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REVIEW

Deciphering the tRNA-dependent lipid aminoacylation systems in bacteria: Novel components and structural advances

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ABSTRACT

tRNA-dependent addition of amino acids to lipids on the outer surface of the bacterial membrane results in decreased effectiveness of antimicrobials such as cationic antimicrobial peptides (CAMPs) that target the membrane, and increased virulence of several pathogenic species. After a brief introduction to CAMPs and the various bacterial resistance mechanisms used to counteract these compounds, this review focuses on recent advances in tRNA-dependent pathways for lipid modification in bacteria. Phenotypes associated with amino acid lipid modifications and regulation of their expression will also be discussed.

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Introduction

The bacterial cell wall exhibits a high net negative charge due to the presence of phosphate as a building block in many of its molecular constituents. The phospholipids that form the membrane, as well as the teichoic acid chains spanning the cell wall in Gram-positive bacteria, contain phosphate groups that give these structures their negative charge. All forms of life- including bacteria, fungi, plants, and animals- have the ability to produce cationic antimicrobial peptides (CAMPs) to inhibit bacterial growth. CAMPs possess antimicrobial properties and exhibit a high affinity for the negatively charged components within the bacterial cell wall.

After a brief introduction on CAMPs and their distinctive modes of action, as well as various mechanisms used by bacteria to counteract CAMP activity, this review will focus on a particular mechanism of CAMP resistance that utilizes aminoacylated tRNAs (aa-tRNAs) to modify lipids in the bacterial envelope. Several reviews describing this mechanism have been published within the past few years.[1-4] This manuscript sets out to review recent advances in the field and outline current insights regarding the structure-function relationship of tRNA-dependent lipid modification systems, and of the flipping mechanism of these modified lipids across the membrane. Examples of novel tRNA-dependent lipid modifications that have recently been discovered, and the enzymes responsible for their synthesis, will be discussed.

Cationic antimicrobial peptides (CAMPs) and bacterial resistance to CAMPs

CAMPs are found in all domains of life. They vary in sequence, size (10–50 amino acids (aa)), and secondary structure, but all share an amphipathic nature and exhibit a net positive charge (usually between +2 and +9) brought about by a lateral chain containing Arg and Lys residues. CAMPs fall into four distinct structural classes: α-helical, β-stranded, linear, and looped. α-helical, β-stranded, and linear CAMPs are mostly encoded in eukaryotic cells, whereas looped CAMPs (i.e., polymyxins) are primarily encoded in bacteria.[5] Some CAMPs are ribosomally synthesized, while others (e.g., polymyxins[6]) are produced by large multifunctional enzymes. The predominant mode of action used by these compounds involves pore formation and general disruption of the bacterial membrane upon binding of CAMPs to negatively charged phospholipids. Certain CAMPs, however, possess a targeted mode of action. For example, bacteriocins (CAMPs secreted by bacteria) such as nisin bind to Lipid II, which is the precursor for peptidoglycan synthesis. Another example, Polymyxin B, forms pores in the outer membrane of gram-negative bacteria after specific interaction with lipid A at the surface of the cell. It is worth mentioning, that at high concentrations, and in the absence of their high affinity molecular targets, both nisin and polymyxin B display general pore forming properties in the cytoplasmic membrane.[7] Beyond direct interaction with membrane components, certain CAMPs pass through the cytoplasmic membrane to inhibit intracellular targets. For instance, the bacterial peptide microcin J25 (MccJ25) inhibits RNA polymerase,[8] while apidaecins (in honeybees), oncocins (in insects), and Bactenecin-7 (in mammalian cells) inhibit bacterial translation by binding to ribosomal proteins.[9,10]

CAMPs secreted as part of the innate immune system in eukaryotes, as well as those produced by bacteria to inhibit growth of other microbial species, are the result of millions of years of co-evolution. It is proposed that the high diversity of structure and function among eukaryotic CAMPs has been

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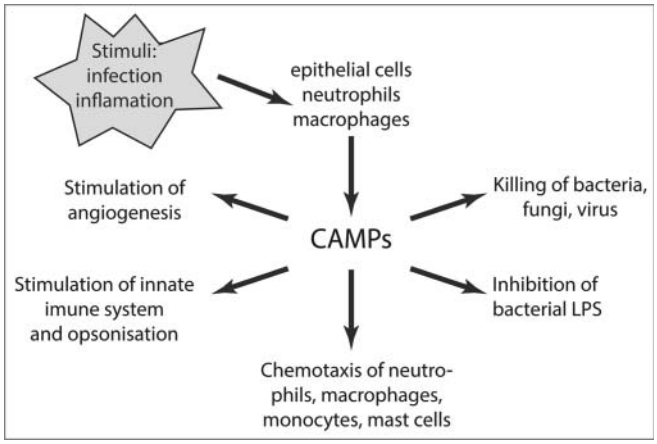

Figure 1. Multiple functions of CAMPs in host defense. CAMPs induce a variety of responses in host innate immune cells. They alter gene expression, induce production of chemokines and cytokines, promote recruitment of immune cells to the site of infection (chemotaxis), and inhibit growth of pathogenic species infecting the host. Selective control of inflammation in response to CAMP activity promotes wound healing and initiation of adaptive immune responses. LPS, lipopolysaccharide. Figure adapted from [14,15,115].

shaped by a variety of crosstalk between host cells and bacterial species.[11,12] The diversity of CAMPs in eukaryotes is illustrated in the Antimicrobial Peptide Database,[13] which lists more than 2,500 CAMPs of eukaryotic origin (122 in human[14]) and only 301 CAMPs of prokaryotic origin (only four were identified in archae). In mammals, CAMPs are produced and secreted by epithelial cells (*i.e.*, skin, gastrointestinal, respiratory, and urogenital cells) and various cells of the immune system (e.g., monocytes, macrophages, neutrophils, and other granulocytes; for review see [15], Fig. 1). These systems use secreted CAMPs to kill bacteria outside the cell as well as intracellular pathogens enclosed inside the phagosome. Constitutive expression of CAMPs is developmentally regulated and influenced by age. Upregulation of CAMPs occurs during inflammation and infection. CAMPs produced in eukaryotic species not only play a direct role in the killing of bacterial pathogens, but also in the modulation of the immune response by, for example, decreasing inflammation and attracting various types of immune cells during infection (i.e., chemotaxis).[15] Additionally, certain CAMPs stimulate production of chemokines and cytokines from a variety of cell types, constituting an indirect mechanism for stimulating the immune response.[15]

