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Roles of tRNA in cell wall biosynthesis

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Abstract

Recent research into various aspects of bacterial metabolism such as cell wall and antibiotic synthesis, degradation pathways, cellular stress, and amino acid biosynthesis has elucidated roles of aminoacyl-transfer ribonucleic acid (aa-tRNA) outside of translation. Although the two enzyme families responsible for cell wall modifications, aminoacyl-phosphatidylglycerol synthases (aaPGSs) and Fem, were discovered some time ago, they have recently become of intense interest for their roles in the antimicrobial resistance of pathogenic microorganisms. The addition of positively charged amino acids to phosphatidylglycerol (PG) by aaPGSs neutralizes the lipid bilayer making the bacteria less susceptible to positively charged antimicrobial agents. Fem transferases utilize aa-tRNA to form peptide bridges that link strands of peptidoglycan. These bridges vary among the bacterial species in which they are present and play a role in resistance to antibiotics that target the cell wall. Additionally, the formation of truncated peptides results in shorter peptide bridges and loss of branched linkages which makes bacteria more susceptible to antimicrobials. A greater understanding of the structure and substrate specificity of this diverse enzymatic family is necessary to aid current efforts in designing potential bactericidal agents. These two enzyme families are linked only by the substrate with which they modify the cell wall, aa-tRNA; their structure, cell wall modification processes and the physiological changes they impart on the bacterium differ greatly.

INTRODUCTION

Aminoacyl-transfer ribonucleic acids (aa-tRNA) both deliver amino acids to the ribosome for translation and participate as substrates in other reactions within the cell.[1] One enzyme family that uses tRNA substrates are the L,F-transferases, which transfer leucine or phenylalanine to the N-terminus of proteins thereby targeting them for degradation via the ClpS-ClpAP-mediated N-end rule pathway.[2] aa-tRNAs are also the substrates for at least two other processes that take place in the cell. These pathways modify existing cell wall structures to evade antimicrobial action. Modification of PG, a component of the lipid bilayer, by the attachment of lysine to form lysylphosphatidylglycerol (LysPG) was first discovered in *Staphylococcus aureus*. The enzyme responsible for this reaction was termed multiple peptide resistance factor (MprF) due to the observation that *mprF* gene disruptions increased susceptibility of *S. aureus* to antimicrobial agents.[3] Homologs of MprF are found in various microorganisms, and some exhibit differential specificity for the amino acid they attach to PG, resulting in a broader classification of these enzymes as aminoacyl-phosphatidylglycerol synthase (aaPGS).[4] This review focuses on the structure and specificity of aaPGSs for their aa-tRNA and lipid substrates. The effect of aaPG formation on bacterial survival in different environments and the regulation of aaPGS in various bacterial species are briefly summarized.

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The Fem proteins use aa-tRNAs to form branched peptides on precursors used in cell wall biosynthesis. These enzymes transfer the amino acid moiety from aa-tRNA to the lysine (Lys), or in some cases diaminopimelic acid (DAP), in the pentapeptide of the peptidoglycan precursor to form branched peptide chains that link these precursors to form the peptidoglycan layer. The peptide bridges formed by Fem proteins differ among bacterial species and their completion is paramount to the structural integrity of the cell wall. Recently, bacterial resistance to β-lactam antibiotics targeting the cell wall has increased as evidenced by the resistance of methicillin resistant *S. aureus* (MRSA) to vancomycin.[5] The efficacy of these antibiotics, which target peptidoglycan formation, can be increased by the formation of truncated peptide bridges that result in inadequately linked peptidoglycan and weak cell walls. As such the Fem family has been the subject of studies to develop novel antibiotics to combat resistant microorganisms. The section on Fem transferases focuses on peptide bridge formation, protein structure, and substrate recognition.

CONTROLLING NEGATIVITY: THE ROLE OF tRNA ADDITION TO PG

Many components of the cell wall contribute to its anionic nature such as lipid A, teichoic acids, and phospholipids. The innate immune system of plants, fungi, and other bacteria target this property by producing cationic antimicrobial peptides (CAMPs), which kill invading bacterial species. Bacteria have developed several mechanisms to circumvent this host defense; the production of secreted proteins to cleave or inhibit the binding of cationic peptides, cationic peptide export, and the modulation of bacterial cell wall charge.[6] The addition of aminoarabinose to lipid A in gram-negative bacteria, alanine to teichoic acid in gram-positives, and lysine to PG across the bacteria, help to neutralize negative cell wall components. aaPGSs catalyze tRNA-dependent PG modification. Homologs of aaPGS are encoded in 348 species of bacteria, spanning 93 genera of gram-positive bacteria (mostly firmicutes and actinobacteria) and gram-negative bacteria (mostly preoteobacteria), and in three archaeal *Methanosarcina* species.[7] Most *mprF* homologs are found in plant and animal pathogens, and soil and plant colonizing microorganisms. Distribution of *mprF* does not occur uniformly across a given genus, for example, *mprF* is only found in 4 of the 42 known *Bacillus* taxa. The transferase domains of the aaPGS homologs group into three types, with more than one domain type found in one bacterial species. Forty-nine of the available genome sequences encode more than one aaPGS homolog. The bacteria that contain more than one homolog are most often gram-positive bacteria, particularly members of the actinobacteria and clostridia.[8] Nonpathogenic soil-dwelling microorganisms are thought to possess this defense in response to bacteriocins produced by other bacterial species inhabiting the same environmental niche.[9] These aaPGS proteins possess varying structures with different substrate specificities. Formation of aaPG confers different physiological effects, and regulation of aaPG content of the membrane is controlled by different mechanisms among bacterial species. The following section focuses on these differences among aaPGS homologs.

aaPGSs Consist of Two Functional Domains

aaPGSs contain an N-terminal integral membrane domain and a C-terminal hydrophilic domain that do not share sequence similarity with other proteins of known function. The membrane domain varies in size and sequence, and is absent in some aaPGS homologs. On the basis of secondary structure predictions the membrane domain consists of a variable number of transmembrane-spanning α helices (between 4 and 14) that orient the hydrophilic C-terminal domain toward the cytoplasm where the aa-tRNA substrate is located.[7] aaPGS homologs are predicted to contain an odd number of helices that orient the hydrophilic domain toward the periplasm,[3,10] which is unlikely due to the presence of aa-tRNA solely in the cytoplasmic compartment and may indicate mis-annotated transmembrane-spanning

domains.[11] Truncation of transmembrane domains in MprF from *Bacillus sub-tilis* and MprF1 from *Clostridium perfringens* produced in *Escherichia* coli results in the formation of LysPG *in vitro*.[7] A similar truncation of *Pseudomonas aeruginosa* AlaPGS resulted in a soluble protein that remains associated with the membrane and is able to produce AlaPG *in vivo* when expressed in *E. coli*.[11] In the case of *B. subtilis* and *C. perfringens*, it is likely that even with the loss of this domain the protein still strongly associates with lipid bilayer allowing access to both substrates and retention of transferase activity. These findings indicate that it is the hydrophilic C-terminal portion of the protein that is responsible for amino acid transfer to PG.

