



Mechanism of Action and Resistance to Daptomycin in *Staphylococcus aureus* and Enterococci

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Lipopeptides are natural product antibiotics that consist of a peptide core with a lipid tail with a diverse array of target organisms and mechanisms of action. Daptomycin (DAP) is an example of these compounds with specific activity against Gram-positive organisms. DAP has become increasingly important to combat infections caused by Gram-positive bacteria because of the presence of multidrug resistance in these organisms, particularly in methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). However, emergence of resistance to DAP during therapy is a well-described phenomenon that threatens the clinical use of this antibiotic, limiting further the therapeutic options against both MRSA and VRE. This work will review the historical aspects of the development of DAP, as well as the current knowledge on its mechanism of action and pathways to resistance in a clinically relevant context.

Lipopeptides refer to a diverse class of compounds that share the general structure of a peptide core attached to a lipid tail, and are produced by a variety of environmental microorganisms including soil bacteria and fungi. This class of compounds possesses a wide therapeutic potential as evidenced by drugs that are currently in clinical use as antimicrobials, including the polymixins (polymixin B and colis-

tin), echinocandins (casposungin, micafungin, and anidulafungin), and daptomycin. The first isolation of a lipopeptide antibiotic occurred in 1953 with the discovery of amphomycin (Heinemann et al. 1953). However, this compound (and related molecules) was not further developed as a result, in part, of complex chemical structure and, in some cases, concerns of toxicity. Of note, there has recently been a resurgence of

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interest in compounds similar to amphomycin, driven by increasing rates of antimicrobial resistance to more traditional therapeutic agents.

Daptomycin (DAP), a lipopeptide antibiotic with in vitro bactericidal activity against Gram-positive bacteria, received approval by the Food and Drug Administration (FDA) in 2003 for soft-tissue infections and in 2006 for *Staphylococcus aureus* bacteremia and right-sided endocarditis. DAP has become a front-line agent in the treatment of challenging infections caused by both methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE) (Munita et al. 2015). Despite its increasing role in the treatment of serious infections by these organisms, details of the precise mechanism of action and the mechanisms by which bacteria develop resistance are incompletely understood. Here, we will provide a brief overview of the structure and synthesis of DAP, explore what is known about its mechanism of action, and discuss the genetic changes associated with DAP nonsusceptibility (hereafter referred to as daptomycin resistance [DAP-R]) in *S. aureus* and the enterococci.

HISTORY, STRUCTURE, AND SYNTHESIS OF DAPTOMYCIN

After the discovery of amphomycin, a variety of lipopeptides with antimicrobial properties were identified over the next decade, including crystallomycin (Lomakina and Brazhnikova 1959), aspertocin (Shay et al. 1960), glumamycin (Shibata et al. 1962), laspertomycin, and tsushimycin (Naganawa et al. 1968; Shoji et al. 1968). Further development of these compounds for study and use was limited by several factors, including (1) the heterogeneous mixture of related molecules isolated from the fermentation of source organisms, (2) the complex chemistry needed to manipulate isolated compounds, and (3) a lack of understanding of the genetics behind their production. By the late 1980s, several important breakthroughs would allow DAP to make the journey from drug discovery to the bedside.

DAP began its journey as a molecule identified as A21987C, a group of lipopeptides pro-

duced by an isolate of *Streptomyces roseosporus* collected from the soil of the slopes of Mount Ararat in Turkey (Eisenstein et al. 2010). It consists of a 13-amino-acid depsipeptide, which harbors a cyclic decapeptide core with three extra-cyclic amino acids attached to an amino-terminal fatty acid tail (Fig. 1A). A distinctive feature of the lipopeptides is the diverse nature of the peptide core. In the case of DAP, the core contains a variety of noncanonical amino acids (kynurenine, ornithine, and 3-methylglutamic acid) and L-enantiomers (D-alanine and D-serine) (Debono et al. 1987). Several of these residues, in particular, kynurenine (of which the carboxyl group is the site of cyclization) and 3-methylglutamic acid have been shown, via substitution, to be important in the antimicrobial activity of the molecule with altered peptides displaying an increase of up to five times the minimum inhibitory concentration (MIC) (Grünwald et al. 2004). Further, six acidic residues in the DAP peptide ring are conserved across other calcium-dependent antimicrobial lipopeptides, highlighting the importance of this inorganic ion in both the mechanism of action and resistance (see below) (Hojati et al. 2002). The fatty acid tail also plays an important role in the activity of the compound, particularly in regard to toxicity. A21987C was found by high-performance liquid chromatography (HPLC) to be a mix of three main constituents differing only in the lipid moiety (with chain lengths of 11, 12, and 13 carbons) at the amino terminus (Debono et al. 1988). It was noted that longer chain lengths correlated with increasing toxicity; however, batch fermentation and subsequent separation was a laborious and difficult task with inefficient yield. The discovery that a penicillin deacylase produced by *Actinoplanes utahensis* (Debono et al. 1988) could remove the lipid tail opened the door for further characterization of the molecule. Using this technique, a semisynthetic derivative of A21987C with an *n*-decanoyl tail, named daptomycin, was found to balance antimicrobial activity with toxicity in a mouse model. Large-scale biosynthesis of DAP was achieved by feeding a controlled amount of decanoic acid to cultures of *S. roseosporus* (Huber et al. 1988).

