MECHANISMS OF RESISTANCE TO LIPOPEPTIDE ANTIBIOTICS IN GRAM-POSITIVE PATHOGENS

by

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Dedicated to my parents.

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by

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MECHANISMS OF RESISTANCE TO LIPOPEPTIDE ANTIBIOTICS

IN GRAM POSITIVE PATHOGENS

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The University of Texas at Dallas, 2017

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Daptomycin (DAP) and surotomycin (SUR) are lipopeptide antibiotics with bactericidal activity against Gram-positive pathogens. Their addition into clinicians' antibiotic repertoire was an important step in combating increasing numbers of multi-drug resistant (MDR) infectious agents. DAP has been shown to have potent activity against vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). However, resistance to DAP has emerged over the last 20 years. Resistance to moderate concentrations of DAP (4-32 µg/mL) has been demonstrated in Gram-positive model organisms such as *S. aureus* and *B. subtilis.* This resistance is normally a step-wise process involving multiple genes working together. Recently, studies on the viridans group streptococci (VGS) and their response to DAP have demonstrated that rapid mechanisms of resistance can develop in these opportunistic pathogens. Specifically, VGS exposed to DAP overnight are capable of acquiring resistance to high concentrations of DAP (> 256 µg/mL). SUR has been approved to treat *C. difficile* associated diarrhea (CDAD), a disease with a high rate of recurrence which places a large economic burden on the healthcare industry. SUR has been shown to be more effective than the current CDAD treatment regimen of

vancomycin. However, like DAP, resistance to SUR has also been observed in laboratory and clinical settings. In my research, I analyzed the genomes of DAP-resistant VGS *S. mitis* and *S. oralis*, as well as SUR-resistant *C. difficile* and *E. faecium*. Using comparative genomics, I identified polymorphisms occurring in resistant populations. Briefly, mutations identified in SUR-resistant *C. difficile* and *E. faecium* are similar to mutations previously identified in studies of DAP resistance, suggesting that these two lipopeptides share a common mechanism of action. Additionally, I identified a mutation in a single gene (*cdsA*) within the VGS that conferred resistance to DAP. To date, mutations in this gene have not been seen, as *cdsA* is considered to be essential for bacterial growth. *cdsA* encodes the enzyme phosphatidate cytidylyltransferase (CdsA), which is responsible for the conversion of phosphatidic acid (PA) to CDP-diacylglycerol (CDP-DAG). CDP-DAG is the common precursor for the synthesis of all major phospholipids found in bacterial membranes. Using lipidomics, I confirm that these *cdsA* mutations result in a loss-of-function phenotype, as demonstrated by the accumulation of PA in the membranes of *S. mitis* and *S. oralis* and the depletion of the lipids phosphatidylglycerol and cardiolipin. Taken together, my research provides critical insight into the mechanisms of resistance to the clinically relevant antibiotics DAP and SUR, as well as providing evidence that *S. mitis* and *S. oralis* share a unique physiology that allows them to survive CdsA loss-of-function mutations.

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ABC	ATP-binding cassette
BHI	Brain heart infusion
C-55P	Undecaprenyl phosphate
CA-SMHA	Calcium supplemented Mueller Hinton agar
CA-SMHB	Calcium supplemented Mueller Hinton broth
CDAD	*C. difficile* associated diarrhea
CDI	*C. difficile* infection
CDP-DAG	Cytidine diphosphate diacylglycerol
CL	Cardiolipin
DAG	Diacylglycerol
DAP	Daptomycin
DHDAG	Dihexosyldiacylglycerol
FA	Fatty acid
FMN	Flavin mononucleotide
gDNA	Genomic DNA
GI	Gastrointestinal
HAI	Hospital associated infection
IE	Infectious endocarditis
LC/MS	Liquid chromatography coupled with mass spectrometry
LPG	Lysyl-phosphatidylglycerol
LTA	Lipoteichoic acid
MDR	Multi-drug resistant
MHDAG	Monohexosyldiacylglycerol
MIC	Minimum inhibitory concentration
MLSA	Multi-locus sequence analysis
MOA	Mechanism of action
MRSA	Methicillin-resistant *Staphylococcus aureus*
ORF	Open reading frame
PA	Phosphatidic acid
PAHN	Phosphatidyl-N-acetylhexosamine
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PS	Phosphatidylserine
RAST	Rapid annotation using subsystems technology
REA	Restriction endonuclease analysis
RIF	Regions of increased fluidity
SE	Single-end
SUR	Surotomycin
TE	Tris-EDTA

THB	Todd Hewitt broth
TmCdsA	CdsA protein from *Thermotoga maritima*
Tn-seq	Transposon mutagenesis with whole genome sequencing
TSA	Tryptic soy agar
VAN	Vancomycin
VGS	Viridans group streptococci
VRE	Vancomycin-resistant enterococci
wHTH	Winged helix-turn-helix

CHAPTER 1

INTRODUCTION

1.1 Author contributions

Hannah Adams prepared this chapter. Hannah Adams and Kelli Palmer edited it.

1.2 Lipopeptide antibiotics: daptomycin and surotomycin

1.2.1 History

In 1985, a novel class of acidic peptide antimicrobial agents was established. The first member of this class was a compound termed A21978C$_1$, a cyclic polypeptide antimicrobial derived from *Streptomyces roseosporus* [1]. Preliminary evidence suggested that this compound had activity against Gram-positive bacteria, including *Enterococcus* and *Streptococcus* species, and

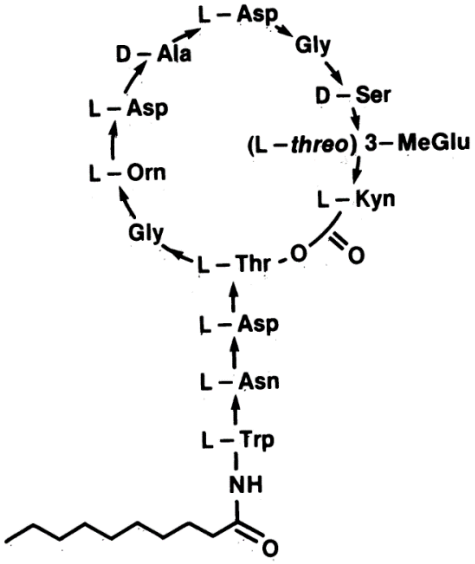

Figure 1.1. Structure of daptomycin. Orn, ornithine; MeGlue, methyl glutamic acid; Kyn, kynurenine. Taken with permission from [2].

methicillin-resistant *Staphylococcus aureus* (MRSA). The original compound was then modified by the replacement of the fatty acid acyl group with an *n*-decanoyl side chain to generate LY146032 [3]. This compound was eventually called daptomycin (DAP; see Figure 1.1).