S. aureus LysPGS contains two functional domains, a membrane-spanning region that flips newly synthesized LysPG to the membrane outer leaflet and a hydrophilic C-terminal region responsible for the transfer of Lys to PG.[12,13] Deletion of the N-terminal region resulted in the accumulation of LysPG in the inner leaflet and increased susceptibility to CAMPs and decreased repulsion of positively charged cytochrome c.[12] Out of the 14 transmembrane-spanning regions encoded by *S. aureus mprF*, only 6 plus the C-terminal hydrophilic domain were necessary for levels of LysPG production adequate to mediate CAMP resistance. These two domains do not need to be co-translated to be functional, as expression of the two domains separately restored bacterial CAMP resistance.[12] Many *mprF* mutations result in gain of function phenotypes that increase resistance to daptomycin, the last drug of choice to treat MRSA infections.[14–18] *mprF* deletion in *S. aureus* results in hypersusceptibility to daptomycin, and recent data indicate that the expression of *mprF*-specific antisense RNA also restores susceptibility in resistant strains.[12,19] Other mutations in the transmembrane domain lead to increased LysPG in the outer leaflet of the lipid bilayer, thereby reducing CAMP binding.[20]

In *Mycobacterium tuberculosis*, aaPGS (*lysX)*, is composed of three domains, a membrane-spanning domain, one with lysyl-transferase activity (LysPG) and one with lysyl-tRNA synthetase (LysRS) activity (*lysU)*.[21] From phylogenetic analyses of *mprF* paralogs, similar fusions of lysyl-transferase with a LysRS domain may also exist in *Streptomyces* species.[22]

aaPGS Amino Acid Specificities and Binding Determinants

Modified species detected in lipid extracts from different bacterial species include alanyl-PG (AlaPG), LysPG, lysyl-cardiolipin (LysCL), and ornithyl-PG (OrnPG).[23–25] Three of the corresponding amino acids, Lys, Ala, and Arg, act directly as substrates for aaPGSs. These activities correspond to distinct proteins that specifically use Ala-tRNA^{Ala}, Arg-tRNA^{Arg}, or Lys-tRNA^{Lys}. These aa-tRNAs bind MprF and elongation factor Tu (EF-Tu) equally well, indicating a possible mechanism of amino acid procurement and transfer to PG in the membrane in parallel with protein synthesis.[22,26,27] The contribution of the tRNA moiety of aa-tRNA to the specificity of aaPGSs appears to be low. An alanylated 12-bp minihelix exhibits unaltered kinetic parameters compared to full-length tRNA with AlaPGS from *C. perfringens*. Furthermore, a non-cognate aa-tRNA, Lys-tRNA^{Asp}, is also active, indicating that recognition of the tRNA moiety is not critical for aaPGS activity.[22] Studies of *P. aeruginosa* AlaPGS indicated that the acceptor stem is critical for recognition, specifically the C5–G68 base pair.[11] Relaxed aaPGS specificity for tRNA is also demonstrated by the ease with which various bacterial (*S. aureus, B. subtilis, C. perfringens, P. aeruginosa*, and *Rhizobium tropici*) pathways can be reconstituted in *E. coli*, which naturally lacks such a PG modification system but possesses similar tRNA substrates.[4,26,28–30]

Lack of recognition of the tRNA moiety of aa-tRNA indicates that the aa moiety serves as a more important determinant for substrate recognition. *β*-Aminoethylcysteienyl-tRNA^{Lys} serves as a substrate for LysPGS, indicating that the alipathic side chain of the lysine moiety is not a discriminatory factor. However, the nature of the amino acid seems critical, as Cys-

tRNA^{Lys} is not recognized by the enzyme.[27] The α-amino group is also an important determinant for the enzyme as neither *N*-acetyl-alanyl-tRNA^{Ala} nor lactyl-tRNA^{Ala} are substrates.[31]

Lipid substrate specificity has been tested through the use of chemically synthesized lipid analogs, which showed that the terminal glycerol phosphate group of the PG head group is critical for LysPGS to transfer Lys from tRNA to the free 3′ hydroxyl group of PG. Assays with *S. aureus* LysPGS determined that 3′deoxy PG was not recognized as a substrate, while 2′deoxy PG is active, indicating exclusive transfer of Lys to the 3′ group.[32,33] Isomerization of the lysyl group between the 3′- and 2′hydroxyl group of PG was shown to be catalyzed under strong acidic conditions *in vitro*.[34] This isomerization may also occur under certain physiological conditions as 3′ and 2′ isomer forms of LysPG have been detected *in vivo*.[35,36] Cardiolipin (CL), also known as diphosphatidylglycerol, possesses a free 2′ hydroxyl group and as such is not recognized by *S. aureus* LysPGS, however, LysCL is formed by the LysPGS of *Listeria monocytogenes*.[10,32,33,37] The polar head group of PG is also important for substrate recognition by AlaPGS of *P. aeruginosa*, indicating homologs with differing amino acid specificities recognize similar lipid elements.[11] On the basis of the mutation of conserved residues in AlaPGS lacking transmembrane-spanning helices, a mechanism of transfer was proposed.[11] Fatty acid chain saturation level and length seem to have little effect on the recognition of PG substrates as many variations are found in the same organism.[11,37,38] A slight preference is shown by the *S. aureus* LysPG for endogenous lipids over PG extracted from egg yolks. This may have to do with variation in fatty acid chain composition, or a difference in the chirality of the PG glycerol moiety, which is a racemic mixture in egg yolk.[33]

Several other species of aaPG have been detected in bacterial cell membranes. L-ornithine lipids are phosphorus-free lipids synthesized under phosphate-limiting stress conditions in a tRNA-independent manner.[39] This allows phosphate normally allocated for cell wall synthesis to be rerouted for use in nucleic acid synthesis via a biosynthetic pathway encoded by the *olsAB* operon.[39] D-AlaPG and D-AlaCL are found in *Vagococcus fluvalis*, GlyPG in *C. perfringens*, and OrnPG in *Bacillus cereus* and *Mycobacterium* but it has yet to be determined if the syntheses of these lipids species are tRNA-dependent.[23,36,40] Multiple aaPG species are found in *Enterococcus faecium*, including AlaPG, PG acylated with two lysines and even L-ArgPG which is made by a dual specific aaPGS (Arg/LysPGS).[7,24,41] The formation of different aaPG species (Figure 1) results in changes in cell membrane properties, allowing bacteria to adapt to changing environmental conditions.