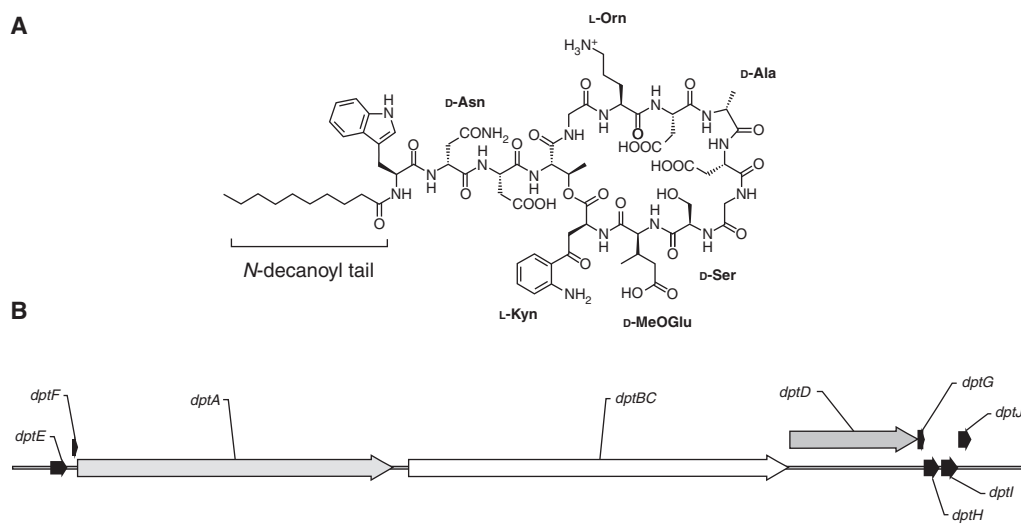


Figure 1. Structure of daptomycin and organization of the daptomycin biosynthesis gene cluster in *Streptomyces filamentosus*. (A) Chemical structure of daptomycin (DAP) with noncanonical amino acids and *N*-decanoyl fatty acid tail labeled. L-Kyn, L-Kynurenine; L-Orn, L-Ornithine; D-MeOGlu, D-3-methylglutamic acid. (B) Organization of the DAP biosynthesis gene cluster (see text for details). (Sequence information from NCBI database, accession number AY787762.1.)

Lipopeptides, similar to many other natural product antimicrobials, are produced by non-ribosomal peptide synthetases (NRPS). These large enzymatic complexes work in an assembly-line fashion to generate a specific peptide sequence. At their core is a series of three enzymatic activities (condensation, adenylation, and thiolation [CAT]) that perform a function analogous to ribosomal polypeptide synthesis, with amino acid specificity determined by the binding characteristics of each adenylation domain rather than an mRNA codon (Marahiel et al. 1997; Fischbach and Walsh 2006). The adenylation domain uses energy from adenosine triphosphate (ATP) to form an aminoacyl-adenosine monophosphate (AMP) intermediary from its cognate amino acid. Next, the AMP is displaced by the formation of a thioester bond coupling the amino acid to the thiolation domain carrier protein. The condensation domain then catalyzes the addition of the growing peptide chain to the amino acid monomer via an amide linkage, resulting in the passage of the nascent chain from one thiolation domain to the next module in the complex, wherein the process is repeated. Additional enzymes aug-

ment this core synthesis machinery, allowing modifications such as the incorporation of D-amino acids and allowing for the cyclic structure of DAP. In *S. roseosporus*, this machinery is organized into three multimodular subunits, DptA, DptBC, and DptD (Fig. 1B), which are responsible for the synthesis, modification, and cyclization of the 13 amino acid core (Baltz 2009). Two genes, *dptE* and *dptF*, located directly upstream of the primary peptide synthesis cluster, show similarity to acyl-CoA ligase and acyl carrier proteins, and are thus predicted to be involved in the addition of fatty acids to the amino-terminal end of DAP (Mchenney et al. 1998; Miao et al. 2005). Downstream are four accessory genes, one of which encodes a protein that shares identity with those known to metabolize tryptophan (a needed step for the synthesis of kynurenine). Another of the accessory genes is predicted to encode a glutamate methyltransferase presumably involved in the production of 3-methylglutarate (Miao et al. 2005).

The understanding of the genetic organization of the DAP NRPS machinery has opened the pathway to further drug modification and discovery. Although the *dpt* locus is transcribed

as a single long mRNA, splitting the DptA, DptBC, and DptD submodules by deletion and subsequent reintroduction into different chromosomal locations (under control of a constitutive *erm* promoter) was not shown to adversely affect the production of DAP (Coëffet-Le Gal et al. 2006). Further, substitution of various CAT domains between lipopeptide synthesis clusters of different *Streptomyces* species has allowed the creation of altered peptide cores to screen for desired characteristics, such as increased activity in the presence of surfactant (Nguyen et al. 2006, 2010). Continued experimentation with novel arrangements of NRPS modules may offer further insights into DAP and may lead to discovery of novel compounds with improved activities.

MECHANISM OF ACTION

DAP shares structural similarities with a group of molecules produced by the mammalian innate immune system known as cationic antimicrobial peptides (CAMPs), specifically the human cathelicidin LL-37. These effectors of the innate immune response possess a wide spectrum of activity against bacteria, fungi, and some encapsulated viruses, and are thought to exert their effect by binding to and disrupting membrane integrity (Bals and Wilson 2003). The structural similarities between DAP and CAMPs have led investigators to postulate that they may share a common mechanism of membrane disruption, as DAP is known to bind the Gram-positive bacterial membrane and initiate a series of events that lead to cell death (Straus and Hancock 2006). Although the precise mechanism of action remains to be fully elucidated, there are at least two important interactions required for DAP to exert its bactericidal effect.

First is the interaction between DAP and calcium. Nuclear magnetic resonance (NMR) data of DAP in solution suggests that DAP complexes with calcium in a 1:1 molar ratio to form small (14–16 molecules) DAP micelles that may aid in antimicrobial delivery to the bacterial membrane (Scott et al. 2007). Changes in the NMR signal of the tryptophan at position 1 and the kynurenine at position 13 on the addition of

calcium were thought to indicate a possible role for these residues in calcium binding or oligomerization of the molecule (Ho et al. 2008). Other divalent cations, such as magnesium, can induce micelle formation at higher concentrations (2.5:1 ratio), but result in decreased antimicrobial activity as evidenced by an increase in MICs by 64-fold (Ho et al. 2008).