Like its predecessor, DAP showed potent activity against Gram-positive pathogens [3-5]. DAP has a minimum inhibitory concentration (MIC) ≤1 μg/mL against *Staphylococcus* and *Streptococcus* species, with the exception of Viridans Group Streptococci (MIC range up to 2 μg/mL); MIC values against *Enterococcus* species ranged up to 4 μg/mL [6].

DAP also showed activity against *Clostridium difficile*, however, it was not fully bactericidal [7]. Derivatives of lipopeptide antibiotics were screened for bactericidal activity against *C. difficile* and compound CB-183,315 was a promising agent [8]. CB-183,315 (surotomycin; SUR) has the same peptide sequence as DAP, but the *n*-decanoyl moiety was replaced with an aromatic ring containing an unsaturated lipid tail (see Figure 1.2). While SUR's efficacy against *C. difficile* infections was comparable to that of vancomycin (VAN) [9], it spared *Bacteroides* spp. in rat gut models, suggesting it might not perturb the human microflora as much as alternative treatment methods [10].

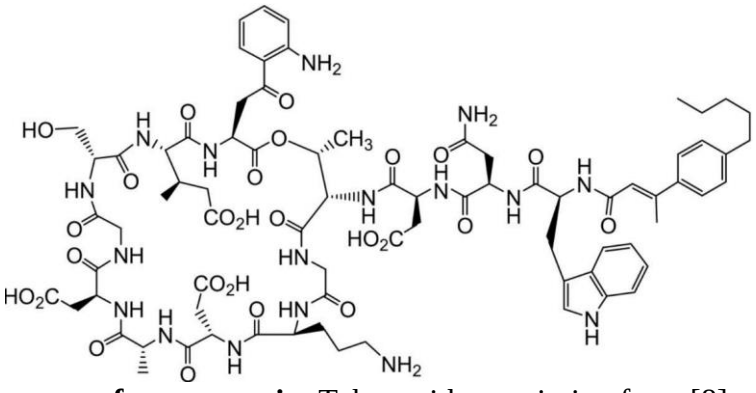

Figure 1.2. Structure of surotomycin. Taken with permission from [8].

1.2.2 Mechanism of action

The exact mechanism of action (MOA) for DAP has yet to be elucidated, as several competing hypotheses have been purposed over the years. It is known that DAP requires calcium supplementation for its activity [1]. Early studies on the MOA of DAP demonstrated that DAP did not affect the incorporation of precursors in protein, RNA, or DNA synthesis [2]. The same study reported that DAP was able to block the incorporation of peptidoglycan precursors, suggesting that inhibition of peptidoglycan synthesis was a potential MOA. In 1990, Canepari et al reported that DAP was able to bind to bacterial cell membranes and cell walls in the presence of Ca^{2+} [11]. They also showed that synthesis of lipoteichoic acid (LTA) was significantly inhibited in the presence of MIC of DAP.

Around the same time, a competing theory on the MOA of DAP was published. These studies suggested that DAP's bactericidal activity was mediated by the dissipation of the membrane potential of *S. aureus* and *Bacillus megaterium* [12-14]. The researchers argued that the collapse of the membrane potential explained the myriad of effects that DAP had on bacterial cells. Laganas et al reevaluated the effect of DAP on the synthesis of LTA in *S. aureus*, *Enterococcus faecalis*, and *Enterococcus hirae* [15]. They demonstrated that the inhibition of LTA caused by DAP was not significantly different than the indirect inhibition of LTA synthesis by rifampin, which prevents transcription by inhibiting RNA polymerase. In addition, they showed that DAP did not bind LTA. These results added credence to the hypothesis that DAP was killing cells via collapse of the bacterial membrane potential.

These data and those described further below led to the development of an early model for DAP MOA (Figure 1.3). Briefly, DAP complexes with free Ca^{2+} ions. This binding increases the overall charge of the DAP molecule, and allows it to interact with the anionic bacterial cell membrane. Jung et al demonstrated that DAP undergoes two conformational changes, the first when DAP complexes with Ca^{2+}, and the second when DAP/Ca^{2+} inserts into the membrane [16]. Insertion of DAP into the bacterial membrane only occurred in negatively charged liposomes, specifically liposomes composed of phosphatidylglycerol (PG), and not liposomes composed of the positively charged phosphatidylcholine (PC) [16]. The model then proposes that DAP oligomerizes at the membrane to cause ion leakage and membrane depolarization. This bactericidal activity is not dependent on cell lysis in *S. aureus* [17]. In addition, DAP is bactericidal against exponential and stationary phase cultures, though the latter requires a higher concentration [18].

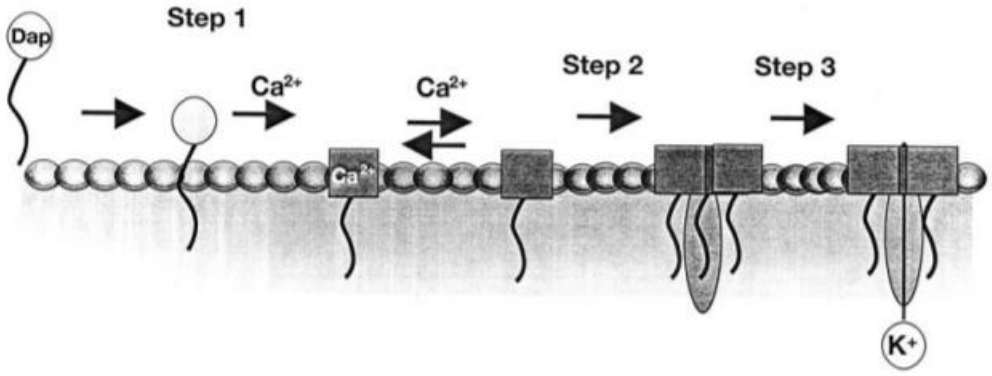

Figure 1.3. One proposed model for the mechanism of action of daptomycin. DAP complexed with Ca^{2+} binds and oligomerizes at the cell membrane, leading to pore formation, ion leakage, and eventual cell death. Figure taken with permission from [14].