When compared with the cell membranes of bacterial species, the cell membranes of multicellular organisms possess many fundamental differences in lipid composition that allow them to be more naturally resistant to CAMPs. The membranes of plant and animal cells are composed of zwitterionic lipids (e.g., phosphatidylethanolamine) that bear no net charge, and are therefore unable to interact efficiently with CAMPs. Eukaryotic membranes also contain lipids such as cholesterol, which stabilize the membrane and contribute to CAMP resistance.[16] In contrast, bacteria exhibit high levels of anionic components in their cell envelopes (phospholipids in the membrane, and teichoic acid chains in the cell walls of Gram-positive bacteria), which support efficient interaction with CAMPs. Because of this, bacteria have developed a battery of mechanisms to increase their resistance to CAMPs (Fig. 2 for review see [17,18]). Several of these mechanisms function by adding positively charged modifications to components of the cell wall to decrease binding to CAMPs.

tRNA-dependent aminoacylation of membrane phosphatidylglycerol

Phosphatidylglycerol (PG) is a ubiquitous anionic phospholipid found in all domains of life and is particularly abundant in bacterial membranes.[19] PG contains a phosphate group, which confers a net negative charge (−1), and a terminal glycerol

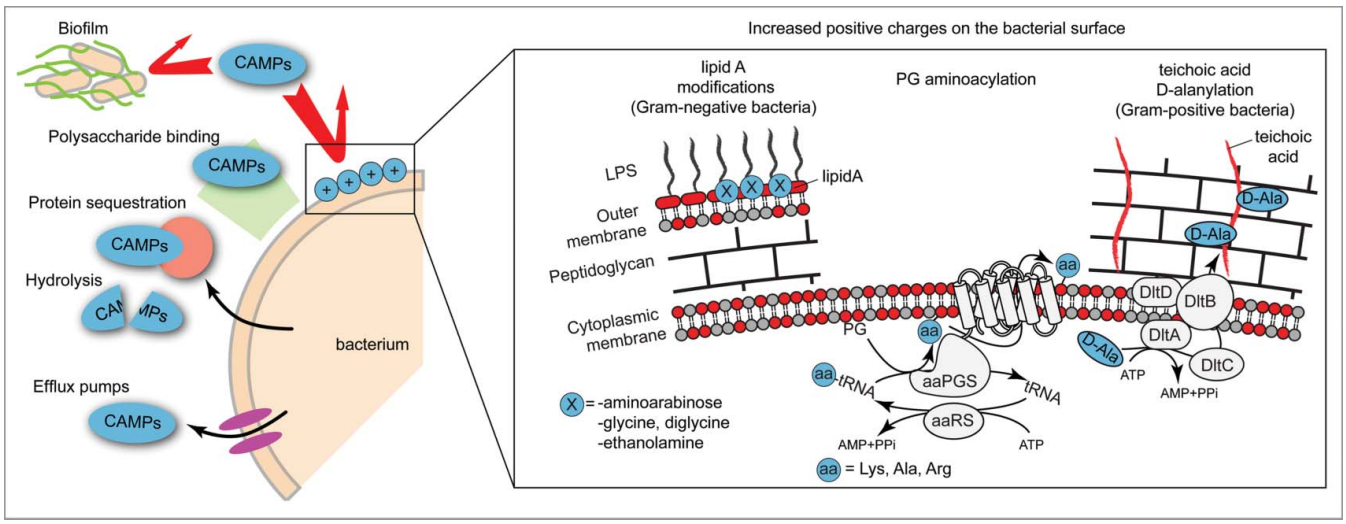

Figure 2. Bacterial defenses against CAMPs and mechanisms for lowering the net negative charge of the cell envelope. CAMP resistance mechanisms include (1) efflux pumps, (2) CAMP-binding or hydrolyzing agents (3) formation of biofilms, and (4) addition of positively charged modifications to negatively charged substructures in the cell wall or lipid membrane. Addition of positively charged groups to the cell wall lowers the net negative charge of the envelope, decreasing binding of cationic compounds such as CAMPs and subsequently increasing bacterial resistance to these antimicrobials. In Gram-negative bacteria, lipid A in the outer membrane is the substrate for various positively charged modifications. Biosynthesis pathways for these modifications are not shown (For details see [116-118]). In certain species of both Gram-positive and Gram-negative bacteria, aminoacyl-phosphatidylglycerol synthases (aaPGSs) transfer aa from aa-tRNA (pre-formed by cytosolic aa-tRNA synthetases, aaRS) to phosphatidylglycerol (PG) in the membrane. In Gram positive bacteria, D-alanylation of lipoteichoic acid chains involves the enzymes encoded by the *dltABCD* operon. Positively and negatively charged molecules are represented in blue and red, respectively.

group characterized by hydroxyls that can be exploited to attach different types of modifications. These modifications include addition of a phosphate or sulfate group to form PG-phosphate or PG-sulfate in Archaea. Addition of a carbohydrate or an amino acid (aa) such as Lys, Ala, or Arg results in formation of the corresponding aminoacyl-PG (aa-PGs) in bacteria. Lastly, condensation of 2 PG molecules to form di-PG (also called cardiolipin, CL) is a process that occurs in all domains of life. Modified PGs provide the cell with a variety of altered components that can affect general properties of the membrane such as surface charge, fluidity, and morphology (for review see [1,2,19]).

Lys-PG was discovered in the membrane of *S. aureus* in the mid 1960s by William Lennarz, who established that synthesis of this modified lipid requires lysyslated tRNA (Lys-tRNA) to serve as an amino acid donor.[20,21] The bacterial enzyme responsible for synthesis of Lys-PG was not determined until 40 y later during a genetic screen aimed at identifying mutants of *S. aureus* that are sensitive to CAMPs.[22] Peschel and coauthors were the first to establish that lysyl-PG synthase (LysPGS), encoded by the gene *mprF* (multiple peptide resistance factor), enhances resistance of *S. aureus* to 10 CAMPs from various origins. The minimum inhibitory concentrations (MIC) for certain CAMPs was decreased by 30-fold in a strain lacking *mprF*.[23] In more recent years, it was shown that LysPGS is only one member of a large family of aminoacyl-PG synthase (aaPGS) homologs spanning nearly all known bacterial phyla. Some homologs are also present in archaeal methanogens,[24] but no examples have been identified in eukaryotic genomes.[1] Aminoacyl-phosphatidylglycerol synthases (aaPGSs), as these enzymes are collectively called, are bifunctional enzymes. They exhibit a cytosolic C-terminal domain bearing the tRNA-dependent aa-PG synthase activity, and an N-terminal integral membrane domain made up of a variable number (2 to 14) of predicted transmembrane helices (TMH). The membrane domain is responsible for flipping neosynthesized aa-PGs across the membrane from the cytoplasmic side where they are formed into the periplasm.[25-28] Interestingly, some Actinobacteria such as *Mycobacterium tuberculosis* display a large aaPGS homolog called LysX. A bioinformatics analysis showed that LysX encompasses, like other aaPGSs, an integral membrane domain and an aaPGS synthase domain, but also includes a lysyl-tRNA synthetase (LysRS) domain at the C-terminus that exhibits a high level of similarity to the LysRS of the cytosolic translation machinery. It has been suggested that this protein is able to mediate each step of the lipid aminoacylation pathway, including tRNA aminoacylation, and by doing so increases the efficiency of lipid modification. The LysRS domain of LysX is required for synthesis of lysylated phosphatidylglycerol in *M. tuberculosis*, suggesting that free Lys-tRNA^{Lys} produced by the translation machinery is not used by LysX for lipid modification.[29]

