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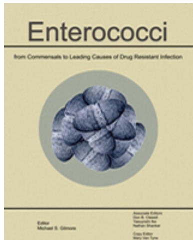

Enterococcal Cell Wall Components and Structures

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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).

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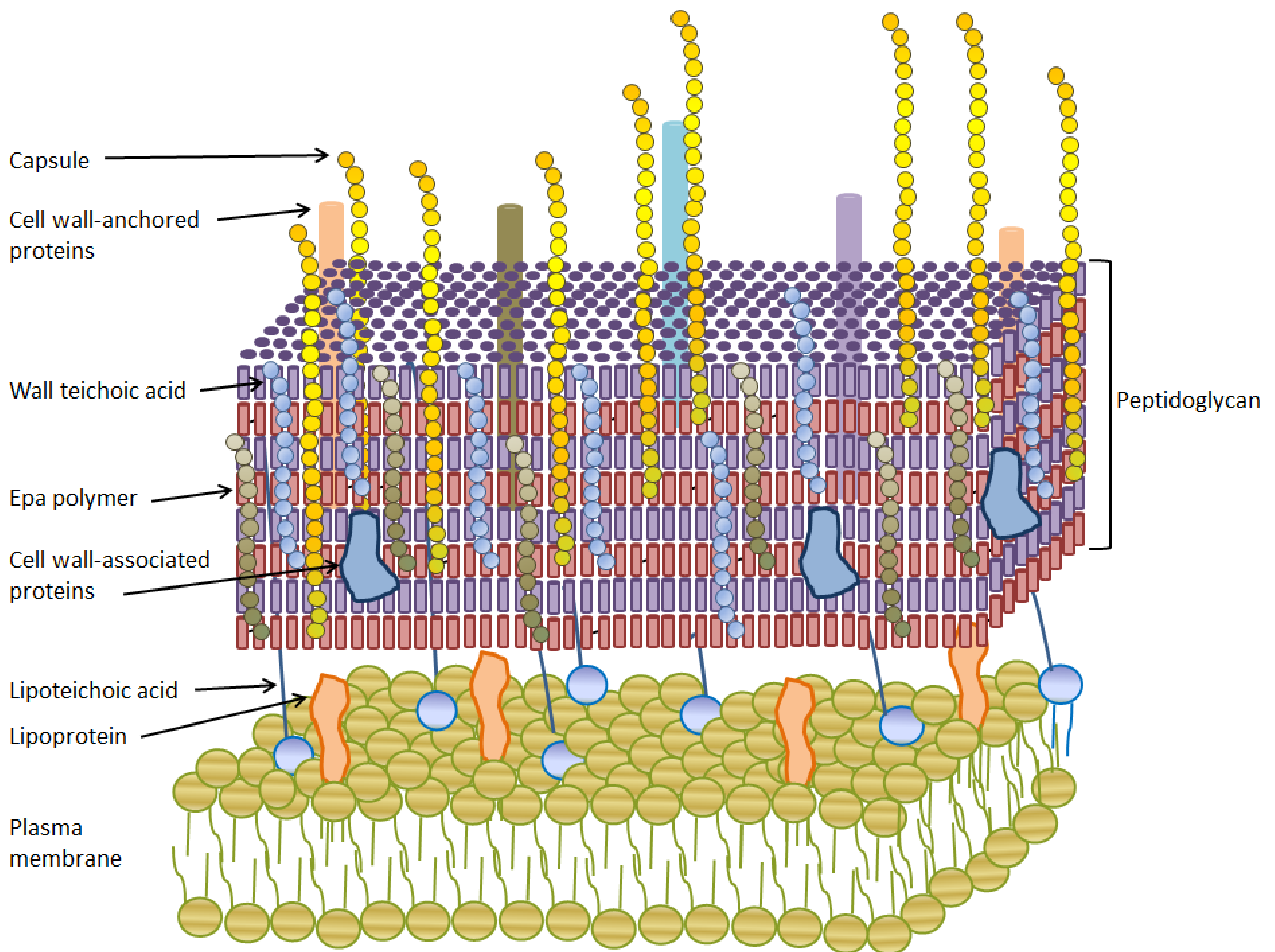

Figure 1. Model of the enterococcal cell wall. The peptidoglycan layer is depicted above the lipid bilayer with membrane bound lipoproteins and lipoteichoic acid. Bound to the muramyl residues of the peptidoglycan are wall teichoic acids, the rhamnopolymer whose synthesis is tied to the *epa* locus, as well as surface-anchored proteins and capsule.

