pMG2200 [i]	106.5	*E. faecalis* NKH15	H	*E. faecalis*	*vanB2_Tn1549, uvr,* Bac41 (cCF10)	Zheng, Tomita, Inoue, & Ike, 2009
	pMG2201	65.7	*E. faecalis* NKH15	H	*E. faecalis*	ermB, Hly/cyl, (cAD1)	Zheng, Tomita, Inoue, & Ike, 2009
	pAM323	66	*E. faecalis* HH2	H	*E. faecalis*	(cAM323)	Murray, An, & Clewell, 1988
	pAM324	53	*E. faecalis* HH2	H	*E. faecalis*	(cAM324)	Murray, An, & Clewell, 1988
	pHKK100	55	*E. faecalis* 228	H	*E. faecalis, E. faecium*	(cHKK100), Tn*1546* (*vanA*)	Handwerger, Pucci, & Kolokathis, 1990
	pOB1	71	*E. faecalis* 5952	H	*E. faecalis*	(cOB1)	Galli & Wirth, 1991; Nakayama, Abe, Ono, Isogai, & Suzuki, 1995
	pYI1/2	58/56	*E. faecalis*	H	*E. faecalis*	(cOB1/cYI2)	Ike & Clewell, 1992; Nakayama, Abe, Ono, Isogai, & Suzuki, 1995
	pYI17	57.5	*E. faecalis* YI717	H	*E. faecalis*	(cYI17)	Tomita, Fujimoto, Tanimoto, & Ike, 1996
	pSL1/2	128.1	*E. faecalis* KV1	H/A (chicken)	*E. faecalis*	(cSL1) Tn*1546* (*vanA*), *ermB, aac(6)-aph(2), ant(6)-Ia, aph(3 ′)-IIIa*	Lim, Tanimoto, Tomita, & Ike, 2006
	EF62pC	55.3	*E. faecalis* 62	HH	*E. faecalis*	*bac, uvr*	Brede, Snipen, Ussery, Nederbragt, & Nes, 2011
	EF62pB	51.1	*E. faecalis* 62	HH	*E. faecalis*	*bac, uvr*	Brede, Snipen, Ussery, Nederbragt, & Nes, 2011
	pTW9	85	*E. faecalis*	H, A (poultry)	*E. faecalis*	Tn*1546* (*vanA*), *erm(B),* bac, IS*4*-b-lactamase B-IS*4*	unpublished AB563188
	pHKK703	55	*E. faecium* R7	H	*E. faecalis, E. faecium*	(cCF10)	Heaton, Discotto, Pucci, & Handwerger, 1996

Table 1. continued from previous page.

	pBRG1	50	*E. faecium* LS10	H	*E. faecalis, E. faecium*	(cCF10-like), Tn*1546* (*vanA*)	Magi, et al., 2003
	pRUM [i]	24.9	*E. faecium* U37	H, HH, F (poultry), A (pig)	*E. faecium*	*Cat (orf25), Δ*Tn*5405, erm(B), uvr, Axe-Tse*	Grady & Hayes, 2003
	pS177	39	*E. faecium* S177	H	*E. faecium*	Tn*1546* (*vanA*),	Halvorsen, Williams, Bhimani, Billings, & Hergenrother, 2011
	p5753B	56.7	*E. faecium* 5753c	H, HH	*E. faecium*	*aac(6´)-aph(2´´), tetM, Δ*Tn*5405, erm(B)*	GenBank: GQ900487.1
	pRUM-like [j]	30-70	*E. faecium*	H, HH	*E. faecium*	Tn*1546* (*vanA*)	Freitas, et al., 2012
	pLG1 [k] pDO2 pDO3 pNB2354	281.0 66.2 251.9 214.3	*E. faecium* UW2774 *E. faecium* DO *E. faecium* DO *E. faecium* B-2354	H, HH, A (pig), F (chicken), SW H H H	*E. faecium* *E. faecium* *E. faecium* *E. faecium*	Tn*1546* (*vanA*), *erm(B), tcrYAZB, ctpA, pilA, aac(6´)Ie-aph(2˝)Ia aadE, sat4,aph2 hly* adhesion *fimH* Type I RM system, diverse traits, Cd, Co, Mn	Laverde-Gomez, et al., 2011; Rosvoll, et al., 2012 Qin, et al., 2012 Qin, et al., 2012 GenBank CP004064.1

Table 1. continued from previous page.

	Untypeable megaplasmidsk	>150kb-350kb	*E. faecium*	H, HH, A, SW	*E. faecium, E. casseliflavus* and *E. gallinarum, E. durans, E. hirae*	Tn*1546* (*vanA*), *erm(B), hyl, aac(6´)-aph(2´´),, crYAZB, different metabolic* (mannitol, glycerol, sorbitol, raffinose, complex carbohydrates) , adhesion fimH	Biavasco, et al., 2007; Freitas, et al., 2011; Freitas, Novais, Cunha, Silveira, Peixe, & Coque, 2010; Freitas, et al., 2010; Hasman, Villadsen, & Aarestrup, 2005; Laverde-Gomez, et al., 2011

Abbreviations: Erm, erythromycin; Gm, high level of resistance to gentamicin; Km, kanamycin; Sm, streptomycin; St, streptothricin; Tet, tetracycline; Van, vancomycin: H, Hospital; HH, healthy humans; HHc, Healthy humans (children); A, animals (farm or healthy animals); WA, wild animals; F, foods; P, pets; SW, sewage.NA: Not available; NI: not identified.

a Plasmid size and backbone of putative Inc18 members are highly variable (30-50kb), due to the frequent acquisition of insertion sequences and transposons and DNA losses which seem to occur by spontaneous deletion after conjugal transfer to different hosts. Cointegration with theta plasmids of different families as pRUM, pheromone responsive, and pLG1-like plasmids from VRE and VSE strains is frequently observed (103, 125, 127, 308, 331, 333).

b Plasmids with unavailable sequences were assigned to a specific family based on homology or incompatibility with other known plasmids.

c p200B (GenBank acc number AB158402) contains a bac32 gene, which is associated with plasmid pTI1 (12.5 kb) (Inoue, Tomita, & Ike, 2006). Bac32 seems to be identical to EntTI (178). p200B and pTI1 are linked to the same publication and the same bacteriocin, so they might be the same plasmid, which is widespread among clinical isolates of E. faecium. To date, it is one of the two known bacteriocins linked to nosocomial E. faecium isolates.

d pEF418b was originally found in E. faecalis (GenBank AF408195.1) but epidemiological studies suggest it is much more frequent among E. faecium (Freitas, et al., 2013; Qin, et al., 2012).

e pEH-1 contains three Rep proteins, one identical to the Inc18 plasmids pVEF1-3 and other two 45 and 87% homologous to pWV05 from lactic bacteria. This plasmid is able to replicate in S. aureus RN4220 (Liu, et al., 2012).

f Inc18::Tn1546 plasmids from Europe are associated with E. faecium isolates (such as pVEF1, pEVF2, pVEF3, and pVEF4) lack a transfer system, and seem to have evolved from pIP816 isolated in France in 1987 (Freitas, et al., 2012; Rosvoll, et al., 2010; Sletvold, Johnsen, Simonsen, Aasnæs, Sundsfjord, & Nielsen, 2007; Sletvold, Johnsen, Wikmark, Simonsen, Sundsfjord, & Nielsen, 2010). Inc18::Tn1546 plasmids from the USA are linked to E. faecalis isolates (pWZ909, pWZ1668, pWZ7140) and contain a complete transfer system (Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008; Zhu, et al., 2010). Differences among enterococcal clonal backgrounds and Inc18 plasmids seem to be important in the spread of Tn1546 to non-enterococcal species (Fitzgerald & Clewell, 1985; Zhu, Clark, McDougal, Hageman, McDonald, & Patel, 2008; Zhu, et al., 2010).

g pEF1 carry a rep identical to that of pRE25 and a relaxase detected among most human E. faecium isolates (more than 90% in different collections analyzed) (Freitas, et al., 2011; Freitas, et al., 2012). Relaxase originally identified in pEF1 (pEF1_p34) (Garcillán-Barcia, Francia, & de la Cruz, 2009) has been detected in plasmids of different RepA_N subfamilies (Freitas, et al., 2013).

h pLG2 contains a rep similar to that of Inc18 plasmids and relaxases related to pAD1 (Laverde-Gomez, et al., 2011).

i pMG2200 is the first pheromone-responsive highly conjugative plasmid that encodes vanB resistance. It is a chimeric plasmid with a prgX-prgQ-traE1 genetic organization in the regulatory region of the pheromone response (prgX and prgQ being the key negative regulatory elements for plasmid pCF10 and TraE1, a key positive regulator of plasmid pAD1), a functional oriT region, and a putative relaxase gene member of the MOBP family, which is found in pheromone-independent pMG1-like plasmids.

j pRUM-related plasmids (such as pRUM, p5373c, pS177, or pDO2) are mosaic plasmids of variable size (30->60kb) that are comprised of sequence fragments from mobile genetic elements of different origins (Tns, ISs, small tetha replicating plasmids, bacteriocin clusters). Members of this group differ in the sequence of RepA_N, the mobilization system, and the presence of the toxin-antitoxin Axe-Txe locus (Freitas, et al., 2013; Freitas, et al., 2012; Grady & Hayes, 2003; Halvorsen, Williams, Bhimani, Billings, & Hergenrother, 2011; Rosvoll, et al., 2010). Plasmids that contain RepA and Axe-Txe from pRUM are globally spread among VRE and VSE E. faecium isolates from human and animal origins (Freitas, et al., 2012; Grady & Hayes, 2003; Halvorsen, Williams, Bhimani, Billings, & Hergenrother, 2011; Rosvoll, et al., 2010; Sung, Khan, & Nawaz, 2008). To date, only the original pRUM plasmid (Grady & Hayes, 2003) and another three pRUM-related plasmids [one of them pDO2 (Qin, et al., 2012) and two that carry vanB (Freitas, et al., 2012)] contain a mobilization system, which corresponds to the one encoded on pC223 from S. aureus (Grady & Hayes, 2003). Plasmids with RepA proteins that are 95% homologous to RepA-pRUM and lacking Axe-Txe are detected among a high percentage of VRE and VSE E. faecium from European countries as Portugal, Spain and Norway (Freitas, et al., 2013; Freitas, et al., 2010)]. They carried a relaxase originally detected in pEF1 (Freitas, et al., 2013; Freitas, et al., 2012; Garcillán-Barcia, Francia, & de la Cruz, 2009; Ruiz-Barba, Floriano, Maldonado-Barragán, & Jiménez-Díaz, 2007). These pRUM-like plasmids frequently contain additional replicons that belong to other RepA_N plasmid families (Freitas, et al., 2013; Freitas, et al., 2012).

k pLG1 contains a Rep protein similar to RepApAD1 (Jensen, Garcia-Migura, Valenzuela, Løhr, Hasman, & Aarestrup, 2010; Laverde-Gomez, et al., 2011)

Table 2. Replication initiator proteins in enterococci

Rep family	Plasmid name	Plasmid GenBank Acc. nº	Bacterial Host	Rep name	Rep initiator protein Genbank Acc. Nº
Rep_trans (RCR)	pJS42	NC_010291	*E. faecium*	RepA	YP_001654986.1
	pRI1	NC_010330.1	*E. faecium*	Rep	YP_001672021.1
	pRE25	NC_008445	*E. faecalis*	pRE25p11	YP_783895.1
	pEFNP1	AB038522.1	*E. faecium*	Orf1	BAA92162.1
	pRUM	NC_005000	*E. faecium*	RepM	NP_863166.1
	pTG9790	NC_015845.1	*E. hirae*	NicK	YP_004739228.1
	pKQ10	U01917.1	*E. faecium*	Orf1	AAB08924.1
Rep_2 (RCR)	pMV158	X15669	*Streptococcus, Enterococcus*	RepB	CAA33711.1
	pJB01	NC_006427.1	*E. faecium*	RepB	YP_138502.1
Rep_1 (RCR)	pNJAKD	NC_015849.1	*E. faecium*	pNJAKD_p5	YP_004747351.1
	pTEF1	NC_004669	*E. faecalis* V583	Rep	NP_816941.1
	pM7M2	NC_016009.1	*E. faecium*	Rep	YP_004849398.1
	pAMα1	NC_005013	*E. faecalis* DS5	RepB	NP_863351.1
RepA_N (θ)	pAD1	L01794.1	*E. faecalis* DS16	RepA	AAB00503.1
	pTEF1	NC_004669	*E. faecalis* V583	RepA-1	NP_816932.1
	EF62pC	CP002494.1	*E. faecalis* 62	PrgW	ADX81274.1
	pBEE99	NC_013533.1	*E. faecalis*	PrgW	YP_003329050.1
	pTEF2	NC_004671	*E. faecalis* V583	RepA-2	NP_817022.1
	pMG2200	NC_011642.1	*E. faecalis*	RepA	YP_002333458.1
	pTW9	NC_014726.1	*E. faecalis*	RepA	YP_004032946.1
	EF62pB	CP002493.1	*E. faecalis* 62	PrgW	ADX81203.1
	pEJ97-1	AJ490170.1	*E. faecalis*	RepA	CAD35304.1
	pAM373	NC_002630.1	*E. faecalis*	pAM373_p02	NP_071995.1
	pCF10	NC_006827	*E. faecalis*	PrgW	YP_195765.1
	pPD1	D78016.1	*E. faecalis*	RepA	BAA11194.1
	pLG1	HM565192.1	*E. faecium*	RepA	ADO66907.1
	pRUM	NC_005000	*E. faecium*	pRUM_p29	NP_863172.1
	pS177	NC_014959.1	*E. faecium*	RepA	YP_004172632.1
	p5753cB	GQ900487.1	*E. faecium*	RepA	ADA62751.1
Inc18 (θ)	pIP501	X17655.1	*Streptococcus, Enterococcus*	RepR	CAA35647.1
	pTEF1	NC_004669	*E. faecalis* V583	RepE	NP_816981.1
	pIP816	NC_011140.1	*E. faecium*	RepE	YP_002128409.1

Table 2. continued from previous page.

	pAMβ1	NC_013514.1	*E. faecalis* DS5	pAMbeta1_p03	YP_003305348.1
	pTW9	NC_014726.1	*E. faecalis*	RepE	YP_004032996.1
	pRE25	NC_008445	*E. faecalis*	RepS	YP_783890.1
	pWZ1668	NC_014475.1	*E. faecalis*	RepE	YP_003864107.1
	pWZ909	GQ484954.1	*E. faecalis*	RepE	ADM24822.1
	pWZ7140	GQ484955.1	*E. faecalis*	RepE	ADM24861.1
	pVEF1	NC_008768.1	*E. faecium*	Rep	YP_976064.1
	pVEF2	NC_008821.1	*E. faecium*	Rep	YP_001019023.1
	pEF-01	NC_014508.2	*E. faecalis*	Rep	YP_003896005.1
	pVEF4	FN424376.1	*E. faecium*	RepE	CAZ67085.1
	pEH-1	JN192453.1	*E. hirae*	RepE	AEV23020.1
	pEFM-1	JN201336.1	*E. faecium*	RepE	AEV23037.1
	pVEF3	NC_010980.1	*E. faecium*	Rep	YP_001974773.1
	pRE25	NC_008445	*E. faecalis*	Orf1	Q9AL28
	pVEF1	NC_008768.1	*E. faecium*	RepR	YP_976089.1
	pVEF2	NC_008821.1	*E. faecium*	RepR	YP_001019046.1
	pVEF3	NC_010980.1	*E. faecium*	RepR	YP_001974806.1
	pIP816	NC_011140.1	*E. faecium*	RepR	YP_002128419.1
	pLG2	HQ426650.1	*E. faecalis*	pLG2_0001	AEF32542.1
	pM7M2	NC_016009.1	*E. faecium*	RepR	YP_004849405.1
	p5753cB	GQ900487.1	*E. faecium*	Rep	ADA62715.1
	pEF1	NC_010880.1	*E. faecium*	RepR	YP_001966128.1
	pTEF3	NC_004670.1	*E. faecalis* V583	RepS	NP_817021.1
	pHTβ	NC_007594.1	*E. faecium*	Rep	YP_398701.1
	pZB18	AB611033.1	*E. faecium*	Rep	BAL40904.1
	pMG1	NC_011364.1	*E. faecium*	Rep	YP_002274355.1
Rep_3 (θ)	pAMα1	NC_005013	*E. faecalis* DS5	RepE	NP_863355.1
	pS86	AJ223161	*E. faecalis*	Rep	CAA11136.1
	pEFC1	D85392.1	*E. faecalis*	Rep	BAA12801.1
	p703/5	AF109375.1	*E. faecalis*	RepA	AAD16983.1
	EF62pA	CP002492.1	*E. faecalis* 62	RepE	ADX81198.1
	pEF-01	NC_014508.2	*E. faecalis*	RepB	YP_003896028.1
	pCIZ2	NC_008259	*E. faecium*	Orf8	YP_691718.1
	pGL	HQ696461.1	*E. durans*	Orf1	ADW93773.1
	pJS33	NC_010290.1	*E. faecium*	pJS33_01	YP_001654983.1
	pEF1071	NC_005010	*E. faecalis*	RepA	NP_863270.1
	p200B	AB158402	*E. faecium*	RepA	BAF44066.1

Table 2. continued from previous page.

	pEF47	AY842500.1	*E. faecalis*	RepE	AAX44237.1
	pB82	AB178871	*E. faecium*	RepA	BAF36632.1
	pHY	AB570326.1	*E. faecium*	RepA	BAK74730.1
	Plasmid	AY063485.1	*E. faecalis* FAIR-E 309	RepA	AAL39167.1
	pEF418	AF408195.1	*E. faecalis*	RepA	AAL05545.1
	pEFR	AF511037.1	*E. faecium*	Rep	AAM44830.1
	pMBB1	U26268.1	*E. faecium*	RepB	AAC44119.1

Neither replication initiators encoded in contigs derived from enterococcal genome sequencing projects, nor replication proteins from cloning vectors, are included.

Table 3. Enterococcal Relaxases

MOB family	Plasmid or Mobile element (ME) name	Plasmid or ME GenBank Acc. Nº	Bacterial Host	Relaxase name	Relaxase GenBank Acc. Nº
MOB_C	pAD1	AF343837	*E. faecalis* DS16	Orf57	AAL59457
	PAI_V583	NC_004668	*E. faecalis* V583	EF0505	NP_814286
	pTEF1	NC_004669	*E. faecalis* V583	EFA0025	NP_816951
	pAM373	NC_002630	*E. faecalis*	Orf8	NP_072012
	pLG2	HQ426665.1	*E. faecalis*	pLG2_0032	AEF32592.1
	pTW9	AB563188.1	*E. faecalis*	Orf39	BAJ34866.1
	EF62pC	CP002494.1	*E. faecalis* 62	EF62pC_0039	ADX81304.1
MOB_Q	pRE25	NC_008445	*E. faecalis*	MobA	YP_783908
	pIP501	L39769	*Streptococcus, Enterococcus*	MobA	AAA99466
	pAMβ1	NC_013514.1	*E. faecalis* DS5	pAMbeta1_p18	YP_003305363.1
	pWZ1668	NC_014475.1	*E. faecalis*	TrsA	YP_003864133.1
	pWZ7140	GQ484955.1	*E. faecalis*	TrsA	ADM24887.1
	pWZ909	GQ484954.1	*E. faecalis*	TrsA	ADM24846.1
MOB_P	pB82	AB178871	*E. faecium*	MobA	BAF36629
	pRUM	NC_005000	*E. faecium*	Rlx protein	NP_863170
	pCIZ2	NC_008259	*E. faecium*	Mob	YP_691715
	p200B	AB158402	*E. faecium*	MobA	BAF44062
	pEF1071	NC_005010	*E. faecalis*	MobA	NP_863268
	Tn*1549*_pIP834	AF192329	*E. faecalis*	Rlx-like protein	AAF72355
	pTEF2	NC_004671	*E. faecalis* V583	relaxase	NP_817049
	pCF10	NC_006827	*E. faecalis*	PcfG	YP_195793
	pGL	HQ696461.1	*E. durans*	MobA	ADW93779.1
	pHY	AB570326.1	*E. faecium*	MobA	BAK74727.1
	pEF1	NC_010880.1	*E. faecium*	pEF1_p34	YP_001966134.1
	pMG1	NC_011364.1	*E. faecium*	pMG1_p45	YP_002274390.1

Table 3. continued from previous page.

	pHTβ	NC_007594.1	*E. faecium*	pHTbeta_34	YP_398676.1
	pZB18	AB611033.1	*E. faecium*	TraI	BAL40943.1
	pLG1	HM565192.1	*E. faecium*	pLG1_0177	ADO66931.1
	pBEE99	NC_013533.1	*E. faecalis*	pBEE99_p32	YP_003329081.1
	EF62pB	CP002493.1	*E. faecalis* 62	EF62pB_0031	ADX81233.1
	pMG2200	NC_011642.1	*E. faecalis*	pMG2200_46	YP_002333406.1
	Tn*1549*_pMG2200	NC_011642.1	*E. faecalis*	pMG2200_26	YP_002333387.1
	BM4518_vanG	AY271782.1	*E. faecalis* BM4518	OrfG11	AAQ16254.1
	V583_P	NC_004668.1	*E. faecalis* V583	EF2303	NP_815959.1
MOB_V	pMV158	X15669	*Streptococcus, Enterococcus*	MobM	AAA25387
	pS86	AJ223161	*E. faecalis*	Mob, ORF4	CAA11139
	pAMα1	NC_005013	*E. faecalis* DS5	MobE	NP_863358
	pAMα1	NC_005013	*E. faecalis* DS5	MobB	NP_863352.1
	EF62pA	CP002492.1	*E. faecalis* 62	MobE	ADX81202.1
	pJS42	NC_010291	*E. faecium*	MobA	YP_001654987
	pRI1	NC_010330.1	*E. faecium*	Disrupted by ISEfa4	
	pNJAKD	NC_015849.1	*E. faecium*	pNJAKD_p4	YP_004747350.1
	pM7M2	NC_016009.1	*E. faecium*	pM7M2_p07	YP_004849396.1
MOB_T	Tn*916*	NC_006372.1	*E. faecalis* DS16	Orf20	YP_133675.1
	Tn*925*_pCF10	NC_006827	*E. faecalis*		
	Tn*5397*	AF333235.1	*Clostridium, Enterococcus*	Orf20	AAO24811.1
	Tn*5386*	DQ321786.1	*E. faecium*	Orf7	ABC48880.1
	Tn*6000*	JN208881.1	*E. faecalis*	Orf20	AEP33205.1
	Tn*6000*	FN555436.1	*E. casseliflavus*	Orf20	CBG92849.1
	OG1RF_CTn	CP002621.1	*E. faecalis*	OG1RF_10792	AEA93479.1
	V583_1	NC_004668.1	*E. faecalis* V583	EF0143	NP_813946.1
	V583_2	NC_004668.1	*E. faecalis* V583	EF1886	NP_815569.1
	V583_3	NC_004668.1	*E. faecalis* V583	EF2338	NP_815993.1
	V583_4	NC_004668.1	*E. faecalis* V583	EF2528	NP_816171.1
	EF62_CTn	CP002491.1	*E. faecalis* 62	EF62_0521	ADX78807.1
	pTG9790	NC_015845.1	*E. hirae*	NicK	YP_004739228.1

Table 4. Insertion sequences among enterococcal genomes and mobile genetic elements

Family group[a]	Nature of the catalytic site	Insertion Sequence	Genetic Element

Table 4. continued from previous page.

IS*6*	DDE	IS*1216*	Tn*1546*, Tn*5405,* Tn*5385* pIP816, pRE25, pRUM, pTEF1, pTEF3, pVEF1-2-3-4, pLG1, pS177, p5753cA, p5753cB, pUW1965, pUW786, pTW9, pSL1, pBEE99, pEF418, pEF-01, Efs-PAI, pA17Sv1, pJH1, pEJ97-1, pJM01, pHKK701, pEF-01, pEH-1, pEFM-1, pM7M2, pMG1-like, pDO1, pDO2, pDO3
		IS*257*	Tn*5384,* Tn*5385,* Tn*924*
IS*256*	DDE	IS*256*	Tn*4001*, Tn*5384*, Tn*5382*, Tn*1547*, p5753cB
			pTEF1, pTEF2, pTEF3, pVEF1-2-3, pWZ1668, pGM1
		IS*16*	Tn*1546*, Tn*1547,* pRUM, pDO2, pDO3
		IS*1191*/IS*905*[c]	Efs-PAI
		IS*1542*	Tn*1546*
IS*3*	DDE	IS*3*/IS*911*	pWZ1668, pBEE99, pLG1, pDO1, pDO2, pDO3, pVEF4, pRE25, pEF1
		IS*150*	Tn*5382*, pUW786
		IS*Enfa3*	Tn*5382*
		IS*153*	
		IS*1485* IS*Efa8*	Tn*1546*, pRE25, p5753cB, pM7M2, pMG1, pBE99, pDO1, pDO3, pVEF4, pWZ1668, pWZ140, pWZ909 pDO1, pDO3, pLG1
		IS*981*	pEF1, pRE25[e], p5753cB
IS*982*	DDE	IS*19* (IS*Efm1*)[d]	Tn*1546*, *vanD* cluster
IS*30*	DDE	IS*6770* (IS*Ef1*)[c]	Tn*1546*, Tn*1549*, pMRC01, pLG1, pBEE99
		IS*1252*	pHKK701, pHKK702, pIP816
		IS*1678*	Tn*1546*, pS177
		IS*1062*	pRE25, pPD1
IS*5* IS*66*	DDE DDE	IS*Efa5* IS*66*	Tn*1546* pLG1, pDO3
IS*110*	Site-specific	IS*110*/*116*/*902*	pEF01-1, pLG1, pDO2
	recombinase	IS*Enfa110*	Tn*5382*
IS*605*	Complex	IS*Efa4*	*vanD* cluster, pRI1
IS*200*	organization*	IS*Enfa200*	Tn*5382*
IS*L3*	Unknown*	IS*L3*	
		IS*204*/IS*1001*/	Tn*1546*, pLG1, pDO3
		IS*1096*/IS*1165*	
		IS*1251*	Tn*1546*, pS177
		IS*1476* IS*Efa11* (IS*1167*)	Tn*1546,* pLG1, pDO3

Table 4. continued from previous page.

IS*1380*	Unknown*	IS*Ecp1*	
IS*NYC*	Unknown*	IS*1182*	Tn*5405*, pRUM, pUW786, pS177, p5753cB

Abbreviations: PAI, Pathogenicity island.
[a] An IS family is defined as a group of ISs with related transposases, strong conservation of the catalytic site, conservation of organization, and similar IRs.
[b] The active sites of IS*200*/IS*605*, IS*L3*, IS*1380*, and IS*NYC* families have not been defined.
[c] IS*905* is an isoform of IS*1191*.
[d] IS*19* and IS*Efm1* and IS*6770* and IS*Ef1* are synonyms.
[e] The truncated version of IS*981* is present on pRE25.

Table 5. Enterococcal Transposons

Transposon	Flanking insertion sequences (Tn backbone)	Size (kb)	Characterized function	Host range	Origin	Comments	Reference
Composite Transposons							
Tn*4001(5281)* and variants	IS*256*-//-IS*256* (IS*256-aac6´-aph2´´*-IS*256*)	4.7	AB^R, Gm (*aac6´-aph2´´*)	*Enterococcus, S. aureus*, *Streptococcus agalactiae*, *Mycoplasma*	H, P	Tn*5381* is almost identical to Tn*4001* from *S. aureus* and Tn*3706* from *Streptococcus* Larger elements identified areTn*5384*, Tn*5385* in enterococci and *SCCmec in Staphylococci*	Hodel-Christian & Murray, 1991; Leelaporn, Yodkamol, Waywa, & Pattanachaiwit, 2008; Simjee, Fraise, & Gill, 1999; Simjee, et al., 2002
Tn*924*	IS*257*-//-IS*257* (IS*257-aac6´-aph2´´*-IS*257*)	27	AB^R, Gm (*aac6´-aph2´´*)	*E. faecalis*	H		Thal, Chow, Clewell, & Zervos, 1994
Tn*5384*	IS*256*-//-IS*256* (IS*256*-Tn*4001*-ΔTn*917-mer*-IS*256*)	26	AB^R, Gm, Erm (*aac6´-aph2´´*, *ermB*) Mercuric chloride (*merX*)	*E. faecalis*	H	Directly repeated copies of IS*256* Larger element identified: Tn*5385*	Bonafede, Carias, & Rice, 1997; Rice, Carias, & Marshall, 1995
Tn*5385*	IS*1216*-//-IS*1216* (IS*1216*-Tn*5381-Tn5384-Tn552*-IS*1216*)	65	AB^R, Gm, Erm, Sm, Tet, Pen (*aac6´-aph2´´*, *ermB, aadE, tetM, blaZ*) Mercuric chloride (*mer*)	*E. faecalis*	H	Directly repeated copies of IS*1216*	Rice & Carias, 1998
Tn*5405* and variants	IS*1182*-//-IS*1182* (IS*1182-aadE-sat4-aphA-3*-IS*1182*)	12	AB^R, Sm, St, Km (*aadE-sat4-aphA-3*)	*Enterococcus, Staphylococcus*, *Streptococcus, Campylobacter*	H, HH, P, A, F, SW	Inverted repeated copies of IS*1182* Larger element identified*:* Tn*4001*-Tn*5405,* Tn*5405*::Tn*5404, ermB*-Tn*5405*	Boerlin, Burnens, Frey, Kuhnert, & Nicolet, 2001; Derbise, Dyke, & el Solh, 1996; Werner, Hildebrandt, & Witte, 2003

Table 5. continued from previous page.

Tn*1547*	IS*16*-//-IS*256*-like (IS*16*-*vanB1*-IS*256*-like)	64	AB^R, Van *(vanB1)*	*Enterococcus*	H	Direct orientation of IS*16* and IS*256*-like	Quintillani, Jr. & Courvalin, 1996
Tn*5482*	IS*1216*-//-IS*1216* (IS*1216*-ΔIS*3*-like-Tn*1546*::*IS1251*-IS*1216*)	26-30	AB^R, Van *(vanA)*	*E. faecium, E. faecalis*	H		Handwerger & Skoble, 1995
Tn*5506*	IS*1216*-//-IS*1252*-*IS1216* (IS*1216V2*-*vanA*-IS*1252*-*IS1216V1*)	39	AB^R, Van *(vanA)*	*E. faecium*	H	IS*1216V2* in inverted direction of IS*1216V1* with IS*1252* insertion	Heaton, Discotto, Pucci, & Handwerger, 1996

Table 5b. Tn*3* and Tn*7* derivatives

Transposon	Family	Integrase type	Size (kb)	Characterized function[a] (genotype)	Host range	Origin	Reference
Tn*917* and variants (Tn*3871*)	Tn*3* (Tn*551*)	NA	5.1-5.4	AB^R, Erm *(ermB)*	*Enterococcus, Staphylococcus, Lactococcus, Streptococcus, Bacillus, Listeria, Paenibacillus*	H, HH, A, F, SW	McDougal, et al., 1998; Novais, et al., 2008; Tomich, An, & Clewell, 1980
Tn*917* and variants (Tn*3871*)	Tn*3* (Tn*551*)	NA	5.1-5.4	AB^R, Erm *(ermB)*	*Enterococcus, Staphylococcus, Lactococcus, Streptococcus, Bacillus, Listeria, Paenibacillus*	H, HH, A, F, SW	McDougal, et al., 1998; Novais, et al., 2008; Tomich, An, & Clewell, 1980
Tn*1546* and variants[d]	Tn*3* (Tn*3*)	NA	10.8	AB^R, Van *(vanA)*	*Enterococcus, Bacillus, Staphylococcus, Oeskorvia, Streptococcus, Rhodococcus, Arcanobacterium haemolyticum, Paenibacillus*	H, HH, A, WA, P, F, SW	Arthur, Molinas, Depardieu, & Courvalin, 1993; Guardabassi, Perichon, van Heijenoort, Blanot, & Courvalin, 2005; López, et al., 2010; Novais, Freitas, Sousa, Baquero, Coque, & Peixe, 2008; Weigel, et al., 2003
Tn552 and variantsd	Tn7	NA	10.8	ABR, beta-lactams (blaZ-blaI-blaR1)	Enterococcus, Staphylococcus	HH	Coudron, Markowitz, & Wong, 1992; Murray, 1990; Rice & Thorisdottir, 1994; Sarti, Campanille, Sabia, Santagati, Gargiulo, & Stefani, 2012

Table 5c. Conjugative transposons/ICEs

Transposon	Family	Integrase type	Size (kb)	Characterized function[a] (genotype)	Host range	Origin	Integration sites	Reference
Conjugative Transposons								
Tn916 [a] and closely related variants showing insertions or deletions	Tn916	Tyr	18	AB^R, Tet (tetM)	Enterococcus, Staphylococcus, Streptococcus, Lactococcus, Lactobacillus,	H, HH, FA, WA, F, SW	A+T rich	Flannagan, Zitzow, Su, & Clewell, 1994

Table 5c. continued from previous page.

					Bacillus, Clostridium, Leuconostoc, Listeria, Mycoplasma, Actinobacillus, Acholeplasma, Acinetobacter, Alcaligenes, Butyrivibria, Citrobacter, Erysipelothrix, Escherichia, Fusobacterium, Granulicatella, Haemophilus, Neisseria, Pseudomonas, Thermus, Ureaplasma, Veillonella, anaerobes			
Tn918	Tn916	Tyr		16	AB^R, Tet (tetM)	Enterococcus faecalis, staphylococci	H	A+T rich	Clewell & Francia, 2004
Tn919 [b]	Tn916	Tyr		15.4	AB^R, Tet (tetM)	S. sanguis, E. faecalis, Lactoccus lactis	HH	A+T rich	Fitzgerald & Clewell, 1985
Tn920 [b]	Tn916	Tyr		23	AB^R, Tet (tetM)	Enterococcus faecalis	H	A+T rich	Murray, An, & Clewell, 1988
Tn925*	Tn916	Tyr		18	AB^R, Tet (tetM)	Enterococcus faecalis, Leuconostoc	H, HH, F	A+T rich	Christie, Korman, Zahler, Adsit, & Dunny, 1987; Hirt, et al., 2005
Tn3702	Tn916	Tyr		18	AB^R, Tet (tetM)	Enterococcus faecalis	H	A+T rich	Horaud, Delbos, & de Cespédès, 1990
Tn5031, Tn5032, Tn5033	Tn916	Tyr		NK	AB^R, Tet (tetM)	Enterococcus faecium	H	A+T rich	Fletcher, Marri, & Daneo-Moore, 1989
Tn5381	Tn916	Tyr		18	AB^R, Tet (tetM)	Enterococcus faecalis	H	A+T rich	Rice & Carias, 1998; Rice, Marshall, & Carias, 1992
Tn5383	Tn916	Tyr		18	AB^R, Tet (tetM)	Enterococcus faecalis	H	A+T rich	Rice, Marshall, & Carias, 1992

Table 5c. continued from previous page.

Tn1545 and variants	Tn916	Tyr	ca. 25	AB^R, Tet, Erm, Kan (tetM, ermB, aphA-3)	Streptococcus, Enterococcus, Staphylococcus, Bacillus, Bacteroides, Clostridium, Lactococcus, Listeria, Escherichia, Eubacterium, Neisseria, Roseburia, Ureaplasma	H, F	A+T rich	Clewell & Francia, 2004; Weaver, Rice, & Churchward, 2002
Tn1549	Tn916	Tyr	30	AB^R, Van (vanB2)	E. faecium, anaerobes	H, HH	A+T rich	Ballard, Pertile, Lim, Johnson, & Grayson, 2005; Garnier, Taourit, Glaser, Courvalin, & Galimand, 2000; Launay, Ballard, Johnson, Grayson, & Lambert, 2006
Tn5382 [c]	Tn916	Tyr	34	AB^R, Van, Amp (vanB2, pbp5)	Enterococcus (E. faecium, E. faecalis, E. gallinarum, E. hirae), Streptococcus, anaerobes	H, A, F	A+T rich	Ballard, Pertile, Lim, Johnson, & Grayson, 2005; Carias, Rudin, Donskey, & Rice, 1998; López, et al., 2009; Torres, et al., 2003
Tn6084, Tn6085a Tn6085b [b]	Tn916	Tyr		AB^R, Tet (tetM)	Enterococcus faecium	H, HH	A+T rich	Rice L. B., Carias, Rudin, Hutton, & Marshall, 2010
Tn5386	Tn916	Tyr	29	Lantibiotics (spa)	Enterococcus faecium	H	A+T rich	Rice L. B., Carias, Marshall, Rudin, & Hutton-Thomas, 2005
Tn6009	Tn916	Tyr	18	AB^R, Tet (tetM) Mercury (mer)	Enterococcus, Streptococcus, Pseudomonas, Klebsiella, Serratia	HHc	Not identified	Soge, Beck, White, No, & Roberts, 2008

Table 5c. continued from previous page.

ICEEfm1 [e] ($espPAI_{Efaecium}$)	Tn916	Tyr	64-104	Esp, partial copy of Tn916 including relaxase	Enterococcus faecium	H, HH, A, SW	Single site, Identified downstream rpsI gene	Leavis, et al., 2004; Top, Sinnige, Majoor, Bonten, Willems, & van Schaik, 2011; van Schaik, et al., 2010
Tn5397	Tn916	Ser	21	AB^R, Tet (tetM)	Enterococcus (E. faecium, E. hirae), Streptococcus, Clostridium difficile, Bacillus subtilis	H, HH, A, WA, F, SW	Single site	Agersø, Pedersen, & Aarestrup, 2006; Novais, Freitas, Baquero, Peixe, & Coque, 2010; Poeta, et al., 2007
Tn5801 [f], CW459 (Tn6086, Tn6014)	Tn916		25	AB^R, Tet (tetM)	Staphylococcus, Enterococcus, Streptococcus mitis, Clostridium perfringens	H, HH, A, F, SW	3′ of GMP synthase gene	de Vries, Christensen, Skov, Aarestrup, & Agersø, 2009; Denapaite, et al., 2010; Novais, Freitas, Baquero, Peixe, & Coque, 2010
Tn6000	Tn916	Related to that of S. aureus PAI SaPIbov and SaPIbov2	33.2	AB^R, Tet (tetS)	Enterococcus (E. faecium, E. faecalis, E. casseliflavus)	H, HH, A	3′ end of gene encoding ribosomal protein L31	Brouwer, Mullany, & Roberts, 2010; Novais, Freitas, Baquero, Peixe, & Coque, 2010; Roberts, Davis, Seville, Villedieu, & Mullany, 2006
Tn950	NI	Not identified	47	AB^R, Erm (ermB)	Enterococcus faecium	H	Not identified	Takeuchi, Tomita, Fujimoto, Kudo, Kuwano, & Ike, 2005
EfaC1	NI	Tyr	25.3	None identified	Enterococcus faecalis	H	3′ end of gene encoding tRNA	Burrus, Pavlovic, Decaris, & Guédon, 2002; Paulsen, et al., 2003

Table 5c. continued from previous page.

EfaC2	NI	Tyr	32.7	None identified	Enterococcus faecalis	H	3′ of GMP synthase gene	Burrus, Pavlovic, Decaris, & Guédon, 2002; Paulsen, et al., 2003
EfaD2	NI	Tyr	ND	None identified	Enterococcus faecalis	H	Unknown	Burrus, Pavlovic, Decaris, & Guédon, 2002; Paulsen, et al., 2003
OG1RF-CTn homologue	NI	Putative phage-related integrase	49	Putative adhesin proteins	*Enterococcus faecalis*	H	Unknown	Bourgogne, et al., 2008

Abbreviations: AB^r, antibiotic resistance; Amp, ampicillin; Erm, erythromycin; Gm, high level of resistance to gentamicin; Km, kanamycin; Sm, streptomycin; St, streptothricin; Tet, tetracycline; Van, vancomycin: Tyr, tyrosine recombinase; Ser, serine recombinase; H, Hospital; HH, healthy humans; HHc, Healthy humans (children); A, animals (farm or healthy animals); WA, wild animals; F, foods; P, pets; SW, sewage.NA: Not available; NI: not identified

[a] Multiple variants that contain insertions or deletions have been described for these CTns. The previously published CTn*916S* (*tetS*), identified in a *Streptococcus*
intermedius human isolate (Genbank accession no.AY534326.1), is in fact an hybrid of CTn*6000* and CTn*916* carrying a mosaic tet(S/M) (Novais, et al., 2012).

[b] Tn*919* and Tn*920* have not been completely characterized.

[c] Tn*5382* is basically Tn*1549* with a cluster of genes that contain *pbp5* inserted upstream of the Tn.

[d] Tn*6084*, Tn*6085a*, and Tn*6085b* contain a 2.7 kb putative group II intron inserted at nucleotide 3913 at the beginning of orf19 in Tn916, whose function remains unknown. Tn*6084* has an *ISEfa11* insertion. Tn*6085a* and Tn*6085b* are identical and therefore were given the designations Tn*6085a* and Tn*6085b*. All three have been identified in the same isolate *E. faecium* strain C68, which also harbors Tn*5382* (*vanB*).

[e] Previously described as a genetic island (Leavis, et al., 2004; van Schaik, et al., 2010). It shares the ***esp*** gene itself and a 10-kb completely conserved gene cluster with the PAI of E. *faecalis* (Top, Sinnige, Majoor, Bonten, Willems, & van Schaik, 2011).

[f] Tn*5801* has been completely characterized in *E. faecium* and *E. faecalis* (Novais, et al., 2013, T.M. Coque personal communication), only integrases have been detected in natural isolates (Novais, et al., 2013).

Table 6. Enterococcal Introns

Name	ORF Name	Host gene	Locus	Locus	Size	Type	GenBank accession nº (nt position)	Species
E.f.I1	RT	Unknown	Plasmid	pHTbeta	638	B	AB105543 (1-2748)	*E. faecalis, E. faecium*
E.f.I2	IepA like protein	TraG like protein	PAI	PAI	653	B	AF454824 (13115-15951)	*Enterococcus faecalis*
E.f.I3	Group II intron RT	MAFF-like protein	Chromosome	Chromosome	628	B	AE016954 (115179-117948)	*Enterococcus faecalis*
E.f.I4	Unnamed	FtsK/ SpoIIIE family protein	Chromosome	Chromosome	584	B	AE016830 (1833893-1836490)	*Enterococcus faecalis*

Table 6. continued from previous page.

En.fm.I1-1	EfaeDRAFT_2438	ORF19	CTn, plasmid	Tn*916*, Tn*6085*a, Tn*6085*, pCF10	638	B	NZ_AAAK03000007 (10877-13634) NC_006372.1	*Enterococcus faecium Enterococcus faecalis, Streptococcus, Staphylococci, Clostridium*
En.fm.I1-2	ORF16	None	CTn, plasmid	CTn*5386*, Inc18 plasmids (pVEF1-4, pIP186, pWZ909, pTW9,	608	B	DQ321786 (12459-15138)	*Enterococcus faecium Enterococcus faecalis, Streptococcus, Staphylococci, Lactococcus*
C.d.I1	ORF16	Unnamed	ORF14	CTn*5397*, CTn*6000*	609	B	X98606 (13-2658)	*Clostridium difficile, Enterococcus casseliflavus, E. faecium,*
L.l.I1[a]	ltrA	ltrB, mobA	Conjugative plasmid	pRS01	599	A1	U50902 (2854-5345)	*Lactococcus lactis*

Updated and completed on the information available at http://www.fp.ucalgary.ca/group2introns/
Abbreviations: PAI, pathogenicity island; CTn, conjugative transposon
[a] L.l.I1 has not been identified in natural enterococcal isolates (see text).

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Enterococcal Bacteriophages and Genome Defense

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Enterococcal Bacteriophages

A brief overview of bacteriophages: Bacteriophages (phages) are viruses that infect bacteria. Similar to the viruses of plants and animals, phages are inert and are unable to propagate themselves in the absence of a host. Phages depend on host metabolism to provide the organic material and machinery necessary for their replication and for the subsequent packaging of the viral genetic material during phage particle biosynthesis. Phages are associated with nearly all known bacterial taxa and, as a result, are found in diverse environments that range from soil to oceans and even in deserts (Prestel, Salamitou, & DuBow, 2008; Prigent, Leroy, Confalonieri, Dutertre, & DuBow, 2005; Srinivasiah, Bhavsar, Thapar, Liles, Schoenfeld, & Wommack, 2008; Wommack & Colwell, 2000). Phages are found either directly associated with their bacterial hosts or in large numbers as free virions in the environment. Since there is a vast distribution of phages across the globe, it is possible to theorize that phages constitute the most abundant biological entities on earth. Their numbers have been estimated to reach as high as 10^{31} particles with the potential for 10^{25} phage infections occurring every second (Pedulla, et al., 2003; Wommack & Colwell, 2000). As many more phage genome sequences have become available in recent years, it is obvious that phages are extremely incongruent at the genomic level. This diversity in genetic makeup is proposed to result from the fastidious replication of phage particles during the infection of highly permissive hosts. During these infections, phages are able to exchange DNA within host genomes through recombination, and continually generate diversity as a result (Hendrix, Smith, Burns, Ford, & Hatfull, 1999).

The vast majority of phages belong to the order of *Caudovirales*, which are tailed phages that have dsDNA and an isometric capsid. *Caudovirales* is comprised of three phylogenetically-related families that are discriminated by tail morphology: *Myoviridae* (long contractile tails), *Siphoviridae* (long non-contractile tails), and *Podoviridae* (short tails) (Ackermann, 2007; Krupovic, Prangishvili, Hendrix, & Bamford, 2011). The most well-studied tailed phages are the coliphages λ (*Siphoviridae*), T4 (*Myoviridae*), and T7 (*Podoviridae*) which infect *Escherichia coli* and which have served as workhorses for elucidating the mechanisms of modern molecular genetics and biochemistry (Johnson, Poteete, Lauer, Sauer, Ackers, & Ptashne, 1981; Miller, Kutter, Mosiq, Arisaka, Kunisawa, & Rüger, 2003; Ptashne, et al., 1980; Tabor & Richardson, 1985). Far less abundant are the non-tailed phages, which encompass numerous families with great morphological distinction; these include phages that are filamentous (long filaments to short rods), polyhedral (vesicular and envelope-like), and pleomorphic (including those that are lemon, droplet, and ampule shaped) (Ackermann, 2007). The nucleic acid content of phage genomes is either DNA or RNA and both double and single stranded DNA and RNA phages have been identified. In addition, the size of the phage genome can range from under ten kilobases to several hundred kilobases.

Phages have evolved replication strategies that can be lytic, lysogenic (temperate), or chronic. Chronic replication results in the continual, non-lethal shedding of virions by protrusion through the membrane. All phages have common life-cycle stages of adsorption, DNA injection and replication, virion production, and release. Tailed phages mediate host cell lysis through the combined action of a holin, which perforates the membrane, and an endolysin (lysin), which hydrolyses cell wall peptidoglycan. Lytic phages are restricted to a

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life-cycle that results in the lysis of their host. Temperate phages have two possible life-cycles: lysis, or the recombination of their genome at a chromosomal attachment site using a phage-encoded integrase. Temperate phages are maintained within the host chromosome by transcriptional repressors that determine when the phage undergoes an infectious or lytic switch. The lytic switch occurs when conditions within their host promote excision. Excision usually proceeds during times of hardship when host health is threatened, either by physical stress or by chemical stress, such as antibiotics, ultraviolet (UV) light, or reactive oxygen species (Allen, et al., 2011; DeMarini & Lawrence, 1992; Little & Mount, 1982). Temperate phages provide key insights into the evolution of bacterial pathogenesis, since many temperate phages encode virulence factors used by pathogenic bacteria during both human and animal infections (Bensing, Siboo, & Sullam, 2001; Brüssow, Canchaya, & Hardt, 2004; Novick, Christie, & Penadés, 2010).

Distribution of phages across the enterococci

The first documented reports of enterococcal phages were published over 70 years ago (Clark & Clark, 1927; Evans, 1934). It was not until the early 1960s that more comprehensive analyses of enterococcal phages began to take shape (Brock, 1964; Rogers & Sarles, 1963). At the time, which was prior to the advent of modern molecular phylogenetics, the enterococci were characterized as group D streptococci. Rogers and Sarles (Rogers & Sarles, 1963) isolated phage-like particles from the intestinal tracts of Sprague-Dawley rats, and identified two phages that were capable of forming plaques on *Streptococcus faecalis* var. *zymogenes.* After careful analysis of host range using intestinal isolates of *S. faecalis,* followed by phage antibody serotyping, Rogers and Sarles captured some of the first images of enterococcal phages by using electron microscopy. They determined that the enterococcal phages appeared to have icosahedral heads and long non-contractile tails (Rogers & Sarles, 1963). One year later, Thomas Brock published a detailed survey of the host range of several enterococcal phages and identified enterococcal lysogens for the first time (Brock, 1964). Furthermore, Brock made the distinction that when testing for plaque formation on *S. faecalis* var. *zymogenes*, *Streptococcus faecalis* var. *liquefaciens,* and *Streptococcus faecium,* a degree of cross-reactivity of the phages for different host strains was present. Interestingly, this promiscuity was unique within *S. faecalis* and *S. faecium,* and phages specific to one species did not otherwise infect the other. When this type of phage resistance does occur, it is often due to inherent differences between species, with respect to their cell wall structure, mechanisms of protection against the acquisition of foreign DNA, and immunity due to lysogeny (superinfection exclusion).

Currently the well-studied enterococcal phages are those that infect and lysogenize *Enterococcus faecalis* and *Enterococcus faecium*. Numerous lytic phages that infect *E. faecalis* and *E. faecium* have been isolated from diverse environments, including sewage and wastewater sites, livestock runoff, and the intestinal tract (Horiuchi, Sakka, Hayashi, Shimada, Kimura, & Sakka, 2012; Lee & Park, 2012; Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010; Otawa, Hirakata, Kaku, & Nakai, 2012; Parasion, Kwiatek, Mizak, Gryko, Bartoszcze, & Kocik, 2012; Rogers & Sarles, 1963). To achieve host lysis phages encode cell membrane holins and peptidoglycan hydrolases. These cell wall hydrolases, most commonly from lytic phages, have great potential as novel therapeutics that can target pathogenic strains of *E. faecalis* and *E. faecium*.

Known phage families found among Enterococci

Until recently, all characterized enterococcal phages belonged to the *Podoviridae*, *Siphoviridae*, or *Myoviridae* families. These phages exhibit considerable genetic and morphological diversity. For example, the genome sequence of *E. faecalis* strain 62, which was isolated from a healthy infant, revealed the presence of a *Podoviridae* phage, EF62Φ, which has a linear extrachromasomal genome. The maintenance of EF62Φ in *E. faecalis* is proposed to be the result of an encoded RepB and a toxin-antitoxin system (Brede, Snipen, Ussery, Nederbragt, & Nes, 2011). Mazaheri Nezhad Fard *et al.* isolated the first non-tailed enterococcal phages that belong to the polyhedral, filamentous, and pleomorphic (PFP) phages. In this study, the authors isolated phages from *E. faecalis*, *E. faecium*, and *Enterococcus gallinarum* strains found in piggery effluent. These phages, which were

similar to PFPs, included a filamentous *Inoviridae* family phage, a polyhedral phage of the *Leviviridae* family, and several abnormally shaped pleomorphic phages that resembled droplet or lemon-like structures and which belong to the *Guttaviridae* and *Fuselloviridae* families (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010). Little is known about the genetic organization of these diverse phages, and it is also unclear whether these phages have lysogenic life-cycles.

Environments where enterococcal phages are found

Enterococcal species reside in the oral cavity and urogenital tract of mammals as well as the digestive systems of mammals and insects. Enterococci are also frequently found in fecal waste sites, such as sewage treatment plants and uncontained areas of fecal contaminated groundwater. Furthermore, enterococci have evolved to become opportunistic pathogens that cause life-threatening infections, including endocarditis, septic bacteremia, and hospital-acquired wound infections. It is quite likely that the phages associated with different enterococcal species are also located within these diverse environments.

Enterococcal phages have been isolated from human and animal origins where the enterococci live as commensal bacteria (Caprioli, Zaccour, & Kasatiya, 1975; Nigutová, Styriak, Javorský, & Pristas, 2008; Rogers & Sarles, 1963). A comprehensive study from the 1970s isolated numerous enterococcal phages from the human urogenital tract, including phages found at urethral, endocervical, and ano-rectal body sites (Caprioli, Zaccour, & Kasatiya, 1975). These phages were highly successful at infecting numerous *E. faecalis* and *E. faecium* strains. Based on their ability to infect and lyse these enterococci, the phages were used to classify isolates of *E. faecalis* and *E. faecium* into 27 and 22 distinct strain types, respectively (Caprioli, Zaccour, & Kasatiya, 1975). This study highlights the importance of environments where enterococci are commensal bacteria as sources of phages. Further studies of these types of phages both within and outside their natural habitats may shed light on the ecology and community dynamics of commensal enterococci.

Enterococcal phages isolated from the intestinal tracts of rodents have been characterized (Rogers & Sarles, 1963); however, studies on enterococcal phage particles from human intestinal contents are limited. One particular study that addresses the composition of intestinal bacterial communities associated with a premature low-weight infant discovered that this infant maintained an amplified clonal population of *Enterococcus* in its intestines (Morowitz, et al., 2010). It was determined that the 16S rRNA sequence of this enterococcal strain, UC1ENC, was identical to the 16S rRNA sequences of several *E. faecalis* strains. Using total bacterial DNA sequences from the infant gut, the authors were able to reassemble the UC1ENC genome. UC1ENC shared ~81% of its protein coding sequence with *E. faecalis* V583. Two UC1ENC chromosomal prophage elements shared DNA sequence similarity with two phages (phage02 and phage04) found in the *E. faecalis* V583 chromosome. A second study further substantiated this evidence, and showed that the rise of *Enterococcus* within premature low-weight infants was not exclusive to the individual infant of the Morowitz *et al*. study. These data showed that in the case of eleven different premature low-weight infants, 75% of the individuals screened were highly populated with *Enterococcus* species within their intestinal bacterial communities (LaTuga, et al., 2011). In this study, numerous phage DNA sequences were also identified from the intestinal contents of the premature low-weight infants; however, this analysis did not elaborate as to whether any of these phages may be associated with the colonizing enterococcal strains. As an effort to better understand the microbial communities of the intestinal tract, the National Institutes of Health–funded Human Microbiome Project has begun to sequence numerous bacterial isolates from the intestine, including many species of enterococci (Proctor, 2011). These enterococcal genome sequences have revealed a large number of putative prophages that may be important to the commensal biology of the enterococci in the intestine.

The enterococci are also commensals of the mammalian oral cavity, and in some instances, have been associated with periodontal disease (Kayaoglu & Ørstavik, 2004). Enterococcal infections of the root canal, especially those caused by *E. faecalis*, are extremely resistant to current therapies (Stuart, Schwartz, Beeson, & Owatz, 2006).

Unlike the intestinal tract, *E. faecalis* phages from the human oral cavity have been isolated and studied in detail. *E. faecalis* isolates have been recovered from the infected root canals of humans for whom therapeutic interventions were unsuccessful (Stevens, Ektefaie, & Fouts, 2011; Stevens, Porras, & Delisle, 2009). Four out of ten of these *E. faecalis* isolates could be induced to produce lytic phages through mitomycin C treatment. Three of these phages resembled *Siphoviridae* phages, and were characterized by long non-contractile tails and spherical heads. The fourth phage identified resembled a phage that was more closely related to the *Myoviridae,* with a contractile tail with tail fibers and an icosahedral head structure. This study was the first to show that enterococcal strains living within the oral cavity can be induced to produce phages. Enterococcal phages have been used to successfully reduce the ability of *E. faecalis* to grow on the surface of human dental roots. A phage multiplicity of infection of 0.1 was sufficient to minimize the ability of *E. faecalis* to colonize dental roots (Paisano, Spira, Cai, & Bombana, 2004). Treatment of *E. faecalis* endontic infection is difficult and recurrent infection is a concern. It has been proposed that phages that infect these bacteria may prove useful as an alternative to current treatment options (Paisano, Spira, Cai, & Bombana, 2004; Stevens, Porras, & Delisle, 2009).

Being members of the intestinal microbiota, *Enterococcus* species are shed from humans and animals in fecal waste. Phages have been instrumental in determining the existence of enterococci within contaminated water environments and are currently being used to monitor fecal contaminated water sources for the presence of enterococcal strains. One method relies on *Enterococcus* isolates from diverse contaminated water sources including waste-water run-off areas of grazing cattle, pigs, or sheep, and municipal waste-water sites (Räisänen, et al., 2007). These enterococcal isolates, which include multiple strains from the species *E. faecalis*, *E. faecium*, *E. gallinarum*, and *Enterococcus casseliflavus*, are used to survey water samples where enterococcal contamination is unknown. Enterococcal contamination within these water samples is indicated by the presence of phages capable of infecting the collection of enterococcal strains. This method, referred to as microbial source tracking, relies on using indicator strains of enterococci to identify infectious phages within water samples as a metric of enterococcal fecal contamination (Purnell, Ebdon, & Tayor, 2011). A second method uses a similar approach; however, a bank of known enterococcal phages termed "enterophages" are used to screen water samples for fecal *E. faecalis* contamination (Bonilla, Santiago, Marcos, Urdaneta, Domingo, & Toranzos, 2010; Santiago-Rodriguez, et al., 2010). Enterophages exclusively infect strains of *E. faecalis* and are present at levels of ~10^2 phages per 100 ml of domestic raw sewage (Bonilla, Santiago, Marcos, Urdaneta, Domingo, & Toranzos, 2010). Enterophages are diverse and include phages similar to the *Siphoviridae* as well as non-tailed phages with icosahedral shaped capsids. It is also thought that enterophages may be indicative of water sources contaminated with human fecal waste, although, enterophages similar to those isolated from contaminated water sources have yet to be identified in human stool samples (Santiago-Rodriguez, et al., 2010).

Enterococcal Temperate Phages and the Impact of Lysogeny

Temperate phages possess an alternative life-cycle that is absent from the reproduction of lytic bacteriophages, whereby the bacterial host harbors the phage genome and then replicates it during cell division. This transmits the phage genome vertically to daughter cells, which subsequently propagate the phage. Lysogens—bacteria acting as phage genome hosts—express resistance to superinfection by the same phage, but not to superinfection by heterologous phages (Birge, 1994). Multiple lysogenic infections, over time and with heterologous temperate bacteriophages, produce polylysogens. Comparative genome analyses of several low G+C genera of the *Bacilli* subbranch, such as *Enterococcus*, *Streptococcus*, *Staphylococcus,* and *Listeria* reveal that polylysogeny is common. During lysogeny, which follows recombination of the phage genome into the host chromosome; most of the phage genes are repressed. Those genes that are expressed are mostly involved in the maintenance of lysogeny, and the expressed proteins have regulatory functions that prevent the transcription of genes encoding replication, morphogenesis, and lytic components responsible for the lytic life-cycle of the phage (Ptashne, 2004). Phage conversion genes that are present on some prophages are often expressed during lysogeny. In numerous prophages of the low G+C Firmicutes (e.g. *Staphylococcus aureus*), phage conversion genes encode

known or proposed virulence and fitness genes (Desiere, Lucchini, Canchaya, Ventura, & Brüssow, 2002; Prévost, et al., 1995; Tormo, et al., 2008; van Wamel, Rooijakkers, Ruyken, van Kessel, & van Strijp, 2006). Lysogeny is not a permanent state and during bacterial growth infective virions arise due to spontaneous prophage induction. The rate at which prophages enter the lytic cycle is phage and host-specific and, in addition, chemical or physical agents that damage DNA, including oxidants, some antibiotics like mitomycin C, and UV radiation, can all induce prophage entry into the lytic cycle.

Temperate phage genomics

The 3.2 Mb chromosome of the vancomycin-resistant *E. faecalis* strain V583 revealed that lysogeny contributed the largest component of horizontally-acquired DNA in this clinical isolate. Seven potential integrated phage–derived sequences were identified that comprised close to 10% of the host cell DNA (Paulsen, et al., 2003). phage01 (EF0303-55), phage03 (EF1417-89), phage04 (EF1988-2043), phage05 (EF2084-145), and phage06 (EF2798-855) have sufficient composition for integration/excision, DNA replication, and capsid/tail morphogenesis to generate functional virions, either alone or synergistically with other prophages. The phage02 region (EF1276-93) identified in the V583 genome, was later described to form part of the core genome (McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007). This region appears to be the remnant of a prophage and this cryptic phage likely retains no capacity for induction into the lytic life-cycle. Similarly, the phage07 region (EF2936-55) of strain V583 appeared to be a cryptic prophage but was recently shown to produce infectious virions (Duerkop, Clements, Rollins, Rodrigues, & Hooper, 2012; Matos, et al., 2013). Phage07 is similar to the phage-related chromosomal islands of Gram-positive bacteria which are mobile elements that utilize the packaging and structural elements of a helper phage for dissemination (Novick, Christie, & Penadés, 2010). Phage07 hijacks phage01 particles in this manner upon excision from the chromosome and has been re-named *E. faecalis* chromosomal island of V583 (EfCIV583) (Matos, et al., 2013). Sequencing of the 2.8 Mb genome of strain OG1RF, the parent of which was originally isolated from the human oral cavity, provided a stark contrast to strain V583, since only one phage element was present (the cryptic phage02). Multiple sequenced *E. faecalis* genomes are deposited in publicly-available databases and the presence or absence of prophages across many of these different genomes has been determined by using DNA microarray-based comparative genomic hybridization and comparative genomic analyses (Lepage, et al., 2006; McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007; Solheim, Brekke, Snipen, Willems, Nes, & Brede, 2011). These studies confirm the variable presence of prophage elements integrated at six regions identified in the V583 genome (phage01, phages 03–06, and EfCIV583).

Lysogenic conversion

In addition to bacterial genome comparison approaches, studies have described the genomics of purified virions liberated by phage-generated lysis after induction (Stevens, Porras, & Delisle, 2009; Yasmin, et al., 2010). The benefit of these studies lies in their capacity to determine the infectious temperate phages present within discrete strain sets, as well as a description of the laterally transferred DNA sequences. In the study by Yasmin et al. (Yasmin, et al., 2010), a collection of 47 clinical *E. faecalis* bacteremia isolates were screened for prophage induction using norfloxacin, mitomycin C, and UV light as stressors. Thirty-four unique phages were induced from these strains, as determined by host range and restriction fragment length polymorphisms (RFLP). Twelve strains in the study (26%) were polylysogens that contained up to five inducible prophages, based upon their host range and the restriction digest of DNA (Yasmin, et al., 2010). Of the phages that were identified, eight were confirmed as *Siphoviridae* by their morphology using electron microscopy indicating their long, non-contractile tails (~200 nm) and isometric capsids (~50 nm diameter) (Figure 1). The genome sequences of these phages were determined using DNA pyrosequencing.

Comparative genomic analyses grouped the eight *Siphoviridae* phages into four phage sequence types (ΦFL1A, B. and C; ΦFL2A and B; ΦFL3A and B; and ΦFL4A). Of these, ΦFL4A shares a high nucleotide identity with the

phage01 region of strain V583, and the integrase proteins of these two phages have 99% amino acid identity (Yasmin, et al., 2010). The ΦFL1, ΦFL2, and ΦFL3 phages all have identical integrase proteins, and two of these phages, ΦFL2B and ΦFL3A, were induced from the same polylysogen host. This finding indicates that polylysogenized genomes are likely to exhibit diversity in the order that phages lysogenize their host. The DNA replication and packaging regions of the ΦFL1 and ΦFL2 phages share sequence identity with the phage03 and phage05 regions of strain V583, but appear to be otherwise distinct (Yasmin, et al., 2010). The study of Stevens et al. (Stevens, Porras, & Delisle, 2009) identified a single *Siphoviridae* phage, ΦEF11, from the lysogen host strain TUSoD11, following induction with mitomycin C, that was morphologically similar with those described by Yasmin et al. (Yasmin, et al., 2010). From these two studies, it appears that the genome organization of the *Siphoviridae* phages of *E. faecalis* is similar to that of many of the *Siphoviridae* phages that infect low G+C Gram-positive bacteria (Stevens, Ektefaie, & Fouts, 2011; Yasmin, et al., 2010). Phage genomes have been described as being modular in their organization (Desiere, Lucchini, Canchaya, Ventura, & Brüssow, 2002; Hatfull, Cresawn, & Hendrix, 2008) and the genomes of the described *E. faecalis Siphoviridae* phages are similarly modular within the three transcriptional units (Figure 2). The first unit of the prophage (i.e., as it appears on the host chromosome) is the leftward-transcribed integrase/cI region for the maintenance of lysogeny. A large rightward-transcribed region encoding proteins for the lytic pathway, replication, packaging, head/tail morphogenesis, and lysis functions is followed by a variable leftward-transcribed region. This terminal leftward-transcribed region between the lysin and the right-hand phage attachment site in many temperate phages of the low G+C Gram-positive bacteria often contains phage conversion genes. For example, the genes located in these regions resemble those that encode innate immune evasion proteins of *S. aureus* (Desiere, Lucchini, Canchaya, Ventura, & Brüssow, 2002; Prévost, et al., 1995; Tormo, et al., 2008; van Wamel, Rooijakkers, Ruyken, van Kessel, & van Strijp, 2006).

Analysis of the ΦEF11 phage genome and the genomes of the ΦFL1, ΦFL2, ΦFL3, ΦFL4 phages identified several potential phage conversion genes, also called cargo (Stevens, Ektefaie, & Fouts, 2011; Yasmin, et al., 2010). The ΦEF11 phage appears to have an extended set of lysins immediately followed by three leftward-transcribed genes, two of which encode a putative membrane protein and a lysM domain-containing protein (Stevens, Ektefaie, & Fouts, 2011). Based on their predicted function, these proteins are likely to be located on the host cell surface, and if expressed during growth, represent *bona fide* phage conversion genes. These proteins could also be involved with lysogeny or immunity to phages—or, as proposed by Stevens *et al* (Stevens, Porras, & Delisle, 2009), they may facilitate host cell lysis. The ΦFL1, ΦFL2, and ΦFL3 phages contain one or more small open reading frames within their cargo regions that have inferred amino acid sequence identity with control proteins of streptococcal phages, which suggests a possible role in the maintenance of lysogeny (Yasmin, et al., 2010). The ΦFL4 phage encodes no potential phage conversion genes in the terminal region of its genome. Further studies remain to be performed to investigate the carriage of phage conversion genes in enterococcal temperate phages.

The initial description of the *E. faecalis* strain V583 genome proposed that phage04 contains a ferrochelatase-encoding gene (EF1989) that could function in heme biosynthesis (Paulsen, et al., 2003). Within phage04, the gene *cspA* (EF1991) encodes a cold shock family protein homologous to that of *E. coli* (Lee, Xie, Jiang, Etchegaray, Jones, & Inouye, 1994). CspA homologs are encoded within a prophage genome of the *Lactococcus lactis* strain ll1403 and the *Streptococcus* phage bIL312 genome. A second copy of *cspA* (EF0781), with a high-sequence identity to the *cspA* homolog on phage04, is found elsewhere in the genome of its strain V583 host. Comparative prophage analyses have revealed that there are clear similarities between many modular phage genes present in *Enterococcus*, *Lactococcus, Streptococcus, and Staphylococcus* species (Paulsen, et al., 2003; Stevens, Ektefaie, & Fouts, 2011; Villion, Chopin, Deveau, Ehrlich, Moineau, & Chopin, 2009; Yasmin, et al., 2010). For example, the ΦFL3A and ΦFL3B phages share 34% and 31% sequence identity, respectively, with prophages of *L. lactis* subsp. *cremoris* SK11 and MG1363 (Yasmin, et al., 2010). As described earlier in this chapter, these bacteria often reside together in similar host environments, foodstuffs, or fecal-contaminated

water sources, which may potentiate the proximal lateral transfer of DNA by phages and other mobile DNA elements.

E. faecium temperate phages

Phages that infect *E. faecium* and *E. faecium* genome–encoded prophages have been described in several studies (Galloway-Peña, Roh, Latorre, Qin, & Murray, 2012; Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010; van Schaik, et al., 2010). The ability to induce prophages from a group of genome-sequenced *E. faecium* strains has recently been demonstrated (van Schaik, et al., 2010). These induced prophages were all *Siphoviridae,* and were morphologically identical to prophages from *E. faecalis*. Within the genome of these seven prophages, 3-5% of the coding sequence was determined to originate from phage DNA. This suggests that the majority of the *E. faecium* prophage sequence contributes potentially novel DNA, which drives the genomic diversity of these strains (van Schaik, et al., 2010).

Other enterococcal temperate phages

Far less is known about the temperate phages of other enterococci; however, current sequencing efforts will facilitate future studies. Non-*faecalis* and non-*faecium* enterococcal species , including *E. casseliflavus* and *E. gallinarum*, respectively, are likely to carry prophage elements, as determined by the presence of phage integrase genes in genome sequences from those strains (Palmer, et al., 2010). The phage genome integrity, boundaries, and organization of these elements are unclear and await annotation. Moreover, induction experiments are required to determine whether potential prophages can be induced from these species to produce infectious phage particles. Recent studies have also identified a number of novel enterococcal phages from diverse environments that can infect *E. gallinarum* and *E. casseliflavus* (discussed above) (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010; Purnell, Ebdon, & Tayor, 2011).

Role of phages in virulence

Comparative genome analyses of the *E. faecalis* V583 strain and of the ΦFL1, ΦFL2, ΦFL3, and ΦFL4 groups of phages revealed that they encoded multiple homologs of the *Streptococcus mitis* phage M1 PblA platelet-binding protein (Bensing, Siboo, & Sullam, 2001; Mitchell & Sullam, 2009). The PblA and PblB phage tail proteins of *S. mitis* were clearly demonstrated to contribute to platelet adhesion via interactions with the α2-8-linked sialic acid residues on gangliosides of platelet membranes (Mitchell & Sullam, 2009). V583 strains harboring singly lysogenized *pbl* gene containing phages (phage01, 04, or 06) adhered to human platelet cells, whereas lysogenized strains carrying only phage03 and 05 or EfCIV583 alone were unable to bind platelets (Matos, et al., 2013). The contribution to phage encoded Pbl proteins during *E. faeclais* diseases such as endocarditis has not yet been tested.

There have been no systematic studies that characterize the role of prophages and cryptic phages in the pathogenesis of *E. faecalis*. A study by Yasmin *et al.* (Yasmin, et al., 2010) revealed that ΦFL3A and ΦFL3B phage lysogens of *E. faecalis* JH2-2 reduced the survival of *Galleria mellonella* caterpillars, as compared to their non-lysogen parent strain. No clear effects were observed in this model for lysogens of the other phages identified. However, this preliminary investigation, using only one insect model of infection, did not study lysogen survival within the *Galleria* caterpillars, or the rates of spontaneous lysis for the various lysogens during growth. Moreover, it will be important to determine the transcription and replication of each of the phage elements during growth in the infection models. In contrast to *E. faecalis*, no comparative genomic analysis of *E. faecium* phages has been undertaken to date, and as a result, their potential contribution to pathogenesis is therefore unknown. The sequential deletion of prophage elements from a lysogenized enterococcal chromosome, or the generation of allelic replacement mutants of phage genes prior to comparative virulence studies, represents a more rigorous approach for future analysis. These types of studies have been performed in *S. aureus* (Bae, Baba, Hiramatsu, & Schneewind, 2006) and *E. coli* (Wang, et al., 2010), and in each case, multiple phage-encoded

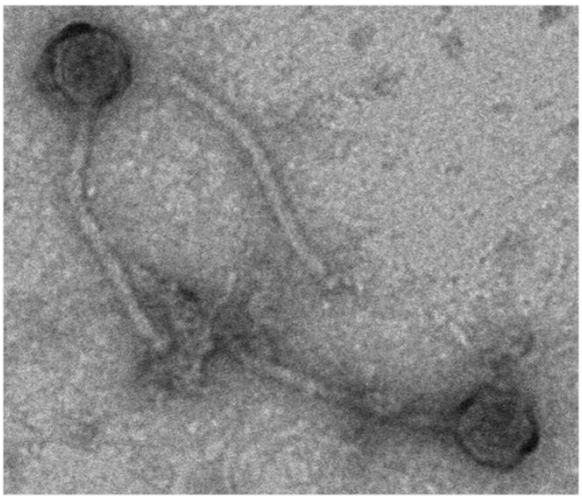

Figure 1. Electron micrograph of induced *Siphoviridae* prophage (ΦFL1A) from a clinical isolate of *E. faecalis*. The long, non-contractile tail (~200 nM in length) enables adsorption for delivery of phage DNA from the isometric capsid (~50 nm in diameter).