Physiological Effects of Cell Wall Charge Modulation

The formation of different aaPGs, such as from addition of Ala and Lys, allows different membrane lipid–lipid interactions to occur and affects the packing and the fluidity of the membrane.[8] Liposomes containing LysPG exhibit increased resistance to Rb^+ ions, while *S. aureus* membranes exhibiting increased levels of LysPG correlate with increased fluidity and decreased permeability to daptomycin.[42,43] While the general trend of resistance to CAMPs is seen for most organisms with PG modifications, PG aminoacylation also seems to confer resistance to a broader range of conditions. The tRNA-dependent addition of Lys to PG was originally discovered in *S. aureus* concurrently by looking for insertional mutations that provided resistance to the CAMP gallidermin and the β-lactam oxacillin.[3,44] This modification in *S. aureus* has been shown to mediate resistance to CAMPs produced by neutrophils and plays an important role in the colonization of mice.[3] Exposure of *S. aureus* to acidic conditions increases LysPG levels, a physiological effect that is also observed in *Enterococcus faecalis* and *R. tropici*.[45] The formation of AlaPG in *P. aeruginosa* is also linked to survival in acidic conditions induced by growth in sodium lactate, as well as

resistance to the β-lactam, cefsulodin, the heavy metal ion Cr^{3+}, and CAMP protamine.[4] While CAMP resistance can be explained by electrostatic repulsion (LysPG) or modulation of membrane fluidity and permeability (LysPG and AlaPG), which differ depending upon the peptide, the mechanism of β-lactam resistance in the presence of LysPG is less obvious.[46] β-Lactam antibiotics are anionic in nature and affect the cross-linking of peptidoglycan in the periplasm during cell wall synthesis. Transpeptidases required for this activity possess a single membrane-spanning domain and carry out reactions on lipid-linked substrates; thus, the presence of LysPG may directly affect the activity of the transpeptidase by interaction with the protein or indirectly by optimally positioning the substrate. Lipid species such as CL enhance the activity of MurG in *E.*coli,[47] linking lipid content and peptidoglycan biosynthesis in regulating cell wall turnover and the cell cycle.[48] Resistance to the charged glycopeptide, vancomycin, which also inhibits peptidoglycan cross-linking, varies among *S. aureus* isolates. The presence of LysPG in these strains variously enhances, diminishes, or has no effect on vancomycin resistance.[44,49,50] In addition, *S. aureus* isolates with wild-type MprF but resistant to daptomycin have also been discovered,[14,51–55] emphasizing the importance of the contribution of the genetic background and regulatory networks to antibiotic resistance against vancomycin and daptomycin.[54]

Regulation of aaPG Formation

aaPGs are expressed during all stages of growth, but their biosynthesis can be further enhanced under certain environmental conditions. AaPGS regulation has not been studied in detail and whether the activity is controlled allosterically at the protein level or regulation occurs at a transcriptional level remains unclear. Expression of *mprF* and two additional CAMP resistance mechanisms, *dltABCD* and *vraFG*, in *S. aureus*, are upregulated by the *apsRSX/GraRSX* system in response to the presence of CAMPs.[56–58] This system consists of a membrane-bound histidine kinase sensor, ApsS/GraS, which is able to bind extracellular CAMPs via a membrane sensor loop that contains anionic amino acid residues.[56,57] The transcriptional response regulator, ApsR/GraR, becomes activated when it is phosphorylated by ApsS/GraS. The third component, ApsX/GraX, is of unknown function. *S. aureus* and *Staphylococcus epidermidis* both possess an *apsRSX* system, and are able to sense different CAMPs by variation in the number of negatively charged amino acid residues in the membrane sensor loop of ApsS. The mechanism behind this preferential binding of certain CAMPs over others is not currently known. In *Staphylococcus* species, this system upregulates the expression of lysine biosynthetic genes, and this is thought to provide excess lysine for PG modification with little impact to translation.[57] Upregulation of *mprF* and *dltABCD* by the *aps* system has been shown to occur during the exponential phase of *S. aureus* growth. The expression of both modification enzymes is repressed by the *agr* two component system, a regulator of the *aps* system that is involved in quorum sensing, during stationary growth. Growth-phase-dependent regulation influences the susceptibility of *S. aureus* to antimicrobial peptides, thus cell surface charge appears to be tightly controlled in this organism.[59] The *apsRSX* system is highly conserved in gram-positive bacterial species including *L. monocytogenes*, *Clostridium dificile*, *Bacillus anthracis*, *Staphylococcus haemolyticus*, and *S. epidermidis*.[58] In *L. monocytogenes*, the related system, *virSR*, appears to regulate genes involved in cell wall stress response, including *mprF* and *dltABCD*.[60] These cell wall modifications have also been implicated in *L. monocytogenes* virulence in a mouse model.[10]

BUILDING BRIDGES: ROLE OF aa-tRNA IN PEPTIDOGLYCAN LINKAGES

Cell wall integrity is critical for bacterial survival; cell wall peptidoglycan serves both as a barrier to osmotic pressure and a scaffold for attachment of various proteins including virulence factors.[61–63] As such, the correct formation of the peptidoglycan layer is the target

of many antibiotics. β-lactam antibiotics target the D,D-transpeptidases that catalyze the final cross-linking step of peptidoglycan synthesis.[64] Resistance to these antibiotics in many gram-positive organisms results from the production of modified D,D- transpeptidases or low-affinity penicillin-binding proteins (PBPs).[65] The addition of a complete side chain to peptidoglycan precursors is necessary for resistance to β-lactams-mediated by low-affinity PBPs found in *S. aureus*, *Streptococcus pneumoniae*, and to a lesser extent *Enterococcus faecalis*.[65–71] As resistance increases to β-lactams, transferases that synthesize peptiodglycan side chains have been targeted for the development of new antibiotics. These proteins mostly belong to the Fem nonribosomal peptidyl transferases that use aa-tRNA as the amino acid donor to synthesize the peptide cross-bridges that link peptidoglycan strands. Another mechanism for tRNA-independent branching is the activation of the β-carboxyl of D-aspartate by phosphorylation and subsequent ligation to cell wall precursor by Rac_{fm} and Asl_{fm}, respectively, in *E. faecium*.72 In *S. aureus*, *femA*, *femB*, and *femX (fmhB)* were shown to be essential for the incorporation of glycine into the side chains of peptidoglycan precursors.[73–75] The *femAB* locus was initially identified as a factor essential for methicillin resistance (fem) in *S. aureus* by insertional mutagenesis of chromosomal genes and a screen for reduced expression of resistance mediated by penicillin-binding protein 2A (PBP2A).[74,76] These Fem proteins are composed of two distinct structural classes that differ in terms of substrate recognition and the peptide linkages they form, as described below.