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 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).

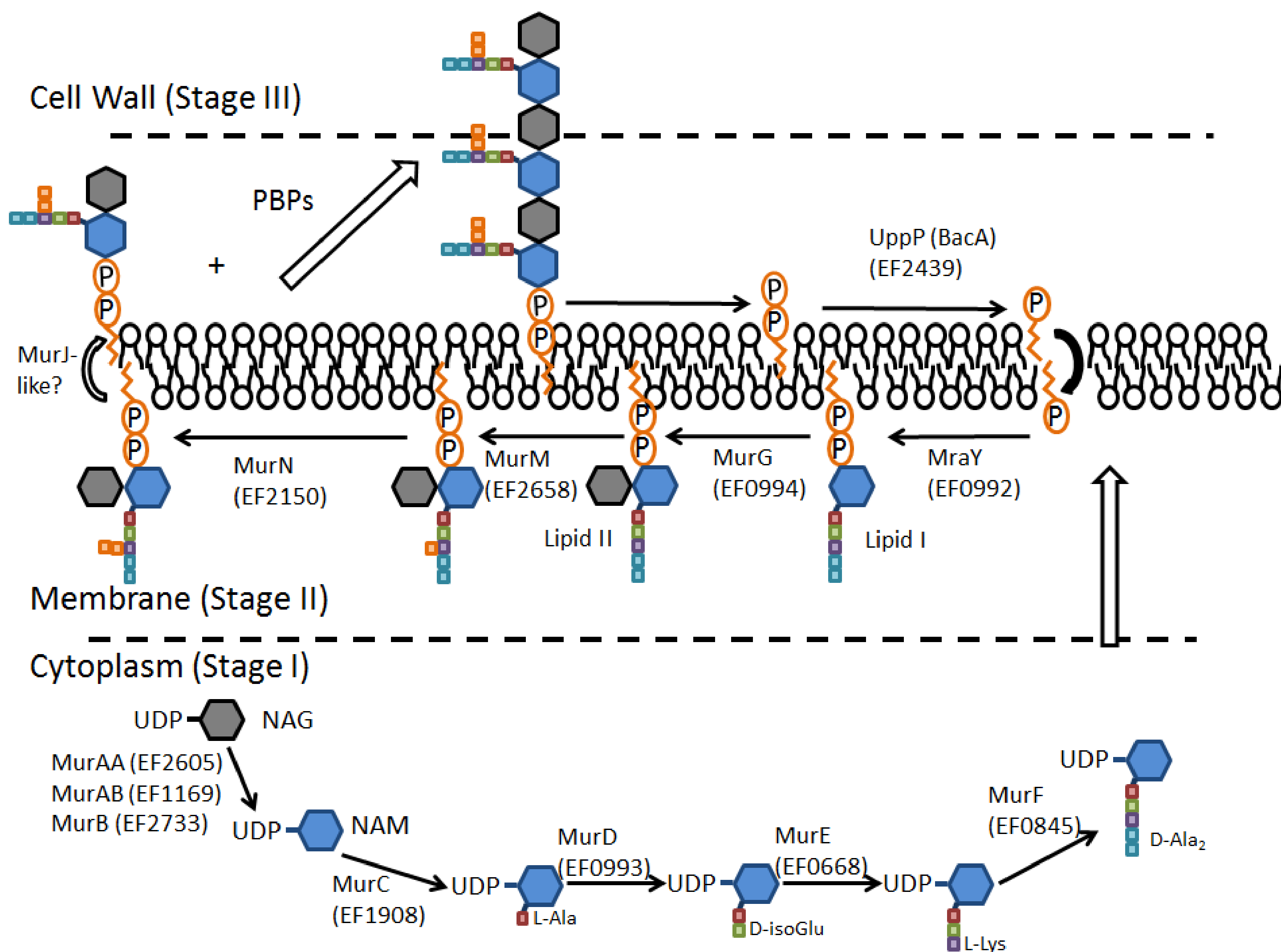

Figure 2. Biosynthesis of peptidoglycan. The common enzyme name for each step in the synthesis is provided along with the *E. faecalis* V583 gene identifier. Adapted from references (10) and (118).