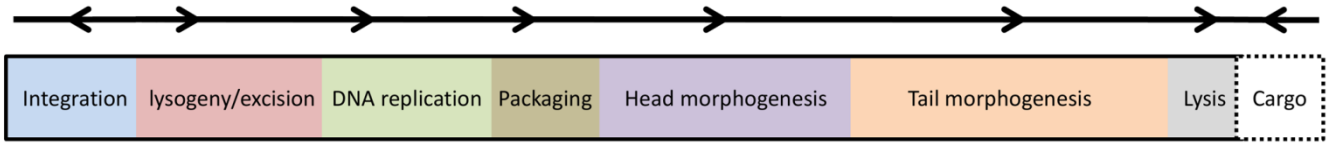

Figure 2. Schematic of *E. faecalis* temperate *Siphoviridae* genomes. Modular organization based upon the different ΦFL1, ΦFL2, ΦFL3, ΦFL4 and ΦEF11 phages (Stevens, Ektefaie, & Fouts, 2011; Yasmin, et al., 2010). The arrows indicate the direction of genes and thus the presumed direction of transcription. Cargo refers to potential phage conversion genes. The image is not drawn to scale.

genes were found to positively contribute to changes in either virulence potential or environmental survival (such as antibiotic resistance).

More recently, a comparative genomic analysis of hospital-associated clonal complex 2 (CC2) strains of *E. faecalis* revealed that there was enrichment of multiple lateral transfer elements in these strains (Solheim, Brekke, Snipen, Willems, Nes, & Brede, 2011). Among 252 genes that were statistically enriched in CC2, as compared to non-CC2 strains, the majority of such genes were found in known lateral transfer elements. This group included 51 genes (nearly the complete genome) of phage03 and several genes of phage04 (Solheim, Brekke, Snipen, Willems, Nes, & Brede, 2011). Most of these genes were expressed based upon transcription analyses; however, no account was recorded for spontaneous lysis from prophages entering the lytic cycle within the cultures, which could account for the observed transcription. Aside from this finding, the determination that phage elements are positively correlated with virulence enhancement supports the future study of phage deletion strains, as described above.

Phage transduction

Temperate phages are well-recognized for their lateral gene transfer capabilities. The bacteriophage-mediated transfer of DNA from a donor cell to a recipient cell is termed transduction, and can be mediated by either lytic or temperate phages. Transducing particles occur during the lytic stage of phage life-cycles and can be of two types: either generalized or specialized transducing phage particles. Generalized transducing particles arise from the packaging of any host bacterial genome sequence of the requisite size with the absence of phage DNA. Specialized transducing phage particles are generated only by a temperate phage that excise and package DNA immediately adjacent to one end of the integrated prophage through covalent attachment (Birge, 1994).

Transduction has the capacity to facilitate lateral gene transfer and was demonstrated in the enterococci by using the lytic phage EFRM1 (discussed below).

It was recently shown that several *E. faecalis* temperate phages were capable of generalized transduction (Yasmin, et al., 2010). Temperate phage transduction in this study was demonstrated by the transfer and recombination of DNA encoding antibiotic resistance in *E. faecalis*. This termperate phage transduction property has implications for the evolution of multi-drug resistant enterococci, as well as being a generally useful tool for laboratory-mediated genetic transfer. The capacity of enterococcal phages to facilitate generalized transduction was extended in a later study, which proposed that phages could mediate inter-species transfer of antibiotic resistance genes between different enterococci, including *E. faecalis, E. faecium*, *E. gallinarum, Enterococcus hirae, and E. casseliflavus* (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2011).

Enterococcal Lytic Phages

By definition, a lytic phage is one that can infect a bacterial host strain, but does not possess the ability to lysogenize. Lytic phages are considered predators of bacteria, as their purpose is to infect, replicate, and lyse the host for transmission. This purpose opposes to that of temperate phages, which can enter a life-cycle of parasitism maintaining chronic infection within their host (Weinbauer, 2004). Of course, prophages themselves can enter the lytic life-cycle under specific inducing conditions and at host strain-specific frequencies. Lytic phages are often devoid of an integrase gene and lack the genes for the maintenance of lysogeny, which is why these phages exist in a perpetually predatory state. Lytic phages and the enzymes that code for host cell wall destruction, including the membrane-permeating holins and peptidoglycan hydrolytic enzymes, called lysins, are debated as alternative therapies against bacteria resistant to multiple antibiotics (Chhibber, Kaur, & Kumari, 2008; Matsuzaki, et al., 2003; McVay, Velásquez, & Fralick, 2007). Several obligate lytic enterococcal phages have been identified (Horiuchi, Sakka, Hayashi, Shimada, Kimura, & Sakka, 2012; Lee & Park, 2012; Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010; Otawa, Hirakata, Kaku, & Nakai, 2012; Parasion, Kwiatek, Mizak, Gryko, Bartoszcze, & Kocik, 2012; Parasion, Kwiatek, Mizak, Gryko, Bartoszcze, & Kocik, 2012); however, due to space restrictions only a few will be discussed in detail.

ϕEF24C

The lytic phage ϕEF24C was isolated from a water channel in Kochi City, Japan after passage in culture with *E. faecalis* strain EF24 (Uchiyama, et al., 2008). Further analysis revealed that ϕEF24C was able to infect and proliferate in multiple strains of *E. faecalis,* including several vancomycin-resistant isolates; however, ϕEF24C was unable to infect or lyse any of the ten *E. faecium* strains that were tested. ϕEF24C shows broad activity for *E. faecalis,* but appears to lack the ability of cross-strain infectivity and lysis. The phylogeny of ϕEF24C places this phage in the *Myoviridae* family. ϕEF24C has an icosahedral head of ~93 nm in diameter and a contractile tail that when fully extended is ~204 nm in length (Uchiyama, et al., 2008). N-terminal degradation sequencing of many of the ϕEF24C structural proteins, including tail and capsid proteins, suggests that ϕEF24C is related to the SPO1 phage genus, which includes members such as *Staphylococcus* phages K and 812, *Lactobacillus* phage LP65, and *Listeria* phage P100, all of which are known lytic phages (Chibani-Chennoufi, Dillmann, Marvin-Guy, Rami-Shojaei, & Brüssow, 2004; Uchiyama, et al., 2008). The genome of ϕEF24C is large, with ~142 kbp of circular double stranded DNA. The ϕEF24C genome houses 221 open reading frames and five tRNA synthetase genes (Uchiyama, Rashel, Takemura, Wakiguchi, & Matsuzaki, 2008). Just fewer than 50% of the open reading frames found in the ϕEF24C are predicted to encode proteins with known functional domains. The ϕEF24C genome is organized into three distinct modules. The first module is structural and includes the genes involved in capsid and tail biosynthesis. The second module contains genes that encode replication proteins. The third module is a short structural module that includes a gene with an immunoglobulin protein motif, which is thought to be structurally associated with the phage particle. Outside of these modular domains are genes that encode putative nucleic acid precursor biosynthesis enzymes, which may be involved in the *de novo* synthesis of

modified nucleotides. Furthermore, the ϕEF24C genome contains no genes homologous to a site specific integrase, which verifies the strictly lytic life-cycle of ϕEF24C (Uchiyama, Rashel, Takemura, Wakiguchi, & Matsuzaki, 2008).

EFRM31

EFRM31 is a double-stranded DNA phage that was originally isolated from runoff water from a pig farm, and was amplified in *E. faecalis* isolates found within piggery waste water (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010). Electron microscopy revealed that EFRM31 is a member of the *Siphoviridae* family and has a long non-contractile tail (206 nm, length) and a spherical capsid (55 nm, width). The EFRM31 genome is a circular ~17 kbp genome and is characterized by low G+C content. There are 87 open reading frames that are predicted to encode proteins, and 17 of these open reading frames are putative morphogenesis genes that encode structural and accessory polypeptides for tail and capsid assembly. EFRM31 also encodes numerous proteins of unknown function (Fard, Barton, Arthur, & Heuzenroeder, 2010). The EFRM31 genome is homologous to the EFAP-1 bacteriophage genome, a lytic phage of *E. faecalis* (Son, et al., 2010).

Outside of encoding proteins for phage particle biosynthesis and phage genome replication, EFRM31 also encodes a putative virulence factor homologous to a zinc metalloprotease, a DNA primase gene, an antibiotic resistance membrane pump, and a polyamine transporter. These genes are clustered together and reside outside of the organized modules for phage particle assembly and genome replication. It is likely that these genes have been acquired from a bacterial host chromosome through recombination (Fard, Barton, Arthur, & Heuzenroeder, 2010). EFRM31 is proficient in packaging DNA, aside from its own chromosome, during phage particle assembly. This can be concluded from studies that measured the ability of EFRM31 to transduce antibiotic resistance markers from *E. faecalis* host strain chromosomal or plasmid DNA to enterococcal host strains where sensitivity to a particular antibiotic had been previously determined (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2011). EFRM31 was highly capable of transducing gentamicin resistance to multiple enterococcal species, and as a result, EFRM31 was also able to undergo inter-species transduction of gentamicin resistance to two *E. faecium* strains and an *Enterococcus* isolate most closely related to the *durans*/*hirae* species (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2011). In some, but not all, of these transfer events, there was an association of an *ant2-I* gene (which codes for an aminoglycoside nucleotidyltransferase) cassette acquisition that is known to confer low level gentamicin resistance in the transduced strain. Since transducing phages are mostly species-specific, EFRM31 is the first example of an enterococcal phage that is capable of inter-species host range generalized transduction.

EFAP-1

EFAP-1 is a lytic phage that was isolated from environmental samples taken from a variety of sources within a cowshed. EFAP-1 infects *E. faecalis*. This phage is a member of the *Siphoviridae* family, and by electron microscopy, was determined to have a non-contractile tail and an isometric capsid. EFAP-1 is most closely related to *Bacillus* phage ϕ105 and *Staphylococcus* prophage ϕPV83 (Son, et al., 2010). The EFAP-1 genome is composed of 24 open reading frames and similar to other related phages, the open reading frames are modular and consist of clusters of genes annotated to be involved in phage structure, host cell lysis, phage genome replication, and electron transport. Several open reading frames within these modules share homology with proteins that are found on bacterial chromosomes. Although these open reading frames may originate from bacterial chromosomes, they may perform functions for the EFAP-1 phage. For example, the gene ORF1 encodes a putative thioredoxin-like superfamily protein that may be involved in phage DNA replication and may also be used during bacterial cell electron transport (Son, et al., 2010). Several open reading frames found in the EFAP-1 genome show no sequence similarity to those found in the current public sequence databases, and as a result, have been delineated as genes of unknown function. This result suggests that, like many other phages, EFAP-1

encodes potentially novel genes that, if expressed, may uniquely aid in determining the biology of this lytic phage.

Many more enterococcal lytic phages have been identified including at least one that is known to infect *E. faecium* as well as several recently identified environmental phages of diverse morphologies that infect *E. faecalis* (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010; Otawa, Hirakata, Kaku, & Nakai, 2012).

Enterococcal phage lysins and therapeutic applications

After replication and packaging of phage particles within a bacterial cell, lytic phages must exit the host cell in order to disseminate to a new host bacterium. To achieve such an exodus, non-filamentous double stranded DNA phage genomes encode enzymes, typically referred to as lysins, that accumulate in the cytoplasm of the host cell and target the destruction of its cell wall (Fischetti, 2005). Lysins act on the peptidoglycan of the bacterial cell wall by binding to polysaccharide or peptide moieties and subsequently cleaving these macromolecules, which aids in the release of phage progeny. Lysins are diverse hydrolases, including endo-β-*N*-acetylglucosaminidase or *N*-acetylmuramidase, that bind to sugar residues of peptidoglycan and endopeptidases that target a peptide ligand of the peptidoglycan cell wall. A fourth type of lysin, *N*-acetylmuramoyl-L-alanine amidase (amidase), hydrolyzes the amide bond between the peptide moiety and the sugar moiety of peptidoglycan (Young R. , 1992). The activity of a lysin is coupled to a second phage protein known as a holin—a transmembrane protein that inserts into the bacterial cell membrane causing disruption (Wang, Smith, & Young, 2000). Following holin insertion, the lysin can then traverse the cell membrane and gain access to the cell wall peptidoglycan to cause lysis. Holins are instrumental in allowing lysins to target the cell wall, because most lysins lack N-terminal signal sequences and cannot independently gain access to the cell wall (Young, Wang, & Roof, 2000).

It has been postulated that phage lysins could be developed into an alternative to antimicrobials, as there is a great need for new antimicrobial agents to fight the steady rise in multi-drug resistant bacteria that has taken place in recent decades. Many phage lysins have been purified and these enzymes have been used in proof of principal experiments to kill pathogenic bacteria both *in vitro* and *in vivo* (Fischetti, 2005; Fischetti, 2003). Lysins as therapeutics have mostly been applied to Gram-positive bacteria, where the lysin has easy access to the peptidoglycan on the outside of the bacterial cell. This process is in direct contrast to Gram-negative bacteria, which usually resist killing by exogenous lysins, due to the peptidoglycan of these cells being protected by the bacterial outer membrane. The enterococci are notorious for their antimicrobial resistance phenotypes (Palmer, Kos, & Gilmore, 2010; Willems & Bonten, 2007). In some cases, enterococcal strains can be resistant to a broad range of antibiotics, including some recently developed antibiotics (Arias, et al., 2011; Palmer, Daniel, Hardy, Silverman, & Gilmore, 2011). Due to their intrinsic antibiotic resistance, the efficacy of enterococcal phage lysins as antimicrobial agents has been tested in various studies.

PlyV12 is a lysin that was identified from a genomic DNA library of the ϕ1 lytic *Myoviridae* family enterococcal phage. This DNA library was screened for lytic factors that had killing activity against the *E. faecalis* strain V12 found in the human urogenital tract (Caprioli, Zaccour, & Kasatiya, 1975; Yoong, Schuch, Nelson, & Fischetti, 2004). The identification of a bacteriolytic polypeptide, PlyV12, that shares homology with *N*-acetylmuramoyl-L-alanine amidase type lysin enzymes, had a high specific lytic activity against both *E. faecalis* and *E. faecium* strains, including vancomycin-resistant strains. Interestingly, PlyV12 was also active in killing strains of *S. aureus*, *Staphylococcus epidermidis*, and multiple groups of streptococci. This established the PlyV12 lysin as a broad spectrum bacteriolysin, which is uncharacteristic of these types of enzymes, as most kill only the species that the parental phage can infect (Fischetti, 2005). Furthermore, it is proposed that PlyV12 may target a cell wall moiety that is conserved within many Gram-positive genera. The amino acid sequence of PlyV12 revealed a protein of ~32 kDa that has an N-terminal catalytic domain, and that shares sequence similarity to *N*-acetylmuramoyl-L-alanine amidase -like lysins from the group A and B streptococci *Streptococcus pyogenes* and *Streptococcus agalactiae,* as well as *Streptococcus pneumoniae* and *Streptococcus mitis* (Yoong, Schuch, Nelson, &

Fischetti, 2004). This similarity in the lysin catalytic domain may account for the broad-spectrum killing ability of PlyV12, although the C-terminal region of PlyV12 where the predicted lysin epitope binding site resides is highly divergent from these streptococcal lysins (Yoong, Schuch, Nelson, & Fischetti, 2004).

A second study focused on a lysin enzyme from the lytic phage EFAP-1. This lysin, designated as EFAL-1, is also predicted to be a cell wall hydrolytic *N*-acetylmuramoyl-L-alanine amidase. EFAL-1 was purified after overexpression in *E. coli* cells, and was found to have broad spectrum killing activity (Son, et al., 2010). EFAL-1 was found to be lytic against 13 *E. faecalis* strains, seven *E. faecium* strains, two strains of *S. agalactiae*, and two strains of *Streptococcus uberis*. This potent activity of EFAL-1 is impressive, considering the parental lytic phage that encodes this lysin, EFAP-1, is only capable of lysing a single *E. faecalis* strain from this group of susceptible bacteria.

A third study characterized the lysin encoded by *orf9* from the lytic enterococcal phage ϕEF24C. Using MALDI-TOF mass spectrometry, Orf9 was shown to specifically cleave peptidoglycan at the peptide sugar linkage, which confirms that Orf9 is also a hydrolytic *N*-acetylmuramoyl-L-alanine amidase (Uchiyama, et al., 2011). Site-specific truncations of the orf9 coding region confirmed that the ability of Orf9 to lyse *E. faecalis* depended on an intact C-terminus. An extensive analysis of the N-terminal region of Orf9 was not possible, as truncations within this region rendered the protein insoluble (Uchiyama, et al., 2011). Unlike the EFAL-1 and PlyV12 lysins, Orf9 was shown to have lytic activity against only *E. faecalis* and *E. faecium*. However, this analysis was only extended to 10 *Staphylococcus* strains, and further screening of other Gram-positive bacteria may reveal similar broad activity.

It is worth noting that phage lysins from non-enterococcal bacteria have been shown to be effective at killing *Enterococcus* species. The temperate phage ϕ-0303 of *Lactobacillus helveticus* encodes a 40 kDa lysin called Mur-LH that functions as a cell wall–degrading *N*-acetylmuramidase (Deutsch, Guezenec, Piot, Foster, & Lortal, 2004). Purified Mur-LH can lyse a diverse array of Gram-positive bacteria, including *E. faecium*. Mur-LH was unable to lyse *E. faecalis*, suggesting that Mur-LH binds to a cell surface epitope that is not shared between these two enterococcal species. *Lactobacillus gasseri* phage ϕgaY encodes an *N*-acetylmuramidase. This lysin, Lys_{gaY}, exhibits broad bacteriolytic activity and can lyse over 20 different species of Gram-positive bacteria, including enterococci (Sugahara, et al., 2007). Purified Lys_{gaY} also causes logarithmically growing Gram-positive bacterial cells to aggregate and form long chains. This phenotype is mostly likely caused by Lys_{gaY} binding to the site of cell wall peptidoglycan synthesis during cell division, which results in the inability of daughter cells to completely partition.

These data emphasize two important points. First, contrary to popular belief, many lysins, specifically those of enterococcal lytic phages, have broad tropism and are capable of intra- and inter-generic killing. Second, bactericidal therapeutics based on highly promiscuous lysins may be powerful tools for combating diverse Gram-positive pathogens. Lysins have been shown to act synergistically with some antibiotics (Loeffler & Fischetti, 2003) and specifically when used in combination with antibiotics that target cell wall synthesis, like penicillin. Bacterial resistance to lysins appears to be a rare event. It is thought that low incidence resistance to phage lysins is caused by phages evolving their lysins to target essential molecules in the bacterial cell wall (Fischetti, 2008). Therapies that combine phage lysins and antibiotics may also decrease the incidence of bacterial resistance through synergistic effects. However, one concern with this course of action is that phage lysins are enzymes, and therapeutic treatments using these types of proteins would result in the production of neutralizing antibodies and the elimination of the lysins (Fischetti, 2005). The efficacy of the bacteriolytic activity of lysins in the presence of neutralizing antibodies has been addressed. A study by Loeffler *et al* showed that a streptococcal phage lysin still maintained killing in the presence of specific neutralizing antibodies, although it was reduced in activity (Loeffler, Djurkovic, & Fischetti, 2003). Furthermore, mice pre-immunized with a streptococcal lysin prior to receiving an intranasal *S. pneumoniae* challenge produced lysin-specific neutralizing antibodies, yet were still protected upon administration of the lysin following infection (Loeffler,

Djurkovic, & Fischetti, 2003). These data suggest that immunogenic phage lysins are still capable of acting against their target in the presence of neutralizing antibodies, and may warrant consideration for development into alternative antimicrobial agents.

Enterococcal Genome Defense Mechanisms

Prokaryotes possess multiple mechanisms that can interfere with phage infection, including receptor/adsorption blocking; abortive infection; clustered, regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) proteins (referred to here as CRISPR-Cas); and restriction modification (RM) (51). CRISPR-Cas and RM can also limit uptake of other mobile elements, such as plasmids (discussed below). Despite clear differences in the abundance of prophages, plasmids, and other mobile elements in enterococcal genomes (Bourgogne, et al., 2008; Leavis, Willems, van Wamel, Schuren, Caspers, & Bonten, 2007; Palmer, et al., 2012; Solheim, Brekke, Snipen, Willems, Nes, & Brede, 2011), little is known about the mechanisms by which some strains appear more susceptible to phage infection and plasmid uptake than others. Recent studies have highlighted potential roles for genome defense systems in the evolution of multidrug resistance and hospital adaptation in the enterococci (Lindenstrauss, Pavlovic, Bringmann, Behr, Ehrmann, & Vogel, 2011; Palmer & Gilmore, 2010; Solheim, Brekke, Snipen, Willems, Nes, & Brede, 2011) and in the transfer of antibiotic resistance genes from enterococci to *S. aureus* (Monk, Shah, Xu, Tan, & Foster, 2012). This section introduces CRISPR-Cas proteins and restriction modification defense, and reviews evidence for these systems in the enterococci. Methods to identify CRISPR-*cas* loci in newly sequenced enterococcal genomes are also discussed. Because no phage receptors have been identified for the enterococci, nor have any phage abortive infection mechanisms been reported, those genome defense strategies are not discussed here.

CRISPR-Cas, a prokaryotic acquired immune system

Clusters of roughly palindromic repeat sequences, referred to as CRISPR, were identified in bacterial and archaeal genomes to be sequenced (Jansen, Embden, Gasstra, & Schouls, 2002; Mojica F. J., Díez-Villaseñor, Soria, & Juez, 2000). The repeats, which can vary in length (typically ~24-48 bp) and palindromic structure (Haft, Selengut, Mongodin, & Nelson, 2005; Kunin, Sorek, & Hugenholtz, 2007), were detected in 83% of archaeal and 56% of bacterial species analyzed by the CRISPI database as of February 2012 (103 archaeal and 956 bacterial species with available genome data) (Rousseau, Gonnet, Le Romancer, & Nicolas, 2009). Thus, CRISPR appear to be both ancient and widespread among prokaryotes. Genes encoding nucleases and other proteins involved in DNA and RNA processing are typically associated with CRISPR, and are called CRISPR-associated genes (*cas* genes) (Jansen, Embden, Gasstra, & Schouls, 2002). The *cas* genes encode Cas proteins. CRISPR-*cas* loci have diverse *cas* gene cohorts and repeat structures, and several classification systems have been reported (Haft, Selengut, Mongodin, & Nelson, 2005; Kunin, Sorek, & Hugenholtz, 2007; Makarova, Grishin, Shabalina, Wolf, & Koonin, 2006; Makarova, et al., 2011). Recently, a unifying nomenclature was established that groups CRISPR-Cas systems into three broad types (I, II, and III) defined by type-specific *cas* genes (Makarova, et al., 2011). Only two conserved *cas* genes, *cas1* and *cas2*, are associated with all CRISPR-*cas* loci that are predicted to be functional (Makarova, et al., 2011). Exemplifying the diversity and complexity of these loci, the three broad CRISPR-Cas types function by distinct molecular mechanisms (Wiedenheft, Sternberg, & Doudna, 2012), and prokaryotes can harbor multiple CRISPR-*cas* loci of varying types in their genomes. The CRISPR-Cas type that appears to be most relevant to the enterococci is Type II, and this chapter focuses mainly on Type II systems. Readers should refer to an excellent and recent review for more information on Type I and Type III systems (Wiedenheft, Sternberg, & Doudna, 2012). Note that the Type II nomenclature was established in 2011 (Makarova, et al., 2011), and that some prior literature referred to these loci as Nmeni (Haft, Selengut, Mongodin, & Nelson, 2005) or Cas4 (Makarova, Grishin, Shabalina, Wolf, & Koonin, 2006) systems.

A representative Type II CRISPR-*cas* locus is shown in Figure 3A. Repeat sequences in Type II loci are typically 36 bp in length, and the unique sequences that occur between repeats, called spacers, are typically 30 bp (Haft,

Selengut, Mongodin, & Nelson, 2005). The final repeat of the array is sometimes degenerate in sequence. A conserved leader sequence located 5′ to the repeat-spacer array drives the expression of the array (Deltcheva, et al., 2011) and likely contains motifs that are important for the addition of new spacers (Yosef, Goren, & Qimron, 2012). Type II CRISPR-*cas* loci typically possess 4 *cas* genes: the type-specific gene *cas9*, the core genes *cas1* and *cas2*, and either *csn2* or *cas4* (Makarova, et al., 2011), although variations exist (Deltcheva, et al., 2011; Palmer & Gilmore, 2010).The *csn2* gene appears to be specific to Type II-A systems, while *cas4* has a wider distribution, being present in both Type I and Type II-B systems (Makarova, et al., 2011).

Catalytic mechanisms and crystal structures for some Cas proteins are known. Cas1 from the Type I CRISPR-Cas system of *Pseudomonas aeruginosa* is a metal-dependent deoxyribonuclease that generates 80 bp double-stranded DNA fragments (note that spacer lengths for this CRISPR-*cas* locus are 32 bp) (Wiedenheft, Zhou, Jinek, Coyle, Ma, & Doudna, 2009). Cas2 proteins from multiple prokaryotes have been characterized, and all are endoribonucleases that preferentially cleave single-stranded RNA targets in U-rich regions (Beloglazova, et al., 2008). Csn2 from *E. faecalis* ATCC 4200 has been purified and its structure characterized; the protein oligomerizes to form a tetrameric ring that binds double-stranded DNA (Nam, Kurinov, & Ke, 2011). The Type II-specific Cas9 proteins are large (~1000 amino acids) proteins that possess predicted RuvC-like nuclease and HNH restriction endonuclease-like domains (Makarova, Aravind, Wolf, & Koonin, 2011). No structural data are currently available for these proteins. Similarly, Cas4 is a predicted nuclease for which no structural data are available.

Genome analysis provided the initial evidence that CRISPR-Cas systems interact with mobile elements. Studies published in 2005 reported that some CRISPR spacers are identical to plasmid and phage sequences; a result that suggests that a prior encounter with mobile elements resulted in spacer acquisition (Figure 3B) (Bolotin, Quinquis, Sorokin, & Ehrlich, 2005; Mojica F. J., Díez-Villaseñor, García-Martínez, & Soria, 2005; Pourcel, Salvignol, & Vergnaud, 2005). A region of a phage, plasmid, or other genome with sequence identity to a spacer sequence is called a protospacer (Deveau, et al., 2008). A relationship between the presence of certain CRISPR spacers and the absence of prophage possessing corresponding protospacers was noted for *S. pyogenes* (Pourcel, Salvignol, & Vergnaud, 2005). These observations contributed to the hypothesis that spacer-derived small RNAs could block phage infection by interfering with phage gene expression via an RNA interference (RNAi)-like mechanism (Bolotin, Quinquis, Sorokin, & Ehrlich, 2005; Makarova, Grishin, Shabalina, Wolf, & Koonin, 2006; Mojica F. J., Díez-Villaseñor, García-Martínez, & Soria, 2005).

In 2007, a role for the Type II CRISPR-Cas as an anti-phage defense system of *S. thermophilus* was experimentally demonstrated (Barrangou, et al., 2007). The *S. thermophilus* type II CRISPR-*cas* locus possesses *cas9*, *cas1*, *cas2*, and *cas4* genes, and for the purposes of this chapter, is referred to as StCRISPR1-Cas system. *S. thermophilus* DGCC7710 was challenged *in vitro* with lytic phages. The authors sequenced the StCRISPR1 of 9 phage-resistant mutants recovered from the phage challenge experiments and found that the loci had acquired new spacer sequences. The new spacer sequences were similar to coding and non-coding genomic sequences from the lytic phages used in the infection challenge. Novel spacer sequences were added only to the leader end of the CRISPR. Most notably, *S. thermophilus* mutant strains that were engineered to lack spacers became phage sensitive; while strains engineered to possess spacers matching phage sequence gained phage resistance.

These experiments confirmed that the newly acquired spacers contributed to phage resistance. One of two *cas* genes queried, *cas9* (formerly *cas5*) (Makarova, et al., 2011) was also required for phage resistance. When taken together, these data demonstrated that the StCRISPR1-Cas system protected a subpopulation of *S. thermophilus* from phage attack through the acquisition of novel, heritable spacer sequences, which in turn provided protection from subsequent attacks by phages with similar sequences by a Cas9-dependent mechanism (Figure 3B and 3D). Additional work has found that StCRISPR1 can acquire spacers from a plasmid resident in *S. thermophilus*, which apparently promotes loss of that plasmid, also by a Cas9-dependent mechanism (Garneau, et al., 2010).

An additional Type II CRISPR-*cas* locus is present in *S. thermophilus* DGCC7710, called CRISPR3-Cas (referred to as StCRISPR3-Cas) (Horvath, et al., 2008). This locus possesses *cas9*, *cas1*, *cas2*, and *csn2* genes, as well as a repeat sequence that is similar (but not identical) to the repeats of StCRISPR1 (Horvath, et al., 2008). Both StCRISPR1 and StCRISPR3 of *S. thermophilus* DGCC7710 obtain new spacers in response to phage challenges (Barrangou, et al., 2007; Horvath, et al., 2008); thus, each of these loci are active in *S. thermophilus* DGCC7710. Despite the presence and activity of StCRISPR3-Cas in *S. thermophilus* DGCC7710, that loci's *cas9* is apparently unable to complement a StCRISPR1-Cas *cas9* mutant (Barrangou, et al., 2007; Garneau, et al., 2010; Horvath, et al., 2008). This indicates that the Cas9 proteins are highly specific to certain repeat sequences and/or other CRISPR structures.

While spacer acquisition has been experimentally demonstrated for the Type II CRISPR-Cas system of *S. thermophilus* (Barrangou, et al., 2007; Deveau, et al., 2008; Garneau, et al., 2010), little of the mechanistic detail is known. It has been proposed that *cas1* and *cas2* are important for spacer addition regardless of CRISPR type, based on their biochemical activities and because of their ubiquity among CRISPR-*cas* loci (Makarova, Aravind, Wolf, & Koonin, 2011). In support of this theory, novel spacer addition occurs when both *cas1* and *cas2* are overexpressed in an *E. coli* strain that possesses a Type I CRISPR but otherwise lacks chromosomally encoded *cas* genes (Yosef, Goren, & Qimron, 2012). In the initial demonstration of StCRISPR1-Cas as an anti-phage system, the authors noted that spacer addition did not occur in strains in which the *cas7* gene (now *cas4*) was insertionally inactivated (Barrangou, et al., 2007). A similar observation was made for anti-plasmid spacer acquisition experiments (Garneau, et al., 2010). Thus it appears that this gene may also play an important role in spacer acquisition.

The selection of novel spacer sequences does not appear to be random. Conserved sequence motifs have been identified immediately adjacent to protospacer sequences targeted by StCRISPR1-Cas (NNAGAAW) and STCRISPR3-Cas (NGGNG) (Deveau, et al., 2008; Horvath, et al., 2008), which suggests that these motifs play a role in spacer selection. In addition to a putative role in spacer selection, these protospacer-adjacent motifs (PAMs) (Mojica F. J., Díez-Villaseñor, García-Martínez, & Almendros, 2009) are important for the interference of StCRISPR-Cas with mobile elements. Mutations in the PAM allow both phages and plasmids to avoid Type II CRISPR interference (Deveau, et al., 2008; Garneau, et al., 2010; Sapranauskas, Gasiunas, Fremaux, Barrangou, Horvath, & Siksnys, 2011), although it appears that the StCRISPR1-Cas system tolerates non-consensus PAM sequences in plasmid, but not in phage targets (Garneau, et al., 2010). More work is required to determine the precise roles of PAMs in CRISPR spacer selection and mobile element interference, as well as why PAMs in the host′s own genome are not readily utilized for spacer selection.

What is the precise role of the CRISPR spacer in mobile element interference? It is now known that Type I, Type II, and Type III CRISPR repeat-spacer arrays are transcribed and processed to generate small guide RNAs called CRISPR RNAs (crRNAs) (Brouns, et al., 2008), which vary in structure depending on their CRISPR type (Wiedenheft, Sternberg, & Doudna, 2012). For Type II systems, transcription of the repeat-spacer array generates a long, pre-crRNA which is processed to short, 39-42 ribonucleotide (nt) crRNAs (Deltcheva, et al., 2011) (Figure 3C). This processing has been observed for Type II loci in *S. thermophilus*, *S. pyogenes*, *Streptococcus mutans*, and *Listeria monocytogenes*, among others (Deltcheva, et al., 2011). A mature Type II crRNA consists of 20 3′ nt of a spacer and 19-22 5′ nt of the following repeat. Thus, each Type II crRNA is a unique guide molecule that consists of a memory of a previous mobile element exposure (a partial spacer) and a conserved handle (a partial repeat) that likely associates with cellular machinery and prevents unproductive self-interference. Based on the structure of mature crRNAs, it appears that the 5′ end of the spacer sequence is less important than the 3′ end for crRNA-mediated interference. This is supported by the observation that when the Type II StCRISPR3-*cas* locus is heterologously expressed in *E. coli*, spacer-protospacer mismatches are tolerated at the 5′ spacer end and the central region (positions 2, 11, 18, and 23 of a 30 nt spacer), but not at the 3′ spacer end (positions 25 and 28) (Sapranauskas, Gasiunas, Fremaux, Barrangou, Horvath, & Siksnys, 2011).

Additionally, StCRISPR1-Cas tolerates a spacer-plasmid protospacer mismatch at the first 5′ position of the spacer sequence (Garneau, et al., 2010).

Processing of the Type II pre-crRNA proceeds via a unique mechanism that is distinct from pre-crRNA processing in Type I and Type III systems (Deltcheva, et al., 2011; Wiedenheft, Sternberg, & Doudna, 2012). A second non-coding RNA associated with the Type II CRISPR-*cas* locus, called the *trans*-activating CRISPR RNA (tracrRNA), is required for pre-crRNA processing (Deltcheva, et al., 2011). For the type II CRISPR-*cas* locus of *S. pyogenes*, tracrRNA is encoded 5′ to *csn1*; however, the location of tracrRNA varies for different Type II loci. tracrRNA contains a repeat-like sequence oriented antisense to the repeats of pre-crRNA. tracrRNA acts as a guide in pre-crRNA processing and forms an RNA-RNA duplex with pre-crRNA repeats, thereby facilitating cleavage within the crRNA repeat/tracrRNA anti-repeat by the housekeeping RNase III. Interestingly, a role for RNase III in Type II crRNA maturation is reminiscent of small interfering RNA and microRNA processing in eukaryotes (Deltcheva, et al., 2011). Certain aspects of the Type II processing mechanism remain to be elucidated, including the factor that is responsible for cleavage within the spacer sequence to liberate a mature crRNA (Deltcheva, et al., 2011). While the *cas9* gene is required for pre-crRNA and tracrRNA processing (Deltcheva, et al., 2011), the precise role of the Cas9 protein in processing is unknown. Cas9 may carry out cleavage within the spacer sequence to liberate a mature crRNA, and/or may stabilize and facilitate base pairing of pre-crRNA and tracrRNA (Deltcheva, et al., 2011). As expected based on their roles in pre-crRNA processing, RNase III, tracrRNA, and *cas9* are each required for *S. pyogenes* CRISPR spacer-dependent interference with plasmid uptake (Deltcheva, et al., 2011).

There is evidence for crRNA-directed cleavage of both RNA and DNA targets in Type III CRISPR-Cas systems (Hale, et al., 2009; Marrafini & Sontheimer, 2008; Zhang, et al., 2012), but there is none thus far for Type II CRISPR-Cas systems. It is clear that DNA is a target of the StCRISPR1-Cas system (Garneau, et al., 2010). Blunt-end cleavage occurs within the target protospacer of double-stranded plasmid DNA, 3 bp 5′ to the PAM (after the 27th nucleotide of a 30 nucleotide protospacer) (Garneau, et al., 2010). The same cleavage site is observed for phage DNA targets (Garneau, et al., 2010). Cleavage occurs 3 bp 5′ to the PAM even if the spacer is < 30 bp, indicating that the cleavage proceeds by a 3′ anchored mechanism (Garneau, et al., 2010). An additional cleavage event occurs within protospacers when spacers target the positive strand of a phage genome. This second cleavage occurs within the protospacer, 19 or 20 bp 5′ to the PAM (35), which corresponds to the 5′ end of mature crRNAs (Deltcheva, et al., 2011). As a result, it appears that the Type II CRISPR-Cas interference machinery can discriminate between DNA strands in the target DNA. Because only a negative strand protospacer has been investigated for plasmid targets (Garneau, et al., 2010), it is unclear whether this strand discrimination is specific to phage targets or is a more general property of Type II CRISPR-Cas interference.

To summarize, Type II CRISPR-Cas are genome defense systems that evolve in response to plasmid and phage attacks and that confer immunity to progeny cells through a genetically programmed memory of these attacks (Figure 3). Despite rapid advances in this field, much remains to be learned about Type II CRISPR-Cas systems. Arguably the most important aspect are the mechanisms by which CRISPR-Cas exclude their own genome's potential spacers from inclusion in CRISPR arrays, and the precise roles of each of the Cas proteins in acquiring new spacers, processing crRNAs, and providing defense. While structural data are available for Type I (Wiedenheft, et al., 2011) and Type III (Zhang, et al., 2012) crRNA interference complexes (i.e. crRNA with its associated protein effectors), this is not the case for Type II. Determining a crystal structure for Cas9 will be especially informative, as this protein acts at both the crRNA processing and interference stages of Type II CRISPR defense. An open question remains as to why a 30 bp spacer is incorporated into a CRISPR array if only 2/3 of that sequence is used in a mature crRNA; perhaps different ruler mechanisms guide spacer addition and crRNA processing.

Finally, while many CRISPR spacers have sequence identity to plasmid and phage sequences, some have identity to the host's own genome These spacers are referred to as self-targeting spacers. Self-targeting spacers were

identified in StCRISPR1 and StCRISPR3 loci of various *S. thermophilus* strains (Horvath, et al., 2008). The role of self-targeting spacers is unclear, although it has been hypothesized that CRISPR systems can erroneously incorporate "self" genome into the spacer array, leading to selection for progeny with degenerate (broken) CRISPR-*cas* loci (Stern, Keren, Wurtzel, Amitai, & Sorek, 2010). An alternative and not necessarily mutually exclusive explanation, depending on the CRISPR type in question, is that self-targeting spacers regulate the expression of chromosomal genes through an RNAi-like mechanism (Horvath, et al., 2008). Despite sequence identities of some CRISPR spacers to known mobile elements and host genomes, most spacers analyzed do not match known sequences—a finding that indicates that prokaryotes are exposed to a diverse variety of uncharacterized phages and plasmids that have not been sampled by genomic and metagenomic (total DNA retrieved from an environmental sample) studies to date.

Enterococcal CRISPR-Cas systems

Enterococcal CRISPR-*cas* was first identified in the *E. faecalis* OG1RF genome (Bourgogne, et al., 2008). A Type II-A locus possessing a CRISPR and *cas9*, *cas1*, *cas2*, and *csn1* genes was identified in OG1RF between homologues of the *E. faecalis* V583 ORFs EF0672 and EF0673, which the authors named CRISPR1 (referred to as the EfsCRISPR1-*cas* locus for the purposes of this chapter, shown in Figure 4). An additional gene of unknown function is encoded 3′ to the CRISPR1 array in OG1RF. A second CRISPR locus that lacks *cas* genes was identified between V583 homologues of EF2062 and EF2063, which the authors named CRISPR2 (EfsCRISPR2 for the purposes of this chapter, and also shown in Figure 4). The consensus repeat sequences for EfsCRISPR1 and EfsCRISPR2 are identical, suggesting that the two loci are functionally linked (Bourgogne, et al., 2008; Horvath, Coûté-Monvoisin, Romero, Boyaval, Fremaux, & Barrangou, 2009). An EfsCRISPR2 locus of identical repeat sequence and different spacer content occurring between EF2062 and EF2063 was subsequently identified in the V583 genome (Horvath, Coûté-Monvoisin, Romero, Boyaval, Fremaux, & Barrangou, 2009; Palmer & Gilmore, 2010). The presence of EfsCRISPR1-*cas* in *E. faecalis* OG1RF was proposed to account for the low prophage content of this strain (OG1RF possesses only the cryptic phage02 which is part of the *E. faecalis* core genome) (Bourgogne, et al., 2008; McBride, Fischetti, Leblanc, Moellering, Jr., & Gilmore, 2007; Palmer, et al., 2012). This is in contrast to *E. faecalis* V583, which possesses seven prophage elements (Paulsen, et al., 2003) and lacks EfsCRISPR1-Cas (Bourgogne, et al., 2008). PCR-based screening for the presence of EfsCRISPR1, *cas1,* and *csn1* in 14 additional *E. faecalis* isolates indicated that this locus is variable in the *faecalis* species (Bourgogne, et al., 2008).

The availability of genome sequence data for an additional 16 *E. faecalis* genomes (Palmer, et al., 2010) allowed for a more comprehensive analysis of CRISPR distribution in the *faecalis* species (Palmer & Gilmore, 2010). As expected, EfsCRISPR1-*cas* was variably distributed among the 16 genomes (present in 5/16), and when present occurred invariably between homologues of EF0672 and EF0673. The number of spacers in the loci varied, as did the presence of the gene of unknown function occurring 3′ to the CRISPR array in *E. faecalis* OG1RF. A second CRISPR-*cas* locus, named CRISPR3-*cas* (referred to as the EfsCRISPR3-*cas* locus for the purposes of this chapter), was identified in two additional *faecalis* genomes between homologues of the V583 ORFs EF1759 and EF1760 (Figure 4). EfsCRISPR3-*cas* encodes a putative Type II CRISPR-Cas system. Its cohort of *cas* genes contains *csn1*, *cas1*, *cas2*, and a gene of unknown function. EfsCRISPR3 repeats are distinct from EfsCRISPR1/CRISPR2 repeats, sharing only 42% nucleotide sequence identity. EfsCRISPR1-*cas* and EfsCRISPR3-*cas* loci did not co-occur in the same strain; thus, 7 of the 16 genomes analyzed possessed a Type II CRISPR-Cas system.

Interestingly, an EfsCRISPR2 locus of conserved repeat sequence and varying spacer content was identified between EF2063 and EF2061 homologues in all 16 *E. faecalis* genomes analyzed (Palmer & Gilmore, 2010). Note that EF2062 homologues are not annotated in these genomes, despite the nucleotide sequence being ≥ 98% conserved. As a result, it appears that an orphan CRISPR locus of varying spacer content is core to the *faecalis* genome, and in 9 of 16 genomes, this locus is maintained in the absence of *cas* genes. EfsCRISPR2 does not appear to confer defense from mobile elements, as genomes possessing only EfsCRISPR2 are significantly larger

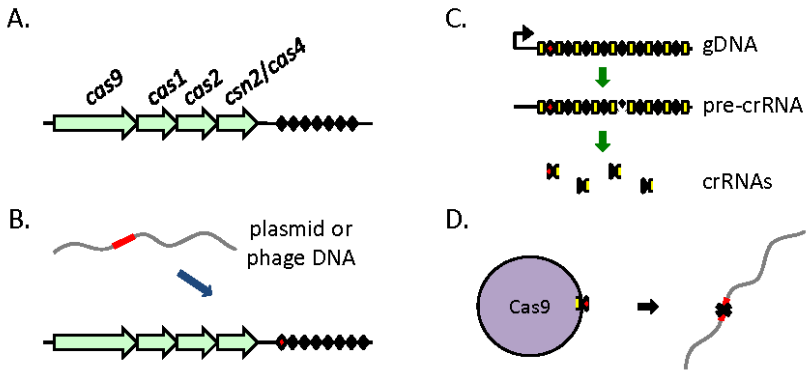

Figure 3. A generalized model of a Type II CRISPR-Cas defense system. (A) A typical Type II CRISPR-*cas* locus. Spacers are shown as black diamonds and repeats are not shown. Either *csn2* or *cas4* may be present as the fourth gene in the *cas* cluster. (B) Upon plasmid uptake or phage attack, a cell incorporates a segment of plasmid or phage DNA into its CRISPR array as a novel spacer (red diamond) that has homology to the protospacer sequence on the incoming phage or plasmid DNA (red rectangle). (C) The CRISPR array is transcribed from the leader sequence to generate a long pre-crRNA which is processed to 39-42 nucleotide crRNAs by RNase III, Cas9, and potentially additional factors in the presence of tracrRNA (not shown). Repeat sequences are shown as yellow boxes. Each mature crRNA consists of 20 nucleotides derived from the 3′ spacer end and 19-22 nucleotides derived from the 5′ repeat end. (D) A mature crRNA guides the Cas9 nuclease to mobile DNA with similar sequence, leading to destruction of the mobile DNA.

and encode more protein domains associated with mobile elements as compared to genomes possessing an EfsCRISPR1-*cas* or EfsCRISPR3-*cas* locus (Palmer, et al., 2012). Further, the absence of *cas9* in EfsCRISPR2-only strains indicates that pre-crRNA generated from EfsCRISPR2 would not be processed to mature crRNAs.

Analysis of 140 spacer sequences extracted from the EfsCRISPR loci of 16 *E. faecalis* genomes revealed identities to phage and plasmid sequences (Palmer & Gilmore, 2010). It appears that the *E. faecalis* Type II CRISPR-Cas systems provide defense from attack by both lytic and temperate phage. Spacers in two strains were similar to sequences from the lytic enterococcal phage ΦEF24C (Uchiyama, et al., 2008), while spacers from seven strains were similar to sequences from the temperate phage ΦFL2B (Yasmin, et al., 2010), or prophages present in the V583 genome (Paulsen, et al., 2003). Spacers from six strains were similar to sequences from pheromone-responsive plasmids and plasmids integrated into the V583 genome, suggesting that *E. faecalis* Type II CRISPR-Cas systems also confer defense against these elements.

Because plasmids are common disseminators of antibiotic resistance genes in *E. faecalis* (Palmer, Kos, & Gilmore, 2010), it is likely that CRISPR-Cas acts as a barrier to the acquisition of these genes. This is supported by the finding that the absence of either Type II EfsCRISPR-*cas* locus is significantly associated with antibiotic resistance acquired by horizontal gene transfer in a collection of 48 *E. faecalis* strains (Palmer & Gilmore, 2010). Further, high-risk *E. faecalis* multilocus sequence typing (MLST) lineages lack EfsCRISPR1-*cas* and EfsCRISPR3-*cas* (Palmer & Gilmore, 2010), and the presence of certain virulence factor genes is associated with EfsCRISPR-*cas* absence in *E. faecalis* (Lindenstrauss, Pavlovic, Bringmann, Behr, Ehrmann, & Vogel, 2011). It appears that the absence of Type II CRISPR-Cas defense in certain enterococcal lineages explains the preponderance of acquired antibiotic resistance genes, prophage, plasmids, and other mobile element traits observed for these strains.

A Type II-A CRISPR-*cas* locus has also been identified in *E. faecium*, occurring in 3 of 8 draft genomes analyzed (Palmer & Gilmore, 2010). This locus, EfmCRISPR1-*cas*, possesses a CRISPR with repeat sequences that are distinct from the *E. faecalis* CRISPR loci, although EfmCRISPR1 appears to be more closely related to EfsCRISPR1 than EfsCRISPR1 is to EfsCRISPR3. EfmCRISPR1-*cas* possesses *csn1*, *cas1*, *cas2*, and *csn2*, as well as an additional gene of unknown function encoded 5′ to *csn1*. Spacers in one of the three strains are similar to predicted phage sequences from *Clostridium novyi* and *L. lactis,* and could be derived from an uncharacterized enterococcal phage. The EfmCRISPR-*cas* locus was absent from three vancomycin-resistant *E. faecium* strains and from three of four multidrug resistant strains, a finding that is consistent with a role for the system in limiting acquisition of antibiotic resistance genes by horizontal gene transfer.

Have Type II CRISPR-Cas systems been lost from certain *E. faecalis* and *E. faecium* lineages, and if so, by what mechanism? It was postulated that *S. thermophilus* Type II CRISPR-*cas* loci could be lost by recombination occurring between a CRISPR repeat and a partial repeat sequence occurring 5′ to the *cas* genes (likely the anti-repeat of tracrRNA (Deltcheva, et al., 2011; Horvath, et al., 2008). This implies the deletion of the CRISPR-*cas* locus, which does not appear to be the mechanism underlying the presence of variable Type II CRISPR-*cas* loci in *E. faecium* or *E. faecalis*. For *E. faecium*, genomic analyses have revealed that what is clinically classified as *E. faecium* is actually composed of two distinct phylogenetic clades (Galloway-Peña, Roh, Latorre, Qin, & Murray, 2012; Palmer, et al., 2012) that possess ~ 4-6% divergence in shared gene sequences (Palmer, et al., 2012). Hospital adaptation appears to have originated from only one of these clades, while the other clade consists of fecal commensal or otherwise non-hospital adapted strains. Recombination can occur between strains from these two clades, generating new strains with hybrid genomes (Palmer, et al., 2012). Strikingly, the EfmCRISPR1-*cas* locus has been identified only in strains from the non-hospital adapted clade, as well as in a hybrid strain that apparently acquired the locus by recombination (Palmer, et al., 2012). This suggests that the ancestral absence of CRISPR-Cas defense in one *E. faecium* clade facilitated the eventual emergence of acquired multidrug resistance and hospital adaptation specifically from that clade.

Unlike *E. faecium*, many *E. faecalis* strains are so closely related in core genome sequence that their relationships to each other cannot be cleanly resolved by whole genome analysis (Palmer, et al., 2012). It is therefore more difficult to postulate that ancestral *E. faecalis* was composed of distinct clades heterogeneous for CRISPR-Cas defense. In the place of the EfsCRISPR1-*cas* locus, strains such as *E. faecalis* V583 possess a novel, ~200 nucleotide sequence found only in strains that lack that locus (Palmer & Gilmore, 2010). Similarly, strains lacking EfsCRISPR3-*cas* possess a novel, ~50 nucleotide sequence found only in strains that lack that locus. The origin and possible function of these novel sequences is unclear. What is clear is that recombination between *E. faecalis* strains can cause the loss of EfsCRISPR1-Cas.

Displacement of the OG1RF EfsCRISPR1-*cas* locus by incoming V583 genomic DNA has been observed for *in vitro* conjugation experiments (Manson, Hancock, & Gilmore, 2010; Palmer & Gilmore, 2010). Pheromone-responsive plasmids resident in the V583 cell can integrate into the chromosome, and from there initiate plasmid transfer functions, ultimately mobilizing large regions of the V583 chromosome into OG1RF recipients (Manson, Hancock, & Gilmore, 2010). These high frequency recombination-like transfers generate transconjugant strains with hybrid V583-OG1RF genomes that lack EfsCRISPR1-*cas* and concomitantly possess acquired antibiotic resistance genes and other clinically relevant traits originating from V583 (Manson, Hancock, & Gilmore, 2010; Palmer & Gilmore, 2010). Conceivably, antibiotic resistant, CRISPR-*cas* deficient strains such as these hybrid strains, as well as high-risk strains such as V583, could be specifically selected for during antibiotic therapy, and could ultimately become the dominant enterococcal lineages in antibiotic-treated patients. Collectively, data from *E. faecium* and *E. faecalis* genome analysis and plasmid transfer experiments indicate that enterococcal CRISPR-*cas* loci can be both disseminated and lost via recombination.

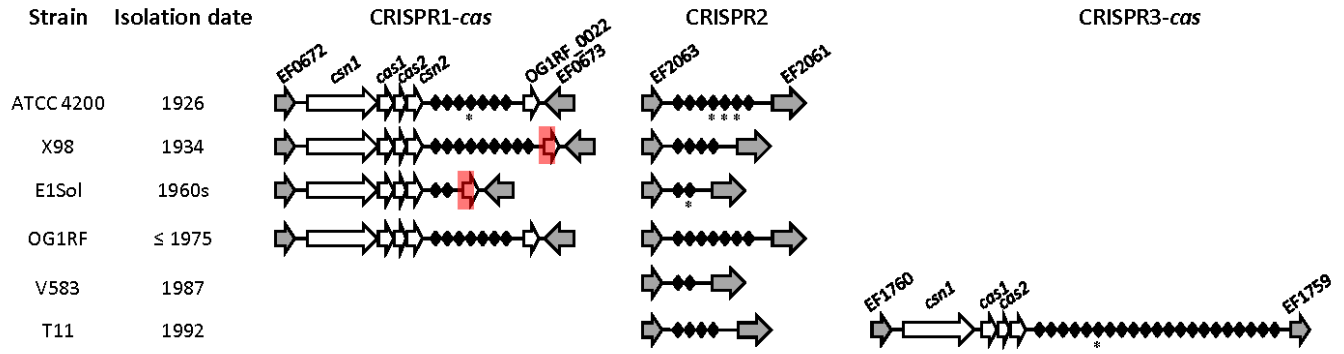

Figure 4. *E. faecalis* CRISPR-*cas* loci. Representative EfsCRISPR1-*cas*, EfsCRISPR2, and EfsCRISPR3-*cas* loci are shown. *E. faecalis* V583 open reading frames and their homologues in other strains are shown in grey. *cas* genes are shown in white. CRISPR spacers (30 nucleotides) are represented by black diamonds. Repeats (36 nucleotides) are not shown. Spacers possessing ≥ 90% to known mobile elements are starred. Red boxes represent a deletion in the X98 and E1Sol CRISPR1-*cas* region relative to OG1RF, which along with ATCC 4200 encodes a gene of unknown function between CRISPR1 and EF0673. Figure is not drawn to scale.

Methods to identify CRISPR-cas loci in enterococcal genomes

Several methods are available to search for CRISPR and *cas* genes in enterococcal genomes. Because the chromosomal locations of EfsCRISPR1-*cas*, EfsCRISPR2, EfsCRISPR3-*cas*, and EfmCRISPR1-*cas* are conserved (Palmer & Gilmore, 2010), those locations can be specifically interrogated for the presence or absence of these loci, either by genomic analysis or by PCR-based screening. To identify novel CRISPR loci, repeat finder programs can be used. A web-based program, CRISPRFinder (Grissa, Vergnaud, & Pourcel, 2007), is useful for the identification of CRISPR candidates. A genome sequence, either draft or complete, can be loaded into the CRISPRFinder web server and analyzed for potential CRISPR content with a short (< 1 minute) turnaround time. Candidates can then be mapped back to the genome sequence and compared to the structure of known CRISPR loci. As a caveat to this approach, small CRISPR loci like EfsCRISPR2 (consisting of 1 repeat, 1 spacer, and a degenerate repeat) are not identified by CRISPRFinder, and require annotation by analysis of a conserved genomic location (Palmer & Gilmore, 2010). If present, *cas* genes may be identified in the vicinity of CRISPR candidates, and can be easily found using genome annotations or by searching for genes encoding conserved protein domains (for example, TIGRFAM domains) that are diagnostic for CRISPR-Cas systems (Makarova, et al., 2011).

To illustrate this approach, analysis of the sequenced genome of *Enterococcus italicus* DSM 15952 (GenBank accession number AEPV00000000) for potential CRISPR-*cas* loci using CRISPRFinder, identified three potential CRISPR arrays: two located on genome contig 74 and one located on contig 75. The CRISPR on contig 75 possesses a 36 nucleotide repeat sequence and 23 spacer sequences with an average size of 30 nucleotides, and is associated with a set of *cas* genes including *csn1*, *cas1*, *cas2*, and *csn2*. Thus, *E. italicus* possesses a Type II-A CRISPR-*cas* locus. The two CRISPR on contig 74 flank a set of *cas* genes that include *cas1*, *cas2*, *cas10*, *csm2*, *csm3*, *csm4*, *csm5*, *csm6*, and *cas6*. *cas10* is the signature gene for Type III CRISPR-*cas* loci, and *csm2* is the signature gene for Type III-A CRISPR-*cas* loci (Makarova, et al., 2011). It appears that in addition to a Type II-A CRISPR-*cas* locus, *E. italicus* also possesses a Type III-A CRISPR-*cas* locus. Whether both the Type II-A and Type III-A CRISPR-Cas systems are active and whether they have overlapping or specialized functions in *E. italicus* genome defense remains to be determined.

Restriction-modification (RM)

There is a small amount of evidence for RM occurring in enterococci. A Type II restriction endonuclease, SfaI, was purified from *Streptococcus faecalis* var. *zymogenes* strain TR (likely an *E. faecalis* isolate) (Wu, King, & Jay, 1978). The recognition sequence of SfaI is GGCC, and cleavage occurs between the G and C (Wu, King, & Jay, 1978). SfaI activity was not identified in other *S. faecalis* and *S. faecium* strains, and based on its variable presence, the authors postulated that the gene encoding SfaI was plasmid-based (Wu, King, & Jay, 1978). Two other *S. faecalis* enzymes, SfaGU and SfaNI, were reported in a survey of RM enzymes, published in 1982 (Roberts R. J., 1984). A RM system (M.SfeI and R.SfeI) encoded by a *Streptococcus faecalis* SE72 plasmid has also been characterized (Okhapkina, et al., 2002). Finally, a methyltransferase with Dam-like activity encoded by the enterococcal VanB-type vancomycin resistance transposon Tn*1549* has been identified (Radlińska, Piekarowicz, Galimand, & Bujnicki, 2005). Available literature suggests that RM does occur in the enterococci, although the systems identified to date are associated with mobile elements or are otherwise strain-specific. It remains to be determined whether these systems act as barriers to the uptake of additional mobile elements.

New England Biolabs maintains a database of computationally predicted and biochemically verified RM proteins, called REBASE (Roberts, Vincze, Posfai, & Macelis, 2010). A search for "*Enterococcus*" in REBASE yields 147 hits (as of March 2012), encompassing putative restriction, modification, and specificity subunits encoded by enterococcal chromosomes, plasmids, transposons, and temperate phages, including ΦFL1, ΦFL2, and ΦFL3. The majority of these RM candidates (100/147) are predicted Type II system components (as compared to 29 hits for Type I, 2 hits for Type III, and 15 hits for Type IV). For some of these proteins, recognition and/or cleavage sites have been identified. In the future, it will be of interest to integrate biochemical data from REBASE with genome analyses to better understand the distribution of these systems across the genus, and how their distributions might relate to prophage abundance, acquired antibiotic resistance, and hospital adaptation.

Perspectives and Future Directions

Enterococcal phage receptors

The receptors utilized by enterococcal phages and the host cell ligands that these receptors bind are mostly uncharacterized. To better understand the biology of enterococcal phages, it will be essential to identify the receptors they use to interact with their host. Peptidoglycan, repeating glycan strands of *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid linked to small tetra-peptides, as well as cell wall teichoic acids, covalently linked to peptidoglycan disaccharides, are likely phage binding site candidates (Osborn, 1969; Tipper & Strominger, 1968). Additional teichoic acids called lipoteichoic acids are also anchored in the phospholipid membrane of enterococci. Teichoic acids in the cell wall of *S. aureus* and *Bacillus subtilis* have been determined to be phage receptors (Lindberg, 1973) and for *S. aureus*, alterations in wall teichoic acids results in phage resistance (Wolin, Archibald, & Baddiley, 1966). Lipoteichoic acids of the *Lactobacillus delbrueckii* cell membrane are also bound by phages. Substitutions in the glycerol backbone of the lipoteichoic acid render *L. delbrueckii* phage incapable of binding to the host cell surface (Räisänen, et al., 2007). Enterococcal teichoic acid structures vary. *E. faecium* U0317 produces a wall teichoic acid polymer of 2-acetamido-2-deoxy-D-galactose, glycerol, and phosphate (Bychowska, et al., 2011), while both poly(glycerol-phosphate) and poly(ribitol-phosphate) wall teichoic acid polymers have been detected in *E. hirae* ATCC 9790 (Armstrong, Baddiley, Buchanan, Davison, Kelemen, & Neuhaus, 1959) *E. faecalis* produces a 1,3 poly(glycerol-phosphate) polymer attached to either D-alanine, kojibiose (α-D-glucopyranosyl-(1,2)-α-D-glucose), or 6,6′-di-alanyl-α-kojibiose (Hogendorf, Bos, Overkleeft, Codée, & Marel, 2010). The sugars and peptides that compose the cell wall teichoic acids and lipoteichoic acids of the enterococci are strong candidates for enterococcal phage attachment structures. Previous work has also shown that the extracts of enterococcal cell walls can inactivate staphylococcal

phages, a finding that suggests that cell wall sugars and/or peptides may be natural ligands for enterococcal phage binding (Rakieten & Tiffany, 1938).

Surface proteins are also likely receptor candidates for enterococcal phages. Numerous types of cell surface proteins have been identified, mainly in *E. coli*, as receptors for phages. These cell surface proteins represent a diverse array of protein types and include peptidoglycan-associated proteins, membrane transport proteins or channel porins, enzymes, substrate receptors used to transport metabolites, and secretion systems (Rakhuba, Kolomiets, Dey, & Novik, 2010). Enterococcal genomes contain many genes whose products are thought to be associated with the bacterial cell surface (Palmer, et al., 2012; Paulsen, et al., 2003). For instance, the *E. faecalis* V583 genome sequence contains greater than 50 putative lipoproteins with predicted signal sequences that may be associated with the *E. faecalis* membrane; approximately 20 different carbohydrate utilization pathways which have identifiable membrane transport proteins; and a large number of virulence related genes that are predicted to be exposed at the bacterial cell surface (Paulsen, et al., 2003). Many of these proteins may be used by enterococcal phages for the transmission of their nucleic acid during infections.

Other cell surface structures may be used for enterococcal phage attachment to the host cell, including capsular polysaccharides and pili. Capsular polysaccharides are secreted from bacterial cells and deposited at the cell surface, creating a layer of carbohydrate slime that covers the cell. In some instances, polysaccharide capsules are protective against phage adsorption; however, other phages utilize cell surface capsular polysaccharide for initial adsorption, a process which is often reversible (Lindberg, 1973; Rakhuba, Kolomiets, Dey, & Novik, 2010). *E. faecalis* encodes two clusters of genes that are known to contribute to capsule biosynthesis: the *epa* and *cps* genes. The *epa* genes are thought to contribute to the production of a rhamnose containing polysaccharide capsule, whereas the *cps* gene cluster polysaccharides are composed of glucose and galactose and are sometimes referred to as diheteroglycan (Hancock & Gilmore, 2002; Theilacker, et al., 2011; Thurlow, Thomas, & Hancock, 2009). The production of the *cps* polysaccharides is variable in *E. faecalis* and is the basis of the group A-D serotyping.

Little is known about capsule biogenesis in other enterococcal species, although *E. faecium*, *E. casseliflavus*, and *E. gallinarum* possess a putative capsule biosynthesis system that is distinct from the *cps* system of *E. faecalis* and is more similar to the capsule biosynthesis system of *S. pneumoniae* (Palmer, et al., 2012). It is possible that enterococcal phages bind to these polysaccharides in a cell trophic manner, due to the variable possession and expression of the capsular polysaccharide synthesis loci between enterococcal strains. If so, this may be a useful way to determine host specificity for particular enterococcal phage groups.

Some phages have also evolved to bind to the bacterial cell surface through interactions with surface appendages, such as flagella and pili (Lindberg, 1973; Rakhuba, Kolomiets, Dey, & Novik, 2010). Enterococci such as *E. gallinarum* and *E. casseliflavus* are flagellated, and many species produce pili (Nallapareddy, et al., 2006; Sillanpää, Prakash, Nallapareddy, & Murray, 2009). Pili receptors for phage adsorption have been identified for pleomorphic-RNA–containing phages and filamentous phages (Rakhuba, Kolomiets, Dey, & Novik, 2010). The recent identification of numerous novel enterococcal phages belonging to the polyhedral, filamentous, and pleomorphic phage group suggests that pili could be cell surface targets for phage adsorption (Mazaheri Nezhad Fard, Barton, & Heuzenroeder, 2010). Future studies on phage receptors of enterococci will be needed to better understand the mechanisms used by enterococcal phages to interact with their hosts, and may yield insight into how these phages influence the community structures of enterococci within natural habitats.

Role of enterococcal phages in mammalian systems

Studies have begun to elucidate the human virome, which is the type and abundance of viral particles found at distinct anatomical sites where commensal bacteria reside, which includes the intestinal tract, oral cavity, and the lungs (Pride, et al., 2012; Reyes, et al., 2010; Willner, et al., 2009). It is clear that the areas of the human body colonized by commensal bacteria are highly populated with phages. It is also evident that these phage populations fluctuate when changes in bacterial community structure occur; for instance, when the human diet

is altered (Minot, et al., 2011). So far, our knowledge of the identity of these phages is limited to metagenomic DNA libraries of phage DNA sequences (i.e. integrases and phage structural genes) and their comparison to known phage and prophage sequences that have been deposited in public nucleotide databases. From these data, it is possible to obtain an idea of the diversity of natural phage populations associated with humans, and, to an extent, determine some information on the relative abundance of certain types of phages (i.e. double- versus single-stranded DNA phages) (Reyes, et al., 2010).

Analysis of metagenomic DNA sequences from the intestinal tract has revealed sequences with similarity to enterococcal phages, specifically those of *E. faecalis* (Duerkop, Clements, Rollins, Rodrigues, & Hooper, 2012; Morowitz, et al., 2010). Studying enterococcal species in the intestinal tract, especially those that are polylysogenic, may provide critical information about the biological significance of enterococcal phages in the intestines. It is possible that phages that infect the enterococci may influence genetic exchange between *Enterococcus* species within the intestine. Phage-mediated exchange of DNA may be another mechanism that enterococcal species use for generating genetic variation to evade the immune system at the intestinal mucosa. Enterococcal phages are a likely source of novel genes. Therefore, phage-mediated transfer of genetic traits may result in the acquisition of genes that contribute to hypervariable phenotypic traits for immune evasion—or, conversely, phages may transfer virulence factors that aid in the transition of enterococci from commensals to pathogens. This type of phage-bacterial interaction could have far-reaching implications for the evolution of pathogenic enterococci, as multiple species have evolved to become successful opportunistic pathogens. Recently, Enterococcal phages have been proposed to play an ecological role in the intestine by aiding in niche competition. In the intestines of gnotobiotic mice, prophage01 and EfCIV583 aided *E. faecalis* V583 during competition with closely related *E. faecalis* strains (Duerkop, Clements, Rollins, Rodrigues, & Hooper, 2012). This suggests that prophage induction could be a means by which certain communities of enterococci compete with neighboring, genetically similar communities for nutrients in the intestine by restricting the invasion of neighboring enterococcal strains through phage-mediated killing.

The use of *in vitro* co-culture systems and mouse models of enterococcal intestinal colonization, including the use of gnotobiotic mice, will be instrumental toward further elucidating the genetic and ecological roles of enterococcal phages within the intestinal tract. Furthermore, these types of studies are not limited to the intestinal tract and can be applied to other environments where enterococci reside, including the oral and vaginal microbiota, as well as endocarditis and bacteremia infections. It is likely that enterococcal phages will profoundly influence enterococcal biology within a multitude of different environments where both commensal and pathogenic enterococci are found, ultimately leading to discoveries relevant to the ways in which commensal and pathogenic enterococci interact with their mammalian hosts.

Enterococcal CRISPR-Cas and RM Systems

Most sequence analysis of enterococcal CRISPR was reported in 2010 (Palmer & Gilmore, 2010), prior to the discovery of tracrRNA and the structure of mature Type II CRISPR crRNAs (Deltcheva, et al., 2011). A strict sequence identity cut-off was used to identify enterococcal protospacer-spacer sequence matches (27/30 identical residues required) (Palmer & Gilmore, 2010), and it is likely that potential targets of the enterococcal CRISPR-Cas systems were missed. Based on current literature on Type II CRISPR-Cas systems, it now appears that 20/30 identical resides at the 3′ spacer end would be a stringent cut-off for spacer analysis. While informative for identifying crRNA targets (especially for *E. faecium*, for which few were identified), additional protospacers would facilitate the identification of consensus PAM sequences for each of the three enterococcal CRISPR-Cas systems. PAM sequences will be important to consider in designing experimental systems interrogating the specific roles of enterococcal CRISPR and CRISPR-Cas in conferring defense against phages and plasmids.

One attribute that distinguishes enterococcal Type II systems from those studied in other bacteria is the EfsCRISPR2 of *E. faecalis*. It is unclear how prevalent orphan CRISPR loci are in other prokaryotes. An orphan

pre-crRNA in *L. monocytogenes* EGD-e appears to stabilize a transcript that encodes iron acquisition proteins (Mandin, Repoila, Vergassola, Geissmann, & Cossart, 2007). This result suggests that EfsCRISPR2 may have a secondary function in *E. faecalis*. Whether EfsCRISPR2 is expressed and processed remains to be determined, although the absence of *cas9* suggests that the generation of mature crRNAs will not occur. A long transcript encoded antisense to the V583 ORF EF2062 and EfsCRISPR2 region has also been detected, which may complicate the analysis of EfsCRISPR2 expression and processing (d'Hérouel, et al., 2011). The significance of this antisense transcript is unknown, although presumably it could contribute to processing and/or stability of the EfsCRISPR2 transcript.

Beyond the CRISPR-Cas defense, it will be important to determine the way in which differential genome modifications and restriction enzyme cohorts affects phage susceptibilities, as well as the frequency of horizontal gene transfer in the enterococci. Restriction modification appears to be a major barrier to gene transfer in *S. aureus* (Monk, Shah, Xu, Tan, & Foster, 2012), and the same may be true for the enterococci. Bioinformatic identification and analysis of candidate restriction-modification and abortive infection systems from available enterococcal genomes will be informative, as very little is known about genome defense in the enterococci. Some therapeutic approaches have already started to explore the use of CRISPR and RM systems (Marrafini & Sontheimer, 2008; Torres, Jaenecke, Timmis, García, & Díaz, 2000).

Concluding Remarks

Enterococcal genomes harbor a diversity of integrated prophage and other mobile elements. Some strains are also susceptible to infection by non-integrating lytic phages. The study of phages and mobile element biology has revealed several exciting areas of enterococcal research. First, enterococcal phage lytic proteins, the lysins, have prospects as novel therapeutics that can target antibiotic resistant strains of enterococci and other Gram-positive bacteria that resist broad-spectrum antibiotic treatment. However, not only lysins, but also CRISPR, RM systems, and bacteriophages have the potential to be used as modern therapeutics. Second, enterococcal phages have great potential as important mediators of genetic exchange through transduction during intra- and inter-species infections. It is these phage-host interactions, along with other mobile elements like plasmids, which facilitate the acquisition of novel traits (i.e. antibiotic resistance determinants and virulence factors). The attainment of genes from various mobile elements helps to uniquely distinguish clonal populations of enterococci. Prophages and plasmids are abundant in the enterococci, even in strains thought to live a strictly commensal lifestyle. Therefore, the contribution of phage and plasmids to the biology of enterococci may provide insight into what role, if any, these elements play in the transition of enterococci from commensals to pathogens. That being said, one idea has surfaced. Many enterococci have evolved mechanisms to protect their genomes from the assault of plasmids and phage by employing CRISPR-Cas regulatory systems. These systems restrict host genome acquisition of foreign mobile elements through their destruction. Notably, those enterococcal strains possessing one or more complete CRISPR-Cas system are parasitized less by phage and other mobile elements which include antibiotic resistance genes. Those strains that lack functional CRISPR-Cas systems retain many more foreign DNA elements. This dichotomy may be one way in which enterococci have evolved to become successful opportunistic pathogens. If true, the lack of CRISPR-Cas systems would represent a legitimate mechanism for the promotion of the commensal to pathogen transition in the enterococci. These studies on enterococcal phage biology, mobile elements, and CRISPR-Cas have just begun to scratch the surface of an emerging field, wide open for new discoveries.

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Transcriptional and Post Transcriptional Control of Enterococcal Gene Regulation

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Introduction

Enterococci are a versatile group of bacteria found in various habitats, which range from a commensal presence within the gastrointestinal tract of mammals and other organisms, to the environment where they have been identified within soil, water, and food supplies. They are also remarkably hardy and are able to withstand drastic changes within their environments, including factors of temperature, salinity, pH, and available nutrients (Klare, Werner, & Witte, 2001). The ability of the enterococci to rapidly respond to growth and environmental conditions is largely achieved by controlling gene expression. In this chapter, we focus on work to date that provides current understanding of the gene regulation mechanisms in *Enterococcus faecalis*, an opportunist pathogen with intrinsic resistance to many antibiotics and a causative agent of nosocomial urinary tract infections, endocarditis, and bacteremia.