The second important interaction takes place between DAP and the anionic phospholipid phosphatidylglycerol (PG). Once in proximity to the bacterial membrane, DAP undergoes a structural transition to insert into the cell membrane (Jung et al. 2004). This process appears to be dependent on the presence of PG in the target membrane (Muraih et al. 2011) and is facilitated by calcium ions, which decrease the DAP concentration needed for membrane insertion by ~50-fold (Chen et al. 2014). Indeed, the presence of PG is an important mediator of DAP aggregation on model membranes. Using excimer fluorescence, excitation of DAP-peryrene conjugants was seen in PG-containing membranes, but was absent from those made exclusively of phosphatidylcholine (Muraih et al. 2012). Thus, the first key steps of DAP membrane insertion and oligomerization rely on both calcium and PG as crucial mediators.

The dependence on specific phospholipids for the mechanism of action of DAP may also explain the antimicrobial spectrum of this drug, as it has potent activity against Gram-positive organisms, but none against Gram-negative organisms. This effect seems to be independent of the permeability barrier of the outer membrane (OM) of Gram-negative bacteria, as *Escherichia coli* protoplasts in which the OM was removed showed a fourfold reduction in MICs to vancomycin (a large glycopeptide antibiotic that would otherwise be excluded from the periplasmic space), but no change in DAP MIC (Randall et al. 2013). This observation has led some to suggest that Gram-negative bacteria are devoid of phospholipids that may interact with DAP. Indeed, the membrane lipid composition of *E. coli* includes 80% of phosphatidylethanolamine (PE) and only 15% PG, as compared with *S. aureus*, which lacks PE and contains 58% PG and 42% cardiolipin (CL)

(Epand et al. 2007). Thus, the differences of phospholipid content may explain the lack of activity of DAP against Gram-negative bacteria.

The series of events that occur after DAP gains access to the membrane are less clear. Early investigations into the mechanism of action of DAP observed that cell-wall synthesis was inhibited by a decreased intracellular pool of UDP-*N*-acetylmuramyl-pentapeptide in *Bacillus megaterium* (Mengin-Lecreux et al. 1990), which the investigators attributed to inhibition of the enzymes involved in the formation of UDP-*N*-acetylglucosamine. This deficit was, however, subsequently found to be caused by impaired active transport of the amino acids required for mu-rein synthesis, an effect associated with dissipation of the membrane electrochemical gradient (Allen et al. 1991). Analysis of major metabolic pathways and macromolecules showed DAP had little effect on the synthesis of DNA, RNA, or proteins. In contrast, cell envelope metabolism was consistently altered, with radiolabeled acetate incorporation into lipids decreased by 50% and lipoteichoic acid synthesis reduced by 93%. Moreover, both enterococci and *Bacillus* species displayed important morphologic changes (elongation) with a relative increase in sidewall synthesis (Canepari et al. 1990) on exposure to DAP. The finding that serial washes

with ethylenediaminetetraacetic acid (EDTA) was unable to remove DAP from bacterial membranes (Canepari et al. 1990) suggested that the cell membrane was the site of action, an observation that fit well with the amphipathic nature of the DAP molecule. Further, DAP is able to exert its bactericidal action against *S. aureus* in stationary phase, under conditions in which active metabolism is quenched and without requiring lysis of the target cell (Mascio et al. 2007; Cotroneo et al. 2008), consistent with disruption of the bacterial cell membrane, rather than inhibition of cell-wall or teichoic acid synthesis.

There are currently two proposed mechanisms of oligomeric DAP action (Fig. 2). One hypothesis, originating from the observed correlation of membrane depolarization and cell death in *S. aureus*, proposes that aggregates of DAP form an oligomeric pore like structure in the membrane, which results in ion leakage and subsequent dissipation of the membrane potential (Silverman et al. 2003). Experimental support for this hypothesis is derived from several studies. Initial stoichiometric calculations using Forester resonance energy transfer showed that ~7–8 DAP subunits associate in a PG-dependent manner for each oligomeric complex (Muraih and Palmer 2012; Zhang

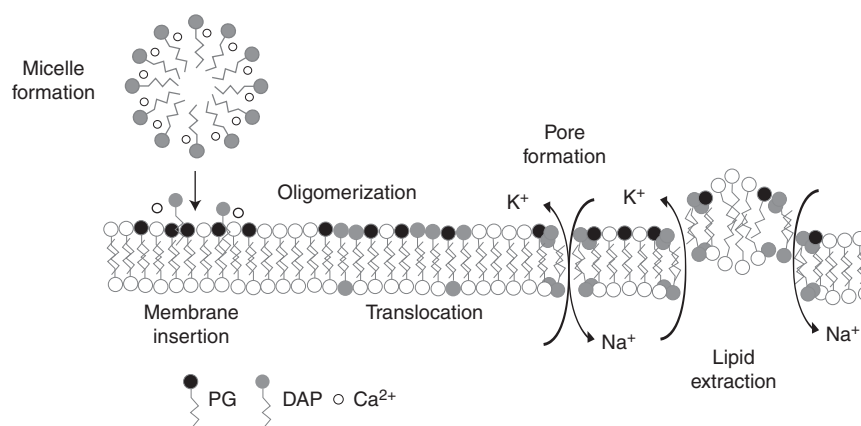


Figure 2. Proposed mechanisms for the action of daptomycin. In solution, daptomycin (DAP) complexes with calcium to form small micelles, and subsequent membrane insertion is dependent on both the presence of calcium and phosphatidylglycerol (PG). Once inserted, DAP oligomerizes and transitions to the inner membrane leaflet. These complexes then align on opposite sides of the membrane to form a pore channel permeable to small cations, or disrupt membrane integrity by extracting lipids and leading to transient ion leakage.