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.

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, 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

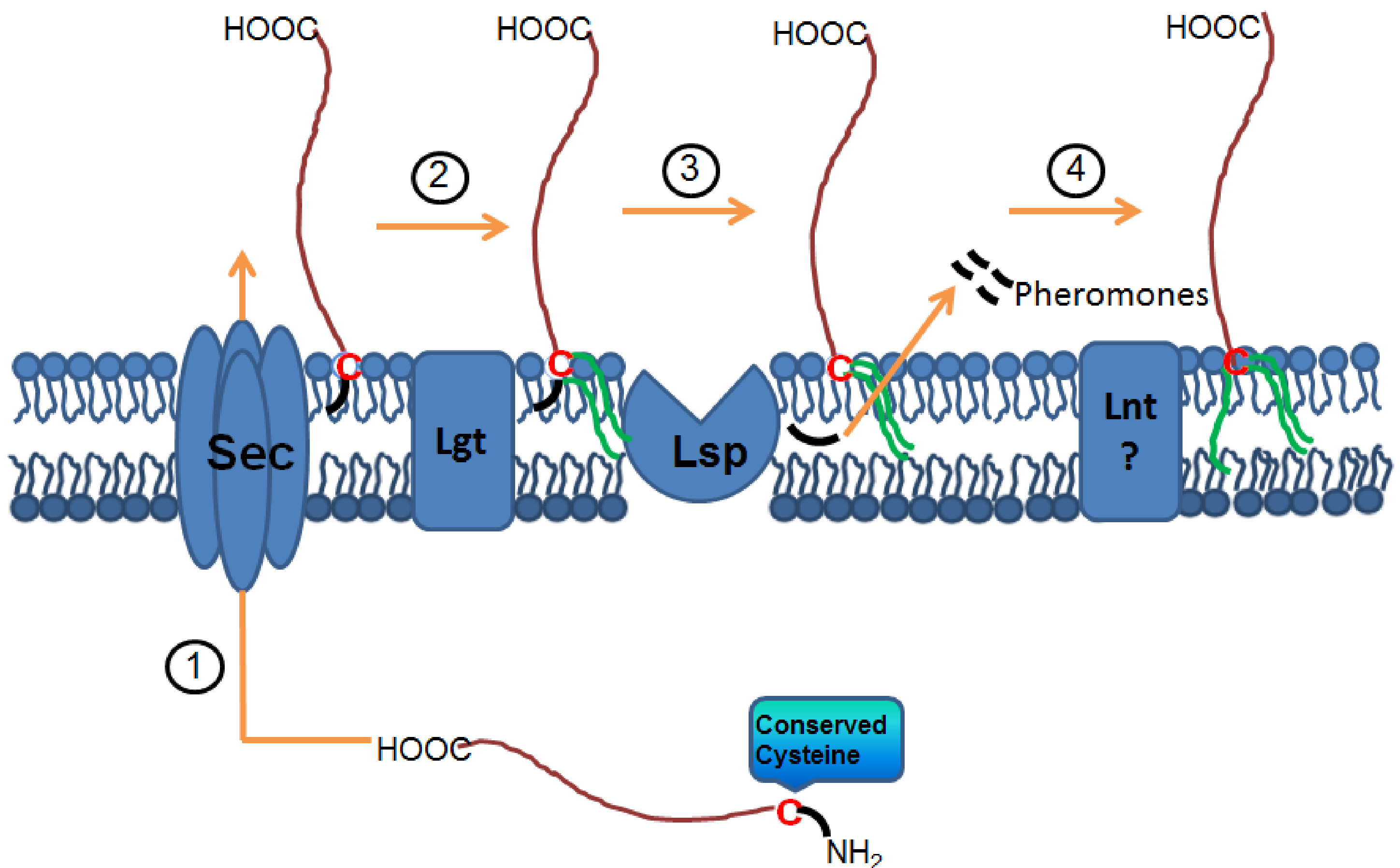

Figure 3. Schematic of lipoprotein synthesis in Gram-positive bacteria. 1) Lipobox domain containing proteins are exported through the sec-dependent pathway. 2) Once exported and by nature of the hydrophobic N-terminus, the protein transiently associates with the membrane where the conserved cysteine 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)