Gene regulation in *E. faecalis* occurs on multiple levels. At the transcriptional level, the initiation of gene transcription is regulated by transcriptional factors which modulate promoter activities. Thanks to advancements in genome sequencing, multiple genomes of enterococcal species have been revealed, which provides the foundation for an understanding of enterococcal gene function and regulation processes. In the case of *E. faecalis* V583, the genome consists of 3337 open reading frames (ORFs) on the chromosome, as well as three plasmids (Paulsen, et al., 2003). Among these, 145 genes are predicted to encode for transcriptional regulators that belong to different protein families, including Cro/CI, GntR, MerR, and other transcription regulator protein families (Paulsen, et al., 2003). These transcriptional factors affect the expression of their effector genes by binding to the promoter region of these genes and positively or negatively affecting transcription, which subsequently alters the abundance of the specific mRNAs and proteins produced. This chapter discusses the mode of action for several transcriptional factors identified in *E. faecalis* and the genes that are regulated by them.

Another broad set of regulation machinery involved in gene expression are the two component systems that sense extracellular signaling molecules, and regulate target gene expression at the transcription level as a result. A typical two-component system consists of a membrane-associated histidine kinase receptor (sensor HPK) and a cognate response regulator (RR) which functions as a transcriptional factor (Stock, Robinson, & Goudreau, 200). Phosphorylation of the response regulator by the histidine kinase upon specific environmental stimuli

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alters its ability to bind to the target DNA sequence or interact with other components of the transcription machinery, which in turn regulates gene expression. In *E. faecalis* V583, at least 15 two-component systems have been identified that play a critical role in *E. faecalis*' ability to response to a wide variety of stimuli, including quorum signals, antimicrobials, nutrients, serum components, and bile salts (Paulsen, et al., 2003). The mode of function of several two-component systems, including quorum-sensing related Fsr and vancomycin-resistant gene regulators VanRS, are further discussed in detail to demonstrate the ways in which bacteria respond to different environmental stimuli through these signal transduction processes.

Other than the typical HPK-RR two-component systems, several other signaling pathways involving transcriptional regulation in *E. faecalis* have been described, including the sex pheromone signaling pathway and the pathway for cytolysin toxin expression. In both of these systems, expression of the target genes are controlled by the balance of extracellular antagonistic peptide pairs, which are shifted upon the presence of recipient cells or host cells, respectively.

Besides transcriptional regulation, control of gene expression can also occur post-transcriptionally through other mechanisms, including mRNA processing, regulation via riboswitches, or modulation by antisense and small regulatory RNA (sRNA). One of the best-studied examples of post-transcriptional regulation is seen in the control of genes involved in ethanolamine utilization (Garsin, 2010). The *E. faecalis* ethanolamine utilization (*eut*) genes are encoded in a complex locus of 18 genes, and include structural, enzymatic, and regulatory components. Upregulation of the *eut* genes is triggered by the presence of two environmental cues—ethanolamine and adenosylcobalamin (AdoCbl). Each of these input signals is perceived and relayed by two different regulatory systems, and synchrony between them is required for expression of the *eut* genes. Both regulatory systems control gene expression at the post-transcription initiation level, and target nascent RNA instead of DNA. Post-transcriptional regulation also occurs at several levels in the control of conjugation of a pheromone response plasmid as previously discussed, in which antisense RNA plays an important role in transcriptional regulation. This RNA-mediated reciprocal regulation is RNAse III dependent, which demonstrates the importance of RNA processing in gene expression regulation. A similar observation is reported for the gene expression of *E. faecalis* Ebp pili, which is positively regulated by the novel endo- and exonuclease RNase J2 (Gao, Pinkston, Nallapareddy, van Hoof, Murray, & Harey, 2010). Small regulatory RNAs have recently been identified as important regulatory elements in bacterial gene expression. Recent transcriptomic analysis of *E. faecalis* sRNA provides valuable information to understand gene regulation through sRNAs. One of the newly identified sRNAs, EF3314_EF3, is potentially involved in the turnover of some abundant proteins, as demonstrated by the ΔEF3314_EF3315 sRNA mutant, a finding that indicates a novel regulation process (Shioya, et al., 2011).

Virulence Related Gene Expression Regulation

Known *E. faecalis* virulence factors include peptidoglycan-anchored surface proteins, such as Ace and the Ebp pili; secreted peptides, such as cytolysin; membrane bound proteins, including aggregation substance; extracellular enzymes, including gelatinase and serine Protease; and other gene products, which play important roles in response to different biological needs that range from surviving environmental changes or evading host defense to adherence and colonization. Similar to other bacterial pathogens, *E. faecalis* has developed sophisticated regulation machinery to tightly control the expression of virulence-related genes to its benefit. Understanding the regulation events in virulence factor expression will contribute significant value in the development of future strategies to battle enterococcal infections.

Transcriptional regulators: SlyA as an example

Proteins of the MarR/SlyA family are known to regulate the expression of virulence genes in many Gram-positive and Gram-negative organisms. Evidence of such a regulator existing in enterococci arose with the

examination of the crystal structure of an *E. faecalis* transcriptional factor (EF3002) in 2003 (Wu, et al., 2003). The 150 amino acid SlyA protein is encoded by an open reading frame that begins with a GTG initiator codon. The crystal structure of SlyA was determined without its DNA target or signal molecule bound, and the unliganded crystal structure revealed that SlyA consists of two monomers in an asymmetric unit (Wu, et al., 2003) (Figure 1). In the SlyA crystal, key arginine residues thought to play a role in DNA binding were found to point away from the major groove, as was determined for the crystal structure of MarR (Alekshun, Levy, Mealy, Seaton, & Head, 2001), but differs from that of the MexR (Lim, Poole, & Strynadka, 2002) transcriptional regulator of *Pseudomonas aeruginosa*. The high degree of flexibility observed between the crystal structures of the various DNA-binding domains provides indirect evidence of the ability of this fold to adapt in order to recognize various DNA targets. Conserved residues among the various transcriptional regulator structures suggest that this fold could make contact with the phosphate groups or bases. Comparison of the SlyA-Ef structure with the structure of MarR and MexR suggest they are similar, which indicates that the MarR/SlyA family shares a common fold and similar DNA-binding properties, despite their low amino acid sequence similarity (Wu, et al., 2003) (Figure 1).

The promoter region of SlyA suggests that it is a part of a bicistronic operon that also serves as the promoter for EF3001, which encodes an N^1-acetyltransferase enzyme. This transcriptional organization is conserved in other Gram positive species (Michaux, et al., 2011). It is thought that over-expression of EF3001 would be an advantage to the Δ*slyA* mutant as it shows homology to PaiA, a protein from *Bacillus subtilis*, that encodes for an N^1-spermidine acetyltransferase (Forouhar, et al., 2005) involved in polyamine homeostasis (excess of this molecule is toxic to the cell).

Recent functional analyses of the SlyA regulator (*ef3002*) (Michaux, et al., 2011; Michaux, Martini, Hanin, Auffray, Hartke, & Giard, 2011) have illustrated that SlyA activity is part of a complex regulatory network. Observations have suggested that the involvement of SlyA is linked to virulence and persistence inside the host (Michaux, et al., 2011). SlyA mutant strains were found to be more virulent than the parental wild type strain in a wax moth (*Galleria mellonella*) model (Michaux, et al., 2011). The immune response of *G. mellonella* has a number of structural and functional similarities to the innate immune response of mammals (reviewed in (Vogel, Altincicek, Glöckner, & Vilcinskas, 2011)). The Δ*slyA* mutant was also found to survive better in mouse organs and in macrophages (Michaux, et al., 2011). DNA microarray experiments revealed that 117 genes were deregulated in the Δ*slyA* mutant as compared to the parental strain, a finding that suggests that SlyA acts as a repressor and activator (Michaux, et al., 2011)—although it should be noted that the expression of most of these genes changed by only two fold. EF3001 was the only gene in this analysis where expression in the Δ*slyA* mutant was up-regulated by 16.62-fold as compared to the parental wild type strain, suggesting that the enzyme it encodes may provide an important advantage during infection (Michaux, et al., 2011). Another gene encoding a helicase (EF3217) in the Δ*slyA* mutant was strongly down-regulated, but its role in virulence is unclear. This particular gene is associated with a mobile element and is not conserved across a number of the sequenced enterococcal genomes (Michaux, et al., 2011).

Further work using a pVEPhoZ-PslyA strain revealed that the expression level of the *slyA* operon was increased in the presence of bile salts (Michaux, Martini, Hanin, Auffray, Hartke, & Giard, 2011). Under the stressed conditions induced by the presence of 0.08% bile salts, the growth of the Δ*slyA* mutant was impaired, with the doubling time of the strain decreased by a factor of two. Under these growth conditions, the bile salt hydrolase (BSH) encoded by EF3005 in the *E. faecalis* V583 genome was found to be induced in the Δ*slyA* mutant by six-fold in comparison to the wild type parental strain, which is somewhat puzzling, given the growth phenotype of the Δ*slyA* mutant in the presence of bile salts (Michaux, Martini, Hanin, Auffray, Hartke, & Giard, 2011). In the *E. faecalis* V583 genome, there are two BSHs encoded by EF0521 and EF3005. In wild-type *E. faecalis* V583, the BSH encoded by EF0521 is induced at a higher level than EF3005, which suggests that the BSH encoded by EF3005 plays a minor role in the bile salt stress response. SlyA may in fact give a selective advantage to bacterial development in the intestine, as *E. faecalis* is naturally present in the gastrointestinal tract (Jones, Begley, Hill,

Gahan, & Marchesi, 2008). However, other experiments using microarrays (Solheim, Aakra, Vebo, Snipen, & Nes, 2007) or a proteomic approach (Bøhle, et al., 2010) have produced contrary results, where the expression of these genes was not seen to be inducible by exposure to bile salt conditions. Differences in the results may be attributable to differences in experimental procedure or the detection limits of the various assays. The down-regulation of a number of genes was also observed, with a number of these being hypotheticals and with a majority related to protein synthesis (i.e. tRNA synthetases). However, expression of one of the tryptophanyl-tRNA- synthetases (EF2679, *trpS*) was found to be up-regulated at all time points studied, as compared to the initial start time. Further work is required to clarify the role of SlyA under different environmental conditions.

Regulation of E. faecalis Ebp pilus

The *E. faecalis* endocarditis and biofilm-associated pili (Ebp) is a major virulence factor that plays an important role in cell adhesion, biofilm formation, and the development of endocarditis. Many environmental factors affect the levels of Ebp surface expression, including culture media (TSB vs. BHI), serum, and bicarbonate levels, which suggests that this is a tightly regulated process (Nallapareddy, et al., 2006; Bourgogne, Thomson, & Murray, 2010). Three genes (*ebpA, B and C*) that encode the *E. faecalis* pilus structural proteins are located in the same polycistron, with the divergent promoter region shared with the *ebpR* gene.The e*bpR* gene encodes a protein that is a member of the AtxA/Mga family of transcriptional regulators. It has been demonstrated that the deletion of the *ebpR* gene leads to reduced levels of *ebpABC* mRNA and Ebp production, as evaluated by qRT-PCR and Western blot analysis, respectively (Bourgogne, Singh, Kox, Pflughoeft, Murray, & Garsin, 2007). As a result, the mutant *ebpR* gene displays phenotypes of reduced biofilm formation and attenuated primary adherence. In a recent study, Bourgogne *et al.* demonstrated that the addition of bicarbonate to culture media enhanced the expression of the *ebpR* and *ebpABC* loci. However, the authors did not observe a strong direct relationship between *ebpR* expression and *ebpA* expression, suggesting that the activation effect of bicarbonate to *ebpABC* may be through other unidentified factors other than *ebpR* (Bourgogne, Thomson, & Murray, 2010). Another recent study by Gao et al. identified RNase J2 as a positive regulator of Ebp expression (Gao, Pinkston, Nallapareddy, van Hoof, Murray, & Harey, 2010). Similar to *ebpR*, the deletion of *E. faecalis rnjB* resulted in a 30-fold decrease in *ebpABC* mRNA expression and significantly decreased Ebp surface display. RT-PCR and a reporter gene assay demonstrated that the mRNA level of *ebpR* is not affected by the deletion of RNase J2, excluding the possibility that RNase J2 affects *ebpR* at the RNA level through transcriptional regulation or RNA processing. It is likely, however, that RNase J2 affects *epbR* expression at the translational level through an as-yet unidentified regulatory or antisense RNA.

Intercellular Signaling Regulated Gene Expression

Quorum sensing is a global regulatory mechanism that bacteria use to communicate with each other through the accumulation and detection of signaling molecules that they release into the environment, which enables individual cells to respond to cell density (Ng & Bassler, 2009). Two major types of signaling molecules are used by different bacteria: Gram-negative bacteria produce N-acyl-homoserine lactones (HSLs) that bind to and activate a transcriptional activator, which in turn activates the transcription of regulated genes; while Gram-positive bacteria use extracellular peptides that are processed from bacteria-encoded proteins, through post-translational processes, as quorum sensing signaling molecules. Upon binding to the specific transmembrane receptors, these autoinducing peptides trigger signal transduction pathways, resulting in the activation or repression of effecter genes. In *E. faecalis*, several quorum-sensing–related regulation systems, which play important roles in biofilm formation, bacteria virulence, and production of cytolysin, toxin, have been identified (Haas, Shepard, & Gilmore, 2002; Hancock & Perego, 2004; Qin X. , Singh, Weinstock, & Murray, 2001). Two well-known examples, the Fsr and Cyl systems, are further discussed in detail.

One of the best-studied Gram-positive quorum-sensing systems is the staphylococcal Agr system. It consists of a transmembrane peptidase AgrB, the autoactivating peptide (AIP) processed from the propeptide AgrD by AgrB,

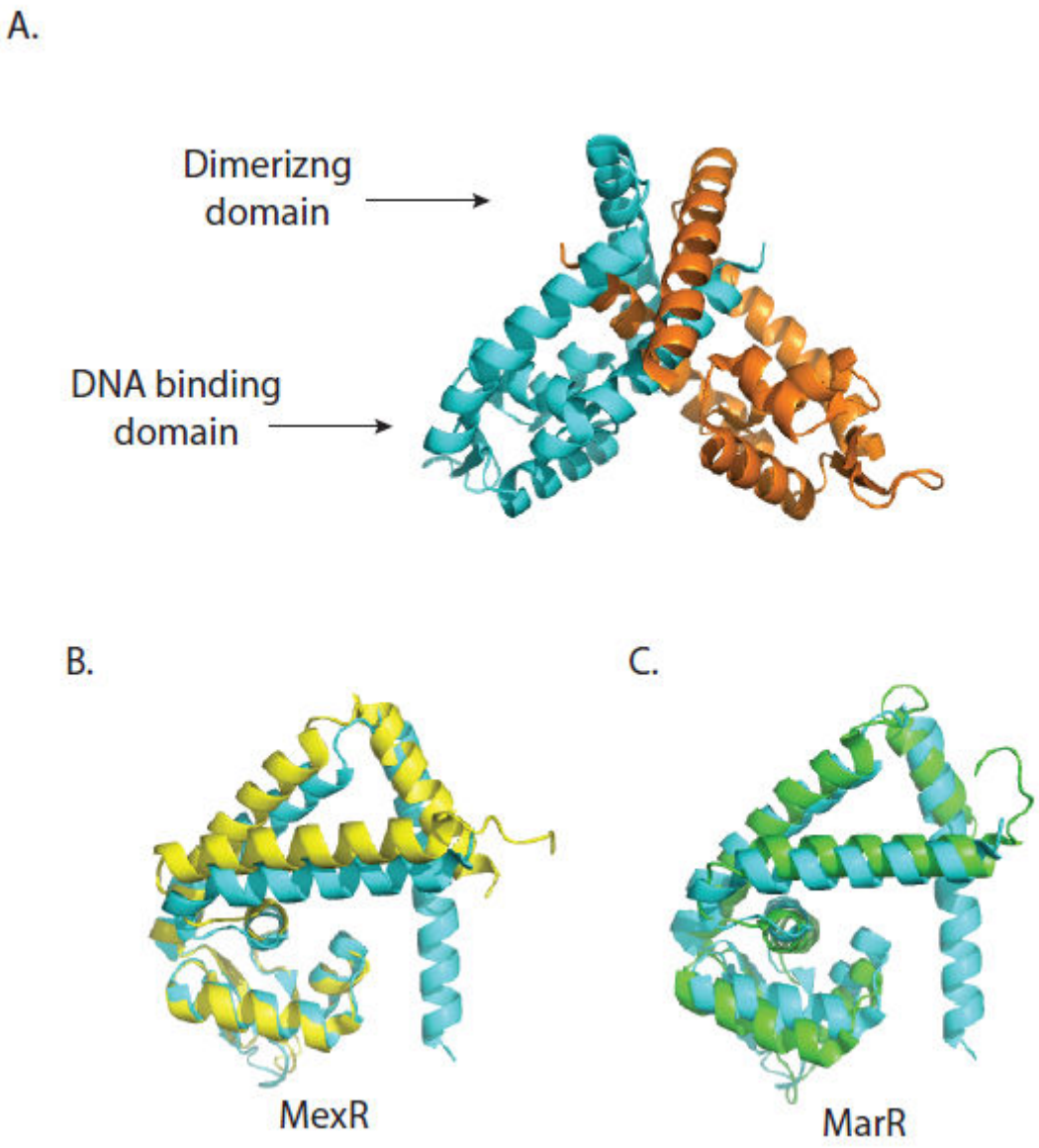

Figure 1. Structure of SlyA dimer and comparison of the structure to the transcriptional factors MexR and MarR. A. The SlyA dimer from *E. faecalis* with the subunits labeled in shades of blue and orange. The structural models were created from the structure under PDB accession numbers 1LJ9 (SlyA), 1LNW (MexR, yellow), 1JGS (MarR, blue), using PyMol (www.pymol.org/).

and a classic two-component signaling module of AgrC (HPK) and AgrA (RR) (Novick & Geisinger, 2008). The homologous system in *E. faecalis* is the Fsr quorum sensing system. The *E. faecalis fsr* gene cluster consists of three genes, *fsrA*, *fsrB*, and *fsrC*, with a recent study revealing that the C-terminus of *fsrB* can be translated individually into a short peptide FsrD, which is the equivalent of AgrD (Nakayama, et al., 2006). Similar to the Agr system, FsrB is responsible for the processing and secretion of the signal peptide gelatinase biosynthesis-activating pheromone (GBAP) from FsrD. The extracellular accumulation of GBAP at high cell density allows binding to the membrane HPK FsrC, and triggers auto-phorsphorylation and dimerization of FsrC, which in turn transfers the phosphate group to the response regulator FsrA. The phosphorylated form of FsrA is able to bind to the promoter of the *fsr* gene cluster and activate the transcription of *fsrBCD*, resulting in the elevated production of GBAP for the further activation of *fsr* genes (Qin X. , Singh, Weinstock, & Murray, 2000). As demonstrated by Northern blot, the *fsrC* mRNA level in cultured *E. faecalis* is low during the early growth phase, but drastically increases in latter growth phases, confirming that *fsr* expression is dependent upon cell density.

Besides binding to its own promoter and self-activating through the positive feedback loop, the staphylococcal Agr system regulates transcription of a global antisense RNA (RNAIII), which in turn regulates expression of exoproteins and pleiotropic regulators at the translational level (Dunman, et al., 2001). Unlike the Agr system, the *E. faecalis* Fsr system does not have the RNAIII component as an effector, and apparently lacks the ability to broadly affect gene expression. In fact, some clinical and environmental *E. faecalis* strains carry a 23.9-kb deletion that covers much of the *fsr* gene locus (Nakayama, Kariyama, & Kumon, 2002). An interesting phenotype identified in some *fsr* mutant strains was that they were gelatinase-negative despite a *gelE*+ genotype. Qin et al. further demonstrated that deletion of the *fsr* gene cluster from the *E. faecalis* OG1RF significantly decreased the expression of two extracellular proteases, gelatinase (GelE) and serine protease (SprE), and that the regulation occurs at the transcriptions level (Qin X. , Singh, Weinstock, & Murray, 2000). As shown in Figure 2, the *gelE*/*sprE* genes are located immediately adjacent to the *fsr* gene cluster, though they are not cotranscribed with the *fsr* genes. A *gelE*-specific promoter (Pe) was detected by transcriptional fusion analysis, and has been identified as *fsr*-dependent (Qin X. , Singh, Weinstock, & Murray, 2001). Altogether, the Fsr system acts as an activator for the expression of *gelE* and *sprE*. Using a gene expression microarray approach, Bourgogne et al. further showed that the deletion of the *fsrB* gene affects the transcription of a number of other genes that encode EF1097, surface proteins EF0750-0757, and enzymes involved in several metabolic pathways (Bourgogne, Hilsenbeck, Dunny, & Murray, 2006). A recent report by Pinkston et al. reported a higher level of surface expression of the collagen adhesion protein, Ace, in *fsrB* mutants in the late growth phase, as compared to wild-type OG1RF (Pinkston, et al., 2011). They demonstrated that regulation of the Ace surface display by the Fsr system is mediated by gelatinase at the post-translational level: The activation of *gelE* expression at a high cell density by the Fsr quorum sensing induces gelatinase production, which in turn cleaves Ace from the cell-surface. Cleavage of Ace significantly inhibits the ability of the bacterial cells to adhere to collagen, which suggests a possible role of this regulation mechanism in *E. faecalis* colonization or dispersion.

Another example of a cell-density–dependent gene regulation mechanism comes from the autoinduction of cytolysin. Cytolysin is a peptide bacterial exotoxin expressed by some strains of *E. faecalis*. As a secreted virulence factor of *E. faecalis*, cytolysin is able to lyse eukaryotic cells, as well as other bacteria that lack the ability to produce cytolysin (Booth, Bogie, Salh, Siezen, Hatter, & Gilmore, 1996). The gene operon involved in the synthesis and regulation of *E. faecalis* cytolysin consists of eight genes: $cylL_L$, $cylL_s$, *cylM, cylB, cylA, cylI, cylR1,* and *cylR2* (Haas, Shepard, & Gilmore, 2002). The $cylL_L$ and $cylL_s$ encode the large and small subunits of cytolysin, respectively, which are further secreted from the cell and processed by gene products of *cylM*, *cylB*, and *cylA*. The divergently transcribed *cylR1* and *cylR2*, however, are not directly involved in the production and maturation of cytolysin peptides. Instead, they compose a novel two-component system that regulates gene expression of cytolysin in response to environmental cues, such as quorum signals or the presence of target cells. Although it has no apparent similarity to the classic two-component systems of HPK/RR pair, *cylR1* functions as the membrane-associated sensor unit, while the DNA-binding protein *cylR2* acts as a response regulator to alter the promoter activity of cytolysin-associated genes. This signal transduction machinery is responsible for the extracellular levels of one of the cytolysin peptides, $cylL_s$. Expression of the cytolysin operon is repressed at subthreshold levels of the autoinducer $cylL_s$ (Coburn, Pillar, Jett, Haas, & Gilmore, 2004). When $cylL_s$ accumulates to threshold levels as a result of either higher cell density or the presence of a target cell which preferentially absorbs the other cytolysin peptide $cylL_L$, derepression occurs leading to high-level cytolysin expression. This quorum-related regulation mechanism provides enterococci with a convenient tool to actively probe and respond to the environment.

Antimicrobial Response

E. faecalis V583 was the first vancomycin-resistant clinical isolate of *E. faecalis* reported in the United States, and was originally isolated from a patient suffering from a persistent bloodstream infection (Sahm, et al., 1989). V583 is rich in numerous virulence traits that have been acquired by horizontal gene transfer, which contribute

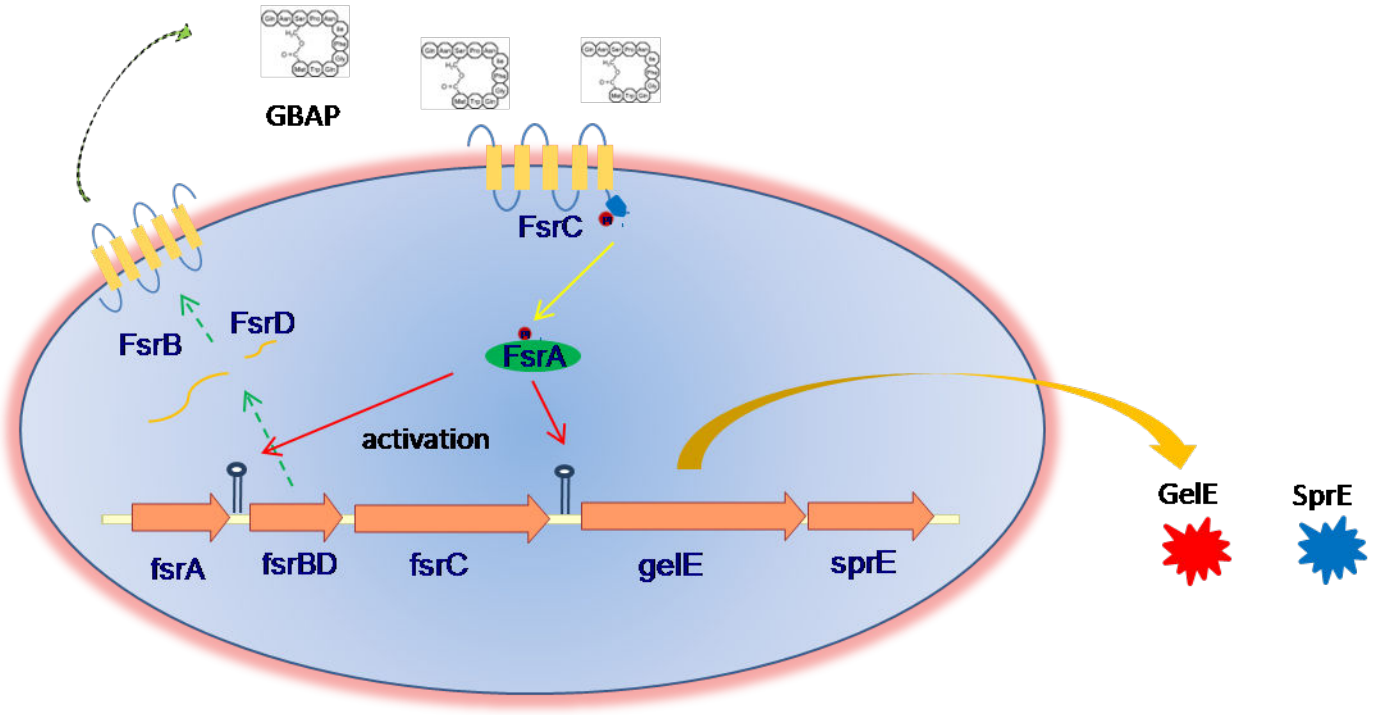

Figure 2. Fsr quorum sensing system in E. faecalis. The fsr locus consists of fsrABCD, which encode the proteins that are responsible for generating and sensing the peptide signal molecule. Once the critical concentration of GBAP is reached, the FsrC/A two-component system is activated, causing the transcriptional activation of fsrBCD and the downstream gene locus gelE/sprE, resulting in elevated expression of extracellular proteases GelE and SprE.

to its success as a hospital pathogen (reviewed in (Paulsen, et al., 2003)). In addition to the acquisition of virulence genes which allow the enterococci to cause infection, V583 is one of the leading causes of hospital-associated Gram-positive infections, through its acquisition of genes that aid in maintaining antibiotic resistance. Antibiotics of various classes induce cell death by targeting different cellular processes, although we have a limited understanding of the extent and effect these antibiotics have on the cell besides binding to or inhibiting their known targets. The response of *E. faecalis* to various antibiotics most likely involves the coordinated interplay between the regulation of virulence factors and multiple genetic traits that govern adaptation of the bacterial cell's physiology. Many transcriptomic studies of enterococci have focused on the response of V583 to different environmental cues. As enterococci continue to be a cause of hospital-acquired infections, it is particularly important to understand the effect of antibiotic pressure. Several studies have been conducted that examined the response of enterococci to various antimicrobial agents, including erythromycin, chloramphenicol, vancomycin, copper, and bacteriocins.

Erythromycin

Erythromycin is a macrolide antibiotic which acts by reversibly binding to the P site of the 50S subunit of the bacterial ribosome. Resistance mechanisms identified in Gram-positives to subvert the inhibitory effects of erythromycin include target modification by methylation, which changes the P site of the rRNA, as well as a macrolide efflux resistance mechanism (Pechère, 2001). *E. faecalis* V583 carries a plasmid known as pTEF1 that encodes an rRNA adenine dimethylase family protein (EF0007), which may aid in its resistance to this drug.

E. faecalis strain V583 is resistant to relatively high concentrations of erythromycin; however, the treatment does retard its growth. A drastic change in gene transcription was seen with erythromycin-treated V583, as compared to untreated cells, over a 90 minute period (Aakra Å. , et al., 2005). In the presence of 50 μg/ml of erythromycin (V583 is resistant to > 400 μg/ml), Aakra et al. (Aakra Å. , et al., 2005) found that 260 genes were down-

regulated at one or more time points, while 340 genes were up-regulated. From this set, genes encoding for hypothetical proteins, as well as genes encoding for transport and binding proteins, were the two most dominant groups of differentially expressed genes. Surprisingly, in this experiment, the EF0007 gene that encodes for an rRNA adenine dimethylase family protein (ErmB), was not up-regulated in the presence of erythromycin, but was constitutively expressed in V583 even in the absence of erythromycin. This would suggest that the expression of genes other than *ermB* is important for the survival and growth of V583 in the presence of erythromycin.

Although changes in transcriptional levels of many genes with differential expression identified upon erythromycin exposure is assumed to occur mainly due to a change in growth conditions (Aakra Å. , et al., 2005), a number of these changes could be linked to survival in the presence of erythromycin. For example, the induction of ribosomal proteins and two protein Ala-acetyltransferases indicate that the overproduction of ribosomal proteins is one way to evade the effects of erythromycin. Interestingly, a MsrC-like protein (EF1413) was up-regulated at all time points studied. MsrC homologs found in *E. faecium* are thought to encode an efflux pump that is involved in low-level macrolide resistance (Portillo, Ruiz-Larrea, Zarazaga, Alonso, Martinez, & Torres, 2000; Singh, Malathum, & Murray, 2001). However, when compared to the MrsC homolog from *E. faecium*, the protein sequence of EF1413 from *E. faecalis* V583 is found to be only 40% identical (60% similar). Other specific genes found to be up-regulated were genes that encode ABC transporters that belong to the MDR family (EF1732 and EF1733) (Aakra Å. , et al., 2005). Another MDR efflux pump, EmeA (EF1078), is also induced at all time points, excluding time zero in V583 exposed to erythromycin, and is most likely involved in mediating resistance. This finding supports previous observations that the efflux pump could extrude erythromycin (Jonas, Murray, & Weinstock, 2001). A number of two-component signal transduction pathways were also found to be up- or down-regulated in the presence of erythromycin—although further work is required to clarify their roles in *E. faecalis*. It is assumed that these factors all play some role in mediating erythromycin resistance.

Another interesting observation made by Aakra et al. (Aakra Å. , et al., 2005) is that in the presence of erythromycin, six genes related to fatty acid and phospholipid metabolism were up-regulated. In Gram-negative bacteria, such intrinsic resistance to macrolides is achieved due to the impermeability of the cellular outer membrane to this hydrophobic drug (Roberts M. C., 2008), and it may be that changes in membrane biochemistry in enterococci also impede the entry of this class of drug into the cell.

Chloramphenicol

Chloramphenicol is a broad-spectrum antibiotic that inhibits the peptidyl transferase reaction of the large subunit of the ribosome. This antibiotic currently has limited use in treating bacterial infections in humans, due to widespread acquisition of resistance mechanisms. Bacterial resistance to chloramphenicol may be mediated by several mechanisms, including inactivation by an enzyme (acetyltransferase or phosphotransferase), the excretion of the drug by an efflux pump, and mutations in the 23S rRNA that result in an altered target site in the cell. The well-studied *E. faecalis* strain V583 is resistant to chloramphenicol, but no specific chloramphenicol resistance genes have been identified in the genome sequence, and resistance cannot be solely attributed to mutations in the 23S rRNA gene. Interestingly, the growth of V583 in the presence of chloramphenicol is decreased, which suggests that chloramphenicol does create stress on the cells. In the presence of chloramphenicol, over 609 genes were up-regulated and down-regulated at one or more of three time points (t=0, 90, and 180 minutes), and these genes were identified through two different methods to undergo transcriptional changes (Aakra A. , et al., 2010).

In response to the addition of chloramphenicol to the growth medium, it would be expected that the bacterial cells would want to adapt to the conditions, potentially through changes in the cell wall, as was observed for treatment with other agents, such as sodium dodecyl sulfate (Solheim, Aakra, Vebo, Snipen, & Nes, 2007) and erythromycin (Aakra Å. , et al., 2005). Two genes (*ef1732* and *ef1733*) that encode potential transporters of

chloramphenicol out of the V583 cells were strongly induced in cells treated with the drug at 90 minutes. These genes encode for an ABC transporter that belongs to the MDR family of transporters, and were also seen to be up-regulated in the presence of erythromycin (Aakra Å. , et al., 2005). Genes involved in fatty acid biosynthesis were up-regulated in *E. faecalis* V583 cells at 180 minutes (Aakra A. , et al., 2010), and a similar response was noted for the growth of *E. faecalis* in the presence of sodium dodecyl sulfate (Solheim, Aakra, Vebo, Snipen, & Nes, 2007) and erythromycin (Aakra Å. , et al., 2005). The up-regulation of these genes suggests that V583 is adapting to the stress induced by chloramphenicol by altering the membrane, which may change the transport capabilities of the cell as a result. This change in membrane composition may also partly explain the relatively low number of induced genes that specify transport and binding proteins. Interestingly, a number of phage genes were also up-regulated in the cells exposed to chloramphenicol, potentially indicating that the potential for cell lysis induces the phage to leave the cell. There are a number of phage-encoded genes in the V583 genome, including one phage that is found in many different *E. faecalis* strains (phage 02); however, the transcriptional profile for the phage 02 genes was not found to change in the time course of chloramphenicol exposure.

The genome of V583 carries the *vanB* operon, which encodes for resistance to vancomycin. In the presence of chloramphenicol, 8 of the 11 genes encoded in the operon were induced, including the genes encoding for the histidine kinase and the cognate response regulator, which are responsible for sensing vancomycin and inducing the expression of the rest of the operon.

The addition of chloramphenicol to the medium led to the up-regulation of the pyrimidine biosynthesis operon (*ef1721* to *ef1712*). Interestingly, the precursor for pyrimidine synthesis, carbamoyl phosphate, is also the precursor of arginine biosynthesis. Arginine biosynthesis in chloramphenicol-treated V583 cells was down-regulated (Aakra A. , et al., 2010). This response is different than what was seen in erythromycin exposed *E. faecalis* V583 (Aakra Å. , et al., 2005), suggesting that it is specific to the presence of chloramphenicol.

As the target of chloramphenicol is the ribosomal protein, it was hypothesized that transcriptional changes would be seen in a number of ribosomal proteins. Although the increased transcription of the ribosomal proteins were noted by Aakra et al. (Aakra A. , et al., 2010), it is also important to keep in mind that microarray analysis may not actually reflect the level of ribosomal proteins in the cells, as many proteins may also be regulated at the translational level.

Vancomycin

Sub-inhibitory concentrations of vancomycin are known to trigger changes in cell wall biosynthesis genes in Gram-positive bacteria, including in *E. faecalis*. Vancomycin is known to inhibit cell wall synthesis by covalently binding to the D-alanyl-D-alanine chain of the cell wall pentapeptide (Reynolds P. E., 1989). Vancomycin resistance in *E. faecalis* and *E. faecium* can be mediated by either the *vanA* or *vanB* operon. Both operons encode for the expression of polypeptides that are involved in aiding in the transposition of the transposon that encodes the *van* genes, as well as genes encoding for the modification of the cell wall pentapeptide to prevent vancomycin from binding to its target (reviewed in (Courvalin, 2006)). The difference between the *vanA* and *vanB* operons is that the *vanA* operon can also be induced by the related glycopeptide antibiotic teicoplanin (Baptista, Depardieu, Reynolds, Courvalin, & Arthur, 1997; Baptista, Rodrigues, Depardieu, Courvalin, & Arthur, 1999).

Transcription of the vancomycin-resistant gene cluster is regulated by the VanRS two-component system that is found at the beginning of the gene cluster (Arthur, Molinas, & Courvalin, 1992; Evers & Courvalin, 1996). Phosphorylation of the VanR response regulator enhances the affinity of the protein for the regulatory regions of the promoter regions of the *vanA* gene cluster; namely, for *vanRS* (P_R) and *vanHAXYZ* (P_H) (Arthur, Depardieu, Gerbaud, Galimand, Leclercq, & Courvalin, 1997; Holman, Wu, Wanner, & Walsh, 1994). A similar setup is also found to regulate the expression of the *vanB* gene cluster (Baptista, Depardieu, Reynolds, Courvalin, & Arthur, 1997; Arthur, Depardieu, Gerbaud, Galimand, Leclercq, & Courvalin, 1997; Wright, Holman, & Walsh, 1993). The response regulators of both *van* operons can also be activated by kinases encoded by the host chromosome

in the absence of their partner sensor (Baptista, Rodrigues, Depardieu, Courvalin, & Arthur, 1999; Arthur, Depardieu, Gerbaud, Galimand, Leclercq, & Courvalin, 1997; Silva, Haldimann, Prahalad, Walsh, & Wanner, 1998).

The activation of the promoters in the *vanA* operon by VanR is inducible by vancomycin, teicoplanin, and moenomycin in wild-type strains of *E. faecalis*, and these are constitutively expressed in *vanS* mutants (Arthur, Depardieu, Gerbaud, Galimand, Leclercq, & Courvalin, 1997). The *vanB* cluster differs with the operon being inducible only by the addition of vancomycin, whereas in a $vanS_B$ mutant, vancomycin, teicoplanin, and moenomycin can all activate transcription of the operon from the two promoter sites (Baptista, Rodrigues, Depardieu, Courvalin, & Arthur, 1999; Evers & Courvalin, 1996). Activation of the *vanA* operon by glycopeptides and moenomycin occurs as these drugs inhibit the transglycosylation reaction, but by different mechanisms, as the structure of moenomycin is unrelated to that of the glycopeptides (Handwerger & Kolokathis, 1990). It is thought that the $VanS_A$ sensor kinase in the *vanA* operon is not involved in direct interaction with the stimulating drugs (Handwerger & Kolokathis, 1990). With the *vanB* operon, the $VanS_B$ sensor kinase does appear to interact with vancomycin, which distinguishes the transcriptional differences between these two operons. Amino acid substitution of the N-terminal sensing domain of $VanS_B$ allows for the induction of the *vanB* operon by the other glycopeptide teicoplanin, but not by moenomycin (Baptista, Depardieu, Reynolds, Courvalin, & Arthur, 1997; Baptista, Rodrigues, Depardieu, Courvalin, & Arthur, 1999). Experiments completed both *in vitro* and *in vivo* have shown that carriage of the transposon encoding for the *vanB* operon, when uninduced, was not significantly costly to the host (Foucault, Depardieu, Courvalin, & Grillot-Courvalin, 2010). Induction of the resistance genes leads to a major reduction in growth rate, colonization, and transmission in *Enterococcus* sp. (Foucault, Depardieu, Courvalin, & Grillot-Courvalin, 2010). Overall, tight regulation mediated by a two-component regulatory system effectively reduces the biological costs that are associated with vancomycin resistance.

Experiments that used sub-inhibitory concentrations of vancomycin on the *E. faecalis* strain V583, which carries the *vanB* operon, revealed that the open reading frame EF2292 (named *vanV*) located 3' to the *vanX* gene exhibited the same level of induction as the vancomycin resistance genes (Ribeiro, Santos, Marques, Gilmore, & de Fátima Silva Lopes, 2011). Analysis of this gene suggests that it might be involved in some regulation process of the *vanB* operon in strains that carry *vanV*, although it is not consistently found in all *vanB*-carrying strains. It also appears that *vanV* does not have an effect on the resistance of the carrying strain to vancomycin (Ribeiro, Santos, Marques, Gilmore, & de Fátima Silva Lopes, 2011). Further screening of *vanB*-resistant enterococci for the presence of this gene is required, and may unravel a particular function for this gene.

It should be noted that many enterococcal species can carry other *van* operons, which encode for low-level or constitutive resistance to vancomycin. One of these operons is the *vanD* operon, which encodes for the constitutive production of the peptidoglycan precursor ending in D-alanyl-D-lactate (Depardieu, Bonora, Reynolds, & Courvalin, 2003). Mutations in the sensor kinase and response regulator, as well as the native D-alanyl-D-alanine ligase (involved in native cell wall synthesis), lead to the constitutive expression of the *van* operon and the cell wall that contains only the D-alanyl-D-lactate pentapeptide structure (Depardieu, Reynolds, & Courvalin, 2003; Depardieu, Kolbert, Pruul, Bell, & Courvalin, 2004).

Another *van* operon carried by *E. casseliflavus*, *E. gallinarum*, and E. *flavescens* is the *vanC* operon. The *vanC* phenotype is inducible and constitutive, and results in the production of a peptidoglycan precursor ending in D-Alanyl-D-Serine (Reynolds & Courvalin, 2005). Other *van* operons that encode for a pentapeptide ending in a serine residue are found in *E. faecalis* and are referred to as the *vanE* and *vanG* operons. The *vanE* operon has an organization identical to the *vanC* operon (Patiño, Courvalin, & Perichon, 2002). The *vanG* operon has a unique gene organization and appears to encode a predicted transcriptional activator upstream of the two component regulatory system (Depardieu, Bonora, Reynolds, & Courvalin, 2003).

The expansion of the *van* operon alphabet is continually expanding with *vanL* (Boyd, Willey, Fawcett, Gillani, & Mulvey, 2008), *vanM* (Xu, et al., 2010), and *vanN* (Lebreton, et al., 2011) gene clusters being reported. Transcriptional regulation of these operons appears to be similarly regulated by a two component transduction system and promoters, as in the *van* operons discussed above.

Bacteriocins

Bacteriocins are peptides secreted by bacteria with the intent of inhibiting the growth of similar or closely related bacterial species. Many lactic acid bacteria produce bacteriocins that have the ability to inhibit the bacterium beyond the genus level, and also have the potential to be used medically for the prevention of growth of unwanted microflora. With the spread of antibiotic resistance, bacteriocins are ideal candidates for use as alternative antimicrobials. In addition, resistance to bacteriocins is not common in nature. Class IIa bacteriocins encompass the non-lantibiotic bacteriocins that have regular amino acid residues. A well-characterized class IIa bacteriocin is pediocin, which is produced by *Pediococcus acidilactici* (Marugg, et al., 1992). Many of these bacteriocins are produced by lactic acid bacteria and have been found to inhibit other Gram-positive organisms, including *Enterococcus* spp. Bacteriocins act by permeabilizing the cell membrane, which leads to the killing of the cell. The target for class IIa bacteriocins is the mannose phosphate transport (*mpt*-PTS), and mutants that lack this system are insensitive to the effects (Diep, Skaugen, Salehian, Holo, & Nes, 2007; Gravesen, et al., 2002; Héchard & Sahl, 2002; Ramnath, Beukes, Tamura, & Hastings, 2000). The PTS is a major uptake system for mannose and glucose, and many of the components are also involved in gene regulation of catabolic operons. In the laboratory, deletion mutants of *mptR* and the *mpt* operon are known to be resistant to the effects of bacteriocins.

Transcriptional analysis has been completed on pediocin-resistant strains of *E. faecalis* generated in the laboratory environment to test the efficacy of this bacteriocin in the treatment of hospital-acquired enterococcal infections. A comparison of wild-type V583, spontaneously resistant isolates, and an Δ*mptD* mutant, which is known to render *E. faecalis strains* resistant to the effects of the class IIa bacteriocin pediocin, was completed (Opsata, Nes, & Holo, 2010). In *E. faecalis*, the *mpt* operon is under transcriptional control of a promoter recognized by σ^{54}, and is dependent upon the activator MptR. The spontaneous bacteriocin resistant isolates contained a mutation in *mptR* (EF0018; A356G mutation) causing the down-regulation of the expression of the *mpt* operon. The expression of several other genes were also down-regulated, and included the components of the *mpt* operon (EF0019-EF0022); the downstream mannose operon regulator (*manO*; EF0024); a major facilitator family transporter (EF0082); glyceraldehyde-3-phosphate dehydrogenase (*gap-2*; EF1964); phosphoglycerate kinase (*pgk* EF1963); triosephosphate isomerase (*triA*; EF1962); enolase (*eno*, EF1961); phosphoglycerate mutase (*gpm,*EF0195); pyruvate kinase (*pyk*; EF1046); and L-lactate dehydrogenase (*ldh-1*; EF0255); a majority of which encode for sugar metabolism. A study by Opsata et al. (Opsata, Nes, & Holo, 2010) found the reduced consumption of glucose as a carbon source in the *mpt* mutants, which demonstrated the important role of Mpt in glucose metabolism in *E. faecalis*. The reduced glucose uptake results in changes in the concentrations of glycolytic metabolites, which subsequently affects the energy status of the cell. Putative carbon catabolite regulation (*cre*)-sites were located upstream of three of the differentially expressed genes, and some with *cre*-sites were located downstream of the genes, supporting the idea that an altered energy status sensed by a histidine protein-kinase/phosphorylase and implemented on the PTS phospho-carrier protein does occur. However, this is also the first time that *cre*-sites have been reported downstream of the regulated gene (Opsata, Nes, & Holo, 2010). In *E. faecalis,* disruption of the mannose PTS altered the transcription of about 90 *cre*-sites, with about 65% of these being involved in the uptake and metabolism of alternative energy sources. A number of these genes were also found to encode transcriptional regulators, and may indicate that a regulatory cascade has been triggered. A number of the genes found to be differentially regulated in the *E. faecalis* V583 *mpt* bacteriocin-resistant mutants were also found to be similarly expressed in a transcriptome study of a *Streptococcus mutans* EIIAB mannose-PTS mutant; however, some differences were found that may be attributed to niche preferences (Opsata, Nes, & Holo, 2010).

Copper

Metal ions are crucial for bacteria to maintain transcriptional control of regulatory networks that govern gene expression. Maintaining homeostasis between the intra- and extracellular concentration of metal ions is critical in adaptations to intracellular survival and replication among pathogenic bacteria. Copper is useful as a cofactor for redox enzymes in reactions, due to its ability to accept and donate electrons; however, this very reactivity also makes it a toxic metal (Urbański & Beresewicz, 2000; Linder & Hazegh-Azam, 1996). It is therefore crucial for cells to maintain control over copper by means of uptake, storage, and efflux.

Copper uptake and regulation in *E. hirae* is a well-studied system. The regulation of intracellular copper in this species is mediated by the *cop* operon, which consists of four open reading frames (Solioz & Stoyanov, 2003) (Figure 3). The four genes encode two P-type ATPase copper transporters (*copA* and *copB*), a copper chaperone protein (*copZ*), and a copper-dependent transcriptional regulator (*copY*) (70). This operon is conserved between all members of the Lactobacillales and includes at least homologues of the *copA* and *copY* genes (Reyes, Leiva, Cambiazo, Méndez, & González, 2006).

The transporters in the *cop* operon are responsible for opposite processes. CopA takes up copper (I) when it is limiting, and structural information for this ATPase is available (Lübben, Portmann, Kock, Stoll, Young, & Solioz, 2009; Tsuda & Toyoshima, 2009). CopB extrudes copper (I) when it reaches toxic concentrations (Odermatt & Solioz, 1995; Solioz & Odermatt, 1995). CopY is a bipartite repressor, where the N-terminal half of the protein exhibits 20% identity to bacterial repressors of β-lactamases, such as MecI (Solioz & Stoyanov, 2003). The N-terminal region also contains a QQ motif found in the phage repressors (lambda and 434), and has been shown to tightly interact with an ACA triplet of the DNA binding site (Anderson, Ptashne, & Harrison, 1987). Mutations of the DNA binding site of CopY abolish the ability of the repressor to interact with DNA *in vitro* (Wunderli-Ye & Solioz, 1999). The C-terminal end of CopY has multiple cysteine residues arranged in a pattern commonly seen in other copper-responsive transcriptional activators that have been studied in yeast ($CxCx_3CxC$) (Zhou & Thiele, 1991; Dobi, Dameron, Hu, Hamer, & Winge, 1995). CopY functions as a dimer (Strausak & Solioz, 1997), binding to an inverted repeat sequence upstream of the *cop* operon when bound to zinc (Zn (II)) (Cobine, Jones, & Dameron, 2002), suppressing transcription. Figure 3 illustrates the role of CopY in the presence of high copper concentrations and low (physiological) copper concentrations. CopZ or another appropriate copper complex can donate two copper (Cu(I)) to CopY, displacing the bound Zn(II), which results in CopY releasing itself from the DNA and allows expression of the downstream *cop* genes (Cobine, Jones, & Dameron, 2002). The interaction of CopY and CopZ carrying Cu(I) is dependent on electrostatic interactions, and transfer of the Cu(I) occurs due to affinity differences for this metal, as well as charged-based interactions between the two proteins (reviewed in (Solioz & Stoyanov, 2003)).An interesting detail pertaining to CopZ is that it more easily degraded when bound to Cu(I) by a cellular protease under high copper stress (Lu & Solioz, 2001). It is hypothesized that CopZ degradation prevents cell damage from occurring as a result of the exposed Cu(I) in the protein metal ion complex, which can participate in Fenton-type reactions leading to formation of reactive hydroxyl radicals and cell damage (Lu, Dameron, & Solioz, 2003; Solioz M. , 2002).

With the emergence of microarray technology, the effect of copper on the transcriptome of enterococci species has been more closely studied. Two recent publications have looked at the transcriptional response of *E. faecalis* OG1RF (Reyes-Jara, et al., 2010) and V583 (Abrantes, Lopes, & Kok, 2011) in the presence of copper. Not surprisingly in both studies, the *cop* operon (EF0297-EF0299) was the most up-regulated operon. In V583, in the presence of 0.05 mM $CuSO_4$, other induced operons included the following: genes involved in potassium transport (*kpdA* and *kpdB*); an operon encoding for two ABC transporters, a hypothetical protein and a GntR family transcriptional regulator (EF1673-EF1676); an operon of V-type ATP enzymes (EF1492-EF1500); nine genes related to cell wall biosynthesis; and several virulence-associated genes, including LemA (EF0468), a cell-envelope associated acid phosphatase (EF3245), and a cell wall surface anchor family protein (EF3314) (Abrantes, Lopes, & Kok, 2011). In the study conducted by Reyes-Jara *et al.* (Reyes-Jara, et al., 2010), the authors

found that an OG1RF *ΔcopY* mutants' response to the presence of 0.5 mM $CuSO_4$ was similar to the wild-type *copY* strain, with the exception of the *cop* operon being constitutively expressed in the absence of copper. Several other transcriptional elongation factors were also induced shortly after the $CuSO_4$ exposure, including GreA (EF2914), Rrf2 (EF3175), the Cro/CI (EF0873), Sor/DeoR (EF1965) and a hypothetical protein of COG class K (EF1752). The induction of these genes may not occur as a result of increased copper concentration, but may be induced in response to a secondary signal, such as the oxidative stress generated by copper exposure. Comparison between the two strains' responses to copper is difficult, given that the experimental conditions were different.

Response to Environmental Cues

The bacterial transcriptome is a dynamic entity that closely reflects the organism's response to the environmental conditions in which it resides. DNA microarray-based transcriptional profiling provides a genome-wide portrait of the transcriptome of the organism, and may disclose important clues on how a bacterium adapts to a particular environmental niche as a result. Much of the effort put into enterococcal transcriptome analysis has consequently focused on stressors relevant to the human host. *In vitro* experimental approaches using a range of concentrations of various body fluids have been carefully designed in an attempt to mimic *in vivo* scenarios, and these studies may provide essential information about the mechanisms involved in an important aspect of the enterococcal life cycle. Indeed, Snyder et al. (Snyder, et al., 2004) observed a considerable overlap between the expression profiles obtained from *Escherichia coli* during colonization of the murine urinary tract and during growth in human urine. In the following section, we will discuss the transcriptional responses of *E. faecalis* (mainly the sequenced V583 strain) when exposed to relevant body fluids.

Bile

Bile is a digestive juice secreted by the liver and stored in the gallbladder, and it functions both to assist the digestion and absorption of fat in the gut and as a means for the body to excrete waste from the blood. The concentrations of bile present in the small intestines range from 0.2–2%, but can get up to 8% in the gall bladder (Bowen, 1998). The ability to resist the deleterious action of various bile components is thus an absolute need for *E. faecalis* in its commensal lifestyle. The mechanisms involved in bile resistance in *E. faecalis* V583 were previously assessed in a genome-wide transcriptional analysis over a time course of 120 min. after the addition of 1% bovine bile to the growth medium (Solheim, Aakra, Vebo, Snipen, & Nes, 2007). The detergent-like action of bile acids was reflected as an enrichment of V583 genes that code for proteins with membrane-associated functions and/or locations among the differentially transcribed genes. In particular, the functional categories of genes involved in fatty acid and lipid metabolism and signal transduction were strongly affected. Studies in lactobacilli (Bron, Molenaar, de Vos, & Kleerebezem, 2006; Pfeiler, Azcarate-Peril, & Klaenhammer, 2007; Whitehead, Versalovic, Roos, & Britton, 2008) and *Bifidobacterium breve* (Ruiz, Zomer, O'Connell-Motherway, van Sinderen, & Margolles, 2012) also suggest that cell envelope stress is one of several aspects of the bile response that is conserved among Gram-positive bacteria.

Active extrusion of bile appears to be another common strategy to counteract bile toxicity in bacteria (Storz, 2000). Transcriptional profiling has previously been used to identify bile efflux proteins in *Lactobacillus reuteri* and *B. breve* (Whitehead, Versalovic, Roos, & Britton, 2008; Ruiz, Zomer, O'Connell-Motherway, van Sinderen, & Margolles, 2012), for example. Several candidate transporters were also reported in *E. faecalis* V583, including two members of the EmrB/QacA subfamily of the major facilitator superfamily of multidrug resistance transporters (Solheim, Aakra, Vebo, Snipen, & Nes, 2007). In *E. coli*, EmrB was shown to play a role in bile resistance and efflux of bile (Lomovskaya & Lewis, 1992; Thanassi, Cheng, & Nikaido, 1997), but the study of Solheim et al. (Solheim, Aakra, Vebo, Snipen, & Nes, 2007) was the first report of a proton motive force dependent transport system involved in bile resistance in Gram-positive bacteria. Shortly after this study, however, a similar induction of a gene coding for an EmrB/QacA-like multidrug resistance transporter (lr1584)

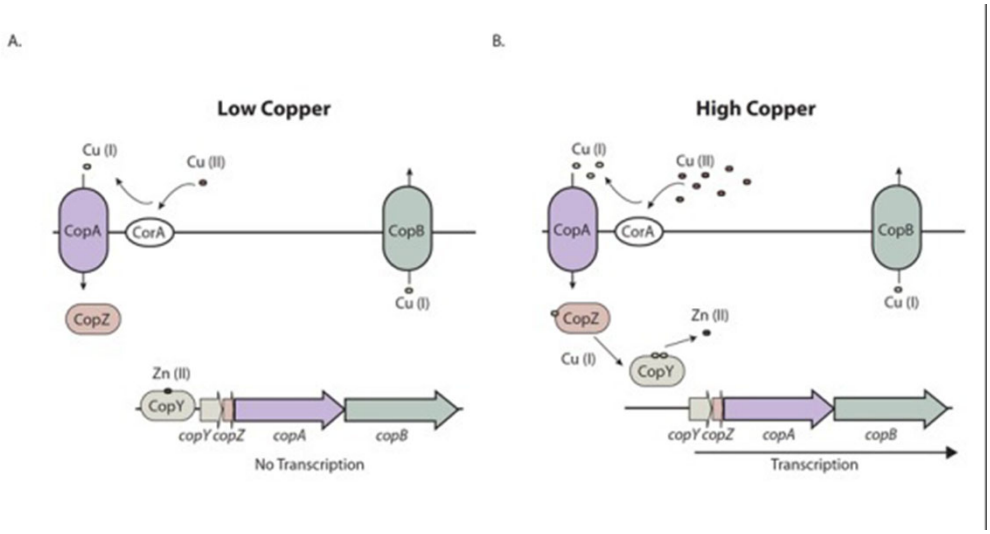

Figure 3. The *cop* operon. A. Regulation of the *cop* operon under low copper conditions (physiological). The CopA repressor is bound to Zn(II) and to the promoter, turning transcription of the *cop* operon down. B. Regulation of the *cop* operon under high copper conditions. An extracellular reductase (CorA) supplies copper (I) for uptake by CopA. Copper inside the cell is then bound by the CopZ chaperone, which carries the copper (I) to the CopY repressor. Zinc is displaced from the active CopY repressor by copper, releasing CopY from the promoter and resulting in the expression of the *cop* genes. Adapted from Solioz &Stoyanov (71).

was observed in *L. reuteri* (Whitehead, Versalovic, Roos, & Britton, 2008). Interestingly, *lr1584* is part of an operon structure which was up-regulated during bile exposure in *Lactobacillus acidophilus*, as well as in *L. reuteri* and *E. faecalis*. Inactivation of both *lr1584* and *lr1582* resulted in an impaired adaptation phenotype in *L. reuteri*. Another V583-encoded transport system (EF2641-42) with significant homology to the well characterized bile responsive *bilE* system in *Listeria monocytogenes* (Sleator, Wemekamp-Kamphuis, Gahan, Abee, & Hill, 2005), also displayed differential expression in V583. This operon codes for proteins involved in glycine betaine uptake. The role of compatible solutes in bacterial stress management, such as glycine betaine, was recently reviewed by Sleator and Hill (Sleator & Hill, 2010).

Bile salt hydrolases (BSH) have also been implicated in bile resistance in other Gram- positive bacteria. These enzymes have been proposed to confer the ability to detoxify bile salts, and studies with a Δ*bsh* mutant revealed an important role for BSH in the intestinal persistence of *L. monocytogenes* (Begley, Sleator, Gahan, & Hill, 2005). None of the two *bsh* homologs in V583 (EF0521 and EF3005) showed significant differential expression during exposure to bovine bile (Solheim, Aakra, Vebo, Snipen, & Nes, 2007); however, the bile-sensitive phenotype of an *E. faecalis* Δ*slyA* mutant was recently connected to the regulation of *ef3005* by the transcriptional regulator SlyA (Michaux, Martini, Hanin, Auffray, Hartke, & Giard, 2011), which suggests the potential role of BSH in *E. faecalis* bile response after all. The diverging result can most likely be attributed to the increased sensitivity associated with real-time PCR, as compared to the microarray-based approach. As a result, further characterization of the loci in question will be needed to conclude whether BSH constitutes a mechanism for bile resistance in *E. faecalis*.

Urine

The urinary tract is among the most common sites of bacterial infection in humans, and urinary tract infections (UTIs) are the type of infection most commonly caused by enterococci (Malani, Kauffman, & Zervis, 2002). In order to identify genetic traits that may distinguish pathogenic and non-pathogenic *E. faecalis* in their ability to cause UTIs, the global transcription signatures of a probiotic (Symbioflor 1) and two pathogenic (MMH594 and OG1RF) strains during cultivation in human urine were compared (Vebø, Solheim, Snipen, Nes, & Brede, 2010). The non-pathogenic strain grew at the same rate as the pathogenic strains, which indicates that the pathogenic

potential is not related to newly required mechanisms that enable growth in this environment. Although the three strains have different strategies in relation to their host, their transcriptional signatures towards urine as a growth medium were surprisingly similar. However, some potentially significant differences were reported. The highest number of differentially expressed genes was observed after 5min in OG1RF, whereas the process took 30min in MMH594 and Symbioflor 1. Consequently, it may be inferred that OG1RF adapts more rapidly to growth in urine. This rationale was further supported by the swift derepression of genes involved in macromolecular biosynthesis (*i.e.* transcription and protein synthesis) in OG1RF, as compared to the two other strains.

More than 50% of all microbial infections have been associated with the formation of biofilms, and biofilm formation appears to also be clinically relevant for UTIs. A particularly problematic aspect of biofilm formation is the ability of sessile bacteria to withstand host defense mechanisms and the increased resistance to antibiotics, biocides, and hydrodynamic shear forces observed for bacteria in biofilm (Bitsori, Maraki, Raissaki, Bakantaki, & Galanakis, 2005). Several genetic determinants with impact on biofilm formation in *E. faecalis* were differentially transcribed during growth in human urine, as compared to the rich medium 2×YT: *bopABCD* (biofilm on plastic; EF0954 to -57) this process was partially up-regulated in OG1RF, while *srtA* (EF3056) was induced in MMH594 and Symbioflor 1. Data from knock-out mutant studies also inferred a role for *salA* (EF3060), *salB* (EF0394) and *altA* (EF0799) in biofilm production, all of which were down-regulated in response to urine (Vebø, Solheim, Snipen, Nes, & Brede, 2010; Mohamed, Tenq, Nallapareddy, & Murray, 2006).

SalB was initially characterized as a stress-inducible protein in *E. faecalis* (Breton, Mazé, Harte, Lemarinier, Auffray, & Rincé, 2002). Its growth in urine also affected the expression of a large number of other genes with a proven or predicted function in stress responses in *E. faecalis*. One of the most pronounced effects was the stimulation of an oxidative stress response. Mn^{2+}-depletion has been reported as a regulator of oxidative stress regulons in other bacteria. Interestingly, the pathogenic strain MMH594 seemed slightly better equipped with genes involved in manganese acquisition than the two other isolates: while *efaABC* and two other genes encoding Mn^{2+}/Fe^{2+} transporters (EF1057 and EF1901) were induced during growth in human urine in all three strains, the putative uptake system for manganese encoded by EF0575 to -78, located on the pathogenicity island, is specific to MMH594. Vebø and coworkers reported the latter system to be induced in MMH594 during cultivation in urine (Vebø, Solheim, Snipen, Nes, & Brede, 2010). Taken together with the differential regulation of several iron transporters, these observations may be indicative of urine as an iron- and manganese depleted environment. This hypothesis was further supported by up-regulation of genes involved in the uptake and assimilation of iron in *E. coli* during growth in the urinary tract (Snyder, et al., 2004).

Surface structures are major determinants of virulence for many pathogens: namely, the presence of a capsule may allow the microorganism to evade the host immune system. Indeed, several cell wall polysaccharides with implications in the pathogenesis of enterococcal infections have been reported (Hancock & Gilmore, 2002; Huebner, et al., 1999; Xu, Murray, & Weinstock, 1998; Xu, Singh, Murray, & Weinstock, 2000), two of which showed differential expression upon the encounter with urine. Both the *cps* (capsular polysaccharide) and the *epa* (enterococcal polysaccharide) loci were down-regulated during growth in urine. The data from the transcriptional analysis also suggested that *E. faecalis* adjusts the fatty acid and phospholipid composition of the membrane in response to urine as a growth medium. This was manifested as an up-regulation of two gene clusters (EF0282-84 and EF2886-75) that were responsible for type II fatty acid biosynthesis (FASII) and isomerization of membrane phospholipids (Vebø, Solheim, Snipen, Nes, & Brede, 2010).

Urine is a complex medium consisting of a mixture of carbon sources (Tasevska, Runswick, McTaggart, & Bingham, 2005; Wishart, et al., 2009) that are likely to change over time, and the ability of the bacteria to exploit available carbon and energy sources may be crucial for virulence. The final cell density reached by *E. faecalis* in urine compared to those in 2× YT growth medium suggests that important growth factors are absent in urine; however, supplementary growth experiments disproved glucose as a major limiting factor for growth of E.

faecalis in urine, as elevated glucose levels alone did not significantly augment bacterial growth (Solheim, unpublished). Nevertheless, the induction of EF3215 to -22 pointed towards citrate as a significant carbon source for E. faecalis during growth in urine. The cit operon is responsive to carbon catabolite repression (CCR) (Blancato, Repizo, Suárez, & Magni, 2008; Suárez, Blancato, Poncet, Deutscher, & Magni, 2011), and this observation is indicative of low glucose levels in urine. Furthermore, the transcriptional data suggested that E. faecalis uses available peptides as a source of amino acids.

The encounter with urine also had a considerable impact on the expression of a number of genes with a potential implication in virulence; however, among the established enterococcal virulence traits, only the *fsr* operon displayed significant changes in transcription. From the microarray data, a modest up-regulation of the *fsrABC* genes (EF1822 to -20) was seen in MMH594 at t_{30}. The *fsrA* gene was also up-regulated at t_5. The regulation of *fsrB* (EF1821) in MMH594 at t_{30} was, however, not confirmed by real time quantitative PCR (QPCR)—thus the importance of this observation remains uncertain. On the other hand, QPCR revealed significant up-regulation of *fsrB* in OG1RF after 30 minutes exposure to urine. As mentioned previously, the *fsr* system modulates expression of *gelE-sprE* operon through a classic quorum sensing signaling cascade. Secretion of these proteases that damage host tissue may contribute to bacterial migration and invasion. The *gelE* gene has been shown to be predominant among clinical enterococcal isolates; however, contradictory results have later been reported (Roberts, Singh, Okhuysen, & Murray, 2004; Coque, Patterson, Steckelberg, & Murray, 1995). Moreover, isogenic strains of *E. faecalis* divergent in gelatinase and serine protease production exhibited attenuated killing in nematode- and mice infection models (Qin X. , Singh, Weinstock, & Murray, 2000; Sifri, et al., 2002). An up-regulation of the *gelE-sprE* operon after prolonged exposure to urine is in line with the work by Shepard and Gilmore (115) which used QPCR to show that *gelE* transcription was 7-fold induced during logarithmic growth in urine.

Blood

E. faecalis is also a significant cause of hospital-acquired bacteremia (Malani, Kauffman, & Zervis, 2002; Wisplinghoff, Bischoff, Tallent, Seifert, Wenzel, & Edmond, 2004), and with this in mind, Vebø et al. conducted a whole-genome transcription study on the adaptation of *E. faecalis* to blood (Vebø, Snipen, Nes, & Brede, 2009). Interestingly, several aspects of the transcriptional signature of *E. faecalis* grown in blood were similar to that of *E. faecalis* grown in urine. An activation of many of the same genes implicated in stress responses was observed, with an overrepresentation of genes involved in oxidative stress adaptation (*e.g. ohr*, *npr*, *sodA*, *ahpFC* and *nox*). Moreover, many of the observed changes in gene expression could also be related to an extensive remodeling of the cell envelope. The changes detected among genes involved in FASII were also particularly pronounced. Other differentially transcribed operons with membrane-associated functions included *lrgAB* (EF3194-93), *cps* (EF2492-84), *epa* (EF2200-2189 and EF2184-77), and *dltABCD* (EF2749-46). D-alanylation of cell wall teichoic acids and lipoteichoic acids by the Dlt complex leads to a reduced negative charge of the bacterial surface. In this case, the reduced content of D-alanine esters in the teichoic acid may have resulted in an increased net negative charge on the bacterial cell surface, which in turn may have affected several bacterial properties, such as susceptibility to cationic antimicrobial peptides and biofilm formation (Theilacker, et al., 2006). The microarray data also showed that genes related to iron acquisition represented one of the major changes related to *E. faecalis*'s adaptation to blood, despite the fact that iron is not essential for the growth of most lactic acid bacteria. A similar trend was also observed during growth in urine, but the transcriptional changes provoked by blood were more pronounced. The induction of the genes responsible for citrate metabolism was observed in both the blood and urine experiments. In addition, genes involved in the breakdown of other CCR regulated substrates also displayed enhanced transcription in blood, including arginine (Barcelona-Andrés, Marina, & Rubio, 2002) and glycerol (Opsata, Nes, & Holo, 2010), along with possibly other C3-glycerides present in blood. As a result, the glucose concentration in body fluids such as blood and urine appears to be below the threshold for release of CCR. As for the established enterococcal virulence factors, QPCR analysis showed that *fsrB* and *gelE* were down-regulated in response to growth in blood, the opposite of what was observed in urine. Whereas cues present in

urine stimulate *fsr* expression, factors present in blood seemingly interfere with its expression. This is the opposite effect from growth in serum by the *E. faecalis* MMH594 strain (Shepard & Gilmore, 2002).

Ethanolamine—An Interesting Case of Post-Transcriptional Regulation

E. faecalis is able to utilize ethanolamine as a source of both carbon and nitrogen, as recently reviewed (Garsin, 2010). This nutrient is abundantly present in the intestinal tract from sloughed-off epithelial cells, resident microflora, and ingested, processed foods. The *E. faecalis* ethanolamine utilization (*eut*) genes are encoded in a complex locus of 18 genes and include structural, enzymatic, and regulatory components. Upregulation of *eut* genes is triggered by the presence of two environmental cues—ethanolamine and adenosylcobalamin (AdoCbl). Each of these input signals is perceived and relayed by two different regulatory systems, and synchrony between them is required for expression of the *eut* genes. Both regulatory systems, as described below, control gene expression at the post-transcription initiation level and target nascent RNA instead of DNA. One is a cis-acting RNA structure, while the other is a response regulator (Garsin, 2010).

EutV, an ANTAR-family member with RNA-binding antiterminator activity

Upregulation of the *E. faecalis eut* genes is partially controlled by a two-component system. This consists of a soluble histidine kinase, *eutW*, and its cognate response regulator, *eutV*. Experiments showed that this two-component system specifically senses ethanolamine. *In vitro*, purified *eutW* underwent autophosphorylation in the presence of ethanolamine and was also able to transfer the phosphate residue to *eutV* (Del Papa & Perego, 2008; Fox, et al., 2009). *In vivo*, *eut* gene expression was demonstrated to be dependent on ethanolamine, *eutV*, and *eutW* (Del Papa & Perego, 2008; Fox, et al., 2009; Baker & Perego, 2011; Ramesh, et al., 2012). *EutV* belongs to the AmiR and NasR transcriptional anti-terminator regulator (ANTAR) family of response regulators, and its output domain controls gene expression by RNA binding (Shu & Zhulin, 2002). ANTAR proteins interact with nascent transcripts to prevent formation of transcriptional terminators that precede certain genes, allowing RNA polymerase to transit into the coding regions. The *E. faecalis eut* locus contains four of these rho-independent terminators, and significant evidence supports that they are the targets for antitermination by the *eutV* ANTAR protein (Fox, et al., 2009; Baker & Perego, 2011; Ramesh, et al., 2012). As already mentioned, expression of the genes downstream of the terminators is dependent on ethanolamine and the two-component system. Deletion of the terminators preceding *eutP*, *eutS* and *eutG* resulted in constitutive expression (Baker & Perego, 2011; Ramesh, et al., 2012). Finally, *eutV* binds to the RNA in a region just upstream and overlapping these terminators (Ramesh, et al., 2012). These data support an antitermination function for *eutV* and validates a model in which the *eut* locus is partially regulated by a series of rho-independent transcriptional terminators interspersed throughout the operon. A major focus of recent studies has been elucidating the details of the antiterminator structure that *eutV* recognizes.

Analysis of the sequences of the 5' untranslated regions (UTR) of the *E. faecalis eut* genes revealed a hairpin structure with a short stem region and a hexanucleotide loop, with the first and fourth loop residues conserved among the *E. faecalis eut* genes (Ramesh, et al., 2012). A similar hairpin structure have been reported in the substrate of a previously characterized ANTAR protein, NasR from *Klebsiella oxytoca* (Chai & Stewart, 1999), and the loop sequences found to be conserved in the *E. faecalis eut* genes were shown to be important for the binding of NasR (Ramesh, et al., 2012; Chai & Stewart, 1999). A second, similar stem-loop structure was identified just downstream of the first hairpin, which also had a hexamer loop with similar conservation of loop residues. This second hairpin overlapped the 5' end of the intrinsic terminator that precedes the ORF, such that formation of the hairpin and the terminator became mutually exclusive. This second stem-loop also contains a motif, called the ANTAR recognition sequence, that was predicted to be the site of recognition by *eutV* based on sequence conservation among *eut* genes present in Firmicutes (Chai & Stewart, 1999). This dual-hairpin motif and its conserved loop residues can be identified in the leaders of the *eut* genes from *Clostridium* species and

Listeria monocytogenes, as well as in the substrates of the previously characterized ANTAR proteins, NasR and AmiR. This led to the hypothesis that upon activation, the *eutV* ANTAR protein bound the *eut* RNA, specifically interacting with the two terminal loops of the dual-hairpin motif in the nascent transcripts to stabilize an antiterminator structure and exclude terminator formation (Ramesh, et al., 2012; Aymerich & Steinmetz, 1992; Babitzke & Gollnick, 2001). This dual-hairpin motif is unique, as compared to the antiterminators employed by other bacterial regulators such as the Sac/Bgl and TRAP proteins (Aymerich & Steinmetz, 1992; Babitzke & Gollnick, 2001). In most previously characterized systems, the antiterminator is a single alternative structure that overlaps with the terminator in a manner such that only a single hairpin can exist.

