

## Mechanisms of drug resistance: daptomycin resistance

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Daptomycin (DAP) is a cyclic lipopeptide with *in vitro* activity against a variety of Gram-positive pathogens, including multidrug-resistant organisms. Since its introduction into clinical practice in 2003, DAP has become an important key frontline antibiotic for severe or deep-seated infections caused by Gram-positive organisms. Unfortunately, DAP resistance (DAP-R) has been extensively documented in clinically important organisms such as *Staphylococcus aureus*, *Enterococcus* spp., and *Streptococcus* spp. Studies on the mechanisms of DAP-R in *Bacillus subtilis* and other Gram-positive bacteria indicate that the genetic pathways of DAP-R are diverse and complex. However, a common phenomenon emerging from these mechanistic studies is that DAP-R is associated with important adaptive changes in cell wall and cell membrane homeostasis with critical changes in cell physiology. Findings related to these adaptive changes have provided novel insights into the genetics and molecular mechanisms of bacterial cell envelope stress response and the manner in which Gram-positive bacteria cope with the antimicrobial peptide attack and protect vital structures of the cell envelope, such as the cell membrane. In this review, we will examine the most recent findings related to the molecular mechanisms of resistance to DAP in relevant Gram-positive pathogens and discuss the clinical implications for therapy against these important bacteria.

**Keywords:** daptomycin resistance; *Bacillus subtilis*; *Staphylococcus aureus*; *Enterococcus*

### Introduction

Antimicrobial resistance is increasingly recognized as a major public health problem that threatens the medical care of patients worldwide. Infections caused by multidrug-resistant (MDR) organisms result in significant increases in mortality and have been associated with a large economic burden. Indeed, a recent report estimated that antibiotic resistance would be responsible for around 300 million premature deaths by 2050, with a loss of up to \$100 trillion to the global economy.<sup>1</sup> Due to this worrisome scenario, a number of governmental agencies, academic societies, and international organizations have issued statements calling for action to tackle the antimicrobial resistance crisis.<sup>2,3</sup> To make things worse, this situation is aggravated by the lack of a robust antibiotic pipeline, resulting in emergence of infections that are almost untreatable

and leaving clinicians with no reliable alternatives to treat these patients.

Daptomycin (DAP), a lipopeptide antibiotic produced by *Streptomyces roseosporus*, has become an important option for the management of MDR infections due to Gram-positive organisms. DAP was initially examined for clinical use in the 1980s, but its development was halted owing to the high frequency of muscle-related toxicity in phase I and II trials.<sup>4</sup> However, subsequent animal studies indicated that muscle toxicity could be markedly reduced by using once-daily dosing (as compared to the original twice-daily dosing regimen). Thus, in 2003, DAP was granted U.S. Food and Drug Administration (FDA) approval for the treatment of complicated skin and soft tissue infections caused by Gram-positive organisms, including vancomycin-susceptible *E. faecalis*. In 2006, DAP

received approval for *Staphylococcus aureus* bacteremia and right-sided infective endocarditis. Similarly, DAP was approved by the European regulatory agency for the same clinical indications in 2006.

DAP is active against a wide-range of Gram-positive bacteria, including most clinically relevant MDR organisms, such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), vancomycin-intermediate *S. aureus* (VISA), and penicillin-resistant *Streptococcus pneumoniae*. The clinical susceptibility breakpoints established by the Clinical Laboratory Standards Institute (CLSI) are  $\leq 1$  mg/L for staphylococci and  $\leq 4$  mg/L for enterococci. No resistance breakpoint has been officially established; hence, isolates exhibiting minimum inhibitory concentration (MIC) values above the susceptibility cutoff are technically labeled as “non-susceptible.” For ease of use, in this manuscript we will refer to this population as DAP resistant. To date, the majority of Gram-positive organisms remain susceptible to DAP (DAP-S). However, the development of DAP resistance (DAP-R) emerging during therapy or as a *de novo* phenomenon has been described in several species.<sup>5</sup> Although the mechanisms of DAP-R remain to be fully elucidated, recent work from different research groups has provided important insights into the mechanistic bases of resistance. Indeed, such mechanisms appear to be complex, diverse, and mainly related to activation of the inherent bacterial self-defense processes in response to the antimicrobial attack. In this manuscript, we will summarize our current understanding of the molecular and biochemical basis of DAP-R. In order to organize the discussion, and considering that the resistance pathways vary among Gram-positive organisms, we will discuss the most relevant mechanisms of DAP-R in each species separately.

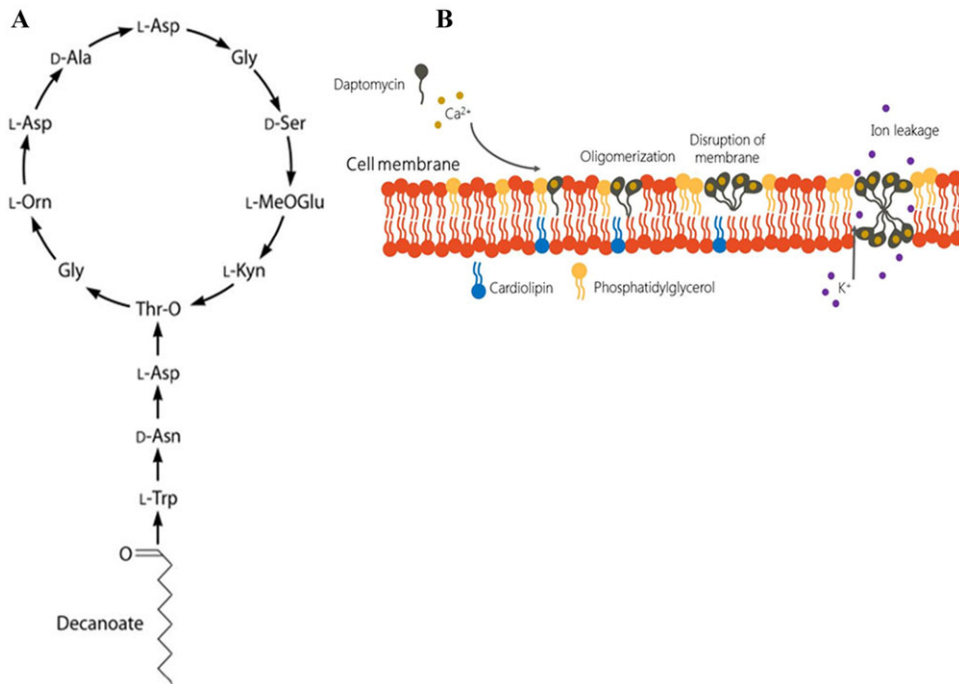
### Mechanism of action of daptomycin

DAP is structurally and functionally related to cationic antimicrobial peptides (CAMPs) produced by the innate immune system. The DAP molecule consists of a cyclic polypeptide core of 13 amino acids attached to a lipophilic tail (a decanoyl fatty acid) (Fig. 1).<sup>6</sup> DAP exerts its bactericidal effect by altering the bacterial cell envelope homeostasis, interacting with phospholipids of the cell membrane (CM) in a process that has not been fully elucidated. The bactericidal activity of DAP depends on

the presence of ionized calcium. Indeed, the positive charge of the DAP–calcium complex is thought to facilitate the insertion of the antibiotic into the bacterial CM. Furthermore, addition of calcium also appears to favor the formation of DAP micellar structures that have been postulated to serve as vehicles for the delivery of DAP to the bacterial CM.<sup>7,8</sup>

The mechanisms that lead to DAP-mediated bacterial cell death are also not fully established, but the antibiotic has been shown to rapidly depolarize cells of *Bacillus* spp. and to inhibit the active transport of amino acids.<sup>9</sup> In addition, it has also been proposed that DAP’s mode of action includes inhibition of peptidoglycan and/or lipoteichoic acid synthesis, through mechanisms that remain poorly understood.<sup>10,11</sup>