Peptide Bridges Differ Among Bacterial Species

Addition of amino acids to form branched peptides occurs at the ε-amino group of L-lysine, meso-DAP, or LL-DAP in the pentapeptide stem linked via a lactyl group to an *N*-acetyl muramic acid (MurNAc) moiety. The pentapeptide bridge consists of (L–Ala)-(D–Glu)-*X*-(D–Ala)-(D–Ala); where *X* is meso-DAP, Lys, or ornithine (Orn), depending on the bacterial species. Addition of glycine to meso-DAP in *M. tuberculosis*, and *Mycobacterium leprae*, and to L,L-DAP in *Streptomyces coelicolor* play important roles in antimicrobial resistance.[77–79] (see also Tables 1 and 2 for further details).

MurM, a FemA homolog found in *S. pneumoniae*, has different activities among bacterial strains. In *S. pneumoniae*, MurM adds either serine or alanine to ε-L-lysine, while a second fem family protein, MurN, adds alanine forming a dipeptide branch.[115] Different strains of *S. pneumoniae* contain different allelic forms of MurM, some of which exhibit increased peptidoglycan branching. The increase in the proportion of branched peptidogylcan in combination with low-affinity PBPs results in penicillin resistance.[87,116]

These branched peptides can be synthesized on either peptidoglycan precursor, UDP-*N*-acetyl-muramyl-pentapeptide (UDP-MurNAc-pentapeptide), free in the cytoplasm or further on in the lipid synthesis process when the peptidoglycan precursors are associated with the membrane. Lipid I (undecaprenylphosphate-MurNAc-pentapeptide) is formed in the first lipid-linked step in cell wall synthesis by MraY, which transfers the soluble UDP-MurNAc-pentapeptide to the lipid carrier undecaprenylphoshpate (C55-P). The translo-case MurG subsequently links UDP-activated *N*-acetylglucosamine (UDP-GlcNAc) to the muramoyl moiety of lipid I, yielding lipid II.[88] MurM is able to use either lipid I or lipid II as a substrate *in vivo*, indicating that the *N*-acetylglucosaminyl group of lipid II is not necessary for MurM lipid precursor recognition.[117] Figure 2 illustrates the peptidogly-can formation and cross-linking portion of cell wall biogenesis including the known Fem enzyme family members and their precursor specificities.

The proteins FemA, FemB, and FemX are responsible for the addition of five glycines to a lipid-bound cell wall precursor in *S. aureus*.[81,118] FemX catalyzes the addition of the first glycine to the ε-amino group of L-Lysine of lipid II, followed by the addition of two glycines by FemA, and two more glycines by FemB.[105,118] In this sequential amino acid

addition reaction, Gly-tRNA^{Gly} was found to be the rate-limiting substrate.[105] Three out of five tRNA^{Gly} isoacceptors encoded in *S. aureus* are recognized by the FemABX proteins but are not used in protein synthesis.[119] The terminal glycine transferred by FemB from Gly-tRNA^{Gly} is linked via D,D-transpeptidase to the fourth D-Ala of a neighboring pentapeptide chain. This addition occurs sequentially, and the presence of FemABX in the same reaction has been shown to inhibit the formation of the full-length glycine chain. This indicates that each of the proteins only recognize the sugar moiety of the peptidoglycan precursor, and bind to substrate equally independent of the number of glycines attached, thereby inhibiting the catalysis of the other proteins in the reaction.[105]

A gene with sequence similarity to *S. aureus fmhB* was characterized in *E. faecalis* and was found to catalyze the addition of L-Ala to free UDP-MurNAc-pentapeptide *in vitro*.[120] Peptidoglycan chains are linked by a branch of two L-Ala, the activity of hep-tapeptide formation was found to be the result of an additional protein, BppA2, the addition of which allowed peptide bridge formation to be reconstituted *in vitro*. The BbpA1 protein of *E. faecalis* is more directly related to the well-characterized Fem family protein, FemX of *Weissella viridescens*, but is an ortholog of MurM from *S. pneumoniae*.[68] FemX of *W. viridescens* is ideal for *in vitro* studies as it is a highly soluble protein that catalyzes the same reaction as FmhB of *E. faecalis* preferentially on peptide precursors that exist as free molecules in the cytoplasm.[121] It is these two characteristics, solubility and the preference of free UDP-MurNAc as a substrate, that initially separated FemX of *W. viridescens* into a different subclass than that of the enzymes found in *S. aureus*, *E. faecalis*, and *S. pneumoniae*. A third distinctive characteristic, the lack of a helical domain, was discovered by protein crystallization and is discussed below.

Enzyme Structure and RNA Substrate Recognition

The structures of two Fem family members have been determined, FemA from *S. aureus*, and FemX from *W. viridescens*.[122,123] As shown in Figure 3, FemA consists of a helical portion and a globular domain that can be separated into two subdomains (domains 1A and 1B). These two domains consist of three-dimensional folds similar to those of the histone acetyltransferase (HAT) domain present in the GCN5-*N*-acetyl transferase (GNAT) protein superfamily.[124] Recent investigation of the structure of MurM using the FemA structure as a basis for modeling found a domain structure similar to *N*-myristoyl transferases (NMT). Each domain consists of a five stranded mixed polarity β-sheet with four α-helices. Two of the α-helices lie on top of the β-sheet parallel with the β-strands, while the other two α-helices are stacked on the bottom, one in parallel, the other at a 60º angle. Superimpositioning of the two subdomains with the HAT domain of *Tetrahymena* GCN5 led to rms (root mean sqaure) deviations of 1.5 and 1.7 Å for residues in common with subdomain 1A and subdomain 1B, respectively. The two subdomains superimposed with each other have an rms deviation of 2.4 Å. The globular domain consists of additional structures not found in the HAT domain, including a pair of β-strands that extend from domain 1A, a pair of α-helices that lie on top of domain 1B, and a C-terminal α-helix. A deep L-shaped channel corresponding to the binding pocket of domain 1B in comparison to a HAT-like domain fold runs across the surface of FemA and is thought to bind to UDP-MurNAc-hexapeptide linked to lipid II.[122] Recent evidence comparing MurM with NMT structural folds suggest that the C-terminal region is critical for activity, which is supported by the loss of transferase activity in truncated MurM and FemA proteins.[87,118] The crystallized protein of FemA is lacking these C-terminal residues as well as a 12-residue loop segment that was not visible on the electron density map, and it is possible the missing residues may bury the proposed peptide-binding cleft, supporting an active site location more similar to that proposed in MurM.[125] The helical arms found in domain 2 are similar to those found in seryl-tRNA synthetases (SerRS), and are responsible for forming a flexible

platform to interact with the tRNA substrate.[126,127] This structure is thought to play a similar role in FemA, by holding Gly-tRNA^{Gly} during Gly transfer to the growing pentaglycine bridge. In MurM, the 30aa variable sequence coincides with the helical arm region found in SerRS and is thought to determine the protein's specificity for either Ala-tRNA$^{Ala\ or}$ Ser-tRNA^{Ser}.[87,125] The FemA homolog, MurM, has been shown to recognize the acceptor stem, and/or the TΨC stem loop of tRNA^{Ala}, the same portion of the tRNA that is bound by the helical arms in SerRS.[116]