Several bacterial species exhibit more than one aaPGS homolog (paralogs), suggesting a functional divergence exists among co-occurring proteins. Thus, in recent years the specificities of various aaPGS homologs have been investigated in different bacterial backgrounds. Several enzymes with altered aa-tRNA and lipid substrate specificities have been identified, and the recently solved crystal structures of two aaPGSs have shed light on the molecular mechanism of tRNA-dependent systems for lipid aminoacylation.[30]

aaPGS mediated lipid remodeling uses several aa-tRNAs as substrates

Clostridium perfringens exhibits two aaPGSs: a LysPGS responsible for synthesis of Lys-PG and an AlaPGS responsible for synthesis of Ala-PG.[31] It has been established *in vitro* that some aaPGSs exhibit promiscuity for aa-tRNA recognition and can utilize multiple aa-tRNAs, while some are specific for a unique aa-tRNA.[32] For example, the LysPGS from *Agrobacterium tumefaciens* and the AlaPGS from *C. perfringens* specifically use Lys-tRNA and Ala-tRNA respectively, while the aaPGS from *Bacillus subtilis* utilizes both Lys- and Ala-tRNA as amino acid donors *in vitro*.[32] Interestingly both Lys-PG and Ala-PG were initially detected in the membrane of *B. subtilis*,[33] but a recent report suggests that D-Ala-PG and not L-Ala-PG is formed in *B. subtilis* by an aaPGS independent pathway.[34] One paralog in *Enterococcus faecium* (referred to as RakPGS or MprF2) displays a more relaxed specificity for its aa-tRNA and can utilize Ala-, Lys-, and Arg-tRNAs.[32,35-37] Organisms that harbor multi-specific aaPGSs produce an expanded repertoire of distinct aa-PGs in their bacterial membranes.

It was established early on that only the acceptor stem of the aa-tRNA is important for aaPGS activity. A minihelix encompassing just the acceptor stem and TYC stem loop of tRNA^{Ala} is as efficiently recognized as full length tRNA by AlaPGS.[31] Further investigations with the AlaPGS from *P. aeruginosa* revealed that a microhelix (a hairpin consisting of 7 base pairs) is the minimal substrate for AlaPGS activity. An in-depth study showed that the Ala moiety and the fifth base pair of the tRNA helix constitute the major elements for efficient recognition of Ala-tRNA^{Ala}.[38] Several studies have tried to determine the phenotypical changes correlated with synthesis of Lys-PG compared with Ala-PG in *S. aureus* and *P. aeruginosa*.[24,30] These investigations showed that substitution of Lys-PG (which confers a net charge of +1) with Ala-PG (which bears a neutral net charge) did not affect bacterial susceptibility to CAMPs such as nisin and gallidermin, or to the CAMP-like antibiotic daptomycin. These findings suggest that the α amino group of the aa moiety of aa-PG alone is able to enhance antimicrobial resistance in bacteria.

The cytosolic domain of aaPGS exhibits a GCN5-like acetyltransferase fold

Until recently, no significant structural relationship was established between aaPGSs and other known proteins using conventional sequence search methodologies (i.e., BLAST). However, recent reports using more sensitive methods for homology detection (i.e., profile Hidden Markov Models, HMM) suggested that aaPGSs exhibited a domain resembling a GCN5-like acetyltransferase (GNAT) fold, which is a fold found in several other aa-tRNA transferases, ([39,40] and see below). These observations were recently confirmed by the crystal structures of aaPGSs from *Bacillus licheniformis* and *Pseudomonas aeruginosa*.[30] In the Protein Family database (Pfam[41]), the GNAT fold is found in 39 families of transferases which altogether constitute the N-acyltransferases clan (CL0257). This clan includes many acyl-CoA and aa-tRNA transferases. Enzymes of the latter category (summarized in

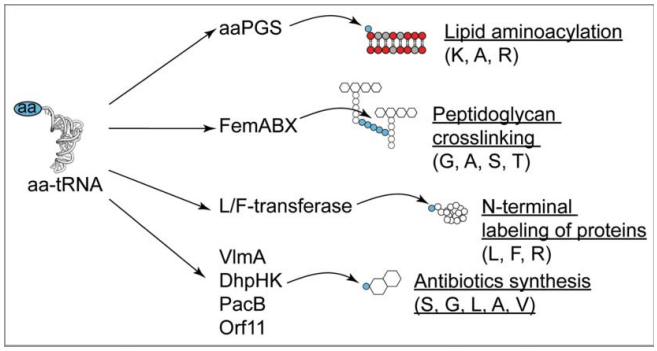

Figure 3. Role of aa-tRNA transferases exhibiting a GNAT fold. aa-tRNA specificities are indicated in brackets. See text for details.