Using a *lacZ*-based reporter assay, it was demonstrated that both stem-loops and the four conserved loop residues are critical for successful antitermination *in vivo* by *eutV* in the presence of ethanolamine and AdoCbl (Ramesh, et al., 2012). This was also supported by *in vitro* EMSA studies. *In vivo* data also showed that the terminal closing base pairs of the first hairpin may result in sequence-specific interactions with *eutV*. In addition, an optimal distance between the two stem-loops was required, and such an alteration resulted in reduced efficiency of antitermination. Hence, the current model for the mechanism of antitermination by the *eutV* ANTAR protein is formation of a protein-RNA complex involving the dual hairpin structure in the *eut* RNA, which prevents the formation of the terminator, resulting in expression of the downstream genes (Ramesh, et al., 2012).

The dual nature of the *eut* RNA substrate hinted at the possibility of interaction with a dimer of *eutV* (123). SEC-MALLS (Size Exclusion Chromatography – Multi-Angle Laser Light Scattering) analysis of full-length *eutV* indicated that it exists as a monomer in the unphosphorylated state. Phosphoryl transfer from the cognate sensor kinase, *eutW*, induced dimerization of *eutV*. DRaCALA (Differential radial capillary action of ligand assay) assays in turn showed that phospho-*eutV* bound the RNA substrate with significantly higher affinity, as compared to the unphosphorylated form of the protein. Interestingly, *eutV* that lacks the N-terminal domain is capable of dimerizing and binding the RNA substrate with higher affinity, as compared to unphosphorylated full-length protein, suggesting that the unphosphorylated receiver domain actively inhibits dimerization and therefore RNA binding. Overall, signal-induced dimerization of the *eutV* ANTAR protein seems to be essential for recognition of a symmetric, dual-hairpin RNA ligand, which is conceptually similar to the molecular mechanism exhibited by many DNA-binding response regulator proteins that dimerize and recognize paired sequences in the DNA. The *E. faecalis eutV*/*eutW* TCS provides, for the first time, a mechanistic model of antitermination employed by ANTAR proteins, validated by *in vitro* and *in vivo* techniques (Ramesh, et al., 2012).

An AdoCbl-Binding Riboswitch

The first step in the degradation of ethanolamine involves the EutBC ammonia lyase that converts ethanolamine into ammonia and acetaldehyde. AdoCbl is an essential co-factor of this enzyme (Roof & Roth, 1988; Roof & Roth, 1989; Scarlett & Turner, 1976). As a result, it is not surprising that AdoCbl also acts as a biochemical cue for the upregulation of the eut genes via an AdoCbl-binding riboswitch found in an intergenic region near the beginning of the locus (Figure 4) (Fox, et al., 2009; Baker & Perego, 2011). Riboswitches are non-coding, cis-acting RNA that serve as ligand-responsive genetic control elements that can modulate the expression of genes in response to changing concentrations of metabolites. They are usually present in the 5' untranslated leader sequences that precede the coding region of genes. Riboswitches typically have two major domains: an aptamer region that binds the ligand with high specificity, and an expression platform that undergoes structural changes upon ligand-binding to control the expression of its target genes (Winkler & Breaker, 2005).

The E. faecalis eut locus has an AboCbl-binding riboswitch in the 5'UTR of the eutG gene (Fox, et al., 2009). A similar riboswitch has also been located in the eut locus of Listeria monocytogenes, which suggests conservation of this element. In-line probing studies have demonstrated that the eut riboswitch specifically binds AdoCbl with

an affinity that is comparable to that of other AdoCbl riboswitches (Fox, et al., 2009). The affinity for cyanocobalamin is significantly poorer, demonstrating the specificity for AdoCbl. Gene control by riboswitches in prokaryotes involves control of either transcriptional termination or translation initiation. In Gram-positive bacteria, the binding of the ligand to its riboswitch typically results in stabilization of an intrinsic terminator, thereby shutting off gene expression (Winkler & Breaker, 2005). However, the eut riboswitch is unique, in that binding of AdoCbl to the aptamer region causes structural changes that prevent the formation of an intrinsic terminator that is located downstream of the riboswitch and upstream of the eutG gene (Fox, et al., 2009). In vitro transcription analysis showed increase in the runoff transcript concentration, with a greater amount of AdoCbl present in the reaction. Hence, the eut riboswitch appears to be a positively acting element that induces gene expression by causing antitermination at the terminator in front of eutG (Fox, et al., 2009). However, this terminator also has the conserved dual-stem loop motif suggestive of regulation by eutV, and it is not clear how both a riboswitch and an antiterminator protein influence formation of this single terminator.

A recent study by Baker and Perego suggested a different mechanism for the way in which the eut riboswitch acts (Baker & Perego, 2011). They identified a promoter region upstream of the eut riboswitch and a second promoter downstream of the riboswitch, but preceding the eutG ORF. Based on their data, they suggest that binding of AdoCbl to the riboswitch causes termination of the transcript that initiates from the first promoter within the riboswitch, rather than causing antitermination at the intrinsic terminator preceding eutG. They speculate that in the absence of AdoCbl, transcripts initiated from the first promoter continue through the second promoter. These read-through transcripts somehow prevent successful initiation at the second promoter. As a result, they suggest that binding of AdoCbl to the eut riboswitch facilitates the initiation of the second, more prevalent transcript by cutting off the interfering first transcript, and in this manner, the action of the AdoCbl riboswitch promotes downstream gene transcription (Baker & Perego, 2011).

Further analysis will be required to resolve the mechanism of action of the AdoCbl riboswitch, and whether or not interaction with AdoCbl promotes antitermination at the T2 terminator as Fox et al. propose; causes termination at a point earlier in the transcript as Baker et al. suggest; or functions differently than either model. Overall, the unique combination of features within the eut operon of E. faecalis makes it a particularly attractive model to investigate the mechanisms of post-transcription initiation regulation.

Conclusion

In discovering the ways in which *E. faecalis* responds to stimuli through transcriptional and post-transcriptional regulation, we begin to build an understanding of the complex mechanisms responsible for the success of the enterococci in adapting to a wide range of host environments. Response of *E. faecalis* to numerous effector signals, often relevant to the human host, have been explored, including nutrient availability, quorum signals, antibiotics, metal ions, bile levels, urine, and blood. Understanding the global transcriptional profile under these conditions provides insight into the dual personality of *E. faecalis* as both a commensal and pathogen.

Looking forward, a rapidly increased understanding of transcriptional regulation will inevitably be linked to next-generation sequencing technology, which may provide a new and improved method for mapping and quantification of microbial transcriptomes. As compared to the microarray-based technology, the sequence-based transcriptomics holds several advantages: (i) The transcriptome of an organism can be studied without any access to its genome sequence; (ii) the technology is not dependent on DNA sequenced probes; (iii) the dynamic range of the sequence technology is better than hybridization based technology; (iv) it is more sensitive and (v) the high throughput expression data are obtained by single nucleotide resolution. Moreover, next-generation sequencing also supplies information on regulatory RNA species as opposed to microarrays, thereby adding a new dimension to our understanding of microbial regulatory networks. Current efforts aimed at reducing the cost of sequencing by several orders of magnitude will further promote sequencing-based technologies, and will likely provide exponential growth in discoveries surrounding transcript regulation.

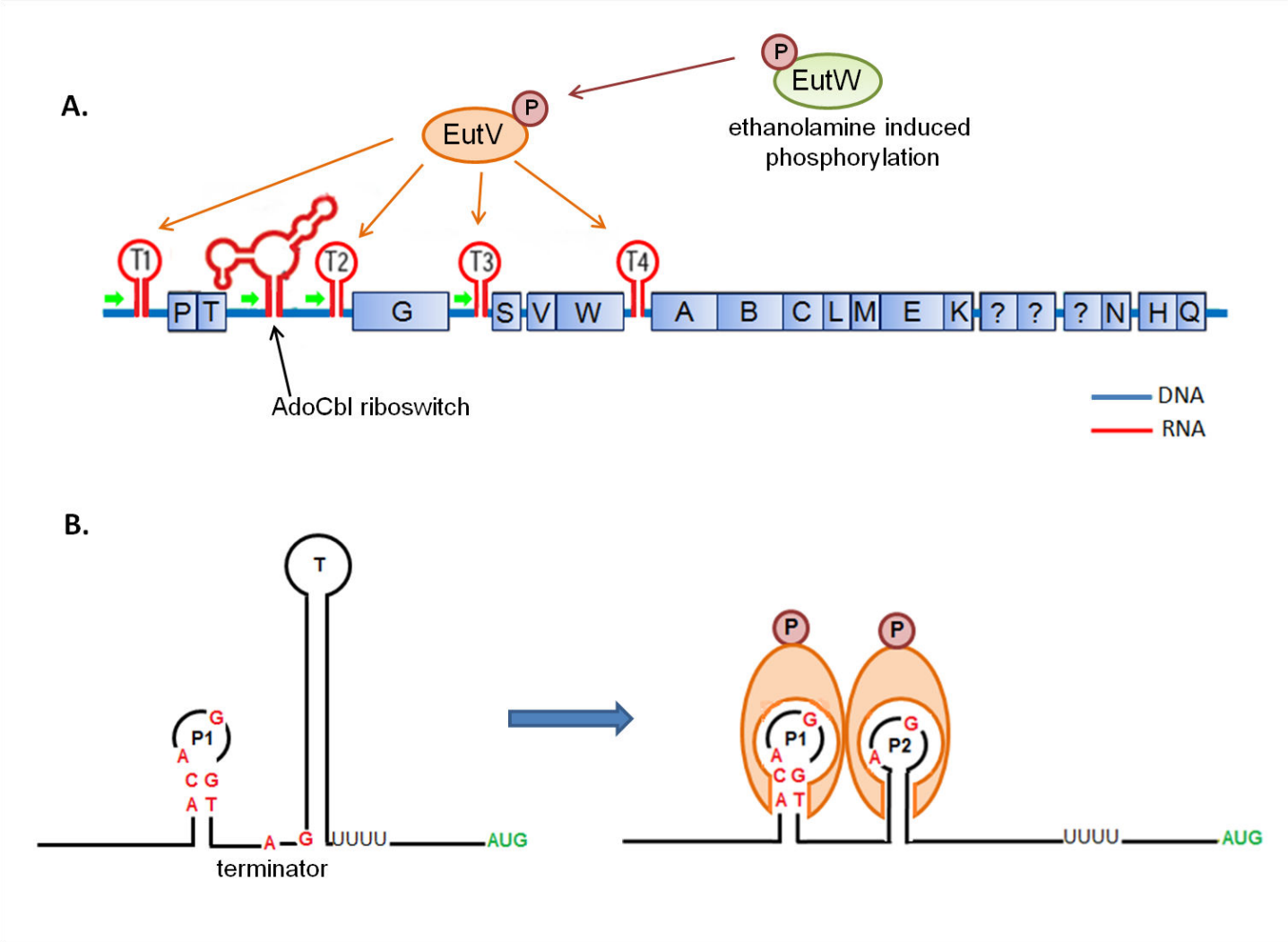

Figure 4. Organization and regulation of the *E. faecalis eut* genes. A) Organization of genes and regulatory elements at the *eut locus* in *E. faecalis*. Promoters indicated by green arrows are present upstream of three genes. Perception of ethanolamine causes autophosphorylation of the *eutW* histine kinase and phosphotransfer to *eutV*. Terminators (T) preceding *eutP*, *G*, *S* and *A* are targets of regulation by antitermination by the activated *eutV* response regulator. The *eutVW* two-component system is part of the third gene cluster. The AdoCbl-binding riboswitch in the 5'UTR of *eutG* binds AdoCbl to coordinate antitermination at T2 in synchrony with the *eutVW* two-component system. B) Model of antitermination mechanism of the *eutV* ANTAR regulator. Phosphorylated *eutV* binds and stabilizes a dual-hairpin motif in the nascent RNA transcript, thereby preventing formation of the intrinsic terminator and allowing for the expression of downstream genes.

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Enterococcal Cell Wall Components and Structures

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Enterococcal Cell Walls

Since the last review of this subject in 2002 (Coyette & Hancock, 2002), we have observed a dramatic expansion in the enterococcal literature related to cell wall components and structures, as well as their underlying genetics. This is largely due to the public availability of genomic sequence information for *E. faecalis* (Bourgogne, et al., 2008; Paulsen, et al., 2003), and more recently for *E. faecium* (Chen, et al., 2012; Lam, et al., 2012; Qin, et al., 2012). Much of this new knowledge arose from pathogenesis studies, as well as studies focused on antibiotic resistance to cell wall active agents. We refer the reader to Pathogenesis and models of enterococcal infection and Enterococcal infection, respectively, for more detailed coverage of the ways in which these cell wall components contribute to these processes. The focus of the present chapter will highlight new information gained over the last decade on the subject of enterococcal cell walls, and will provide particular focus on the ancillary proteins and other cell wall polymers that build on the framework of the cell envelope.

For nearly half a century, biochemical studies have focused on the cell wall of the genus *Enterococcus* (Salton, 1964). A survey of the enterococcal cell envelope (consisting of the cell membrane, as well as cell wall components) allows us not only to examine those pathways that are shared among many Gram-positive bacteria, but also to highlight the differences that make enterococci unique among the Firmicutes (Figure 1). We begin with coverage of peptidoglycan synthesis and then examine the associated proteins and carbohydrate-based polymers of the cell wall and membrane. We also include a section on the emerging importance of lipoproteins and the known processing enzymes that serve to anchor these important proteins to the cell membrane. The cell wall of Gram-positive bacteria is primarily composed of three major constituents: a peptidoglycan backbone, anionic polymers (teichoic acids and cell wall polysaccharides), and wall-associated and wall-anchored proteins (Bhavsar & Brown, 2006). The peptidoglycan backbone and anionic polymers comprise nearly 90% of the total cell wall weight, with the protein content comprising less than 10% of the cell wall weight. For a more detailed analysis of the enterococcal cell wall structure and assembly, as well as related Gram-positive bacteria, we refer the reader to several reviews on this topic (Coyette & Hancock, 2002; Archibald, Hancock, & Harwood, 1993; Huycke & Hancock, 2011).

Peptidoglycan

Structure

The major constituent of the enterococcal cell wall is the peptidoglycan (PG) (Coyette & Hancock, 2002). Peptidoglycan consists of the repeating disaccharide *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine (MurNAc-GlcNAc) (Navarre & Schneewind, 1999). The strands of these repeating sugars, which generally range in size from 5-30 subunits (Archibald, Hancock, & Harwood, 1993), are cross-linked together by the presence of stem peptides that are attached to MurNAc (NAM) residues as part of the assembly process. For decades, researchers have attempted to determine the solution structure of peptidoglycan, but the complexity and absence of pure and discrete structures made this an elusive endeavor. Work by Mobashery and colleagues (Meroueh, et al., 2006) has shed new light on the overall structure of the cell wall peptidoglycan, as these investigators synthesized a segment of pure peptidoglycan that contained a tetrasaccharide cell wall segment with the typical

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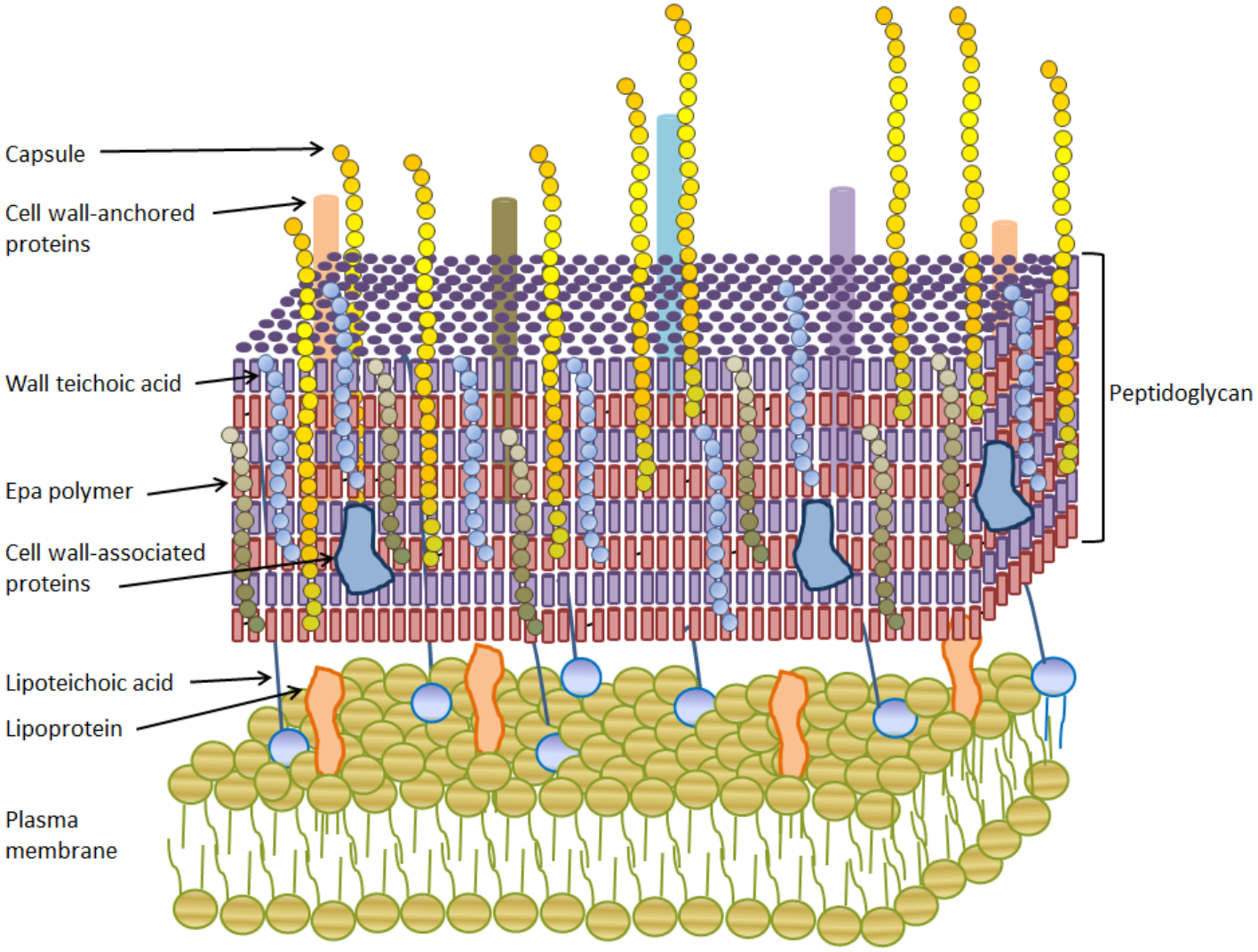

Figure 1. Model of the enterococcal cell wall. The peptidoglycan layer is depicted above the lipid bilayer with membrane bound lipoproteins and lipoteichoic acid. Bound to the muramyl residues of the peptidoglycan are wall teichoic acids, the rhamnopolymer whose synthesis is tied to the *epa* locus, as well as surface-anchored proteins and capsule.

Gram-positive stem peptide anchored to the NAM residues. In determining this structure by NMR, these authors discovered that the cell wall peptidoglycan takes on an ordered, right-handed helix comprised of three NAG-NAM pairs per turn of the helix, which orients the stem peptides with three-fold symmetry around the axis. This symmetry allows a single peptidoglycan strand to be cross-linked to three neighboring strands, and depending on the extent of cross-linking, also allows for various pore sizes to be present within the peptidoglycan lattice-work. A so-called honeycomb pattern would generate pores of ~70 Å when fully cross-linked. This predicted pattern is in excellent agreement with atomic-force microscopic images of the *Staphylococcus aureus* cell wall, in which pores ranged in size from 50 to 500 Å (Touhami, Jericho, & Beveridge, 2004). This model would also build peptidoglycan out away from and orthogonal to the cell membrane, rather than parallel to the membrane, as has been the accepted dogma in "textbook" descriptions of these processes.

The short stem peptides are connected to NAM residues through amide linkages between the terminal amino group of the stem peptide L-alanine and the carboxyl group of the D-lactyl moiety of each MurNAc, with the stem peptide comprised of alternating L- and D- amino acids. Like many related Gram-positives, the enterococcal stem peptide generally consists of L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. These stem peptides cross-link adjacent strands with an interpeptide bridge from the ε-amino group of the L-Lys residue in position 3 to the

carboxyl group of D-Ala in position 4 of an adjacent strand. This covalent modification results in the removal of the terminal D-Ala residue at position 5. The overall differences in the peptidoglycan structure of Gram-positive organisms stems from the variation in the amino acid sequence that forms the interpeptide bridge, which is commonly referred to as the crossbridge. For most species in the genus *Enterococcus*, this crossbridge is comprised of a single D-Asp residue (Schleifer & Kilpper-Bälz, 1987). *E. faecalis* appears to be an exception to this theme, as it possesses a cross-bridge of 2-3 L-Ala residues (Schleifer & Kandler, 1972).

Biosynthesis

The synthesis of cell walls by Gram-positives is generally divided into discrete stages that are principally based upon the cellular compartmentalization; namely, stage 1= cytoplasm, stage 2 = membrane, and stage 3 = cell wall (Navarre & Schneewind, 1999). Figure 2 depicts the various stages of cell wall synthesis.

Stage I

The initial steps of cell wall biosynthesis involve the conversion of the UDP-derived N-acetyl glucosamine [UDP-NAG] to UDP-NAM, which is catalyzed by MurA and MurB (van Heijenoort, 1998), along with phosphoenol pyruvate. In the MurA-catalyzed reaction, UDP-NAG receives enolpyruvate from PEP, and this intermediate is subsequently reduced by MurB to a lactoyl moiety on UDP-NAM (Gunetileke & Anwar, 1968). Of note, many Gram-positive bacteria possess two independent MurA homologues, including *E. faecalis* (Paulsen, et al., 2003) and *E. faecium* (Qin, et al., 2012). Vesic et al. (Vesić & Kristich, 2012) recently showed that MurAA (and not MurAB) was responsible for the intrinsic cephalosporin resistance in *E. faecalis*, but the reason why two MurA homologues exist remains a mystery. Following the synthesis of UDP-NAM, the stepwise addition of the first three amino acids in the stem peptide is accomplished through the concerted action of MurC, MurD, and MurE synthetases to produce UDP-NAM-L-Ala-D-isoGlu-L-Lys. The final two amino acids (D-Ala-D-Ala) of the peptide stem are added by the MurF transferase to produce UDP-MurNac-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala (al-Bar, O'Connor, Giles, & Akhtar, 1992; Neuhaus F. C., 1962; Neuhaus & Struve, 1965). This molecule is commonly referred to as Park's nucleotide, and is the last step of Stage I cell wall synthesis (Park, 1952).

Stage II

Movement of the soluble Park's nucleotide to the membrane requires the lipid carrier undecaprenol, and the transfer of the UDP-NAM plus stem peptide from UDP to the C55-isoprenoid carrier by the catalytic action of MraY (Bouhss, Mengin-Lecreulx, Le Beller, & van Heijenoort, 1999). This exchange reaction occurs at the inner or cytoplasmic face of the cell membrane to produce lipid I (C55-PP-NAM-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala) (Anderson & Strominger, 1965). MurG then catalyzes the addition of soluble UDP-NAG to Lipid I to generate lipid II [C55-PP-NAM(-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala)-β-1-4-NAG] (Ha, Walker, Shi, & Walker, 2000).

In *E. faecalis*, the addition of the cross-bridge peptides to Lipid II also occurs at the cytoplasmic membrane, and is initiated by the transfer of L-Ala from a charged tRNA, to the epsilon-amino group of lysine in the stem peptide by MurM (Lloyd, et al., 2008). MurN then catalyzes the addition of L-Ala to the Lipid II-L-Ala precursor in an analogous fashion to that observed in *Streptococcus pneumoniae* (De Pascale, et al., 2008). As the crossbridge in other enterococcal species, including *E. faecium*, possesses D-isoAsp (Lamont, Staudenbauer, & Strominger, 1972; Staudenbauer & Strominger, 1972; Staudenbauer, Willoughby, & Strominger, 1972), these species depend on a cytoplasmic D-Asp racemase, with the incorporation of D-Asp to lysine in the stem peptide by D-aspartyl transferase activity (Bellais, et al., 2006). A two-gene cluster of *E. faecium* encodes aspartate racemase (Rac_{fm}) and ligase (Asl_{fm}) for the incorporation of D-Asp into the side chain of the peptidoglycan precursor. The conversion to the isoAsp form must occur after the Asl_{fm} catalyzed addition, as isoAsp was not a substrate for the Asl_{fm} enzyme. It is yet to be determined whether this reaction occurs spontaneously or is enzymatically driven.

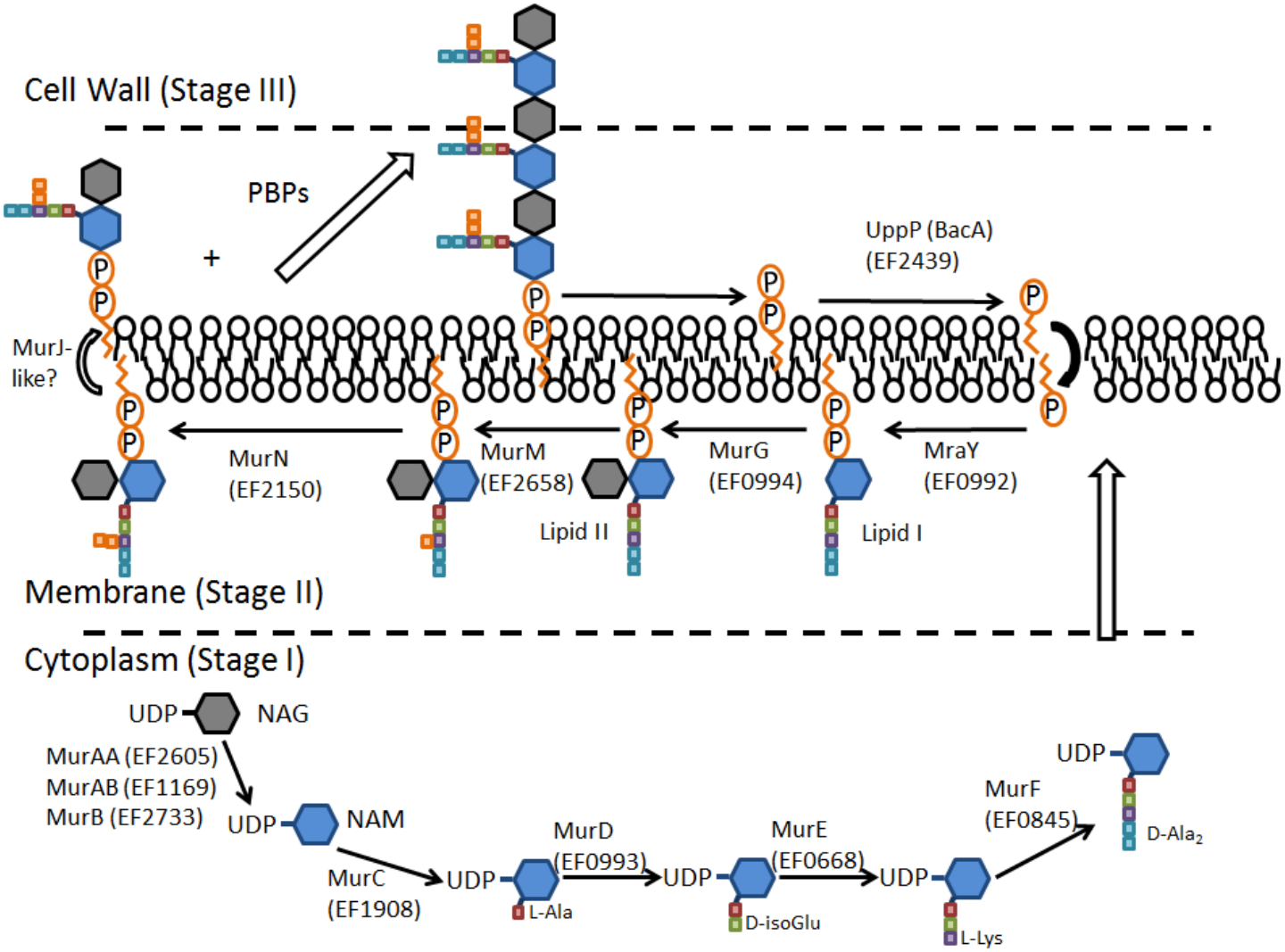

Figure 2. Biosynthesis of peptidoglycan. The common enzyme name for each step in the synthesis is provided along with the *E. faecalis* V583 gene identifier. Adapted from references (10) and (118).

After the addition of cross-bridge peptide(s) to lipid II, these derivatives must be transferred by an unknown mechanism to the outer face of the membrane, presumably through a lipid flipping reaction carried out by the *E. coli* MviN (MurJ) in Gram-negative bacteria, where it is now positioned as a substrate for assembly of PG (Ruiz, 2008). However, in *B. subtilis* and presumably other Gram-positive bacteria, including enterococci, four MurJ homologues exist and all appear to be non-essential, which is in contrast with the essential nature of MurJ in *E. coli* (Fay & Dworkin, 2009). There may be additional (redundant) proteins that flip Lipid II in Gram-positives, or that MurJ has an accessory role in Lipid II flipping that is essential in *E. coli*, but not in Gram-positives. Further elucidation of the lipid II flippase awaits additional biochemical tests.

Stage III

The final stage of assembly occurs on the outside face of the cell membrane and is catalyzed by penicillin binding proteins (PBPs) (Ghuysen, 1991). Following the removal of the peptidoglycan precursor from the lipid carrier through a transglycosylation reaction by PBPs onto the nascent peptidoglycan chain, the undecaprenyl pyrophosphate (UPP) is returned to the monophosphate form by the action of UppP (a phosphatase) (El Ghachi, Bouhss, Blanot, & Mengin-Lecreulx, 2004). Undecaprenyl monophosphate (UMP) traverses the membrane to once again serve as a lipid carrier for cell wall synthesis.

PBPs can be classified based on their ability to polymerize nascent glycan strands to the disaccharide precursor (transglycosylation) or the ability to cross-link wall peptides between adjoining glycan strands (transpeptidation) (Archibald, Hancock, & Harwood, 1993; Ghuysen, 1991; Zapun, Contreras-Martel, & Vernet, 2008; Ghuysen, 1968; Rogers, Perkins, & Ward, 1980; Delcour, Ferain, Deghorain, Palumbo, & Hols, 1999). PG polymerization in all eubacteria is catalyzed by membrane-bound PBPs, and these enzymes are specific targets for β-lactam antibiotics (Ghuysen, 1991; Rogers, Perkins, & Ward, 1980; Ghuysen & Dive, 1994). In enterococci, they can be used for identification, as each species possesses a specific pattern of at least five PBPs (Williamson, Gutmann, Horaud, Delbos, & Acar, 1986).

PBPs can be further divided into two groups: the multimodular, high molecular mass–PBPs (> 60 kDa, HMM-PBPs), and the monofunctional, low molecular mass–PBPs (LMM-PBPs) (Ghuysen & Dive, 1994; Goffin & Ghuysen, 1998; Ghuysen, et al., 1996). The HMM-PBPs can be further categorized based on their functional activity. Class A HMM-PBPs promote both polymerization of the glycan chain and cross-linking of wall peptides (Nakagawa, Tamaki, Tomioka, & Matsuhashi, 1984; Rice, et al., 2009), whereas Class B HMM-PBPs primarily possess transpeptidase activity and include the low affinity PBP5 in *E. faecium* and *E. faecalis* (36, 44), which are responsible for increased resistance to ampicillin (see Enterococcal infection). It should be noted that the transglycosylation reaction can also be carried out by monofunctional glycosyltransferases. These enzymes are not considered to be PBPs, but may share sequence similarity with the glycosyltransferase domain of class A PBPs (Wang, Peery, Johnson, Alborn, Yeh, & Skatrud, 2001), or, in some cases, may appear to be a novel class of glycosyltransferases (Rice, et al., 2009; Arbeloa, et al., 2004).

New glycan chains are cross-linked to existing PG in the wall by transpeptidation reactions. Cross-linking also depends on the availability of D-Ala-D-Ala terminated stem peptides. As not all stem peptides are cross-linked to neighboring PG strands, it is likely that the degree of cross-linking is regulated by the action of LMM-PBPs that exhibit carboxypeptidase activity (Waxman & Strominger, 1983). As was observed by the structural work of the cell wall by Mobashery and colleagues (Meroueh, et al., 2006), the extent to which peptidoglycan is fully cross-linked is predicted to confer different pore sizes to the lattice-work of the peptidoglycan. Carboxypeptidases are responsible for the removal of the terminal D-Ala residue from the stem pentapeptide, which prevents the resulting peptide from serving as a substrate in further transpeptidation reactions, and would therefore play a role in regulating the porosity of the cell wall. Enterococci possess four to eight HMM-PBPs and usually one LMM-PBP (Williamson, Gutmann, Horaud, Delbos, & Acar, 1986). *E. faecalis* has three class A and three class B PBPs as well as one LMM-PBP (Williamson, Gutmann, Horaud, Delbos, & Acar, 1986; Arbeloa, et al., 2004; Duez, Zorxi, Sapunaric, Amoroso, Thamm, & Coyette, 2001). *E. faecium* and *E. hirae* each have three class A and three class B PBPs, as well as one LMM-PBP (Williamson, Gutmann, Horaud, Delbos, & Acar, 1986; Rice, et al., 2009; Coyette, Ghuysen, & Fontana, 1980). There appears to be functional redundancy, in that not all PBPs appear to be essential for cell wall maintenance at any given time. Antibiotic exposure may select for variants that retain cell wall function while reducing affinity for the drug. For example, PBP3 is the major PBP required for proper cell division, but is inhibited by cefotaxime (Coyette, Somze, Briquet, Ghuysen, & Fontana, 1983). In the absence of PBP3, PBPP5, a low-affinity PBP protein, confers resistance to β-lactam antibiotics (el Kharroubi, Jacques, Piras, Van Beeumen, Coyette, & Ghuysen, 1991; Fontana, Cerini, Longoni, Grossato, & Canepari, 1983). Consistent with overlapping roles for the multiple PBPs in transglycosylation and transpeptidation reactions, the low-affinity PBPs are not essential for cell viability under laboratory conditions (Mainardi, Legrand, Arthur, Schoot, van Heijenoort, & Gutmann, 2000; Sapunaric, Franssen, Stefanic, Amoroso, Dardenne, & Coyette, 2003).

Cell Wall Associated Proteins

In addition to the polymerization of glycan strands and the extensive cross-linking of strands through transpeptidation reactions, cell wall synthesis and turnover is also regulated by autolytic enzymes, which are commonly referred to as muramidases. These enzymes behave in a fashion analogous to host-derived lysozymes,

in that they can target NAG-NAM residues for cleavage. Shockman and coworkers were the first to describe such enzymes in enterococci in studies dating back to the 1960s (Shockman & Martin, 1968). More recently, three muramidases (AtlA, AtlB and AtlC) have been described for *E. faecalis* (Eckert, Lecerf, Dubost, Arthur, & Mesnage, 2006; Mesnage, Chau, Dubost, & Arthur, 2008; Béliveau, Ptvin, Trudel, Asselin, & Bellemare, 1991), while two different muramidases, M1 and M2, have been characterized in *E. hirae* ATCC9790 (Shockman G. D., 1992; Shockman, Dolinger, & Daneo-Moore, 1988; Kariyama & Shockman, 1992). In *E. hirae*, muramidase-1 is produced as a 130-kDa latent proprotein that becomes proteolytically activated to an 87-kDa form. M1 appears to favor the turnover of *E. hirae*-derived cell walls, in contrast to the M2 muramidase, which is noted for enhanced activity on *Micrococcus luteus* cell walls (Shockman G. D., 1992; Shockman, Dolinger, & Daneo-Moore, 1988; Kariyama & Shockman, 1992).

E. faecalis AtlA (Atn) was first identified by Beliveau, by screening an *E. faecalis* genomic bank cloned in *E. coli* for cell wall lytic activity on *Micrococcus lysodeikticus* cell walls (Béliveau, Ptvin, Trudel, Asselin, & Bellemare, 1991). In addition to activity on micrococcal cell walls, the expressed gene product also exhibited activity on *E. faecalis* cell walls. Qin et al. generated an *atn* insertion mutant (Qin X. , Singh, Xu, Weinstock, & Murray, 1998), and observed that the mutant formed longer chains, relative to the wild-type parental strain. Eckert et al. (Eckert, Lecerf, Dubost, Arthur, & Mesnage, 2006) showed that the *E. faecalis* major autolysin Atn, which they renamed AtlA, is an N-acetylglucosaminidase. Similar to the proteolytic maturation of the *E. hirae* autolytic enzymes, AtlA also undergoes proteolysis to achieve its active form. Thomas et al. (Thomas, Hiromasa, Harms, Thurlow, Tomich, & Hancock, 2009) showed that the secreted gelatinase (GelE) and serine protease (SprE) are key contributors to the activation of AtlA. The activation of AtlA by GelE is thought to be the mechanism by which GelE contributes to eDNA-dependent biofilms (Thomas, Hiromasa, Harms, Thurlow, Tomich, & Hancock, 2009; Thomas, Thurlow, Boyle, & Hancock, 2008), as the deletion of *atlA* results in similar defects in biofilm architecture as that observed for a *gelE* mutant (Thomas, Hiromasa, Harms, Thurlow, Tomich, & Hancock, 2009; Guiton, et al., 2009). SprE can also cleave AtlA (Thomas, Hiromasa, Harms, Thurlow, Tomich, & Hancock, 2009), but the precise mechanism by which these two proteases compete to regulate AtlA activity and biofilm formation in an eDNA-dependent fashion is still unknown (see Enterococcal Biofilm Structure and Role in Colonization and Disease for additional details). The remaining autolysins of *E. faecalis*, namely AtlB and AtlC, are prophage-encoded enzymes and are not present in all *E. faecalis* strains (Bourgogne, et al., 2008; Paulsen, et al., 2003). The fact that these proteins are encoded by genes residing on known mobile elements likely explains the absence of correlation (Qin X. , Singh, Weinstock, & Murray, 2000) of the GelE-phenotype with cellular chain length, as was first described by Waters et al. (Waters, Antiporta, Murray, & Dunny, 2003).

Cell Envelope Dynamics

Enterococci, like other related Gram-positive organisms, decorate their PG and cell membrane with a variety of polysaccharides and proteins. Most of these are directly tethered to the cell wall PG through covalent linkages (polysaccharides, teichoic acids, and surface-anchored proteins), while lipoteichoic acid and lipoproteins are anchored to membrane lipids by covalent attachment. We will first discuss the lipid-anchored moieties lipoproteins and lipoteichoic acid.

Lipoproteins

Research on enterococcal lipoprotein biology is still at an early stage of development. The understanding that peptide pheromones used in conjugal plasmid mating systems are derived from lipoprotein signal sequences has also spurred interest in understanding their biological function (Antiporta & Dunny, 2002; Clewell, An, Flannagan, Antiporta, & Dunny, 2000). Rince and colleagues (Reffuveille, Leneveu, Chevalier, Auffray, & Rincé, 2011) recently undertook a bioinformatic analysis of the *E. faecalis* V583 genome database to identify predicted lipoproteins, based on a predicted lipobox with a conserved cysteine at the amino terminus of the protein with the consensus sequence L_{-3}-[A/S/T]$_{-2}$-[G/A]$_{-1}$-C_{+1} (75). Their analysis predicted 90 lipoprotein-encoding genes

in V583. Furthermore, a surface proteome study by Bøhle et al. (Bøhle, et al., 2011) identified lipoproteins as a highly abundant group of surface-exposed proteins on the surface of V583. Lipoproteins in Gram-positive bacteria are known to capture and facilitate transport inside the cell of small molecules, such as heme-bound iron and manganese. The genes that encode lipoproteins are often genetically arranged with those that encode ABC-transporters (Hutchings, Palmer, Harrington, & Sutcliffe, 2009).

Lipoprotein anchoring to the outer leaflet of the cell membrane is achieved through the covalent addition of diacylglyceride to the conserved cysteine residue in the lipoprotein signal peptide, and follows protein secretion across the cytoplasmic membrane by the Sec or Tat pathway. After translocation, lipoprotein biogenesis in Gram-positive bacteria is thought to require two steps. The first step is catalyzed by Lgt (EF1748), a prolipoprotein diacylglyceryl transferase which transfers a diacylglyceryl moiety from a glycerophospholipid onto the thiol group of the conserved cysteine. In the second step, the signal peptide is cleaved by the type II signal peptidase, Lsp (EF1723) at the conserved cysteine residue in the lipobox, which leaves the lipid-modified cysteine at the N-terminus of the mature lipoprotein anchored to the membrane with the protein moiety and its carboxyl terminus exposed at the surface (Hutchings, Palmer, Harrington, & Sutcliffe, 2009) (Figure 3).

In Gram-negative bacteria like *E. coli*, an additional lipid-anchoring enzyme the apolipoprotein N-acyltransferase (Lnt) acylates the exposed amino group of the cleaved cysteine, which results in tri-acylated lipoproteins (Robichon, Vidal-Ingigliardi, & Pugsley, 2005). To date, a homolog of Lnt has not been identified in the low GC Gram-positives, suggesting that lipoproteins in these organisms are only di-acylated. However, recent biochemical studies by Kurokawa et al. (Kurokawa, et al., 2012) identified di- and tri-acylated lipoprotein forms in *S. aureus* and *E. faecalis*, suggesting that an Lnt-like activity exists in Gram-positives. However, the identification and characterization of such an enzyme has not yet been discovered.

In Gram-positive bacteria, lipoproteins are thought to function within a subcellular region defined by the plasma membrane and PG, and can be considered functional equivalents of periplasmic proteins of Gram-negative bacteria (Hutchings, Palmer, Harrington, & Sutcliffe, 2009). Evidence for a bacterial periplasm in Gram-positive bacteria appears to be gaining favor with recent advances in electron microscopy techniques (Matias & Beveridge, 2005; Matias & Beveridge, 2006; Merchante, Pooley, & Karamata, 1995). Lipoproteins would be positioned in this defined space to facilitate the capture of imported nutrients, such as iron, or to localize detoxifying enzymes, such as β-lactamase, in staphylococci and enterococci (Navarre, Daefler, & Schneewind, 1996).

What is apparent from studies in related Gram-positive bacteria is that the enzymes responsible for lipoprotein biogenesis are not essential to cell viability, but instead contribute to pathogenesis in disease models (Khandavilli, Homer, Yuste, Basavanna, Mitchell, & Brown, 2008). Reffuveille (Reffuveille, et al., 2012) recently constructed a deletion mutant of the *E. faecalis lgt* (*ef1748*), and this mutant displayed an attenuated phenotype in a *Galleria mellonella* infection model, which is consistent with an important role for lipoproteins in the pathogenesis of infection. The study of lipoprotein biology in the enterococci will continue to be an exciting area of investigation for years to come.

Lipoteichoic acid

Beginning in the early 1930s, Rebecca Lancefield developed a serologic typing scheme to identify and type the known streptococcal species that are important in human infection (Lancefield, 1933; Lancefield, 1940). According to Lancefield's typing scheme, enterococci were classified as group D streptococci, along with *Streptococcus bovis*, based on their distinct lipoteichoic acid structure (LTA) (Wicken, Elliott, & Baddiley, 1963; Elliott, 1962). More recently, Theilacker et al. solved the NMR structure of the *E. faecalis* 12030 LTA (Theilacker, et al., 2006). The structure consists of a glycerol phosphate teichoic acid polymer with kojibiose substitutions (disaccharide of 1, 2-linked glucose), with decorations of D-alanylation interspersed along the glycerol polymer backbone. LTA is anchored to the lipid membrane by the enzymes BgsA and BgsB (Theilacker, et al., 2009; Sava,

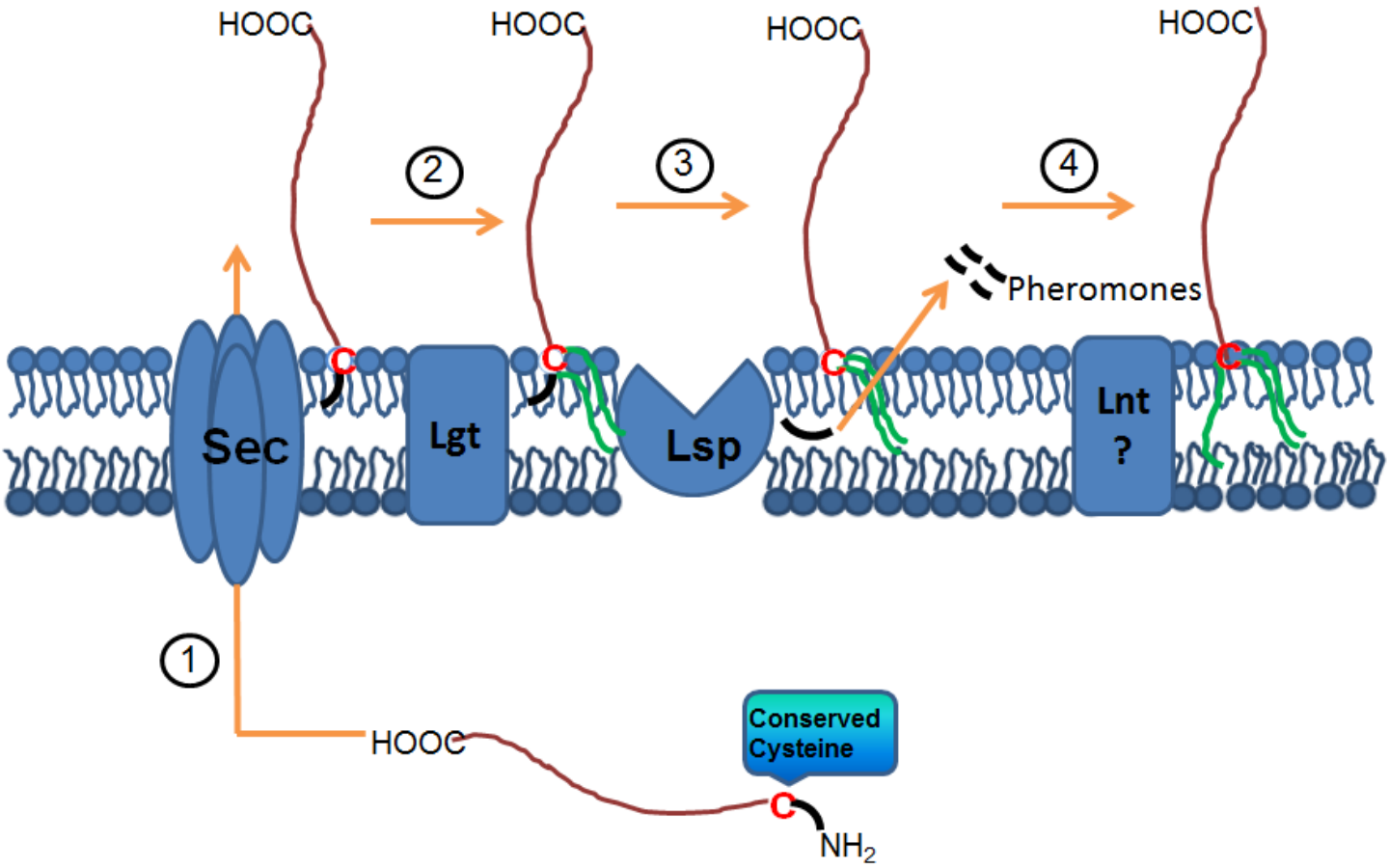

Figure 3. Schematic of lipoprotein synthesis in Gram-positive bacteria. 1) Lipobox domain containing proteins are exported through the sec-dependent pathway. 2) Once exported and by nature of the hydrophobic N-terminus, the protein transiently associates with the membrane where the conserved cysteine is di-acylated by Lgt. 3) The amino terminus is removed by Lsp, a signal peptidase II enzyme, cleavage at the acylated cysteine and 4) the free amino group from the cysteine is thought to be further acylated by an unknown enzyme (Lnt-like ?). After Lsp cleavage, the small amino terminal peptide is further processed to yield the peptide pheromones. Adapted from (Thomas, Hiromasa, Harms, Thurlow, Tomich, & Hancock, 2009)

et al., 2009). These catalyzed steps produce the LTA precursor diglucosyl-diacylglycerol or 1-kojibiosyl diglyceride (Toon, Brown, & Baddiley, 1972), to which the growing polyglycerol phosphate is anchored. The linkage of lipoteichoic acids to the cell membrane contrasts with the attachment of cell wall teichoic acids, which occur through N-acetyl muramic acid residues in the PG (Hancock & Poxton, 1988).

Work out of the Schneewind lab has identified LtaS as the key polymerizing enzyme in LTA biosynthesis (Gründling & Schneewind, 2007; Gründling & Schneewind, 2007; Lu, Wörmann, Zhang, Schneewind, Gründling, & Freemont, 2008). The rationale behind this identification centered on screening a *S. aureus* genomic library cloned in *E. coli* for production of LTA, since this polymer does not exist in Gram-negatives (Gründling & Schneewind, 2007). Furthermore, since LTA is essential in Gram-positives, the authors showed that the inducible expression of LtaS resulted in viability, whereas cells depleted of LtaS failed to grow (Gründling & Schneewind, 2007).

LTA modifications: Glycosylation and D-alanylation

Because the nature of the LTA glycosylation is unique to enterococci, the enzymes responsible for this modification have yet to be identified. This is in contrast to the known genetic conservation of functions responsible for D-alanylation of LTA in the low GC Gram-positives (Nakao, Imai, & Takano, 2000; Clemans, et

al., 1999; Debabov, Heaton, Zhang, Stewart, Lambalot, & Neuhaus, 1996; Neuhaus, Heaton, Debabov, & Zhang, 1996; Perego, Glaser, Minutello, Strauch, Leopold, & Fischer, 1995), including *E. faecalis* (Fabretti, et al., 2006). We refer the reader to a review by Neuhaus and Baddiley for a detailed overview of the D-alanylation system in Gram-positives (Neuhaus & Baddiley, 2003). Briefly, a four-gene operon designated *dltABCD* encodes the machinery to D-alanylate LTA. The first gene product is DltA (EF2749), a D-alanine:D-alanyl carrier ligase that ligates D-alanine through the hydrolysis of ATP to the product of *dltC* (*ef2747*), a D-alanine carrier protein (Dcp). Dcp is channeled through the membrane by the product of *dltB* (*ef2748*), and the *dltD* (*ef2746*) gene encodes a membrane protein involved in bringing Dlc and Dcp together. Once outside the cell membrane, Dcp transfers the carried D-alanyl moiety to the 2nd carbon of the glycerol backbone within the so-called "periplasmic space".

The role of D-alanylation of LTA is thought to play a major role in the maintenance of cationic homeostasis, particularly with magnesium, as well as modulating autolytic activities in the cell. D-alanylation of LTA has been shown to affect the rate of autolysis in *B. subtilis* (Wecke, Perego, & Fischer, 1996), acid sensitivity in *S. mutans* (Boyd, et al., 2000), and intrageneric coaggregations in *S. gordonii* DL1 (Challis) (Clemans, et al., 1999). In *E. faecalis*, the deletion of *dltA* resulted in increased sensitivity to cationic peptides (polymyxin B and nisin), as well as diminished biofilm production, with defects in adhesion to eukaryotic cells (Fabretti, et al., 2006).

Cryo-electron microscopy studies using labeled LTA in *B. subtilis* (Matias & Beveridge, 2008) suggest that LTA is a major constituent of the "periplasmic space" of Gram-positive cells and may in fact constitute the periplasmic boundary of the cell, giving further credence to its important role in cellular function. One item that this model does not explain is the way in which LTA can serve as a major surface antigen in serotypes A and B of *E. faecalis* (Theilacker, et al., 2011), and be agglutinated by LTA-specific antiserum (Thurlow, Thomas, Fleming, & Hancock, 2009) at the surface of the cell, but still retain sub-cellular properties consistent with a role in marking the periplasmic boundary. Additional study is needed to address this challenging hypothesis on LTA's role in the cell.

Cell-Wall Teichoic Acids

Cell wall teichoic acids from Gram-positive bacteria confer similar anionic surface charge to the cell wall as those imposed by lipoteichoic acid. These wall teichoic acid (WTA) polymers consist of either glycerolphosphate or ribitolphosphate repeating units, which are substituted by glycosylation and D-alanylation, as is the case for the LTA backbone. The chains of LTA and WTAs are generally synthesized by separate enzyme systems. LTA uses glycerol 1-phosphate (phosphatidyl glycerol) derived from lipid substrates and whose synthesis therefore is likely to occur on the outer layer of the cytoplasmic membrane. This is consistent with membrane topology predictions for membrane-bound LtaS (Lu, Wörmann, Zhang, Schneewind, Gründling, & Freemont, 2008). In contrast, WTA utilizes glycerol 3-phosphate or ribitol phosphate derived from CDP-glycerol or CDP-ribitol and nucleotide activated sugars for its biosynthesis and likely occurs in the cytoplasm, where it must be shuttled to wall locations through membrane transporters, where it is ultimately covalently linked to NAM residues on the PG (Bhavsar & Brown, 2006; Archibald, Hancock, & Harwood, 1993). NMR structures for purified wall teichoic acids have recently been elucidated for both *E. faecium* (Bychowska, et al., 2011) and *E. faecalis* (Theilacker, Holst, Lindner, Huebner, & Kaczyński, 2012). The *E. faecium* WTA is comprised of a repeating glycerol phosphate polymer with two molecules of N-acetyl galactosamine. In contrast, the *E. faecalis* WTA had a more complex structure containing glucose, galactose, N-acetyl galactosamine, N-acetyl glucosamine, and ribitol phosphate. The underlying genetics for these two structures is not known at the present time. The well-conserved WTA biosynthetic operons from *Bacillus* and *Staphylococcus* appear to only be partly present in enterococcal species; either that, or their functions are encoded in separate cell wall polymer genetic pathways (i.e. *epa* genes). As an example, the initial step in WTA synthesis for Gram-positive bacteria is catalyzed by TagO in *B. subtilis* (D'Elia, Millar, Beveridge, & Brown, 2006) and TarO in *S. aureus* (D'Elia, et al., 2006), and these genes show a similar sequence to that of *ef2198* (*epaA*). These collective gene products catalyze the addition

GlcNAc-1-phosphate to the lipid carrier to initiate polymer synthesis on the cytoplasmic face of the membrane. The second step in the synthesis is catalyzed by TagA or TarA (D'Elia, Henderson, Beveridge, Heinrichs, & Brown, 2009), and involves the addition of *N*-acetylmannosamine to the GlcNAc-1-phosphate residue on the lipid carrier. TagA and TarA were shown to be dispensal for growth, which highlights the synthesis of the lipid carrier-disaccharide as the first committed step in WTA synthesis. A four gene operon designated *tagBACD* in *E. faecalis* V583 encodes a TagA-like protein (EF1173). Of note, a European consortium recently constructed a library of insertion mutations in the V583 background, including *ef1173*, and they observed increased susceptibility to phagocytic killing with this mutant (Rigottier-Gois, et al., 2011). We recently discovered that this operon was also critical for capsule production (Iyer, VS and Hancock, LE unpublished data). Bioinformatics also revealed that this operon was only found in encapsulated (*cpsC-K*) genetic backgrounds (Palmer and Gilmore, personal communication). Taken together, these two pieces of evidence provide a rational basis for why deletion of this operon (*ef1172-1175*) would render the bacterium more susceptible to opsonophagocytosis, as it likely impacts capsule synthesis.

It was previously thought that these early *tag/tar* genes were indispensable for cell viability. Targeted deletion mutants revealed that, while the other teichoic acids genes were indispensable, viable mutants could be obtained for both TagO and TarO. This may be due to the dependence of both PG and WTA biosynthesis on the lipid-carrier bactoprenol. The initial step of WTA biosynthesis appears to be reversible, which allows for the efficient recycling of the lipid carrier. Blocks at later stages in WTA synthesis prevent efficient recycling of the bactoprenol, with cells no longer viable as they are unable to synthesize cell wall PG. This knowledge has led to the recent discovery of small molecule inhibitors targeting WTA biosynthesis in related Gram-positive bacteria (Swoboda, et al., 2009). While some WTA gene functions are conserved in enterococci, many appear to be absent or lack strong homologs to WTA machinery from *Bacillus* or *Staphylococcus*. The overall basis for WTA biosynthesis in enterococci is not clear at present, and further investigation into enterococcal WTA synthesis may reveal potential drug targets (Swoboda, et al., 2009) that may be useful in the treatment of this drug-resistant pathogen.

Enterococcal Polysaccharide Antigen (Epa)

The enterococcal polysaccharide antigen (or *epa*) gene locus was discovered by screening a library of *E. faecalis* genes cloned into *E. coli* for antigens using sera from patients with *E. faecalis* endocarditis; this locus was later defined as an 18-gene cluster extending from *epaA* to *epaR* (Teng, Singh, Bourgogne, Zeng, & Murray, 2009; Xu, Jiang, Murray, & Weinstock, 1997; Xu, Murray, & Weinstock, 1998) (Figure 4). See Pathogenesis and models of enterococcal infection for the role of *epa* in virulence and virulence-associated phenotypes. The *epa* cluster contains genes that are predicted to encode proteins involved in synthesis of nucleotide sugar precursors, formation and polymerization of repeating units, and their export to the cell surface. Analysis of the purified Epa polysaccharide from *E. faecalis* OG1RF showed that it is composed of glucose, rhamnose, N-acetyl glucosamine, N-acetyl galactosamine, and galactose (Teng, Singh, Bourgogne, Zeng, & Murray, 2009), and disruption of *epaA*, *epaB*, *epaE*, *epaM*, or *epaN* all resulted in a change in this Epa polysaccharide content (Teng, Singh, Bourgogne, Zeng, & Murray, 2009; Teng, Jacques-Palaz, Weinstock, & Murray, 2002). Further analysis of the polysaccharide produced by the *epaB* mutant revealed the replacement of rhamnose with mannose in the overall sugar composition, which suggests that the glycosyl transferases encoded by the *epaBCD* operon contribute to the incorporation of rhamnose into the Epa polysaccharide (Teng, Singh, Bourgogne, Zeng, & Murray, 2009). The same study also reported that all of these *epa* mutations resulted in a more round cell shape, as compared to the more oval-shaped cells of wild-type OG1RF, suggesting that alterations in the Epa polysaccharide affect the structure or integrity of the cell envelope. The structure of Epa or its location and attachment mechanism to the cell wall are not known, but it has been suggested that this polysaccharide may be buried within the cell envelope (Hancock & Gilmore, 2002). Western immunoblot analysis detected a similar Epa polysaccharide pattern from

OG1RF and 100+ diverse *E. faecalis* isolates (Teng, Singh, Bourgogne, Zeng, & Murray, 2009; Teng, Jacques-Palaz, Weinstock, & Murray, 2002), which suggests that the *epa* cluster is highly conserved.

A recent study of the first completed genome sequence of *E. faecium* (strain TX16, also referred to as DO) reported the presence of a homologous *epa* cluster in this species (Qin, et al., 2012) (Figure 4). Of the 18 *E. faecalis epa* genes, 15 were found to have a homolog, although with a slightly different gene order. Also, similarities between *epa* homologs of the two species varied widely, from 31% to 92% amino acid identity. The 15-gene *E. faecium epa* cluster was also found with high sequence and gene order conservation in the 21 available draft genomes of other *E. faecium* strains (Qin, et al., 2012). The genetic differences between the *epa* regions of *E. faecalis* and *E. faecium* suggest that their Epa polysaccharide compositions are also likely to differ to some degree. The same study also reported the presence of a cluster of additional genes, which are predicted to be involved in polysaccharide biosynthesis immediately downstream of the *E. faecium epa* cluster. An *epa* "extension" was found in all 22 *E. faecium* genomes, although with highly variable polysaccharide gene compositions. Notably, a second, highly variable cluster of predicted polysaccharide biosynthesis genes was found at another chromosomal location in 21 of the 22 genomes, and is consistent with the analysis described by Palmer et al. (Palmer, et al., 2012), wherein they describe a novel capsule locus in *E. faecium* strains. Although the production of these polysaccharides and their structures are yet to be characterized, the hyper variability of these two gene clusters raises the possibility of antigenically diverse surface polysaccharides that could contribute to the immune evasion of *E. faecium*.

Capsular Polysaccharide (Cps)

Serologic studies dating back to Maekawa (Maekawa & Habadera, 1996; Maekawa, Yoshioka, & Kumamoto, 1992) suggested that *E. faecalis* isolates could be grouped into a limited number of dominant serologic types with 4 serotypes [serotypes 1, 2, 4 and 7] accounting for 72% of all typeable isolates. The most predominant serotype among clinical isolates was type 2, and this serotype accounted for 30% of all typeable isolates. Taking a shotgun sequence approach to a clinical isolate of *E. faecalis* MMH594, Hancock and Gilmore identified two genetic loci responsible for polysaccharide biosynthesis (Hancock & Gilmore, 1997). The first pathway mapped to the aforementioned *epa* locus, whereas the second locus appeared to encode a variable polysaccharide, as not all *E. faecalis* strains possessed this genetic pathway. Through a series of mutagenesis studies, as well as Southern hybridization analysis, the genetic basis for the Maekawa serotype 2 antigen was mapped to a 9-gene operon consisting of *cpsC-K* (Hancock, Shepard, & Gilmore, 2003) (Figure 4). This antigen exhibited capsule properties, as mutants were more susceptible to phagocytic killing, and exhibited attenuated persistence in a mouse model of subcutaneous infection (Hancock & Gilmore, 2002). More recently, Thurlow et al. (Thurlow, Thomas, & Hancock, 2009) showed that all but two genes (*cpsF* and *cpsH*) in the operon were indispensable for the production of capsular material, as detected by cationic dye stains following native PAGE. It was also shown that a *cpsF* mutant failed to react with Maekawa type 2 antisera, suggesting that the presence of CpsF formed the serologic basis for differences between Maekawa type 2 and type 5 capsule antigens (Thurlow, Thomas, & Hancock, 2009). Both serologic types (type 2 = CPSC and type 5 = CPSD) (Hufnagel, Hancock, Koch, Theilacker, Gilmore, & Huebner, 2004) possess a *cps* gene cluster and differ by the presence (type 2) or absence (type 5) of *cpsF* (Hufnagel, Hancock, Koch, Theilacker, Gilmore, & Huebner, 2004). The capsule structure for the Maekawa serotype 2 was reported by Thielacker and colleagues (Theilacker, et al., 2011) to consist of a novel diheteroglycan with a repeating unit of →6)-β-Galf-(1→3)-β-D-Glcp-(1→ with O-acetylation in position 5 and lactic acid substitution at position 3 of the Galf residue (Theilacker, et al., 2011). The structure of this glycan was found to be consistent with that produced by the *cps* operon, because absorption experiments with an acapsular *cps* insertion mutant *(::cpsI)* failed to remove opsonic antibodies directed at the capsular material.

The gene products for the capsule operon begins with CpsC as a glycerolphospho-transferase, which likely explains the dependency of the *cps* operon for the 4 gene operon *ef1172-ef1175* in *E. faecalis* capsule production (Iyer et al., unpublished data), as CpsC may initiate capsule synthesis on an existing lipid-saccharide carrier

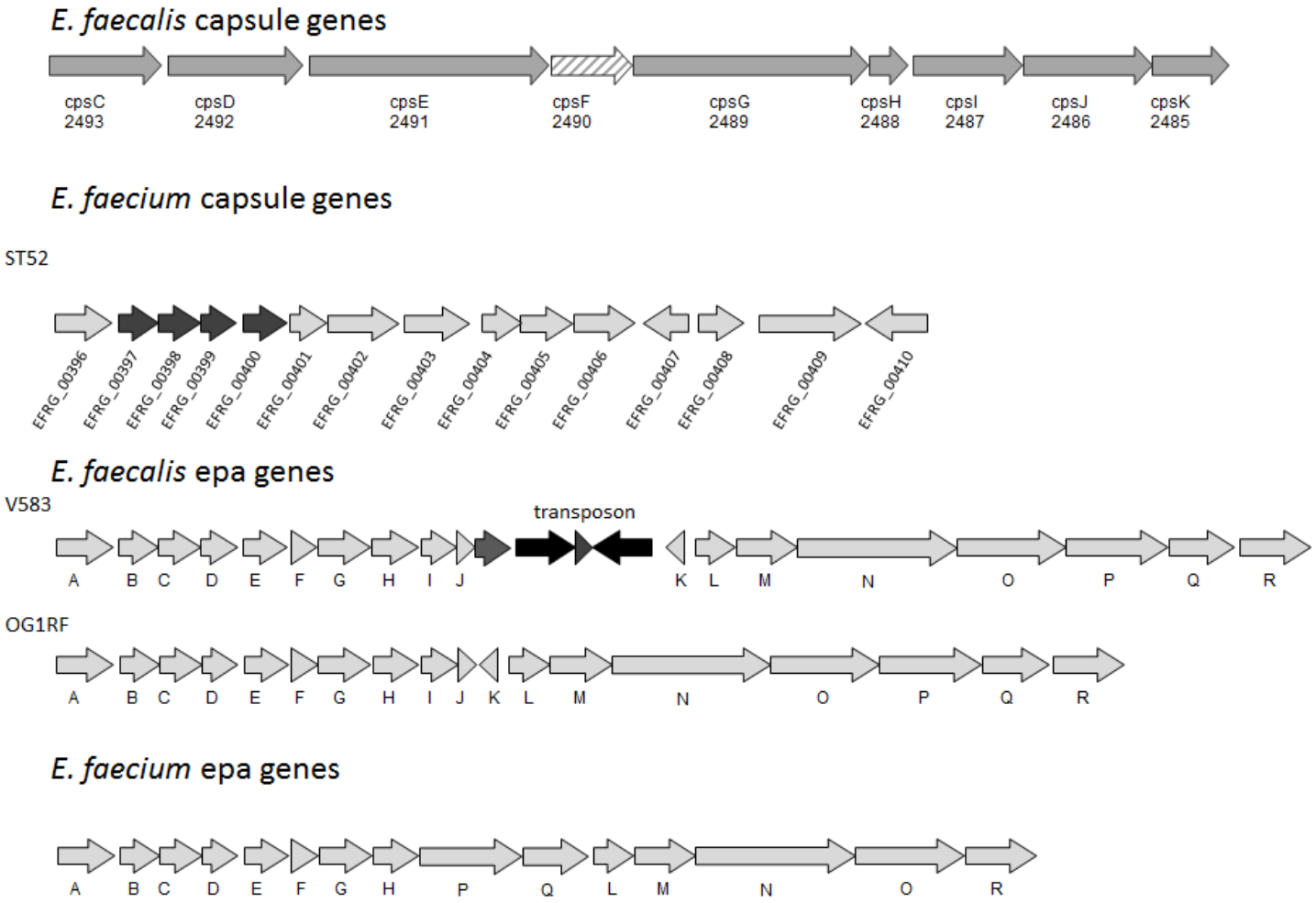

Figure 4. Polysaccharide loci in *Enterococcus faecalis* and *faecium*. The Cps capsule locus of V583 is shown, note the hashed marks for *cpsF*, as the presence of this gene confers serospecificity. A representative capsule locus for *E. faecium* (see reference 126 for further details). Comparison of the *epa locus* in *E. faecalis* V583 and OG1RF, as well as *E. faecium*.

provided by the enzymatic activity of the EF1172-75 proteins. CpsD is a predicted glycosyl transferase, as is the bifunctional glycosyl transferase CpsE. While CpsF possesses no identifiable homologs in the database, its presence in CPSC strains was predicted to behave as a glycosyl transferase (Thurlow, Thomas, & Hancock, 2009). However, the recent determination of the CPSC structure suggests that this enzyme might be responsible for O-acetylation on the Galf residue, but additional studies are needed to determine the exact enzymatic nature of CpsF. CpsG, a MurB homolog, would be predicted to contribute the lactic acid substitution on Galf, and CpsI converts galactose from the pyranose to furanose form consistent with the structural identification of Galf in the repeating backbone. As CpsH is not essential to capsule production, its present function remains elusive. The capsule transporters CpsJ and CpsK are responsible for capsule secretion.

A detailed genetic analysis by McBride et al. (McBride, Fischetti, Leblanc, Gilmore, & Gilmore, 2007) of over 100 strains of *E. faecalis* spanning 100 years, including the pre-antibiotic era, demonstrated that the most pathogenic enterococcal lineages possess the capsule operon along with multiple virulence and antibiotic resistance traits, which suggests that the capsule plays an important role in pathogenic interactions of *E. faecalis* with the host. These predictions were confirmed by Thurlow et al. (Thurlow, Thomas, Fleming, & Hancock, 2009) when they compared a strain with a markerless deletion in *cpsC* to the wild-type strain, and observed that the unencapsulated (Δ*cpsC*) strain was more readily phagocytosed, as compared to the isogenic parent strain or a strain with a deletion in *cpsF*. It is important to also remember that a deletion in *cpsF* retains the synthesis of the

capsule operon, but changes the capsule structure to a type 5 or CPSD serotype (Thurlow, Thomas, & Hancock, 2009).

The emergence of genomic data for eight strains of *E. faecium* through the Broad Institute allowed Palmer et al. (Palmer, et al., 2012) to identify a novel polysaccharide cluster that resembles capsule synthetic genes from related Gram-positives. This region encodes a predicted phosphoregulatory system that is conserved among all species in the enterococcal sequence collection except for *E. faecalis*. Proteins encoded by this conserved locus are similar to the CpsBCD proteins of *Streptococcus pneumoniae*, which are tyrosine kinase/dephosphorylase regulatory systems that regulate UDP-glucose dehydrogenase activity and capsule production (Kadioglu, Weiser, Paton, & Andrew, 2008). The predicted phosphoregulatory system is 5′ to a variable set of polysaccharide biosynthetic genes in *E. faecium*, and this genetic arrangement is reminiscent of *S. pneumoniae* capsule loci (Bentley, et al., 2006) (Figure 4). The degree to which this locus contributes to evasion of the host immune response awaits further study, but the presence of an anti-phagocytic capsule in *E. faecium* would be consistent with historic findings by Arduino et al. (Arduino, Jacques-Palaz, Murray, & Rakita, 1994), in which these authors showed that a sodium-periodate sensitive polysaccharide was responsible for the resistance of some *E. faecium* strains to neutrophil-mediated phagocytosis.