The structure of FemX from *W. viridescens* varies from FemA by one major structural detail; it is the first characterized Fem protein lacking the antiparallel coiled-coil domain formed by the helical domain that is expected to bind Gly-tRNA^{Gly}.[123] This feature further sorts it into its own subclass along with substrate preference and protein cellular localization, separating it from the other 50 FemABX-related sequences that form subclass I. *W. viridescens* FemX also consists of a globular domain with two structural subdomains separated by a cleft proposed to be the UDP-MurNAc-pentapeptide binding site as evidenced by co-crystallization with this molecule. Domain 1A consists of a six-stranded mixed polarity β-sheet surrounded by five α-helices. Domain 1B consists of a seven-stranded β-sheet surrounded by six α-helices. The two domains can be superimposed with rms deviation of 2.0 Å for common atoms. Superimpositioning of FemX with FemA yields an rms deviation of 2.8 Å for the 302 common Cα atoms. FemX differs from FemA in two minor and the major structural detail previously mentioned. The N-terminal β-strand, β1, of FemA is absent in FemX. Second, there is no equivalent found in FemX for the β-hairpin β6–β7, which lengthens the β-sheet of domain 1B and strengthens the junction between the two domains. The strand β1 is replaced in FemX by a loop. The lack of the β6–β7 hairpin can be explained by a sequence deletion in FemX, which is also found in other members of the Fem proteins such as FmhB from *S. aureus*.[81] An additional structural variation is that the coiled-coil domain 2 of FemA is replaced in FemX by a small loop but this deletion does not affect the global fold of the protein. The central core is comparable to FemA, and consists of a four-stranded mixed polarity β-sheet connected by two helices. This central core domain appears to be a functionally conserved feature common to both the FemABX and GNAT superfamilies. Binding of the UDP-MurNAc-pentapeptide, which was co-crystallized with FemX, occurs in the cleft formed at the interface between the two domains, and is mostly in contact with domain 1A. The binding site can be divided into four regions that interact with the peptidoglycan precursor including the long loop β5–β6, the end of helix α11, the loop β2–β3, and the end of helix α2.[123] Domain 1B residues implicated in binding as a result of co-crystallization are located in loop α7–α8. Structural differences between the complexed and uncomplexed FemX are minor, with notable spatial differences observed for Lys36, Tyr215 of α8, and Tyr256 of β10. In this structure, binding of the UDP-MurNAc-pentapeptide orients FemX in a manner that exposes the lysine side chain to solvent, while the electron density of the ε-amino group of this lysine is missing from the crystal structure, indicating that this may not be a key feature in the mechanism of FemX.

On the basis of mutational analysis of the hypothetical UDP-MurNAc-pentapeptide binding pocket the complex hydrogen bonding network formed by Lys36, Arg211, and a lesser extent, Tyr215 were shown to be essential for FemX transferase activity in *W. viridescens*.[128] Interactions between Lys36 and Arg211 and UDP-MurNAc-pentapeptide (both phosphate groups and both D-Ala residues) maintain the substrate in a bent conformation essential for catalysis. The Tyr215Phe change resulted in a 25-fold loss of transferase activity, indicating that the hydrogen bond network formed by this residue is important but not essential for activity. FemX Tyr215Leu was insoluble suggesting that stacking interactions between Tyr215 and Arg211 play a role in protein folding. Despite the integral role of Lys36 and Arg211 in FemX they are not highly conserved in members of the Fem family. Mutational studies based on chemical modification of *W. viridescens* FemX

when bound to Ala-tRNA^{Ala} showed Asp109 to be an absolutely conserved residue in fem ligases that when mutated to asparagine results in a 230-fold loss of activity.[129] Earlier studies of the catalytic mechanism of FemX from *W. viridescens* based on sequencing of the protein and mutation of conserved residues led to the identification of Gln29 as being the only conserved potentially catalytic residue.[121] Additionally, mutations in Gly292 and Pro110 also led to loss of activity, but normal protein yields indicated no conserved residue that might plausibly form acyl intermediates thought to be crucial in the catalytic activity of FemX.[121] This study also found Phe305 and Tyr216 to be important aromatic residues, potentially forming aromatic–aromatic interactions with the tRNA substrate.[121] Also, as previously mentioned, truncation of the C-terminus of FemX led to inactivity, implicating a role of these residues in catalytic activity.[121]

Fem Recognition of aa-tRNA Differs from aaRS

In order for tRNAs to be used as substrates by Fem proteins they must first escape their primary role in the cell, ribosomal protein synthesis. In *S. aureus*, this complication is avoided by three encoded tRNA^{Gly} isoacceptors that bind poorly to EF-Tu, therefore avoiding the cellular translational machinery.[130] These three tRNA^{Gly} isoacceptors, out of five encoded tRNA^{Gly} molecules, have a UCC anticodon, while the remaining two have UCC and GCC anticodons. In *S. epidermidis*, the tRNA^{Gly} utilized for peptidoglycan synthesis has the universally conserved D-loop residues G18 and G19 changed to UU or CC, rendering these charged tRNA molecules inactive in protein synthesis. Additionally, the tRNA substrate of fem transferases in *S. epidermidis* has an additional base pairing in the anticodon loop between C32 and G38, which is thought to sufficiently distort the anticodon loop to render it incapable of binding to the translation machinery. Ser is also incorporated into *S. epidermidis* peptide linkages, however, no distinguishing mutations in tRNA^{Ser} able to synthesize these bridges have been identified. Identification of *W. viridescens* FemX recognition elements of the tRNA moiety was determined by site-directed mutagenesis of the tRNA^{Ala} acceptor stem based on the FemA crystal structure. This led to the identification of two cytosines, C72 and C71, which are essential for transferase activity using chemically acylated minihelix mimics of the tRNA^{Ala} acceptor stem as substrates. Mutation of the G30-U70 wobble base pair did not affect enzyme activity, indicating that this key identity element for AlaRS is not recognized by FemX.[131] Experiments conducted using Ser-tRNA^{Ala}, Gly-tRNA^{Ala}, and Ala-tRNA^{Ala} resulted in Gly and Ala incorporation into the pentapeptide indicating that the enzyme preferentially transfers Ala over the larger amino acid Ser, and shows flexibility in its use of Ala by transferring both the L and D stereoisomers.[132] This is due to the unfavorable interactions that take place with the acceptor stem when larger amino acids are attached to the tRNA body. FemX is able to discriminate against the smaller amino acid, Gly, via recognition elements present in the tRNA body. Minihelices mimicking the acceptor stems of tRNA^{Ala}, tRNA^{Ser}, and tRNA^{Gly} were chemically acylated with Ala; while Ala-tRNA^{Ser} was a viable substrate, Ala-tRNA^{Gly} was discriminated against as well as the cognate Gly-tRNA^{Gly}, whose acceptor stem contains C72 and G71.[131,132] Figure 4 highlights differences in tRNAs used in peptidoglycan synthesis versus isoacceptors used in translation. Inhibitors are being developed by the synthesis of stable tRNA analogs, 2′-deoxy-adenosine, adenosine 3′-phosphate analogs, and aryl sulfanilamides, all of which mimic the tetrahedral transition state thought to be part of the MurM catalyzed reaction.[133–136]