Fig. 3) include the Fem proteins, which utilize aa-tRNAs to synthesize a connecting peptide to bridge peptidoglycan chains.[42] This bridging peptide exhibits up to 5 aa. Depending on the bacterial species, the connecting peptide can be made of Gly, Ala, Ser, or Thr. These aa are sequentially added to the cytoplasmic precursor of peptidoglycan (i.e., UDP-N-acetyl-muramyl-pentapeptide) by distinct Fem proteins (for review see [3,43]). This pathway is an alternate route to the direct bridging of peptidoglycan chains, and uses a set of penicillin binding proteins exhibiting a low affinity for β-lactam antibiotics (e.g., protein mecA in methicillin resistant *S. aureus*).[42] Another family of tRNA-dependent enzymes exhibiting a GNAT fold are Arg- and Leu/Phe-transferases, which transfer Arg, Leu, or Phe to the N-termini of proteins, thereby targeting them for degradation via the ClpS-ClpXP-mediated N-end rule pathway (see [44] for review). The GNAT fold was recently identified in many more tRNA-dependent transferases in *Streptomyces* species, which utilize specific aa-tRNAs for synthesis of broad-spectrum antibiotics. For instance, the protein VlmA utilizes Ser-tRNA^{Ser} for synthesis of the antibiotic valanimycin[45]; DhpH and DhpK utilize Gly-tRNA^{Gly} and Leu-tRNA^{Leu} for biosynthesis of tripeptide phosphonate[46]; the protein PacB utilizes Ala-tRNA^{Ala} in the biosynthetic pathway for pacidamycin[47]; FzmI uses Val-tRNA^{Val} for synthesis of fosfazinomycin[48]; Orf11 uses Gly-tRNA^{Gly} for the synthesis of a streptothricin-related antibiotic.[49]

The structures of the transferase domains of the AlaPGS from *P. aeruginosa* and the LysPGS from *B. licheniformis* (in complex with the Lys analog lysinamide) revealed tandem repeated GNAT folds (Fig. 4A). The strongest homologies with aaPGS structures were found within proteins of the Fem family. The active site of aaPGS is composed of two cavities located on opposite sides of the protein linked by a short bottlenecked tunnel.[30] One cavity, which is presumably facing the cytoplasm, is responsible for binding the aa born by the tRNA. The second cavity is more hydrophobic and, opening onto the membrane side, is responsible for binding to PG.[30] This structural work, in combination with biochemical experiments using protein variants and substrate analogs, showed that catalysis takes place at the bottleneck between the two substrate-binding pockets (Fig. 4B). Regarding catalysis, an Arg residue, bound to the α carbonyl group of the co-crystalized amino acid analog, is proposed to increase the electrophilicity of the ester bond of the aa-tRNA mediating the nucleophilic episode of the 3′hydroxyl group of PG. A similar role in catalysis was proposed for a Lys residue in the structure of the alanyl-tRNA transferase FemX,[50] which is the strongest structural homolog of both crystallized aaPGSs.[30] These structural investigations also confirmed that the acceptor stem, the aminoacyl moiety of the aa-tRNA, and the terminal glycerol group of PG constitute the main determinants for substrate recognition. Comparison of the aaPGS structure to that of FemX in complex with an aa-tRNA analog showed that binding of the acceptor stem of the tRNA is mediated through interactions of a positively charged helix in the protein born by one GNAT fold, which represents an important structural feature common to all aa-tRNA transferases (i.e., Fem proteins, L/F-transferases, and aaPGS).[30]

Flippase domain of aaPGS

It was only recently that the lipid flippase activity of aaPGS was directly demonstrated.[27] The flippase domain of aaPGS is an integral membrane domain consisting of a variable number (up to 14) of predicted TMHs. The aaPGS flippase activity has been investigated with the *S. aureus* LysPGS, whose membrane domain consists of 14 TMHs. In this organism, flipping of Lys-PG from the inner to the outer leaflet of the cytoplasmic membrane is necessary for optimal resistance to CAMPs.[27] Although the synthase domain of *S. aureus* LysPGS exhibits a strict specificity for its aa-tRNA, the flippase domain is able to flip Ala-PG or Lys-PG.[28] A recent study verified the membrane localization of the flippase domain, as well as the topology and oligomerization state of the protein inside the membrane.[25] This work showed that the region encompassing TMHs 1 through 6 supports the flippase activity of LysPGS, whereas TMHs 7 through 14 enhance LysPGS activity most likely by stabilizing the synthetic domain at the surface of the membrane, or by participating in selection and binding of PG. Mutagenesis of the protein revealed residues involved in the flippase mechanism. These residues, mostly hydrophilic, are thought to form a hydrophilic cleft within the flippase domain to facilitate the passage of the polar head of aa-PG through the membrane. Interestingly, using a bacterial 2-hybrid assay *in vivo*, one study showed that LysPGS forms dimers, and possibly higher oligomeric structures within the membrane. These oligomeric states are mediated through interactions between the membrane and cytoplasmic domains of each subunit.[25]

Beyond PG modification: Aminoacylation of cardiolipin and diacylglycerol

Besides PG, three additional lipids (cardiolipin, lysophosphatidylglycerol, and diacylglycerol) have been identified as substrates participating in tRNA-dependent pathways for lipid modification by addition of amino acids (Fig. 5).

- Aminoacylation of cardiolipin (CL)

It was discovered early on that LysPGS from *Listeria monocytogenes* exhibits dual specificity for its lipid substrate. This enzyme is able to lysylate PG as well as the PG derivative CL, which exhibits a single hydroxyl group available for aminoacylation[51-53](Fig. 5). The role of Lys-CL is not clear, but several studies showed that Lys-PG and Lys-CL increase resistance of *L. monocytogenes* to CAMPs[53] and osmolytic stress. Some