The specific interactions between the DAP–calcium complex and the CM are still a matter of active research, but several steps are thought to be important (Fig. 1). In the first step (after insertion into the CM), the DAP–calcium complexes oligomerize in the outer leaflet of the CM in a process that appears to be dependent on the presence of the phospholipid phosphatidylglycerol (PG). Indeed, experiments using liposomes with different composition of phospholipids have indicated that PG is crucial for DAP’s oligomerization into the CM,<sup>12,13</sup> which is necessary for its bactericidal activity. Furthermore, DAP-R has been consistently associated with a decrease in PG content or an increase in the conversion of PG to its positively charged derivative lysyl-PG (L-PG) in the CM of several Gram-positive organisms, such as *B. subtilis*, *S. aureus*, and *E. faecalis* (see below). The second step involves translocation of DAP oligomers into the inner leaflet of the CM in a model in which two opposing structures (located in the outer and inner CM leaflet, respectively) form a functional pore-like structure.<sup>14</sup> Indeed, using fluorescence resonance energy transfer (FRET), Muraih and Palmer<sup>15</sup> confirmed that DAP forms oligomers on PG-containing membranes and that the oligomers contain approximately six to seven subunits. Furthermore, characterization of DAP pores shows that they are cation and size selective, with the highest permeability to  $\text{Na}^+$ ,  $\text{K}^+$ , and alkali metal ions.<sup>14</sup> Interestingly, the translocation of DAP oligomers from outer to inner leaflets of the CM appears to be influenced by the presence of cardiolipin (CL), a negatively charged phospholipid that plays important roles in



**Figure 1.** (A) Structure of daptomycin and (B) proposed mechanism of action (B). See text for details.

CM homeostasis in bacteria. Indeed, enrichment of liposomes with 10% CL was sufficient to prevent translocation of DAP oligomers, disrupting the formation of pore-like structures.<sup>16</sup> These findings suggest that increased CL concentrations might prevent, at least in part, the antibacterial effect of DAP.

Chen *et al.*<sup>17</sup> recently postulated an alternative model for DAP's mechanism of action. Using microscopy imaging of giant unilamellar vesicles (GUVs) formed by different lipid compositions, the authors evaluated DAP's activity and provided evidence that suggested that interaction of DAP with the CM results in a marked alteration of phospholipid content, an effect that they designated "lipid extracting effect." The authors showed that, in the presence of low concentrations of DAP, an initial expansion of the GUV occurs due to binding of the antibiotic molecule to the surface. At higher DAP concentrations, this expansion is followed by a decrease in outer surface area of the GUVs, which is associated with lipid-peptide aggregates exuding from the surface of GUVs (i.e., lipids are removed from the lipid bilayer, a phenomenon designated the lipid-extracting effect). This phenomenon was only seen when binding of DAP increased the

membrane surface area to >3%. Importantly, the lipid-extracting effect was only observed when PG was present in the GUVs and when calcium was added to the solution.<sup>17</sup>

Although these data have increased our understanding of the molecular events that occur when DAP inserts into the CM, the specific mechanisms leading to bacterial cell death in DAP-treated organisms remain obscure. Using a *B. subtilis* model, Pogliano *et al.*<sup>18</sup> showed that interaction of DAP with the CM preferentially occurs at nascent septa and induces marked changes in cell shape. The exposure of *B. subtilis* to sublethal concentrations of DAP produces bent and patchy areas of the CM at sites of interactions of the DAP molecule with the CM target. These areas of CM "damage" appear to trigger mislocalization of cell division proteins (including the essential protein DivIVA of *B. subtilis*) and induce localized synthesis of peptidoglycan.<sup>18</sup> On the basis of these observations, a refined model of the DAP mechanism of action was proposed, in which DAP binds to the bacterial CM in the presence of PG, causing local alterations in membrane curvature. The changes in membrane structure are recognized by DivIVA, which incorrectly identifies this area as a potential site of division, triggering

**Table 1.** Proposed mechanisms and genes associated with daptomycin resistance in *B. subtilis*, *S. aureus*, and enterococci

Organism	Gene	Predicted function	Proposed mechanism of resistance	References
<i>B. subtilis</i>	<i>pgsA</i>	Phosphatidylglycerol synthase	Repulsion	24,34
	<i>liaFSR</i>	Three-component regulatory system as part of the cell envelope stress response		24,28,34
<i>S. aureus</i>	<i>dltABCD</i>	Proteins that are involved in D-alanylation of cell wall teichoic acids	Repulsion	63–68
	<i>mprF</i>	A bifunctional enzyme that catalyzes the lysinylation of phosphatidylglycerol and translocation of lysyl-phosphatidylglycerol from the inner to the outer leaflet of the cell membrane		46–62
	<i>yycFG</i>	Two-component regulatory system of cell wall synthesis and homeostasis		57,75,76
	<i>cls</i>	Cardiolipin synthase		74,77–80
<i>E. faecalis</i>	<i>pgsA</i>	Phosphatidylglycerol synthase	Diversion	80
	<i>liaFSR</i>	Three-component regulatory system as part of the cell envelope stress response		89,91,94
	<i>cls</i>	Cardiolipin synthase		89,91,97
<i>E. faecium</i>	<i>gdpD</i>	Glycerophosphoryl diester phosphodiesterase	Repulsion	89,91
	<i>liaSR</i>	Histidine kinase and response regulator of three-component regulatory system		89,98,100,105,106,125
	<i>cls</i>	Cardiolipin synthase		89,101–105,108
	<i>yycFG</i>	Two-component regulatory system of cell wall synthesis and homeostasis		101,104,108

peptidoglycan biosynthesis. At high DAP concentrations, the overwhelming changes in CM homeostasis (and perhaps cell wall synthesis) lead to leakage of ions and loss of CM potential.<sup>18</sup>

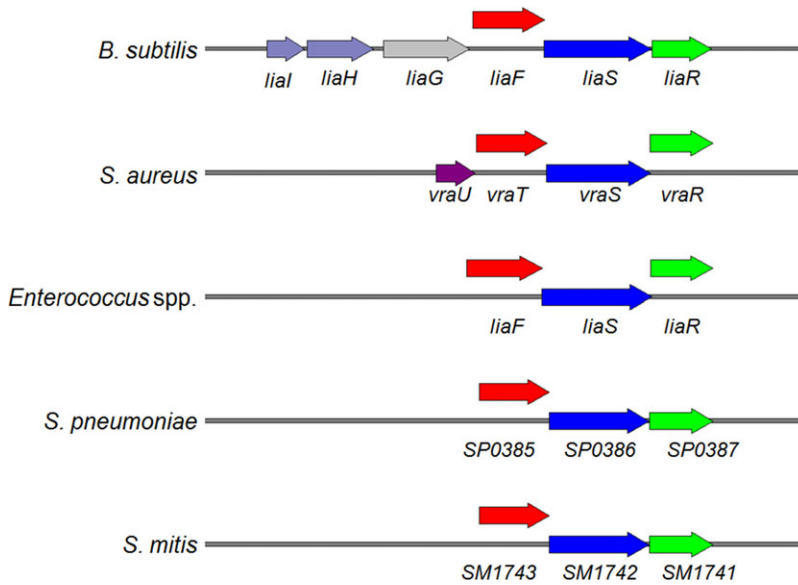
### Mechanisms of daptomycin resistance

Through years of evolution, Gram-positive bacteria have developed a myriad of sophisticated mechanisms to survive hostile environments. In order to succeed in the human host, bacteria need to adapt and, particularly, resist the attacks orchestrated by the immune system. CAMPs produced by the innate immune system are among the first potent antibacterial molecules and the first line of defense against bacterial “invaders.” In order to respond to the CAMP challenge, bacterial pathogens have devised a cadre of very complex mechanisms to counteract this attack and prevent disruption of pivotal cellular process such as cell wall synthesis and membrane homeostasis. As will be discussed below, resistance to DAP frequently results in decreased activities of CAMPs, suggesting con-

vergent mechanisms. Although some environmental, nonpathogenic organisms have been found to harbor enzymes with the ability to hydrolyze and inactivate the DAP molecule, this type of resistance has not been shown in clinically important bacteria to date. However, these genes coding for DAP-inactivating enzymes could be a potential source of resistance determinants if they could be captured by pathogenic bacteria, as it has been previously reported for vancomycin and  $\beta$ -lactam resistance determinants (*van* gene clusters originating in *Paenibacillus* spp. and CTX-M enzymes from *Kluyveromyces* spp., respectively). A summary of proposed mechanisms of DAP-R in *B. subtilis*, *S. aureus*, and enterococci is presented in Table 1.