Cell-Wall Anchored Proteins

The cell envelope of Gram-positive bacteria contains a large number of proteins, which, after secretion through the cell membrane, become attached to the cell wall and are then displayed toward the external environment. Cell-wall anchored (CWA) proteins are an important class of these proteins, which contain C-terminal LPXTG or LPXTG-like motifs. These motifs are recognized by membrane-bound transpeptidase enzymes, called sortases, which are responsible for the covalent anchoring of CWA proteins to the cell wall and also for the polymerization of pilus subunit proteins (Mazmanian, Liu, Ton-That, & Schneewind, 1999; Ton-That & Schneewind, 2003; Schneewind, Fowler, & Faull, 1995). Crystal structures of several sortases from different Gram-positive bacteria have revealed mostly similar structures with a conserved TLXTC sequence in their active sites, in addition to other conserved residues that are necessary for sortase activity (Hendrickx, Budzik, Oh, & Schneewind, 2011). Analyses of bacterial genomes have revealed a plethora of sortases that represent almost all Gram-positive bacteria (Pallen, Lam, Antonio, & Dunbar, 2001). Based on their sequence similarities, substrate cleavage motifs, and the target molecules to which CWA protein substrates become linked, sortases have been divided into four different structural categories, which are designated as classes A-D (Hendrickx, Budzik, Oh, & Schneewind, 2011; Dramsi, Trieu-Cuot, & Bierne, 2005). Class A sortases are also referred to as housekeeping sortases; they covalently anchor the majority of the multitude of Gram-positive bacterial CWA proteins of this class to the cell wall peptidoglycan. Class B sortases have been found in a much more limited number of Gram-positive genera, such as *Staphylococcus, Bacillus*, and *Listeria*, but have not been reported in enterococci (Bierne, et al., 2004; Maresso, Chapa, & Schneewind, 2006; Zong, Mazmanian, Schneewind, & Narayana, 2004). These sortases catalyze the cell-wall anchoring of proteins involved in heme-iron scavenging from the outside environment, and recognize a distinctly different C-terminal motif, NP(Q/K)TN. Class C sortases are also known as pilin-specific sortases; they can recognize a variety of LPXTG-like motifs [(I/L)(P/A)XTG] and direct the cross-linking of pilus subunit proteins into a polymerized fibrous structure (see below). Unlike housekeeping sortases, genes coding for pilin sortases are typically located next to or within clusters of structural pilin genes, often on acquired elements. In some cases, including in streptococci and corynebacteria, several class C sortases have been found as part of the same pilus gene cluster (Ton-That & Schneewind, 2004; Telford, Barocchi, Margarit, Rappuoli, & Grandi, 2006). There are also reports of several pilus gene clusters in a single strain: for example, there are five class C sortases of *E. faecium* TX16, four of which are located in four separate pilus gene clusters and one is located elsewehere in the chromosome (Sillanpää, et al., 2008). The fourth group, which consists of class D sortases, is involved in recognition and anchoring a specific set of sorting motifs (LPNTA) during the formation of spores in *Bacillus* spp. (Marraffini & Schneewind, 2006; Marrafini & Schneewind, 2007).

Following the synthesis of a precursor form of a CWA protein in the cytoplasm, its transport across the cell membrane via the Sec machinery is guided by an N-terminal signal sequence, which is removed by a type I signal peptidase during translocation. The C-terminal end of a CWA protein contains a tripartite CWA domain, which consists of an LPXTG or LPXTG-like motif, a hydrophobic transmembrane domain, and a short stretch of positively charged residues (Navarre & Schneewind, 1999). As discussed above, the majority of these CWA proteins are covalently anchored to the cell wall by the class A housekeeping sortase. This transpeptidase recognizes and cleaves the LPXTG motif between the threonine and glycine residues, and forms an acyl-enzyme intermediate between the CWA protein threonine and a cysteine of the TLXTC motif in the sortase reactive site (Hendrickx, Budzik, Oh, & Schneewind, 2011; Marrrafini, Dedent, & Schneewind, 2006). A subsequent nucleophilic "attack" by an amino group of the peptidoglycan precursor lipid II then creates a covalent link between the CWA protein threonine and lipid II. As a last step, this CWA protein-lipid II precursor is incorporated as part of the peptidoglycan cell wall by penicillin-binding proteins. Although this finding has yet to be characterized in detail, it has been proposed that secretion and sortase processing of CWA proteins is linked with the cell wall synthesis process at discrete locations near the site of cell division, similar to a report of the pilin sortase Bps of *E. faecalis* (Kline, et al., 2009).

MSCRAMMs

Ace (adhesin to collagen of *E. faecalis*) was the first microbial surface component recognizing adhesive matrix molecules (MSCRAMM) adhesin identified in enterococci (Rich, et al., 1999; Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000); its role in pathogenesis is discussed in Pathogenesis and models of enterococcal infection. Similar to various staphylococcal MSCRAMMs (Patti & Höök, 1994), Ace contains an LPXTG motif, with an N-terminal signal peptide followed by a non-repetitive segment, called the A-domain, and a variable number (2.4 to 5.4) of sequence repeats, which are referred to as the B-repeats (Nallapareddy S. R., Singh, Duh, Weinstock, & Murray, 2000). The A-domain of Ace has 46% similarity with the corresponding region of the *S. aureus* MSCRAMM Cna (Rich, et al., 1999). Binding studies with ELISA and surface plasmon resonance spectroscopy (SPR) showed that a His_6-tagged recombinant version of the Ace A-domain bound to multiple regions in collagen type I, although with distinctly different kinetics than the collagen-binding A-domain of Cna (Rich, et al., 1999; Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000). In contrast to Cna, rAce A-domain also showed concentration-dependent and saturable binding to collagen type IV and laminin (Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000). Crystal structure analysis of this ligand-binding A-domain of Ace revealed two subdomains of similar size, N1 and N2, both of which adopt the DEv-IgG (DE variant-IgG) fold, previously found in staphylococcal MSCRAMMs (Zong, et al., 2005; Deivanayagam, et al., 2002), with the putative collagen-binding surface at the interface between the two subdomains (Liu, et al., 2007). This structure is generally similar to the collagen-binding subdomains of Cna, for which a unique ligand-binding mechanism called the "collagen hug" has been proposed (Rogers, Perkins, & Ward, 1980). The "collagen hug" model predicts that there is an equilibrium between an open, ligand-binding conformation and a closed conformation of N1N2 that cannot bind ligands. When these subdomains are in an open conformation, a collagen triple helix extending from damaged collagen fibers on damaged tissues can associate with the ligand-binding trench of the N2 domain, resulting in a structural rearrangement where the N1N2 subdomains and their inter-domain linker peptide wrap around the collagen triple helix, which transitions the N1N2 subdomains to the closed conformation. The tunnel-like space created between the subdomains secures the ligand in place. Finally, a C-terminal extension of the N2 subdomain complements a beta-sheet in the N1 subdomain and functions as a "latch" that stabilizes the adhesin-ligand complex (Zong, et al., 2005; Liu, et al., 2007). Mutations of conserved residues within the proposed ligand-binding site of Ace altered its interaction with collagen, which indicates that these residues are involved in ligand binding (Liu, et al., 2007). Furthermore, introduction of an internal disulfide bond stabilizing Ace in the closed conformation abrogated collagen binding. Finally, point and truncation mutations in the N2 extension confirmed the importance of this "latch" for ligand binding (Liu, et al., 2007). Although only moderately homologous, these studies have shown that the A-domains of Ace and Cna are

structurally and functionally similar, and support the proposed collagen hug model for Ace.In contrast, Ace does not have the third Ig-folded subdomain, N3, which is present in the A-domain of Cna but whose function is not known. Furthermore, the B-repeats of Ace and Cna have virtually no sequence similarity (Nallapareddy S. R., Singh, Duh, Weinstock, & Murray, 2000). Sequencing of *ace* in a worldwide collection of 26 *E. faecalis* isolates found it present in all isolates, as well as indicating that the Ace A-domain was highly conserved (≥97.5% amino acid identity) (Nallapareddy S. R., Singh, Duh, Weinstock, & Murray, 2000). However, four different size variants of Ace were found to relate to the number of otherwise well conserved B-repeats. The function(s) of these repeat regions and the significance of their size variation have yet to be determined.

Although screening with anti-Ace antibodies or hybridizations with *ace* probes failed to identify an *ace* homolog in *E. faecium* strains, analysis of the genome sequence of *E. faecium* TX16 identified a gene whose highest identity is to *cna* of *S. aureus* (Nallapareddy, Weinstock, & Murray, 2003). The encoded protein, which was named Acm (adhesin of collagen from *E. faecium*), has a similar overall domain organization as the previously described MSCRAMMs, with an N-terminal signal peptide, A-domain and B-repeats, and a C-terminal LPXTS cell-wall anchoring domain. The Acm A-domain has 60% similarity to that of Cna, but only 47% similarity to the Ace A-domain. Molecular modeling suggested that the A-domain of Acm also consists of N1 and N2 subdomains, each with a DEv-IgG fold (Nallapareddy, Weinstock, & Murray, 2003) that is consistent with the β-sheet-rich secondary structure revealed by CD spectroscopy (Sillanpää, et al., 2008). The A-domain in Acm is also 166 aa longer than in Ace and predicted to fold into an additional Ig-folded subdomain, N3, similar to Cna. The recombinant Acm A-domain was shown to bind collagen but, unlike Ace, with a higher affinity to collagen type I than IV and with no binding to laminin (Nallapareddy, Weinstock, & Murray, 2003). Analysis of the collagen binding of a series of recombinant N1N2N3 segments of Acm mapped the N1N2 subdomains, including the predicted C-terminal latch sequence in N2, as a high-binding region. This N1N2(+latch) region showed higher affinity to collagen type I than the full Acm A-domain (N1N2N3), which resembles previous findings with Cna (Nallapareddy S. R., Sillanpää, Ganesh, Hook, & Murray, 2007). Antibodies against different segments of the Acm A-domain (Nallapareddy S. R., Sillanpää, Ganesh, Hook, & Murray, 2007) as well as a recombinant protein segment consisting of the Acm A-domain (Nallapareddy, Weinstock, & Murray, 2003) dose-dependently inhibited adherence of *E. faecium* to collagen, corroborating the specificity of the Acm-collagen interaction; the highest inhibition was seen with anti-N1N2 antibodies, which suggests the possibility of blocking Acm-mediated *E. faecium* adherence as a therapeutic or prophylactic strategy.

The B-repeat region of Acm has significant similarity with that of Cna, unlike Ace, which only shares homology with Acm in the A-domain. As with Ace, the number of Acm B-repeats varied from strain to strain (Nallapareddy, Weinstock, & Murray, 2003). Screening of a large number of *E. faecium* isolates found *acm* in almost all isolates studied (99%, although frequently as a pseudogene in non-clinical isolates), unlike *cna*, which was present in only some of *S. aureus* isolates (38-56%) (Ryding, Flock, Flock, Söderquist, & Christensson, 1997; Smeltzer, Gillaspy, Pratt, Jr., Thames, & Iandolo, 1997; Switalski, Patti, Butcher, Gristina, Speziale, & Höök, 1993). This, together with the similarity of Acm and Cna, led to a suggestion that *acm* could be a distant ancestor of *cna* (Nallapareddy, Weinstock, & Murray, 2003).In summary, the two major enterococcal pathogens in humans, *E. faecalis* and *E. faecium*, both have an MSCRAMM adhesin that is ubiquitously present and highly conserved among isolates. This adhesion is responsible for the majority of their adherence to collagen/laminin, and is also important for pathogenesis in animal models (Lebreton, et al., 2009; Nallapareddy, Singh, & Murray, 2008; Singh, Nallapareddy, Sillanpää, & Murray, 2010), as discussed in Pathogenesis and models of enterococcal infection. The presence of a structurally related collagen-binding adhesin in several other Gram-positive bacteria, including *S. aureus* (Zong, et al., 2005), *Streptococcus equi* (Lannergård, Frykberg, & Guss, 2003), *Erysipelothrix rhusiopathiae* (Shimoji, et al., 2003), *Streptococcus mutans* (Sato, Okamoto, Kagami, Yamamoto, Igarashi, & Kizaki, 2004), and *Streptococcus gallolyticus* (Sillanpää J. , et al., 2009), suggests that host ligand binding by a collagen hug-like mechanism may be widespread among Gram-positive bacteria, and beneficial for their interactions with the host. Bioinformatics analyses have identified 17 (including Ace) LPXTG-motif cell-wall anchored proteins with predicted MSCRAMM-like folding into multiple Ig-like domains in the genome of

E. faecalis V583 (12 of which are present in the genome of *E. faecalis* OG1RF (Bourgogne, Hilsenbeck, Dunny, & Murray, 2006)) and 15 such proteins (including Acm) in *E. faecium* TX16 (Sillanpää, et al., 2008; Sillanpää, Xu, Nallapareddy, Murray, & Höök, 2004). Three of the *E. faecalis* MSCRAMM-like proteins, Fss1 (for *E. faecalis* surface protein; EF0089), Fss2 (EF2505), and Fss3 (EF1896), were found to contain regions with structural similarity to the ligand-binding domains (N2N3) of previously described staphylococcal fibrinogen-binding MSCRAMMs (Sillanpää J. , et al., 2009). Recombinant proteins of the Fss1-, Fss2-, and Fss3 N2N3-like regions were shown to bind to immobilized fibrinogen with variable apparent affinities, but differed in their specificities to the three polypeptide chains that make up the fibrinogen molecule (Sillanpää J. , et al., 2009). Using an isogenic *fss2* insertion mutant and its complementation derivative, Fss2 was further shown to function as an adhesin to fibrinogen on *E. faecalis* OG1RF cells, while the disruption of *fss1* led to only a marginal reduction in fibrinogen adherence, and *fss3* was not found in this strain (Sillanpää J. , et al., 2009). However, even greater reduction in fibrinogen adherence was seen with an *ebp* pilus mutant of OG1RF (see Pathogenesis and models of enterococcal infection) (Nallapareddy S. R., Singh, Silanpaa, Zhao, & Murray, 2011). The presence of multiple fibrinogen adhesins is similar to the functional redundancy seen with *S. aureus*, in which several MSCRAMMs and other adhesins that bind to fibrinogen have been described (Rivera, Vannakambadi, Höök, & Speziale, 2007). This raises the possibility that these adhesins might be expressed at different tissue locations or stages of infection, and that their potential binding to different sites on the fibrinogen molecule could affect tissue tropism. In addition to Acm, two of the 15 MSCRAMM-like proteins identified from *E. faecium*, Scm (Fms10) and EcbA (Fms18, a homolog of *E. faecalis* Fss3), have been shown to bind to collagen as recombinant proteins, and rEcbA also binds to fibrinogen (Sillanpää, et al., 2008; Hendrickx, et al., 2009). However, both of these proteins bound to immobilized collagen type V, differing from Acm in their collagen-type specificities and pointing to the possibility of similar flexibility in collagen adherence of *E. faecium*, as above with the fibrinogen adhesins of *E. faecalis*. The non-repetitive N-terminal A-domain was identified as the collagen-binding region of Scm; however, its ligand-binding mechanism, as well as that of EcbA, is not known.

A subset of the MSCRAMM genes identified above are clustered together in loci with an adjacent gene that is predicted to code for a class C sortase. Two such loci have been identified in *E. faecalis* (Sillanpää, Xu, Nallapareddy, Murray, & Höök, 2004; Nallapareddy S. R., et al., 2006; Tendolkar, Baghdayan, & Shankar, 2006) and four in *E. faecium* (Sillanpää, et al., 2008; Hendrickx, van Wamel, Posthuma, Bonten, & Willems, 2007). Studies of *Corynebacterium diphtheriae* were the first to show that these loci encode filamentous surface appendages called pili (Ton-That & Schneewind, 2003; Ton-That & Schneewind, 2004); similarly organized gene clusters have been found in a number of other Gram-positive bacteria. The corynebacterial pili were found to be encoded by three structural pilin genes; one of the encoded proteins, the major pilin, was shown to form the backbone or shaft of the pilus filament, while one of the minor pilins is located at the pilus tip, and another functions as a cell-wall anchor subunit that links the pilus to the bacterial cell wall (Ton-That & Schneewind, 2003; Mandlik, Das, & Ton-That, 2008). Similar roles have since been reported for pilins from streptococci (Nobbs, Rosini, Rinaudo, Maione, Grandi, & Telford, 2008; Smith, et al., 2010). These studies have led to a pilus assembly model, in which pilin polymerization is typically initiated by cleavage of the LPXTG sorting signal of the tip pilin by the pilin-specific class C sortase, which leads to the formation of an acyl intermediate between the tip pilin and the pilin sortase. In the next step, this intermediate is resolved by a nucleophilic "attack" by the ε-amino group of a conserved lysine residue in a pilin motif of the major subunit. As a result of this reaction, the tip pilin becomes covalently attached to the pilin motif lysine of the major pilin via the threonine residue of its sorting signal. Pilin polymerization then continues with similar cross-linking reactions of additional major pilins to the growing polymer, catalyzed by the pilus-specific sortase. Pilus elongation finally ends when an anchor subunit, presented on the housekeeping sortase as an acyl-enzyme intermediate, is cross-linked to the growing end of the pilin polymer through a lysine residue in the anchor pilin, thus transferring the pilus-polymer to the housekeeping sortase. In a similar process as with cell-wall anchored proteins, the pilin polymer (now attached to the housekeeping sortase via the anchor pilin), is then tethered by this sortase to the cell wall peptidoglycan (Mandlik, Das, & Ton-That, 2008). A variation of this model has been proposed for the two-component pili of

Bacillus spp.; in this model, the major pilin has an additional role as the cell-wall anchor subunit, while the minor subunit is located at the pilus tip (Budzik, Oh, & Schneewind, 2008).The endocarditis and biofilm-associated pili (Ebp) pilus-encoding locus first identified in *E. faecalis* consists of three genes, *ebpA* (EF1091), *ebpB* (EF1092), and *ebpC* (EF1093), in addition to the biofilm and pilus-associated sortase (*bps*) EF1094, which encodes a putative class C sortase. A clue to the function of the *ebp* locus was suggested by the finding of a conserved pilin motif in EbpC (Ton-That & Schneewind, 2003) and, surprisingly, also in EbpB and EbpA; all these pilin motifs also contained a conserved lysine (Nallapareddy S. R., et al., 2006). Using anti-Ebp antibodies, Nallapareddy et al. (Nallapareddy S. R., et al., 2006)demonstrated that *E. faecalis* OG1RF produces pilus-like appendages on the cell surface, and that these structures are composed of EbpA, EbpB and EbpC (Nallapareddy S. R., et al., 2006). Further studies with a set of *ebp* and *bps* mutants showed that both EbpC and Bps are needed for polymerization of these pili, while disruption of *srtA*, which encodes the housekeeping sortase, had no effect on Ebp pilus production (Nallapareddy S. R., et al., 2006). Hence, these studies indicated EbpC as the primary pilin that forms the majority of the pilus shaft, and Bps as the pilus-specific sortase responsible for pilin polymerization. More recent data point to EbpA as a tip pilin, although it is present in large amounts on the cell surface, and EbpB as the cell-wall anchor subunit, which is linked to the cell wall by the housekeeping sortase, SrtA (Nielsen, et al., 2013; Sillanpää, et al., 2013). While assembly of the Ebp pilus appears to mostly conform to the general pilus assembly model, deletion of *ebpA* reduced the overall production of high molecular weight pilin polymers and led to the formation of very long pili, suggesting a role for *ebpA* in both initiation and termination of pilus polymerization (Sillanpää, et al., 2013).

Recent studies with Gram-positive cocci have indicated that proteins with Sec-dependent signal sequences are translocated through the Sec machinery at distinct sites on the cell surface. Localization of SecA in secretion domains, termed ExPortal in *S. pyogenes* (Rosch & Caparon, 2005), has also been reported with *E. faecalis* (Kline, et al., 2009). Furthermore, both the housekeeping sortase (SrtA) and pilin sortase (Bps) were found to co-localize with SecA at the same foci on the cell surface of derivatives of *E. faecalis* OG1. In the absence of Bps, pilins were shown to accumulate at these sites, suggesting that pilin translocation and their sortase processing are spatially coordinated. The N-terminus of SrtA and the C-terminus of Bps were found to contain a positively charged amino acid segment, similar to the positively charged C-terminal tail of CWA proteins (Kline, et al., 2009). This segment was shown to be important for the localization of Bps to distinct foci, and was proposed to function as a general retention signal for compartmentalization of membrane proteins at these secretion domains (Kline, et al., 2009).

Gene hybridization studies have shown that the *ebp* pilus-encoding locus is carried by the vast majority of *E. faecalis* isolates (472 of 473 diverse isolates tested; genome comparisons suggested that the 473rd strain, TX1346, has a 12-kb region, which includes the *ebp* locus, replaced by a transposase) and, as a result, this locus is considered to be part of the core genome of this species (Nallapareddy S. R., et al., 2006). Comparison of *ebp* gene sequences among 55 available *E. faecalis* genomes representing >30 multi-locus sequence types (MLSTs) showed high conservation within each of the three *ebp* genes, with DNA identities ranging from 98.4% to 99.99% (Nallapareddy S. R., et al., 2011). A second pilus gene cluster of *E. faecalis*, designated *bee* (biofilm enhancer in *Enterococcus*) due to its association with a high-biofilm phenotype, has also been reported from *E. faecalis*; however, the structure and assembly of these pili remain to be characterized (Tendolkar, Baghdayan, & Shankar, 2006). In contrast to *ebp*, the *bee* locus was located on a conjugative plasmid and was found to be carried by a minority (~1%) of *E. faecalis* isolates (Nallapareddy S. R., et al., 2011). The presence of a single and highly conserved pilus-encoding locus in nearly all *E. faecalis* isolates, across clonal complexes and regardless of their isolation source or geographical origin, is in direct contrast to many other pathogenic Gram-positive species, such as *S. pyogenes*, *S. agalactiae*, *S. pneumoniae*, and *C. diphtheriae*; among these species, pilus loci are typically either highly variable among strains, represented by two or more distinctly different pilus types, and/or are located on genomic islands (Soriani & Telford, 2010; Mandlik, Swierczynski, Das, & Ton-That, 2008; Kline, Dodson, & Caparon, 2010).

As mentioned above, analysis of the *E. faecium* TX16 genome identified four predicted pilus-encoding gene clusters, each with an adjacent sortase gene (Sillanpää, et al., 2008; Hendrickx, Bonten, van Luit-Asbroek, Schapendonk, Kragten, & Willems, 2008).One of these, designated *ebpA$_{fm}$-ebpB$_{fm}$-ebpC$_{fm}$* (also known as *pilB*) due to its partial similarity with the *ebp* locus of *E. faecalis*, has been shown to produce a single mRNA transcript and high-molecular-weight protein complexes (Sillanpää, et al., 2008). Both the *ebpA$_{fm}$-ebpB$_{fm}$-ebpC$_{fm}$* operon and the *fms21 (pilA)-fms20* locus have been demonstrated to encode pilus filaments on the *E. faecium* cell surface, using antibodies against their predicted major subunits, EbpC$_{fm}$ and Fms21 (PilA) (Hendrickx, Bonten, van Luit-Asbroek, Schapendonk, Kragten, & Willems, 2008; Sillanpää J. , et al., 2010). Interestingly, the *fms21-fms20* cluster was found to be located on a large, transferable plasmid, which also contains an ortholog for a housekeeping sortase, in addition to a pilin sortase-encoding gene (Kim, et al., 2010).

Screening of a large number of diverse *E. faecium* isolates found the *ebp$_{fm}$* genes, as well as *scm*, *ecbA*, a functional *acm* gene (see above), and several of the other MSCRAMM- and pilin-encoding *fms* genes whose potential host targets are not yet known, to be significantly more common in isolates of the hospital-associated (HA) clade of *E. faecium* versus the community-associated clade (CA) (Sillanpää, et al., 2008; Hendrickx, van Wamel, Posthuma, Bonten, & Willems, 2007; Sillanpää, prakash, Nallapareddy, & Murray, 2009). In addition, many of the MSCRAMMS differ in sequence between isolates of the HA clade and the CA clade (Galloway-Peña, Roh, Latorre, Qin, & Murray, 2012); these differences are usually between 85-94% amino acid identity (except for more distantly related *fms13* and *fms20* homologs). Other recent analyses of the available *E. faecium* genomes reported a ~3.5-4.2% difference between 100 core genes of HA- and CA-clade strains (192), and a 3-10% difference between these two clades among four surface protein-encoding genes (*pbp2, pbp5, gls20* and *wlcA*) (Galloway-Peña, Rice, & Murray, 2011). Thus, the above MSCRAMM sequence variation is in the range of what was previously reported for the genes of the two clades, pointing to fundamental differences at both the core and accessory genome levels between these clades which are estimated to have diverged at least hundreds of thousands of years before the modern antibiotic era (Galloway-Peña, Roh, Latorre, Qin, & Murray, 2012; Galloway-Peña, Rice, & Murray, 2011).

Aggregation substance (AS) refers to a family of surface proteins involved in aggregation of *E. faecalis* (Wirth, 1994; Kozlowicz, Dworkin, & Dunny, 2006). As discussed in Extrachromosomal and Mobile Elements in Enteroocci, members of this family are encoded on pheromone-inducible conjugative plasmids, which also have a quorum-sensing mechanism that detects a short peptide pheromone that plasmid-free (recipient) cells release into the surrounding environment; this, in turn, leads to the expression of AS, which mediates donor-recipient aggregation and facilitates plasmid transfer (Wirth, 1994; Kozlowicz, Dworkin, & Dunny, 2006). AS has typical features of a CWA protein, including an N-terminal signal sequence and a C-terminal CWA domain with an LPXTG sorting motif. Although several AS sequence variants have been described, including Asc10 (encoded by plasmid pCF10), Asp1 (pPD1), and Asa1 (pAD1), they are generally highly conserved with over 90% amino acid identity, excluding a variable region with 30 to 50% identity in the N-terminal one third of the protein (Wirth, 1994). An exception to this is Asa373 (pAM373), which has little overall similarity to the other characterized AS proteins and likely exhibits a different aggregation mechanism (Muscholl-Silberhorn, 1999). Unlike many other enterococcal CWA proteins, no pronounced repeat region has been identified in AS (Wirth, 1994). Studies with insertion and/or deletion mutants identified two domains in the N-terminal half of AS that are necessary for aggregation, while the C-terminal remainder of the protein was suggested to be dispensable in this process (Waters & Dunny, 2001; Waters, Hirt, McCormick, Schlievert, Wells, & Dunny, 2004; Muscholl-Silberhorn, 1998). Both of the aggregation domains were also reported to be necessary for the internalization of *E. faecalis* into intestinal epithelial cells (HT-29) (Waters, Hirt, McCormick, Schlievert, Wells, & Dunny, 2004; Waters, Wells, & DunnyqGary, 2003). Using purified Asc10 protein fragments, the more N-terminally located aggregation domain was shown to bind to lipoteichoic acid (LTA), a likely bacterial surface receptor for AS-mediated donor-recipient aggregation, while the second, more central aggregation domain was shown to be non-essential to LTA binding (Waters, Hirt, McCormick, Schlievert, Wells, & Dunny, 2004). Both the LTA-binding and central aggregation domains were recently shown to contribute to experimental rabbit endocarditis

(Chuang, Schlievert, Wells, Manias, Tripp, & Dunny, 2009). The AS protein contains two RGD motifs; in eukaryotic systems, the RGD motif is known to mediate the binding of surface proteins to cell surface receptors known as integrins, as well as interactions between ECM proteins. The RGD to RAD mutation of both AS motifs led to more significant attenuation in the above endocarditis model than the disruption of the two aggregation domains (Chuang, Schlievert, Wells, Manias, Tripp, & Dunny, 2009), although these motifs were not necessary for *E. faecalis* adherence and internalization into intestinal epithelial cells (Waters, Wells, & DunnyqGary, 2003). Furthermore, there is some evidence that these two RGD motifs may contribute to the resistance of *E. faecalis* cells to PMN-mediated killing (Vanek, Simon, Jacques-Palaz, Mariscalco, Dunny, & Rakita, 1999). The presence of AS has also been associated with increased *E. faecalis* adherence to several ECM proteins, such as fibronectin, collagen, thrombospondin, and vitronectin (Rozdzinski, Marre, Susa, Wirth, & Muscholl-Silberhorn, 2001). In the same study, the central variable region of Asa1, located between the two aggregation domains, was implicated in binding to fibronectin (Rozdzinski, Marre, Susa, Wirth, & Muscholl-Silberhorn, 2001). Collectively, the above studies have identified AS as a multifunctional protein with several domains or regions that affect *E. faecalis* aggregation and/or host-pathogen interactions. Finally, although sortase-mediated anchoring of AS has yet to be described in detail, a recent study by Kline et al. (Kline, et al., 2009) showed that SecA and the housekeeping sortase (SrtA) co-localize in single foci on the surface of *E. faecalis*. Furthermore, AS was found to accumulate at comparable surface locations on a *srtA* deletion mutant, implying that secretion and sortase-mediated cell-wall anchoring of AS are coordinated at single-secretion loci, similar to sortase-processing of the Ebp pili (Kline, et al., 2009).

An enterococcal surface protein (Esp) was first identified in *E. faecalis* by Shankar et al. (Shankar, Baghdayan, Huycke, Lindahl, & Gilmore, 1999) and has been associated with virulence in animal models, biofilm formation, and several other phenotypes related to pathogenesis (see Pathogenesis and models of enterococcal infection). This large LPXTG-motif–containing CWA protein (~200 kDa) has an N-terminal signal sequence followed by a variable N-terminal domain, and a large region consisting of three types of repeat sequences, A, B, and C. Both A and C repeats are arranged as multiple tandem repeating units, while only two separately located B repeats were found (Shankar, Baghdayan, Huycke, Lindahl, & Gilmore, 1999). Together, these repeats make up ~50% of the total protein length. The overall domain organization of Esp is similar to several other surface proteins that contain highly repetitive regions, including another protein, EF3314, from *E. faecalis*, Rib and C alpha proteins from *S. agalactiae*, R28 from *S. pyogenes*, and the biofilm-associated Bap from *S. aureus* (Creti, et al., 2009; Cucarella, Solano, Valle, Amorena, Lasa, & Penadés, 2001; Stålhammar-Carlemalm, Areschoug, Larsson, & Lindahl, 1999; Stålhammar-Carlemalm, Stenberg, & Lindahl, 1993; Michel, Madoff, Olson, Kling, Kasper, & Ausubel, 1992). While extensive sequence similarity between Esp, Rib, C alpha, and Bap was only found between the highly reiterated C-repeats, especially within a 13-amino acid C-repeat motif, Esp and Bap were found to share additional sequence similarity in their N-terminal domains (Shankar, Baghdayan, Huycke, Lindahl, & Gilmore, 1999; Eaton & Gasson, 2002). An *esp* homolog (*esp*$_{fm}$) has been identified in *E. faecium*; similar to *E. faecalis*, it has been located in a pathogenicity island and was found more commonly, albeit in quantities usually less than 70%, among clinical versus non-clinical isolates (Shankar, Baghdayan, Huycke, Lindahl, & Gilmore, 1999; Eaton & Gasson, 2002; Woodford, Soltani, & Hardy, 2001; Leavis, et al., 2004). Esp$_{fs}$ and Esp$_{fm}$ have a similar global organization, with up to 90% overall amino acid identity, which suggests that they may also have similar functions (Eaton & Gasson, 2002). The numbers of A and C repeats in both Esp$_{fs}$ and Esp$_{fm}$ were found to vary among isolates, similar to the B-repeats of the collagen-binding MSCRAMMs Ace and Acm (see above) (Shankar, Baghdayan, Huycke, Lindahl, & Gilmore, 1999; Eaton & Gasson, 2002). Combined with additional sequence divergence within the N-terminal and C-terminal nonrepeat regions, this variation has led to the assignment of four Esp$_{fm}$ types among *E. faecium* isolates (Leavis, et al., 2004). At the C-terminal end, both Esp$_{fs}$ and Esp$_{fm}$ have a typical sortase-dependent CWA domain, although with a variant (Y/F)PXTG motif.

Studies by Tendolkar et al. (Tendolkar, Baghdayan, & Shankar, 2005) on regions in Esp important for biofilm formation found that an in-frame deletion mutant, expressing a truncated Esp$_{fs}$ without the non-repeated N-terminal domain, produced less biofilm than a strain expressing the wild-type protein (Tendolkar, Baghdayan, &

Shankar, 2005). Furthermore, heterologous surface display of the Esp_{fs} N-terminal domain resulted in similar biofilm formation as that of full-length Esp_{fs} (Tendolkar, Baghdayan, & Shankar, 2005). Finally, a recombinant protein consisting of the N-terminal domain of Esp_{fm} inhibited initial adherence of *E. faecium* to a polystyrene surface (Van Wamel, Hendrickx, Bonten, Top, Posthuma, & Willems, 2007). Hence, the non-repeated N-terminal domain appears to have an important role in Esp-associated biofilm formation. However, heterologous expression of full-length Esp_{fs} in *L. lactis* or *esp*-negative *E. faecium* failed to enhance their ability to form biofilm, which led the authors to propose a potential requirement for some additional *E. faecalis*-specific factor(s) (Tendolkar, Baghdayan, & Shankar, 2005). In contrast, no functional role has so far been assigned to the large repeat region of Esp.

Genome analyses of *E. faecalis* and *E. faecium* have also identified a number of LPXTG-motif CWA proteins without the predicted Ig-like folding characteristic of MSCRAMMs (Sillanpää, et al., 2008; Sillanpää, Xu, Nallapareddy, Murray, & Höök, 2004; Hendrickx, van Wamel, Posthuma, Bonten, & Willems, 2007). One such protein is SgrA (serine-glutamate repeat containing protein A; Fms2 (*faecium* surface protein)), which was found in *E. faecium* TX16 and was shown to be enriched among clinical isolates (Sillanpää, et al., 2008; Hendrickx, van Wamel, Posthuma, Bonten, & Willems, 2007). A recombinant segment of SgrA was reported to bind to fibrinogen and nidogen (entactin), an ECM protein present in basal lamina (Hendrickx, et al., 2009). Furthermore, an *sgrA* mutant showed reduced adherence to immobilized fibrinogen and nidogen; this mutant was also impaired in biofilm formation (Hendrickx, et al., 2009). Although SgrA, with no predicted Ig-like folding, is likely to be structurally different from typical MSCRAMMs, this protein has a comparable general organization to a short non-repeated N-terminal region followed by sequence repeats (Hendrickx, et al., 2009).

In an earlier study by Teng et al. (Teng, Kawalec, Weinstock, Hyrniewicz, & Murray, 2003), screening for antigens with sera from endocarditis patients led to the identification of a major antigen from *E. faecium*, SagA (Teng, Kawalec, Weinstock, Hyrniewicz, & Murray, 2003). Recombinant SagA was shown to bind to fibrinogen, fibronectin, collagen, and laminin. However, this broad-spectrum adhesin, which lacks an identifiable CWA domain, was found to be secreted (Teng, Kawalec, Weinstock, Hyrniewicz, & Murray, 2003), thus resembling secreted fibrinogen-binding proteins from *S. aureus*, such as Eap and Efb (Rivera, Vannakambadi, Höök, & Speziale, 2007).

Conclusion and Future Perspectives

The last decade has seen an explosion in the literature relevant to enterococcal cell wall structures and their underlying genetics. We now know the genetic basis for capsule typing in *E. faecalis.* The capsule structure for the most prevalent pathogenic lineage of *E. faecalis* has been solved (CPSC or Maekawa type 2). Recent NMR analysis has also solved the wall teichoic acid structure for both *E. faecium* and *E. faecalis* isolates. Unique pilin loci have been described and characterized. The complete genome sequence of *E. faecium* has led to the discovery of a variable capsule locus, which suggests rich antigenic diversity in this emerging nosocomial pathogen. Interest in the importance of lipoproteins to enterococcal biology is also emerging. The next decade promises to be just as exciting as the last, as investigators unravel more detail about the cell wall architecture of this collective group of superbugs. Such discoveries will hopefully lead to advances in the treatment of multi-drug resistant infections.

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Enterococcal Biofilm Structure and Role in Colonization and Disease

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Biofilm Formation by Enterococci: Concepts and Caveats

This chapter discusses biofilm formation in enterococci and its role in the biology of these organisms, especially in relation to opportunistic infections. As is the case for many microbes, the pace of enterococcal biofilm research has quickened in the past few years, and numerous genes and gene products affecting biofilm formation have been identified. At the same time, this research has not resulted in a comprehensive understanding of the critical steps in the process, particularly those steps that are involved in initiating the transition from planktonic growth to biofilm growth. Both the physical/chemical environment and the type of surface substratum on which the biofilm develops have a tremendous impact on the process, even with a single strain. A cursory scan of 15-20 years of publications on enterococcal biofilms indicates that the number of different experimental conditions employed to measure biofilm formation is comparable to the number of published papers on the topic. Clearly, these efforts have contributed to the discovery of different biofilm determinants and processes by different research groups, and in some cases, have provided contradictory reports and conclusions. Therefore, we begin this chapter with a brief summary of central principles, definitions, and questions to provide a framework for subsequent sections that contain more detailed descriptions of relevant research in this area. We conclude with a discussion of future research directions that may clarify and increase the level of understanding of this important microbial activity.

Biofilms are microbial communities resulting from the adherence of planktonic organisms to an abiotic surface, usually followed by growth. It is generally recognized that microbial species spend much of their time in the biofilm state, and that biofilm formation plays a critical role in infections. Thus, research on enterococcal biofilms is driven by its potential to yield new insights into the pathogenesis of opportunistic infections, their treatment, and their prevention. It is commonly hypothesized that the formation of biofilms represents a developmental process that involves a shift in physiology from planktonic growth, organization of the adherent bacteria into structured communities physically linked by an extracellular matrix, and where communication between members of the community coordinates gene expression and metabolic activity (O'Toole, Kaplan, & Kolter, 2000). While this model has been a useful paradigm to guide experimental investigations, it has not been completely validated, especially for non-sporulating bacteria, and strong arguments have been made for considering alternatives (Monds & O'Toole, 2009). The developmental model implies that there are critical functions specific for biofilm formation, and it can also be inferred that there may be conservation of these functions among different bacteria.

Examination of the published literature suggests that many, if not most, determinants of enterococcal biofilm formation identified to date are members of global networks important for adaptation to a variety of environments, and that factors such as the medium composition, physical/chemical conditions, and type of surface used for cultivation of biofilms may have a larger influence on the “biofilm functions” than biofilm growth itself. Investigators have also used different criteria to quantify biofilm formation. Conclusions about optimal biofilm formation can vary greatly depending on whether total biomass, enumeration of cell counts by

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microscopy or plate counting, or the ratio of biofilm biomass to bacterial growth in the planktonic phase is used as the primary criterion (Di Rosa, et al., 2006; Creti, Koch, Fabretti, Baldassarri, & Huebner, 2006; Kristich, Li, Cvitkovich, & Dunny, 2004; Hufnagel, Koch, Creti, Baldassarri, & Huebner, 2004; Sandoe, Witherden, Cove, Heritage, & Wilcox, 2003; Baldassarri, et al., 2001). Given these discrepancies, researchers interested in linking biofilm formation to pathogenesis need to carefully consider the ways in which their laboratory assays closely reflect conditions in an animal or human host, to compare results using different in vitro assays, and to examine the role of biofilm determinants of interest in the context of a relevant animal model. Pathogenesis and models of enterococcal infection discusses the use of various animal models to investigate enterococcal virulence in more detail. While it is conceivable that biofilm defects could result in the attenuation of virulence in any animal model, the rabbit cardiac catheterization model for experimental endocarditis (Frank, Barnes, Grindle, Manias, Schlievert, & Dunny, 2012; Singh K. V., Nallapareddy, Sillanpää, & Murray, 2010; Chuang, Schlievert, Wells, Manias, TRipp, & Dunny, 2009; Nallapareddy, Singh, & Murray, 2008; Nallapareddy S. R., et al., 2006) and the mouse urinary tract infection models (Sillanpää, et al., 2010; Guiton, Hung, Hancock, Caparon, & Hultgren, 2010; Singh, Nallapareddy, & Murray, 2007; Shankar, Lockatell, Baghdayan, Drachenberg, Gilmore, & Johnson, 2001) may be especially informative in examining the role of biofilm formation in the host on pathogenesis.

Epidemiology of Biofilm-Related Enterococcal Infections

The earliest reports of enterococci in association with infection-related biofilms were probably from the studies that identified *Enterococcus faecalis* in infected vascular ports from patients (Reed, Moody, Newman, Light, & Costerton, 1986) and in a urinary stone (Nickel, Reid, Bruce, & Costerton, 1986). Later, the expression of two *E. faecalis*-specific surface antigens, shown to be enriched when the bacteria were cultured in serum or brain heart infusion *in vitro*, or during growth on silastic discs in the rabbit peritoneum (but not in a chemically defined broth), were correlated to the potential to form biofilms during infection (Lambert, Shorrock, Aitchison, Dominique, Power, & Costerton, 1990). A number of studies in the early 1990s evaluated bacterial communities that were associated with indwelling catheters, and frequently isolated either *E. faecalis* alone or as part of a polymicrobial species on these devices (Reid, Denstedt, Kang, Lam, & Nause, 1992; Jansen, Goodman, & Ruiten, 1993; Stickler, King, Winters, & Morris, 1993; Jass, Phillips, Allan, Costerton, & Lappin-Scott, 1994; Keane, Bonner, Johnston, Zafar, & Gorman, 1994; Koivusalo, et al., 1996).

In an effort to examine approaches to combat biofilm-associated infections, numerous studies evaluated multiple antimicrobials, either alone or in combination, as agents used to coat catheter surfaces to prevent microbial adhesion. For instance, Farber *et al* (Farber & Wolff, 1993) reported that salicylic-acid–coated catheters showed diminished adherence of many Gram-negative bacteria, yeast, and *E. faecalis*. In another study, Raad *et al* (Raad, Darouiche, Hachem, Sacilowski, & Bodey, 1995) found that the inhibitory activities of catheters coated with minocycline and rifampin against *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *E. faecalis* strains, for example, were significantly better than those of catheters coated with vancomycin ($P < 0.05$). In other studies, the efficacies of clinical dosing schedules of once-daily versus thrice-daily regimens of aminoglycosides in treating endocarditis were correlated to the killing of *E. faecalis* coated on glass beads, and concluded that no differences existed between the two regimens (Schwank & Blaser, 1996). Static *in vitro* biofilms perfused with the glycopeptide antibiotics vancomycin and teicoplanin, either alone or in combination, showed slower growth in the presence of either antibiotic, and the effect of the combination was a further 2-3 log reduction in growth rate and a 3-log reduction in viability of the biofilm (Foley & Gilbert, 1997). In other related studies, Bowers *et al* (Bower, Daeschel, & McGuire, 1998) explored the potential of peptide antimicrobials, such as lysozyme and nisin, to inhibit *E. faecalis* adhesion to treated catheters. The studies reported above established the fact that *E. faecalis* is a prominent bacterium encountered in biofilms, especially in catheter-related infections, and raised the concern that the therapeutic options to treat such infections might be somewhat limited.

Later studies attempted to correlate the ability of enterococcal isolates from clinical settings to biofilm formation *in vitro*, and also began to associate specific bacterial factors with this phenotype. One prospective study in Italy

determined that 80% of *E. faecalis* clinical isolates and 48% of *E. faecium* isolates formed biofilms (Baldassarri, et al., 2001), while another study of 47 clinical isolates found the biofilm phenotype to be associated with 87% of *E. faecalis*, but only 16% of *E. faecium* strains (Duprè, Zanetti, Schito, Fadda, & Sechi, 2003). A later study in the same geographical region found that 96% of 52 *E. faecalis* isolates from orthopedic infections produced biofilms *in vitro* (Baldassarri, et al., 2006). A study in Spain found that a little over one-half of 152 *E. faecalis* clinical isolates were able to form biofilms *in vitro* with no biofilm phenotype exhibited by *Enterococcus faecium*, *Enterococcus gallinarum*, or *Enterococcus avium* strains (Toledo-Arana, et al., 2001). Studies in the UK analyzed 109 isolates from bloodstream infections and showed that, while all of the *E. faecalis* isolates (n=71) formed biofilms, less than one-half (16/38) of the *E. faecium* isolates did so (7). This study further found that *E. faecalis* isolates from catheter-related bloodstream infections (CRBSI) produced more biofilm, as compared to non-CRBSI isolates. Another study in Europe also found a significantly higher ability to form biofilms among *E. faecalis* isolates, but not from the four other enterococcal species studied (Dworniczek, Wojciech, Sobieszczańska, & Seniuk, 2005). A total of 163 *E. faecalis* isolates (51 from outside the US) were evaluated in a US study, which concluded that biofilm formation is common among *E. faecalis* clinical as well as fecal isolates. This study also found that the percentage and degree of biofilm formation are significantly greater ($P = 0.001$) among endocarditis isolates than among isolates from other sources. All 352 samples of *E. faecalis* isolated from urinary tract infections in one study in Japan were capable of forming biofilms and could be grouped into weak, medium, or strong biofilm formers (Seno, Kariyama, Mistuhata, Monden, & Kumon, 2005). A study of 171 clinical isolates of enterococci at a tertiary care hospital in South India concluded that about a quarter of the *E. faecalis* strains (n=44) were capable of forming biofilms *in vitro*, in contrast to none of the 25 *E. faecium* isolates in that cohort (Prakash, Rao, & Parija, 2005).

Enterococci play a role in endodontic failure and are often isolated from the root canal system. The results of one study showed that out of 100 root-filled teeth with apical periodontitis, 69% of the isolated bacteria were facultative and 50% of those were enterococci (Dahlén, Samuelsson, Molander, & Reit, 2000). *E. faecalis* is responsible for the vast majority of human enterococcal endodontic infection, and is usually the only *Enterococcus* species isolated from the obturated root canal (Love, 2001). The biofilm-forming ability of *E. faecalis* isolated from the oral cavity has also recently been evaluated (Duggan & Sedgley, 2007). This study revealed that unlike other clinical isolates, especially endocarditis strains, the *E. faecalis* from oral and endodontic sources were poor biofilm formers. In contrast, another recent study observed that surface conditioning of dentin with saliva and starvation can enhance the adherence of *E. faecalis* to dentin (George & Kishen, 2007). No significant difference in the prevalence of this species between subgingival biofilm (34.6%) and saliva (35.1%) samples was observed in another study (Souto & Colombo, 2008). *E. faecalis* was detected significantly more often in saliva and subgingival samples of periodontitis patients (40.5% and 47.8%, respectively) as compared to controls (14.6% and 17.1%, respectively; $P < 0.05$), but the correlation with the biofilm phenotype is unclear. The consensus from the epidemiological studies above is that *E. faecalis* strains are better biofilm formers overall than *E. faecium* strains, and that the biofilm phenotype is an important contributory factor to enterococcal pathogenesis. These observations set the stage to explore the role of specific bacterial factors that mediate biofilm formation in *E. faecalis* and *E. faecium* in greater depth.

Factors that Affect Biofilm Formation

The past decade has seen the identification of a number of enterococcal genes that play a role in biofilm formation and maturation, especially in *E. faecalis* (Paganelli, Willems, & Leavis, 2012). These include surface adhesions, such as cell wall-associated proteins, autolysins, and glycolipids, which predominate early during the adhesion phase, and extend to polysaccharides, lipoteichoic acid, extracellular DNA, and proteases, which contribute to biofilm maturation. A brief description of each of these biofilm factors is presented below and summarized in Table 1.

Growth conditions

An indirect observation of the influence of growth medium on the ability of *E. faecalis* to form biofilms was probably made in 1990 by Lambert *et al* (Lambert, Shorrock, Aitchison, Dominique, Power, & Costerton, 1990), who observed that the expression of two *E. faecalis* surface antigens was enriched when the organisms were cultured in serum or brain heart infusion *in vitro*, or during growth on silastic discs in the rabbit peritoneum, but not in a chemically defined broth. Kristich *et al* (Kristich, Li, Cvitkovich, & Dunny, 2004) carefully evaluated different media with respect to biofilm formation by *E. faecalis* OG1RF and revealed a significant effect of growth medium on biofilm dynamics. While growth in TSB, M17, and M9YE medium caused biofilm accumulation to slow after 6-8 hours of growth, biofilm production in brain-heart infusion (BHI) or Todd-Hewitt yeast extract reached a plateau at 4 hours and subsequently declined. It must be noted that this study defined biofilm formation as the ratio of optical density in biofilm to optical density in planktonic mode, while most other studies below have relied on absolute optical densities of biofilms.

The influence of additional sugar, such as glucose, in the growth medium used to cultivate enterococci for biofilm assays became apparent early on, when adhesion to polystyrene microtiter plates was adopted as the method of choice for evaluating biofilm formation *in vitro* (Toledo-Arana, et al., 2001). In these initial studies, trypticase soy broth (TSB) supplemented with either 0.25 or 0.5% (w/v) of glucose was used to cultivate the bacteria. Subsequent studies indicated that the addition of 1% glucose (w/v) to TSB enhanced biofilm production by some strains, as compared to growth without additional glucose (Baldassarri, et al., 2001; Pillai, Sakoulas, Eliopoulos, Moellering, Jr., Murray, & Inouye, 2004). Another study in agreement with this phenomenon noted optimal biofilm formation with 0.5% glucose added to TSB, as compared to 0.2% or no glucose added (Kristich, Li, Cvitkovich, & Dunny, 2004). The enhancement of biofilm formation by *E. faecalis* OG1RF was noted with 1% glucose added to TSB, but the same effect was not evident in a *fsr* mutant or a *gelE* mutant (Pillai, Sakoulas, Eliopoulos, Moellering, Jr., Murray, & Inouye, 2004). One possible explanation suggested for this observed effect was that a glucose-dependent transcriptional regulator controlled *fsr*, either directly or indirectly, and that *fsr* exerts catabolite control over biofilm formation through downstream proteases GelE and SprE. The *bopABCD* operon (biofilm on plastic) in *E. faecalis*, deemed to have a putative maltose metabolism function, is regulated by *fsr* and important for biofilm formation (Hufnagel, Koch, Creti, Baldassarri, & Huebner, 2004; Bourgogne, Hilsenbeck, Dunny, & Murray, 2006). It has been shown that maltose can influence a *bopABC* triple-deletion mutant to produce more biofilm than the wild type in a medium containing 1% glucose, whereas in the presence of 1% maltose, the mutant only produced about 4% of biofilm with respect to the wild type (Creti, Koch, Fabretti, Baldassarri, & Huebner, 2006). While these observations suggest that the availability of different sugars in the growth environment can drastically alter biofilm production in *E. faecalis*, it remains to be established if this process is related to catabolite repression or the total availability of fermentable carbon sources.

The addition of 10% human serum to the culture medium has been reported to enhance the adhesion of *E. faecalis* ATCC 29212 to glass and silicone surfaces (Gallardo-Moreno, González-Martín, Perez-Giraldo, Bruque, & Gómez-García, 2002). Although a specific mechanism for the observed effect has not yet been proposed, another study examining a *salB* mutant noted enhanced biofilms when grown in TSB supplemented with 0.25% glucose and 10% serum (Mohamed, Teng, Nallapareddy, & Murray, 2005). The same effect was not seen with the wild-type OG1RF strain. A recent study has also revealed the enhanced expression of the Ebp pilus in the presence of 0.1 M sodium bicarbonate added to TSB containing glucose (Bourgogne, Thomson, & Murray, 2010), although the molecular basis of this is unclear. Along these lines, the *E. faecalis* pathogenicity island encoded AraC-type transcriptional regulator (PerA) was suggested to be a repressor of biofilm formation (Coburn P. S., Baghdayan, Dolan, & Shankar, 2008). Comparison of wild type *E. faecalis* E99, a *perA*-insertion mutant, and the complemented strain showed that when grown in Todd-Hewitt broth (THB) containing 1% glucose, biofilm formation of the mutant in vitro was significantly greater ($P < 0.0001$) relative to the wild type, as well as the complement. However, a similar effect was not seen when these strains were grown in TSB that

contained 1% glucose (Coburn P. S., Baghdayan, Dolan, & Shankar, 2008). Additional studies are needed to understand the mechanism behind these observations, but they highlight the importance of how the growth environment can significantly affect biofilm formation in *E. faecalis*.

Enterococcal Surface Adhesins

Aggregation substance

Aggregation substance (AS) is a surface adhesin that mediates cell-cell contact during pheromone responsive mating of donor and recipient *E. faecalis* cells, which is crucial for plasmid transfer (Clewell & Weaver, 1989). It has been suggested that AS may facilitate the translocation of *E. faecalis* across the intestinal epithelium, due to its involvement with adhesion to and invasion of intestinal epithelial cells (Wells, Moore, Hoag, Hirt, Dunny, & Erlandsen, 2000). AS has also been reported to mediate adherence to renal epithelial cells (Süßmuth, Muscholl-Silberhorn, Wirth, Susa, Marre, & Rozdzinski, 2000) and components of the extracellular matrix (Rozdzinski, Marre, Susa, Wirth, & Muschol-Silberhorn, 2001), as well as increase survival in polymorphonuclear leukocytes (Rakita, et al., 1999; Vanek, Simon, Jacques-Palaz, Mariscalco, Dunny, & Rakita, 1999) and macrophages (Süßmuth, Muscholl-Silberhorn, Wirth, Susa, Marre, & Rozdzinski, 2000). Deletion of the N-terminal region or the RGD domain of AS resulted in significantly reduced virulence of *E. faecalis* in the endocarditis model. Interestingly, while antibodies to the N-terminal region were not protective (McCormick, Hirt, Waters, Tripp, Dunny, & Schlievert, 2001), polyclonal antibodies to the full-length AS inecreased the severity of endocarditis (Schlievert, Chuang-Smith, Peterson, Cook, & Dunny, 2010). The same study also showed that Fab fragments of IgG from rabbits immunized against AS were able to reduce total vegetation size and microbial counts when passively administered to rabbits prior to challenge with *E. faecalis*-expressing AS. *In vitro*, the Fab fragments also prevented enterococcal aggregation. While AS did not appear to play a role in colonization in an ascending model of mouse urinary tract infection (Johnson, Clabots, Hirt, Waters, & Dunny, 2004), it has been reported to increase the severity of infective endocarditis by increasing *E. faecalis* vegetation (biofilm) weights on aortic heart valves (Chuang, Schlievert, Wells, Manias, TRipp, & Dunny, 2009). In recent work using an *ex vivo* porcine heart valve adherence model (Chuang-Smith, Wells, Henry-Stanley, & Dunny, 2010), it has been shown that AS significantly accelerated biofilm development on this biotic surface, relative to abiotic membranes, which implies that there is a significant role for AS *in vivo*. Reconciling all of these observations into a single model for the contribution of aggregation substance to the molecular pathogenesis of infection is an ongoing process, but it seems fairly certain to promote interbacterial association, which directly or indirectly contributes to increased adherence to human tissues, increasing the likelihood of formation of a quorum of E. faecalis cells and subsequent expression of factors that are dependent on quorum-sensing signals, such as the cytolysin or gelatinase.

Enterococcal Surface Protein, Esp

One of the first cell wall-associated proteins implicated in biofilm formation *in vitro* was the enterococcal surface protein Esp. First identified in *E. faecalis* as a large surface-anchored protein enriched among infection-derived isolates (Shankar V. , Baghdayan, Huycke, Lindahl, & Gilmore, 1999), it led to the discovery of a homolog in *E. faecium* (Willems, et al., 2001; Coque, Willems, Cantón, Del Campo, & Baquero, 2002; Eaton & Gasson, 2002). Subsequent studies localized the *esp* gene to pathogenicity islands in both species (Leavis, et al., 2004; Shankar, Baghdayan, & Gilmore, 2002). An early report suggested that there was a strong correlation of *E. faecalis esp* with the ability to form biofilms, with 93.5% of *esp*-positive isolates forming biofilms on polystyrene, while none of the *esp*-negative isolates did so (Toledo-Arana, et al., 2001). Biofilm formation was impaired in two *esp* insertion mutants, but not in a third strain tested, leading the authors to conclude that while *esp* was important for biofilm formation, it was probably one among many other factors in *E. faecalis* that mediated this phenotype.

Using a genetic approach, Tendolkar *et al* (Tendolkar, Baghdayan, Gilmore, & Shankar, 2004) showed increased biofilm formation by two *E. faecalis* strains OG1RF and FA2-2 (natively lacking the *esp* gene) when transformed with plasmid constructs expressing *esp*. However, a similar effect was not observed when *esp* from *E. faecalis* MMH594 was expressed in *E. faecium* or *L. lactis*, which led the authors to conclude that Esp may act in concert with other surface factors unique to *E. faecalis*. In a follow-up study, the same authors also showed that the non-repeat N-terminal region of mature Esp was sufficient for biofilm enhancement (67). Related studies in *E. faecium* established a high degree of sequence identity between Esp from *E. faecalis* and *E. faecium,* and further showed that primary adherence and biofilm formation correlated with levels of Esp expression at the cell surface (Leavis, et al., 2004; Van Wamel, Hendrickx, Bonten, Top, Posthuma, & Willems, 2007).

The multifactorial nature of biofilm formation by enterococci was borne out by one study that demonstrated biofilm formation by *E. faecalis* strain OG1RF lacking *esp* (Kristich, Li, Cvitkovich, & Dunny, 2004) and another that made the observation that 77 of 89 *esp*-negative clinical isolates of *E. faecalis* were also able to form biofilms categorized as weak, medium, or strong (69). However, all 74 *esp*-positive isolates in this study formed biofilms, strengthening the argument that, when present, *esp* significantly enhances biofilm formation. A number of other recent reports that examined *esp*-positive and *esp*-negative *E. faecalis* and *E. faecium* have made similar observations (Di Rosa, et al., 2006; Sandoe, Witherden, Cove, Heritage, & Wilcox, 2003; Duprè, Zanetti, Schito, Fadda, & Sechi, 2003; Dworniczek, et al., 2003; Raad, et al., 2005; Ramadhan & Hegedus, 2005). Murine intestinal colonization models failed to show a definitive role for *esp* in *E. faecalis* or *E. faecium* in gut colonization or persistence (Pultz, Shankar, Baghdayan, & Donskey, 2005; Heikens, et al., 2009). However, in a mouse ascending urinary tract infection model, *esp*-deficient *E. faecalis* were recovered in lower numbers from the bladder and urine of infected mice as compared to the wild type strain, which suggests that Esp may facilitate persistence and colonization at this site (Shankar, Lockatell, Baghdayan, Drachenberg, Gilmore, & Johnson, 2001).

Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs)

Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (MSCRAMMs) facilitate the colonization of host tissues and binding of bacterial cells to indwelling abiotic surfaces that become coated with host-derived extracellular matrix components. Both *E. faecalis* and *E. faecium* appear to have more than a dozen MSCRAMMs apiece, as deduced from genome sequences currently available (Sillanpää, Nallapareddy, Prakash, Qin, Höök, & Weinstock, 2008; Sillanpää, Xu, Nallapareddy, Murray, & Höök , 2004). Adhesion of collagen from *E. faecalis* (Ace) was the first of three MSCRAMMs that have been extensively characterized in enterococci, and have been shown to bind to collagen type I, collagen type IV, laminin, and dentin (Kowalski, Kasper, Hatton, Murray, Nallapareddy, & Gillespie, 2006; Nallapareddy S. R., Singh, Duh, Weinstock, & Murray, 2000; Nallapareddy, Qin, Weinstock, Höök, & Murray, 2000). An Ace homolog in *E. faecium*, Acm, predominantly interacts with collagen type I and interacts somewhat weakly with collagen type IV (Nallapareddy S. R., et al., 2006; Nallapareddy & Murray, 2006; Nallapareddy, Weinstock, & Murray, 2003). A second collagen adhesion of *E. faecium*, Scm, was shown to mediate binding to collagen type V and fibrinogen (Sillanpää, Nallapareddy, Prakash, Qin, Höök, & Weinstock, 2008). These three MSCRAMMs were ubiquitous among both clinical and non-clinical isolates tested; however, *E. faecium* isolates of non-clinical origin lacked binding to collagen type I and showed an insertion element disruption of the *acm* gene (Nallapareddy S. R., Singh, Okhuysen, & Murray, 2008). The structural features of these enterococcal MSCRAMMs have been described in a recent review (Hendrickx, Willems, Bonten, & van Schaik, 2009).

In vitro studies have shown that microspheres coated with the A domain of *E. faecalis* Ace can mediate binding to human intestinal and umbilical vein endothelial cells, a process that could be blocked by either soluble collagen or Ace-specific antibodies (Hall, et al., 2007). An *ace*-deletion mutant has recently been shown to be significantly attenuated, as compared to wild-type OG1RF in a mixed-infection rat endocarditis model ($P <$

0.0001), while no differences were observed in a peritonitis model (Singh K. V., Nallapareddy, Sillanpää, & Murray, 2010). In these experiments, Ace appeared to be important to the early colonization of heart valves, and rats actively immunized against *r*Ace were less likely to develop *E. faecalis* endocarditis ($P = 0.0001$) and showed fewer counts in vegetations ($P = 0.0146$). A deletion mutant of *ace* has also been shown recently to be less virulent in an insect (*Galleria mellonella*) virulence model (Lebreton, et al., 2009) and attenuated in an experimental UTI model (87). Likewise, an *acm* deletion mutant has been shown to be less virulent in a rat endocarditis model (Nallapareddy, Singh, & Murray, 2008).

Two other MSCRAMMs in *E. faecium* have been recently reported (Hendrickx, et al., 2009). The surface adhesion SgrA binds to extracellular matrix molecules nidogen 1 and nidogen 2, while EcbA mediates binding to collagen type V. An *E. faecium sgrA* insertion mutant displayed reduced binding to both nidogens and fibrinogen. SgrA did not mediate binding of *E. faecium* to human intestinal epithelial, bladder, or kidney cells, but did contribute to *E. faecium* biofilm formation *in vitro*.

Pili

Expression of pili at the bacterial cell surface facilitates adhesion, which is the first step in the biofilm process. In Gram-positive organisms, the synthesis of pili is a complex process that involves the assembly of pilin subunits into a pilus polymer, which is eventually anchored to the cell wall through the action of housekeeping and dedicated sortase enzymes (Mandlik, Swierczynski, Das, & Ton-That, 2008). Genes for pilus biosynthesis are generally clustered as operons called pilin gene clusters (PGCs). The pilin protein subunits are known to contain MSCRAMM-like features, as well as conserved pilin motifs (HLYPK) and E box motifs (ETxAPExY) that facilitate assembly of the pilus. Gram-positive pili appear to play a major role in mediating cell-cell contact, colonization of host tissue, and biofilm formation (Telford, Barocchi, Margarit, Rappuoli, & Grandi, 2006).

Pilus-like structures in *E. faecalis* JH2 were reported long before the genetic basis for pilus biogenesis was worked out (Handley & Jacob, 1981). Subsequent to the sequencing of multiple enterococcal genomes, it became apparent that both *E. faecalis* and *E. faecium* may harbor PGCs that encode proteins bearing the classic motifs identified in pilin subunits (Nallapareddy S. R., et al., 2006; Tendolkar, Baghdayan, & Shankar, 2006; Hendrickx, Bonten, van Luit-Asbroek, Schapendonk, Kragten, & Willems, 2008). Two PGCs have been reported in *E. faecalis*, the ubiquitous endocarditis and biofilm-associated pili (*ebp*) operon (Nallapareddy S. R., et al., 2006; Cobo Molinos, Abriouel, Omar, López, & Galvez, 2008), and the much less frequently detected and plasmid encoded *bee* locus (Tendolkar, Baghdayan, & Shankar, 2006). In E. faecium, four PGCs have been described, all of which appear to be enriched among hospital-derived *E. faecium* isolates (Hendrickx, Bonten, van Luit-Asbroek, Schapendonk, Kragten, & Willems, 2008).

The detection of antibodies to Ebp subunits in the sera isolated from endocarditis patients suggests that this pilus plays an important role in this biofilm-type infection (Nallapareddy S. R., et al., 2006). Mutants defective in expression of one or more of the subunits or the dedicated sortase, SrtC, were found to be attenuated in attachment and biofilm formation *in vitro*. Further, recent data has also revealed a role for the Ebp pilus in mediating binding to human platelets (Nallapareddy, et al., 2011) and fibrinogen, with a lesser role in binding to collagen (Nallapareddy S. R., Singh, Sillanpää, Zhao, & Murray, 2011). The *ebp* locus was also found to contribute to virulence in a mouse ascending UTI model (Singh, Nallapareddy, & Murray, 2007), although it appeared that other factors may play a role in this model.

The roles of housekeeping sortase SrtA and Ebp pilus-specific sortase Bps (SrtC) have been evaluated for their relative contribution to biofilm formation in *E. faecalis* (Kemp, Singh, Nallapareddy, & Murray, 2007). A *srtA* deletion mutant of OG1RF showed little effect on biofilm formation ($P = 0.037$) as compared to the wild type, whereas both a *bps* deletion mutant and *srtA-bps* double deletion mutant showed significantly impaired biofilm formation ($P < 0.001$) relative to its parent strain. These data were interpreted as suggesting that Bps, and/or the protein(s) that it anchors, may compensate for some of the SrtA functions. Examining biofilm formation under

static and hydrodynamic conditions others have determined that SrtA primarily functions during the initial attachment phase, which involves the initiation of biofilm formation (Guiton, et al., 2009).

Although it is ubiquitous among *E. faecalis* isolates, the expression of the Ebp pilus varies widely within a population of cells (30-72%), depending on growth conditions (Nallapareddy, et al., 2011). The *ebp* locus is under positive control of the EbpR regulator, which in turn is under the control of *fsr* (BourgogneA., Singh, Fox, Pflughoeft, Murray, & Garsin, 2007). A significant enhancement in the expression of the Ebp pilus was noted when cells were exposed to bicarbonate—although the precise mechanism for this effect remains to be deciphered (Bourgogne, Thomson, & Murray, 2010). An effect of RNA processing on Ebp pilus expression was also noted, whereby deletion of *rnjB* encoding RNase J2 decreased transcript levels for the *ebp* operon (Gao, Pinkston, Nallapareddy, van Hoof, Murray, & Harvey, 2010).

The *bee* locus was identified as a cluster of five genes encoded on a large conjugative plasmid in an *E. faecalis* strain E99 (Tendolkar, Baghdayan, & Shankar, 2006; Coburn P. S., et al., 2010). The structural subunits of the Bee pilus are Bee-1, Bee-2, and Bee-3, which bear the cell wall sorting and pilin motifs characteristic of pilin subunits. Unlike in the *ebp* operon, the *bee* structural genes are followed by two sortase genes in tandem. While pilus expression at the cell surface has been confirmed by electron microscopy in strain E99, as well as in *E. faecalis* OG1RF and JH2-2 transconjugants, the role of the Bee pilus in infection models remain to be established, although a pilot study concluded that the Bee pilus did not contribute to virulence or persistence in a mouse model of UTI (unpublished). However, in *in vitro* biofilm assays, the Bee pilus has been shown to confer a high biofilm phenotype upon *E. faecalis* (Tendolkar, Baghdayan, & Shankar, 2006).

E. faecium clinical isolates, including those from endocarditis, harbor genetic loci that encode two pilus-like structures, PilA and PilB (Hendrickx, Bonten, van Luit-Asbroek, Schapendonk, Kragten, & Willems, 2008; Hendrickx, Schapendonk, van Luit-Asbroek, Bonten, van Schaik, & Willems, 2010). The *pilA* locus is encoded on a large conjugative plasmid (Kim, et al., 2010) and appears to be a minor pilus, while the *pilB* gene was found to be part of the *ebpABC(fm)* cluster (Sillanpää, et al., 2010) that encodes the major pilus PilB. The differential expression of *pilA* and *pilB* was noted, with the former being expressed only on solid growth media, while the latter was also expressed in broth. Loss of PilB was evident in a *ebpABC* deletion mutant with concomitant reduction in *in vitro* biofilm formation and attenuated virulence of the mutant in a murine UTI model, as compared to wild type (Sillanpää, et al., 2010).

Polysaccharides

An *E. faecalis* polysaccharide antigen locus (*epa*) has been described (Teng, Jacques-Palaz, Weinstock, & Murray, 2002) as being involved in synthesis of cell wall-associated polysaccharides, which contributes to biofilm formation, among other virulence properties. Biofilm formation was specifically attenuated by mutations in *epaB* and *epaE*. A more recent characterization has defined the *epa* locus to be a 26-kb region comprising genes *epaA* to *epaR* (Teng, Singh, Bourgogne, Zeng, & Murray, 2009). Similar to that observed with epaB and epaE mutants, disruptions in *epaA*, *epaM*, and *epaN* resulted in the alteration of Epa polysaccharide content and decreased biofilm formation.

A putative sugar binding transcriptional regulator, BopD, which shows sequence similarity with a number of proteins involved in maltose metabolism, has also been shown to influence biofilm formation in *E. faecalis* (Hufnagel, Koch, Creti, Baldassarri, & Huebner, 2004). By comparing a transposon insertion mutant and a deletion mutant, a sugar-specific effect on biofilm production was observed (Creti, Koch, Fabretti, Baldassarri, & Huebner, 2006). When grown in medium containing 1% glucose, the transposon mutant produced less biofilm and the non-polar deletion mutant produced more biofilm than the wild type. However, in the presence of 1% maltose in the medium, the transposon mutant produced more biofilm than the wild type and biofilm formation by the deletion mutant was abrogated. These data suggested that BopD was likely to be a maltose-sensitive negative regulatory protein that may repress both *bopABC* and the divergently transcribed *malT* operon. The

lower expression of *bopD* in the transposon mutant could thus lead to the derepression of *bopA* (a maltose phosphorylase) and of the sugar transport gene *malT,* which may also lead to the increased utilization of maltose and enhanced biofilm formation when bacteria are grown in maltose.

Secreted factors, autolysin and eDNA

The zinc metalloprotease GelE is ubiquitous among *E. faecalis* strains, and is a significant factor in promoting the formation of biofilm (Hancock & Perego, 2004). The *gelE* determinant is adjacent and upstream of *sprE,* the determinant that encodes a serine protease. Both genes are cotranscribed and are under the control of the *fsr* quorum sensing system (Qin, Singh, Weinstock, & Murray, 2001). A detailed description of the organization and regulation of this operon is presented later in this chapter. The influence of gelatinase and the *fsr* locus in relation to biofilm development have been described (Mohamed, Huang, Nallapareddy, Teng, & Murray, 2004; Hancock & Perego, 2004; Thomas, Thurlow, Boyle, & Hancock, 2008; Carniol & Gilmore, 2004), and both GelE and SprE act in concert with autolysin (AtlA) processing to regulate cell death and eDNA release by a process akin to fratricide (Guiton, et al., 2009; Thomas, Thurlow, Boyle, & Hancock, 2008; Thomas, Hiromasa, Harms, Thurlow, Tomich, & Hancock, 2009; Thomas & Hancock, 2009). Additional confirmation of the importance of GelE in biofilm formation was put forth by Kristich *et al* (Kristich, Li, Cvitkovich, & Dunny, 2004), who showed that conditioned media from OG1RF (gelatinase positive) promoted biofilm formation by *E. faecalis* JH2, a poor biofilm former, as did GelE expressed under a nisin-inducible promoter in JH2.

In biofilm-associated infection models, both GelE and SprE were found to be important for virulence in experimental rat endocarditis (Singh K. V., Nallapareddy, Nannini, & Murray, 2005). A double *gelE*/*sprE* mutant were found to be more attenuated for virulence then a *fsr* mutant affecting GelE production in this model; however, the opposite effect was seen in the rabbit endophthalmitis (Mylonakis, et al., 2002) and *C. elegans* (Sifri, et al., 2002) models. It has also been shown in the rabbit endocarditis model that GelE contributes to increased bacterial counts at disseminated sites of infection (Thurlow, Thomas, Narayanan, Olson, Fleming, & Hancock, 2010).

Other enterococcal biofilm mediators

A number of different studies have attempted to identify additional factors that mediate biofilm formation. Inactivation of the biofilm-associated glycolipid synthesis A (*bgsA*) gene that encodes a putative glycosyltransferase in *E. faecalis* had a significant impact on initial adherence and biofilm formation on plastic, adhesion to colonic epithelial cells, and virulence in a mouse bacteremia model (Theilacker, et al., 2009). No changes were observed in levels of surface protein expression, autolysis, or sensitivity to antimicrobial peptides. In similar studies, a *dltA* mutant of *E. faecalis* 12030 lacking D-alanine esters in lipoteichoic acid (LTA), due to the disruption within the *dltABCD* operon, showed reduced *in vitro* biofilm formation and binding to eukaryotic cells (Fabretti, et al., 2006). However, a *dltA* mutant in *E. faecalis* OG1RF failed to show the same effect. Two secreted *E. faecalis* proteins, SalA and SalB, homologous to the *E. faecium* SagA protein, have been implicated in *in vitro* biofilm formation on polystyrene (Mohamed, Teng, Nallapareddy, & Murray, 2005; Breton, Mazé, Hartke, Lemarinier, Auffray, & Rincé, 2002). The effect was more pronounced in the *salB* mutant, which exhibited marked morphological changes, as well as a 54% reduction in biofilm production. A putative *E. faecalis* surface-exposed antigenic protein EF3314 that showed a delayed effect on biofilm after culturing in medium containing glucose diminished adherence of the deletion mutant to HeLa cells and less killing in a *C. elegans* model (Creti, et al., 2009).