et al. 2014a). Further, the introduction of DAP into the outer leaflet induces a local membrane stress that increases levels of lipid flip-flop, an exchange of lipids between the inner and outer membrane leaflets (Jung et al. 2004), including the transition of DAP from the outer to inner leaflet. In the presence of PG, DAP associates into two oligomers of four units each opposite each other on the membrane, bending the membrane and establishing a pore like structure (Zhang et al. 2014a). Using model liposomes, exposure to DAP was found to make the membrane permeable to small cations such as sodium and potassium, and less so to anions or larger organic acids, suggesting that an influx of sodium ions abolished the membrane potential and served as the effector of DAP action (Zhang et al. 2014b). Interestingly, the presence of another phospholipid (PL), CL, in liposomes containing PG served to inhibit the translocation of DAP from the outer leaflet to the inner one, resulting in tetrameric complexes on the outer surface only (Zhang et al. 2014a). As we will discuss below, alterations of enzymes involved in PL metabolism are a common feature of resistance to DAP in some bacteria, consistent with the important role of PL metabolism in its mechanism of action.

A second hypothesis centers on a newly described phenomenon termed the lipid extracting effect. Using giant unilamellar vesicles (GUV), Chen et al. (2014) observed that DAP insertion into the membrane results in an initial expansion of vesicle surface area. As DAP concentrations continue to increase, there is a rapid aggregation of lipid on the membrane surface, while at the same time the overall surface area of the vesicle decreases, implying that the lipid clusters are extracted and “released” from the vesicle membrane. Interestingly, this phenomenon is dependent on both calcium and PG, and displays a threshold concentration of DAP required to initiate the membrane changes, which the investigators postulate may correlate with MIC values in bacterial isolates. Further, the extraction of lipids results in the formation of transient water pores, which could theoretically explain the ion leakage observed experimentally (Gurtovenko and Vattulainen 2007). This effect

may also explain the observations of Pogliano et al. (2012) in *Bacillus subtilis* showing that DAP binding to the membrane near the cell septum induced a patchy aggregate of lipid, altering cell morphology to a bent “L” shape and mislocalizing the essential cell division protein DivIVA. Indeed, abnormal septation and thickened cell walls are common features of DAP-R bacteria, and may be because of recognition of altered lipid membranes as signals for new peptidoglycan synthesis away from the septum.

It is important to note that the two hypotheses are not mutually exclusive because both pore formation and lipid extraction may be playing a role once DAP makes contact with the bacterial membrane and could explain the broad effects of the antibiotic in bacterial permeabilization, cell division, and metabolism.

DAPTOMYCIN RESISTANCE

DAP-R in *S. aureus* and the enterococci has been well documented and it is a serious concern for the treatment of serious infections caused by these organisms (Bayer et al. 2013; Miller et al. 2014). Given the clinical burden of disease that these organisms represent, an understanding of the mechanisms by which they subvert the DAP “attack” is likely to provide novel insights into the manner that bacteria protect their cell membrane and adapt to the antimicrobial challenge. Detailed analyses of both DAP-R laboratory and clinical isolates have revealed several common pathways associated with resistance, namely, alteration of regulatory systems responsible for the bacterial cell envelope stress response, as well as enzymes involved in phospholipid metabolism and membrane homeostasis. Despite the genetic similarities, the mechanisms by which these changes drive DAP-R seem quite varied and are adapted to the biology of each organism, a fascinating feature of bacterial evolution. Thus, we will discuss each relevant species separately.

DAP-R IN *Staphylococcus aureus*

S. aureus use several strategies to circumvent the DAP effect, the most common appears to in-

volve the alteration of the cell-surface charge (Fig. 3A). Indeed, *S. aureus* seems to primarily respond to the DAP attack by producing a more positive overall cell-surface charge, presumably to prevent the positively charged DAP–calcium insertion by electrostatic repulsion. This phenotype is classically associated with mutations in *mprF* (multiple peptide resistance factor), which encodes a bifunctional enzyme that contains a carboxy-terminal cytoplasmic tail responsible for lysinylation of PG and an amino-terminal domain, which consists of eight transmembrane domains. The amino-terminal domain encodes a “flippase” activity, which is responsible for the translocation of lysyl-PG (LPG) from the inner to the outer membrane. A central domain of four transmembrane helices seems to assist with both lysinylation and flippase activities (Ernst et al. 2009).

In DAP-R *S. aureus*, a number of *mprF* mutations have been described that result in amino acid changes clustering in the central bifunctional region that overall confer a “gain-of-function” of the enzyme (Bayer et al. 2015). Thus, the net result is an increased synthesis and expression of positively charged LPG on the outer membrane. Strong evidence for the role of *mprF* in DAP-R are studies in which expression of *mprF* with DAP-R associated mutations (but not wild-type *mprF*) in *trans* could restore elevated DAP MICs to strains of *S. aureus* in which *mprF* had been deleted from the chromosome (Yang et al. 2013). Moreover, inhibition of MprF protein synthesis in DAP-R strains harboring gain-of-function mutations by antisense RNA (directed against *mprF* transcripts) was able to reverse DAP-R in vitro (Rubio et al. 2011).