#### DAP resistance in *B. subtilis*

Although *B. subtilis* is not a common human pathogenic species, an important amount of work has been performed in this organism as a model for studying the Gram-positive cell envelope stress response (CESR). Therefore, it deserves to be discussed in detail. The CESR of *B. subtilis* is mediated



**Figure 2.** The *lia* genes in some Gram-positive bacteria with low G + C content. The loci are drawn to scale. Gene names follow National Center for Biotechnology Information entries of the published genome sequences.

by two-component regulatory systems (TCSs) and extracytoplasmic function (ECF)  $\sigma$  factors.<sup>19–21</sup> The *B. subtilis* response to antibiotics that target the cell wall (such as vancomycin and bacitracin) and CAMPs is mediated by activation of several regulatory networks. Among them, the most studied are those that involve  $\sigma^M$ , the TCSs LiaSR and BceRS (for bacitracin), and  $\sigma^W$  (for vancomycin).<sup>21,22</sup> In the case of DAP, exposure to the antibiotic appears to induce a general response mediated by ECF  $\sigma$  factors  $\sigma^V$ ,  $\sigma^M$ ,  $\sigma^W$ , and  $\sigma^X$  (that are overexpressed by approximately threefold) and a specific response that involves the TCS LiaSR.<sup>23</sup> Indeed, expression of LiaSR appears to be upregulated >400-fold in the presence of DAP, a value that is several orders of magnitude higher than that of any other regulatory systems.<sup>23,24</sup> Interestingly, molecules that are structurally similar to DAP, such as the cyclic lipopeptide friulimicin B, did not trigger the same response seen with DAP, suggesting that activation of LiaSR may be a specific signature of DAP CM disruption.<sup>23</sup> Additionally, LiaSR has been described as part of a complex general network that orchestrates the cell envelope response to a wide range of stresses, including detergents, ethanol, organic solvents, and antibiotics.<sup>25</sup>

LiaSR (for lipid II-interacting antibiotics) is named because it was initially identified after

exposing *B. subtilis* to antibiotics that affect lipid II and CM undecaprenol cycles (e.g., bacitracin, nisin, ramoplanin, and vancomycin), which are crucial steps in cell wall synthesis.<sup>21</sup> The *liaRS* genes are clustered with an additional gene designated *liaF*, which encodes a transmembrane protein that appears to strongly inhibit LiaR-dependent gene expression.<sup>26</sup> The LiaFSR three-component regulatory system is well conserved in Gram-positive pathogens with low G + C content (Fig. 2).<sup>25,26</sup>

Characterization of the *lia* locus of *B. subtilis* indicates that it consists of six genes, designated *liaIH–liaGFSR*, that are differentially expressed.<sup>26</sup> In uninduced conditions, low-level expression of *liaGFSR* is driven by a weak constitutive promoter ( $P_{liaG}$ ) located upstream of *liaG*. Upon activation (i.e., exposure to antibiotics), high expression of the *liaIH* operon is observed by induction of the promoter  $P_{liaI}$  (upstream of *liaIH–liaGFSR*), a phenomenon that is strictly dependent on the presence of the response regulator LiaR.<sup>21,25–28</sup> Indeed, LiaR not only induces *liaH* expression but also upregulates its own expression by readthrough of the entire *liaIH–liaGFSR* locus.<sup>21,26,27</sup> Furthermore, gene expression studies and proteomic analysis have shown that *liaIH* seems to be the only relevant target locus of LiaR.<sup>28</sup>

Stoichiometry studies of the LiaFSR system indicate that different amounts of the three proteins are produced under uninduced conditions at a ratio of 18:4:1 for LiaF, LiaS, and LiaR, respectively.<sup>29</sup> As mentioned above, *liaF* encodes a membrane protein that acts as a specific inhibitor of the LiaRS TCS by exerting its activity through LiaS.<sup>26,29</sup> LiaS is a bifunctional kinase that functions as a phosphatase in uninduced conditions, preventing the phosphorylation of LiaR and hence keeping the system “off” ( $P_{liaI}$  activity).<sup>29</sup> In the presence of cell envelope stress (e.g., exposure to antibiotics), alterations in the stoichiometry of LiaFSR or overexpression of LiaS switches LiaS into its kinase mode, which results in phosphorylation of LiaR and strong induction of  $P_{liaI}$ .<sup>21,26,29</sup> Notably, LiaR can also be phosphorylated by acetyl phosphate (a small molecule phosphor donor produced as part of the cellular metabolism) in the absence of its cognate LiaS or when LiaR is produced in excess, illustrating different levels of regulation in response to cell envelope stressors.

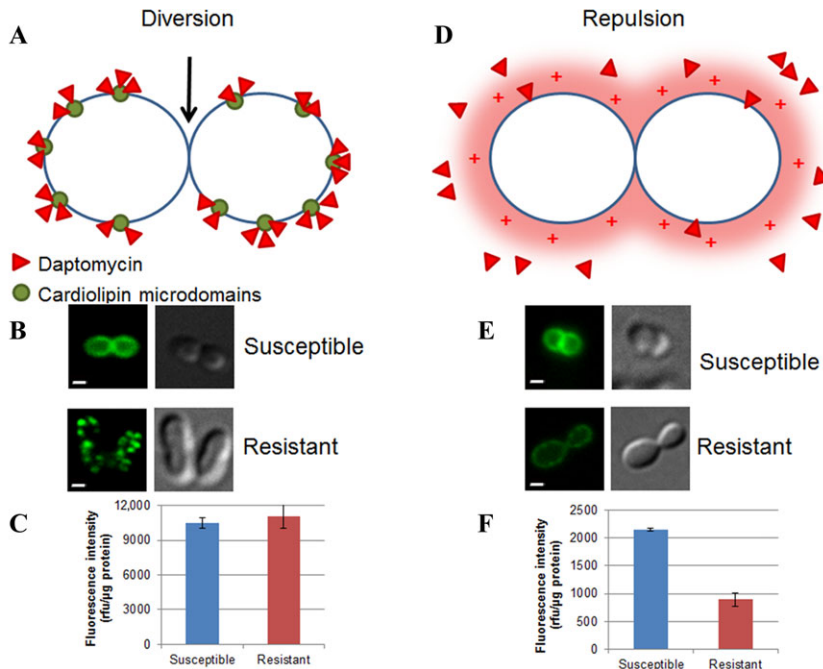
Recent data have shed light into the function of *liaIH* (the target of LiaR). The LiaI gene (*liaI*) encodes a small membrane protein with two trans-membrane regions whose N- and C-termini are cytoplasmic, while LiaH is a homologue of the phage-shock protein described in Gram-negative bacteria and involved in the CM response to the attack by bacteriophages.<sup>30</sup> Indeed, LiaI appears to function as a membrane anchor for LiaH through its C-terminal domain in cell envelope stress conditions. Under normal conditions (i.e., nonstressed), LiaI remains highly motile and can move about the cytoplasmic membrane in a fast and random fashion, presumably to scan for perturbations. In the presence of an inducing trigger (e.g., antibiotics, including DAP), LiaI stalls in distinct foci while it recruits LiaH to the CM. Interestingly, another feature of LiaH is the formation of large oligomeric ring-like structures, comparable to the structure observed in phage-shock proteins described in *E. coli*. Of note, the genome of *B. subtilis* also contains a LiaH paralog, PspA, which suggests functional redundancy of these phage-shock proteins. The dynamics of the Lia system via LiaIH highly resembles the Psp system described in *E. coli* and *Yersinia enterocolitica*.<sup>31,32</sup> The Psp system is a highly conserved system that functions as an adaptive mechanism to respond to conditions that can affect the cell envelope adversely.<sup>31–33</sup>

The strong induction of the Lia system by DAP appears to protect *B. subtilis* against stress caused by this antibiotic. Deletion in the *liaIH* operon (*liaH* or *liaIH*) increased *B. subtilis* susceptibility to DAP, while deletion of *liaF* appeared to have no effect on DAP MIC.<sup>24,28</sup> Since *B. subtilis* is a model organism, the mechanism of DAP resistance has only been studied in laboratory settings after *in vitro* serial passage.<sup>34</sup> DAP-resistant *B. subtilis* displayed increased expression of the LiaRS TCS and its operon, including LiaH, but was not associated with mutations in any of its components.<sup>34</sup> Phenotypically, development of *in vitro* DAP-R in *B. subtilis* has been associated with aberrant septum placement, thickened cell wall, and cross-resistance to vancomycin, moenomycin, and bacitracin. DAP-resistant *B. subtilis* cells also displayed reduced binding of the antibiotic molecule (compared to its susceptible counterpart) (Fig. 3). Interestingly, DAP-resistant *B. subtilis* was found to have a marked decrease in the concentration of PG in the CM.