However, the hypothesis that DAP was causing cell death via membrane depolarization remained contentious, as some researchers argued that membrane depolarization was a side effect of cellular death rather than the cause [16]. Researchers began to try and reconcile the differing effects DAP

had on bacterial cells, i.e. membrane depolarization and inhibition of cell wall synthesis. In *Bacillus subtilis*, it was shown using fluorescent microscopy techniques that DAP oligomerization at the membrane caused patches of altered membrane curvature, leading to the recruitment of the membrane protein DivIVA [19]. DivIVA plays a large role in regulating the proper septation of actively dividing *B. subtilis* [20]. These data led to the creation of a revised model for the mechanism of action of DAP (see Figure 1.4).

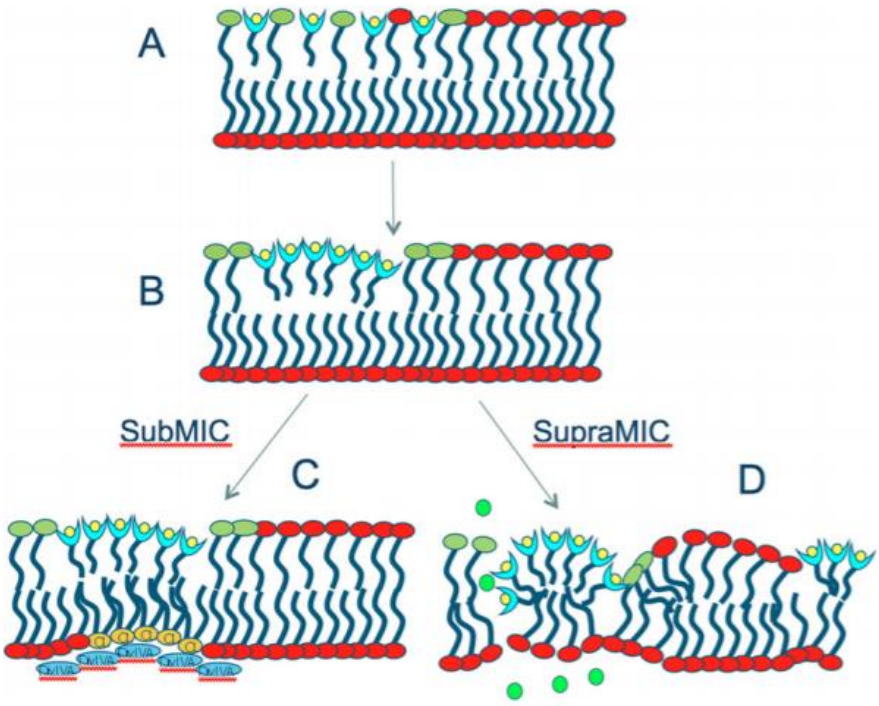

Figure 1.4. Daptomycin causes alterations to the membrane curvature and structure. Blue cup, DAP; green lipids, PG; yellow lipids, cardiolipin; blue ovals, DivIVA; green circles, potassium ions. Taken with permission from [19].

Briefly, DAP binds and oligomerizes at the membrane as previously thought. The shape of the DAP molecule causes patches of altered membrane curvature, which is compensated for by reorganization of membrane lipids. These curved regions recruit the cell division protein, DivIVA,

which incorrectly identifies the region as a site for cellular division, thereby affecting downstream processes including peptidoglycan biosynthesis [19]. In higher concentrations of DAP, it is hypothesized that the abundance of aberrant curvature regions cannot be compensated for, and the membrane integrity is compromised, leading to cell leakage and depolarization [19].

Recent evidence suggests that the true MOA is more nuanced. Using the model organism *B. subtilis*, Müller et al attempted to address the individual components of previous models [21]. The researchers found no evidence that DAP caused discrete membrane pores. Membrane depolarization did occur in sub-lethal concentrations of DAP, but it occurred over the course of ~30 minutes. Perhaps most strikingly, they found no evidence to suggest that DAP altered membrane curvature, and suggested that the previous findings with DivIVA were due to complexed GFP molecules rather than aberrant membrane curvature. Using fluorescently labeled DAP, Müller et al found that DAP preferentially binds to the membrane in regions of increased fluidity (RIFs). It stands to reason that RIFs would be better able to accommodate DAP insertion due to their less rigid composition. This discovery led to the most recent proposed model of DAP MOA (see Figure 1.5). Essentially, the binding of DAP at RIFs results in membrane reorganization and displacement of integral membrane proteins involved in peptidoglycan and phospholipid synthesis (MurG and PlsX, respectively). Recruitment of fluid lipids to the DAP binding sites increases the rigidity of the remaining membrane, and prevents accurate localization of MurG and PlsX, which leads to inhibition of their biosynthetic processes. This model explains how membrane binding by DAP results in inhibition of cell wall synthesis and eventual cell death and membrane depolarization. It is unknown at this time whether results in *B. subtilis* are broadly generalizable to other Gram-positive bacteria, or whether species-specific variations in MOA occur.