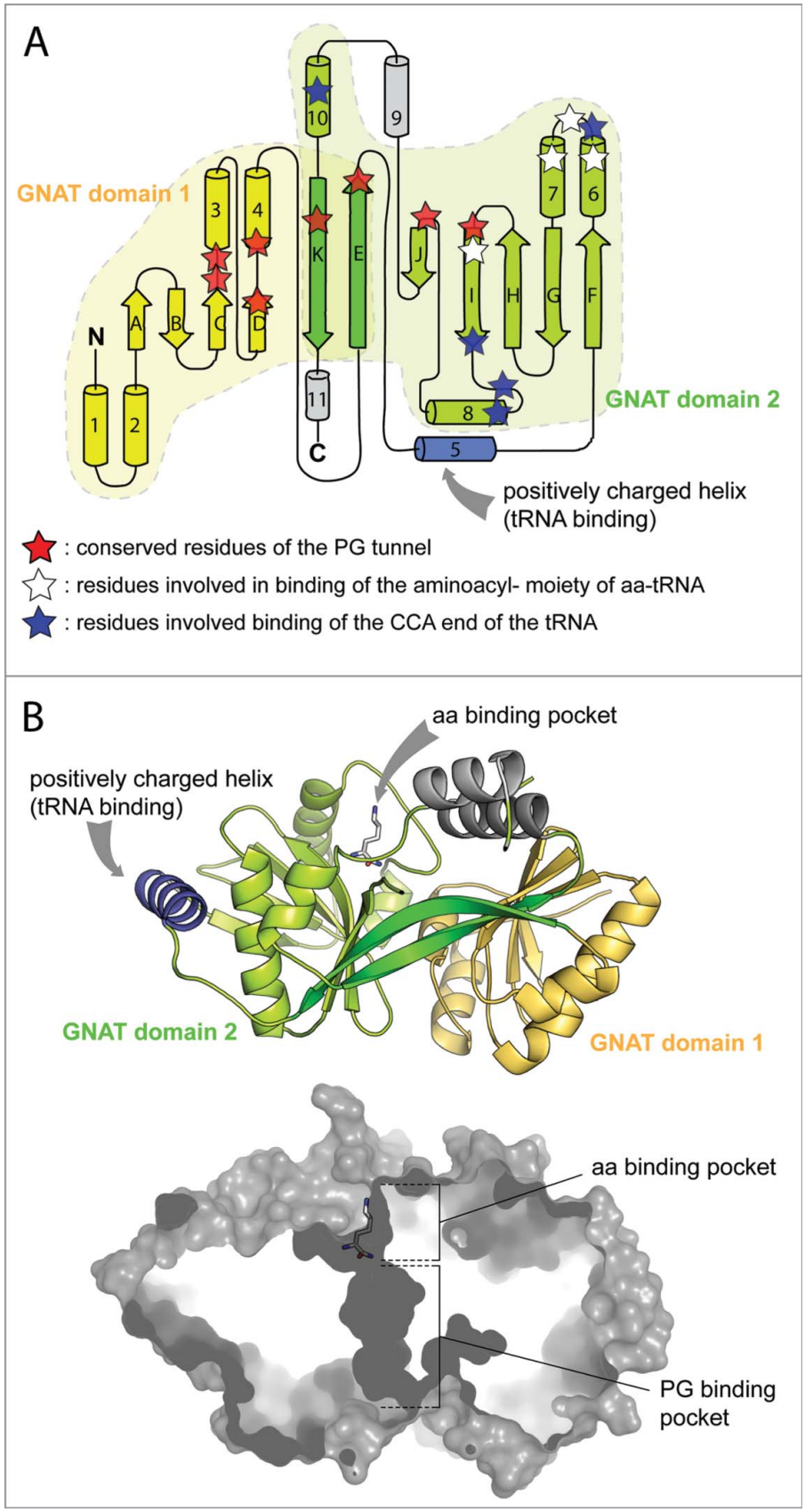

Figure 4. X-ray structures of the catalytic domains of AlaPGS from *P. aeruginosa* and LysPGS from *B. licheniformis*. A. Topology of the tandem GNAT fold repeat in aaPGSs. B. Structure of LysPGS with the Lys analog L-lysinamide bound to the aa binding pocket. Superposition of the FemX structure in complex with the tRNA[50] reveal a possible binding mechanism of the tRNA, with helix 5 exhibiting basic residues. The cross-section of the structure shows the aa and PG binding pockets; the active site is located at the interface between the 2 pockets.

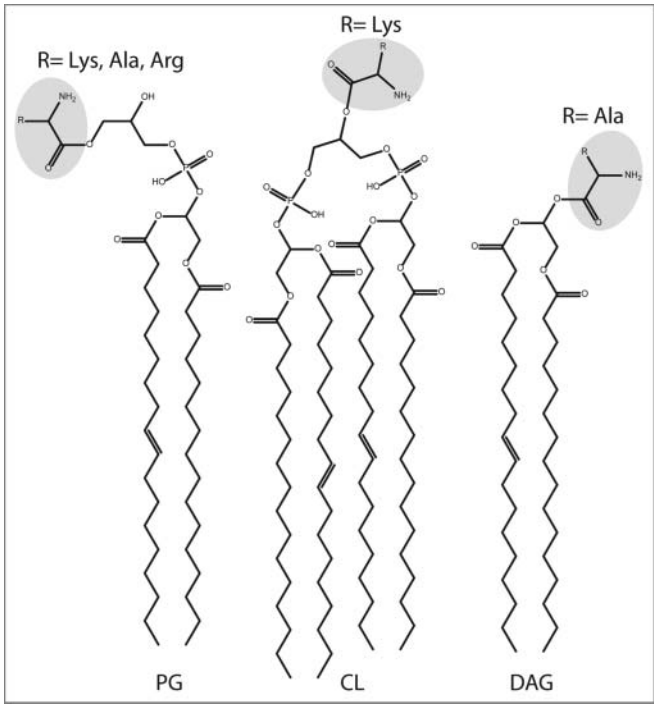

Figure 5. Lipid and aa substrates of lipid aminoacylation pathways. Phosphatidylglycerol (PG), Cardiolipin (CL), and diacylglycerol (DAG). See text for details.

evidence suggests that the two modified lipids may additionally influence expression of membrane proteins.[54]

- Aminoacylation of diacylglycerol (DAG)

A recent phylogenetic analysis revealed 7 primary types of aaPGS homologs.[55] aaPGS homologs of types I and II are found in proteobacteria and firmicutes, respectively, and have been found to be specific for aminoacylation of PG and, more rarely, CL. Biochemical studies revealed the function of a novel type of aaPGS homolog found exclusively in Actinobacteria, a bacterial phylum comprising several important human pathogens. Specifically, various species of *Corynebacterium* exhibit an alanyl-diacylglycerol synthase (AlaDAGS) that uses Ala-tRNA^{Ala} and diacylglycerol (DAG, Fig. 5) to form Ala-DAG. Interestingly, co-expression of AlaDAGS with a protein called PesT (a putative esterase encoded in operon with *alaDAGS*) induces formation of both Ala-DAG and Ala-PG in *C. glutamicum*. The role of PesT in this process is not clear. Since synthesis of Ala-PG requires co-expression of PesT and AlaDAGS, it has been proposed that PesT supports either i) an Ala-DAG/PG transferase activity (using Ala-DAG to form Ala-PG), or ii) an Ala-DAG hydrolase activity that allows formation of Ala-PG by AlaDAGS[55] (Fig. 6A).