Another gene that has been implicated in DAP-R in *B. subtilis* is *mprF*, which encodes an enzyme with the ability to modify membrane phospholipids by lysinylation of PG (leading to formation of L-PG), increasing the amount of positively charged PLs (similar to *S. aureus*, see below). Deletion and overexpression of *mprF* both lead to changes in DAP-S in *B. subtilis*.<sup>24,35</sup> However, changes in *mprF* were not found in adapted DAP-resistant *B. subtilis*. Rather, a mutation was found in the gene encoding the PG synthase (*pgsA*), and genetic reconstruction of this *pgsA* mutation demonstrated that this gene contributes significantly to the development of DAP-R in *B. subtilis*, leading to a 20-fold increase in DAP MIC. Mutation in other genes, including *mreB* (cell shape-determining protein) and *relA*, were also seen in a DAP-resistant derivative. However, their roles appear to be compensatory rather than major determinants of the resistance phenotype.

#### DAP-R in staphylococci

Although DAP-R in staphylococci is uncommon in clinical practice, development of this phenomenon during therapy has been widely described. Cases of DAP-resistant staphylococci are generally seen in high-inoculum infections (e.g., infective endocarditis and abscesses) and when lower doses of the drug have been used (i.e.,  $\leq 6$  mg/kg/day).<sup>36–38</sup> In addition, the prior use of vancomycin associated



**Figure 3.** Proposed mechanisms of DAP-R in Gram-positive organisms. Two main mechanisms of resistance have been postulated. The first is diversion (*Enterococcus faecalis* only) of the antibiotic from the preferential binding site of DAP at the septum (black arrow), resulting in ineffective binding of DAP (A). Images of cells treated with BODIPY-labeled DAP (a fluorescent derivative of DAP) demonstrate binding of the antibiotic to the septum in DAP-S. When exposed to the same concentration, DAP binding does not appear to occur at the septum in a DAP-resistant isolate (B). Panel C evaluates the amount of DAP bound to CMs of *E. faecalis* by measurement of fluorescence intensity normalized to protein content. As shown, no change in fluorescence intensities were noted between DAP-S and DAP-R, indicating similar binding of the antibiotic molecules to the CM. The second mechanism, seen in *Bacillus subtilis*, *Staphylococcus aureus*, and *Enterococcus faecium*, is electrostatic repulsion of the positively charged DAP–Ca<sup>2+</sup> complex from the CM (D). Binding of BODIPY-labeled DAP is decreased in a DAP-resistant isolate compared to its susceptible counterpart (E), demonstrated by *E. faecium*. Lower fluorescence intensity is also noted in DAP-R versus DAP-S strains (F) as described in *E. faecium*. DAP, daptomycin; rfu, relative fluorescence unit. Bar = 1 μm.

with development of the VISA phenotype has also been linked with increased resistance to DAP during therapy.<sup>36,38–46</sup> The mechanisms of DAP-R in *S. aureus* have yet to be completely understood. A prevailing phenomenon that mechanistically links all the pathways of DAP-R in staphylococci appears to be “repulsion” of the DAP antibiotic molecule from the cell surface, which is generally associated with an overall change in the net charge of the bacterial surface (toward a more positive CM) (Fig. 3). However, as will be discussed below, the repulsion hypothesis does not explain the emergence of DAP-R in all *S. aureus* isolates.

#### *MprF* and the repulsion theory

One of the genes most consistently implicated in the development of DAP-R in *S. aureus*, both *in vivo* and *in vitro*, is *mprF*, which codes for a bifunctional

enzyme (MprF, for multiple peptide resistance factor) that catalyzes the lysinylation of PG and the translocation of L-PG from the inner to the outer leaflet of the CM. MprF is composed of a cytosolic C-terminal domain that uses lysyl-tRNA as a substrate to add lysine residues to negatively charged PG, resulting in L-PG. The positively charged nature of L-PG appears to be a major contributor to the change of surface charge that helps repel the DAP antibiotic molecule from the surface.<sup>47–54</sup> In order to alter surface charge, L-PG must be transferred from the inner to the outer leaflet of the CM, an activity that appears to be catalyzed by the first six to eight (out of a total of 14) hydrophobic transmembrane domains in the N-terminal domain of the protein (flipase activity).<sup>55,56</sup> Thus, an increase in L-PG in the outer leaflet of the CM (and subsequent increase in net positive charge) appears to be

of paramount importance to prevent the binding of the DAP–calcium complex to the CM.

In an *in vitro* passage experiment that exposed *S. aureus* to ascending sublethal concentrations of DAP, changes in MprF were one of the first genetic changes identified in DAP-resistant derivatives.<sup>57</sup> A number of *mprF* mutations have been described in at least 12 different loci, but the exact role of each mutation has not been established. Nonetheless, mutations appear to localize in “hot spots” of the enzyme (mainly in the central transmembrane domains, but also one in the C-terminal cytosolic domain) and are associated with a gain of function.<sup>58,59</sup> Bayer *et al.*<sup>58</sup> recently analyzed over 30 DAP-susceptible and DAP-resistant *S. aureus* isolates and demonstrated that, although *mprF* single-nucleotide polymorphisms were not uncommon in DAP-susceptible bacteria, none of them were localized in the hot spots. Conversely, all DAP-resistant isolates with *mprF* mutations harbored the changes within the already identified hot spots. Furthermore, only mutations in these specific locations were correlated with the expected gain of enzymatic function and with an increase in the positive charge of the CM. Of note, these mutational changes in MprF have also been correlated with a decrease in PG content.<sup>58</sup> Also of interest, using a model of *S. aureus* prosthetic joint infections in rabbits, Mishra *et al.*<sup>53</sup> recovered DAP-resistant isolates harboring mutations in the same MprF hot spots from animals not exposed to any antibiotics, and suggested that this phenotype was presumably driven by the exposure to endogenous host defense antimicrobial peptides.

The role of MprF in the development of DAP-R has been further supported by experimental evidence showing that *mprF* deletion mutants exhibited increased susceptibility to DAP and other CAMPs.<sup>60,61</sup> Likewise, translation blockade using an antisense strategy was able to reverse DAP-R in a strain harboring *mprF* mutations.<sup>62</sup> Furthermore, trans-complementation of *mprF* deletion mutants with mutated alleles was followed by an increase in DAP MICs.<sup>46</sup> Interestingly, some data suggest that the main function of MprF might not be specifically related to an increase in the positive net charge of the CM, since not all DAP-resistant isolates with *mprF* mutations exhibit changes in cell surface charge.<sup>40,63</sup> It has been postulated that MprF may be controlling the concentration of PG (by converting

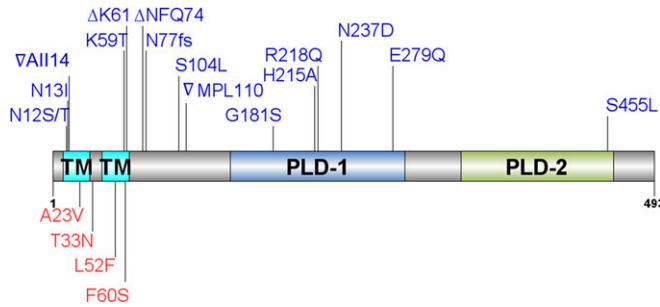
it to L-PG) and thus altering the interactions of DAP and CAMPs with their target CM.<sup>46</sup> Thus, the reduction in PG content (as a result of conversion to L-PG) may impair the ability of DAP to oligomerize in the outer leaflet of the membrane, compromising its antibacterial activity.

Another strategy used by *S. aureus* to change the surface charge and, therefore, alter DAP activity is overexpression of the *dlt* operon.<sup>63–68</sup> The *dlt* genes are involved in the introduction of the positively charged amino acid D-alanine to cell wall teichoic acids. Thus, the alanylation of these surface-exposed structures results in an increase in the net positive charge of the CM, similar to what is observed in DAP-resistant strains with mutated *mprF* alleles.