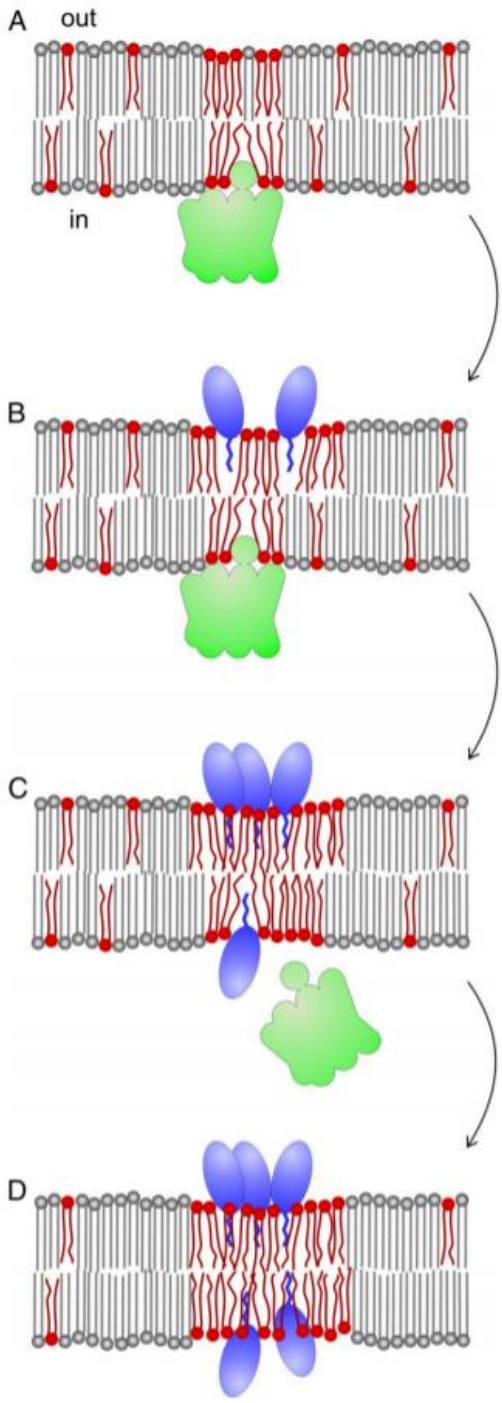

Figure 1.5. Daptomycin binds to membrane fluid domains and alters membrane structure. Fluid lipids are depicted in red, DAP is depicted in blue, integral membrane proteins are depicted in green. See text for step-by-step details. Taken with permission from [21].

Like DAP, SUR has potent activity against many multi-drug resistant (MDR) Gram-positive pathogens, including MRSA, VAN-resistant enterococci (VRE), and *Clostridium* species [22]. Few studies on the MOA have been reported. Most studies have focused on its ability to target *C. difficile* infections (CDI). Classically, VAN and metronidazole have been used to treat CDI, and in comparison, SUR out-performs both drugs in the killing of exponential- and stationary-phase *C. difficile* [23]. Studies have also demonstrated that SUR is capable of dissipating the membrane potential of *S. aureus* [8] and *C. difficile* [24] without inducing pore formation in the membrane. Researchers argued that SUR would have a similar MOA as that of DAP, due to the similarity in the structures of the antibiotics [8]; however, it is important to note that recent studies are suggesting a MOA of DAP that focuses on membrane fluidity and displacement of integral membrane proteins to cause cell death. More in-depth studies are needed to confirm the MOA of SUR since the consensus on the MOA of DAP has evolved over time.

1.3 Gram-positive pathogens investigated in this dissertation

1.3.1 *Clostridium difficile*

C. difficile is a Gram-positive, anaerobic bacillus capable of producing toxins and forming spores. It was originally linked to disease in 1978 when it was found in the stool samples of patients with antibiotic-associated diarrhea [25]. *C. difficile* infection (CDI; or *C. difficile* associated diarrhea, CDAD) occurs after the normal gastrointestinal microbiota has been perturbed, most commonly by antibiotic therapy [26, 27]. This fact has led CDI to be considered a hospital-associated infection (HAI). However, recent studies have identified a strain of *C. difficile* capable of causing

community-associated CDAD; patients reported in these studies had no prior health-care facility exposure despite suffering from CDAD [28, 29].

A novel strain of *C. difficile* was identified in association with epidemic outbreaks in Canada with increased mortality rates [30]. This strain, named NAP1/027, was found to produce higher levels of toxin A and B, most likely contributing to the increased severity of infection. Analysis of the fully sequenced infectious *C. difficile* 630 [31] revealed resistances to tetracycline, erythromycin, and β-lactams, among others [32]. The economic burden of CDAD in the US has been estimated to reach over $1 billion annually [33, 34]. Due to economic and healthcare associated burdens of CDAD, continuing research on novel and effective treatments methods are of great importance.

1.3.2 *Enterococcus faecalis* and *Enterococcus faecium*

E. faecalis and *E. faecium* are Gram-positive, opportunistic pathogens which normally reside as commensal inhabitants of the human gastrointestinal tract. Enterococci are capable of causing life-threatening endocarditis, catheter-associated urinary tract infections, and central line-associated bloodstream infections [35, 36]. In a study of HAIs recorded between 2009 and 2010, *Enterococcus* species were reported to be the 2nd leading cause of nosocomial infections, with *E. faecalis* and *E. faecium* being responsible for 6.8% and 4.1% of infections, respectively [36]; note that *C. difficile* infections were not included in this study. While *E. faecium* was not as prevalent as *E. faecalis*, it can prove much more difficult to treat, as over 80% of *E. faecium* infectious isolates are resistant to the last-line antibiotic VAN (compared with ~9% of *E. faecalis* isolates which are VAN resistant; [36]).

E. faecium is intrinsically resistant to β-lactam antibiotics, making the evolution of VAN resistance particularly concerning [37]. DAP has potent activity against Gram-positive pathogens, including *E. faecium*, however, resistance is beginning to arise (see section 1.4). The prevalence of enterococcal infections, specifically those caused by MDR *E. faecium*, highlights the importance of research into novel treatment methods and mechanisms of resistance.

1.3.3 Viridans group streptococci

The Viridans Group Streptococci (VGS) are a genetically diverse group of Gram-positive bacteria which are normal inhabitants of the human oropharyngeal tract. The VGS are opportunistic pathogens and are responsible for life-threatening bacteremia and infective endocarditis in immuno-suppressed patients [38, 39]. To date, there are six groups of VGS: *Streptococcus mutans* group, *S. salivarius* group, *S. anginosus* group, *S. mitis* group, *S. sanguinis* group, and *S. bovis* group [40]. Generally, VGS are classified based on the following phenotypic characteristics: catalase-negative, leucine aminopeptidase positive, pyrrolidonylarylamidase negative, and failure to grow in 6.5% NaCl [41]. Despite these parameters, there are still misidentification of the VGS as *S. pneumoniae* isolates depending on the biochemical assay used [42].

More recently, laboratories have attempted to use molecular sequencing methods to identify *Streptococcus* species, namely 16S rDNA sequencing. However, this method has proved insufficient for the identification of the clinically relevant *S. pneumoniae*, *S. mitis*, and *S. oralis*, as these strains share 99% sequence similarity in their 16S regions [43]. Multilocus sequencing analysis (MLSA) concatenates internal sequences of 6 to 7 house-keeping genes and uses the nucleotide sequences to classify isolates to a species level [44]. MLSA has been shown to be

effective for identification of VGS [45], however, this method is not feasible for routine usage in a clinical laboratory [46]. Single-gene analysis techniques have been shown to be reliable and feasible for the identification of VGS to the species level [47, 48], however, the possibility of rampant horizontal gene transfer between members of the streptococci can limit these methods [49, 50].