Homeostasis and utilization of aa-PG in the membrane

Three types of semi-conserved and structurally-unrelated hydrolases (AhyD, VirJ, and the putative esterase, PesT) have been identified.[35] Although the function of PesT remains to be clarified (see above), the functions of VirJ (found in various Gram-negative proteobacteria) and AhyD (found in several genera of Gram-positive bacteria) were recently revealed. Both of the latter enzymes are aa-PG hydrolases encoded in operon with aaPGS.[35,56] These hydrolases are involved in controlling the levels of aminoacylated lipids in the membrane (Fig. 6B), and both proteins were found to increase bacterial resistance to antimicrobial compounds.[35,56,57] While the cellular localization of AhyD remains undefined, VirJ is known to be anchored to the periplasmic surface in the cytoplasmic membrane of the gram-negative bacterium *P. aeruginosa*. VirJ exhibits broad substrate specificity and can hydrolyse the Ala-, Gly-, and Lys- moieties of artificial substrates.[56]

In an interesting development, the modified lipid Lys-PG was shown to serve as a substrate for an additional membrane modification. A recent report showed that Lys-PG in *B. subtilis* is used to produce N-succinylated Lys-PG.[33] The biologic significance of and enzyme responsible for this modification are not known. However, it was proposed that a homolog of the enzyme

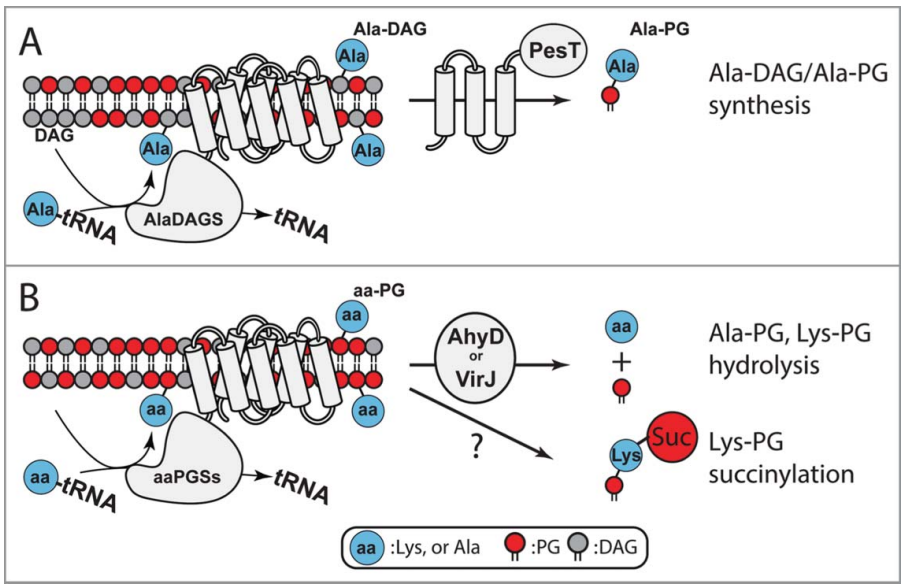

Figure 6. Homeostasis and utilization of aa-PG and Ala-DAG. A. In Corynebacterium, AlaDAGS utilizes Ala-tRNA^{Ala} and DAG to synthesize Ala-DAG. PesT, in concert with AlaDAGS, synthesizes Ala-PG. The role of PesT in this process is unknown.[55]B. AhyD (in certain Firmicutes) and VirJ (in some proteobacteria) are aa-PG hydrolases. In *B. subtilis*, Lys-PG is N-succinylated by an unknown mechanism.

responsible for protein succinylation, recently discovered in bacteria, may be accountable for Lys-PG modification. It is worth noting that attachment of succinyl to Lys reverses the net positive charge of Lys-PG (+1), yielding a net negative charge that is the same as that of unmodified PG (−1). This system may represent a means for bacteria lacking an aa-PG hydrolase (e.g., AhyD or VirJ are not found in *B. subtilis*) to reverse the net charge of the membrane without hydrolysing Lys-PG.

Increased virulence and antibiotic resistance linked to lipid aminoacylation

The effects of aa-PG on antibiotic resistance have been investigated in several important human pathogens such as *S. aureus* (for review see [4]), *L. monocytogenes*,[53] *B. anthracis*,[58] *P. aeruginosa*,[24,59] *M. tuberculosis*,[29,60] *Enterococcus* species,[35,36,61] and *B. subtilis*[62,63](for review see [1]). aa-PGs in these organisms primarily enhance bacterial resistance to positively charged compounds targeting the membrane (such as CAMPs) and last resort lipopetides like daptomycin.[4,58] aa-PGs have been found to modestly enhance bacterial resistance (e.g., a 2-fold increase in the MIC) to other classes of antimicrobials such as β-lactams and glycopeptides. Lys-PG, for example, increases resistance of *S. aureus* to several β-lactams,[64,65] and vancomycin.[66] Similarly, resistance phenotypes against β-lactams were observed in *P. aeruginosa*, which produces Ala-PG.[59] In *M. tuberculosis*, Lys-PG was shown to increase resistance to vancomycin.[29] β-lactams and vancomycin are antibiotics that do not interact with the cytoplasmic membrane; both compounds target distinct components of the peptidoglycan synthesis machinery inside the periplasm (i.e., penicillin binding proteins and peptidoglycan connecting pentapeptides, respectively). Increased resistance to these antibiotics may be due to indirect effects of the aaPGS lipid modification process on the homeostasis and general properties of the cell wall. For instance, Ala-PG found in the outer membrane of the Gram-negative bacterium *P. aeruginosa* may modulate the activity of porins in the outer membrane that are necessary for β-lactams to reach their periplasmic targets.[59] Lys-PG synthesis in *S. aureus* on the other hand might modulate the activity of proteins involved in peptidoglycan synthesis, which would indirectly affect resistance to β-lactams and vancomycin. These hypotheses are supported by several studies that demonstrated that the lack of aa-PG in the membranes of *S. aureus* and *L. monocytogenes* induces significant changes in the membrane proteome and its function.[54,67,68]

aaPGSs have been recognized as virulence factors for prominent pathogens in several animal models. For instance, the lack of LysPGS in *S. aureus* decreases its capacity to proliferate in an endocarditis model in rabbits,[69] and in a sepsis model in mice.[23] The mortality rate of mice infected with a LysPGS deletion strain was significantly diminished.[23] Likewise, deletion of LysPGS in *L. monocytogenes* lowered the bacterial counts detected in the spleens and livers of infected mice as compared with those infected with the wild-type strain.[53] In *M. tuberculosis*, deletion of LysPGS (i.e., *lysX*) decreases bacterial growth in the lungs of guinea pigs relative to the wild-type strain.[29]