Two independent genome-wide approaches have recently identified a number of new and novel genetic determinants in the core genome of *E. faecalis* OG1RF that influence biofilm formation (Ballering, Kristich, Grindle, Oromendia, Beattie, & Dunny, 2009; Kristich, Nguyen, Le, Barnes, Grindle, & Dunny, 2008). The first approach employed a modified mini-mariner transposable element (EfaMarTn) that produced mostly single insertions distributed throughout the genome (Kristich, Nguyen, Le, Barnes, Grindle, & Dunny, 2008). Mutants

were screened for impaired biofilm formation *in vitro* on polystyrene, and led to the identification of not only a number of the previously identified loci, but also additional genes, such as those that encode heat-shock proteins (GrpE, DnaK, and DnaJ), as well as putative regulators (EF0676 and EF0983) that belong to the ArgR family. In the second study, a recombinase-in vivo expression technology (RIVET) approach was used to identify chromosomally-encoded biofilm determinants (Ballering, Kristich, Grindle, Oromendia, Beattie, & Dunny, 2009). This study identified 68 different promoters that were active at different stages of biofilm growth, with at least 17 conserved hypothetical genes, and 7 of them were predicted to encode DNA-binding proteins not previously associated with biofilms. Using the same approach, a more recent study has highlighted the way in which the RIVET screen may provide information on temporal activation of genes during infection (Frank, Barnes, Grindle, Manias, Schlievert, & Dunny, 2012). In this study, deletions in the *in vivo*-activated *eep* gene (encoding a membrane metalloprotease, Eep) affected the cellular organization of *in vitro* biofilms, and the deletion mutant was severely attenuated in a rabbit endocarditis model. Microscopic analysis of *in vitro* biofilms revealed numerous small aggregates not present in wild-type biofilms. Another independent study that evolved from the results of the RIVET screen has shown that in the presence of uric acid, which is freely available in both urine and blood, *E. faecalis* may shift from a planktonic to biofilm mode of growth (Srivastava, Mallard, Barke, Hancock, & Self, 2011). Enhanced biofilm production was seen to be a selenium-dependent process, driven through the production of peroxide, and mutations in the *selD* (selenophosphate synthetase) and *xdh* (xanthine dehydrogenase) genes that reside within the selenium-dependent molybdenum hydroxylase (SDMH) operon both abolished biofilm formation.

Regulation of Biofilm Development

The recent increase of literature on the topic of enterococcal biofilms has coincided with the emergence of accessible genomic information, starting with the published sequence of the first enterococcal genome in 2003 (Paulsen, et al., 2003). Since that time, various enterococcal factors have been shown to contribute to *in vitro* biofilms (Paganelli, Willems, & Leavis, 2012), while only a handful of factors have also been shown to play a key role in pathogenesis (Frank, Barnes, Grindle, Manias, Schlievert, & Dunny, 2012; Nallapareddy S. R., et al., 2006; Singh, Nallapareddy, & Murray, 2007; Shankar, Lockatell, Baghdayan, Drachenberg, Gilmore, & Johnson, 2001; Singh K. V., Nallapareddy, Nannini, & Murray, 2005; Thurlow, Thomas, Narayanan, Olson, Fleming, & Hancock, 2010). Pili and Esp are known to be important adhesins for attachment to biotic and/or abiotic surfaces, but the subsequent steps that lead to organization of the community structure of the biofilm are still poorly defined. Here we focus on what is known about such mechanisms, as well as raise the specter of additional signaling pathways that may facilitate such development. Because *E. faecalis* causes the majority of enterococcal infections, much of our understanding of the way in which cell signaling influences biofilm development comes from studies on this species.

Signal Transduction in Enterococcal Biofilms

In microbial species, the best characterized signal transduction system involves a two-component system comprised of a histidine kinase and its cognate response regulator (Hoch & Silhavy, 1995). As the histidine kinase perceives a sensory signal, it undergoes autophosphorylation and subsequently transfers a phosphoryl group to the response regulator. The phosphorylated form of the response regulator allows it to regulate gene expression. An analysis of the *E. faecalis* V583 genome sequence revealed that it possesses 17 paired two-component systems and an additional orphan response regulator (Hancock & Perego, 2002). Systematic inactivation and phenotypic characterization of the 18 response regulator mutants in V583 showed that mutant RR15 was attenuated in its biofilm-forming ability (Hancock & Perego, 2004). The response regulator disrupted in this mutant was previously characterized by Qin et al. (Qin, Singh, Weinstock, & Murray, 2001) as being part of the quorum-sensing signal transduction system Fsr, which responds to the accumulation of GBAP, an 11 amino acid peptide lactone (Nakayama, et al., 2001). The Fsr quorum system of *E. faecalis* partially resembles the staphylococcal Agr system (Novick & Geisinger, 2008), where a histidine kinase (FsrC) responds to the

accumulation of a quorum peptide (GBAP) to phosphorylate the cognate response regulator (FsrA). Del Papa and Perego (Del Papa & Perego, 2011) recently showed that the phosphorylated form of FsrA is required in order to recognize its target DNA sequence to promote transcription. The production of GBAP arises from the processing of a 53 amino acid precursor propeptide encoded by *fsrD* (Nakayama, et al., 2006). Processing of the propeptide and export by the membrane-localized FsrB drives the external accumulation of GBAP. Several genes have been shown to be directly regulated by the Fsr system (Bourgogne, Hilsenbeck, Dunny, & Murray, 2006; Qin, Singh, Weinstock, & Murray, 2001) and include autoregulation of the *fsrBDC* operon, as well as the co-transcribed *gelEsprE* genes, which encode two extracellular proteases, gelatinase (GelE) and serine protease (SprE).

Several independent laboratories have established a role for the Fsr system and proteases in biofilm formation (Kristich, Li, Cvitkovich, & Dunny, 2004; Pillai, Sakoulas, Eliopoulos, Moellering, Jr., Murray, & Inouye, 2004; Mohamed, Huang, Nallapareddy, Teng, & Murray, 2004; Hancock & Perego, 2004). Thomas et al. (Thomas, Thurlow, Boyle, & Hancock, 2008) went on to show that the production of GelE, a zinc metalloprotease, resulted in the release of extracellular DNA (eDNA), presumably through cell lytic processes. Consistent with observations from other bacterial pathogens (Montanaro, et al., 2011), eDNA proved to be an important early biofilm matrix component, as treatment of developing *E. faecalis* biofilms with DNase I significantly reduced biofilm accumulation at 6 and 12 hours, but not at 24 hours. A role for eDNA as a matrix component in *E. faecalis* OG1RF was also reported by Guiton et al. (Guiton, et al., 2009), who showed that the major autolysin (Atn), AtlA, also contributed to eDNA release. A role for Atn in biofilm development was initially reported by Mohamed et al. (Mohamed, Huang, Nallapareddy, Teng, & Murray, 2004), and was subsequently confirmed by a mariner-based transposon screen to identify biofilm defective mutants (Kristich, Nguyen, Le, Barnes, Grindle, & Dunny, 2008). From a pool of ~15,000 transposon mutants, 25 were biofilm-defective, and three mapped to transposon insertions in the *atn* gene (Kristich, Nguyen, Le, Barnes, Grindle, & Dunny, 2008). This same screen further highlighted the importance of the Ebp pilus in biofilm formation, as nearly one-third of the biofilm-defective mutants mapped to this locus (EbpR, EbpABC or SrtC).

Thomas et al. presented a mechanism for how a quorum response mediated through Fsr could give rise to a lytic process that governs biofilm development (Thomas, Thurlow, Boyle, & Hancock, 2008; Thomas, Hiromasa, Harms, Thurlow, Tomich, & Hancock, 2009). These studies implicated GelE as being a pro-lytic effector of lysis, as the deletion of *gelE* rendered the cells less susceptible to autolysis. In contrast, the deletion of *sprE* resulted in a more rapid rate of lysis, which suggests that it possessed anti-lytic properties. The observation that these two proteases governed the lytic properties of the cell in opposing ways suggested that they might target the same downstream effector. The use of cell wall zymography identified AtlA as being differentially targeted by GelE and SprE. The targeting of AtlA by GelE results in multiple AtlA active forms, whereas SprE processes AtlA to a discrete ~ 62 kDa active form that has a high affinity for cell walls and renders this AtlA form immune to further processing by GelE. Mutation of *atlA* also gives rise to a cellular chaining phenotype (Qin, Xu, Singh, Weinstock, & Murray, 1998), and this phenotype is also partially observed in a *gelE* mutant (Waters, Antiporta, Murray, & Dunny, 2003), which provides further evidence for an association of these proteins.

Fratricide

The observation that cell-cell communication through quorum signaling resulted in the co-transcription of genes whose products trigger opposing roles in cell death is reminiscent of competence development in *Streptococcus pneumoniae* (Claverys & Håvarstein, 2007). In the pneumococcal model, termed fratricide (Gilmore & Haas, 2005), the competence-stimulating peptide serves as a quorum molecule to activate the early competence regulon, which includes cell wall lytic enzymes and bacteriocins, as well as immunity proteins that prevent self-induced killing. In the *E. faecalis* model of fratricide (Figure 1) and biofilm development, cells that respond to GBAP enhance expression of both GelE (effector) and SprE (immunity), whereas quorum non-responders fail to express these proteins and would therefore be lysis-susceptible. Consistent with a requirement

for a bimodal population of quorum responders and non-responders, roughly 10-15% of the cells failed to respond to the quorum signal in stationary phase cultures, as noted by FACS analysis with a *gelE*-GFP reporter fusion (Thomas, Hiromasa, Harms, Thurlow, Tomich, & Hancock, 2009). Non-responders would then be more susceptible to activation of their AtlA by GelE diffusing from a neighboring cell in the absence of SprE. This was demonstrated through co-culture experiments, in which a GFP-tagged prey population ($GelE^-SprE^-$) was mixed with various predator populations. When $GelE^+$ strains served as the predator, lysis of the prey occurred, and this lytic activity was enhanced in the absence of SprE activity. A dependence on functional AtlA is also required in this model, as mutation of *atlA* in both the predator and prey populations is necessary to abolish lysis, suggesting that soluble forms of AtlA derived from the predator can also mediate prey lysis. The susceptibility of the prey or non-responder population to lysis would release eDNA to serve as a biofilm matrix component. The rates of diffusion away from producing cells, as well as an affinity for AtlA by GelE and SprE, would likely govern the extent of non-responder death. As death in *E. faecalis* biofilms occurs in only a small minority of cells, it suggests that this process is highly regulated to favor survival.

Cell-cell communication

In addition to the aforementioned Fsr quorum signaling in cell-cell communication, one of the hallmarks of *E. faecalis* biology is the use of peptide pheromones secreted by recipient cells to induce the conjugative apparatus of the donor cell to mediate the transfer of pheromone responsive plasmids (reviewed in Extrachromosomal and Mobile Elements in Enteroocci). A recent study by Frank et al. (Frank, Barnes, Grindle, Manias, Schlievert, & Dunny, 2012) identified the regulated intramembrane protease Eep as being important to the endocarditis model of infection, as an *eep* mutant was attenuated by 3-logs compared to the wild-type OG1RF strain. Eep was originally identified by Clewell's group (An, Sulavik, & Clewell, 1999) as an enhancer of enterococcal pheromones. As peptide pheromones are known to be derived from the signal sequence of putative lipoproteins (An & Clewell, 2002; Antiporta & Dunny, 2002), Eep is required to further process the signal peptide into the active pheromone (Chandler & Dunny, 2008). The fact that an *eep* mutant is highly attenuated in the endocarditis model, and that this model provides an ideal environment to study *in vivo* biofilms, raises the intriguing prospect that peptide pheromones might be playing an important signaling role in biofilm development (Frank, Barnes, Grindle, Manias, Schlievert, & Dunny, 2012). This potential peptide pool arising from the numerous lipoprotein signal peptides could provide a treasure trove of signaling molecules by which biofilm development could be modulated. Consistent with this prediction, *in vitro* imaging of *eep* mutant biofilms compared to the isogenic parent OG1RF revealed altered biofilm architecture in the mutant with small cellular aggregates. Whether peptide pheromones play roles in biofilm development beyond the mating response awaits further investigation.

A large number of bacterial species, including enterococci, possess the capacity to produce autoinducer-2 (AI-2) (Schauder, Shokat, Surette, & Bassler, 2001; Federle, 2009). Unlike other cell-cell signaling pathways reserved for intraspecies communication, AI-2 signaling has evolved to permit crosstalk among bacteria from varying genera that might occupy the same environmental niche (Kaper & Sperandio, 2005). The production of AI-2 is driven by the enzyme LuxS, which is centered on the metabolism of S-adenosyl methionine as it converts ribose-homocysteine into homocysteine and 4.5-dihydro-2,3-pentanedione, which spontaneously cyclizes in the presence of water to produce furanone, the precursor of AI-2 (Schauder, Shokat, Surette, & Bassler, 2001). AI-2 signaling is known to be important in biofilm formation in firmicutes closely related to enterococci, such as *S. pneumoniae* (Vidal, Ludewick, Kunkel, Zähner, & Klugman, 2011). A recent study has linked AI-2 signaling with fratricide and competence development in pneumococci (Trappetti, Potter, Paton, Oggioni, & Paton, 2011). The known link between fratricide and enterococcal biofilms raises the question of whether AI-2 signaling might also play a role in this process, but this examination will await further study. What is clear is that the study of cell-cell communication in enterococcal biofilm development portends an exciting and potentially fruitful area of research.

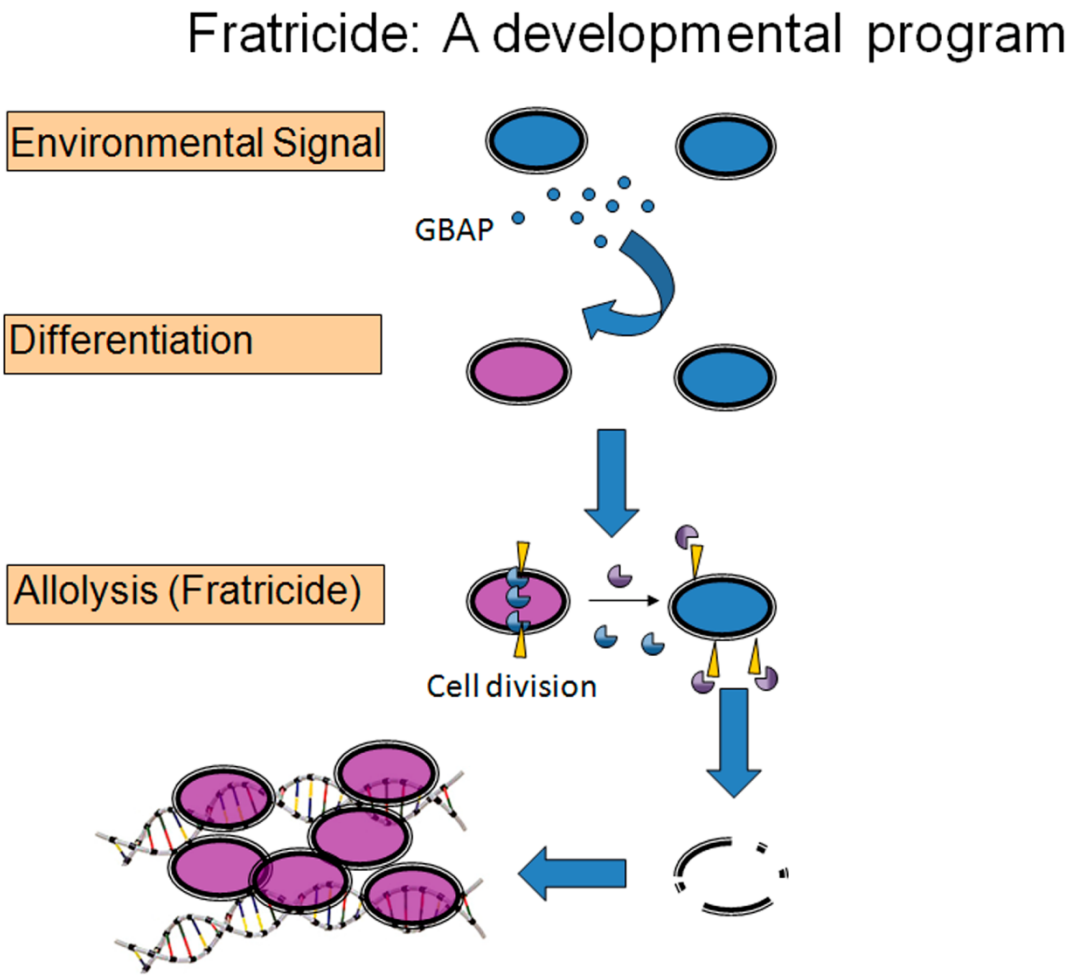

Figure 1: Model of fratricide mediated lysis in *E. faecalis* biofilm development. Cells within a population respond differently to the quorum signaling molecule GBAP. Responder cells (shaded in purple) induce the production of GelE ( ) and SprE ( ). Non-responders (shaded in blue) fail to induce expression of GelE and SprE. Bioinformatic evidence suggest that SprE, by nature of a YSIRK motif in the signal peptide sequence, initially localizes at the division septum of the responder cells to assist in processing of AtlA ( ) to a mature form, rendering AtlA resistant to further proteolytic processing by GelE. Targeting of AtlA to the septum assists in cell division. The production of GelE and SprE by responder cells diffuses to target AtlA bound on the surface of the non-responder population. Processing of AtlA by GelE produces several enzymatically active variants which cause the lysis of the non-responder cell. Genetic and biochemical evidence suggests that SprE can also protect non-responder cells, but the mechanism is less clear. In addition, a GelE gradient away from the responder cell may also regulate the extent of AtlA activation and turnover to prevent lysis of the responder population. Ultimately, the release of nutrients as well as extracellular DNA from lysed cells serves as a scaffold on which responder cells initiate biofilm formation (Antiporta & Dunny, 2002).

Future Directions

Since the publication of the first edition of this book, many more databases and experimental tools have become available for studies of enterococcal biofilms. These have great potential to address many current gaps in our understanding, including the following areas.

Genetic basis for biofilm formation

While a great deal of progress has been made in the identification of biofilm determinants, a comprehensive list of these determinants has not been generated. We still do not know whether unique biofilm functions actually exist, what the relative contributions of the conserved core genome versus mobile elements to biofilm-formation

are, or the true impact of biofilm growth on expression and transfer of antibiotic resistance. Several researchers have suggested that new paradigms for antimicrobial development are needed to overcome the rapid increases in resistance to currently available antimicrobials. Strategies such as preventing disease production without directly killing or inhibiting growth, or targeting the genetic transfer machinery, should be considered in this regard (Rasko & Sperandio, 2010; Baquero, Coque, & de la Cruz, 2011). Gene products involved in biofilm growth and biofilm-associated antibiotic resistance certainly represent potential targets for alternative approaches to drug development.

Biofilms and pathogenesis

The "gold standard" for implicating a particular bacterial determinant in pathogenesis is the demonstration of attenuation of virulence in an animal model upon disruption of the determinant. It will be essential to continue to examine the effects of mutations that affect biofilm formation *in vitro* in relevant animal models, such as experimental endocarditis and urinary tract infections. Comparative studies using these models, in conjunction with ex vivo tissue, tissue culture cell, insect, or nematode systems, may be informative in determining the relative contributions of attachment and surface growth functions with evasion of host immunity. For example, it has been shown that AS expression is associated with resistance to phagocytic killing (Süßmuth, Muscholl-Silberhorn, Wirth, Susa, Marre, & Rozdzinski, 2000; Rakita, et al., 1999), as well as with enhancing attachment and biofilm development on heart valves *in vitro* (Chuang-Smith, Wells, Henry-Stanley, & Dunny, 2010). Thus, it is likely that the AS-mediated enhancement of virulence in experimental endocarditis results in multiple functions of the protein, which are likely encoded in separate domains (Chuang, Schlievert, Wells, Manias, TRipp, & Dunny, 2009).

Sensing and signaling

A previous section of this chapter described the important role of *fsr*-mediated quorum sensing in regulation of proteases controlling fratricidal cell lysis and DNA release *E. faecalis* biofilm development. This is the only well-documented example of an important effect of cell-cell signaling in enterococcal systems that fits well with the developmental model for microbial biofilms. However, recent work has shown that biofilm growth affects the dynamics of peptide pheromone signaling as well as its control of plasmid transfer (Cook, Chatterjee, Barnes, Yarwood, Hu, & Dunny, 2011). Given the genetic potential for *E. faecalis* to produce a plethora of extracellular peptide signals, it would not be surprising to find new cell-cell communication systems that affect biofilm development. In addition, the mechanism(s) by which the initial attachment of a planktonic cell to a surface triggers the physiological transition from planktonic to biofilm growth is not known. The answer to this question may represent the "holy grail" for all biofilm researchers, and finding it will require analysis of events that occur very early in biofilm development. Comparatively little analysis of the initiation of the process has been done, and the tiny amount of cell-associated microbial biomass present at this stage likely precludes standard "-omics" approaches. Success in addressing this topic will likely come from the creative use of genetic and microscopic tools. The most attractive drug or vaccine targets may be those required to complete the initial transition to biofilm growth, so their identification and characterization is critical.

The extracellular matrix

Based on work with other bacteria, it is likely that polysaccharides, DNA, and protein comprise a substantial portion of the enterococcal biofilm matrix, and the importance of eDNA in the structure and adhesive function of the enterococcal matrix is well documented, as noted above. High-resolution scanning electron microscopy (Figure 2) and transmission electron microscopy suggest an important structural role of anionic polysaccharides in the enterococcal matrix (Erlandsen, Kristich, Dunny, & Wells, 2004), and genetic evidence is also supportive of this finding (Teng, Jacques-Palaz, Weinstock, & Murray, 2002; Teng, Singh, Bourgogne, Zeng, & Murray, 2009; Singh, Lewis, & Murray, 2009). However, a detailed biochemical characterization of the extracellular matrix of

an enterococcal biofilm has not been presented, and studies of the way in which the matrix changes temporally or in different growth conditions have not been reported. Likewise, while emerging information from *Staphylococci* point to mechanisms of biofilm disassembly that involve the production of extracellular proteases, deoxyribonucleases, and surfactants, little such information is known in enterococci (Boles & Horswill, 2011). It will be essential to obtain this information in the future to complement continuing genetic analyses of biofilm formation and dispersal.

Biofilm and the evolution of the adaptable enterococcal life style

Perhaps the most remarkable aspect of enterococci is their ability to survive and proliferate in a remarkable diversity of host-associated and environmental niches that are lethal to many phylogenetically-related pathogens. How does biofilm-forming ability impact the fitness of enterococci in these diverse environments, and how many genetic determinants important to one niche play a role in others? Since much of the evolution of the core genomes of these organisms occurred outside a mammalian host, further study of biofilms and their effects on enterococcal ecology in diverse environments, and on strain and gene transmission between niches should be a high priority. Given the availability of many new tools for genetic, physiological, and evolutionary studies, there is unlimited potential for this area of research. There is a tremendous amount to be learned about the role of biofilm formation in the intestinal versus extra-intestinal ecology within the human host, both in healthy individuals and in hospital patients. In the long run, the insights gained from these studies may prove as useful in prevention and control of enterococcal diseases as those obtained in more focused studies of pathogenesis.

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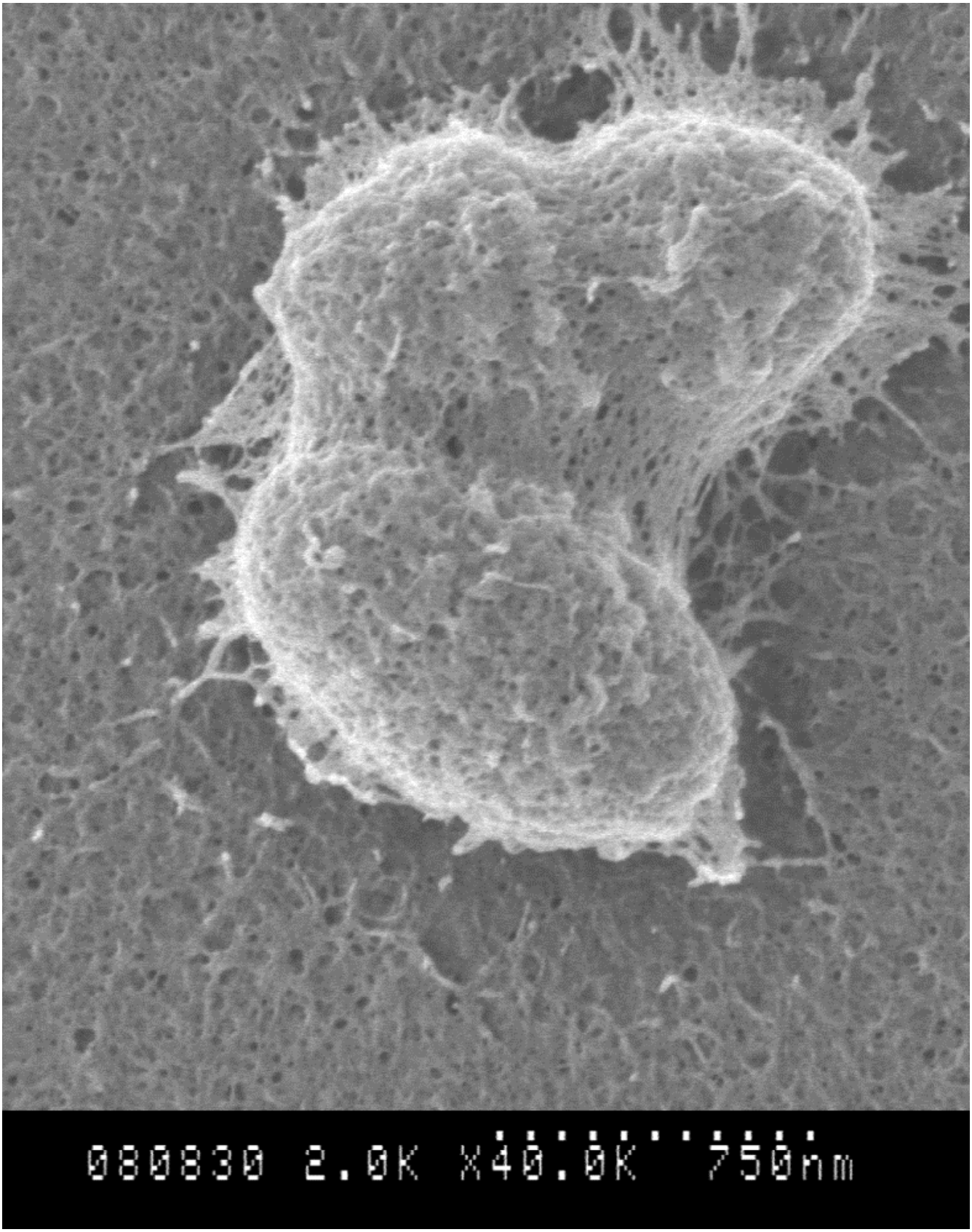

Figure 2: Early biofilm growth of *E. faecalis* OG1RF. A cellulose dialysis membrane was submerged for 2h in a liquid culture, the non-adherent bacteria were removed by rinsing, and the adherent organisms were aldehyde-fixed in the presence of Alcian Blue, and examined by high resolution scanning electron microscopy as described (Baldassarri, et al., 2006). Although the density of adherent cells is very low, abundant extracellular material is already evident. Micrograph was prepared by Shen Dong and Katie Ballering, University of Minnesota.

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The Physiology and Metabolism of Enterococci

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Introduction

When possible, the authors have provided open reading frame (ORF) numbers (EF####) from the extensively annotated E. faecalis V583 genome sequence. This information can be easily accessed at enterocyc.broadinstitute.org. If applicable, non-V583 enterococcal sequences with homology to the annotated ORF have been supplied. Additionally, we have included IUBMB nomenclature for most reactions, which are available at *http://www.iubmb.org*.

Enterococci have been isolated and characterized for more than 113 years (MacCallum & Hastings, 1899). During the past century, the classification of this genus has been refined, with the most significant change occurring in 1984 when most members of the Group D streptococci, including Streptococcus faecalis and Streptococcus faecium, were included in the new genus Enterococcus (Schleifer & Kilpper-Bälz, 1984). This genus currently consists of 37 species that occupy a broad range of habitats that include the gastrointestinal microbiota of nearly every animal phylum (See Enterococcus Diversity, Origins in Nature, and Gut Colonization for details). An ability to widely colonize is due, at least in part, to their metabolic versatility and intrinsic resistance to inhospitable conditions. Despite being unable to form spores, enterococci are highly tolerant to desiccation and can persist for months on dried surfaces. Enterococci also tolerate extremes of pH, ionizing radiation, osmotic and oxidative stresses, high heavy metal concentrations, and antibiotics. Enterococci survive or grow over a wide range of temperatures for mesophilic bacteria, from 10 to 45°C. These bacteria, as highly evolved commensals, have been extensively used in the food industry and as probiotics to prevent or ameliorate disease. Finally, rogue strains of enterococci have emerged on the worldwide stage as multidrug-resistant and hospital-acquired pathogens.

Enterococci are often simply described as lactic-acid–producing bacteria—a designation that understates their vast metabolic potential. The ubiquitous nature of enterococci in our environment implies this potential. Investigations into the remarkable physiology of these bacteria have fluctuated over the past century. Prior to publication of The Enterococci (Huycke M. M., 2002), and now with this volume, the last formal comprehensive review of enterococcal metabolism was written in 1964 (Deibel, 1964). At that time, substantial efforts by Gunsalus, Sokatch, Gale, Niven, and Deibel, among others, focused on the central metabolism of enterococci. Since then, research into enterococcal physiology has increasingly used the tools of molecular biology and has shifted toward understanding antibiotic resistance, pathogenesis, and genomics. With this new information, there has been increasing recognition that many metabolic genes and pathways vary, even within single species, and led investigators to question the concept of a uniform core metabolism for enterococci. To address this perspective and update the available information on enterococcal physiology, this chapter compiles and reviews the most recent findings from laboratories around the world, and integrates those results with the older literature. As will be evident, the rapid growth of genomic databases continues to offer valuable insights into the physiology of enterococci and greatly facilitates experimental designs into their metabolism.

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Sugar Metabolism

The ability to utilize hexose and pentose carbohydrates is a key component of metabolism for a majority of the eubacteria. Carbohydrate fermentation by enterococci allows this genus to thrive in diverse environments. At least 13 sugars are metabolized by all *Enterococcus* species and over 30 more are utilized by at least two members of the genus. The tremendous expansion of genomic sequencing by current *Enterococcus* researchers assures the discovery of additional carbohydrates that have the potential to be metabolized by this genus. These discoveries will also occur because of a remarkable ability for enterococci to share new carbon utilization mechanisms between strains and species, often on mobile elements. The metabolism of a broad array of carbohydrates provides enterococci with a robust advantage in colonizing competitive environments, especially the gastrointestinal tracts of nearly every phylum within the animal kingdom. Carbohydrate sources include not only a diverse array of monomers, but also many naturally abundant carbohydrate polymers.

Carbohydrates

Each of the three routes of intermediary carbohydrate metabolism—the Embden-Meyerhof-Parnas (glycolysis), Entner-Doudoroff, and pentose phosphate (phosphogluconate) pathways—are present in enterococci, or at least in E. faecalis, where appropriate studies have been performed (Sokatch & Gunsalus, 1657). The Embden-Meyerhof-Parnas and Entner-Doudoroff pathways are similar in that hexoses are phosphorylated and subsequently cleaved by aldolases to form the triose phosphate intermediate, glyceraldehyde-3-phosphate, for subsequent metabolism by the glycolytic pathway. These pathways provide the cell ATP via substrate phosphorylation. In contrast, the multifunctional pentose phosphate pathway not only ferments hexoses, pentoses, and other sugar acids for energy (Goddard & Sokatch, 1964), but also generates NADPH for biosynthetic reactions and channels pentoses into nucleotide biosynthesis.

The branch point from the Embden-Meyerhof-Parnas pathway to the pentose phosphate pathway occurs when glucose-6-phosphate is oxidized to 6-phosphogluconate which, in turn, is decarboxylated and oxidized to D-ribulose-5-phosphate. Few enzymes in these pathways have been characterized for enterococci. One exception is the *E. faecalis* 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44–EF3142 and EF1049), which comprises two distinct enzymes that use NADP or NAD as cofactors (Brown & Wittenberger, 1972). The NADP-linked dehydrogenase is inhibited by fructose-1,6-bisphosphate, but not by ATP, while the NAD-linked enzyme is inhibited by ATP, but not by fructose-1,6-bisphosphate. Although these enzymes are undoubtedly important to carbon flow through the pentose phosphate and the Embden-Meyerhof-Parnas pathways (Wittenberger, Palumbo, Bridges, & Brown, 1971), little work in this area has been performed using enterococci.

In comparison to the relative lack of investigation into the central carbon metabolism for enterococci, considerable effort has focused on the *E. faecalis* phosphoenolpyruvate phosphotransferase system (PTS). This system senses sugars outside the cell and couples their uptake with phosphorylation (Postma, Lengeler, & Jacobson, 1993). The sugars most commonly metabolized by enterococci are substrates for PTS. Novel PTS components have been observed on mobile elements in *E. faecalis*, which suggests their importance in virulence (Paulsen, et al., 2003). Most low-GC content Gram-positive bacteria that synthesize ATP by substrate-level phosphorylation under anaerobic conditions also express PTS. This ubiquitous system allows organisms to couple carbohydrate transport to phosphorylation using a mechanism that is more efficient for monosaccharides than non-PTS systems, which can ultimately expend more than one ATP per imported sugar versus the consumption of a single phosphoenolpyruvate. For enterococci and other bacteria, PTS helps regulate glycerol metabolism (via HPr[his15-P]) and is involved in inducer expulsion, inducer exclusion, and catabolite repression.

The initial reaction in PTS-mediated sugar translocation is phosphorylation of a small, soluble cytoplasmic protein by enzyme I (EI, E.C. 2.7.3.9–EF0710) (Figure 1). For *E. faecalis*, EI is a constitutive 140 kDa homodimer

phosphorylated at a specific histidyl residue by the energy-rich glycolytic intermediate phosphoenolpyruvate (Alpert, Frank, Stüber, Deutscher, & Hengstenberg, 1985). Phosphorylated EI (EI-P) transfers its phosphate to a constitutive 9.6 kDa histidine-containing phosphocarrier protein (HPr–EF0709). The three-dimensional structure of the *E. faecalis* HPr is similar to that of other microorganisms, with the phosphate acceptor located on a histidine residue at position 15 (HPr[his15-P]) (Maurer, Döker , Görler, Hengstenberg, & Kalbitzer, 2001). Unlike subsequent reactions that use the sugar-specific enzymes II (EIIs), EI and HPr are general PTS proteins. EIIs can consist of up to four polypeptides or domains (A, B, and C, or A, B, C, and D) with EIIC and EIID being integral membrane proteins (Deutscher, Francke, & Postma, 2006). EII enzymes catalyze the last step in sugar transport by acting as a phosphorelay from HPr to the sugar, which adds a phosphoryl group at the 6 carbon during hexose sugar transport. The only EII enzymes that have thus far been characterized for enterococci include mannitol, maltose, and gluconate (Fischer, von Strandmann, & Hengstenberg, 1991; Le Breton, Pichereau, Sauvageot, Auffray, & Rincé , 2005; Brockmeier, et al., 2009).

The site of HPr phosphorylation is an important regulatory mechanism for sugar metabolism in *E. faecalis*. EI-P exclusively phosphorylates HPr at his15 (HPr[his15-P]) to initiate a cycle of events that leads to sugar uptake. This phosphorylation occurs in both Gram-negative and Gram-positive bacteria. In Gram-positive bacteria, especially those with low G+C content, HPr can also be phosphorylated at a seryl residue at position 46 (HPr[ser46-P]) (Deutscher, Francke, & Postma, 2006). This phosphorylation is reversible and both reactions are catalyzed by the bifunctional enzyme HPr Kinase/Phosphatase (*hprK*, E.C. 2.7.1., and E.C. 3.1.3.–EF1749). Phosphorylation only occurs when ATP levels are elevated, as might happen during active sugar metabolism (Kravanja, et al., 1999). Phosphorylation at this site greatly attenuates EI-P phosphorylation at his15, and as a consequence inhibits PTS-mediated sugar uptake. Conversely, during periods of ATP limitation, a second enzymatic site on HprK hydrolyzes the serine phosphate to free HPr for phosphorylation at his15 by EI-P. This uniquely dual function of HprK under ATP control presumably coordinates the metabolic demand by adjusting the ratios of active (HPr[his15-P]) to inactive (HPr[ser46-P]) HPr.

Bacteria, including enterococci, repress and/or inhibit alternate carbon source metabolism during growth on rapidly fermentable carbon sources like glucose. This phenomenon is called catabolite repression (CR) (Fig. 1). Serine-phosphorylated HPr is a key negative regulator for low-GC content Gram-positive bacteria. Other components of CR include a *trans*-acting factor called the catabolite control protein A (CcpA–EF1741) and *cis*-acting nucleotide sequences that are termed catabolite responsive elements (*cre*s). CcpA is a DNA-binding protein that was first identified in *Bacillus subtilis* and regulates expression via *cre* sequences either within or near promoters of target genes. Both gene activation and repression have been described as a consequence of CcpA binding. The cofactor required for CcpA binding to *cre* is HPr[ser46-P], but not non-phosphorylated or histidine-phosphorylated forms of HPr. A 36 kDa CcpA homologue for *E. faecalis* was recently identified (Leboeuf, Auffray, & Hartke, 2000). The *ccpA* gene product restored glucose repression for *cre*-responsive genes in *B. subtilis*, which demonstrated CR function. 2-D protein electrophoresis assays determined that *E. faecalis* CcpA regulated 22 individual gene products, including glycerol dehydrogenase and dihydroxyacetone kinase. Additionally, an *E. faecalis ccpA* knockout mutant de-regulated catabolite repression in the presence of glucose, which allowed this strain to simultaneously co-metabolize citrate and glucose (Rea & Cogan, 2003). Another study demonstrated the presence of 63 putative *cre*-sites in the promoters of genes involved with several metabolic pathways, including those for citrate, sucrose, lactose, galactose, serine, and arginine (Opsata, Nes, & Holo, 2010). Another form of catabolite-dependent regulation is inducer expulsion. This process involves de-phosphorylating and exporting other sugars during growth on glucose (Ye, Minarcik, & Saier, Jr., 1996). Inducer expulsion requires a small membrane-associated sugar-phosphate phosphatase (Pase II) to dephosphorylate cytosolic sugars prior to export, and is positively regulated by HPr[ser46-P].

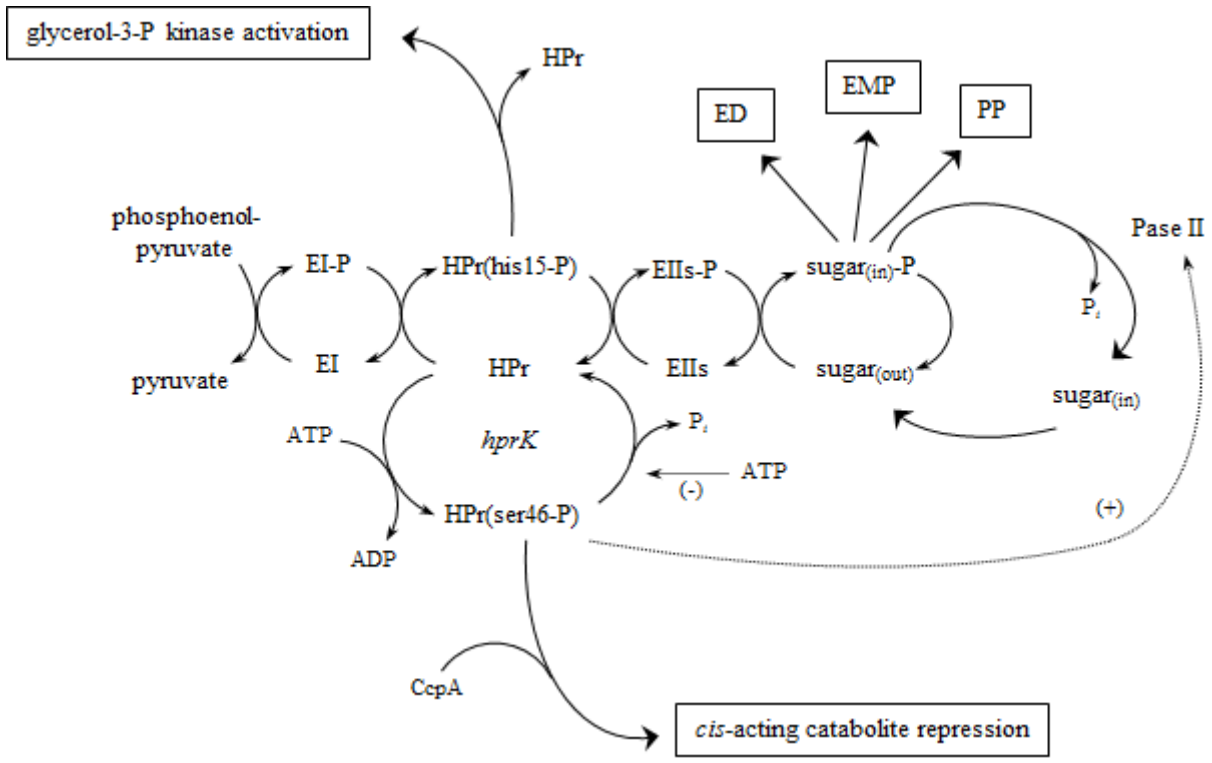

Figure 1 Phosphoenolpyruvate phosphotransferase and catabolite repression. Phosphorylation of *E. faecalis* phosphocarrier protein (HPr) by enzyme I (EI) at histidine residue 15 forms HPr(his15-P) and couples sugar uptake to sugar phosphorylation through carbohydrate-specific enzymes II (EIIs). Phosphorylated sugars are then channeled into glycolytic pathways. HPr(his15-P) also regulates glycerol metabolism (see Figure 2) through glycerol-3-phosphate kinase. The bifunctional HPr kinase/phosphatase enzyme (HprK) regulates HPr activity through phosphorylation at serine residue 46 to form HPr(ser46-P), which is unable to phosphorylate EIIs. HPr(ser46-P) also activates catabolite repression through the catabolite control protein A (CcpA), and up-regulates inducer expulsion through phosphatase II (Pase II, hashed arrow). The HprK phosphatase is attenuated by ATP (hashed arrow).

Glycerol metabolism

Glycerol metabolism is of importance as a pathway for the synthesis of lipids and (lipo)teichoic acids in many Gram-positive bacteria, including *E. faecalis* (Coyette & Hancock, 2002). Glycerol can also be a carbon/energy source for several pathogenic bacteria. For example, *Listeria monocytogenes* use glycerol-catabolizing enzymes for intracellular growth (Joseph, et al., 2006). Furthermore, studies on *Mycoplasma* sp., bacteria that are adapted to life within eukaryotic hosts through reductive evolution as evident by a minimalistic genome, still rely on a handful of carbon sources, including glycerol (Halbedel, Hames, & Stülke, 2004).

Among enterococci, *E. faecalis* appears to have the most diverse glycerol metabolism. Members of this species can ferment glycerol under aerobic as well as anaerobic conditions (Bizzini A. , et al., 2009; Gunsalus & Sherman, 1943). Genome sequences for *E. faecalis* reveal two pathways for glycerol catabolism (Fig. 2). One begins with the ATP-dependent phosphorylation of glycerol by glycerol kinase (GlpK / EF1929) to yield glycerol-3-phosphate (glycerol-3-P). In Gram-positive bacteria, such as *Bacillus subtilis*, *E. faecalis*, and *E. casseliflavus*, GlpK activity is inhibited by fructose-1,6-bisphosphate and activated by phosphorylation through the general PTS (phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system) regulator (HPr[his15-P]), which leads to 10- to 15-fold increased activity (Deutscher, Francke, & Postma, 2006). Detailed structural studies with the GlpK of *E. casseliflavus* in the presence or absence of glycerol have been performed (Yeh, et al., 2004). The glycerol-3-P formed is subsequently oxidized to dihydroxyacetone phosphate (DHAP) by glycerol-3-P oxidase (GlpO–EF1928). This enzyme uses molecular oxygen as an electron sink and leads to the formation of H_2O_2. Exogenous catalase increases the aerobic growth of *E. faecalis* on glycerol, which indicates that this oxidant can

accumulate to growth-inhibiting concentrations under these conditions (Bizzini A. , et al., 2009). Inactivation of the *E. faecalis npr* gene (EF1211) that encodes NADH peroxidase or inactivation of the *ahpCF* operon (EF2739 and EF2738) that encodes alkyl hydroperoxide reductase led to reduced growth on glycerol under aerobic conditions. These enzymes catalyse the NADH-dependent dismutation of H_2O_2 to oxygen and water (La Carbona, et al., 2007). Genes that encode GlpK and GlpO are contained in an operon structure that also encodes the *glpF* gene (EF1927) for glycerol transport. This integral membrane protein belongs to the aquaporin family of molecular water channels and allows glycerol to enter the cell through facilitated diffusion (13). The PrfA-like transcriptional regulator Ers (EF0074) has been indirectly implicated in regulating the *glpK*-operon (Riboulet-Bisson, Hartke, Auffray, & Giard, 2009). The *glpK*-operon appears to be conserved among enterococci, with identical elements present in genome sequences for other enterococcal species.

The second pathway for glycerol catabolism in *E. faecalis* involves the oxidation of glycerol to dihydroxyacetone by a soluble NAD^+-dependent glycerol dehydrogenase (GldA1/ EF1358). Dihydroxyacetone is then phosphorylated to DHAP by dihydroxyacetone kinase (DhaK/ EF1360). Anaerobic growth of *E. faecalis* on glycerol depends on fumarate as an electron acceptor to allow for the reoxidization of NADH. In this reaction, fumarate is reduced to succinate by a membrane-associated fumarate reductase (30). Genes for these activities are encoded in an operon structure (EF1358 to EF1361) that includes GldA1, a small hypothetical protein, and two subunits for DhaK (DhaK and DhaL). Interestingly, *E. faecium* harbours a similar operon that lacks *gldA*, and this likely explains why the species is unable to grow anaerobically on glycerol. The putative sequence of a small hypothetical polypeptide on the *dhaK* operon has significant homology to the DhaM protein of *Lactococcus lactis* (Zurbriggen, et al., 2008). *L. Lactis* DhaM contains an EIIA domain for the mannose family of PTS and is also conserved in the DhaM-like *E. faecalis* protein. Recently it has been demonstrated that the *E. faecalis* DHA kinase is phosphorylated in a PEP-dependent phosphotransfer reaction, which includes the DhaM enzyme (Sauvageot, et al., 2012) that is similar to *L. lactis* (Fig. 2).

Mutants involving both pathways have been analysed for growth under both aerobic and anaerobic conditions (Bizzini A. , et al., 2009). Comparisons using several *E. faecalis* isolates demonstrate an impressive diversity of growth behaviours on glycerol under these conditions, which is not due to differing gene content, but rather a modulation of gene expression. Some strains preferentially use the *glpK* pathway, others the *dhaK* pathway; or glycerol is catabolised simultaneously under aerobic conditions. These findings clearly demonstrate that *E. faecalis* displays heterogeneous modes of aerobic glycerol catabolism (Bizzini A. , et al., 2009). However, under anaerobic conditions, glycerol is exclusively metabolized via the DhaK pathway.

Two other genes with annotated roles in glycerol metabolism are found in genome sequences for *E. faecalis* and include a second putative glycerol dehydrogenase (EF0895) and a putative glycerol-3-phosphate dehydrogenase (EF1747). However, these activities do not appear to be involved in glycerol catabolism (Bizzini A. , et al., 2009). Finally, a second putative glycerol facilitator (EF1828) is found in few *E. faecalis* strains besides the V583 strain, but its significance to glycerol transport remains to be determined.

Citrate

Enterococcal citrate metabolism is commercially valuable and presumed to contribute to “flavor compound” production in numerous cheeses. Citrate can be fermented as the sole source of carbon and energy for *E. faecalis* and *E. durans*. Enterococcal growth on citrate requires transportation across the cell membrane, followed by cleavage into acetyl-coA and oxaloacetate by citrate lyase. Oxaloacetate is then decarboxylated to pyruvate. End-products produced by *E. faecalis* grown on citrate as a sole carbon source include acetate, formate, and, to a lesser degree, lactate, ethanol, and acetoin (Sarantinopoulos, Kalantzopoulos, & Tsakalidou, 2001; Campbell & Gunsalus, 1944). Other minor products include α-acetolactate, diacetyl, and 2,3-butanediol. These metabolites, in addition to the previously listed compounds, contribute to flavor production in commercial fermentations.

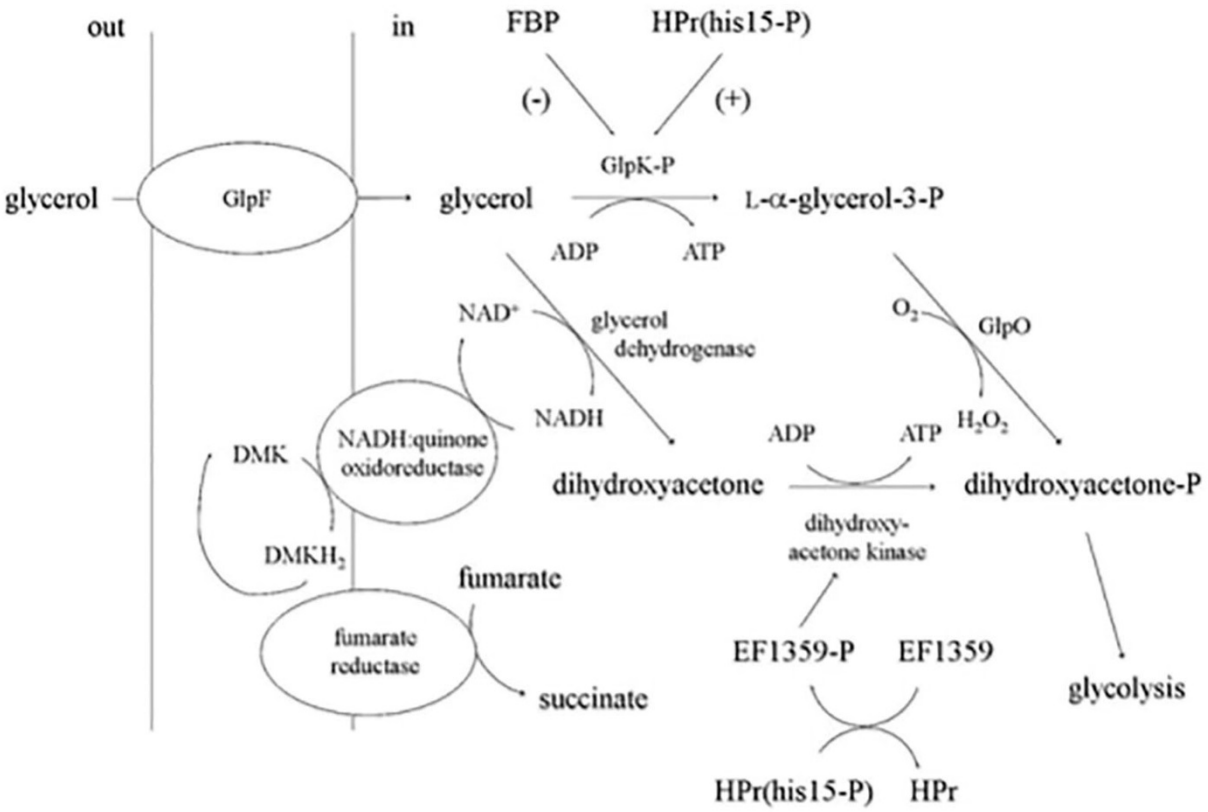

Figure 2. Glycerol metabolism. Glycerol uptake in *E. faecalis* occurs through an energy-independent diffusion facilitator (GlpF). Enzymes for glycerol dissimilation include: ATP-dependent glycerol kinase (GlpK); H_2O_2-producing L-α-glycerophosphate oxidase (GlpO); an NAD^+-dependent glycerol dehydrogenase; and dihydroxyacetone kinase (DHA-kinase). Once inside the cell, glycerol can be dissimilated by two pathways. It is either first phosphorylated by GlpK and then oxidized by GlpO, or is initially oxidized by G-dehydrogenase and then is phosphorylated by DHA-kinase. In both cases, the final end product is dihydroxyacetone phosphate (DHAP), which enters the glycolytic pathway. Both pathways are connected to the PTS: G-kinase is activated by the general PTS compound HPr(his15-P) and phosphorylation of dihydroxyacetone (DHA) by DHA-kinase is mediated by phosphotransfer via EI, HPr, and EF1359 (DhaM) with PEP as the phosphoryl donor. Under anaerobic conditions, only the G-dehydrogenase/DHA-kinase pathway is functional, and requires coupling through a putative NADH:quinone oxidoreductase to an alternate electron acceptor, such as fumarate.

The regulation of citrate metabolism has been characterized in *E. faecalis* (Blancato, Repizo, Suárez, & Magni, 2008). Citrate is presumably transported by the CitH transporter (EF3327) and leads to the increased affinity of a CitO protein for non-coding regulatory regions upstream of the *citHO-oadHDB-citCDEFX-oadA-citMG* operons. These genes comprise the citrate regulon and have significant sequence homology to genes for citrate fermentation in other lactic acid bacteria. Together these genes encode products necessary for citrate transport (CitH–EF3327), citrate-dependent regulation (CitO), citrate lyase (CitD-F–EF3318 to EF1322), oxaloacetate decarboxylase (CitM–EF3317), and the oxaloacetate decarboxylase complex (OadABD H). Citrate is metabolized in the presence of lactose, although catabolism is inhibited by PTS via catabolite repression, and additional unidentified regulatory elements are involved in this process (Suárez, Blancato, Poncet, Deutscher, & Magni, 2011).

Mucin degradation

Enterococci likely derive carbon and energy by fermenting non-absorbed sugars in the gastrointestinal tract. Other sugars, however, are also available in this environment as heavily glycosylated mucins produced by specialized epithelial cells. Mucins are large, cysteine-rich proteins that have been highly decorated with carbohydrates (70-80% by weight). These complex molecules form the primary component of the adherent mucous layer that protects the intestinal epithelium from luminal contents and acts as a mechanical lubricant. The ability to degrade mucin and liberate their attached sugars would be advantageous to members of the intestinal microbiota. However, relatively few enteric bacteria can degrade mucins. This is due, in part, to poly-*O*-acetylation and *O*-sulfation of sialic acids on outer non-reducing ends of mucin chains. These modifications protect mucin from most forms of bacterial degradation.

Only a few enteric bacteria can completely hydrolyze mucins and use them as a source of carbon and energy, including *Escherichia coli*, *Bifidobacterium* spp., *Bacteroides* spp., and *Ruminococcus* spp. These bacteria overcome acetylated and sulfated sialic acid residues by expressing cell-bound and extracellular sialate *O*-acetylesterase and arylesterase, in addition to sialidase, β-*N*-acetylglucosaminidase, and β-*N*-acetylgalactosaminidase. This allows *E. coli* to rapidly grow in cecal mucus, as compared to luminal contents (Poulsen, Licht, Rang, Krogfelt, & Molin, 1995). Evidence for enterococcal growth on mucin as a sole carbon source is lacking, although the sulfatase components of mucin degradation are observed in *E. faecalis* (Bøhle, et al., 2001). The growth of vancomycin-resistant enterococci in mucin is enhanced by the generation of monosaccharides from mucin through the mucin-degrading activities of either *Ruminococcus torques* or a human fecal consortia (Pultz, Hoskins, & Donskey, 2006). Apart from these studies, little work is available on mechanisms by which enterococci might degrade and grow in mucin.

Plant Carbohydrates and Errata

The diverse habitat range of the *Enterococcus* genus suggests that these bacteria are exposed to vast array of carbon and energy sources, both as planktonic bacteria and in gastrointestinal environments. While this information is incomplete, enterococci appear able to degrade numerous biological polymers and modified carbohydrates and use them as carbon and energy sources. Many plant carbohydrate polymers are resistant to decomposition by the human digestive system and can therefore provide a source of nutrients to the gastrointestinal microbiota. Of these polymers, cellulose is the most abundant and is composed of repeating β(1-4) glucose linkages. *E. saccharolyticus*, *E. faecalis* and *E. gallinarum* can grow in a pure or mixed consortium on cellulose as a carbon source (Adav, Lee, Wang, & Ren, 2009; Chassard, Delmas, Robert, & Bernalier-Donadille, 2010; Wang, Gao, Ren, Xu, & Liu, 2009). At least four putative β-glucosidases necessary for cellulose decomposition are encoded on the *E. faecalis* V583 genome (EF1020, EF1238, EF1243, and EF1606). Many more are likely present in other strains and species within the genus. Hemicellulose-containing compounds such as lignin are second in abundance to cellulose, but its degradation and utilization by enterococci has yet to be investigated.

Raffinose is a trisaccharide found in many plant tissues that cannot be digested by human enzymatic activity. Recently, raffinose-utilization genes were identified on a megaplasmid in *E. faecium* (Zhang, Vrijenhoek, Bonten, Willems, & van Schaik, 2011). This activity may provide a means for establishing a niche in the complex gastrointestinal milieu. *E. faecium* also possesses enzymes for the initial steps in fructosamine catabolism, which potentially allows it to grow on this plant-derived carbon source (Wiame, Lamosa, Santos, & Van Schaftingen, 2005). Pectin is another abundant plant-derived polysaccharide that is only partially digested by most mammals, at best. Pectin is composed of several sugars that most commonly include D-galacturonate in α(1–4) linkages. Human enterococcal isolates can utilize pectin *in vitro*, and dietary pectin from heavy fruit consumption has been linked to increased intestinal enterococci (Shinohara, Ohashi, Kawasumi, Terada, & Fujisawa, 2010).

Maltose is an α(1–4) linked glucose disaccharide produced by amylase degradation of starches and can be readily utilized by *E. faecalis* as a growth substrate. Recent activity has demonstrated that this disaccharide is transported by a PTS system that uses the maltose permease MalT and generates maltose-6-P similar to that of *Bacillus subtilis* (Le Breton, Pichereau, Sauvageot, Auffray, & Rincé , 2005). However, *E. faecalis* does not hydrolyse maltose-6-P into glucose-1-P and glucose-6-P like *B. subtilis*. Instead, *E. faecalis* dephosphorylates maltose-6-P using a novel enzyme MapP (Mokhtari, et al., 2013). Maltose is then cleaved by MalP into glucose and glucose-1-P, which can enter glycolysis after conversion to glucose-6-P by phosphoglucomutase.

Trehalose is an α(1–1) linked glucose disaccharide that is found primarily in insects and plant materials. Trehalose metabolism resembles maltose uptake and utilization, and involves a trehalose PTS system that converts this disaccharide into trehalose-6-P during transport. Trehalose-6-P is split by a trehalose-6-phosphate phosphorylase (TPP) enzyme into glucose-6-P and β-glucose-1P. β-glucose-1P is isomerized by β-phosphoglucomutase into glucose-6-P, and both sugars then enter glycolysis (Andersson & Rådström, 2002). Enzymatic activity for this pathway in enterococci has been verified, and putative gene sequences that encode these enzymes are found within the *E. faecalis* genome.

In addition to trehalose, enterococci are exposed to the β(1-4) linked N-acetylglucosamine (GlcNac) polymer chitin during growth on insect-associated material, as well as fungi. A recent study showed that *E. faecalis* can grow on insoluble chitin as a sole source of carbon and energy (Vaaje-Kolstad, et al., 2012). Chitin degradation requires several enzymatic steps with individual GlcNac monomers imported through PTS prior to entry into glycolytic pathways. *E. faecalis* secretes two enzymes encoded by a putative operon that functions as a chitinase and chitin-binding protein with chitinase activity (EF0361–EF0362). These enzymes degrade chitin into the disaccharide $GlcNac_2$,which is then degraded into GlcNac monomers by a secreted chitobiase (E.C. 3.2.1.52 - EF0114). GlcNac is then transported by an undetermined PTS system prior to deacetylation by either NagA-1 (EF1317) or NagA-2 (E.C. 3.5.1.25, EF3044) deamination into fructose-6-P (NagB, E.C. 3.5.99.6, EF0466), as well as entry into glycolytic pathways.

Ethanolamine catabolism is specific to *E. faecalis* and is obtained from cell membrane lipids through phosphatidylethanolamine degradation. Further degradation of lipid-derived fatty acids has not been previously demonstrated, and a survey of enterococcal genomic databases does not reveal the presence of any homologous gene sequences that encode for known β-oxidation enzymes. Ethanolamine catabolism is extensively covered in Pathogenesis and models of enterococcal infection.

α-Keto Acid Metabolism

Pyruvate is a methyl-α-keto acid product, which is a potentially significant source of energy for growth. Among enterococci, pyruvate-adapted growth is possible only for *E. faecalis* (Deibel & Niven, Jr., 1964). This trait requires exogenous lipoic acid in its role as an essential cofactor for the dihydrolipoyl acetyltransferase subunit (E2) of the pyruvate dehydrogenase complex (Delk, Nagle, Jr., Rabinowitz, & Straub, 1979). Pyruvate dissimilation follows several pathways that lead to at least five fermentation end-products, depending on growth conditions: lactate, acetoin, formate, ethanol, and acetate (Figure 3) (Snoep, Joost, de Mattos, & Neijssel, 1991). *E. faecalis* and other enterococci notably lack a tricarboxylic acid cycle. There is no evidence for fumarase, aconitase, isocitrate dehydrogenase, succinate thiokinase, or succinate dehydrogenase genes in the genome database. The absence of this pathway, however, does not explain why *E. faecalis* has enhanced pyruvate metabolism, since this metabolic limitation is common among all enterococci. The following section reviews α-keto metabolism and emphasizes those features unique to *E. faecalis*.

Pyruvate synthesis

For *E. faecalis*, at least four metabolic pathways lead to the formation of pyruvate. Sugar fermentation produces pyruvate through dephosphorylation of phosphoenolpyruvate by pyruvate kinase (*pyk*, EC 2.7.1.40–EF1046).

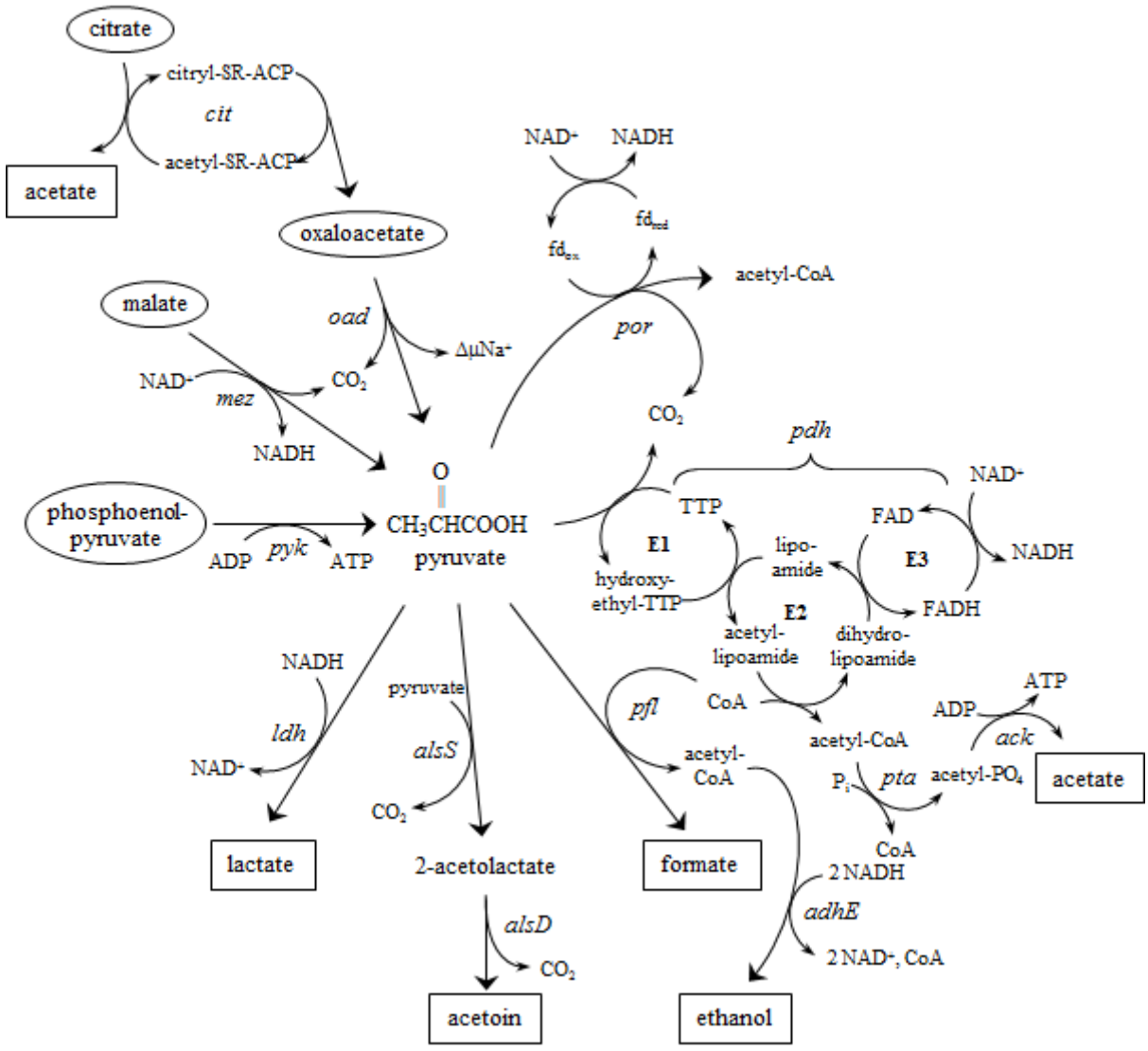

Figure 3. Pyruvate metabolism. Circled compounds represent substrates for pyruvate synthesis. Major end-products are boxed. *E. faecalis* genes that code enzymes for reactions, cofactors, and gradients are shown: *pyk*, pyruvate kinase; *mez* malic enzyme; *cit*, citrate lyase; ACP, acyl carrier protein (γ-subunit), *oad*, oxaloacetate decarboxylase; $\Delta\mu NA^+$, transmembrane sodium gradient; *por*, pyruvate decarboxylase; fd_{red}, reduced ferredoxin; fd_{ox}, oxidized ferredoxin; *pdh* pyruvate dehydrogenase complex including E1, E2 and E3 subunits; TTP, thiamine pyrophosphate; *ldh* L-(+)-lactate dehydrogenase; *alsS*, α-acetolactate synthase; *alsD*, α-acetolactate decarboxylase; *pfl*, pyruvate formate-lyase; *adhE*, aldehyde-alcohol dehydrogenase; *ack*, acetokinase; *pta*, phosphoacetyltransferase. Exact stoichiometry is not indicated and reaction details are omitted for clarity.

This reaction forms ATP and is the final step in the glycolysis. Malic enzyme (E.C. 1.1.1.39) constitutes another pathway leading to pyruvate. This enzyme catalyzes the NAD^+-dependent oxidative decarboxylation of malate and, unlike other bacteria, does not participate in biosynthetic reactions that utilize pyruvate as a carbon source. Instead, malate is only catabolized for energy by *E. faecalis*. Not surprisingly, *E. faecalis* does not express a malolactic enzyme often found in lactic acid bacteria that directly converts malate to lactate. Malic enzyme has been identified in *E. faecalis* (MaeE–EF3316) and is essential for malate catabolism, as well as pH homeostasis under acidic conditions (Espariz, et al., 2011). Malate metabolism is regulated by HPr(Ser-P)-dependent catabolite repression in the presence of sugars and glycolytic intermediates. Furthermore, this gene is induced by the activation of the *maeR* gene product encoded on the *maeKR* operon (EF1209-EF1210) (Mortera, et al., 2012). Growth on malate is promoted by oxygen or fumarate as terminal electron acceptors, as well as lipoic acid.

Oxaloacetate decarboxylase (*oad*, E.C. 4.1.1.3) is a sodium transport decarboxylase and a member of a family that includes methylmalonyl-coenzyme A (CoA), glutaconyl-CoA, and malonate decarboxylases (Bott, 1997). These multicomponent biotin enzymes couple exergonic decarboxylation with sodium export to produce a transmembrane sodium gradient ($\Delta\mu Na^+$). This potentially assists other sodium transport systems (see Ion Transport section). At least one oxaloacetate decarboxylase has been identified in *E. faecalis* (CitM–EF3317); however, it remains to be determined whether this is the sole enzyme responsible for oxaloacetate catabolism (Espariz, et al., 2011).

Pyruvate dehydrogenase complex

Dehydrogenation of pyruvate by *E. faecalis* can occur through one of three reactions, each of which produces acetyl-CoA. Acetyl-CoA then proceeds by a two-step reduction to generate ATP, or directly enters into fatty acid biosynthesis. The pyruvate dehydrogenase complex (*pdh*) is one dehydrogenation pathway for this methyl-α-keto acid. Pdh is a member of a family of multienzymes that catalyze oxidative decarboxylation of α-keto acids. The *E. faecalis* Pdh is an ordered collection of multiple copies of three enzymes, pyruvate dehydrogenase (E1, E.C. 1.2.4.1), dihydrolipoyl acyltransferase (E2, E.C. 1.8.1.4), and dihydrolipoamide dehydrogenase (E3, E.C. 1.8.1.3) (Perham, 1991). Substrate specificity for Pdh resides in E1 and E2 subunits, with the E3 subunit serving to reoxidize enzyme-bound dihydrolipoamide. The *E. faecalis* Pdh dihydrolipoyl acyltransferase (*aceF*–EF1355, E2 subunit) is a hollow dodecahedral 60-meric enzyme with icosahedral symmetry by x-ray crystallography (Izard, et al., 1999). The E2 subunit contains two lipoyl domains, a peripheral subunit-binding domain, and an acetyltransferase that is linked by a polypeptide enriched for alanine and proline (Allen & Perham, 1991). The lipoyl group is a swinging arm that conveys substrates between three successive active sites. The *E. faecalis* E2 subunit is similar to human E2 (two lipoyl domains), but not E2s for yeast (one lipoyl domain) or other bacteria (one or three lipoyl domains). In this respect, the *E. faecalis* Pdh differs from all other bacterial pyruvate dehydrogenases.

The E3 subunit is a homodimer that contains noncovalently bound FAD and a redox-active disulfide bond. The E1/E2/E3 stoichiometry for *E. faecalis* Pdh is 1.5:1:1 repeated 60 times to give a complex with an estimated mass of 14 million Da (Snoep, et al., 1992). A unique feature of the *E. faecalis* Pdh complex is continued activity, despite strong reducing conditions. NADH/NAD^+ ratios that completely inhibit *E. coli* Pdh have no effect on *E. faecalis* Pdh. In this regard, the *E. faecalis* E3 subunit seems far less susceptible to over-reduction than other E3 subunits and this, coupled with full expression of *E. faecalis* Pdh under anaerobic conditions, may explain the resistance of Pdh to anaerobiosis (Snoep, et al., 1992). This unusual characteristic likely provides *E. faecalis* with considerable metabolic flexibility during aerobic-anaerobic transitions.

Pyruvate formate-lyase

Dehydrogenation of pyruvate by *E. faecalis* and *E. faecium* can occur under anaerobic conditions by pyruvate formate-lyase (or formate acetyltransferase; *pflB*, E.C. 2.3.1.54–EF1613). This enzyme converts pyruvate and CoA to formate and acetyl-CoA through the unusual mechanism of a protein radical intermediate. Pfl has been partially purified for *E. faecalis* and is similar to *E. coli* Pfl. Under anaerobic conditions, *E. coli* is activated through the introduction of a stable free radical onto glycine at position 734 (Sawers & Watson, 1998; Wagner, Frey, Neugebauer, Schäfer, & Knappe, 1992). Activated Pfl is extraordinarily sensitive to oxygen, with exposure causing peptide-bond cleavage near the C terminus of the subunit that contains the glycyl radical. This leads to irreversible inactivation of the enzyme. Pfl is also acid sensitive, with growth of *E. faecalis* on pyruvate possible only at pH 7.5 or higher, when Pdh has been rendered non-functional (Snoep, van Bommel, Lubbers, Teixeira de Mattos, & Neijssel, 1993). Activated Pfl is protected from oxygen by a specific activating enzyme (*pflA*, E.C. 1.97.1.4–EF1612).