1.4 Mechanism of resistance to lipopeptide antibiotics in Gram-positive pathogens

There have been many studies on the mechanism of resistance to DAP in *S. aureus*, *B. subtilis*, and the enterococcal species *E. faecalis* and *E. faecium*. The literature supports the hypothesis that changes in membrane composition can lead to DAP resistance [51-56]. Specifically, mutations which reduce the level of PG in the membrane lead to increased DAP MIC values. Originally, it was shown that oligomerization of DAP was dependent on the presence of PG in bacterial and artificial membranes [57, 58]. Since then, several researchers have shown that mutations which alter the membrane lipid composition or the cell's response to membrane stress result in DAP resistance. This section will discuss the common mutations found in DAP-resistant strains of *B. subtilis*, *S. aureus*, and the enterococci.

Hachmann et al originally demonstrated a correlation between PG and DAP activity in *B. subtilis* [57]. In continuation of their work, they evolved a highly DAP-resistant (27.5 µg/mL) strain of *B. subtilis* and identified a point mutation in the PG synthesis gene *pgsA* [54]. Analysis of total lipid content confirmed that this mutation resulted in a reduction of PgsA function, as the resistant strain had >5-fold lower levels of PG when compared to the wild-type strain. Introduction of the wild-

type allele for *pgsA* in the resistant mutant restored DAP susceptibility and PG levels, indicating that PG content is directly related to DAP susceptibility.

In 2006, Friedman et al evolved three DAP-resistant strains of *S. aureus* from the susceptible parental strain MW2 [59]. Each of the three derived strains possessed mutations in *mprF*, *yycG*, and either *rpoB* or *rpoC*, with the *mprF* mutation occurring either before or on the day of an increasing shift in the DAP MIC value. While the role of *yycG* and *rpoBC* mutations in DAP resistance is still unclear, several researchers have linked mutations in *mprF* to DAP resistance in *S. aureus* [52, 60-62]. It is important to note, however, that not all DAP-resistant *S. aureus* isolates possess an *mprF* mutation, indicating that there are multiple routes to resistance in *S. aureus* [60, 62]. *mprF* encodes for Multiple Peptide Resistance Factor, which is responsible for the lysinylation of PG with L-lysine to generate lysylphosphatidylglycerol (LPG) [63, 64]. The mutations in *mprF* lead to a gain-of-function as indicated by increased expression levels and increased levels of LPG within the membrane [65, 66].

The common DAP resistance-associated mutations in the enterococci differ from than those identified in *B. subtilis* and *S. aureus*. This section will focus on two genes or operons (*cls* and *liaFSR*) that have been associated with DAP resistance in *E. faecalis* and *E. faecium*. The first studies to identify mutations associated with DAP resistance in the enterococci occurred in 2011 [51, 56]. Arias et al utilized a DAP-susceptible and a DAP-resistant strain pair isolated from a patient after DAP therapy [51]. Whole genome sequencing identified deletion mutations in *liaF*, which encodes one part of a three-component regulatory system that plays a role in cell membrane stress response to antimicrobials, and in *cls*, which encodes cardiolipin (CL) synthase. Allelic

replacement in the DAP-susceptible parent with the mutated *liaF* sequence resulted in a DAP MIC increase. Palmer et al evolved DAP-resistant *E. faecalis* via serial passaging. Analysis of whole genome sequencing data identified nonsynonymous mutations in *cls* [56]. There is experimental evidence that *cls* mutations also occur in DAP-resistant *E. faecium* [51, 56, 67].

The exact mechanism by which these mutations confer resistance is still debated. Palmer et al identified a R218Q substitution in the Cls protein of a DAP-resistant derivative of *E. faecalis* V583 [56]. This mutation was also seen in other DAP-resistant strains of *E. faecalis* and *E. faecium* [51, 68]. In *E. faecalis*, overexpression of the mutated allele in a DAP-susceptible background led to DAP resistance [56], however, allelic replacement with the same mutation in *E. faecium* did not significantly alter the DAP MIC levels [68]. This could be attributed to the differences in membrane content between *E. faecalis* and *E. faecium*. Additional studies on the Cls^{R218Q} enzyme indicate that these mutations confer increased activity [53]. An increase in the activity of CL synthesis could alter the membrane composition enough to effect DAP binding and function, perhaps by reducing the overall levels of PG. These data suggest that there is more to DAP resistance in enterococci than a single amino acid substitution; perhaps many mutations working in concert are required for DAP resistance in these bacteria.

Since the identification of the *liaF* mutation in *E. faecalis*, studies have shown a strong correlation with increased DAP MICs and mutations in the *liaFSR* operon [69]. Tran et al demonstrated that the deletion of isoleucine 177 in LiaF results in the redistribution of CL domains within the cell membrane, thus altering the location of DAP binding [70]. Follow-up studies indicate that this redistribution is mediated through the LiaR response regulator; deletion of *liaR* in a *liaF177* mutant

background restored wild-type CL distribution [71]. It is still unclear whether or not the *liaFSR* stress response system plays a direct role in DAP resistance or if it indirectly leads to these phenotypic changes through its downstream regulon. As the exact mechanism of action of DAP becomes better understood, the role each of these mutations play in DAP resistance should become clearer.

1.5 Goals and summary of dissertation

Due to the increasing incidence of antibiotic resistance in pathogenic bacteria, the need for novel treatments is as pressing as ever. A deeper understanding of the mechanisms of resistance to antibiotics is a necessary step in identifying potential therapeutic targets and understanding the pitfalls of antibiotic usage. SUR is a novel treatment method for CDI and has been suggested as an adequate alternative antibiotic in the treatment of MDR *C. difficile* and enterococci. Even though resistance to SUR is still rare, we sought to identify genes associated with SUR resistance in the hopes of better understanding the MOA of SUR. Using whole genome sequencing techniques and comparative bioinformatics, we were able to identify polymorphisms in SUR-resistant strains of *C. difficile*, *E. faecalis*, and *E. faecium* (see Chapter 2). In each species, we identified mutations that were previously associated with DAP resistance, indicating that SUR possesses a similar mechanism of action.