#### *Vancomycin nonsusceptibility and development of DAP-R in S. aureus*

Several lines of research have suggested a connection between the molecular pathways leading to the development of the VISA phenotype and DAP-R in *S. aureus*. First, a number of studies have demonstrated that an important number of VISA strains exhibit DAP MICs above the susceptibility breakpoint, with percentages of DAP-R as high as 80%.<sup>39</sup> Furthermore, development of DAP-R *in vivo* was documented in an *S. aureus* VISA strain that failed vancomycin therapy and was never exposed to DAP.<sup>45</sup> Second, several independent studies have reported that DAP-resistant *S. aureus* strains frequently exhibit phenotypic changes that parallel those typically observed in VISA isolates; the most important is a marked increase in the thickness of the cell wall. Furthermore, the degree of thickness of the cell wall has been positively correlated with the increase in DAP MIC in VISA isolates.<sup>41,42</sup> Third, transcriptional analysis studies have shown that one of the most consistent findings after DAP exposure is the upregulation of the cell wall stimulon, similar to what is observed with the response to vancomycin and other cell envelope–targeting drugs.<sup>46,69,70</sup> Likewise, comparisons of DAP-susceptible and DAP-resistant *S. aureus* strain pairs have located changes in genes that have been associated with the VISA phenotype, indicating that genes involved in cell wall synthesis and/or homeostasis play important roles in both vancomycin and DAP resistance.<sup>66</sup>

Among the most relevant group of genes involved in VISA and DAP-R phenotypes, two gene



**Figure 4.** Schematic representation of cardiolipin synthase (Cls). Predicted N-terminal transmembrane (TM) domains and phospholipase (PLD) domains are indicated. Lines refer to positions of amino acid changes associated with daptomycin resistance in *Enterococcus* spp. (blue) and *Staphylococcus aureus* (red).

clusters encoding TCSs (*vraSR* and *yycFG* (*walkR*)) are the most studied. Of note, *VraSR* is the ortholog of *LiaSR* in other Gram-positive organisms (Fig. 2). Both systems play important roles in cell envelope homeostasis and have been consistently implicated in the development of the VISA phenotype, along with an additional regulatory system designated *GraSR*.<sup>71,72</sup> Mehta *et al.*<sup>73</sup> showed that construction of a *vraRS* null mutant resulted in reversion of DAP-R (and decrease in cell wall thickness) in *S. aureus*. Trans-complementation with *vraSR* was able to restore resistance to DAP, with MICs restored to the values determined in the original resistant strain. In addition, upregulation of *vraSR* expression and its regulon have been reported in DAP-resistant *S. aureus* selected *in vitro*.<sup>74</sup>

*YycFG* (*WalkR*) is an essential TCS of cell wall synthesis and homeostasis that has also been implicated in DAP-resistant enterococci (see below). Indeed, analysis of *in vitro*-derived DAP-resistant *S. aureus* mutants revealed changes affecting the histidine kinase of the system (*YycG*).<sup>57,75</sup> Furthermore, Howden *et al.*<sup>76</sup> demonstrated that the *YycFG* system plays a major role in the *in vivo* evolution of the VISA phenotype and DAP-R in *S. aureus*. Several mutations in different locations of the *yycFG* operon (or in its regulatory genes *yycH* and *yycI*) have been reported, although the exact contribution to DAP-R is unclear. However, a single amino acid change in *YycG* (K208R) was sufficient to increase the DAP MIC from 0.5 to 2 mg/L, a value that is sufficient for clinical DAP resistance.<sup>76</sup>

#### Role of genes involved in phospholipid metabolism

Other genes strongly implicated in the development of DAP-R in *S. aureus* are those encoding

enzymes involved in phospholipid metabolism (other than *MprF*). In bacteria, cardiolipin synthases (Cls) are the critical enzymes for the synthesis of CL, often using two molecules of PG as substrate.<sup>77</sup> Changes in these enzymes are likely to play a role in DAP-R by altering the pool of PG/CL in the CM (see above). Importantly, *S. aureus* (and other Gram-positive organisms) harbors two (or more) *cls* genes (*cls1* and *cls2*), and their differential expression is thought to vary depending on the presence of different stress conditions.<sup>78,79</sup> Using a whole-genome approach, Peleg *et al.*<sup>80</sup> analyzed clinical and laboratory-derived strain pairs of *S. aureus* and reported that three clinically derived DAP-resistant *S. aureus* strains harbored amino acid changes (F60S, A23V, and L52F) in *Cls2*, all of which were found in conjunction with mutations in *mprF* (Fig. 4). Similarly, *Cls2* was also found to have an amino acid substitution (T33N) in two laboratory-derived DAP-resistant mutants, but in this case the mutation was the only genetic change reported and its presence was sufficient to increase the DAP MIC from 0.5 to  $\geq 2$  mg/L (above the clinical breakpoint).<sup>80</sup> The role of Cls in DAP-R was further supported by a transcriptomic analysis of isogenic strains obtained after DAP passage, in which the expression of *cls* was significantly downregulated in the resistant mutant as compared to its susceptible parental strain.<sup>74</sup>

Another gene involved in phospholipid metabolism and implicated in the DAP-R phenotype is *pgsA*, which encodes a CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase that is involved in the production of PG. As mentioned above, *PgsA* has also been reported as an important determinant of DAP-R in *B. subtilis*. In the genomic analysis performed by Peleg

*et al.*,<sup>80</sup> mutations in *pgsA* were often observed in DAP-resistant laboratory derivatives but not in DAP-resistant clinical isolates. Of note, one particular amino acid change (A64V) was seen in two different isolates, and its presence was sufficient to increase the DAP MIC above the established breakpoint. The role of the mutations is unclear, but they likely impair or abolish the enzymatic activity, decreasing the overall pool of PG.

#### ***Other genes and phenotypic changes associated with DAP-R in S. aureus***

As mentioned, the most consistent phenotypic changes reported in DAP-resistant strains of *S. aureus* are an increase in the positive charge of the CM and a thickened cell wall, which have been found in clinical pairs of DAP-susceptible/DAP-resistant MRSA and methicillin-susceptible *S. aureus* isolates.<sup>64,67</sup> The net increase in positive charge has been related to an increase in L-PG (generally associated with a gain in function of MprF, see above) or to the alanylation of cell wall teichoic acids, which is secondary to the overexpression of the *dlt* operon. This electrostatic change results in a decreased binding of DAP to the CM, reducing its antibacterial properties. On the other hand, the increase in cell wall thickness has been correlated with higher levels of expression of *tagA*, a gene that is directly involved in the early steps of the synthesis of cell wall teichoic acids.<sup>64,67</sup>

However, other phenotypic changes have also been reported, albeit less frequently. For instance, changes in membrane fluidity have been correlated with the development of DAP-resistant *S. aureus* strains. Interestingly, strains that developed DAP-R *in vivo* have been found to have membranes with increased fluidity, whereas mutants obtained *in vitro* had more rigid membranes.<sup>75,81</sup> Therefore, it has been suggested that an optimal amount of fluidity might be necessary for specific CAMPs to exert their action and that changes in any direction (higher or lower fluidity) could play important roles in resistance to these compounds.<sup>46</sup>

Another important component of *S. aureus* CM associated with DAP-R is staphyloxanthin, a carotenoid pigment that gives *S. aureus* its iconic golden color. A study by Liu *et al.*<sup>82</sup> demonstrated that blockage of staphyloxanthin synthesis resulted in colorless bacteria with increased susceptibility to H<sub>2</sub>O<sub>2</sub> and killing by human whole blood. In addition,

inhibition of staphyloxanthin synthesis was associated with rapid clearance of *S. aureus* by the innate immune system.<sup>82</sup> Mishra *et al.*<sup>83</sup> provided evidence suggesting a role of carotenoid in DAP-R. Indeed, when carotenoid production was increased by plasmid induction in methicillin-susceptible *S. aureus*, the DAP MICs also increased. In a follow-up study, reduced carotenoid content in a MRSA was associated with DAP-R.<sup>5,84</sup> Thus, it is postulated that carotenoid content influences CM order (rigidity) and fluidity, a phenomenon that may affect susceptibility to a variety of CAMPs. Further clarification of the role of staphyloxanthin and CM fluidity is the object of active investigation.