Several studies demonstrated the importance of lipid aminoacylation in the interaction of pathogens with different types of cells in infected hosts. Lipid aminoacylation was also shown to increase the extra and intracellular survival of pathogens. For instance, LysPGS increases resistance of *S. aureus* to killing by neutrophils and leukocytes.[23,70] In contrast, Lys-PG did not affect *S. aureus* survivability after phagocytosis by human neutrophils.[23] When deprived of Lys-PG, the obligate intracellular species *L. monocytogenes* poorly infects epithelial cells and macrophages, but is not compromised in cell-to-cell spreading ability.[53] Likewise, Lys-PG increases the survivability of *M. tuberculosis* inside phagocytic cells,[71] and is critical for maintaining intracellular pH levels while in activated macrophages.[60]

Although each of the examples summarized above suggests that lipid aminoacylation increases resistance to CAMPs and virulence of pathogens, the associated phenotypes and their relative intensities are largely dependent on bacterial context. For example, lipid aminoacylation in *Enterococcus faecalis* does not affect virulence in a mouse bacteremia model, and only modestly affects the MICs of various CAMPs (up to a 4-fold increase). Mutants of this species defective for lipid aminoacylation do, however, exhibit increased resistance to opsonic killing, and a 42% decrease in biofilm formation compared with the wild-type strain.[36]

Single nucleotide polymorphisms: Gain of function mutations in LysPGS of multi-resistant S. aureus

In recent years, as many multi-resistant strains of *S. aureus* have emerged, multiple independent studies have identified strains exhibiting single-nucleotide polymorphisms (SNPs) in *mprF* that are linked to resistance against daptomycin, a last resort CAMP-like pore forming lipopeptide used to combat acute microbial infections. Several studies identified SNPs in the *mprF* gene of *S. aureus* clinical isolates, or laboratory strains evolved *in vitro* under antibiotic pressure.[72-83] Genome analysis revealed additional SNPs in genes such as *rpoB* (a polymerase subunit), *capB* (for capsular polysaccharide synthesis), and the promoter region of the *dltABCD* operon which is known to increase CAMP resistance (Fig. 2). However, SNPs in *mprF* were the most frequently encountered, and were accompanied by an increase in resistance to daptomycin and other CAMPs (nisin and human CAMPs e.g., [75,79]). Most *mprF* gain-of-function SNPs are located in the flippase domain, and they generally increase the total amount of Lys-PG in the membrane as well as the amount of Lys-PG translocated to the outer surface of the cytoplasmic membrane (e.g., [77,78]). Interestingly, SNPs in the *lysX* gene in *M. tuberculosis* were recently discovered; this finding further supports the hypothesis that this system plays a role in virulence.[84] The precise role of SNPs in the catalytic activities of aaPGSs (aa-PG synthesis and flipping) is not well understood, but they are predicted to increase both processes affecting aminoacylated lipids.

Physiological significance of lipid aminoacylation

Several studies showed that the lack of lipid aminoacylation in mutants of *S. aureus* and *L. monocytogenes* induces changes in the proteome of the membrane compared with that of wild-type strains. This indicates that aa-PGs not only modify the electrostatic properties of the membrane surface, but also affect expression and activity of membrane proteins.[54,67]

Interestingly, LysPGS in *B. subtilis* is localized at the septum of the bacterium, along with other components involved in membrane and cell wall biogenesis.[85] A recent study showed that LysPGS is also localized at the septum in *E. faecalis*, and demonstrated that human β-defensins (i.e., hBD2 and hBD3) interact with *E. faecalis* near this region to target the excretory system consisting of protein translocase (SecYEG) and sortase enzymes (SrtA). Altogether, these findings show that aa-PGs can provide positively charged lipid microdomains that protect the septum, along with important components involved in cell wall biosynthesis, from inhibition by CAMPs.[86]

Most studies on lipid aminoacylation systems have emphasized their role in protecting bacteria from stresses located outside the cell. A single study reported the potential role of lipid aminoacylation in processes occurring inside the cell. Ichiahashi and coauthors demonstrated that the first step during genomic DNA replication (involving the protein DnaA) is inhibited by synthesis of Lys-PG in *S. aureus*.[87] These investigations suggest that stresses in the environment trigger expression of Lys-PG, not only to increase resistance to external challenges, but also to repress DNA replication and regulate cell cycling events.

Another important concept that has emerged in recent years, is the idea that low levels of aminoacylated lipids in the bacterial membrane may alter general membrane protein composition. In Firmicutes such as *L. monocytogenes, S. aureus*, and various *Bacillus* species, aminoacylated lipids represent a significant portion of the total membrane lipid content, ranging from 10–75% of the total.[52-54,88,89] In *M. tuberculosis*, Lys-PG is a minor lipid constituent, representing less than 0.3% of the total lipids. Despite its low abundance, Lys-PG in this species is important for maintaining membrane potential, virulence, CAMP resistance, and survival in mononuclear phagocytes.[29,71,90] It was determined that the phenotypes observed in mutants deprived of Lys-PG may, in fact, be due to indirect effects of the modified lipids on phospholipid catabolism and cell division.[90] These findings indicate that PG-lysylation in *M. tuberculosis* plays a broader role outside of the typical electrostatic effects associated with aa-PGs, which might be relevant to many bacterial species.

Regulation of PG aminoacylation

Studies performed in various bacterial backgrounds have shown that lipid aminoacylation is triggered by environmental stimuli. For instance, in bacterial symbionts of plants such as *Rhizobium tropici* and *Sinorhizobium medicae*, Lys-PG formation is triggered by low pH and is required for acid tolerance and colonization of root nodules. Likewise, in species like *B. subtilis*,[91,92] *Bacillus megaterium*,[89] *S. aureus*,[93,94] *and E. faecium*,[35,94,95] acidic conditions increase the amount of aa-PG produced in the membrane.[55,93,94,96] Because aa-PG does not provide any apparent tolerance to acidic conditions in *E. faecium*, it was recently proposed that lipid aminoacylation confers tolerance to inhibitors that are produced during exposure to acidic conditions, rather than to low pH levels themselves. For instance, aa-PGs enhance resistance of *P. aeruginosa* and *E. faecium* to lactic acid,[35,59] a toxic osmolyte known to diffuse passively through the cytoplasmic membrane of bacteria.[97]