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, &

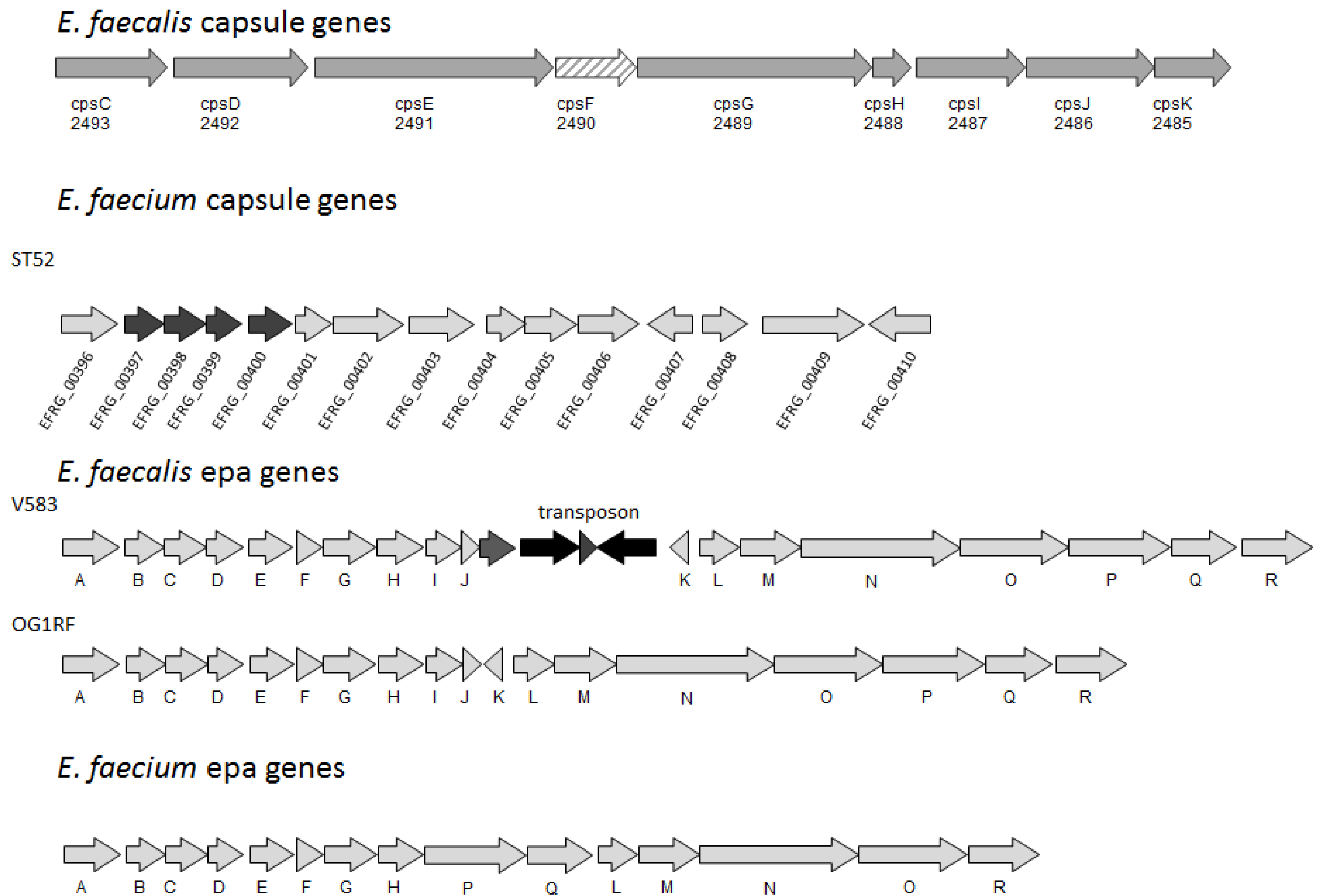

Figure 4. Polysaccharide loci in *Enterococcus faecalis* and *faecium*. The Cps capsule locus of V583 is shown, note the hashed marks for *cpsF*, as the presence of this gene confers serospecificity. A representative capsule locus for *E. faecium* (see reference 126 for further details). Comparison of the *epa locus* in *E. faecalis* V583 and OG1RF, as well as *E. faecium*.

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 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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