Pyruvate flavodoxin/ferredoxin oxidoreductase

A third, distinct enterococcal pyruvate dehydrogenation pathway has been reported, but only for *E. faecalis* (Yamazaki, Watanabe, Nishimura, & Kamihara, 1976). This activity is detectable under reduced conditions, converts pyruvate to acetyl-CoA, is unlike Pfl, and is insensitive to catabolite repression. The enzyme most likely responsible for this activity is pyruvate flavodoxin/ferredoxin oxidoreductase (E.C. 1.2.7.1–EF2559). A gene that codes for this enzyme is present in the *E. faecalis* and *E. faecium* genome databases and is nearly identical to *por* for *Entamoeba* spp., *Giardia* spp., and many other Gram-negative bacteria (Samuelson, 1999). No reports, however, have characterized this gene or its product in enterococci. Much like Pdh, it is likely that this enzyme catalyzes the oxidative decarboxylation of pyruvate using thiamine pyrophosphate, followed by an acetyl transfer to form acetyl-CoA. In this fashion, it differs from Pdh in the use of ferredoxin or flavodoxin as an electron acceptor with reducing equivalents transferred to NAD^+.

Alcohol dehydrogenase

Fermentation of mannitol or glucose by *E. faecalis* under nutrient-limited conditions produces not just formate and lactate, but also ethanol (Sarantinopoulos, Kalantzopoulos, & Tsakalidou, 2001; Snoep, Joost, de Mattos, & Neijssel, 1991). Ethanol-forming enzymes have not been identified for enterococci. One likely candidate, however, is the multifunctional alcohol dehydrogenase (*adhE*, E.C. 1.1.1.1 and 1.2.1.10–EF0900). A gene with similarity to *adhE* for other bacteria can be found in the *E. faecalis* and *E. faecium* genomic databases (Arnau, Jørgensen, Madsen, Vrang, & Israelsen, 1998). AdhE catalyzes the conversion of acetyl-CoA to ethanol through acetaldehyde dehydrogenase and alcohol dehydrogenase. Reducing equivalents are provided by NADH. This enzyme, which is expressed under anaerobic conditions in *E. coli* and *L. lactis*, is a gene fusion product, with the N terminus homologous to the family of aldehyde:NAD^+ oxidoreductases and C-terminus homologous to iron-dependent alcohol:NAD^+ oxidoreductases.

Acetate metabolism

Acetyl-CoA formed by Pdh, Pfl, and the putative Por can generate one additional molecule of ATP through reactions catalyzed by phosphotransacetylase (*eutD*, E.C. 2.3.1.8–EF0949) and acetate kinase (*ackA*, E.C. 2.7.2.1–EF1983). The first step in this energy-yielding process involves the replacement of acetyl-CoA with phosphate to produce acetylphosphate and CoA. AckA catalyzes the second step to generate ATP from acetylphosphate. This pathway appears to be reversible in *E. faecalis* when cytosolic lactate dehydrogenases (ldh, E.C. 1.1.1.27) are inactivated (Jönsson, Saleihan, Nes, & Holo, 2009; Rana, Gente, Rincé, Auffray, & Laplace, 2012).

Branched chain α-keto acid catabolism

An energy-yielding metabolic pathway has been described for *E. faecalis* that generates ATP from non-pyruvate α-keto acids. This pathway is encoded by the *bkd* operon and decarboxylates derivatives of branched-chain amino acids to produce corresponding acyl-CoAs. Compounds used in this pathway include α-ketoisovalerate (valine), α-ketoisocaproate (leucine), and α-keto-β-methylvalerate (isoleucine) (Rüdiger, Langenbeck, & Goedde, 1972; Ward, Ross, van der Weijden, Snoep, & Claiborne, 1999). Keto acids, like pyruvate, are energy-rich molecules that produce ATP through sequential reactions. The initial step involves an α-keto acid dehydrogenase complex that is encoded by the *bkd-DABC* operon. This large multienzyme complex is similar to Pdh and requires NAD^+, CoASH, and lipoic acid, much like Pdh requires these same cofactors. *bkdA* (EF1660), *bdkB* (EF1659), *bkdC* (EF1658) and *bkdD* (EF1661) encode proteins homologous to the E1α, E1β, E2, and E3 subunits of the branched-chain α-keto acid dehydrogenase complex from *B. subtilis*, and function to decarboxylate α-keto acids with the generation of corresponding acyl-CoAs. Amongst enterococci, the *bkd* operon appears to be unique to *E. faecalis* (Palmer, et al., 2012).

After the formation of branched-chain acyl-CoAs, reactions catalyzed by *ptb* and *buk* gene products form ATP (Ward, Ross, van der Weijden, Snoep, & Claiborne, 1999). These enzymes use acyl-CoAs to produce isovalerate, isobutyrate, and methylbutyrate as end-products. Substrate specificity of the *ptb* gene product is broad, and includes C_2 to C_8 straight chain acyl-CoAs, along with branched-chain acyl-CoAs. The *buk* gene product is not well characterized, but shows kinase activity coupled to ATP formation using acetyl-phosphate and butyrylphosphate as substrates. Growth of *E. faecalis* in pyruvate-limited culture with α-ketoisovalerate, α-ketoisocaproate, or α-keto-β-methylvalerate results in a significantly greater biomass, and corresponding branched-chain carboxylic acids are recovered in the growth medium. This pathway results in 0.5 mole of ATP per mole of catabolized α-keto acid (Ward, et al., 2000). Although one gene similar to the family of branched-chain amino acid aminotransferases is present in the *E. faecalis* genome, when branched-chain amino acids were tested for growth enhancement, none was observed (Ward, Ross, van der Weijden, Snoep, & Claiborne, 1999).

Acetoin metabolism

Acetoin (3-hydroxy 2-butanone) is a neutral compound that allows bacteria to degrade sugars without substantial acidification of the growth medium (Sarantinopoulos, Kalantzopoulos, & Tsakalidou, 2001; Dolin & Gunsalus, 1951). Production is measured by the Voges-Proskauer reaction and is positive for all enterococci (Fertally & Facklam, 1987). There is no evidence for a conversion of acetoin to diacetyl or 2,3-butanediol by enterococci. Acetoin is formed from pyruvate by α-acetolactate synthase (*alsS*, E.C. 4.1.3.18–EF1213) and α-acetolactate decarboxylase (*alsD*, E.C. 4.1.1.5–EF1214), with both present in the E. *faecium* and *E. faecalis* genomes. α-acetolactate synthase is a thiamine pyrophosphate-containing enzyme that forms a pyruvate-thiamine adduct. Pyruvate is initially decarboxylated prior to attack on the keto group by a second pyruvate molecule to form α-acetolactate. This enzyme has a low affinity for pyruvate and is only active when intracellular pyruvate concentrations are high. Acetoin is then formed from α-acetolactate by α-acetolactate decarboxylase. Regulatory mechanisms for these genes have been extensively studied in many bacteria, including *B. subtilis* (Cruz Ramos, et al., 2000), but have only recently been studied in *E. faecalis* (Mehmeti, et al., 2011).

Lactate Production

Lactate is the major end product of enterococcal fermentation during growth on excess glucose under reducing conditions. It is generated by the reduction of pyruvate to regenerate NAD^+ for ongoing glycolysis. *E. faecalis* possesses two cytosolic NAD^+-dependent L-(+)-lactate dehydrogenases (*ldh*, E.C. 1.1.1.27), which are deemed *ldh*-1 and *ldh*-2 (EF0255 and EF0641). These isoenzymes are activated by the glycolytic intermediate fructose-1,6-bisphosphate (Ldh-2 > Ldh-1) and are further regulated by the combined effects of intracellular pH and phosphate (Feldman-Salit, et al., 2013). This intermediate regulation is a specific need for these enzymes that otherwise show little or no activity without it as an activator. Ldh-1 is more active and is responsible for the bulk of lactate production during growth on hexoses. Recent evidence has suggested that Ldh-1 is extensively regulated and post-transcriptionally dependent upon culture growth rate, possibly to allow *E. faecalis* to rapidly adapt to nutrient variations in its environment (Mehmeti, et al., 2012). The deletion of Ldh-1 or both Ldh-1 and 2 causes a significant increase in mixed acid fermentation end-products (Mehmeti, et al., 2011). Ldh-2 contributes to lactate formation, but is insufficient for normal growth on glucose by itself (Jönsson, Saleihan, Nes, & Holo, 2009). Interestingly, although growth under laboratory conditions is comparable, *ldh1* and *ldh1/ldh2* double mutants demonstrate decreased fitness in stressing environments and are attenuated in virulence (Rana, et al., 2013).

Deiminase Catabolism

The amino acid arginine and its decarboxylated derivative agmatine are alternate energy sources for a few species of enterococci (namely, *E. faecalis* but not *E. faecium*) and several other Gram-positive microorganisms (Deibel, 1964). Compared to glycolysis, the energy gain from arginine or agmatine catabolism is low, with only 1

mol of ATP per mole of substrate. The growth of *E. faecalis* on semidefined medium with arginine or agmatine as a sole energy source still requires a small concentration of fermentable carbohydrate (0.05%), most likely for biosynthesis (Deibel, 1964).

Arginine and agmatine catabolism is distinct, and comprises separate operons and pathways (Figure 4). The arginine deiminase operon in *E. faecalis* consists of five genes (*arcABCRD*–EF0104 to EF0108) that encode three enzymes, an ornithine-arginine antiporter, and regulators (Barcelona-Andrés, Marina, & Rubio, 2002). In comparison, homologous genes in the agmatine deiminase operon appear to have evolved separately from the arginine operon, with four genes currently recognized (*agcABCD*–EF0732 to EF0735) (Llácer, et al., 2007). Energy for arginine and agmatine uptake is supplied by inwardly directed gradients, while end-product export occurs through outwardly directed gradients (Poolman, Driessen, & Konings, 1987). Thus, movement of substrates and byproducts does not require additional energy that would otherwise be limited when these pathways are used for growth.

The first step in arginine catabolism occurs through arginine deiminase (ArcA–E.C. 3.5.3.6; EF0104), whereas agmatine is catalyzed by agmatine deiminase (AgcA–E.C. 3.5.3.12; EF0734), to form citrulline and carbamoylputrescine, respectively, along with ammonia. The operon for arginine catabolism is induced by arginine and repressed by glucose, fumarate, and aerobiosis, while enzymes for agmatine catabolism are induced by agmatine and are repressed by glucose and arginine (Simon & Stalon, 1982). The second step in deiminase catabolism involves the phosphorolysis of citrulline by ornithine carbamoyltransferase (ArcB; EF 0105) to form carbamoyl phosphate and ornithine (for arginine), and carbamoylputrescine by putrescine carbamoyltransferase (AgcB; EF0732) to form putrescine and carbamoyl phosphate (for agmatine). Carbamoyl phosphate has a high-energy phosphate bond that can be used to generate ATP, with CO_2 and ammonia as byproducts. In *E. faecalis*, these reactions are catalyzed by two distinct carbamate kinases (ArcC and AgcC; EF0106 and EF0735, respectively). Nucleotide sequences for the *E. faecalis* and *E. faecium* arginine-induced carbamate kinases are identical and show similarity to carbamoyl phosphate synthetase, an enzyme that forms carbamoyl phosphate as a precursor molecule for pyrimidine and arginine biosynthesis (Marina, et al., 1998).

Respiration

Several enterococcal species express an electron transport chain that enables them to respire. Aerobic respiration is best characterized for *E. faecalis* and depends on the supply of heme (Ritchey & Seeley Jun, 1974; Pritchard & Wimpenny, 1978) that serves as a cofactor for cytochrome *bd* (see below). Analogous heme-dependent respiration also occurs in *Lactococcus lactis*, a lactic-acid–producing bacterium that is important to the food industry (Rezaïki, et al., 2004). In the absence of heme, respiration is blocked in these bacteria and extracellular superoxide is generated along with other reactive oxygen species (Figure 5) (Rezaïki, et al., 2004; Huycke M. M., et al., 2001). Other components of the *E. faecalis* respiratory chain include demethylmenaquinone, fumarate reductase, and the F_0F_1-ATP synthase. Oxidation of lactate for energy is also potentially linked to respiration, and is therefore discussed.

Demethylmenaquinone

Quinones are membrane-embedded electron carriers essential to all respiratory processes. In bacteria, quinones are derivatives of either ubiquinone or menaquinone. *E. faecalis* synthesizes a modified menaquinone that lacks a 2-methyl group. This derivative is termed demethylmenaquinone (Baum & Dolin, 1965). A comprehensive survey of quinones in enterococci identified demethylmenaquinone in *E. faecalis*, *E. casseliflavus*, and *E. gallinarum*, but not *E. faecium* or *E. durans* (Collins & Jones, 1979). No enterococcal strain has been shown to express ubiquinone or menaquinone. The midpoint potential for demethylmenaquinone ($\Delta E_{m,7}$ = +36 mV) is halfway between ubiquinone ($\Delta E_{m,7}$ = +113 mV) and menaquinone ($\Delta E_{m,7}$ = -74 mV) (Gennis & Stewart,

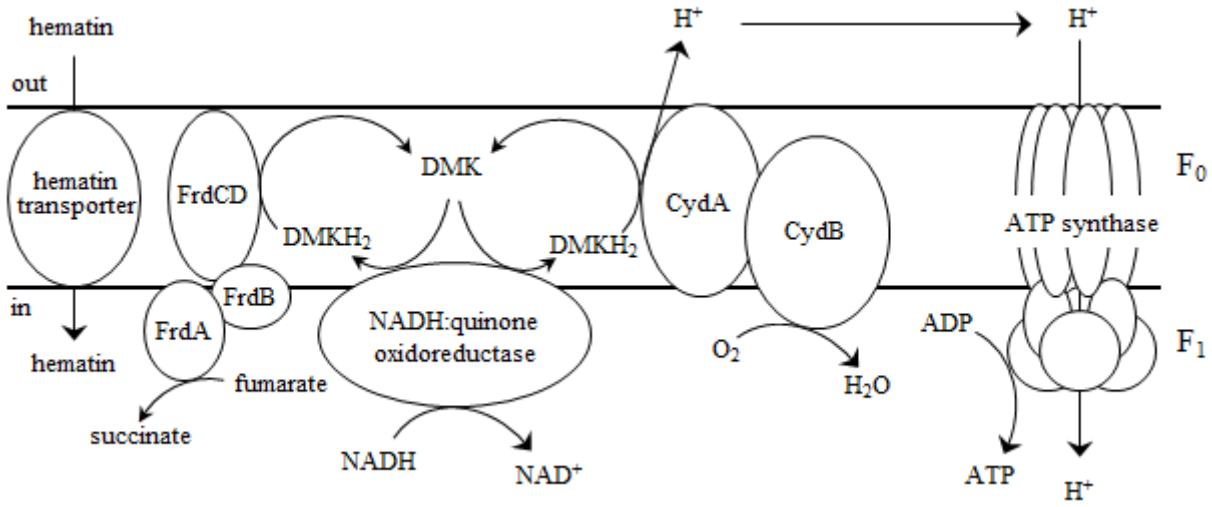

Figure 4. Deiminase catabolism. Arginine and agmatine pathways both lead to a high-energy carbamoyl phosphate intermediate. A third reaction forms ATP from carbamoyl phosphate. Specific, gradient-dependent antiporters provide for the import and export of substrates and products. Genes that code enzymes for reactions are shown: *arcA*, arginine deiminase; *arcB*, ornithine carbamoyltransferase; and *arcC*, arginine-induced carbamate kinase. Genes for agmatine deiminase (EF0734), putrescine carbamoyltransferase (EF0732), and agmatine-induced carbamate kinase (EF0735) have been putatively identified in the *E. faecalis* V583 genome.

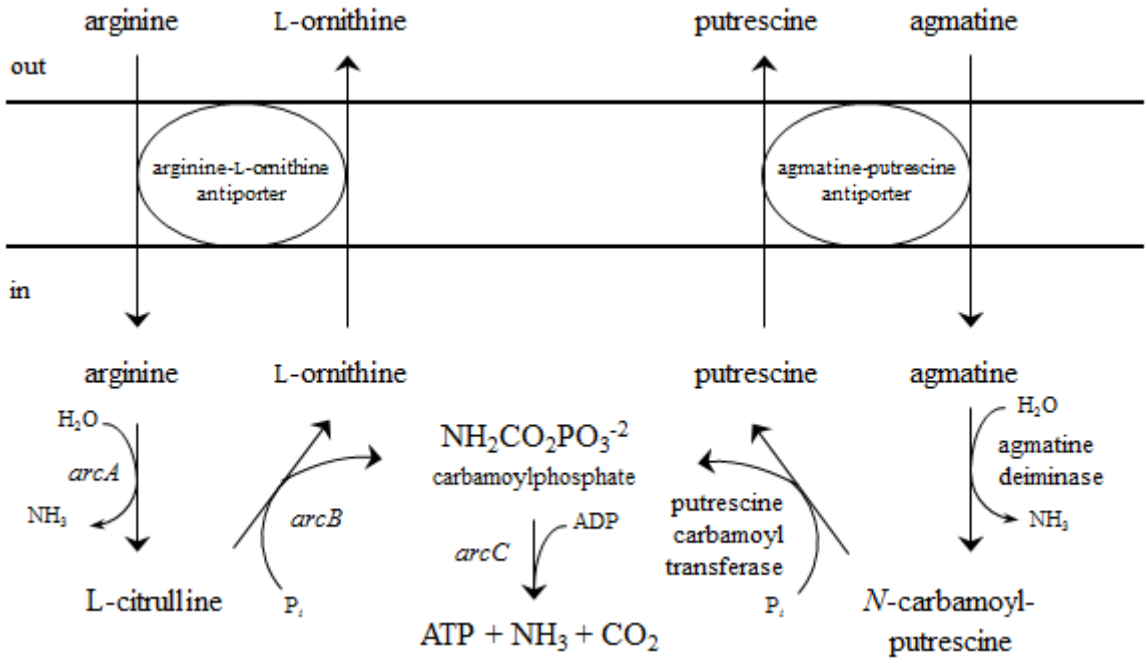

Figure 5. Respiration. A conceptualized model of *E. faecalis* respiratory components. A putative transporter facilitates hematin uptake for incorporation into cytochrome *bd* (CydAB). Cytosolic reducing equivalents are transferred to demethylmenaquinone (DMK) through a putative NADH:quinone oxidoreductase. Fumarate reductase (FrdABCD) and cytochrome *bd* are terminal demethylquinol ($DMKH_2$) oxidases that generate succinate from fumarate and H_2O from O_2, respectively. Cytochrome *bd* translocates one proton per electron to establish a proton motive force. F_0F_1-ATP synthase couples proton movement into the cell to form ATP. A putative L-lactate:quinone oxidoreductase for lactate oxidation is not shown.

1996). This characteristic confers respiratory flexibility and allows redox reactions to use fumarate or oxygen as electron acceptors under anaerobic and microaerophilic conditions.

Cytochrome bd

Enterococci, like streptococci, lack the ability to synthesize heme because porphyrin precursors cannot be produced, due to a missing tricarboxylic acid cycle. As a result, *E. faecalis* does not ordinarily express cytochromes, but instead relies on fermentation for growth. Among streptococci, enterococci, and lactococci, only *E. faecalis* and *L. lactis* are known to express cytochromes. This occurs only in the presence of heme, where aerobic growth leads to oxidative phosphorylation and enhanced production of ATP (Ritchey & Seely, Jr. , 1976; Winstedt, Frankenberg, Hederstedt, & von Wachenfeldt, 2000).

Cytochrome *bd* is the key respiratory enzyme for *E. faecalis*. This cytochrome is a widely distributed terminal quinol oxidase (Borisov, Gennis, Hemp, & Verkhovsky, 2011) that contains two subunits (CydA and CydB–EF2061 and EF2060) with three distinct cytochromes (b_{558}, b_{595}, and *d*). The low-spin b_{558} heme in CydA is the site of quinol oxidation. Substrate protons that arise from quinol oxidation are released outside the cell and help provide a proton-motive force by transmembrane charge separation. The high-spin b_{595} heme is located near heme *d* in CydB and forms a bimetallic center for the four-electron reduction of O_2 to H_2O. *cydC* and *cydD* (EF2059 and EF2058) are necessary for cytochrome *bd* expression. These genes code for proteins with similarity to ATP-binding cassette transporters, and are implicated in heme transport and/or assembly. The loss of cytochrome *bd* in *E. coli* leads to a pleiotropic phenotype that is characterized by stationary-phase arrest, as well as hypersensitivity to elevated temperatures and high concentrations of H_2O_2 and zinc (Goldman, Gabbert, & Kranz, 1996). Similar effects have not been seen with *E. faecalis* and seem unlikely, since this organism tolerates diverse and severe stresses (see below) despite *in vitro* growth without heme.

Fumarate Reductase

Nonoxidative respiration for prokaryotic organisms substitutes a variety of electron acceptors—nitrate, nitrite, sulphite, iron (III), CO_2, and fumarate—for O_2. The only system so far identified for *E. faecalis*, and a few strains of *E. faecium*, is fumarate reductase (Frd) (Deibel, 1964; Huycke M. M., et al., 2001; Aue & Diebel, 1967). This membrane-associated enzyme catalyzes the reduction of fumarate, a four-carbon dicarboxylic acid, to succinate. Although the term respiration is used loosely to describe Frd activity, this enzyme cannot catalyze a net transfer of protons across the cell membrane (Hederstedt, 1999). Although Frd can form fumarate from succinate *in vitro*, this reverse oxidation reaction has not been observed *in vivo*. Frd is composed of a large flavin adenine dinucleotide (FAD)-containing domain on the cytosolic surface of the cell membrane (subunit A; EF2556), three iron-sulfur clusters (subunit B), and small hydrophobic polypeptide anchors (subunits C and C). Frd appears to be constitutively expressed.

F0F1-ATP Synthase

A primary ion pump for mitochondria and many bacteria is the proton-translocating F0F1-ATP synthase, and F0F1-ATP synthases from diverse biological sources are nearly identical. These complexes are one of the three major classes of ion-motive ATP synthases that have the distinctive characteristic of coupling ATP synthesis to an electrochemical gradient of protons across the cell or mitochrondrial membrane. This system is reversible, and the enzyme can act to either synthesize ATP, or to use ATP to extrude protons and maintain a gradient for transport of other substrates or control cytoplasmic pH. Enterococci can use F0F1-ATP synthase for both purposes.

The first description of a membrane-associated ATP synthase was the F0F1-ATP synthase isolated from E. hirae (formerly Streptococcus faecalis) (Abrams, McNamara, & Bing, 1960). This large enzyme has an F1 moiety with five subunits (α, β, γ, δ, and ε) and a membrane-spanning F0 component that consists of three subunits (a, b, and c) (Shibata, Ehara, Tomura, Igarashi, & Kobayashi, 1992). The structure of the F0F1-ATP synthase for E. hirae is similar to other F0F1-ATP synthases, although the enterococcal enzyme is highly resistant to azide. This enzyme is also encoded by E. faecalis (AtpA) and its contribution to ATP synthesis through oxidative phosphorylation is

certain, based on several studies (Ritchey & Seeley Jun, 1974; Pritchard & Wimpenny, 1978). E. hirae, in contrast, does not express a cytochrome and therefore cannot couple the enzymatic activity of the F0F1-ATP synthase to a proton motive force. Instead, for E. hirae and non-respiring enterococci, the F0F1-ATP synthase assists in cytosolic alkalization in acidic environments, like the intestine.

L-Lactate Oxidation

Lactate is not just a fermentation end-product, but yet another energy source for the aerobic growth of *E. faecalis* (Pritchard & Wimpenny, 1978; Clarke & Knowles, 1980; London, 1968). This unusual feat of metabolism is a phenotypic characteristic of *E. faecalis* and is distinct from the activity of cytosolic lactate dehydrogenases. Current evidence suggests that a membrane-associated L-lactate:quinone oxidoreductase, analogous to the *E. coli* D-lactate dehydrogenase, is responsible for lactate oxidation. Evidence for this metabolic activity includes: (i) heme-grown *E. faecalis* oxidizes lactate 10-20 times faster than bacteria grown without heme (Pritchard & Wimpenny, 1978); (ii) uncoupling ionophores repress lactate oxidation, which links this to the proton motive force (Pritchard & Wimpenny, 1978); and (iii) brisk production of O_2^- occurs when isolated *E. faecalis* membranes are exposed to L-lactate (Huycke M. M., et al., 2001). Genes that encode enzymes for lactate oxidation have not been identified in enterococci.

Ion Transport

Enterococci are characteristically tolerant of extreme alkaline pH and high salt concentrations. These traits require cation transport to maintain the constant cytosolic ion composition that is essential for homeostasis. The *E. faecalis* V583 genome contains 14 predicted metal ion P-type ATPases (Paulsen, et al., 2003). Apart from genomic sequence data, investigators have commonly used *E. hirae* (formerly *Streptococcus faecalis*) to study ion transport mechanisms. Of note, *E. hirae* is an uncommon intestinal commensal without the necessary components necessary for respiration. Although *E. hirae* is metabolically limited, as compared to *E. faecalis*, much of the knowledge on inorganic cation transport and energy transduction for prokaryotic organisms has been developed using this species as a model system (see (Kakinuma Y. , 1998) for review).

Sodium

Salt and alkali tolerance are enterococcal phenotypes due, in part, to several independent mechanisms of sodium transport. All cells must expel excess sodium to maintain cytosolic concentrations in a homeostatic range. *E. hirae* expresses both a Na^+/H^+ antiporter that uses a proton motive force for activity (102) and a vacuolar-type ATPase that uses ATP hydrolysis to pump out sodium (103). A proton gradient used by the Na^+/H^+ antiporter can be generated by the F_0F_1-ATPase in cytochrome-free *E. hirae* or from *E. faecalis* grown without hematin and that expresses a non-functional apo-cytochrome *bd*. Under alkaline conditions when the proton motive force is dissipated, the Na^+/H^+ antiporter is inhibited and the V_0V_1-ATPase functions instead to extrude sodium and maintain homeostasis (103, 104).

Several genes that code for putative enterococcal Na^+/H^+ antiporter have been identified in genome databases (*nhaC-1*–EF0402; *nhaC-2*–EF0636; and EF1574), and one has been cloned from *E. hirae* (105). This gene, termed *napA*, encodes an extremely hydrophobic protein with 11 or 12 predicted transmembrane helices that shows little in common to other bacterial Na^+/H^+ antiporters, but is instead strikingly similar to the glutathione-regulated K^+ efflux system (KefC) of *E. coli* (106). *E. hirae* mutants that only express NapA, and not the vacuolar ATPase (see below), grow in 0.5 M sodium, but only at a pH less than 9.5. Sodium sensitivity at high pH values reflects an inadequate proton-motive force for proton-driven sodium extrusion. NapA expression in an *E. coli* strain with inactivated Na^+/H^+ antiporters restores growth under high sodium concentrations. Growth above pH values of 9.5, however, requires the ATP-driven V_0V_1-ATPase sodium extrusion system, because NapA is nonfunctional when the proton motive force has been dissipated (107).

Vacuolar-ATPases are members of a class of widely distributed proton pumps found in acidic vacuoles of fungi and plants, endosomes of animal cells, and selected bacteria. Both F_0F_1- and V_0V_1-ATPases are multisubunit enzymes that consist of a hydrophilic catalytic portion (F_1 and V_1) and a membrane-associated proteolipid segment that contains the proton or sodium channel (F_0 and V_0, respectively). Significant similarity for major subunits of these enzymes suggests that they have a close evolutionary relationship. The *E. hirae* V_0V_1-ATPase is an exceptional member of the vacuolar class of enzymes because it translocates sodium, rather than protons, out of the cell. The "Na^+"-translocating activity is encoded by the *ntpFIKECGABD* operon and consists of nine Ntp proteins (Murata, Kawano, Igarashi, Yamato, & Kakinuma, 2001). The *E. hirae ntp* operon is transcribed as a single mRNA and is induced by high intracellular sodium concentrations that occur with excessive extracellular sodium, by high pH when the Na^+/H^+ antiporter is nonfunctional, and when the proton motive force has been dissipated by ionophores or a loss of the F_0F_1-ATPase synthase (Murata, Kawano, Igarashi, Yamato, & Kakinuma, 2001). V_0V_1-ATPase activity is maximal at pH 8.5 to 9.0 and undetectable at a pH of 6.0, which fits well with its importance in sodium homeostasis under alkaline conditions. These observations highlight the importance of this ATPase to sodium homeostasis under alkaline conditions. Finally, the mechanism for sodium (or proton) extrusion by V_0V_1-ATPases involves coupling ATP hydrolysis to cation extrusion through rotational catalysis (for a review, see (Murata, Yamato, & Kakinuma, 2005)).

Potassium

Potassium is the major intracellular cation. For *E. hirae* cytosolic concentrations range from 0.4 to 0.6 M. Potassium is essential for cellular metabolism—it neutralizes intracellular anions, activates diverse enzymes, and regulates cytosolic pH. Maintenance of high intracellular concentrations, especially when potassium is in limited supply, requires active uptake. *E. hirae* expresses at least three potassium transporters to perform this task: KtrI, KtrII, and a low-affinity transporter (Kawano, Igarashi, & Kakinuma, 1999; Kawano M. , Abuki, Igarashi, & Kakinuma, 2001). In addition, there is the Kep system for potassium extrusion (Kakinuma & Igarashi, 1988).

The primary potassium uptake system for *E. hirae* is KtrI (in *E. faecalis*, *kdpABC*–EF0567, EF0568, and EF0569). This activity is likely due to symport of K^+ and H^+, although this has yet to be directly demonstrated (Bakker & Harold, 1980). The pH values optimal for KtrI is 6 to 7, with an apparent K_m of 0.2 mM for K^+, which allows KtrI to establish a gradient from inside to outside of 10^5 (Bakker & Harold, 1980). KtrI is regulated by an ATP-dependent modification, requires a membrane potential, and is active under neutral or acidic, but not alkaline conditions. These features are strikingly similar to the Trk K^+ potassium transport system of *E. coli*, which also has a low affinity, high-capacity mechanism for K^+ accumulation, coupled to ATP hydrolysis and a membrane potential (Epstein, 2003). KtrI activity appears to be constitutive, although purification, cloning, and analysis of regulatory mechanisms are not reported.

A second potassium uptake system for *E. hirae* is KtrII. Unlike KtrI, this transporter does not require a membrane potential or ATP. The K_m for K^+ is 0.5 mM and its optimal pH value is near 9. KtrII is not constitutively expressed, but is instead induced by high intracellular concentrations of sodium. This uptake system operates under conditions that otherwise render KtrI nonfunctional, such as a high pH. KtrII requires an integral membrane protein, called NtpJ. This protein exhibits strong similarity to KtrB. Interestingly, *ntpJ* is the final open reading frame in the V_0V_1-ATPase *ntpFIKECGABDJ* operon, but is not a functional subunit of this sodium transporter (Murata, Takase, Yamato, Igarashi, & Kakinuma, 1996). Studies with *E. hirae* strains that are defective in NtpJ suggest that this protein mediates not just K^+, but also Na^+, uptake. Indeed, KtrII may be a major reentry pathway for Na^+ under alkaline conditions (Kawano M. , Abuki, Igarashi, & Kakinuma, 2000). A third low affinity/high rate K^+ transport system has been reported that functions at pH values greater than 10, although corresponding genes remain to be identified (Kawano M. , Abuki, Igarashi, & Kakinuma, 2001).

Kep is a potassium expulsion system that exports K^+ against a concentration gradient in exchange for H^+. The Kep K^+/H^+ antiporter is constitutively expressed, but only functions at alkaline pH values. K^+ extrusion stops at pH values less than 8. Mutants defective in antiport are unable to grow at a pH value greater than 8.5 (Kakinuma

& Igarashi, 1999). Thus, in an alkaline environment, this system regulates not only cytosolic K^+, but also pH. K^+/H^+ antiport activity requires ATP, but attempts to detect differences in K^+-activated ATPase in membrane vesicles of wild-type and mutant strains of *E. hirae* have been unsuccessful. Isolation of the Kep antiporter and cloning its gene has yet to be reported.

Copper

Copper is an essential cofactor in a large number of redox-active respiratory, metabolic, and stress enzymes, because of redox activity between Cu^+ and Cu^{2+} oxidation states. This reactivity can be toxic, especially for enterococci that generate O_2^-, as well as derivative-reactive oxygen species, such as H_2O_2 and hydroxyl radicals. Copper, like iron, readily promotes free radical reactions by Haber-Weiss and Fenton chemistry. To minimize damaging oxidation from copper (or iron), cytosolic concentrations must be tightly regulated. This is achieved through proteins called copper chaperones and copper-ATPases that regulate uptake and export. Copper chaperones bind free copper to protect against redox toxicity and deliver this metal to target proteins. A system of copper homeostasis was first identified and characterized for *E. hirae* by Solioz and colleagues and is now recognized as being widely distributed among prokaryotic and eukaryotic organisms (Solioz & Stoyanov, 2003). *E. hirae* is a model organism for copper metabolism and has been the subject of intense study (see (Magnani & Solioz, 2005) and (Solioz & Stoyanov, 2003) for reviews). Human copper ATPases are remarkably similar to the *E. hirae* transport enzyme. Defects in these genes have been implicated in Menkes' and Wilson's diseases of copper metabolism.

The *cop* operon consists of four genes, *copYZAB* (EF0297 to EF0299 and EF0875; Figure 6). Both *copA* and *copB* genes encode transporters that are CPx-type ATPases (E.C. 3.6.1.) that pump heavy metals and belong to a larger class of P-type ATPase ion pumps (Lutsenko & Kaplan, 1995). The "P-type" refers to a phosphorylated intermediate that forms during the catalytic cycle and distinguishes these enzymes from V- and F_0F_1-type ATPases. CopA catalyzes the uptake of Cu^+ under copper-limiting conditions. Since this ion is largely insoluble at a neutral pH, an extracellular copper reductase appears to generate Cu^+ from Cu^{2+}. CopZ presumably accepts Cu^+ from CopA, although interactions between CopZ and CopA have not been directly demonstrated, nor have mechanisms been elucidated for Cu^+ transfer between chaperone and ATPases or target proteins. CopB is another P-type ATPase that functions to export Cu^+ or Ag^+ from cytosol to the cell exterior.

CopY is a copper-responsive repressor that binds an upstream promoter and regulates the expression of all other *cop* genes. CopZ is a 69-amino-acid hydrophilic protein that belongs to a family of copper chaperones that includes MerP, ATX1 from yeast, and HAH1 for humans (Rosenzweig & O'Halloran, 2000). Excess or limited copper induces *cop* operon expression (Solioz & Stoyanov, 2003). When cytosolic copper rises, two Cu^+-CopZ molecules deliver copper to CopY, a copper-responsive repressor that controls the expression of *cop* genes. CopY is a homodimer that, when coordinated with Zn^{2+}, binds to two distinct 28-bp sequences near the translational start of the *cop* operon. CopZ transfers Cu^+ ions to CopY, which then releases Zn^{2+} and causes CopY to dissociate from the promoter. This results in operon induction (Solioz & Stoyanov, 2003). In contrast, to control *cop* expression during copper excess, copper-limited conditions do not cause the release of CopY from the promoter, and operon induction must occur through a second, as yet unknown mechanism. The unusual coregulation of *copA* and *copB* is believed to be necessary to protect the cell from copper toxicity by ensuring that an export pump is available to expel excess copper when the import pump is induced. In *E. hirae*, copper-dependent proteolytic degradation of CopZ is an additional novel mechanism for copper homeostasis (Magnani & Solioz, 2005).

Iron

Iron is an essential nutrient for most microorganisms, including enterococci (Lisiecki & Mikucki, 2006). Despite the exceedingly low concentration of free iron at physiological pH (10^{-18} M) (Andrews, Robinson, & Rodríguez-Quiñones, 2003), acquisition is possible for enterococci, because these microorganisms synthesize and secrete

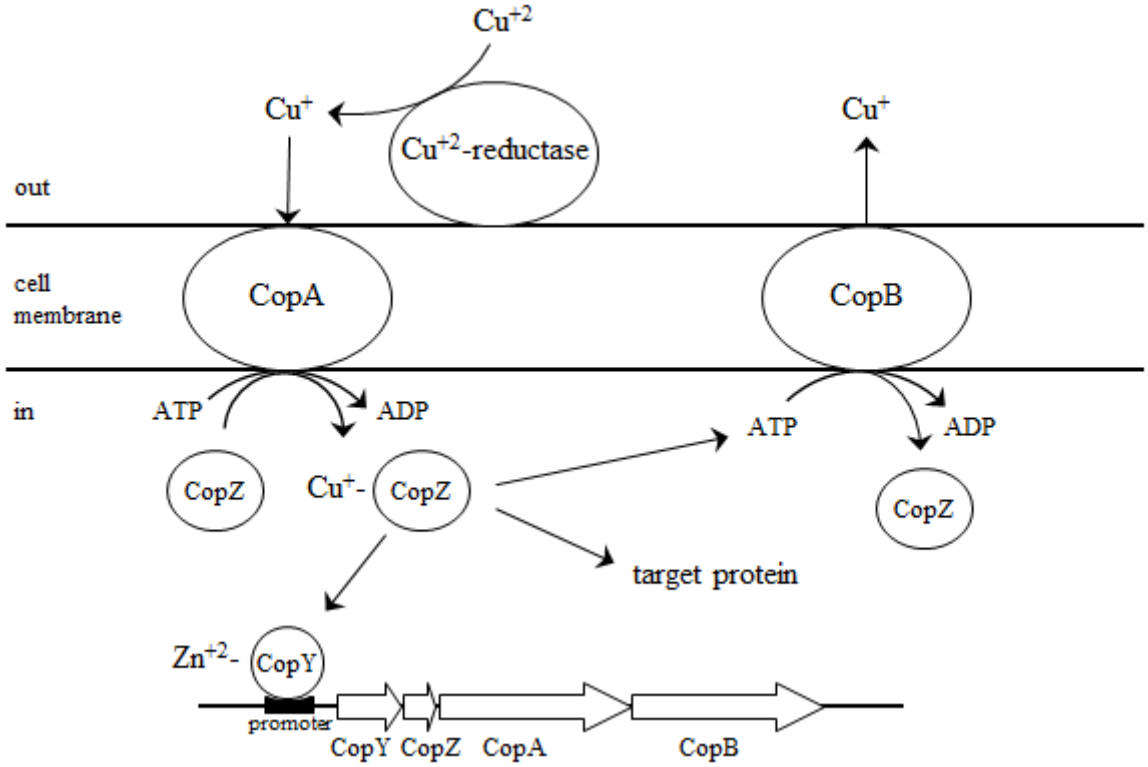

Figure 6. Copper metabolism. The *E. hirae* CopZ copper chaperone is central to copper homeostasis. Cu^{2+} is extracellularly reduced to Cu^{+} by a putative Cu^{2+}-reductase prior to import via a P-type ATPase, termed CopA. CopZ transports bound Cu^{+} to target proteins and the CopY repressor that controls *cop* expression. CopB is a P-type ATPase that accepts Cu^{+} from CopZ for export.

iron-binding compounds called siderophores. Siderophores are a varied group of low-molecular-weight chelators that specifically bind Fe^{+3}, and are made by cells in response to iron deprivation. Membrane-associated siderophore receptors complete a high affinity system for iron acquisition. Most enterococci tolerate iron deprivation through the secretion of linear trihydroxamate or citrate hydroxamate siderophores (Efthymiou, Saadi, Young, & Helfand, 1987; Lisiecki, Wysocki, & Mikucki, 2000; Maskell, 1980). Although the structural diversity of hydroxamate siderophores is enormous, all use ornithine as a common precursor. In addition to siderophore secretion, several strains of *E. faecalis* and *E. faecium* assimilate iron by transporting 2-oxo acids when complexed to Fe^{+3} (*e.g.*, pyruvic acid or 2-oxo-3-methylvaleric acid) (Lisiecki & Mikucki, 2006).

Beyond limited work on siderophore secretion by enterococci, there has been no other report on iron acquisition by these microorganisms. The *E. faecalis* V583 genome database includes several iron uptake mechanisms that are homologous to other bacteria (Paulsen, et al., 2003). At least three putative operons are present. One encodes Fe-chelator ABC transporters (EC 3.6.3.34), another is homologous to *feoA* (EF0475) and *feoB* (EF0476) that encode for ferrous iron uptake, and a third composed of *fur* (ferric uptake regulator)-like sequences. The ABC transporters *feuA* (EF0188) and *fatB* (EF3082), as well as the ferrous transporter *feoB*, all appear to be involved in iron acquisition during growth in blood (Vebø, Snipen, Nes, & Brede, 2009). While experimental evidence for the role of these genes in iron metabolism remains to be determined, *E. faecalis* appears to encode the necessary machinery for both ferric and ferrous iron transport.

Manganese

Manganese is a trace metal with variable oxidation states that serves as a cofactor for numerous enzymes and regulators in metabolic pathways, signal transduction, and response to oxidative stress (Jakubovics & Jenkinson, 2001). In stark contrast to iron and copper, this ion does not catalyze Fenton chemistry to generate damaging

hydroxyl radicals. This attribute has important implications for the resistance of enterococci to desiccation and ionizing radiation. The accumulation of high concentrations of intracellular manganese in *E. faecium* results in a high manganese-to-iron ratio that renders the species nearly as resistant to γ-irradiation as the prototypic radiation-resistant *Deinococcus radiodurans* (Daly M. J., et al., 2004). This degree of radiation tolerance is 10-fold greater than for most other bacteria and involves intracellular manganese protecting DNA repair proteins from oxidative damage caused by ionizing radiation (Daly M. J., et al., 2007). This mechanism helps explain how non-spore-forming enterococci (and *Deinococcus* spp.) are also able to survive extreme oxidative stresses associated with desiccation (Krisko & Radman, 2013).

Manganese can also modulate the virulence of enterococci. The *E. faecalis efaCBA* operon encodes a putative ATP-binding cassette transporter that is regulated by manganese through EfaR, a Mn^{++}-responsive transcriptional regulator (Low, Jakubovics, Flatman, Jenkinson, & Smith, 2003). Genome-wide analysis of *E. faecalis* V583 that was grown in high concentrations of manganese shows the induction of many genes, including numerous transporters (Abrantes, Lopes, & Kok, 2011). Finally, EfaA is a lipoprotein component of this transporter, and was highly expressed in patients with *E. faecalis* endocarditis. A knockout of this gene was associated with delayed mortality in mice (Singh, Coque, Weinstock, & Murray, 1998). Additional investigations of manganese physiology are needed to help better define its role in enterococcal stress responses and virulence.

Lactate

Lactate is the predominant fermentative end-product and is one of the most abundant ions secreted for enterococci under anaerobic conditions with excess glucose. Lactate, with a pK_a' of 3.8, is anionic at all metabolic pH values and thus cannot freely pass the cell membrane. As a result, lactate efflux is necessary and occurs in symport with protons by a carrier-mediated process that translocates the protonated species, lactic acid (Harold & Levin, 1974). Recent evidence using ^{1}H-NMR techniques that distinguish between intracellular and extracellular lactate compartments shows the concentration of free cytosolic lactate in *E. faecalis* is in exact balance with the proton electrochemical-potential gradient over a wide pH range (Hockings & Rogers, 1997). These data identify a pool of tightly bound intracellular lactate at high external pH values and dispel any notion that end-product efflux is an energy-yielding process.

Nucleotide Biosynthesis

Two classes of heterocyclic nitrogenous compounds, pyrimidines and purines, occur in cells as nucleic acids, ribo- or deoxyribonucleoside mono-, di-, and triphosphates, and nucleotide-containing coenzymes. NTPs and dNTPs are precursors for nucleic acids and certain coenzymes. The pathway for purine biosynthesis in enterococci has not been investigated. In contrast, key enzymes for pyrimidine biosynthesis, along with regulation of the *pyr* operon, have been evaluated for *E. faecalis* (Turnbough, Jr. & Switzer, 2008). Pyrimidines are derivatives of (UMP) uridine 5′-monophosphate (the precursor for all pyrimidine nucleotides) and include thymine and cytosine. A cytosolic pool of both purines and pyrimidines is required for the ongoing formation of RNA and DNA.

Purine Biosynthesis

Despite a lack of the biochemical characterization of purine biosynthesis in enterococci, some information on this pathway is available in the *E. faecalis* genome database (Paulsen, et al., 2003). *E. faecalis* purine synthesis is similar in reaction schema to that of other prokaryotes. The first step in purine biosynthesis is catalyzed by amidophosphoribosyltransferase (PurF, E.C. 2.4.2.14–EF1781) that forms 5-phosphoribosylamine from 5-phospho-α-D-ribose-1-diphosphate and L-glutamine. The *E. faecalis* PurF amino acid sequence is 62% identical to PurF for *B. subtilis* (Makaroff, Zalkin, Switzer, & Vollmer, 1983). After the formation of 5-phosphoribosylamine, biosynthesis continues through ten additional reactions, until the central intermediate inosine monophosphate (IMP) is formed. Purine biosynthesis branches from IMP into pathways that produce

guanosine and adenosine monophosphate (GMP and AMP). The genes for purine biosynthesis resemble the *pur* operon in *B. subtilis*, with many encoded in a single putative transcriptional unit (EF1787 to EF1777).

Pyrimidine Biosynthesis

The pyrimidine biosynthetic pathway is similar in all bacteria and begins with glutamine, bicarbonate, and ATP. A single branch point occurs at the initial reaction where carbamoyl phosphate, an intermediate in arginine biosynthesis and catabolism, is formed by carbamoyl phosphate synthetase (CarAB or aspartate transcarbamoylase–EF1716). The *E. faecalis* carbamoyl phosphate synthetase is unusual when compared to the enzymes for other bacteria, because ATP is not activating and pyrimidines are not inhibitory. Instead, this enzyme has an allosteric activator site that is sensitive to many anions, including substrates and products of the reaction (Chang & Jones, 1974).

Genes for *E. faecalis* pyrimidine biosynthesis were first characterized during a study of enterococcal virulence factors (Li, Weinstock, & Murray, 1995). Unlike *E. coli*, where pyrimidine biosynthesis genes are spread throughout the chromosome, *E. faecalis pyr* genes are clustered (EF1715-EF1721) with coordinate regulation that is similar to the *B. subtilis pyr* operon (Turnbough, Jr. & Switzer, 2008). The 5′ end of the *pyr* operon encodes a regulatory protein, termed PyrR, which attenuates *pyr* transcription by binding its own mRNA transcript (Ghim, et al., 1999). In comparison, there are three PyrR binding sites in the *B. subtilis pyr* operon. Exogenous uracil represses the *pyr* operon, while UMP stimulates PyrR binding to a conserved anti-antiterminator sequence in the 5′ leader mRNA.

The oxidation of dihydroorotate to orotate is an intermediate reaction in the biosynthesis of UMP from aspartate and carbamoyl phosphate (Turnbough, Jr. & Switzer, 2008). Dihydroorotate dehydrogenase catalyses the formation of this aromatic intermediate and is the only redox reaction in the pathway. Two families of dihydroorotate dehydrogenase enzymes are known. One is membrane-associated, uses ubiquinone as an electron acceptor, and is found in Gram-negative bacteria and eukaryotic cells. The other family consists of two enzymes (types A and B) described for Gram-positive bacteria, including *E. faecalis*. The *E. faecalis* type A dihydroorotate dehydrogenase is a homodimer encoded by *pyrD-1* (EF0285), and uses fumarate, oxygen, and quinones as electron acceptors (Marcinkeviciene, et al., 2000). In contrast, the *E. faecalis* type B dihydroorotate dehydrogenase (*pyrD-2*–EF1714) is a heterotetramer, with single FMN, FAD, and Fe-S redox sites. Since these enzymes are essential for bacterial growth, effective inhibitors are potential candidates for drug development.

Coenzymes and Prosthetic Groups

The vitamin requirements of enterococci are numerous. Except for folate, lipoic acid, demethylmenaquinone, and hematin, little is known about this topic in the enterococci. Most enterococci require biotin, cobalamin, nicotinate, lipoic acid, pantothenate, riboflavin, and pyridoxine (Sherman, 1938). Characteristically, *E. faecium* requires folate while *E. faecalis* does not. Both species presumably synthesize thiamine, as genes for enzymes in this biosynthetic pathway are present in the genome databases. Examples of vitamin requirements are lipoic acid for the E2 subunit of Pdh, hematin for cytochrome *bd* and catalase, and riboflavin for fumarate reductase.

Folate utilization by *E. faecalis* is one area of enterococcal vitamin metabolism that has provided insights into mechanisms by which Met-tRNA^{fMet} initiates ribosomal protein synthesis. Folate derivatives are one-carbon donors for many cellular reactions: tetrahydrofolate during serine biosynthesis; N^5,N^{10}-methylenetetrahydrofolate during thymidine monophosphate (TMP) and pantothenate formation; N^{10}-formyltetrahydrofolate in purine biosynthesis and formylation of Met-tRNA^{fMet}; and N^5-methyltetrahydrofolate during methionine biosynthesis. The synthesis of folate requires *para*-aminobenzoic acid from chorismate and the pteridine portion from guanosine 5′-triphosphate (GTP). Reduction of dihydrofolate, or folic acid, as the end-product of *de novo* biosynthesis to tetrahydrofolate is necessary, since folic acid is not active. Finally, glutamyl residues are added to form tetrahydropteroyltriglutamate to help bind folate cosubstrates to target

enzymes. Enterococci presumably synthesize folate, although most steps in this process have not been studied. The *E. faecalis* dihydrofolate reductase (E.C. 1.5.1.3) has been purified (Albrecht, Palmer, & Hutchison, 1966), and its gene, *folA* (EF1577), has been cloned and sequenced (Coque, Singh, Weinstock, & Murray, 1999). Many microorganisms that synthesize folate are unable to import this vitamin, and are thus susceptible to folate synthesis inhibitors. Enterococci in particular are sensitive to dihydrofolate reductase inhibitors like trimethoprim but, compared to most other bacteria, are easily rescued by exogenous folate (Zervos & Schaberg, 1985). These observations suggest that there is a mechanism for folate uptake, although this transport system remains to be investigated.

The R strain of *E. faecalis* has a metabolic defect such that it cannot synthesize folate (see (Kersten, 1984) for a review). It grows readily in a semidefined medium supplemented with folate-dependent metabolites: serine, methionine, thymine, and adenine or guanine. Under normal conditions, *E. faecalis* initiates protein synthesis like other bacteria using formyl-methionine-tRNA^{fMet}. All tRNAs contain modifications in the ribosomal-binding region at positions 54, 55, and 56 that allow peptide elongation. This sequence, TψC, is invariant among Gram-positive and Gram-negative microorganisms, with the methyl group for T transferred to the maturing tRNA in Gram-positive organisms by a N^5,N^{10}-methylenetetrahydrofolate- and flavin-dependent enzyme (Delk, Nagle, Jr., Rabinowitz, & Straub, 1979). Although this tRNA-modifying enzyme has not been purified for *E. faecalis*, a likely candidate is thymidylate synthase (*thyA*, E.C. 2.1.1.45–EF1576). This enzyme catalyzes the reductive methylation of dUMP using N^5,N^{10}-methylenetetrahydrofolate to yield dTMP and dihydrofolate. A lack of folate in strain R inhibits the conversion of U to T at position 54 in the tRNA ribosomal binding site and simultaneously blocks formylation of Met-tRNA^{fMet}. Strain R, however, can still initiate RNA translation in a folate-free medium using nonformylated Met-tRNA^{fMet}. This feat is due to differences in the tRNA^{fMet} and appears to also be due to the UψC ribosomal binding region that compensates for the nonformylated-Met-tRNA^{fMet}. This permits normal initiation of binding at the ribosomal P site. Such flexibility in RNA translation may benefit *E. faecalis* in folate-restricted environments, and can potentially contribute to antimicrobial resistance.

Stress Responses

Bacteria have limited abilities to modify their environment and must cope with fluctuations in habitat by programmed responses to stresses. Microorganisms with highly variable lifestyles, like enterococci, are frequently confronted with environmental stresses. As members of the intestinal microbiota, enterococci are exposed to variations in pH, bile salts, fluctuations in osmolarity, and pressures from other inhabitants of the intestinal tract. After release by the host, enterococci must adapt to drastic changes in temperature and entry into oligotrophic conditions. In addition, in the food industry, these bacteria are exposed to temperature extremes and high osmolarity, and in hospitals to high temperatures, detergents, desiccation, antibiotics, and oxidants.

Enterococci have intrinsic resistance to many environmental stresses. Sherman reported in 1937 that these bacteria grow under numerous harsh conditions (Sherman, 1937). An examination of stress responses for these bacteria show extraordinary resistance to acidic and basic pH values, high temperatures, detergents, oxidants, heavy metals, and high osmolarity, as well as survival during long periods of starvation or under oligotrophic conditions (Giard, et al., 2003; Rincé, et al., 2003). Stress resistance is typically enhanced by pretreatment with sublethal exposure to the same (or heterologous) agent. Furthermore, multiple, non-specific stress resistances are established in non-growing cells. For some stresses, resistance is increased by several orders of magnitude, as compared to untreated controls (Giard, et al., 2003). Enterococci are also able to survive for months in a desiccated state on surfaces (Kramer, Schwebke, & Kampf, 2006). Altogether, the remarkable intrinsic ruggedness of these bacteria likely explains their successful persistence and spread within health care settings.

Analysis of *E. faecalis* by two-dimensional gel electrophoresis following stresses demonstrates adaptation, with major switches in gene expression. Approximately 200 polypeptides show enhanced synthesis under several stressful conditions (Giard, et al., 2003; Bøhle, et al., 2010). Many of these polypeptides have been identified by N-terminal Edman degradation or mass spectrometry, and the majority are only specifically induced by a given stress, although a few show enhanced synthesis following many different stress or starvation signals. These generalized stress proteins are likely to be important in protecting enterococci against the challenges of changing environments. Two of these generalized stress proteins correspond to GroEL and DnaK chaperones and are termed Gsp66 and Gsp67, respectively. Gsp65, another stress response protein, is homologous to bacterial organic hydroperoxide reductases (see subsequent section on metabolism of reactive oxygen species). Gls24 was initially identified as a generalized stress protein of unknown function and has since been linked to virulence (see Pathogenesis and models of enterococcal infection). Gsp62 is induced by many different stresses, as well as at the onset of stationary growth. However, a *gsp62* (EF0770) mutant showed no significant change in phenotype following stresses that induce its expression (Rincé, et al., 2002). Two other generalized stress proteins (Gsp63 and Gsp64) do not show homology to other genes and remain uncharacterized.

The stringent response

The stringent response is a global cellular signalling mechanism in which bacteria adapt to adverse environmental conditions, such as nutrient starvation. This response is mediated by an accumulation of alarmones. These small molecules are synthesized by the phosphorylation of GDP and GTP, using ATP as the pyrophosphate donor, and include guanosine-5′-diphosphate-3′-diphosphate (ppGpp) and guanosine-5′-triphosphate-3′-diphosphate (pppGpp). In *E. coli*, RelA and SpoT are two proteins with alarmone synthase activity (Dalebroux, Svensson, Gaynor, & Swanson, 2010). The SpoT protein additionally functions as a (p)ppGpp hydrolase. E. faecalis harbors a homologue to the bifunctional SpoT enzyme, termed RelA, and another small monofunctional RelA-like synthase fragment, termed relQ (EF1974 and EF2671, respectively). In two *E. faecalis* strains, OG1RF (Abranches, et al., 2009) and V583 (Yan, et al., 2009), RelA is responsible for (p)ppGpp accumulation. In contrast, RelQ appears responsible for maintaining basal levels of alarmone in OG1RF, but not V583, and may be important for the timely activation of the stringent response (Gaca, Abranches, Kajfasz, & Lemos, 2012).

E. faecalis accumulates (p)ppGpp during amino acid starvation, heat, and alkaline shock, but not following exposure to acid pH (Abranches, et al., 2009), high osmolarity, or hydrogen peroxide (Yan, et al., 2009). A strong stringent response is observed in cells treated with vancomycin, a glycopeptide antibiotic that blocks cell-wall synthesis, but not with ampicillin, which also inhibits cell-wall synthesis (Abranches, et al., 2009). *relA* mutants of *E. faecalis* strains OG1RF and V583 show adverse growth effects under high osmolarity. The *relA* mutant for OG1RF, but not V583, is also sensitive to growth inhibition at a pH value of 5.0 and with 2 mM H_2O_2. In a *relAQ* double mutant that lacks (p)ppGpp, wild-type resistance to high osmolarity and low pH is restored for strain OG1RF and the double knockout is more tolerant to H_2O_2 stress. These OG1RF *rel*-mutants show less biovolume and decreased long-term survival in biofilms, in comparison to wild-type strains (Chávez de Paz, Lemos, Wickström , & Sedgley, 2012). In contrast to this enhanced sensitivity to environmental stress, OG1RF *relA* mutants grew faster with subinhibitory concentrations of vancomycin and showed a higher tolerance to this glycopeptide in time-course experiments compared to a wild-type strain. On the other hand, the *relAQ* double mutant and, to a lesser extent the *relQ* mutant, were more sensitive in these experiments. Of note, the *relA* mutant of strain V583 was slightly more resistant to lethal concentrations of ethanol, acid, and bile salts in comparison to the parent and *relA* complemented strains. The virulence of the *relAQ* double mutant (but not single mutants) was significantly attenuated in *Caenorhabiditis elegans* (Abranches, et al., 2009) and *Galleria mellonella* infection models (Yan, et al., 2009; Gaca, Abranches, Kajfasz, & Lemos, 2012), and survived less well in murine macrophages (Gaca, Abranches, Kajfasz, & Lemos, 2012).

These results indicate that *E. faecalis* (p)ppGpp alarmones are key for adaption to diverse stresses, resistance and tolerance to glycopeptides, and virulence. Like other bacteria, the stringent response triggers a complex re-programming of gene expression in *E. faecalis*, such as repression of genes associated with cell growth and the replication and activation of genes involved in amino acid biosynthesis, nutrient transport, and stress survival (Gaca, Abranches, Kajfasz, & Lemos, 2012). However, the physiological behavior of *relA*, *relQ*, and *relAQ* mutants is complex and without an obvious correlation between the RelA-dependent accumulation of (p)ppGpp and stress resistance or antibiotic tolerance. Rather, it seems the inability of the *relA* mutant to hydrolyse (p)ppGpp (as synthesized by RelQ), which potentially increases basal levels of (p)ppGpp, is responsible for the sensitization of *E. faecalis* to sublethal stresses and might increase resistance or tolerance to antibiotics (Abranches, et al., 2009). Therefore, to better understand the relationship between the stringent responses, stress responses, and antibiotic tolerances, study of the additional mutants that are only affected in one of the two RelA activities might be helpful. Recently, a *relA* mutant with a large C-terminal deletion named *relAsp* was characterized. A mutant expressing this truncated protein accumulated (p)ppGpp during amino acid starvation. In addition, this mutant was resistant to environmental stresses and was more virulent in the *G. mellonella* model of enterococcal infection (Yan, et al., 2009).

Viable but nonculturable state

Among enterococci, the extremes of temperature, starvation, osmotic concentration, and solar radiation can induce a metabolic state that is termed viable but nonculturable (VBNC). Bacteria with this phenotype do not grow on laboratory media, but are otherwise alive and capable of resuscitation. This metabolic state was initially reported for *E. coli* and *Vibrio cholera*, but has since been described for many eubacteria, including *E. faecalis*, *E. faecium*, and *E. hirae* (Oliver, 2010). The VBNC phenotype is a primary survival strategy for bacteria in natural environments. Cells in the VBNC state have lowered metabolism, maintain cell membranes, contain high levels of intracellular ATP, and continue gene expression with the persistence of mRNAs for as long as 50 days. Metabolism during VBNC results in distinctive protein profiles for *E. faecalis*. *E. faecalis*, *E. faecium*, and *E. hirae* enter the VBNC state after approximately two to six weeks of exposure to an inducing stress, show extensive cross-linking of cell wall peptidoglycan, and increase the expression of autolysins (Pfeffer, Strating, Weadge, & Clarke, 2006). This stress response and key survival strategy results in a general increase in antibiotic resistance, although antibiotics also appear capable of inhibiting resuscitation when cells reenter favorable environments (Lleò, Benedetti, Tafi, Signoretto, & Canepari, 2007). The VBNC phenotype likely renders routine culture techniques inadequate for accurately monitoring enterococci in environmental samples (Signoretto & Canepari, 2008).The potential role of the VBNC state in nosocomial transmission of enterococci, however, has yet to be investigated. Finally, although the expression of stress regulators, DNA polymerases, and virulence factors have been reported during the VBNC state for Gram-negative pathogens, including *E. coli*, *Helicobacter pylori*, *Vibrio vulnificus*, and *V. cholerae* O1 (Oliver, 2010), the metabolic and genetic basis for this phenotype in enterococci remains unknown.

Redox Metabolism

Enterococci are potent producers and scavengers of reactive oxygen species (ROS). A remarkable reducing capacity was noted in original descriptions of enterococci over 100 years ago (Andrewes & Horder, 1906). This activity has been ascribed, at least in part, to the production of extracellular O_2^- (Huycke M. M., et al., 2001; Falcioni, Coderoni, Tedeschi, Brunori, & Rotilio, 1981). This phenotype is rare among eubacteria and is only known for *E. faecalis*, *E. casseliflavus*, a few *E. faecium* isolates, *Lactococcus lactis*, and several mycoplasmas. Although *E. coli* also produces extracellular O_2^-, concentrations are 1000-fold less than those observed *in vitro* for enterococci (165). Enterococci also produce H_2O_2 during aerobic metabolism of glycerol as a byproduct of α-glycerophosphate oxidase (Fig. 2). These pro-oxidant activities necessitate potent antioxidant defenses. *E. faecalis* amply demonstrates evidence for these capabilities. For example, a 30-min adaptation to 2.5 mM H_2O_2 confers greater than 200-fold increased tolerance to subsequent challenges with 45 mM H_2O_2 (Flahaut, Laplace,

Frère, & Auffray, 1998). As will be discussed, such impressive resistance to oxidative stress, as well as a capacity to survive inside phagocytic cells (Gentry-Weeks, Karkhoff-Schweizer, Pikis, Estay, & Keith, 1999) results from the expression of diverse anti-oxidative proteins that include peroxidases, oxidases, peroxiredoxins, and alkyl hydroperoxidases, along with glutathione.

Extracellular superoxide

The production of extracellular O_2^- by "group D *Streptococcus*" was initially described by Falcioni and colleagues in 1981 (164). These observations were extended to several other species of enterococci and lactococci (Winters, Schlinke, Joyce, Glore, & Huycke, 1998; Huycke, Joyce, & Gilmore, 1995), as well as Gram-negative bacteria, to a more limited extent (Korshunov & Imlay, 2006). O_2^- results from the univalent reduction of O_2. As an anion, this radical is impermeant to passive diffusion and remains extracellular when generated on the outer side of a cell membrane. Although O_2^- is relatively non-reactive, it can readily dismute to form more powerful oxidants, such as H_2O_2 and a hydroxyl radical. Disruption of the pathway for demethylmenaquinone synthesis blocks extracellular O_2^- production by *E. faecalis* and suggests membrane-associated quinones as the source of these radicals (Huycke M. M., et al., 2001). The most probable mechanism involves univalent oxidation of reduced quinone, or quinol-to-labile semiquinone radicals that spontaneously react with O_2 to form O_2^- (Figure 7).

For *E. faecalis*, functional cytochrome *bd* suppresses the production of extracellular O_2^- (Huycke M. M., et al., 2001). Thus, when these bacteria are grown with heme, the sole apo-cytochrome expressed by this species becomes functional and radical production is abolished. Similarly, inactivation of the cytochrome *bd* by gene knockout or withholding heme is permissive to extracellular O_2^-. Presumably, actively cycling cytochrome *bd* efficiently reoxidizes demethylmenaquinol and minimizes semiquinone reactivity with O_2. Frd is another *E. faecalis* quinol oxidase that also attenuates extracellular O_2^- production. This effect, however, only occurs in the presence of fumarate, the substrate for this membrane-bound enzyme (Huycke M. M., et al., 2001). Electron-spin resonance studies of intestinal contents and the detection of hydroylated isomers of tyrosine in rodents colonized with *E. faecalis* indicate that conditions exist in the mammalian intestinal tract that allow for O_2^- formation (Huycke & Moore, 2002; Moore, Kotake, & Huycke, 2004). These observations show that commensals can be potential sources for oxidant stress on the intestinal epithelium. Indeed, mice colonized with *E. faecalis* develop colonic epithelial cell DNA damage (Huycke, Abrams, & Moore, 2002; Wang & Huycke, 2007). Oxidative stress from *E. faecalis* also causes chromosomal instability in mammalian cells (Wang & Huycke, 2007; Wang, et al., 2008). Finally, interleukin-10 knockout mice colonized with these bacteria develop inflammation and colorectal cancer (Balish & Warner, 2002; Kim, et al., 2005). The formation of tumors in these mice depends on extracellular O_2^- production (Wang, et al., 2012). These findings have profound implications for the role of intestinal commensals in general, and enterococci in particular, in inflammatory bowel disease and colorectal cancer (Sinicrope, 2007).

Manganese superoxide dismutase

The univalent reduction of O_2 to produce O_2^- is a common minor byproduct of numerous intracellular sources that include autoxidizable small molecules, oxidoreductive enzymes, and subcellular organelles, like mitochrondria (Fridovich, 1999). If not properly scavenged, O_2^- can damage thiols, tetrahydropterins, ascorbate, poly-unsaturated fatty acids, [4Fe-4S] clusters in dehydratases, and DNA. O_2^- can also form other damaging oxygen and nitrogen compounds, like H_2O_2, hydroxyl radical, and peroxynitrite. Superoxide dismutase (SOD) and peroxidases are the primary defenses against the cascade of oxidative injuries initiated by O_2^-.

Virtually all facultative and aerobic organisms express one or more types of SOD. SOD rapidly converts O_2^- to H_2O_2 and molecular oxygen. Depending on the cofactor, these metalloenzymes are classified as iron, manganese, or copper-zinc SODs. Iron and manganese SODs share a common ancestor, while copper-zinc enzymes appear to have evolved independently (Zelko, Mariani, & Folz, 2002) and, among bacteria, are most

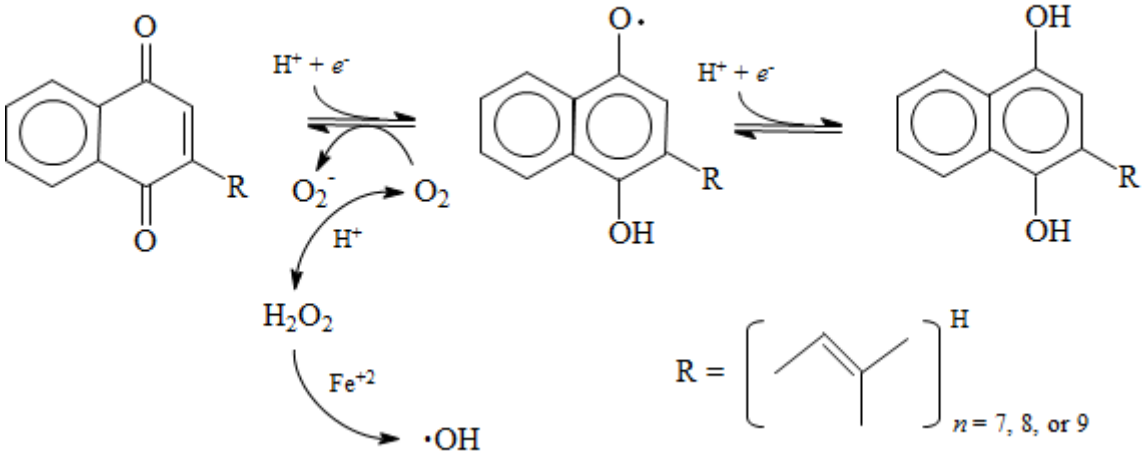

Figure 7. Extraceulluar superoxide production. Model of extracellular O_2^- production by *E. faecalis* demethylmenaquinone. Cytosolic reducing equivalents transfer to demethylmenaquinone (left) through oxidoreductases that form demethylmenaquinol (right). Normally, reduced demethylmenaquinone binds terminal quinol oxidases, such as fumarate reductase or cytochrome *bd*. In the absence of fumarate or hematin, extracellular O_2^- is generated by the univalent reduction of O_2 with electrons donated by unstable semiquinone radicals (middle). Under acidic conditions O_2^- spontaneously dismutes into H_2O_2 and, in the presence of transition metals like iron or copper, catalytically forms damaging hydroxyl radicals (·OH).

often found in the periplasm of Gram-negative organisms. *E. faecalis* contains a single manganese SOD (SodA, EF0463) that is induced by O_2 (Britton, Malinowski, & Fridovich, 1978). No evidence exists for iron or copper-zinc forms. Mutants in *sodA* are more sensitive to oxidative stress and show reduced resistance to killing by macrophages (Verneuil, et al., 2006). *E. faecalis* is highly tolerant to many bactericidal drugs. Recently, SodA was functionally shown to eliminate the bactericidal activity of penicillin and vancomycin (Bizzini A. , Zhao, Auffray, & Hartke, 2009). This clinically important finding is presented in further detail at the end of this chapter (see the section on the relation between oxidative stress and antibiotic tolerance).

NADH oxidase

E. faecalis expresses an unusual FAD-dependent cytosolic enzyme that catalyzes the direct reduction of oxygen to H_2O. NADH oxidase (Nox, EC 1.6.99.3–EF1586) completes this reaction through a four-electron reduction of O_2, without the release of any O_2^- or H_2O_2 (Schmidt, Stöcklein , Danzer, Kirch, & Limbach, 1986). This reaction requires NADH as the preferred electron acceptor. The main physiological role for Nox is to regenerate NAD^+ for glycolysis. This allows the synthesis of additional ATP by converting pyruvate to acetate instead of lactate. Since O_2 is consumed by this reaction, Nox can also act as an antioxidant. A Nox homologue is present in *E. faecium*, but appears to be absent in *E. casseliflavus* and *E. gallinarum*. The *E. faecalis* Nox is a homodimer containing one FAD and, like the NADH peroxidase (Npr, see below), a nonflavin cysteine-sulfenic acid (Cys-SOH) redox center (Mallett & Claiborne, 1998). The *nox* gene for *E. faecalis* is 44% identical to *npr* (see below) and, as with NADH peroxidase, conserves the Cys-SOH at position 42, which suggests similarity in catalysis. Coordinate regulation of NADH oxidase has not been described, although this enzyme is induced by oxygen and is partially repressed by exogenous heme (Pugh & Knowles, 1982). The potential role for Nox in enterococcal pathogenesis remains to be investigated.

Glutathione

Few Gram-positive bacteria synthesize glutathione (γ-GluCysGly, GSH), a thiol tripeptide that protects against oxidative stress and serves as an essential cofactor for many metabolic reactions (Masip, Veeravalli, & Georgiou,

2006). Instead, most bacteria simply import GSH. *E. faecalis* and *E. faecium*, however, can not only import GSH, but can also synthesize it *de novo* (Gopal, et al., 2005; Newton, et al., 1996). GSH is usually made by two enzymes in separate steps: γ-glutamylcysteine formation from glutamate and cysteine, and glutathione formation by the subsequent addition of glycine. In enterococci, however, these reactions appear to occur through an unusual bifunctional enzyme that exhibits both γ-glutamylcysteine synthetase and glutathione synthetase activity (Gopal, et al., 2005; Janowiak, Hayward, Peterson, Volkman, & Griffith, 2006). An enzyme with similar activity has been isolated from *S. agalactiae*, *Listeria monocytogenes*, and *Pasteurella multocida* (Gopal, et al., 2005). A homologous gene termed *gshAB* (or *gshF*–EF3089) is annotated in genome databases for *E. faecalis* and *E. faecium*. However, the enterococcal gene product remains to be characterized.

Many enzymes utilize GSH as a cofactor, including glutathione peroxidase, glutathione *S*-transferases, and glutathione reductase. *E. faecalis* expresses glutathione reductase (Gor, EF3270), an FAD-containing enzyme, and a poorly characterized glutathione peroxidase that scavenges H_2O_2 (Patel, Marcinkeviciene, & Blanchard, 1998). A survey of available genomic sequences indicates that genes that encode proteins with amino acid identities of 34% (*E. casseliflavus, E. gallinarum*) to 47% (*E. faecium*) to EF1211 are present in other enterococcal species. In contrast, Gor is conserved among enterococci and catalytically reduces GSSG to GSH. The reaction equilibrium for glutathione reductase is such that cytosolic ratios of GSH to GSSG remain high, which provides an anaerobic-like cytosolic environment. As such, glutathione reductase serves a primary role in the defense against oxidants. Glutathione reductase is induced by O_2, although aerobiosis does not significantly change the concentration of intracellular GSH, which suggests that GSH synthesis is not coordinately regulated.