The full regulatory network controlling aaPGS expression is not completely understood, but it has been studied in *L. monocytogenes, R. tropici* (see[1] for review), and more extensively, in *S. aureus*. Various bacterial inhibitors targeting the cell envelope trigger expression of *mprF* in *S. aureus*. For instance, antibiotics such as methicillin, vancomycin, penicillin-G, D-cycloserine, and bacitracin, which all target the cellular envelope, induce expression of Lys-PG. Antibiotics such as chloramphenicol and puromycin, which target the translation machinery, do not induce Lys-PG synthesis.[98-100] Recent studies showed that the 2-component system, LytSR, thought to be responsible for maintaining electrical potential and CAMP sensing at the surface of the cell membrane in *S. aureus*, does not control expression of *mprF* and other genes involved in maintenance of the cell surface charge.[101] Instead, the 2-component system GraSR (a.k.a. the antimicrobial peptide sensing system; aps) was shown to

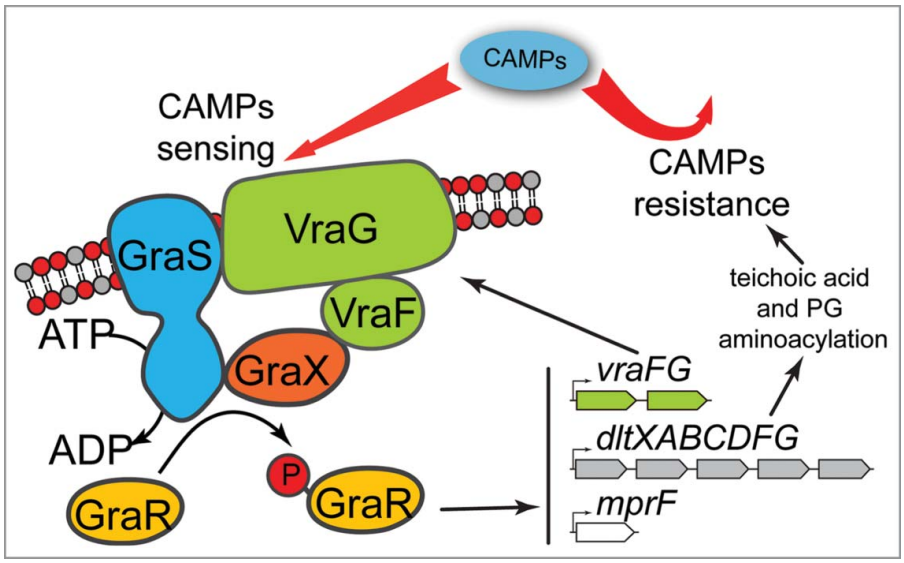

Figure 7. The 5-component signal transduction network controlling CAMP sensing and resistance pathways in *S. aureus*. CAMPs are sensed by VraFG and the signal is transduced to GraS through a mechanism that is likely to involve interaction between VraG and GraS. Activation of the GraSR system leads to increased transcription of the *dltABCD* operon and the *mprF* gene, leading to cell wall remodeling and CAMP resistance (figure adapted from [107]).

modulate expression of *mprF*, as well as the surface charge remodeling operon *dltABCD* involved in D-alanylation of teichoic acid chains inside the cell wall. This system also regulates expression of enzymes responsible for biosynthesis of Lys (a substrate of LysPGS), and VraFG, a putative ATP dependent CAMP efflux transporter.[102-105] The cell surface sensor GraS binds to CAMPs using an extracellular loop at the surface of the membrane and activates the transcription factor GraR. The extracellular loop of GraS contains several anionic residues that interact with various CAMPs including polymyxin B, nisin, and histatin. A recent study shed light on the molecular mechanism used by GraS to sense certain CAMPs with more sensitivity than others.[106] Interestingly, the GraSR locus encodes a third subunit (GraX) that is predicted to form a part of the sensing mechanism.[102-104] Together with previous observations showing that expression of *mprF* and *dltABCD* is dependent on a functional VraFG, new studies have shown that GraX is the central component of a "5-component system" for sensing of CAMPs (Fig. 7,[107]). The GraSR sensing system requires both GraX and the VraGF transporter to function. In this complex signal transduction pathway VraGF does not play a role in detoxification of the cell as previously thought, but instead completes the GraSR sensing mechanism via its interaction with GraX to efficiently induce expression of *dltABCD* and *mprF*.[105]

Conclusion and outlook

Developments in recent years have shown that the tRNA-dependent system for lipid modification is more complex than originally hypothesized. Several novel aa-tRNA and lipid substrates were recently discovered, and new enzymes have been added to the repertoire of aaPGS homologs. Several hydrolases were found that are involved in maintaining the homeostasis of aminoacylated lipids. Lipid aminoacylation affects the biochemistry of the bacterial cell membrane and is involved in cellular adaptation to stress and other changes in the environment. In spite of recent progress, many open questions remain unanswered regarding the mechanism of aa-PG synthesis, flipping across the membrane, and the physiologic relevance of these modifications in bacteria. It is possible that, investigated through the prism of biomedical relevance, the fundamental physiologic role of lipid modifications has partially escaped previous investigations.

Another open question pertains to the relevance of aaPGS as a valid drug target. Sources in the literature state that lipid aminoacylation might represent an appealing drug target for which inhibitors would make pathogenic species more susceptible to existing drugs, or to natural defenses of the immune system of an infected host. Although several studies cited in this review have shown that aa-PG synthesis significantly enhances antimicrobial resistance and virulence in some pathogens, these effects are modest in the context of other bacterial species. Moreover, aa-PGs are not essential for bacterial growth, and one could argue that because the effects of aminoacylated lipids on antimicrobial resistance are weak, these modification systems might not represent practical targets for anti-infective strategies. With the rise of bacteria that are increasingly resistant to current drugs, new antimicrobials and/or drug combinations must be identified to find effective treatments of managing infections.[108,109] In the last decade, many antibiotics that target bacterial systems that enhance antimicrobial resistance have been developed to potentiate the effect of existing antibiotics (e.g., [110,111]). For instance, multidrug efflux pumps, which have been shown to increase antibiotic resistance, have become a popular target. Indeed, recent studies suggest that multidrug transporters are a major determinant for the efficacy of both new and old antibiotics.[112] Efforts have also begun toward discovery of inhibitors of the lipid aminoacylation pathway. A high throughput assay was recently established to screen libraries of potential inhibitors of the aaPGS active site.[113] Also, a targeted antisense RNA strategy developed by Cubist Pharmaceuticals demonstrated that modulation of *mprF* expression in *S. aureus* can increase susceptibility to daptomycin.[114] More work is needed to identify lead compounds that might serve as inhibitors of lipid aminoacylation, and to determine whether inhibition of this pathway increases the efficacy of new and existing therapeutic strategies.

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