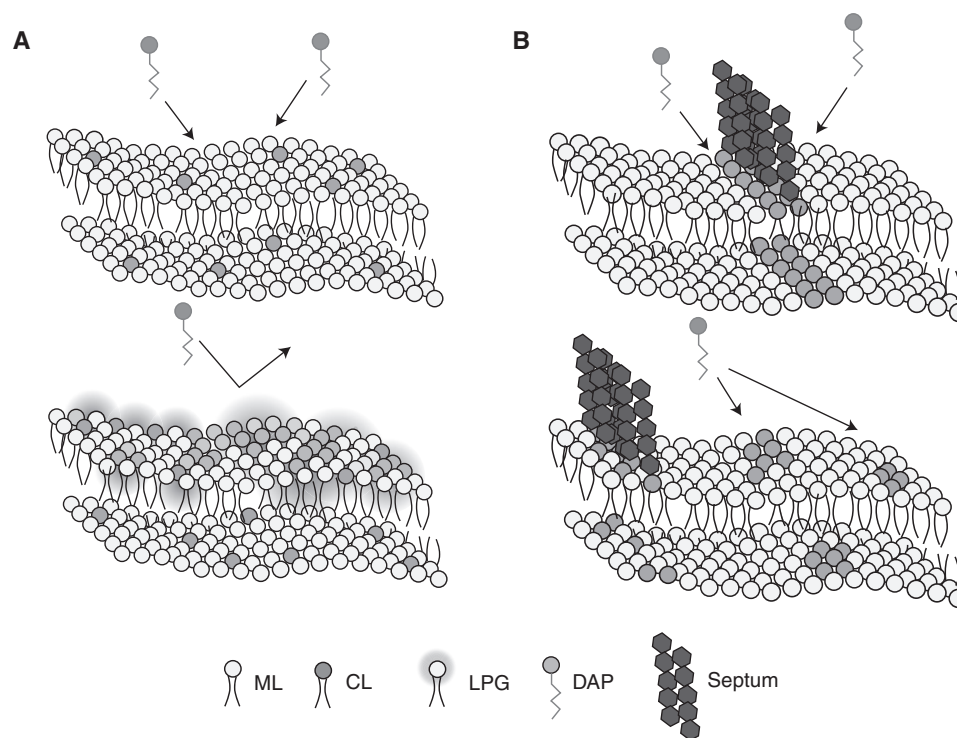


Figure 3. Strategies for resisting daptomycin membrane attack. (A) Repulsion: In *Staphylococcus aureus* and *Enterococcus faecium*, changes in cell-surface charge and membrane phospholipid content block daptomycin (DAP) membrane association and oligomerization. (B) Diversion: In *E. faecalis* sensitive to DAP cardiolipin (CL) clusters at the division septum. In resistant isolates, redistribution of CL microdomains “traps” DAP away from the septum. ML, membrane lipid; LPG, lysylphosphatidylglycerol.

An alternative pathway for DAP-R in *S. aureus* that results in an increase of cell-surface charge is the overexpression of the *dlt* operon (Yang et al. 2009; Cafiso et al. 2014). This operon produces the machinery responsible for attaching the positively charged amino acid alanine to cell-wall teichoic acid (WTA), leading to an increase in positive cell-surface charge in a manner similar to increased LPG synthesis. Up-regulation of WTA synthesis (as observed by increased transcription of *tagA*) and the *dlt* operon were also associated with increased cell-wall mass, another common phenotype observed in DAP-R staphylococci (Bertsche et al. 2011, 2013). However, despite the strong association between mutations in *mprF* and increased expression of *dlt* with increases in net positive cell-surface charge, these changes do not seem to correlate with changes in DAP MICs in all strains (Mishra et al. 2014). Indeed, an in vitro study generated DAP-R isolates with alterations in both *mprF* and *dlt*, but the net positive charge of the DAP-R mutants was less than the parent strain (Mishra et al. 2009). Thus, additional characteristics must also play a role in mediating DAP-R in staphylococci.

A second mechanism associated with DAP-R in staphylococci is the alteration of membrane phospholipid composition, which is postulated to either decrease the amount of PG available at the membrane interface or to change the fluidity of the membrane, thus interfering with DAP binding and subsequent oligomerization. Interestingly, by analyzing the action of a membrane active antimicrobial peptide on GUVs, it was found that increases in LPG were not associated with decreased peptide binding (as might be expected in a charge repulsion mechanism) but rather with inhibition of intravesicular dye leakage after binding takes place, consistent with a membrane integrity protective effect (Killee et al. 2010). Further, as discussed above, other phospholipid species, such as CL, may also play a protective role in preventing DAP translocation once inserted in the membrane (Zhang et al. 2014a). The enzyme responsible for cardiolipin synthesis, cardiolipin synthase, joins two molecules of PG to make CL (Short and White 1972). Thus, it is tempting to spec-

ulate that mutations producing changes in enzyme function may play a role in DAP-R by altering the ratio of PG to CL in the cell membrane. Indeed, genomic analysis of 33 DAP-R strains indicated that, among others, mutations in *pgsA* (which encodes an enzyme involved in PG synthesis) and *cls2* (cardiolipin synthase) were associated with DAP-R (Peleg et al. 2012). Additionally, membrane fluidity (which is highly dependent on PL and fatty acid composition) may also be an important factor that influences the DAP-R phenotype in certain strains. (Jones et al. 2008; Mishra et al. 2011). Interestingly, membranes of DAP-R clinical isolates are more fluid, whereas laboratory isolates tend to have more rigid membranes (Mishra et al. 2009), suggesting that DAP requires an optimal membrane order for insertion and oligomerization and perturbations of this order to either side may be protective. Along these lines, changes (both increase and decrease) in the production of staphyloxanthin, the carotenoid responsible for the golden color of *S. aureus*, was associated with DAP-R and was postulated to be a result of its influence on membrane fluidity (Mishra and Bayer 2013).

Global regulatory changes in genes modulating cell envelope stress and maintenance in *S. aureus* have also been associated with development of DAP-R (Utaiida et al. 2003; Rose et al. 2012). Interestingly, DAP challenge induces important changes in global gene expression. These genomic pathways are similar to those associated with resistance to other antibiotics such as vancomycin and seem to affect the expression of the cell-wall “stimulon.” Two important two-component regulatory systems (TCS) have been involved in DAP-R, namely, *VraSR* and *YycFG* (Muthaiyan et al. 2008; Mehta et al. 2012). Of note, DAP was also found to induce a group of genes that was previously associated with exposure to carbonyl cyanide *m*-chlorophenylhydrazone, a proton ionophore, reflecting its ability to disrupt the membrane and induce ion leakage (Muthaiyan et al. 2008).