Finally, a recent publication analyzed the differences in physiologic fitness and metabolic pathways between six DAP-resistant/DAP-susceptible isogenic strain pairs. Utilizing a metabolomic approach, Gaupp *et al.*<sup>85</sup> found that DAP-resistant strains exhibit a decrease in the tricarboxylic acid cycle activity and a preference for the pentose phosphate pathway and purine/pyrimidine metabolism. The latter pathways are associated with cell wall teichoic acid and peptidoglycan biosynthesis.<sup>85</sup>

#### ***DAP resistance in enterococci***

Enterococci are less susceptible to DAP than staphylococci and streptococci and exhibit higher MICs (e.g., the current CLSI breakpoint is fourfold higher than that of *S. aureus*). Despite this difference, most enterococcal isolates remain susceptible to the antibiotic.<sup>86</sup> However, the emergence of DAP-resistant enterococci during and after therapy has been extensively documented in both *E. faecalis* and *E. faecium*. Interestingly, DAP-R has also been reported in isolates recovered from patients without any exposure to DAP or any other lipopeptide.<sup>87,88</sup>

Most genes implicated in DAP-R in enterococci can be grouped into two broad categories: (1) genes encoding regulatory systems that orchestrate cell envelope homeostasis and stress response and (2) genes coding for enzymes involved in the metabolism of CM phospholipids. Although some of the genes implicated in resistance in *E. faecalis* and *E. faecium* code for proteins of similar function, there seem to be important differences in the genetic and biochemical routes leading to DAP-R among enterococcal species. Furthermore, it is also likely that multiple different pathways to resistance exist within a same species, and that

specific isolates demonstrate a preference for one resistance pathway over the other.

### DAP resistance in *E. faecalis*

Phenotypically, DAP-R in *E. faecalis* has been associated with thickened cell wall, aberrant septal placement, increase in relative cell surface positive charge, and reduction of the ability of DAP to depolarize the CM.<sup>89</sup> These findings parallel those of *S. aureus* and other Gram-positive species (see above). Analysis of CM phospholipid content of a clinical strain pair of DAP-susceptible and DAP-resistant *E. faecalis* revealed a significant decrease in PG content accompanied by an increase in negatively charged glycerophosphoglycolipid (glycerophospho-diglycodiacylglycerol (GP-DGDAG)), without changes in CL or positively charged aminophospholipids.<sup>90</sup>

An important consideration is that the mechanism of resistance to DAP in *E. faecalis* appears to be distinct from that in *S. aureus*. Indeed, using BODIPY-FL-labeled DAP, it has been shown that repulsion of DAP from the cell surface is not the main strategy used by this organism to withstand the DAP bactericidal effect. Instead, it appears that *E. faecalis* has developed a sophisticated strategy to divert DAP from its principal septal target toward other CM areas (Fig. 3). This diversion of the antibiotic is associated with redistribution of CL microdomains away from the septum.<sup>91</sup> Although the specific biochemical steps mediating this strategy are not fully understood, the LiaFSR system seems to play a very prominent role in the steps leading to CM adaptation.

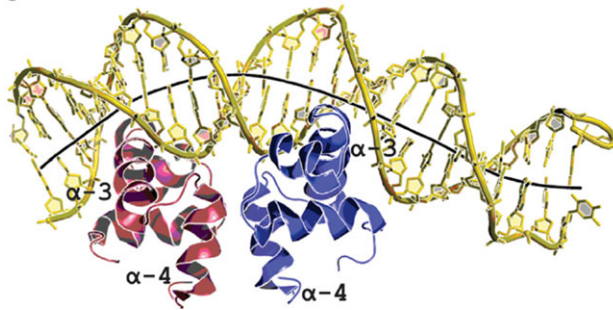
In *E. faecalis* (and *E. faecium*, see below), the LiaFSR system has similar components as described above for *B. subtilis* and other Gram-positive organisms (Fig. 2).<sup>25,26</sup> LiaF is a transmembrane protein thought to be a negative regulator of the system; LiaS and LiaR are the histidine kinase and response regulator of the system, respectively. Using a quantitative experimental evolutionary approach, Miller *et al.*<sup>92</sup> demonstrated that alterations in *liaFSR* were the initial pivotal step leading to DAP-R in *E. faecalis*. Furthermore, genomic analyses of a clinical strain pair of DAP-susceptible and DAP-resistant *E. faecalis* recovered from the bloodstream of a patient treated with DAP found a single deletion of an isoleucine at position 177 of LiaF in the DAP-resistant strain. Introduction of the altered *liaF* allele into the DAP-

susceptible *E. faecalis* isolate increased the DAP MIC from 1 to 4  $\mu\text{g}/\text{mL}$ . More importantly, this amino acid change was sufficient to abolish DAP's *in vitro* bactericidal activity.<sup>89,93</sup> Of note, the single introduction of the mutated *liaF* allele was sufficient to produce CL microdomain redistribution, suggesting that the LiaFSR system plays an important role in CM homeostasis.

Further underscoring the critical role of LiaFSR in DAP-R, a nonpolar deletion of *liaR* (encoding the response regulator of the system) was obtained in a clinical DAP-resistant strain of *E. faecalis*. The deletion resulted in reversion of DAP-R and restoration of the normal distribution of CM CL-rich microdomains.<sup>94</sup> Interestingly, deletion of *liaR* also resulted in a marked increase in the activity of several unrelated CAMPs and a parallel decrease in the MIC of telavancin (a CM-acting antibiotic available clinically). Moreover, a *liaR* knockout mutant generated in an *E. faecalis* laboratory strain (OG1RF, DAP susceptible) resulted in hypersusceptibility to DAP with a marked decrease in the MIC, supporting the important role of LiaR in DAP-R.<sup>94</sup>

Recently, Davlieva *et al.*<sup>95</sup> used a combination of structural and biophysical experiments to examine the molecular basis of LiaR-mediated DAP-R. Indeed, activation of the response regulator LiaR (by phosphorylation or mutations in LiaR that mimic the phosphorylated state) triggers a transition of dimer to tetramer that appears to significantly increase the affinity of the protein for the target DNA (Fig. 5).<sup>95</sup> With this approach, it was confirmed that activated LiaR appears to bind to its own (*liaFSR*) promoter, but it also regulates a cluster of genes that encode a putative soluble protein of 533 amino acids (designated LiaX) and two transmembrane proteins of 107 (LiaY) and 118 (LiaZ) amino acids, respectively. Although the function of these proteins remains to be elucidated, it is interesting to note that a frameshift mutation on LiaX has been shown to cause DAP-R *in vitro*.<sup>92</sup>

A second group of genes implicated in *E. faecalis* DAP-R code for enzymes involved in the metabolism of CM phospholipids. Among them, the two most prominent enzymes associated with the DAP-R phenotype are CIs (which synthesizes CL) and a glycerophosphoryl diester phosphodiesterase (GdpD) that is involved in glycerol turnover for phospholipid biosynthesis. Indeed, mutagenesis experiments confirmed the additive



**Figure 5.** Crystal structure of the DNA-binding domain of LiaR of *Enterococcus faecalis* bound to a DNA sequence upstream of its target genes. The  $\alpha 4$  helices form part of the molecular recognition surface responsible for formation of the functional dimer required for DNA binding. The  $\alpha 3$  DNA-recognition helices in the dimer are positioned to create a large electropositive DNA-binding surface. The LiaR–DNA complex structure shows a strong bend in the DNA, as shown by its helical axis (gray). Adapted from Ref. 95.

effect of *gdpD* and *cls* mutations in the development of DAP-R in a genetic background of a strain with changes in LiaFSR.<sup>89</sup> Interestingly, a mutation in *gdpD* did not affect DAP-S on its own, but when accompanied by a LiaF substitution resulted in marked increases of DAP MICs, suggesting a synergistic effect of these changes. It has been postulated that mutations in genes involved in phospholipid metabolism are likely to occur in later stages of development of DAP-R that occur after the LiaFSR system is activated (triggering the CESR).<sup>96</sup> Thus, changes in phospholipid homeostasis are likely to potentiate the resistance phenotype by completing CM adaptation to the antimicrobial peptide attack.