As with *C. difficile* and *E. faecium*, antibiotic resistance in the VGS is on the rise. Resistance to classically used antibiotics such as penicillin [72], the fluoroquinolones [73], and macrolides [74] has been reported in VGS isolates. Novel treatment methods for VGS infections are required. In addition, due to their commensal nature, the VGS will come into contact with antibiotics used to

treat other infectious agents, and their response to clinically relevant antibiotics is of concern. Since DAP has been shown to be effective in the treatment of other MDR Gram-positive bacteria, its efficacy against VGS was of interest. In addition, the possibility of the commensal VGS becoming reservoirs for DAP resistance was a concern. In previous studies, exposure of *S. mitis* and *S. oralis* to DAP led to the development of high-level resistance overnight [75, 76].

In my doctoral research, I aimed to identify the mechanism of resistance to DAP in *S. mitis* and *S. oralis*. I generated three independent VGS strains (one *S. mitis* and two *S. oralis*) with high-level (>256 µg/mL) resistance to DAP (see Chapter 3). These strains were subjected to whole genome sequencing and analysis in order to identify polymorphisms associated with DAP resistance in the VGS. These analyses revealed that all three strains possessed a nonsynonymous mutation in the gene *cdsA*. Bioinformatic analyses predicted that all of these mutations led to a loss-of-function of the expressed protein, phosphatidate cytidylyltransferase (CdsA). CdsA is responsible for the enzymatic conversion of phosphatidic acid (PA) to CDP-diacylglycerol (CDP-DAG), which is the precursor for phospholipid synthesis in prokaryotes. Using liquid chromatography coupled with mass spectrometry to analyze the lipid content of the DAP resistant VGS, the loss-of-function in CdsA was confirmed, as evidenced by the accumulation of PA and the depletion of phospholipids such as PG and CL. These data point to a novel mechanism of DAP resistance not seen in previous studies of *Enterococcus*, *Bacillus*, and *Staphylococcus* species. In addition, the inactivation of *cdsA*, which is considered to be an essential gene [77-83], suggests that *S. mitis* and *S. oralis* possess unique physiology compared to other Gram-positive model organisms.

To date, this research is the only to investigate mutations associated with SUR resistance, and supports previous research that suggests SUR has a MOA similar to that of DAP. In addition, my studies on DAP resistance in the VGS is one of the first to identify a novel mechanism of resistance and suggest that *cdsA* is not essential in certain VGS species. Together, this research provides critical insight into the mechanisms of resistance to the last line antibiotics DAP and SUR, and contributes to the understanding of the MOA of these drugs.

CHAPTER 2

MUTATIONS ASSOCIATED WITH REDUCED SUROTOMYCIN SUSCEPTIBILITY IN *CLOSTRIDIUM DIFFICILE* AND *ENTEROCOCCUS* SPECIES

2.1 Author contributions

The original manuscript was published along with Xiang "Farry" Li (FL), Carmela Mascio (CM), Laurent Chesnel (LC), and Kelli L. Palmer (KP). Hannah Adams (HA) and FL contributed equally to this work. Experimental design was carried out by HA, FL, and KP. *C. difficile* serial passaging and gDNA extraction were carried out by CM and LC [84]. Bioinformatic analysis of *C. difficile* 2994 and *E. faecium* strain pairs was carried out by HA. FL performed the bioinformatics analysis for *C. difficile* 2179, 2989, and *E. faecalis* 201 strain pairs. Analysis of *E. faecalis* 807 was performed by KP. HA, FL, and KP prepared the manuscript. The republication of the manuscript was done with permission from FL and ASM per the ASM Journals Statement of Author's Rights. volume 59, 2015, pages 4139-4147, doi: 10.1128/AAC.00526-15.

2.2 Abstract

Clostridium difficile infection (CDI) is an urgent public health concern causing considerable clinical and economic burdens. CDI can be treated with antibiotics, but recurrence of the disease following successful treatment of the initial episode often occurs. Surotomycin is a rapidly bactericidal cyclic lipopeptide antibiotic that is in clinical trials for CDI treatment and that has demonstrated superiority over vancomycin in preventing CDI relapse. Surotomycin is a structural analogue of the membrane-active antibiotic daptomycin. Previously, we utilized *in vitro* serial

passage experiments to derive *C. difficile* strains with reduced surotomycin susceptibilities. The parent strains used included ATCC 700057 and clinical isolates from the restriction endonuclease analysis (REA) groups BI and K. Serial passage experiments were also performed with vancomycin-resistant and vancomycin-susceptible *Enterococcus faecium* and *Enterococcus faecalis*. The goal of this study is to identify mutations associated with reduced surotomycin susceptibility in *C. difficile* and enterococci. Illumina sequence data generated for the parent strains and serial passage isolates were compared. We identified nonsynonymous mutations in genes coding for cardiolipin synthase in *C. difficile* ATCC 700057, enoyl-(acyl carrier protein) reductase II (FabK) and cell division protein FtsH2 in *C. difficile* REA type BI, and a PadR family transcriptional regulator in *C. difficile* REA type K. Among the 4 enterococcal strain pairs, 20 mutations were identified, and those mutations overlap those associated with daptomycin resistance. These data give insight into the mechanism of action of surotomycin against *C. difficile*, possible mechanisms for resistance emergence during clinical use, and the potential impacts of surotomycin therapy on intestinal enterococci.

2.3 Introduction

Clostridium difficile is a Gram-positive anaerobic spore-forming bacterium and a causative agent of health care-associated infections (HAIs). *C. difficile* infection (CDI; alternatively called *C. difficile*-associated diarrhea) can occur after disruption of the normal microbiota of the gastrointestinal (GI) tract, thereby decreasing colonization resistance and allowing for *C. difficile* outgrowth [27]. Toxin production by *C. difficile* can lead to severe disease. Over the last two decades, the incidence of CDI has increased dramatically, especially among elderly health

care patients [27, 85, 86]. A recent Centers for Disease Control and Prevention report on antibiotic resistance threats in the United States classifies *C. difficile* as an urgent public health threat, as it causes an estimated 250,000 infections per year, leading to an estimated 14,000 deaths and at least $1 billion in medical costs [86].