In general, TCS consist of a membrane-bound sensor histidine kinase (HK) responsible for detecting a particular stimulus or cellular

perturbation, and a DNA-binding response regulatory (RR) that alters transcription of target genes (Dubrac et al. 2008). Mutations in these proteins can lead to altered expression of the system's regulon, profoundly affecting membrane homeostasis. The essential TCS YycFG (also known as WalkR) is involved in the control of peptidoglycan biosynthesis in *S. aureus*, mainly through the regulation of expression of two major autolysins, LytM and AltA (Dubrac and Msadek 2004). The genes encoding this system are clustered with two other genes, *yycHI*, that are "accessory" to the function of YycFG. Both YycH and YycI are amino-terminal transmembrane proteins with extracellular carboxy-terminal domains that in *B. subtilis* have been shown to repress the activity of the YycG HK (Szurmant et al. 2007). In nondividing cells, the entire YycFGHI complex remains in the peripheral cell wall, presumably in an inactive state. However, under growth conditions, YycG is recruited to the site of septal formation, whereas YycH and YycI remain in the periphery (Fukushima et al. 2011).

Using an inducible promoter to control YycFG expression, Dubrac et al. (2007) showed that low levels of YycFG expression were associated with decreased peptidoglycan turnover, increased cross-linking, and increased glycan chain length. Interestingly, low levels of YycFG were also associated with increased resistance to lysis by the detergent Triton X-100. By varying the temperature of model lipid membranes, it was shown that the activity of the HK YycG was impacted by membrane fluidity, with the system turned off under highly fluid conditions (Türck and Bierbaum 2012). The investigators suggested a mechanism by which YycG senses changes in membrane fluidity and responds by adjusting cell-wall cross-linking to compensate for stresses caused by osmotic pressure. Of note, in DAP-R isolates, several mutations in *yycFG* affecting multiple domains of both YycG HK and YycF RR (Friedman et al. 2006; Howden et al. 2011) have been described. Additionally, mutations in the accessory genes have also been noted. For example, a mutation resulting in a frameshift and truncation of ~10% of the accessory protein YycH (which in *B. subtilis* is associated

with regulating YycF signaling) was associated with DAP-R (Szurmant et al. 2005; Mwangi et al. 2007). Given that the DAP-R phenotype displays some similarities to the YycFG-deficient phenotype (e.g., thickened cell walls, increased membrane fluidity, and resistance to membrane disruption), it is tempting to speculate that the observed changes in YycFG impair the functioning of the operon, down-regulating cell-wall homeostasis to survive the DAP-mediated attack.

The *VraSR* TCS is orthologous to the *LiaSR* system of *B. subtilis* and enterococci (discussed below) and is conserved across the low G+C bacteria (Jordan et al. 2006). It is up-regulated by both vancomycin and DAP exposure, and is associated with cell-wall biosynthesis via transcription of *pbp2* (penicillin binding protein 2), *tagA* (WTA synthesis), *prsA* (a chaperone), and *murZ* (UDP-*N*-acetylglucosamine enolpyruvyl transferase), among others (Kuroda et al. 2003; Mwangi et al. 2007; Camargo et al. 2008). Structural studies have shown that on activation by phosphorylation, the *VraR* RR undergoes a conformational change allowing for dimerization and a subsequent increase in its binding affinity for target DNA (Leonard et al. 2013). Experimental evidence supports a role for this system in DAP-R, as deletion of the *vraSR* operon from a DAP-R strain of *S. aureus* resulted in a DAP-sensitive (DAP-S) phenotype, which could be reversed by supplying the genes in *trans* (Mehta et al. 2012). Additional mutations associated with the DAP-R phenotype include genes encoding the RNA polymerase subunits *rpoB* and *rpoC* (Friedman et al. 2006; Peleg et al. 2012). A mutation in *rpoB*, resulting in the amino acid change A621E, was associated with increased expression of the *dlt* operon and correlated with an increase in positive cell-surface charge, whereas *RpoB* mutations A621E and A477D were both linked to activation of cell-wall biosynthesis and increased cell-wall thickness (Cui et al. 2010; Bæk et al. 2015).

The pathway to DAP-R also results in significant cellular metabolic shifts. Analysis of six strain pairs of *S. aureus* under normal growth conditions and DAP exposure revealed that there is a decrease in activity of the tricarboxylic

acid (TCA) cycle and, instead, carbon sources are redirected into the pentose phosphate pathway (Gaupp et al. 2015). This is corroborated by prior work that had shown levels of succinate dehydrogenase, an enzyme involved in the TCA cycle, were lower in a DAP-R strain when compared with its DAP-S counterpart (Fischer et al. 2011). Additionally, mutations noted upstream of acetyl-CoA synthetase in DAP-R isolates (Friedman et al. 2006) may affect the production of acetyl-CoA, which is involved in lipid synthesis and may also feed into the TCA cycle. Redirection of the flow of metabolites results in the formation of larger pools of amino sugar precursors, which can be used for peptidoglycan, teichoic acid, and nucleotide synthesis (Gaupp et al. 2015). Thus, a metabolic shift primes DAP-R isolates to build larger stores of cell envelope precursors allowing them to weather the storm of DAP-induced membrane stress.