CONCLUSION

The ability to modify PG in the membrane by the addition of amino acids is important for bacterial survival under different conditions. Modulation of net membrane charge plays a role in resistance to several antibiotics, such as daptomycin. Although the physiological

effects of these aaPG modifications have been studied in detail, an aaPGS structure is now required to further characterize the catalytic residues of the hydrophilic transferase domain. Perhaps once one of the enzymes is crystallized, structural alignment will correlate paralog sequence diversity to the broad range of aa-tRNA substrates recognized by these enzymes. Substrate docking or co-crystallization will also help clarify aaPGS recognition elements of the tRNA moiety, which to date points to primary recognition of the amino acyl moiety with little recognition of the tRNA body.[22,27,31,32] Additionally, in order to investigate the role of aaPGS in antibiotic resistance, a better understanding of the function of the flippase domain *in vivo* for additional bacterial strains is necessary. A study of the residues and substrate recognition elements of the flippase domain may provide an additional basis for the development of novel antibiotics. As shown in *S. aureus* this domain is critical for cationic peptide resistance and it appears to be the sole flippase responsible for aaPG translocation across the membrane.[13] However, variation in the flippase domain among bacterial species could complicate the development of broad range inhibitors. The development of new antibiotic compounds is critical as mutations in LysPGS of MRSA enhance resistance to daptomycin, one of the last drugs in the antibiotic arsenal used to treat such multidrug resistant strains. Furthermore, the identification of aaPGS homologs responsible for other amino acid modifications of PG is necessary. These modifications are of interest due to the fact that the presence of aaPG has different effects on bacterial strains in varying environmental conditions. Identification of the conditions under which these modifications play a role in survival may lead to the identification of aaPG species *in vivo* that to date have only been found *in vitro*.

Although functional homologs, Fem proteins bear no structural similarity to aaPGSs. Research on the structure of this class of enzymes is more advanced due to the crystallization of FemA of *S. aureus* and Fem X of *W. viridescens*. Substrate-binding elements for UDP-MurNAc-pentapeptide have been identified via co-crystallization and site-directed mutagenesis.[122,123,128] Similar mutagenesis studies have been carried out for the aa-tRNA substrate, but co-crystallization with this second substrate of Fem has yet to be carried out.[132] This is of some importance as the two structures differ by the presence or absence of a helical domain, which is purported to bind tRNA in the context of FemA. Also, a more in-depth mutational study of tRNA body and amino acyl moiety has been carried out for FemX recognition of Ala-tRNA^{Ala} than FemA recognition of its cognate substrate Gly-tRNA^{Gly}.[132] In order to definitively characterize the recognition elements of the aa-tRNA and UDP-MurNAc-pentapeptide substrates in the formation of branched chain peptides further studies of the two currently crystallized Fem proteins are needed. Additional structures of Fem family members with varying aa-tRNA specificities would be integral for full functional characterization of this cell wall modification. Also, to date, the investigation into recognition elements of all Fem proteins responsible for peptide synthesis in a single organism has yet to be determined. Fully understanding this process will allow for the development of more potent inhibitors of cell wall synthesis, and perhaps negate bacterial resistance to some current antimicrobial agents.

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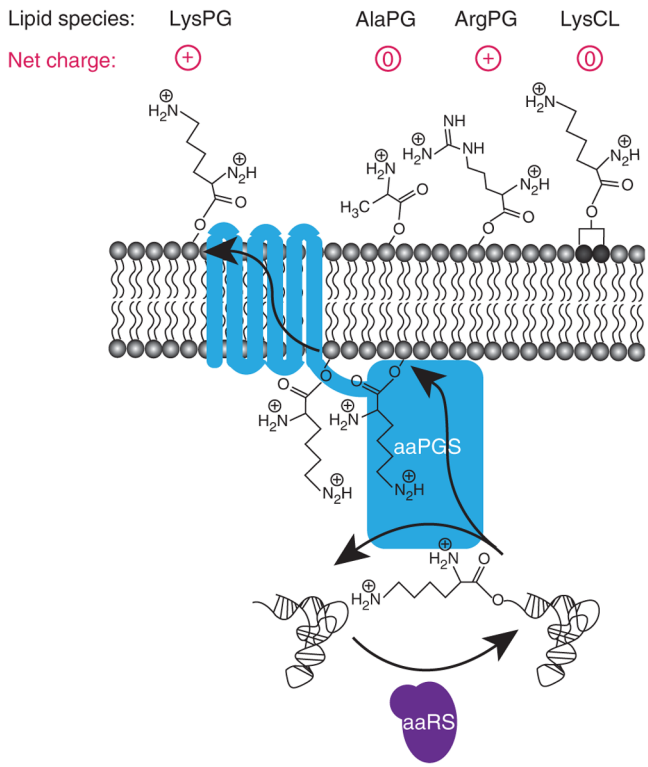

FIGURE 1.

Aminoacyl-phosphatidylglycerol synthase (aaPGS) modifications contribute to the charge of the cell wall. Shown are the known modifications and the resulting charge of the modified phosphatidylglycerol (PG).