CDI can be treated with antibiotics, but recurrence can occur after a successful therapy and is a persistent concern [85]. Vancomycin, metronidazole, and the recently FDA-approved drug fidaxomicin are used to treat CDI [27, 85, 87]. Surotomycin (CB-183,315) is a cyclic lipopeptide antibiotic and daptomycin analogue that is currently in phase 3 clinical trials for CDI treatment. Surotomycin is dosed orally, not absorbed, and rapidly bactericidal against *C. difficile* [8, 84]. Surotomycin is likely to have a mechanism of action similar to that of daptomycin, as treatment with either antibiotic leads to membrane depolarization but not increased membrane permeability in *Staphylococcus aureus* and *C. difficile* [8, 88]. Importantly, the MIC values of surotomycin for normal GI tract microbiota such as *Bacteroides* species and enterobacteria are higher than surotomycin concentrations recorded *in situ* during clinical trials [89]. This suggests that surotomycin therapy leaves these commensal populations intact, which could restore colonization resistance and reduce the likelihood of CDI relapses. In accordance with this, surotomycin was superior to vancomycin in CDI recurrence prevention in phase 2 clinical trials [90]. Note that although *C. difficile* is susceptible to daptomycin *in vitro* [7, 91], the drug is not approved to treat CDI. The oral bioavailability of daptomycin given intravenously is limited, probably due to poor GI permeability (Cubist Pharmaceuticals, unpublished data).

A recent study utilized *in vitro* serial passage experiments to derive *C. difficile* strains with reduced surotomycin susceptibilities [84]. *Enterococcus faecalis* and *Enterococcus faecium* were

also investigated in that study [84]. Like *C. difficile*, enterococci colonize the human GI tract [92], are common causative agents of HAIs [36, 93, 94], and are susceptible to surotomycin [22, 84]. Further, daptomycin is commonly used to treat difficult enterococcal infections, and several studies have identified genetic variations in enterococci that are associated with reduced daptomycin susceptibility [55]. This makes enterococci useful comparators for surotomycin susceptibility studies. Here, we used Illumina sequencing to identify mutations associated with reduced surotomycin susceptibility in *C. difficile*, *E. faecalis*, and *E. faecium*. These data give insight into the mechanism of action of surotomycin against *C. difficile*, possible mechanisms for resistance emergence during clinical use, and the potential impacts of surotomycin therapy on GI tract enterococci.

2.4 Materials and methods

2.4.1 Bacterial strains

Bacterial strains used in this study are shown in Table 2.1, and their derivation was previously described [84]. Parent strains were used as inocula for *in vitro* serial passage experiments with surotomycin. Pass strains are isolates obtained by colony purification from day 15 of serial passage experiments. Pass strains have decreased susceptibility to surotomycin relative to the parent strains. Prior to genome sequencing, pass strains were passaged three times in drug-free medium and retested for surotomycin susceptibility to ensure stability of the surotomycin MIC. Surotomycin MICs of parent and pass strains are shown in Table 2.1.

2.4.2 Enterococcal genomic DNA (gDNA) isolation

Enterococcal strains were cultured on brain heart infusion (BHI) agar at 37°C, and isolated colonies were used to inoculate BHI broth. Genomic DNA (gDNA) was isolated from overnight cultures using a modified version of a previously published protocol [95]. Cells were washed once with Tris-EDTA (TE) buffer (pH 8.0; 10 mM Tris-HCl, 1 mM EDTA) and resuspended in 180 µl enzymatic lysis buffer (pH 8.0; 20 mM Tris-HCl, 2 mM sodium EDTA, 1.2% [vol/vol] Triton X-100) amended with 20 mg/ml lysozyme, 10 µl of a 2.5 kU/ml mutanolysin stock, and 15 µl of a 10 mg/ml preboiled RNase A stock. Samples were incubated at 37°C for 1 to 2 h prior to proteinase K treatment and column purification of gDNA using the Qiagen DNeasy blood and tissue kit per the manufacturer's instructions.

2.4.3 *C. difficile* gDNA isolation

C. difficile stocks were passaged once on brucella agar, and a single colony was used to inoculate thioglycolate broth. Liquid cultures were grown for 48 h prior to gDNA extraction using a modified phenol-chloroform prep. Three milliliters culture was split into three 1.5-ml microcentrifuge tubes and pelleted at 14,000 × *g* for 2 min. Pellets were resuspended in 500 µl of phosphate-buffered saline and combined into one tube for a second round of centrifugation. The cell pellets were then resuspended in 570 µl of a resuspension buffer (60 µl of 500 mM EDTA, 30 µl of a 20-mg/ml lysozyme stock, 30 µl of a 10-kU/ml mutanolysin stock, 30 µl of a 10-mg/ml lysostaphin stock, 420 µl water). Solutions were incubated at 37°C for 30 min, and then 30 µl 10% (wt/vol) SDS was added. Solutions were incubated at 37°C for an additional 30 min. An equal volume of TE-saturated phenol-chloroform-isoamyl alcohol (Sigma) was added, tubes were mixed by inversion,

and aqueous and organic layers were separated by centrifugation. The aqueous fraction was extracted again and then extracted once with chloroform alone. The gDNA was ethanol precipitated and the pellet resuspended in 100 µl of DNA rehydration solution. Prior to Illumina sequencing, *C. difficile* gDNA samples were treated with RNase A as described above and then treated with proteinase K and column purified using the Qiagen DNeasy blood and tissue kit per the manufacturer's instructions.

2.4.4 Illumina genome sequencing

Illumina library preparation and sequencing were performed at the Tufts University DNA Core Facility. All gDNA samples were treated as low-abundance samples, and libraries were prepared using the NuGen Ovation Ultralow DR Multiplex System 1-96 kit. The average library fragment size was ~390 bp, with high variability in fragment sizes due to low DNA inputs. Libraries were multiplexed and sequenced using the Illumina HiSeq 2500, yielding paired reads of 151 × 2 bases. Single-end (SE) reads were used for analysis.

2.4.5 Mutation detection in pass genomes

SE reads from parent strains were assembled into draft contigs using CLC Genomics Workbench default parameters. Contigs were annotated using the Rapid Annotation using Subsystem Technology (RAST) server with default parameters (rast.nmpdr.org) [96, 97]. Draft genome assemblies are summarized in Table A.1 in the supplemental material. SE reads from pass strains were mapped to RAST-annotated parent assemblies using CLC Genomics Workbench with default mapping parameters. Sequence variations were identified using the "Quality-based variant detection" tool in CLC Genomics Workbench with default parameters (≥10-fold coverage of the

reference position and sequence variation in ≥35% of mapped reads). All variations were manually analyzed. All variations that fell within two read lengths (300 bp) from a contig end were removed from consideration. Next, the variant frequency was considered. All sequence variations occurring with a frequency of ≥90% (occurring in ≥90% of mapped reads) were confirmed to be true mutations by additional PCR and Sanger sequencing (discussed below). For variations occurring with <90% frequency, parent and pass strain read assemblies were manually inspected. If sequence variation was observed in the pass but not parent strain assembly, PCR and additional Sanger sequencing were performed. In all instances, either these were found to not be true sequence variations or sequence heterogeneity occurred in both parent and pass strains (data not shown). Finally, the "Find low coverage" tool in CLC Genomics Workbench was used to identify regions of zero coverage in pass strain read mappings, which represent possible large deletions. These regions were manually inspected, and PCR and additional Sanger sequencing were performed where appropriate.