Cls is a membrane-bound enzyme harboring two putative N-terminal transmembrane domains (Fig. 4) and two phospholipase domains (PLD1 and PLD2) that catalyze the formation of CL from two PG molecules. Changes in different domains of the Cls protein (transmembrane, linker, and PLD domains) have been associated with DAP resistance, but the biochemical consequences of these alterations and their contribution to the DAP-R phenotype remains unclear.<sup>89,92,97</sup> Of note, overexpression in trans of a mutated *cls* allele harboring an amino acid change in the PLD1 domain resulted in a four-fold increase in the DAP MIC and was sufficient to yield a DAP-R phenotype (from 4 to 64 mg/L).<sup>97</sup>

### DAP-R in *E. faecium*

*E. faecium* is the most drug-resistant and recalcitrant of the enterococcal species, and DAP has become a key antibiotic to treat infections caused by these organisms. Description of DAP-R in

*E. faecium* is becoming a serious clinical problem since DAP has become a first-line option to treat severe *E. faecium* infections. Most worrisome is the scenario of DAP tolerance (lack of bactericidal activity) in strains reported “susceptible” by standard susceptibility testing, which has been noted in DAP-susceptible *E. faecium* clinical strains with MICs close to the breakpoint (3–4  $\mu\text{g}/\text{mL}$ ).<sup>98</sup> Development of DAP-R in this species has also been associated with increased surface charge, increased cell wall thickness, and decreased depolarization after DAP exposure.<sup>90,99</sup> However, in contrast to what was described above for *E. faecalis*, the development of DAP-R in *E. faecium* appears to be similar to that in *S. aureus*, in which repulsion of the antibiotic from the cell surface seems to be the predominant mechanism of resistance (Fig. 3). Additionally, and also different from *E. faecalis*, DAP-R in *E. faecium* is not associated with redistribution of CM CL microdomains, despite the fact that similar genes (i.e., *liaFSR*) appear to be involved in the resistance phenotype.<sup>100,101</sup> This mechanistic difference suggests independent evolutionary trajectories in two species within the same bacterial genus.

In terms of phospholipid content of the CM, the most relevant change associated with DAP-R in *E. faecium* strains (where PLs have been examined) appears to be a marked decrease in the content of PG without major alterations of CL content.<sup>90</sup> Genomic analyses of several clinical strain pairs of *E. faecium* have shed light into the genetic determinants of DAP-R in this organism,<sup>102–104</sup> although the specific role of the majority of genes involved remains to be fully elucidated. In a recent

genomic analysis of 19 unrelated *E. faecium* strains with different DAP MICs (3–48 µg/mL) in which mutations in 43 genes previously associated with DAP-R were investigated, Diaz *et al.*<sup>105</sup> showed that mutations in *liaFSR* were the most common changes observed in these strains. Among the identified changes, substitutions in LiaS (T120A) and LiaR (W73C) were the most frequently observed changes. Interestingly, these two amino acid substitutions were always found together, suggesting that they might have coevolved. Furthermore, the same LiaSR substitutions were also found in *E. faecium* isolates recovered from the bloodstream of patients exhibiting DAP MICs in the higher range of susceptibility (between 3 and 4 µg/mL), but were absent in isolates with DAP MIC ≤ 2 µg/mL.<sup>98</sup> More importantly, these mutations were sufficient to abolish the *in vitro* bactericidal activity of DAP and associated with DAP failure in a neutropenic patient who presented with VRE bacteremia caused by a DAP-susceptible isolate (MIC 3 µg/mL).<sup>106</sup> Moreover, deletion of *liaR* reversed DAP resistance in *E. faecium* isolates independent of the genetic background or changes in LiaFSR, supporting the notion that LiaR is the master regulator of the CESR and plays a universal role in enterococci regardless of the genetic paths of DAP-R.<sup>107</sup>

Another TCS that has been found to be involved in DAP-R in *E. faecium* is the YycFG system and its accessory proteins (YycHII), as described in *S. aureus*.<sup>107</sup> In *E. faecium*, mutations in *yycFG* (or accessory genes) have been found in both DAP-resistant and DAP-tolerant (lack of *in vitro* DAP bactericidal activity) strains.<sup>104,105</sup> Interestingly, a single mutation in YycG was not sufficient to raise DAP MICs, unless accompanied by changes in genes encoding phospholipid enzymes.<sup>101,108</sup> YycFG is an essential regulatory system involved in cell wall homeostasis in *S. aureus*; however, its role in DAP-R in enterococci has not yet been fully characterized.<sup>57,75,107,109–111</sup>

Similar to what has been found in *E. faecalis*, mutations in *cls* have also been commonly associated with DAP-R in *E. faecium* (Fig. 4). Biochemical characterization of the impact of two independent PLD1 mutations (R218Q (also described in *E. faecalis*) and H215R) in Cls showed that both changes resulted in an increase in enzymatic activity (gain-of-function mutation).<sup>112</sup> Of note, using an allelic replacement strategy, Tran *et al.*<sup>104</sup> demonstrated

that introduction of a mutated *cls* (resulting in the R218Q substitution) associated with DAP-R did not affect the DAP MIC, suggesting that Cls changes alone are not sufficient to mediate resistance in *E. faecium*.

#### *DAP-R in other Gram-positive pathogens*

Although DAP-R is well described in *S. aureus* and enterococci, it remains bactericidal against a wide range of other Gram-positive pathogens. As such, DAP has been increasingly used for treatment of infections caused by Gram-positive bacteria, especially in cases of severe or deep-seated infections. Thus, reports of DAP-R in other clinically relevant Gram-positive species have emerged in recent years. In this section, description of DAP-R in two pathogens, *Corynebacterium* spp. and *Streptococcus mitis*, will be discussed.

Reports of DAP-R in *Corynebacterium* spp. is limited to three cases, one of *C. jeikeium* and two of *C. striatum*. DAP-resistant *C. jeikeium* was recovered from the blood of a neutropenic patient who underwent cord blood transplantation for secondary acute myeloid leukemia.<sup>113</sup> *C. striatum* strains were recovered from two patients, one with a left ventricular assist device who presented with bacteremia and another with native valve endocarditis.<sup>114,115</sup> All *Corynebacterium* isolates exhibited DAP MICs ≥ 256 µg/mL. Intriguingly, all patients were exposed to DAP before isolation of *Corynebacterium* spp., including two (one *C. jeikeium* and one *C. striatum* case) patients who had been receiving DAP therapy for infections caused by other Gram-positive pathogens. These cases serve as cautionary tales for the selection of DAP-R in a genus that comprises organisms considered to be of low pathogenicity and generally regarded as human commensals. Of note, McElvania Tekippe *et al.*<sup>114</sup> described the emergence of DAP-R in seven of 12 clinical isolates of *C. striatum* after only 24 h of DAP exposure with MICs > 256 µg/mL. *C. striatum* also displayed heterogeneous high-level DAP resistance in one case described by Tran *et al.*<sup>115</sup> The two DAP-resistant derivatives did not show any alterations in cell surface charge but exhibited decreased CM depolarization induced by DAP. As mentioned, the emergence of DAP-resistant *Corynebacterium* spp. appears to be related to prolonged courses of DAP. No further evaluation of the genetic basis of DAP-R in this genus has been reported.

Despite earlier studies that reported a low prevalence of DAP-R in streptococci,<sup>116,117</sup> certain isolates have been shown to have the ability to adapt and rapidly develop high-level DAP-R.<sup>118–121</sup> This phenomenon was initially described in two patients. The first was a case of breakthrough bacteremia caused by DAP-resistant *S. anginosus* in a patient who was treated with DAP for a previous MRSA infection.<sup>120</sup> Another case involved a patient with native valve *S. oralis* endocarditis whose DAP-resistant isolate emerged during treatment with DAP.<sup>121</sup> An independent finding by another research group found emergence of DAP-R within 24 h of antibiotic exposure in viridans-group streptococci exhibiting high levels of resistance (MIC  $\geq$  256  $\mu$ g/mL).<sup>119</sup> Therapy with DAP alone in a rabbit endocarditis model also produced DAP-R after 48 h of exposure. Similarly, DAP exposure in a simulated endocardial vegetation (SEV) model corresponded to a rapid development of high-level DAP-R after only 24 h at a high concentration, up to 8  $\times$  MIC.<sup>118</sup> Like studies in *Corynebacterium*, the mechanism of DAP-R in streptococci remains to be elucidated.