Catalase

Catalase is a cytosolic hemoprotein that catalyzes the dismutation of H_2O_2 to molecular oxygen and water. Catalase (KatA, EF1597) activity is detected in *E. faecalis* during aerobic growth in the presence of heme (Whittenbury, 1964; Pugh & Knowles, 1983), but a homologous gene is absent in *E. faecium, E. casseliflavus* and *E. gallinarum*. Because *E. faecalis* cannot synthesize heme, and because the incorporation of heme into apoenzyme KatA (apo-KatA) is O_2 dependent, KatA only becomes functional when exogenous heme is provided. In the absence of heme, KatA remains a nonfunctional apoenzyme. As is typical for most catalases, KatA is inhibited by azide and cyanide (Whittenbury, 1964). The *katA* gene and its corresponding protein have been isolated and characterized for *E. faecalis* (Frankenberg, Brugna, & Hederstedt, 2002; Baureder, Reimann, & Hederstedt, 2012).

NADH Peroxidase

E. faecalis NADH peroxidase (Npr, EF1211), like KatA, catalyzes the decomposition of H_2O_2 to molecular oxygen and water. A survey of available genomic sequences indicates that genes that encode proteins with amino acid identities of 34% (*E. casseliflavus*, *E. gallinarum*) to 47% (*E. faecium*) to EF1211 are present in other enterococcal species. This activity was originally considered to be due to a "pseudocatalase," because the responsible protein did not contain heme. The nonheme nature of Npr renders it insensitive to azide and cyanide, and permits continued enzymatic activity, despite exposure to high concentrations of H_2O_2 (Whittenbury, 1964). Npr is a flavoprotein disulfide reductase that is similar to lipoamide dehydrogenase, glutathione reductase, and thioredoxin reductase (Claiborne, et al., 1999). A peroxidase with similar reactivity has been described in *E. hirae* (Miller, Poole, & Claiborne, 1990), although this particular species does not encode an *npr* gene with sequence homology to that of *E. faecalis* or other enterococci. In the absence of functional KatA, Npr is absolutely required for aerobic growth on glycerol and for maximal growth on lactose, galactose, or ribose (La Carbona, et al., 2007). In addition, Npr contributes to the survival of enterococci against exogenous H_2O_2 (La Carbona, et al., 2007) generated, for example, by other facultative or anaerobic bacteria in the intestine (Gordon, Holman, & McLeod, 1953). Sequence analysis of *npr* reveals highly conserved segments that correspond to three of the four structural domains for glutathione reductase (Ross & Claiborne, 1992). A

putative OxyR protein in *E. faecalis* that binds to an upstream region of *npr* potentially assists with coordinate regulation of genes following oxidative stresses (Ross & Claiborne, 1997).

Peroxiredoxins

A serious consequence of oxidative stresses is the formation of organic hydroperoxides that subsequently initiate free-radical chain reactions, which lead to DNA damage. Peroxiredoxins use cysteine thiols to detoxify such harmful peroxides and thereby protect cells from mutation. This is especially important for *E. faecalis*, as it generates endogenous ROS through multiple mechanisms. The first peroxiredoxin system was characterized in *Salmonella typhimurium* (Aph) (Jacobson, Morgan, Christman, & Ames, 1989). The catalytic site for reductase activity in this bacterium (AhpC, EF2739) contains cysteines that react with peroxides to yield corresponding alcohols. AhpCs reduce a broad range of peroxides from H_2O_2 to complex organic hydroperoxides. The activities of these enzymes depend on small FAD-containing thioredoxin reductases (AhpF, EF2738) that use NADPH to recycle the reductase. The *E. faecalis ahpCF* operon is expressed following oxidative stress and controlled by the *hy*drogen *p*eroxide *r*egulator (HypR–EF2958) that also regulates genes for SodA, KatA, glutathione reductase, and thiol peroxidase (Verneuil, et al., 2004). Notably, HypR does not contain cysteines that are commonly used to sense oxidative stress (Verneuil, et al., 2004). This transcriptional regulator for oxidative stress is therefore functionally unrelated to OxyR. Finally, AhpC and AhpF represent important defenses against oxidative stresses, as shown for *E. faecalis* when grown on glycerol (La Carbona, et al., 2007). Corresponding homologous operons are also present in other sequenced enterococcal species.

Thiol peroxidases (Tpx; EF2932) are members of another peroxidase class that confers broad substrate specificity. The *E. faecalis* Tpx is typical of members in this class and contains two cysteine residues that align with catalytically important residues found in the *E. coli* Tpx (La Carbona, et al., 2007). For *E. faecalis*, this antioxidant enzyme appears more important than Npr or AhpC for survival in macrophages and in promoting virulence using a murine peritonitis model. Homologous enzymes with approximately 60% amino acid identity are present in *E. casseliflavus* and *E. gallinarum* genome databases, but are lacking for *E. faecium*.

Organic hydroperoxide resistance (Ohr) comprises a third class of peroxiredoxins that, similar to AhpC, contain invariant cysteine residues that help reduce peroxides. Ohr genes occur in only a few eubacteria, including *E. faecalis* (Ochsner, Hassett, & Vasil, 2001). In the genome database for *E. faecalis* V583, two genes (EF0453 and EF3201) encode Ohr family proteins. EF0453 appears to be a general stress response protein, termed Gsp65, with significant homology to Ohr proteins from other organisms (Rincé, Giard, Pichereau, Flahaut, & Auffray, 2001). Knockout of *gsp65* is deleterious to *E. faecalis*, with mutants showing increased susceptibility to *tert*-butylhydroperoxide and ethanol. Proteins with approximately 50% amino acid identity to EF0453 are present in other sequenced enterococci.

Relationship between oxidative stresses and antibiotic tolerance

The most effective antimicrobials function to rapidly kill pathogens, not just inhibit them, and thereby limit the severity of infections and emergence of resistance. *Enterococci*, however, are intrinsically tolerant to many bactericidal drugs, including β-lactams and glycopeptides. Mechanisms that confer protection against the lethal effects of these drugs have recently been investigated (Bizzini A. , Zhao, Auffray, & Hartke, 2009; Ladjouzi, et al., 2013). Singly- and multiply-deficient mutants of the *E. faecalis* strain JH2-2 affected in oxidative stress defense activities or DNA repair were screened for loss of tolerance to bactericidal antibiotics. The only mutant that was efficiently killed by vancomycin or penicillin (but not by bacteriostatic drugs) was deficient in manganese SOD (Bizzini A. , Zhao, Auffray, & Hartke, 2009). This dependence of tolerance on active SOD was confirmed for another *E. faecalis* strain (OG1RF) and the manganese SOD (SodA) of *E. faecium* also appeared to be key for tolerance to bactericidal antibiotics (Ladjouzi et al. 2013). The combined results implied that these drugs increase intracellular superoxides. Although SodA efficiently detoxifies O_2^- to H_2O_2, this latter ROS must be less problematic, even in peroxidase- or catalase-deficient strains, since mutants with deficiencies in these enzymes

were still tolerant to these antibiotics. In *sodA* mutants, however, O_2^- presumably accumulates to toxic levels that damage cellular targets and cause cell death. The mechanisms by which bactericidal antibiotics promote intracellular superoxide production remain to be determined. Finally, *E. faecalis* can also generate extracellular superoxide through the leakage of electrons from membrane-associated demethylmenaquinone (Huycke M. M., et al., 2001). However, this site of O_2^- production does not seem to play a role in the bactericidal activity of antibiotics, since mutants that lacked demethylmenaquinone (i.e., extracellular O_2^- production) along with SodA were no less sensitive to these antibiotics than the Δ*sodA* mutant alone (Bizzini A. , Zhao, Auffray, & Hartke, 2009).

Conclusion

Enterococci are commercially and medically important bacteria that are commonly found in close association with nearly every type of animal life. Their colonization range is enabled by intrinsic tolerances to many forms of stress and diverse metabolic capabilities. While the study of enterococcal metabolism has been ongoing for over a century, it is only within the last several years that we have begun to understand the ways in which enterococci adapt to diverse environments and colonize numerous host ranges.

The recent explosion of next-generation sequencing technology and genomic information has diverted many researchers away from present work on bacterial metabolism and physiology. However, as data accumulate, the necessity for a thorough knowledge of gene function, especially in regards to metabolism, is of greater importance than ever. The initial sequencing efforts for the pathogenic, antibiotic-resistant *E. faecalis* V583 strain have revealed a tremendous amount of genomic information on mobile elements (Paulsen, et al., 2003; Shankar, Baghdayan, & Gilmore, 2002). Acquisition of new capabilities through exchange of these elements may allow enterococci to transition from commensals to pathogens, and suggests the possibility of enhanced ability to colonize new hosts. The genomic sequence of the *E. faecalis* commensal strain OG1RF demonstrates a different view on this species, as its genome contains comparatively few mobile elements (Bourgogne, et al., 2008). Recent efforts by Palmer *et al*. highlight additional strain variation within *E. faecalis* (Palmer, et al., 2012; Palmer, et al., 2010).

An important goal for future research involves establishing a true core metabolism for *E. faecalis*. Of even greater importance is the identification of those core metabolic functions that can unite all *Enterococcus* species. Our initial forays into these subjects suggest that *E. faecalis* V583 utilizes sugar compounds through a mobile element that expresses PTS genes that are not found in other strains. Additionally, *E. faecalis* OG1RF can catabolize inositol, a sugar carbon source not used by other strains. The possession of substrate specific genes may reveal ancestry and habitat preferences within enterococcal lineages that could be exploited for containment or treatment. Understanding the core metabolism of all Enterococci will allow us to design specific treatments for this robust organism. While much remains to be discovered, we owe a tremendous debt to the previous generation of investigators. The quality and quantity of their body of work is humbling and encourages us to carry on their efforts by using techniques both new and old.

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Enterococcal Bacteriocins and Antimicrobial Proteins that Contribute to Niche Control

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Introduction

Enterococci, which belong to the group of lactic acid bacteria (LAB), have received increased attention in recent years for various reasons (Fisher & Phillips, 2009; Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011; Leavis, Bonten, & Willems, 2006). While lactobacilli, another group of LAB, have been shown to confer numerous benefits and are often regarded as health-bringing organisms, enterococci have become more recognized as emerging human pathogens despite the fact that they are as numerous as the lactobacilli in our gastrointestinal tractgastrointestinal tract. *Enterococcus faecalis* is the dominant *Enterocuccus* in the gastrointestinal tract, followed by *E. faecium*; however, *E. avium* and *E. hirae*, as well as other enterococcal species, are frequently found in human stool samples. The commensal/probiotic role of enterococci in humans and animals has evolved through thousands of years in mutual coexistence—but the ability of the enterococci to behave in a way that causes problems to human health is only beginning to be understood. Virulence, which may evolved as an adaptation to the "modern lifestyle," including the profligate use of antibiotics in medical practice and animal husbandry, needs to be understood and limited where possible. On the other side, enterococci have many positive traits that have been appreciated in food fermentation and preservation, and may also serve as probiotics to promote health.

Bacteriocin-producing bacteria are found in all environments. In many LAB isolates, bacteriocin production has been examined by biochemical and genetic studies, and the bacteriocins produced by enterococci are often similar to those produced by other lactic acid bacteria. A classification scheme has been developed for bacteriocins produced by Gram-positive bacteria, and most of this information is based on findings from LAB. Although classification is still a disputed issue, two major classes of heat-stable, ribosomally synthesized antimicrobial peptides are well defined. Class I constitutes the lantibiotics, while Class II constitutes the unmodified non-lantibiotics.

Bacteriocins within different classes and subclasses also have been isolated and characterized in enterococci (Cotter, Hill, & Ross, 2005; Nes, Diep, Håvarstein, Brurberg, Eijsink, & Holo, 1996; Nes, Yoon, & Diep, 2007). One of the most striking findings so far is the almost complete absence of lantibiotics among enteroccci, with the only exceptions being cytolysin and enterocin W (Coburn & Gilmore, 2003; Cox, Coburn, & Gilmore, 2005; Sawa, et al., 2012). Most of the characterized enterocins belong to the Class II bacteriocins and a few are heat-labile lytic enzymes. The latter were previously classified as bacteriocins, but are now included in a distinct class of antimicrobials (Cotter, Hill, & Ross, 2005).

The hemolytic bacteriocin (cytolysin), the circular AS-48, and bacteriocin 21 have been known as *E. faecalis* bacteriocins for a long time, and they have been genetically and biochemically well characterized (Clewell, 1981; Gilmore, 1991; Gilmore, Coburn, Nallapareddy, & Murray, 2002; Gilmore, Segarra, Booth, Bogie, Hall, & Clewell, 1994; Haas, Shepard, & Gilmore, 2002; Ike, Clewell, Segarra, & Gilmore, 1990). Many bacteriocin

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producers have been identified and investigated from infection-derived *E. faecalis* or *E. faecium* isolates. From *E. faecalis* isolates, for example, bacteriocin 31(Tomita, Fujimoto, Tanimoto, & Ike, 1996) and bacteriocin 41 (Tomita, Kamei, & Ike, 2008) have been studied; and from *E. faecium*, there are bacteriocin 43 (Todokoro, Tomita, Inoue, & Ike, 2006), bacteriocin 32 (Nes, Diep, Håvarstein, Brurberg, Eijsink, & Holo, 1996), and bacteriocin 51(Yamashita, Tomita, Inoue, & Ike, 2011). Besides enterococcal bacteriocins of clinical origin, bacteriocins from enterococci of food origins have been studied, and several bacteriocins from *E.faecium* isolates have been identified and characterized. These include enterocin L50A/L50B (Cintas, Casaus, Holo, Hernandez, Nes, & Håvarstein, 1998), enterocin Q (Cintas L. M., et al., 2000; Criado, et al., 2006), enterocin A (Aymerich, Holo, Håvarstein, Hugas, Garriga, & Nes, 1996; Nilsen, Nes, & Holo, 1998), enterocin P (Cintas L. M., Casaus, Håvarstein, Hernández, & Nes, 1997; Kang & Lee, 2005), enterocin B (Casaus, Nilsen, Cintas, Nes, Hernández, & Holo, 1997) and others.

Many enterocins have also been characterized from various enterococcal species and from many environments, and the most thoroughly characterized enterocins are summarized in Table 1. Most of the characterized enterocins are from *E. faecium* and *E. faecalis*, but enterocins have also been isolated from *E. muntii*, *E. avium*, *E. hirae*, and *E. durans* (see Table 1). The bacteriocin-producing enterococci are by and large isolated from food, waste, and the feces and gastrointestinal tract of humans and animals, but may also be isolated from other sources. Fermented food, specimens from human infections, and feces from healthy babies seem to be particularly good niches for isolating bacteriocin-producing enterococci (see Figure 1). It seems likely that most enterococci originate from the digestive tract of humans and animals, a notion which is in line with the finding that the same bacteriocins are identified in enterococci isolated from many environments, which most often include those of human origin.

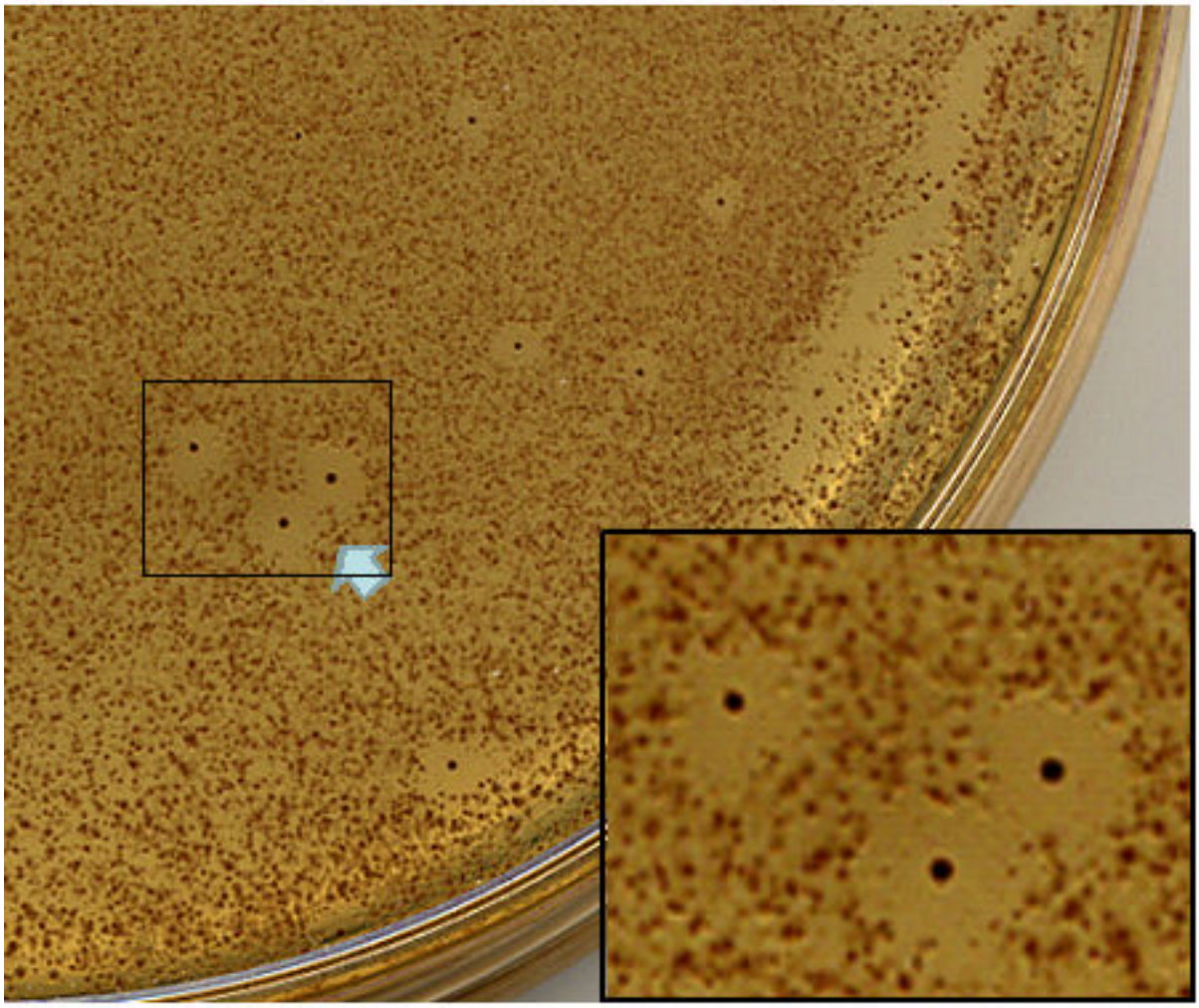

Figure 1. Plating of a fecal sample from a healthy 6-month-old breast fed baby on a MRS agar plate anaerobically. No indicator bacteria are added and only endogenous fecal lactic acid bacteria are grown on the plate. Some bacteriocin-producing fecal bacteria inhibit growth of other fecal bacteria. The arrow indicates bacterial growth inhibitory zones due to the bacteriocin producing endogenous lactic acid bacteria (enterococci) present in the feces. The insert shows magnification of some bacteriocin-producing colonies.

Table 1. Classification of Enterocins

			Source of isolation[a)]	Mol. Weight (amino acids)	References
Class I: Lantibiotics					
E. faecalis	Cytolysin Cyl_L, and Cyl_S	two-peptide lantibiotic	Clinical isolates	3,458 (38), 2,032 (21)	(Gilmore, Segarra, Booth, Bogie, Hall, & Clewell, 1994)
E. faecalis	Enterocin Wα and Wβ	two-peptide lantibiotic	Thai fermented fish	3,256 (30) and 2,728 (29)	(Sawa, et al., 2012)
Class II: Bacteriocins					
Class IIa: Antilisteria-Pediocin-like bacteriocins					
E. faecium	Enterocin A	double-glycine leader	Spanish dry fermented sausage	4,829 (47)	(Aymerich, Holo, Håvarstein, Hugas, Garriga, & Nes, 1996)
E. faecium	Enterocin P	sec -leader	Spanish dry fermented sausage	4,493 (44)	(Cintas L. M., Casaus, Håvarstein, Hernández, & Nes, 1997)

Table 1. continued from previous page.

Class II: Bacteriocins					
E. faecium	Bacteriocin GM-1	sec-leader	Feces infants	4,630 (44)	(Kang & Lee, 2005)
E. faecalis	Bacteriocin 31	sec-leader	Clinical isolate	(43)	(Tomita, Fujimoto, Tanimoto, & Ike, 1996)
E. mundtii	Mundticin KS, enterocin CRL35, mundticin QU2	double-glycine leader	Grass silage, artisanal cheese	4,287 (43)	(Kawamoto, et al., 2002; Saavedra, Minahk, de Ruiz Holgado, & Sesma, 2004)
E. faecalis	Enterocin SE-K4	sec-leader	Grass silage	5,356 (43)	(Eguchi, et al., 2001)
E. faecalis	Enterocin MC4-1	sec-leader	Macaque monkey	(43)	(Flannagan, Clewell, & Sedgley, 2008)
E. faecium	Bacteriocin T8	sec-leader	Vaginal secretion of children infected with HIV	5,090 (44)	(De Kwaadsteniet, Fraser, Van Reenen, & Dicks, 2006)
E. avium	Avicin A	double-glycine leader	Feces from babies	4,289 (43)	(Birri, Brede, Forberg, Holo, & Nes, 2010)
E. hirae	Hiracin JM79	sec-leader	Wild mallard duck	5,093 (44)	(Sánchez, Diep, Herranz, Nes, Cintas, & Hernández, 2007)
E. faecium	Bacteriocin RC714	sec-leader Identical to bacteriocin 31	Human exudate	(43)	(del Campo, et al., 2001)
E. faecium	Bacteriocin 43	sec-leader Identical to bacteriocin 31	Clinical isolate	(44)	(Todokoro, Tomita, Inoue, & Ike, 2006)
E. durans	Duracin GL			(43)	gb | ADW93772.1|
Class IIb: Two-peptide bacteriocins					
E. faecalis	Enterocin C	Human colostrum	4,286 (39) 3869 (35)	(Maldonado-Barragán, Caballero-Guerrero, Jiménez , Jiménez-Díaz, Ruiz-Barba, & Rodríguez, 2009)	
E. faecalis	Enterocin 1071 A and B	Feces from mini-pigs	4,286 (39), 3,899 (35)	(Balla, Dicks, Du Toit, Van Der Merwe, & Holzapfel, 2000)	
E. faecium	Enterocin X a and b	Sugar apples	4420 (40), 4068(37)	(Hu, Malaphan, Zendo, Nakayama, & Sonomoto, 2010)	
Class II: circular bacteriocins					
E. faecalis	AS-48, Bacteriocin 21, enterocin 4	Clinical isolate	7,166 (70)	(Joosten, Nunez, Devreese, Van Beeumen, & Marugg, 1996; Martínez-Bueno, et al., 1994; Samyn, et al., 1994; Tomita, Fujimoto, Tanimoto, & Ike, 1997)	
Class II: leaderless bacteriocins					
E. faecalis	MR10A MR10B / (343)Ent7A Ent7B	Bird uropyal glands, Beef	5,202 (44), 5,208 (43)	(Liu, et al., 2011; Martín-Platero, et al., 2006)	
E. faecium	Enterocin L50A, L50B / 62A , 62B	Dry fermented sausage, Human vagina	5,190 (44), 5,178 (43)	(Cintas L. M., Casaus, Holo, Hernandez, Nes, & Håvarstein, 1998; Dezwaan, Mequio, Littell, Allen, Rossbach, & Pybus, 2007)	
E. faecium	Enterocin Q	Dry fermented sausage,	3,980 (34)	(Cintas L. M., et al., 2000; Criado, et al., 2006)	
E. faecalis	Enterocin EJ97	Municipal waste water	5,328 (44)	(Gálvez, et al., 1998; Sánchez-Hidalgo, Maqueda, Gálvez, Abriouel, Valdivia, & Martínez-Bueno, 2003)	
E. faecium	Enterocin RJ-11	Rice bran	5,049 (44)	(Yamamoto, Togawa, Shimosaka, & Okazaki, 2003)	

Table 1. continued from previous page.

Class II: leaderless bacteriocins				
Class II: Other bacteriocins				
E. faecium	Bac 32, enterocin IT	Clinical isolate (VRE), ryegrass	6,390 (54)	(Inoue, Tomita, & Ike, 2006; Izquierdo, et al., 2008; Izquierdo, Cai, Marchioni, & Ennahar, 2009)
E. faecium	Bacteriocin 51	Clinical isolate (VRE)	105 aa heat stable	(Yamashita, Tomita, Inoue, & Ike, 2011)
E. faecium	Enterocin B	Spanish dry fermented sausage	5,479 (53)	(Casaus, Nilsen, Cintas, Nes, Hernández, & Holo, 1997)
E. faecalis	Enterocin 96	Munster cheese	5,179 (theoretical) (48)	(Izquierdo, Wagner, Marchioni, Aoude-Werner, & Ennahar, 2009)
Heat label enterolysins				
E. faecalis	Enterolysin A	Fish , milk	34,501 (343)	(Hickey, Twomey, Ross, & Hill, 2003; Nilsen, Nes, & Holo, 2003)
E. faecalis	Bacteriocin 41	Clinical isolate	(595)	(Tomita, Kamei, & Ike, 2008)

This indicates the first source of isolation.

Class I: Lantibiotics

Lantibiotics are rarely found in enterococci. Until now, only two two-peptide lantibiotics have been purified and genetically characterized, both from *E. faecalis* isolates (Booth, Bogie, Sahl, Hatter, & Gilmore, 1996; Coburn, Hancock, Booth, & Gilmore, 1999; Sawa, et al., 2012).

The cytolysin is the most thoroughly characterized enterococcal lantibiotics, and it exerts antimicrobial activity against a broad range of Gram-positive bacteria, but it also antagonizes certain eukaryotic cells, such as erythrocytes from various animals, and is therefore often referred to as a hemolysin. Cytolysin also lyses retinal cells, polymorphonuclear leukocytes, and human intestinal epithelial cells (Coburn & Gilmore, 2003). The role of cytolysin in the pathogenesis of enterococcal infection has been reported in mice (Ike, Hashimoto, & Clewell, 1984). Isogenic mutants of cytolysin, which is encoded by the pheromone-responsive plasmid pAD1, have been used in a murine intraperitoneal lethal challenge model. *E. faecalis* strains that express cytolysin of wild type pAD1 are more than 10 times more toxic than that of the *E. faecalis* cytolysin-negative mutant. The 50% lethal dose of *E. faecalis* strains that express cytolysin was one order of magnitude lower than that of a non-cytolysin producing strain. Experiments using a rabbit endocarditis model, in which valvular damage was induced by catheter insertion, demonstrated that the mortality was higher when both the plasmid-determined aggregation substance and the cytolysin were expressed (Chow, et al., 1993; Galli & Wirth, 1991). In a rabbit experimental endophthalmitis model, it was shown that the cytolysin significantly determined the course and severity of the disease (Jett, Jensen, Nordquist, & Gilmore, 1992).

The activity of cytolysin entails two unique peptides that possess modifications and physiochemical characteristics of lantibiotic bacteriocins. Both peptides are needed for antimicrobial activity. Expression of the cytolysin is tightly controlled by a two-component regulatory system, which is often referred to as quorum-sensing regulation (Coburn, Pillar, Jett, Haas, & Gilmore, 2004; Gilmore, Coburn, Nallapareddy, & Murray, 2002; Haas, Shepard, & Gilmore, 2002). In this regulatory network, the smaller peptide of the cytolysin induces the high-level expression of the cytolysin genes through binding to a membrane-bound histidine protein kinase (receptor protein), followed by a phosphorylation relay resulting in a phosphorylated response activator that, in turn, activates genes involved in the biosynthesis of the cytolysin (Coburn, Pillar, Jett, Haas, & Gilmore, 2004).

The second two-peptide lantibiotic characterized so far is enterocin W, which shares the strongest homology to plantaricin W, a bacteriocin from *Lactobacillus plantarum* (Sawa, et al., 2012). The amino acid sequences of the two prepeptides of enterocin W are 63.3% and 44.7% identical to plantaricin Wα and Wβ prepetide counterparts, respectively (Sawa, et al., 2012). Enterocin W is active against several Gram-positive bacteria and its optimal antimicrobial activity is achieved at equimolar concentration of the two peptides. The target bacteria are killed at peptide concentrations below 1 μM.

The recent advent of high through-put DNA sequencing technologies has generated a wealth of genomic information in public domains that represents an important source for bioprospecting of bioactive substances. Indeed, in silico screening has identified a gene cluster of a one-peptide lantibiotic within the genome of *E. faecalis* Fly1 (Marsh, O'Sullivan, Ross, Cotter, & Hill, 2010). This lantibiotic shows significant homology to a number of epidermin-like (type 1) lantibiotics (Marsh, O'Sullivan, Ross, Cotter, & Hill, 2010), but has not been purified or studied further.

Class II Bacteriocins

Class II comprises a diverse group of bacteriocins that still is under debate with respect to their classification. The Class II bacteriocins are defined as non-modified and heat stable bacteriocins, although some modifications (e.g., disulfide bridging, circularization, and methionine formylation) are actually found within some of these bacteriocins. However, there is a general consensus that the Class IIa (the pediocin-like) and IIb (the two-peptide bacteriocins) are well-defined groups. This is also the case for the circular bacteriocins, but it has been proposed that this group should be included in a separate class (Maqueda, et al., 2004). In addition, a subgroup of leaderless bacteriocins (enterocins) has been defined; and finally, some enterocins do not fall into any of the recognized subgroups.

Class IIa: the pediocin-like and strong anti-listerial enterocins

Class IIa constitutes the largest subclass of Class II bacteriocins, which are apparently the most abundant bacteriocins in LAB. They are found among many species of enterococci, while identical and closely related enterocins are encountered in different enterococcal species, though they have been given different names. Presently, Class IIa bacteriocins have been identified in six enterococcal species: *E. faecalis. E. faecium, E. mundtii, E. avium, E. hirae*, and *E. durans* (Table 1). Class IIa bacteriocins from all genera vary in length between 37 to 58 amino acid residues, while the Class IIa enterocins vary from 43–47 residues, but they all share the following consensus sequence in their N-terminal half: KYYGNGL/VXCXKXXCXVDW (Drider, Fimland, Héchard, McMullen, & Prévost, 2006; Eijsink, Skeie, Middelhoven, Brurberg, & Nes, 1998; Nes, Diep, Håvarstein, Brurberg, Eijsink, & Holo, 1996).

The two cysteines in the N-terminal consensus sequence form a disulfide bridge, which is a prerequisite for antimicrobial activity. These bacteriocins are of particular interest because they strongly inhibit the growth of *Listeria monocytogenes* and enterococci. At least thirteen different Class IIa bacteriocins have been isolated and characterized in enterococci, and *E. faecium* is the most frequent species found to be in the Class IIa enterocin producers (Table 1). It is interesting to note that enterocin A is among the most potent antimicrobial peptides in this subgroup (Eijsink, Skeie, Middelhoven, Brurberg, & Nes, 1998). It has been shown that the high antibacterial potency of enterocin A is due to the presence of two disulfide bridges, where the second bridge is located in the C-terminal part (Eijsink, Skeie, Middelhoven, Brurberg, & Nes, 1998; Fimland, et al., 2000; Uteng, et al., 2003). This structural feature is also found in some of the other Class IIa bacteriocins, which all exert strong antimicrobial activity. NMR analysis has shown that the C-terminal disulfide bridge stabilizes a folded-back structure in this region, which is required for enhanced antimicrobial activity. In fact, introducing a C-terminal disulfide bridge in similar position in Class IIa bacteriocins that originally enclose only the N-terminal disulfide bridge significantly enhances their antimicrobial activity (Fimland, et al., 2000). Temperature studies

have also shown that the Class IIa bacteriocins without the C-terminal disulfide bond are 30-50 times less active at 37°C, as compared to the activity at 25°C, while bacteriocins with the C-terminal disulfide bond do not exhibit such a temperature-dependent antimicrobial activity (Fimland, et al., 2000; Kaur, Andrew, Wishart, & Vederas, 2004; Uteng, et al., 2003).

Class IIa bacteriocins can use two different secretion systems. Some are secreted by a dedicated ABC transporter that recognizes the double-glycine leader in the N-terminal part of the prebacteriocin while others are synthesized with a *sec*-dependent leader, and are consequently secreted by a *sec*-system (see Table 1).

Some of the Class IIa bacteriocins with a double glycine leader, which also includes a few enterocins, are regulated by a three-component regulatory system that encompasses a peptide pheromone, also referred to as an autoinducing peptide (which often resembles a bacteriocin molecule but with fewer amino acid residues and have very low, if any, antimicrobial activity), a membrane-bound histidine protein kinase that serves as receptor for the peptide pheromone, and finally, a response regulator protein that activates the operons participating in the bacteriocin biosynthesis upon phosphorylation (96,97). Among the Class IIa enterocins, it has been shown that such a regulatory system is involved in the production of enterocin A and B, as well as avicin A (Birri, Brede, Forberg, Holo, & Nes, 2010; Nilsen, Nes, & Holo, 1998).

Mundticin KS-producing *E. mundtii* was originally isolated from grass silage and its genetic determinants are encoded on the 50 Kbp-plasmid pML1 (Kawamoto, et al., 2002). In contrast to the enterocin A and avicin A strains, the accessory protein for the ABC transporter is apparently missing, and the three-component regulator system for the bacteriocin production is not found in the mundticin KS-producing strains. It has also been shown that mundticin KS is produced by an *E. faecium* isolate obtained from Peruvian cheese (Aguilar-Galvez, Dubois-Dauphin, Campos, & Thonart, 2011).

The *sec*-dependent leader Class IIa bacteriocins, except enterocin P, share a high degree of amino acid sequence homology (more than 70% identity) to bacteriocin 31. The peptide sequence of enterocin P shares less identity with other bacteriocins that have a *sec*-dependent leader, which suggests that enterocin P belongs to another sub-group of the *sec*-leader dependent bacteriocins. The genetic organization of *sec*-dependent Class IIa bacteriocin is included in one operon that consists of the structural gene encoding the bacteriocin preprotein and its dedicated immunity gene, while the *sec*-dependent transporter system is situated in a different location on the bacterial genome.

The crystal structure of the immunity protein of enterocin A and mundticin KS has been determined (Jeon, Noda, Matoba, Kumagai, & Sugiyama, 2009; Johnsen, Dalhus, Leiros, & Nissen-Meyer, 2005). The immunity proteins of enterocin A (ImEntA) and mundticin KS (ImMunKS) consist of 98 and 103 amino acid residues, respectively. Based on sequence homology, Class IIa bacteriocins have been classified into three subgroups (A, B, and C) (Eijsink, Skeie, Middelhoven, Brurberg, & Nes, 1998; Fimland, Eijsink, & Nissen-Meyer, 2002). The two structurally determined immunity proteins belong to different subgroups: ImEntA to subgroup A and ImMunKSto subgroup B. The structures of both immunity proteins fold into an antiparallel four-helical bundle with a flexible C-terminal part, which is also shown with the immunity proteins of other Class IIa bacteriocins. This finding indicates that this is a conserved structural feature common for all pediocin-like immunity proteins, independent of their subclassification. The current model of mode of action suggests that the C-terminal half of the immunity protein contains a region that recognizes the C-terminal half of the cognate bacteriocin, and the flexibility in the C-terminal end of the immunity protein might therefore be an important characteristic that enables the immunity protein to interact directly or indirectly with its cognate bacteriocin (Fimland, Johnsen, Dalhus, & Nissen-Meyer, 2005). It has also been shown that the Class IIa immunity proteins, together with their cognate bacteriocins, form a strong ternary complex with the mannose-phosphotransfer-system (manPTS), and thereby prevent permeabilization and killing of the target cell, as seen in Figure 2 (Diep, Skaugen, Salehian, Holo, & Nes, 2007).

It was recently observed that the conjugative plasmid antibiotic-resistant pAMS1 encodes a Class IIa bacteriocin, designated MC4-1 (Sedgley, Clewell, & Flannagan, 2009). Interestingly, the production of the bacteriocin from its original host *E. faecalis* MC4-1 was not detectable in plate assays but when pAMS1 was transferred to an *E. faecalis* JH2-2 host, production of bacteriocin MC4-1 was observed. It was also shown that transconjugants of only some *E. faecalis* strains gave rise to detectable bacteriocins. It was not detectable in plasmid hosts that produced gelatinase (protease), which is not produced in the JH2-2 strain. It was also found that while bacteriocin production and related immunity occurs readily as cells enter the stationary phase, production is not expressed during exponential growth; thus, strains growing on plates as an early lawn (i.e., exponentially growing) exhibit sensitivity to bacteriocins produced by stabs from sibling colonies (Flannagan, Clewell, & Sedgley, 2008; Sedgley, Clewell, & Flannagan, 2009). This phenomenon is referred to as siblicidal activity (sibling killing).

Enterocin A is most often produced in conjunction with other bacteriocins, and often in combination with enterocin B (Casaus, Nilsen, Cintas, Nes, Hernández, & Holo, 1997). Occasionally, enterocin P, which is another Class IIa bacteriocin, and the leaderless enterocins L50 and Q, as well as other enterocins, are coproduced with enterocin A (Aguilar-Galvez, Dubois-Dauphin, Campos, & Thonart, 2011; Cintas L. M., et al., 2000; Strompfová, Lauková, Simonová, & Marcináková, 2008). Enterococci seem to commonly have the genetic ability to produce more than one bacteriocin, as also seen among some other LAB (Diep, Håvarstein, & NEs, 1996; van Belkum, Kok, & Venema, 1992). It is interesting to note that many Class II bacteriocins use a typical double-glycine leader for export, which is facilitated by a dedicated ABC transporter, but many Class IIa enterocins have a *sec* leader and consequently apply a *sec*-dependent secretion.

Bacteriocins target a defined group of bacteria that are often closely related to the producers. This constriction in the inhibitory spectrum suggests that a specific receptor is being targeted in sensitive cells. However, very little is known about receptors for most LAB bacteriocins. Some lantibiotics, including nisin, employ the cell wall precursor lipid II as a docking molecule. Depending on their concentration, nisin and related bacteriocins can either inhibit cell wall biosynthesis (at low bacteriocin concentrations) or form lethal pores on target cells (at high bacteriocin concentrations) (Bierbaum & Sahl, 2009). For Class II bacteriocins, the sugar transporter man-PTS has been shown to serve as a receptor for the pediocin-like bacteriocins (Class IIa) and for some linear non-pediocin-like bacteriocins, such as lactococcins A and B. The man-PTS, which is a major permease for glucose in most bacteria, is composed of three proteins where the IIC and the IID proteins form a membrane-embeded complex, and the cytosolic IIAB entity is reversibly associated with its membrane-located partners. Only the membrane-located components (IIC and IID) are required for the receptor to function (83). Moreover, a region of about 40 amino acid residues which encloses a predicted extracellular loop of IIC appears to enclose the specific interaction site with the Class IIa bacteriocins (see Figure 2A). This region contains a sequence signature present only in bacterial species/genera that are known to be sensitive to pediocin-like bacteriocins, but is absent in the corresponding IIC protein of the man-PTS in Gram-negative cells, such as bacilli, clostridia, and other bacterial cells that are known to be insensitive to the pediocin-like bacteriocins (Kjos, Salehian, Nes, & Diep, 2010). Thus, this region is definitely involved in defining the spectrum of sensitive cells targeted by pediocin-like bacteriocins.

It has been clearly demonstrated that pediocin-like bacteriocins not only require man-PTS as a target molecule, but also that they destroy the functionality of man-PTS by keeping it irreversibly open as depicted in Figure 2B. However, this finding has not yet been experimentally proven—but there are some strong pieces of additional evidence that favor this model. First, each producer cell expresses an immunity protein to protect itself from self-destruction. Upon purification of this immunity protein, it was found that it was tightly associated and co-purified with components of the receptor (man-PTS) and the bacteriocin (Diep, Skaugen, Salehian, Holo, & Nes, 2007; Kjos, Nes, & Diep, 2009), which suggests that this bacteriocin was most likely trapped within the protein complex, as shown in Fig. 1C. This complex formation is probably needed to prevent the bacteriocin from causing the irreversible leakage of intracellular solutes, as seen in the absence of an immunity protein. Second,

upon exposure to higher concentrations of bacteriocin, an immunity clone (expressing the immunity gene) appeared to grow less efficiently on glucose than on galactose (Diep, Skaugen, Salehian, Holo, & Nes, 2007; Kjos, Nes, & Diep, 2011); the second sugar does not use the man-PTS for transport. This result strongly indicates that man-PTS is impaired or less functional when bacteriocin immune cells are challenged by the bacteriocin. This mechanism of action is applied not only to the pediocin-like bacteriocins but also to lactococcin A—but lactococcin A recognizes only lactococcal man-PTS, and not the man-PTS that are recognized by the pediocin-like bacteriocins (Diep, Skaugen, Salehian, Holo, & Nes, 2007; Kjos, Nes, & Diep, 2009; Kjos, Salehian, Nes, & Diep, 2010).

Class IIb: The two-peptide bacteriocins

The first two-peptide, lactococcin G, was isolated from *Lactococcus lactis*, while the first identified two-peptide enterocin was enterocin 1071, which originates from *E. faecium* (Franz, et al., 2002; Nissen-Meyer, Holo, Håvarstein, Sletten, & Nes, 1992). The two peptides in a two-peptide bacteriocin do not share sequence identity toward each other, but significant peptide sequence identity was observed between the homologous peptides of lactococcin G and enterocin 1071. A two-peptide bacteriocin needs both peptides, which are genetically co-localized, in order to exert antimicrobial activity. In a few cases, individual peptides show some antimicrobial activity, but this activity is far less when compared to the combined activity of both peptides in equal molar concentration. It is worth noting that the peptides of a two-peptide bacteriocin are different from the leaderless peptide bacteriocins (which have been occasionally and incorrectly assigned as two-peptide bacteriocins) in that, the leaderless peptides within a bacteriocin share high sequence identity with each other and the individual peptides exert significant antimicrobial activity (see below). It has therefore been suggested that the genes that encode a two-peptide bacteriocin probably evolved independently, while genes for the leaderless peptides of a bacteriocin evolved by gene duplication. Another important criterion of a two-peptide bacteriocin is that only a single immunity protein is involved to protect the producer from committing suicide.

Enterocin 1071, enterocin C, and enterocin X are included in the group of two-peptide bacteriocins. Enterocin 1071, which is composed of the peptide enterocin 1071A, and enterocin 1071B were first characterized in *E.faecalis* BFE1071, an isolate obtained from fecal samples of a minipig. The enterocin-1071–encoding genes are situated on a 50kbp conjugative plasmid (Balla, Dicks, Du Toit, Van Der Merwe, & Holzapfel, 2000). Enterocin C, which is composed of the two peptides enterocin C1 and C2, is produced by *E.faecalis* C901 (Maldonado-Barragán, Caballero-Guerrero, Jiménez , Jiménez-Díaz, Ruiz-Barba, & Rodríguez, 2009). The genes encoding Ent C1, Ent C2, and the putative immunity protein Ent CI are located on the 9-kbp plasmid pEntC1. Full antimicrobial activity requires the complementary action of Ent C1 and Ent C2. The Ent C1 peptide is identical to the Ent 1071A peptide, while the Ent C2 peptide diverges from the Ent 1071 peptide by a single amino acid residue in position 17, where alanine is replaced by threonine. Based on antimicrobial testing with a cell-free supernatant, it was shown that enterocin C differs from enterocin 1071 in a few major aspects, which include the complementary activity of the two peptides as well as its broader inhibitory spectrum (Maldonado-Barragán, Caballero-Guerrero, Jiménez , Jiménez-Díaz, Ruiz-Barba, & Rodríguez, 2009). However, such investigations should be performed on purified peptides, since bacteria often produce multiple bacteriocins, which might affect the bacteriocin activity measurements and hamper the results and conclusions.

Enterocin X is composed of two peptides, EntX_α and EntX_β, and is produced by *E. faecium* KU-B5 (Hu, Malaphan, Zendo, Nakayama, & Sonomoto, 2010). There are no homologies between Ent X_α/X_β and other reported bacteriocins. When X_α and X_β were mixed beforehand in equimolar amounts, the combined antibacterial activity displays variable antibacterial activity toward a panel of indicators compared to the individual peptides.

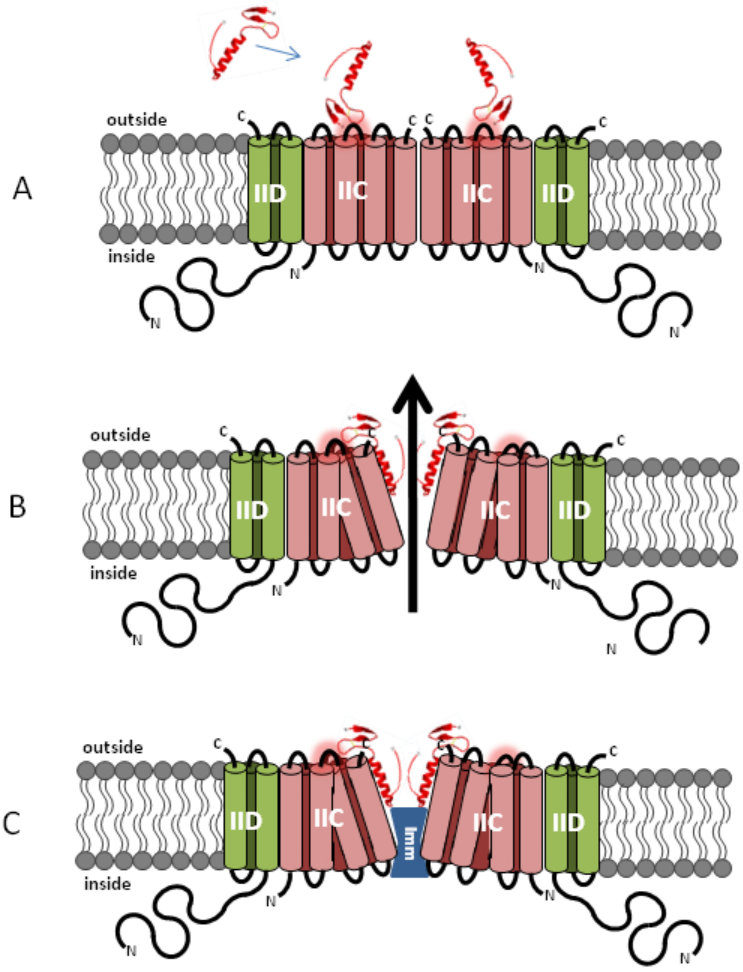

Figure 2. Model of target recognition, mode of killing, and immunity of Class IIa bacteriocins. A) a Class IIa bacteriocin specifically targets an extracellular loop of IIC, one of the two membrane-embedded components (IIC and IID, also called ManCD) of man-PTS; and B) the initial interaction leads to further interactions with some membrane helices of man-PTS, somehow causing the channel of the sugar permease to remain open, leading to leakage of solutes, destruction of membrane integrity, and eventually cell death. C) In producer cells, the cognate immunity protein binds to IICD and locks the bacteriocin in a tight complex, thereby preventing the bacteriocin from opening the pore.

Class II: Circular bacteriocins

Circular bacteriocins have often been included in the Class II bacteriocins, but grouping them into a separate class has been proposed (Class IV bacteriocins) (Maqueda, Sánchez-Hidalgo, Fernández, Montalbán-López, Valdivia, & Martínez-Bueno, 2008). These bacteriocins differ from most Class II bacteriocins in that they do not have free ends and are circularized by the α-amino group of one residue linked to the carboxyl group of the terminal residue of the peptide as a peptide bond. Circular bacteriocins are synthesized with a leader sequence that varies between 3 and 20 amino acids. After removal of the leader, the resulting N-terminal residue is covalently linked to the C-terminal residue and finalized in a compact structure of four or five helical bundles structured with a hydrophobic core.

Not many circular bacteriocins are found among LAB, but enterocin AS-48 is among them (Cobos, et al., 2001; Gálvez, Giménez-Gallego, Maqueda, & Valdivia, 1989; González, et al., 2000). AS-48, which was originally isolated from *E. faecalis*, has been studied in great detail with respect to genetics, biochemical features, and

structure, as well as its mode of action. AS-48 has also been called bacteriocin 21 in some studies (Tomita, Fujimoto, Tanimoto, & Ike, 1997; Tomita, Fujimoto, Tanimoto, & Ike, 1996). The genetic foundation of AS-48 production and its immunity function relies on the coordinated expression of ten genes located in two operon structures (Maqueda, et al., 2004).

Studies on modes of action have been performed on the circular bacteriocins, including AS-48, by using liposomes and lipid bilayers, and it was concluded that membrane receptors were not engaged in antimicrobial activity (Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1991). Furthermore, it has been shown that AS-48 activity is not dependent on a membrane potential (Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1991). It is important to note that these mode-of-action studies were performed on synthetic membranes/lipids, in which a potential receptor was absent, and that the active concentrations of bacteriocins used on synthetic systems were far higher (100–1000 fold) than the active concentrations used on live sensitive cells. Thus, it is not possible to rule out that a specific receptor/target/mediator for a high sensitivity of live cells is implicated in some killing mechanisms. Indeed, it was quite recently shown that another cyclic bacteriocin, garvicin ML from *Lactococcus garvieae*, needs a membrane-located maltose transporter to exert maximum killing efficiency (Gabrielsen, Brede, Hernández, Nes, & Diep, 2012). It remains to be seen whether this is true for other cyclic bacteriocins, including AS-48.

Class II: Leaderless bacteriocins

Most Class II bacteriocins contain an N-terminal leader peptide, which directs the secretion of the bacteriocin and is cleaved off during the secretion process. Their leaders belong to the so-called double-glycine leader type, or, in some cases, to a *sec*-dependent leader type, and as a result, the *sec* secretion system is employed for the latter. However, some bacteriocins are not synthesized with a leader-peptide, and their secretion seems to be performed by dedicated ABC transporters with sequence features that are notably different from the ones that externalize the double-glycine leader and the *sec*-leader bacteriocins. A bacterium can produce a single or multiple leaderless bacteriocins. When several leaderless bacteriocins are produced by the same strain, the bacteriocins often share strong sequence homology and their genes are located next to each other. The individual peptides possess antimicrobial activity, but when combined, they exhibit increased potency. However, enhanced antimicrobial activity among peptides varies, and are target-dependent, as seen with the two leaderless peptides (L50A and L50B) of enterocin L50 (Cintas, Casaus, Holo, Hernandez, Nes, & Håvarstein, 1998). In one strain of *Staphylococcus aureus*, up to five homologous antimicrobial peptides have been found (100). Among enterococci, single leaderless peptides and two homologous leaderless peptides have been identified so far (Cintas, et al., 2000; Cintas, Casaus, Holo, Hernandez, Nes, & Håvarstein, 1998). Dedicated ABC transporters seem to manage the transport of such bacteriocins (Criado, et al., 2006).

Ent L50A and L50B, which are encoded on the plasmid pCIZ1(50kbp), are produced by *E. faecuim* L50 isolated from Spanish dry-fermented sausage. Enterocin L50A and Enterocin L50B display 72% sequence identity and consist of 44 and 43amino acids, respectively. Both bacteriocins possess individual antimicrobial activity, with EntL50A being the most active. A synergistic antimicrobial activity was observed by combining the two bacteriocins (Ent L50A and Ent L50B). In addition to the production of EntL50, *E. faecium* L50 also produces enterocin Q, which is a single leaderless peptide whose genes are located on another plasmid pCIZ2 (7.4kbp), as well as enterocin P, which is chromosomally encoded (Cintas, et al., 2000; Criado, et al., 2008).

Several characterized leaderless enterocins have been shown to be identical or highly homologous to Ent L50A/L50B (Dezwaan, Mequio, Littell, Allen, Rossbach, & Pybus, 2007; Izquierdo, et al., 2008; Kang, et al., 2009; Liu, et al., 2011; Yamamoto, Togawa, Shimosaka, & Okazaki, 2003). The two respective peptides of the leaderless enterocins 7A/7B and MR10A/10B are identical and share strong homology to EntL50A and Ent L50B peptides (Kang, et al., 2009). However, amino acid sequences of enterocin Q and enterocin EJ97 have no strong homology to other bacteriocins.

A recent study has shown that leaderless bacteriocins might possess a formyl group in their N-terminal methionine (Liu, et al., 2011). The two leaderless bacteriocin peptides, enterocin 7A (Ent7A) and enterocin 7B (Ent7B), were analyzed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry and electrospray infusion tandem mass spectrometry analyses. The data and DNA sequence analysis showed that both peptides are produced without N-terminal leader sequences but the observed masses for Ent7A and Ent7B were 5200.80 and 5206.65 Da, respectively, which are larger than the theoretical masses. Further experiments provided evidence that both Ent7A and Ent7B are formylated in the N-terminal methionine residue (Liu, et al., 2011). It seems likely that N-terminal formylation is a general feature for such leaderless bacteriocins.

Other small heat-stable bacteriocins

Some bacteriocins fall outside the classification scheme described above, because they do not share the basic classification features of bacteriocins from any of the above mentioned classes/subclasses. Two such enterocins have presently been characterized. Enterocin B is a 53-amino-acid residue, linear, non-pediocin-like peptide that employs a double glycine-leader for export. This bacteriocin is often co-expressed with enterocin A and they act synergistically (Casaus, Nilsen, Cintas, Nes, Hernández, & Holo, 1997; Ennahar, Asou, Zendo, Sonomoto, & Ishizaki, 2001; Franz, et al., 1999). The second one is bacteriocin 32, which is a sec-dependent bacteriocin encoded on the mobile plasmid pTI1 (12.5kbp) of the vancomycin-resistant *E. faecium* (VRE) 200 strain. It is active against *E. faecium, E. hirae*, and *E. durans*, but is not active against *L. monocytogenes* (Inoue, Tomita, & Ike, 2006). The *bac32* operon is composed of two genes, *bacA* and *bacB*, which encode the bacteriocin preprotein with a *sec*-dependent leader peptide and the immunity protein, respectively (Inoue, Tomita, & Ike, 2006; Yamashita, Tomita, Inoue, & Ike, 2011).

Bacteriolysins (previously named Class III bacteriocins)

Years ago, a group of heat-labile bacteriocins from LAB were included in the separate Class III of heat-labile bacteriocins (Klaenhammer, 1993). Since then this class has been redefined to include heat-labile antimicrobial proteins that performed enzymatic degradation of the cell wall of targeted bacteria, and it seemed reasonable to name this group of antimicrobial proteins bacteriolysins (Cotter, Hill, & Ross, 2005). Such antimicrobial proteins are apparently easy to define because of their heat-labile characteristics, as well as their ability to degrade the cell walls of susceptible target strains.

Enterolysin A has been characterized as a Class III bacteriocin in *E. faecalis* (Hickey, Twomey, Ross, & Hill, 2003; Nilsen, Nes, & Holo, 2003). The mode of action of enterolysin A is quite different from the previous mentioned heat-stable bacteriocins, because it attacks susceptible bacteria by degrading the cell wall structure, which eventually leads to lysis of the cells. Enterolysin A exhibits a similar identity to cell wall-degrading enzymes that are produced by different Gram-positive bacteria. Sequence analysis of enterolysin A suggested that this bacteriocin consists of two separate domains: an N-terminal catalytic domain, and a C-terminal substrate recognition domain. The specificity of enterolysin A indicates that the target of its putative recognition domain is different from the previously described targets, and this is consistent with the fact that the putative, non-catalytic C-terminal part of enterolysin A shows no sequence homology to the proteins active against staphylococci and streptococci. However, the C-terminal domain of enterolysin A exhibits high levels of sequence identity to a lysin from bacteriophage A2 and an *N*-acetomuramoyl-L-alanine amidase from bacteriophage PL-1, both of which are bacteriophages of *L. casei*. The regions of these lysins that are homologous to the C-terminal part of enterolysin A are thought to be responsible for its binding to their cell wall substrate. It is also worth mentioning that the N-terminal part of enterolysin A is linked to a putative C-terminal recognition domain by a threonine-proline-rich region, which shows significant sequence identity to the two previously mentioned bacteriophage lysins (Klaenhammer, 1993).

A new antimicrobial compound, named bacteriocin 41, was isolated in a recent study (Tomita, Kamei, & Ike, 2008). This bacteriocin consists of three components: a lysin-like molecule (BacL1), an activator (BacL2) of

BacL1, and a third protein, which is needed for the antimicrobial activity (BacA). In addition, an immune protein (BacI) that prevented lysis of the producer was identified. While BacL1 showed homology to several bacteriophage lysins and a muramidase, Bac L2 was homologous to the holin of a bacteriophage. BacA, which is the largest protein (726 aa) in the antimicrobial protein complex, showed homology to two *B. subtilis* proteins (Ybfg and YkuG) of unknown function. A putative peptidoglycan binding domain was identified in the N-terminal part of BacA. The mode of action of bacteriocin 41 is not presently known, but its antimicrobial activity is certainly heat-labile. However, the target is most likely the cell wall, and killing apparently occurs by cell wall degradation. Consequently, it seems most reasonable to include bacteriocin 41 in the class of bacteriolysins.

Because of the presence of conserved domain-like structures involved in the translocation, receptor binding, and antimicrobial activity, homologous bacteriolysins can readily be identified in genome databases by performing homology searches.

The Ecology of Enterocins

Bacteriocin-producing enterococci are isolated from a broad range of environments. It is of interest to note that food is a common source of enterococci (*E. faecium* in particular), but *E. faecalis* is also frequently found in fermented food commodities (Giraffa, 2003; Hugas, Garriga, & Aymerich, 2003). Enterococci isolated from different environments, including food, most likely originate from the commensal gut flora of humans and animals. Enterococci are one of the dominant fecal LAB in humans, though a great fluctuation in numbers, depending on age and lifestyle of the host (above 10^8 enterococcal cells per gram of feces in babies, but usually significant less in adults, have been observed). Several studies have shown that there is a high frequency of bacteriocin production among enterococci, and it has also been reported that a major fraction of the isolated enterococci from stool samples do produce bacteriocins (Forberg, 2005; Herrera, Brede, Salehin, Holo, & Nes, 2006).

Hospital isolates of vancomycin-resistant enterococci (VRE) have been shown to be frequent producers of bacteriocins (del Campo, et al., 2001). In one study, 636 VRE isolates were tested for bacteriocin production, and 44% of them were shown to be bacteriocinogenic (Inoue, Tomita, & Ike, 2006). The frequency of bacteriocinogenic enterococci appears to be higher for certain bacteriocins (Bac41, Bac32) among clinical isolates than those of non-clinical isolates (Ike, Inoue, Yamashita, & Tomita, 2010; Inoue, Tomita, & Ike, 2006). It is important to note that many of those clinical isolates are not clonal. In one study, approximately 40% of clinical *E. faecium* / *E. faecium* VRE produced Bac32, while this was the case for only 2% of *E. faecium* isolates from stools samples of healthy students. In another study, about 50% of *E.faecalis* clinical isolates were Bac41 producers (Ike, Inoue, Yamashita, & Tomita, 2010).

An increased hemolytic phenotype among clinical-derived *E. faecalis* has been reported (Ike, Hashimoto, & Clewell, 1987). About 60% of *E. faecalis* clinical isolates are cytolysin (Hly/Bac) producers. In contrast to the high frequency of hemolysin producers among parenteral isolates, isolates derived from fecal specimens of healthy individuals exhibited a low (17%) incidence of hemolysin production. In one study, *E. faecalis* blood isolates were probed for the serine protease activator of cytolysin (*cylA*) (Huycke & Gilmore, 1995), and it was found that *cylA* occurred more frequently among bacteremia isolates [34 of 68 (50%)] than isolates from endocarditis [4 of 35 (11%)] or stool samples [0 of 14]. But when clonality was taken into account, it was concluded that no significant enrichment for *cylA* was present among clonally unrelated bloodstream isolates (Huycke & Gilmore, 1995). However, the clonally related hemolytic strains demonstrated an increased propensity to cause bloodstream infections (Huycke & Gilmore, 1995). Bacteriocin-activities are also observed in other hemolytic peptides obtained from staphylococcus isolates (Donvito, Etienne, Denoroy, Greenland, Benito, & Vandenesch, 1997; Watson, Yaguchi, Bisaillon, Beaudet, & Morosoli, 1988).

As previously mentioned, LAB, including enterococci, often produce multiple bacteriocins. It has been shown that enterocins A and B are commonly found in the same isolates (Nilsen, Nes, & Holo, 1998), but they are also

found together with other enterocins, such as enterocins P, Q, and L50 (Cintas L. M., et al., 2000). It has also been shown that the multiple-enterocin-producing *E. faecium* L50 strain expressed bacteriocin activity within a broad temperature interval, with different combinations of bacteriocins produced at different temperatures. *E. faecium* L50 produced EntP and EntQ in the temperature range between 16 ^{O}C to 47 ^{O}C, and maximal activity was observed between 37 ^{O}C to 47 ^{O}C, while EntL50A and EntL50B are maximally synthesized between16 ^{O}C to 25 ^{O}C, and no activity was detected at 37 ^{O}C or above (Cintas, et al., 2000). The mechanism and the biological significance of temperature-regulated bacteriocin production are not known and should be investigated further.

Many of the same bacteriocins are found to be produced by enterococci isolated from quite different environments, which suggests that the bacteriogenicity is not a feature to promote growth in specific environments, but might be advantageous for a bacterium in any environment. The presence of identical bacteriocins in strains from different environments may indicate that the enterococci originate from a common source. For example, bacteriocin31 or bacteriocin31-type bacteriocins have been isolated from enterococci isolates from human sources (i.e., Bac31, Bac RC714, Bac43 and BacT8), grass silage from Thailand (Ent SE-L4), and from a wild Mallard duck (Hiracin JM79). Enterocin 7A/7B, enterocinMR10A/10B, enterocin 62-6A/B, and enterocin RJ-10, which are enterocin L50A/L50B-type bacteriocins, have been found among enterococci isolated from diverse environments. On the other hand, cytolysin-producing *E. faecalis* strains are predominantly located in human clinical isolates, and bacteriocin 32 and bacteriocin 41 determinants seem to be more frequently identified in human clinical isolates of *E. faecium* or *E. faecalis*. Enterocin A and enterocin B were originally isolated from *E. faecium* isolates from Spanish dry fermented sausage, but they are also among the most common bacteriocins found in enterococci obtained from stool samples of healthy babies. These findings suggest that some of the dominant type of bacteriocins in enterococcal clinical isolates might differ from the dominant type of bacteriocins found in enterococcal isolates that are predominantly found in fermented foods and commensal enterococci, and that the type of bacteriocins that dominate a given environment is influenced by nutritional and microbial factors.

Such a prevalence of bacteriocin-producing enterococci implies an efficient way for bacteriocins to transfer genes horizontally. It has been known for years that conjugative plasmids often carry bacteriocin genes and such mobile elements might promote the dissemination of bacteriocins. Three types of conjugative plasmids have been identified and analyzed in enterococci. Two of these plasmid types include pheromone responsive plasmids and non-pheromone responsive plasmids, which are efficiently transferred in broth mating (Clewell, 1981; Clewell, 1993; Ike, Tanimoto, Tomita, Takeuchi, & Fujimoto, 1998; Tomita, et al., 2003). The pheromone-responsive plasmids were originally identified in *E. faecalis* strains (Dunny, Brown, & Clewell, 1978) and comprise a narrow host range; they appear to transfer primarily within *E. faecalis* strains. The pheromone-responsive plasmid pAD1 (60kbp) that encodes cytolysin is representative, and is one of the most well-analyzed pheromone-responsive plasmids (Clewell, 1993; Clewell & Dunny, 2002; Dunny, Brown, & Clewell, 1978; Galli & Wirth, 1991). The bacteriocin determinants of *E. faecalis* are frequently encoded on pheromone-responsive plasmids. For example, cytolysin, AS48, bacteriocin 21, bacteriocin 31, bacteriocin 41, and enterocin EJ97 are encoded by pheromone-responsive plasmids pAD1 (60kbp) (Clewell, 1981; Gilmore, 1991; Gilmore, Coburn, Nallapareddy, & Murray, 2002; Ike, Clewell, Segarra, & Gilmore, 1990), pMB2 (58kbp) (Martínez-Bueno, Gálvez, Valdivia, & Maqueda, 1990), pPD1 (59kbp) (Fujimoto, Tomita, Wakamatsu, Tanimoto, & Ike, 1995; Tomita, Fujimoto, Tanimoto, & Ike, 1997), pYI17 (57.5kbp) (Tomita, Fujimoto, Tanimoto, & Ike, 1996), pYI14 (61kbp) (Tomita, Kamei, & Ike, 2008), and pEJ97 (60kbp) (Gálvez, et al., 1998), respectively. About 90% of conjugative plasmids in *E. faecalis* resemble the pAD1- type (Ike, Hashimoto, & Clewell, 1987).

The cytolytic phenotype is common in *E. faecalis* clinical isolates. About 60% of *E. faecalis* clinical isolates are hemolytic (Ike, Hashimoto, & Clewell, 1987). The cytolysin of about 50% of the cytolytic strains is encoded on pheromone-responsive conjugative plasmids. The cytolysin is also found to be encoded by a pathogenicity island found on the bacterial chromosome (Ike & Clewell, 1992; Shankar, Baghdayan, & Gilmore, 2002).

Enterocin 1071A/B isolated from *E.faecalis* BFE1071 was encoded on plasmid EF1071(50kbp), which can be transferred by filter mating. Enterocin L50A/B and Bac T8 from *E. faecium*, and mundticin KS from *E.mundtii* are encoded by the non-conjugative plasmids pCZ1(50kbp), 7kbp plasmid, and pML1(50kbp), respectively. Bacteriocin 32, bacteriocin RC714, bacteriocin 43, and bacteriocin 51, which were all isolated from *E. faecium* strains, are encoded by conjugative plasmids and are mobilized to the recipient strain by filter mating.

What is the biological role of bacteriocins? It is presently believed that the production of bacteriocins make their producer more competitive in certain ecological niches (Eijsink, Axelsson, Diep, Håvarstein, Holo, & Nes, 2002). This might be reflected by the high frequencies of bacteriocin-producing enterococci in the confined environment of the GI tract.

Large populations of bacteriocin-producing enterococci exist in human intestinal flora. As previously mentioned, the frequency of bacteriocin-producing isolates was reported to be higher among human clinical enterococcal isolates than among isolates from human fecal samples. In contrast, just a few clinical *E. faecium* bacteriocin producers were reported.

A different bacteriocinogenic profile was observed among enterococci obtained from fecal samples of babies. In one study, it was observed that among healthy Norwegian babies, bacteriocin-producing *E. faecium* was frequently observed, while significant fewer *E. faecalis* isolates were shown to produce bacteriocins (Birri, Brede, Forberg, Holo, & Nes, 2010; Nes, Forberg, Salehian, & Holo, 2005). Avicin A is produced by two different *E. avium* strains (XA83 and 208) isolated from feces of two healthy human infants from Norway and Ethiopia, respectively. The genetic determinants are located on the chromosome. Though avicin A producers are rare in babies, they have been isolated from two fecal stool samples of babies from such disparate areas as Ethiopia and Norway (Birri, Brede, Forberg, Holo, & Nes, 2010). This observation indicates that the bacteriocin producers have become a dominating LAB in the GI-tract of these babies (Birri, Brede, Forberg, Holo, & Nes, 2010). These results imply that the bacteriocins might provide a competitive advantage and promote dominance and colonization of the bacteriocinogenic strains in bacterial ecological environments, such as the human intestine. It is possible that bacteriocinogenic strains contribute to the pathogenic potential of virulent enterococci, while bactericnogenic commensal enterococci promote and maintain a healthy entercoccal flora.

Bacteriocin genes in *E. faecalis* are often carried by mobile genetic elements, including conjugative plasmids/transposons, which might serve an efficient way to disseminate such traits to a larger population of bacteria. Antibiotic-resistance genes and other accessory genes which are necessary for the bacterial host in certain growth conditions or ecological niches are often located on low-copy-number plasmids, and these traits can easily be lost during non-selective conditions. In such circumstances, it is feasible to think that their co-located bacteriocin genes might act as a toxin/antitoxin system to allow the low copies of plasmids to endure and pass on to daughter cells.

In some bacteria, bacteriocins may play a role to supply naked DNA for uptake and genetic recombination. It has been proposed that for certain streptococci, such as *Streptococcus mutants*, the production of bacteriocins is activated during DNA competence development (Claverys & Håvarstein, 2007; Perry, Jones, Peterson, Cvitkovitch, & Lévesque, 2009). It is suggested that the bacteriocins, in combination with certain lytic enzymes, lyse a subpopulation of the culture to make DNA available for the competent bacteria, a process referred to as fratricide. However, it has not been conclusively shown that competence development occurs in enterococci, and therefore the role(s) of bacteriocins in enterococci are likely to be involved in other functions.

Future Perspectives

Many bacteriocins have been purified or genetically analyzed in numerous enterococci (Table 1), and even more bacteriocinogenic isolates have been found, but their active entities have yet to be characterized. It is surprising to observe that among the fecal LAB flora, *E. faecium* is probably the most frequent producer of bacteriocins

(Nes, Forberg, Salehian, & Holo, 2005). In addition, enrichment of bacteriocinogenic *E. faecium* is found among vancomycin-resistant (VRE) hospital isolates (Inoue, Tomita, & Ike, 2006; Todokoro, Tomita, Inoue, & Ike, 2006). The many genome sequences that have been published the last few years have also confirmed the great abundance of bacteriocins that encode genes in enterococci. It is interesting to note that identical bacteriocins are encoded by *E. faecalis* and *E. faecium*. The genome sequences also suggest that bacteriocins that encode genes are found not only in hospital *E. faecalis*, but also in hospital isolates of *E. faecium*.

The reason why enterococci have developed such an ability to produce antimicrobial peptides is not known, but one might speculate that bacteriocin production is a beneficial trait (probiotic trait) in some environments (such as the human gut). However, the co-enrichment of antibiotic resistance and certain bacteriocinogenic traits among enterococci of clinical origins suggests that bacteriocin production might be a beneficial asset that allows enterococcal adaption to various environmental conditions. The bacteriocin system might serve as a toxin-antitoxin system to increase plasmid stability, thereby preventing the loss of the plasmid-encoding antibiotic-resistance genes under non-selective conditions. The characterized bacteriocins associated with antibiotic resistance seem to be different from the bacteriocins found in enterococci isolated from healthy individuals and babies (Birri, Brede, Forberg, Holo, & Nes, 2010). In the former case, bacteriocin 41 was frequently found in clinical isolates, but was absent in non-clinical isolates. Furthermore, the bacteriocin 32 was found at high frequencies among resistant VRE and clinical isolates, but at much lower frequencies among fecal isolates of healthy students. The genetic constituents of bacteriocin 32 were localized on a different plasmid than the vancomycin resistance genes, and it has also been shown that this bacteriocin can be co-localized with enterocin L50 (Inoue, Tomita, & Ike, 2006; Izquierdo, Cai, Marchioni, & Ennahar, 2009).

It is tempting to speculate that transferable antibiotic resistance takes advantage of bacteriocin systems to make such bacteria more competitive for growth and survival in an environment with other closely related bacteria—but more studies must be performed to support such a hypothesis.

The human gastrointestinal tract appears to be an excellent environment for bacteriocin-producing enterococci. We and others have shown that bacteriocin-producing enterococci are found in most stool samples of healthy babies at relatively high frequencies, suggesting that bacteriocin-producing enterococci are important in the development of the gut flora in infants.

Though most of the focus of enterococci today is on their pathogenicity and VRE, enterococci are a major LAB component in our gastrointestinal tract, and they are also a major bacterial entity of many endogenous dairy and meat products, as well as fermented food products (Hugas, Garriga, & Aymerich, 2003).

The biological role(s) of bacteriocins in nature are still not well understood. Are bacteriocins important for a healthy gut flora, could they be considered to be probiotics, or is their presence an indicator of virulence or antibiotic resistance? These are some of the urgent questions and scientific challenges that should be investigated. Only detailed insight in host-*Enterococcus* interaction can give us answers for how to safely appreciate their potential positive traits and how to deal with their problematic features.

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