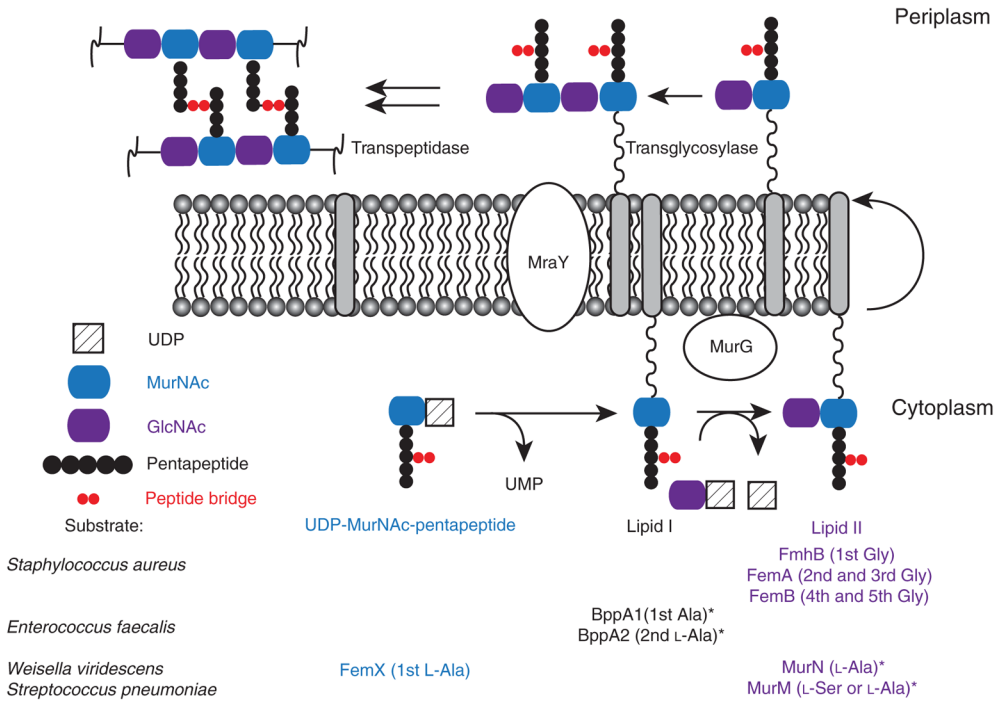

FIGURE 2.

Formation of the peptidoglycan layer in some bacterial species involves peptide bridges formed by Fem proteins. Shown is a schematic of cell wall biogenesis, below each precursor the amino acid specificity of the Fem enzyme(s) found in the organism listed to the left is indicated in the same color text as the precursor used by the enzyme for amino acid transfer. For *Enterococcus faecalis* the distinction of which lipid precursor that is preferred by the enzyme has not been determined. For *Weissella viridescens*, FemX only adds the first amino acid, the other two are added by a second unknown enzyme or enzymes.

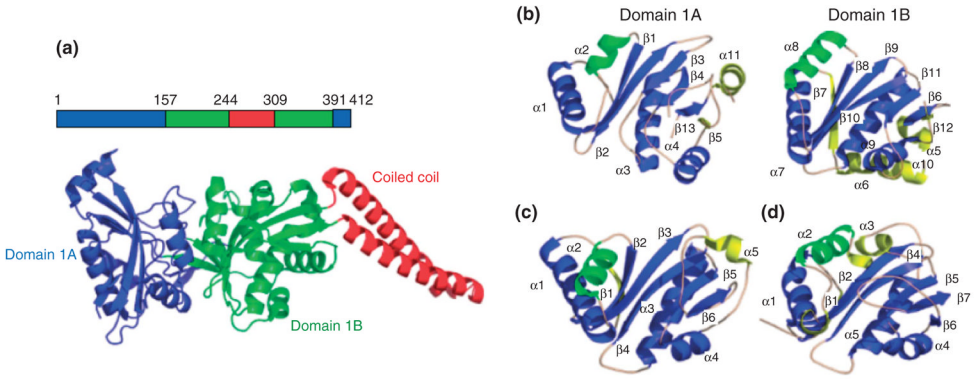

FIGURE 3.

Structural comparisons of *Staphylococcus aureus* FemA and *Weissella viridescens* FemX. (a) Sequence and structure of FemA with an additional coiled coil absent in FemX. (b) Domains 1A and 1B of *W. viridescens* FemX. The two proteins can be structurally compared to the catalytic domains of the histone acetyltransferase of *Tetrahymena thermophila* (c), and the serotonin acetyltransferase of *Ovis ovaries* (d). From a review published by Mainardi et al.[70]

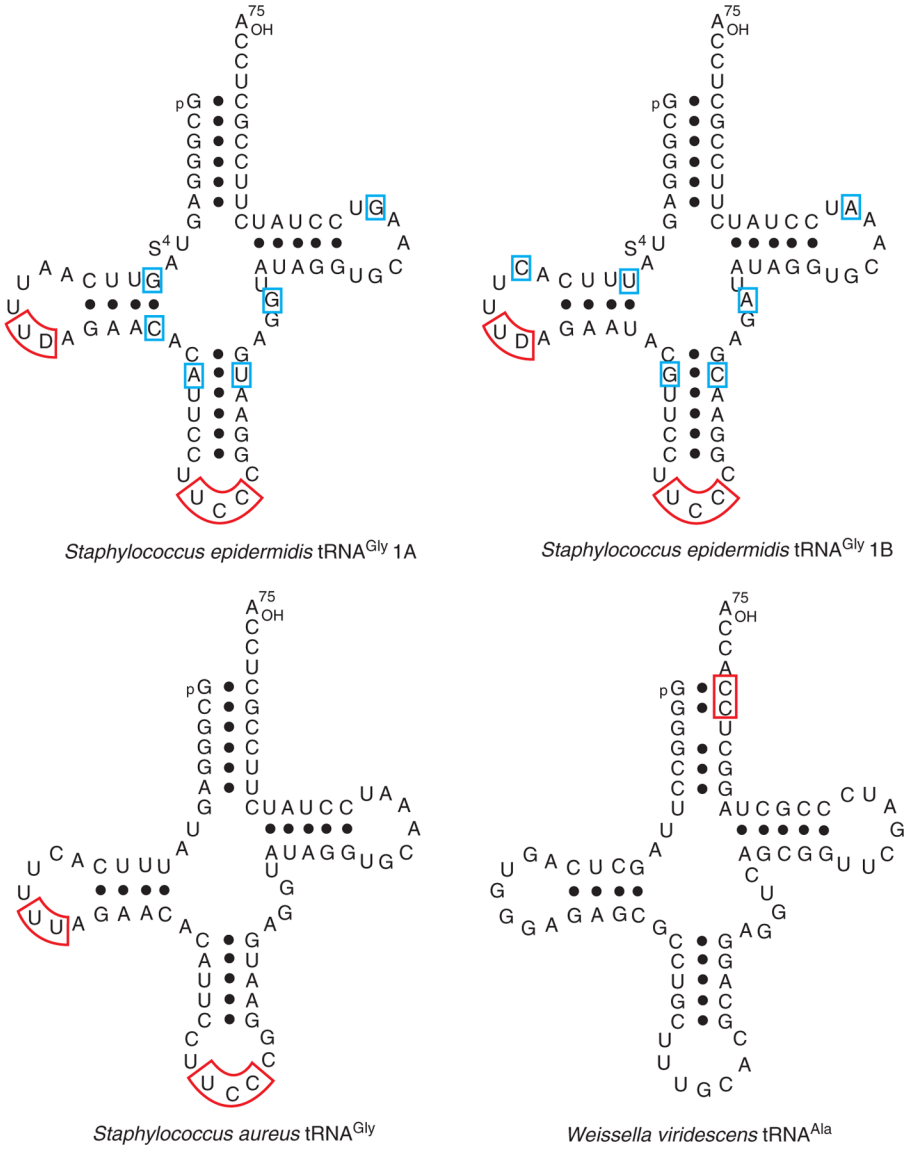

FIGURE 4.