DAP-R IN ENTEROCOCCI

The introduction of DAP provided clinicians with an agent that possessed in vitro bactericidal activity against enterococci, and it quickly became a front-line antibiotic for recalcitrant VRE infections, despite the lack of FDA approval for this indication. Even in early development of DAP, it was noted that longer acyl chain lengths (13–14 carbons) tended to improve activity against enterococci, but with the trade-off of increased toxicity (Debono et al. 1988). Thus, DAP (with its *n*-decanoyl fatty acyl side chain) is less potent against enterococci, a fact that is reflected in the clinical breakpoints, which are fourfold higher for enterococci compared with *S. aureus* (4 µg/mL vs. 1 µg/mL). Similar to what has been discussed in staphylococci, development of DAP-R in enterococci seems to affect two important groups of genes, namely, those controlling the cell membrane stress response and phospholipid metabolism. Despite the genetic similarities, the two clinically relevant species, *Enterococcus faecalis* and *E. faecium*, seem to display distinctive phenotypic differences in their response to DAP challenge and, thus, we will discuss them separately.

Daptomycin Resistance in *E. Faecalis*

The genetic bases of DAP-R in *E. faecalis* were mapped using whole-genome sequencing of both in vitro and clinical isolates that had developed resistance in the presence of the drug (Arias et al. 2011; Palmer et al. 2011). Using a strain pair from a patient with *E. faecalis* bacteremia who failed DAP therapy, Arias et al. (2011) mapped the genetic changes to genes encoding the LiaFSR system (a conserved TCS associated with DAP-R in *B. subtilis*) and two enzymes involved in phospholipid metabolism, cardiolipin synthase (Cls), and a glycerophosphoryl diester phosphodiesterase (GdpD). Phenotypic changes associated with the DAP-R phenotype included increased thickness of the cell-wall and abnormal septations. Additionally, the DAP-R derivative was found to have a decrease in the proportion of PG and increased rigidity of the cell membrane (Mishra et al. 2012). However, in contrast to both *S. aureus* and *E. faecium*, a distinct characteristic of DAP-R *E. faecalis* is a rearrangement of cell membrane PL microdomains. Indeed, DAP-S *E. faecalis* shows prominent concentration of anionic PLs (including CL) at the division septum and in polar areas. Development of DAP resistance markedly changes the architecture of these PL microdomains, moving them away from the division septum, the principle site of DAP action (Tran et al. 2014). This reorganization in *E. faecalis* seems to be crucial for full expression of the DAP-R phenotype. It is postulated that these PL aggregates may serve as “sink holes” for DAP, diverting the antibiotic away from the vital septal area of the membrane (the diversion hypothesis) (Fig. 3B). Indeed, compelling experimental data suggest that DAP-R *E. faecalis* strains do not “repel” DAP from the cell surface as shown previously by *S. aureus* (Tran et al. 2014).

Detailed studies on the molecular basis of the DAP-R phenotype in *E. faecalis* has identified the LiaFSR system as a major contributor to the adaptive response against DAP and antimicrobial peptide “attack.” This system is conserved across the Firmicutes (VraSR is its ortholog in *S. aureus*, see above) and has been

well-characterized in the model organism *B. subtilis* (Jordan et al. 2006; Schrecke et al. 2013). The HK LiaS responds to as-yet-unidentified membrane stressors induced by DAP or other membrane active agents. LiaS phosphorylates its cognate RR LiaR, which contains a DNA-binding motif and alters expression of target genes. LiaF serves a regulatory role by inhibiting the activation of LiaR through interactions with LiaS in the absence of membrane stress. The *liaFSR* operon in *E. faecalis* consists of only three open reading frames. However, in *B. subtilis*, an additional three genes, *liaG*, *liaH*, and *liaI*, are targets of LiaR (Wolf et al. 2010) and mediate resistance to antimicrobial peptides via a response that appears to be similar to that described for the phage shock protein (PSP) response of Gram-negative organisms (Brissette et al. 1990; Yamaguchi et al. 2013).

Several lines of experimental evidence point to an activation of the LiaFSR system and its downstream effectors as mediators of DAP-R in *E. faecalis*. Mutations in the predicted inhibitor LiaF have been associated with increases in DAP MIC, presumably caused by increased activity of the system. A deletion of isoleucine at position 177 of LiaF, (identified in a clinical isolate of *E. faecalis*) was sufficient to increase the DAP MIC of a susceptible isolate from 1 to 4 $\mu\text{g}/\text{mL}$ and resulted in redistribution of membrane phospholipid microdomains (Tran et al. 2014). Further, this same change was noted to abolish the bactericidal action of DAP in vitro (loss of a three \log_{10} decrease in time–kill curve colony counts), despite the MIC being within the “susceptible” range (Munita et al. 2013). In an experimental evolution of a polymorphic population of *E. faecalis* maintained in continuous culture, changes in the LiaFSR system emerged as the first step in the pathway to DAP resistance (Miller et al. 2013). The most frequently observed mutations involved either insertion or deletion of the isoleucine at position 177 in LiaF (suggesting the importance of this residue for the inhibitory function of LiaF) and appeared after ~ 2 weeks as MICs rose into the 3–4 $\mu\text{g}/\text{mL}$ range.

Because of the major role of LiaFSR in DAP and antimicrobial resistance, Davlieva et al.

(2015) sought to investigate the structural bases of DAP-R associated with mutations in LiaR (which have been commonly identified in clinical isolates of DAP-R enterococci). These studies showed that a substitution of asparagine for aspartate at position 191 of LiaR mimics phosphorylation and changes the oligomeric state of LiaR. Indeed, “wild-type” unphosphorylated LiaR seems to exist as a dimer. When the protein is phosphorylated or harbors mutations that mimic phosphorylation LiaR tetramerizes, increasing the binding affinity for its own and other promoters by 100-fold (Davlieva et al. 2015), resulting in constitutive activation of the LiaFSR system. Furthermore, deletion of the *liaR* gene results in a “hypersusceptible” phenotype (MICs of 0.047 $\mu\text{g}/\text{mL}$) that is independent of the genetic background into which it is introduced (Reyes et al. 2015). Thus, LiaFSR seems crucial in orchestrating the specific response to a variety of membrane active agents and overexpression of this system results in a membrane protective effect that results in DAP-R.