### Clinical implications of DAP resistance and tolerance

From a clinical perspective, DAP is a potent and bactericidal antibiotic that, in the case of VRE, is one of the drugs of last resort to treat these organisms. Therefore, the development of resistance during therapy is a serious threat because it leaves clinicians without options for these severe infections. Our current knowledge on the mechanism of action, resistance, and pharmacological aspects of DAP is now at a stage at which innovative approaches may be considered to preserve the activity of DAP until new molecules become available. We have discussed that the development of DAP-R during therapy involves important adaptive responses that are triggered by a variety of environmental cues that do not only depend on the presence of DAP (bacterial cell envelope adaptation). Thus, it would be wise to consider that development of DAP-R is a natural process and would be likely under therapy, since bacterial pathogens have “learned,” through evolution, how to respond to the antibiotic challenge. Therefore, devising therapeutic strategies to prevent development of resistance by weakening the bacterial CM adaptive response would be an inno-

vative approach. This strategy could not only preserve the activity of DAP but may also enhance the activity of other cell envelope-acting agents that have become obsolete in the treatment of Gram-positive infections. Additionally, it may contribute to the clearance of bacteria by the innate immune system.

A plethora of molecular and pharmacological data indicate that DAP is a concentration-dependent antibiotic. Neutropenic murine thigh models have demonstrated that the area under the concentration–time curve (AUC)/MIC and maximum concentration ( $C_{\max}$ )/MIC ratios are important pharmacodynamic parameters to determine the *in vivo* activity of DAP against Gram-positive organisms.<sup>122</sup> Indeed, the amount of active DAP molecules available to bind to the CM at any given point is of paramount importance for the bactericidal effect of the antibiotic. The two types of mechanisms of resistance discussed above (i.e., repulsion of the antibiotic from the cell surface and diversion of DAP from the septum) could potentially be overcome by increasing concentrations of the antibiotic, although the amount of drug necessary in these scenarios may be limiting owing to toxicity. Using a SEV model, Rose *et al.*<sup>123</sup> compared DAP monotherapy in doses equivalent to 6 and 10 mg/kg (the former is the FDA-approved dose for *S. aureus* bacteremia) and demonstrated that the use of higher concentrations of DAP was able to prevent the appearance of resistant mutants in *S. aureus*. Similarly, two studies using the same SEV model to treat vancomycin-resistant *E. faecium* and *E. faecalis* showed that the only regimen that did not select for DAP-resistant mutants was 12 mg/kg.<sup>124,125</sup> Furthermore, Sakoulas *et al.*<sup>126</sup> utilized a rat IE model and performed population analysis of bacterial isolates recovered from vegetation. They were able to show that higher DAP doses (equivalent to a human dose of 6 vs. 4 mg/kg) were able to prevent the emergence of DAP heteroresistance in MRSA isolates *in vivo*. Therefore, it appears that optimizing the amount of drug delivered to the target site may improve its activity and also prevent, at some level, the development of resistance.

Another interesting observation is that, once Gram-positive organisms become DAP resistant, they appear to markedly increase their susceptibility to other cell wall-targeting antibiotics such as the

$\beta$ -lactams (the so-called “see-saw” effect).<sup>127–131</sup> This observation is not unique to DAP and has been previously documented in the development of the VISA phenotype (low-level resistance to vancomycin). Similar to DAP, as the cells decrease susceptibility to vancomycin, there is a concomitant increase of susceptibility to  $\beta$ -lactams (even if the isolate is fully resistant to these compounds *in vitro*).<sup>132–136</sup> Several *in vitro* and *in vivo* experiments support the use of the combinations of DAP and  $\beta$ -lactams to treat recalcitrant Gram-positive infections and achieve therapeutic success and prevent the development of resistance.<sup>105,106,127,137–159</sup> Yang *et al.*<sup>127</sup> utilized a rabbit model of aortic IE to evaluate the combination of DAP plus oxacillin against MRSA clinical strain pairs of DAP-susceptible and DAP-resistant derivatives recovered from patients who failed DAP therapy. They showed that the combination was highly effective against DAP-resistant strains and were able to demonstrate the “see-saw” effect with DAP and oxacillin.<sup>127</sup> Moreover, the combination of DAP and  $\beta$ -lactams has been used successfully in several cases of recalcitrant *S. aureus* bacteremia that fails DAP and other therapies. For instance, a recent report documented 24 cases (20 MRSA, two MSSA, and two VISA strains) in which the combination of DAP plus ceftaroline was successfully used to clear the bloodstream of patients who had failed other therapeutic strategies (i.e., high-dose DAP monotherapy or vancomycin).<sup>139</sup>

The combination of DAP plus  $\beta$ -lactams has also shown promising results in recalcitrant enterococcal infections. For example, the addition of subinhibitory concentrations of ampicillin prevented development of DAP-R in an *in vivo* model of experimental endocarditis.<sup>158</sup> Additionally, the addition of ceftriaxone to DAP (6 mg/kg) in a SEV model was able to prevent the appearance of DAP-resistant isolates of both vancomycin-resistant *E. faecalis* and *E. faecium*.<sup>140</sup> With enterococci, the two  $\beta$ -lactams that appear to be the best at obtaining synergism are ampicillin and ceftaroline.<sup>105,137,151,157</sup> Importantly, the additive effect with ampicillin was observed regardless of the presence of high-level ampicillin resistance,<sup>157</sup> and the addition of ceftaroline has been shown to restore DAP susceptibility in DAP-resistant *E. faecium* and *E. faecalis*.<sup>138,151,160</sup> The evidence for the use of other  $\beta$ -lactam com-

pounds is less abundant, but Smith *et al.*<sup>138</sup> recently performed time-kill assays with DAP plus several members of the  $\beta$ -lactam family and concluded that cefepime, ceftriaxone, and ertapenem were also synergistic against a DAP-resistant *E. faecium* strain, while ceftazidime and ceftotaxime were not. In terms of clinical evidence, there are a handful of cases in which the addition of ampicillin<sup>106,144,157,161</sup> or ceftaroline<sup>151</sup> was successfully used as salvage therapy in patients who were otherwise failing DAP treatment.

The mechanistic bases of the DAP– $\beta$ -lactam synergistic effect are not well understood. It has been postulated that exposure to  $\beta$ -lactams results in a decrease in the net positive charge of the bacterial surface, which results in an increase of DAP binding to the CM target.<sup>147,151,157</sup> Of note, two important caveats of this synergistic effect deserve to be discussed. First, Berti *et al.*<sup>141</sup> provided evidence showing that the additive effect of  $\beta$ -lactams might not be a drug class effect, but rather is compound specific. Furthermore, they observed that the synergistic effect was mostly seen with  $\beta$ -lactam molecules that target PBP-1 in *S. aureus* and suggested that the specific PBP activity profile of each compound would play an important role in the ability to potentiate DAP's antibacterial effect.<sup>141</sup> Second, recent reports by Diaz *et al.*<sup>105</sup> and Hindler *et al.*<sup>137</sup> independently showed that the synergistic effect of the ampicillin–DAP combination was only observed against DAP-resistant/tolerant *E. faecium* isolates harboring *liaFSR* mutations, but no such effect was described in the presence of changes in the YycFG system (and absence of *liaFSR* mutations). Thus, synergistic activity may depend on the genetic pathway leading to DAP-R in each particular strain. Interestingly, ampicillin enhanced the activity of LL-37 (a human CAMP) against DAP-resistant *E. faecium*, regardless of the genetic pathway.<sup>137</sup>

Finally, evidence to support the use of combination therapy with DAP and other antimicrobials is scarce. Steed *et al.*<sup>145,162</sup> analyzed the use of DAP plus trimethoprim sulfamethoxazole in an SEV model and reported synergism against DAP-resistant MRSA, including hVISA and VISA strains. In addition, the same combination was used to manage two cases of vertebral osteomyelitis caused by a DAP-resistant VISA isolate.<sup>162</sup> Lastly, a case of DAP-resistant MRSA bacteremia was successfully treated

with DAP plus rifampin after failing DAP monotherapy (6 mg/kg).<sup>163</sup>

## Concluding remarks

Studies of the mechanisms of action and resistance of DAP have uncovered an intricate relationship between Gram-positive bacterial adaptive processes and host innate immune response. The development of DAP-R appears to be a “built-in” response in these bacteria to the presence of CAMPs, including DAP. Although our understanding of DAP has evolved in the last several years, data related to the molecular mechanism(s) of resistance, treatment of DAP-resistant infections, and alternative strategies to overcome DAP-R remain limited. As DAP usage continues to increase in clinical settings, especially in severe infections caused by Gram-positive pathogens, the threat of DAP-R will likely only escalate. Studies on the mechanistic bases of DAP-R are likely to yield novel insights into bacterial CM adaptation and lead to development of innovative therapeutic strategies to combat MDR Gram-positive infections.

## Conflicts of interest

The authors declare no conflicts of interest.

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