tRNA isoacceptors predominantly utilized by Fem proteins in peptidoglycan biosynthesis. Differences found to be important for Fem recognition or features that are detrimental to protein synthesis in red. In blue are the differences between the two co-purified tRNA species shown to be preferred substrates of *Staphylococcus epidermidis* FemA.

TABLE 1

Peptide Bridge Composition of Bacterial Species with ε-L-Lysine at Position 3 of the Pentapeptide

Interpeptide bridge composition	Species	Figure	References	Transferase Proteins	References
Peptidoglycan variation type A3α with D-alanine at position 4 and ε-L-lysine at position 3					
→Gly→	*Borrelia* sp.		80	AAB91521	81
→Gly→	*Treponema pallidum*		82	AAC65773	81
→Gly→	*Streptococcus toyocaensis*		83	StaO	81
→Gly→	*Clostridium perfringens*		84	BAB87796	81
→Gly→	*Deinococcus radiodurans*		84	AAF10635	81
→Gly_2→	*Staphylococcus aureus* Copenhagen	8	85		
→Gly_5→	*S. aureus* Copenhagen	8	85, 86	FmhB, FemA, FemB, FmhA, FmhC	87–89
→Gly (L-Ser)→	*Micrococcus mucilaginosus*	16	84		
→L-Ala (L-Ser)→	*Micrococcus mucilaginosus*	16	84		
→L-Ala→	*Arthrobacter crystallopoietes*	21c	90		
→$L\text{-}Ala_2$→	*Lactobacillus coprophilus*	21c	91		
→$L\text{-}Ala_{2–3}$→	*Staphylococcus zooepidemicus*		84	Zif	92
→$L\text{-}Ala_{2–3}$→	*Staphylococcus pyogenes*		84		
→$L\text{-}Ala_{2–3}$→	*Staphylococcus thermophilus*		84		
→$L\text{-}Ala_{2–3}$→	*Enterococcus faecalis*		84		
→$L\text{-}Ala_2$→	*Streptococcus pneumoniae*	21c	84	FibA, FibB, MurMN	66, 93
→$L\text{-}Ala_3$→	*Micrococcus roseus*	13a	94		
→$L\text{-}Ala_3$→	*Staphylococcus thermophilus*	13a	95		
→$L\text{-}Ala_4$→	*Arthrobacter ramnosus*	13a	96		
→$L\text{-}Ala_5$→	*Micrococcus* sp. 7425		84		
→Gly→L-Ala→	*Peptostreptococcus* sp.	19d	97		
→$Gly_{4–5}$→L-Ala→	*Staphylococcus epidermidis*	12b	98	FemA, FemB	99
→Gly_2→Ser→Gly→Ser→	*Staphylococcus simulans*		100	Lif/Epr, FemABX	101, 102
	Staphylococcus capitis				
→$Gly_{2–4}$→$L\text{-}Ser_{2–}$4→Gly→	*S. epidermidis*	12c	103		
→L-Ala→$Gly_{5–6}$→	*Staphylococcus roseus*	14e	84		

Interpeptide bridge composition	Species	Figure	References	Transferase Proteins	References
→L-Ala→Gly→L-Ala2→	*Streptococcus* sp.	19e	84		
→L-Ala (L-Ser)→L-Ala_2→	*Streptococcus* Group G	19c	84		
→L-Ala (L-Ser)→L-Ala→	*Staphylococcus agalactiae*	19b	84		
→L-Ala→L-Ser→	*Weisella viridescens*	21a	104	FemX	105
→L-Ala→L-Ser→L-Ala→					
→L-Ser (L-Ala)→L-Ala→	*S. pneumoniae*	1	106	MurM, MurN	79
→L-Ser→L-Ala (D-Ser)→	*Leuconostoc gracile*	21a	107		
→L-Ala_2→L-Ser→	*Leuconostoc cremoris*	21b	104		
→Gly→L-Thr→	*Staphylococcus salivarius*	20b	108		
→L-Ser (D-Ala)→L-Thr→	*Staphylococcus bovis*	20a	108		
→L-Ser (D-Ala)→L-Thr→	*Staphylococcus equinus*	20a	108		
→L-Ala→L-Thr→	*Streptococcus* sp.	25a	84, 95		
→L-Ala_2→L-Thr→	*Arthrobacter citreus*	26a	84		
→L-Ala_3→L-Thr→	*Micrococcus roseus* R 27	13b	94		
→L-Ala_2→L-Ser→L-Thr	*Micrococcus ruber*		84		
→L-Ala→L-Thr→L-Ala→	*Arthrobacter aurescens*	26b	84		
→L-Ala→L-Thr→L-Ser→	*Arthrobacter polychromogenes*	26c	84		
→L-Thr→L-Gly→	*Streptococcus milleri*		84	MilF	109
→L-Thr→L-Ala→	*Streptococcus mutans*		84		

TABLE 2

Peptide Bridge Composition of Bacterial Species with δ-L-Ornithine or ω-L,L-Diaminopimelic Acid at Position 3 of the Pentapeptide

Interpeptide bridge composition	Species	Figure	References
Peptidoglycan variation type A3β with D-alanine at position 4 and δ-L-ornithine at position 3			
→$Gly_{2–3}$→	*Micrococcus radiodurans*	14f	110
→L-Ala_3→	*Bifidobacterium globosum*	22c	111
→L-Ala→L-Thr→L-Ala→L-Ser→Gly→	*Bifidobacterium longum*	22b	112
→L-Ala→L-Thr→L-Ala→L-Ser→Gly→	*Propionibacterium petersonii*	15a	113
→Gly→	*Mycobacterium tuberculosis*		76
→Gly→	*Mycobacterium leprae*		77
→Gly→	*Streptomyces coelicolor*		78
→Gly_5→	*Streptomyces roseochromogenes*	15a	114
→Gly_5→	*Acanthocephalus tumescens*	27a	96