Once established, LiaFSR mutations allow the accrual of additional genetic changes resulting in the full resistance phenotype (Miller et al. 2013). Mutations in genes affecting membrane phospholipids, particularly *cls*, have been frequently associated with DAP-R. In *E. faecalis*, introduction of the altered *cls* alleles in *trans* bearing the R218Q substitution or the N77-Q79 deletion were able to confer resistance to the laboratory strain OG1RF (Palmer et al. 2011). Mutations in GdpD had no effect on DAP MICs in isolation, but when introduced along with LiaF mutations, they resulted in a fully resistant phenotype (Arias et al. 2011). Genes in the LiaR regulon bear similarities to the Psp system mediated by *liaI* and *liaH* in *B. subtilis*, although these genes (named *liaXYZ*) seem to be organized into an independent operon in the *E. faecalis* genome distant from *liaFSR* (Miller et al. 2013). Interestingly, point mutations in this group of three genes, specifically a frameshift disrupting the carboxy-terminal end of LiaX and a second frameshift mutation in LiaY have been associated with DAP-R in enterococci both in vitro and in clinical isolates (Palmer et al. 2011; Humphries et al.



2012). Additional mutations in *yybT*, a cyclic dinucleotide phosphodiesterase predicted to be involved in cell stress and signaling, and *gshF*, a glutathione synthase, have been described, although their contributions to DAP-R are not well understood (Miller et al. 2013).

Daptomycin Resistance in *E. Faecium*

Although a number of genetic determinants of DAP-R in *E. faecium* have been identified, the biochemical bases for their effect on the DAP resistance phenotype are not well understood. Unlike *E. faecalis*, *E. faecium* does not display a visible alteration or rearrangement of anionic phospholipids in the membrane, even in isolates with mutations in the LiaFSR system (Tran et al. 2015). Instead, it appears that the impact of mutations in *E. faecium* results in phenotypic changes that are more akin to those associated with DAP-R in *S. aureus* (Mishra et al. 2012). Indeed, the overall mechanism for DAP-R in *E. faecium* appear to involve repulsion of the antibiotic from the cell surface.

Analysis of the genomes of 19 clinical isolates of *E. faecium* with DAP MICs ranging from 3 to 48 $\mu\text{g}/\text{mL}$ revealed that the majority of the strains harbored mutations in either LiaFSR or YycFG and that either pathway can lead to DAP-R (Diaz et al. 2014). In the LiaFSR system, the most common mutation was a W73C change in LiaR accompanied by a T120A substitution in LiaS, suggesting that these changes coevolve during the development of DAP-R (Munita et al. 2012). Four strains also harbored various mutations in LiaE, although these changes did not affect the isoleucine at position 177 as described in *E. faecalis*. The importance of LiaFSR changes in *E. faecium* was shown by deletion of the *liaR* gene from clinical strains harboring mutations in both the LiaFSR and YycFG pathway (Panesso et al. 2015). In both cases, strains developed a “hypersusceptible” phenotype with increased binding of fluorescently labeled DAP to the cell membrane in the absence of *liaR*. The presence of LiaRS substitution has also been associated with clinical failure of DAP and loss of bactericidal activity of the antibiotic (Munita et al. 2014). Changes in the YycFG pathway are

commonly localized to the YycG HK as well as both accessory proteins YycH and YycI (Diaz et al. 2014); however, the role of such mutations in the development of DAP-R remains to be established.

As in both *S. aureus* and *E. faecalis*, mutations in *cls*, the gene encoding cardiolipin synthase, are common in DAP-R *E. faecium*. They are often found with substitutions in LiaFSR or YycFG and, in this setting, they may contribute to the progression of an isolate from DAP-tolerant to DAP-resistant (Diaz et al. 2014). Exchange of the R218Q *cls* allele from a DAP-R strain into a susceptible one was not able to increase the MIC, further suggesting that this change alone is not sufficient for the development of a DAP-R phenotype in *E. faecium* (Tran et al. 2013). Biochemical characterization of Cls proteins from a susceptible and resistant strain pair of *E. faecium* showed that the R218Q and H215R substitutions mapped to the PLD1 phospholipase catalytic domain resulted in an increase in the V_{max} of the enzyme (Davlieva et al. 2013). This is consistent with an enzymatic gain-of-function and may allow for a more rapid depletion of the available PG by shunting this PL to the CL pool during times of membrane stress. Mutations in a *pspC*-like protein (the above mentioned LiaY), *cfa* (a cyclooxygenase that catalyzes the addition of a methyl group to unsaturated fatty acids), *dlt*, and *mprE*, among others, have been associated with DAP-R *E. faecium*. However, they appear to be rare in clinical isolates and their role in resistance is currently difficult to assess (Humphries et al. 2012; Tran et al. 2013; Diaz et al. 2014).

CONCLUDING REMARKS

Over the last decade, the increase of multidrug-resistant Gram-positive organisms has brought DAP into the spotlight as a therapeutic option for severe infections. DAP has potent bactericidal activity and a unique mechanism of action, which have made it a useful addition to the clinician’s antibiotic repertoire. As its clinical use continues to increase reports of resistance are becoming more common. To preserve the use of this and other antimicrobial compounds,

a deeper understanding of the robust and redundant pathways that mediate the mechanism of resistance may shed light on the biology of bacterial membrane adaptation, including the response to the innate immune system. With continued efforts to unravel the complex networks that mediate DAP-R, additional insights into the coordination of cell envelope synthesis machinery are sure to provide new therapeutic targets to exploit against recalcitrant Gram-positive infections in the future.

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