2.4.6 Analysis of Efs807 sequence reads

A complete reference sequence is available for Efs807 (V583) [98]. Single-end reads from the Efs807 parent and pass samples were mapped to the Efs807 chromosome (GenBank accession number AE016830) and three plasmids (pTEF1, GenBank accession number AE016833; pTEF2, GenBank accession number AE016831; and pTEF3, GenBank accession number AE016832) using CLC Genomics Workbench with default mapping parameters. Read assemblies are summarized in Table A.2 in the supplemental material. Sequence variations were identified using the "Quality-based variant detection" tool in CLC Genomics Workbench with default parameters as described above. Variants occurring in nonspecific regions of the read assemblies (rRNA

operons, insertion sequences, etc.) were ignored. Previously described sequence variations occurring in the Efs807 parent strain relative to the GenBank reference [56] were removed from analysis.

2.4.7 Confirmation of mutations

Primers were designed to amplify genomic regions that included putative mutations. Primers used are shown in Table A.5 in the supplemental material. gDNA was used as the template for a 50-µl PCR volume with Phusion polymerase (Fermentas). Reactions were performed for parent and pass strains to confirm absence of variation in the parent strains. PCR products were purified with the Qiaquick PCR purification kit (Qiagen) and sequenced at the Massachusetts General Hospital DNA Core Facility (Boston, MA).

2.4.8 Bioinformatic analysis of candidate genes

Transmembrane helices were predicted by TMHMM, version 2.0 [99]. Subcellular location was predicted by Psortb, version 3.0 [100]. RNA secondary structure prediction was from RNAfold [101]. Pfam 27.0 [102] and NCBI Conserved Domains were used for analysis of conserved protein domains and for substrate-binding and catalytic sites. *C. difficile* 630 [32, 103], *E. faecalis* V583 [98, 104], and *E. faecium* DO [105] are commonly used model strains, and we identified homologues of our genes of interest in those strains using NCBI BLAST (Table 2.2 and Table A.3 in the supplemental material). For consistency with existing literature on *C. difficile* 630, in the text we express locus identifiers in the "CD####" format.

2.4.9 Sequence data generated in this study

Sequence reads generated in this study have been deposited in the NCBI Sequence Read Archive, and the accession numbers can be found via BioProject record number PRJNA281633.

2.5 Results and discussion

2.5.1 Strains used for Illumina sequencing analysis

In a previous study, *C. difficile*, *E. faecalis*, and *E. faecium* strains were serially passaged in broth in the presence of surotomycin for a period of 15 days [84]. The protocol for and results of the serial passage experiments were previously reported [84]. *C. difficile* strains used in passage experiments included an American Type Culture Collection quality control strain (ATCC 700057; referred to as Cdi2179 in this study) and two clinical isolates obtained during the phase 2 dose-ranging study (LCD-DR-09-03) [90]. One clinical isolate (Cdi2989) is from the restriction endonuclease analysis (REA) type BI, representing the epidemic *C. difficile* strain [27, 85], and the other (Cdi2994) is REA type K. Vancomycin-resistant and vancomycin-susceptible *E. faecalis* and *E. faecium* strains obtained from the ATCC were similarly used as parents for serial passage (Table 2.1). For genome sequencing, a single colony isolate obtained from each serial passage culture at day 15 was passaged three times in drug-free medium and then retested for surotomycin MIC to confirm stable reduced susceptibility to the drug. We refer to these strains as pass strains (Table 2.1). Note that strains with other genotypes may have coexisted in the day 15 serial passage cultures (i.e., representing other mutational paths to reduced surotomycin susceptibility). Therefore, our selection of only a single isolate from each serial passage experiment for genome sequencing is a possible limitation of our study.

Table 2.1. Bacterial strains used in this study[a]

Bacterium and Strain[b]	Description	MIC (µg/mL) SUR	VAN	DAP	FDX	MET
C. difficile						
Cdi2179 (ATCC 700057)	Quality control strain for MIC testing	0.5	1	1	0.125	0.25
Cdi2179 Pass	Day 15 serial passage isolate	8	1	8	0.0625	0.25
Cdi2989	REA type BI isolate from phase 2 clinical trial	0.5	1	1	0.125	0.5
Cdi2989 Pass	Day 15 serial passage isolate	8	1	16	0.0625	0.125
Cdi2994	REA type K isolate from phase 2 clinical trial	0.5	2	1	0.0625	0.25
Cdi2994 Pass	Day 15 serial passage isolate	4	2	8	0.0625	0.25
E. faecalis						
Efs201 (ATCC 49452)	VSE	0.5	1	2	NT	NT
Efs201 Pass	Day 15 serial passage isolate	2	1	16	NT	NT
Efs807 (ATCC 700802)	VRE	1	64	2	NT	NT
Efs807 Pass	Day 15 serial passage isolate	4	>64	32	NT	NT
E. faecium						
Efm14 (ATCC 6569)	VSE	0.5	0.5	4	NT	NT
Efm14 Pass	Day 15 serial passage isolate	8	0.25	64	NT	NT
Efm277 (ATCC 51559)	VRE	1	>64	4	NT	NT
Efm277 Pass	Day 15 serial passage isolate	16	0.5[c]	>64	NT	NT

[a]Abbreviations: SUR, surotomycin; VAN, vancomycin; DAP, daptomycin; FDX, fidaxomicin; MET, metronidazole; VSE, vancomycin-sensitive *Enterococcus*; VRE, vancomycin-resistant *Enterococcus*; NT, not tested.
[b]Alternate strain names are shown in parentheses. Pass strains were generated by Mascio et al. [84].
[c]Efm277 became vancomycin sensitive over the course of serial passage [84].

MIC data for parent and pass strains are shown in Table 2.1. Susceptibility to ampicillin and rifampin was not affected in pass strains (data not shown). For all strain pairs, pass strains have elevated surotomycin and daptomycin MICs relative to their parents.

To identify mutations occurring in pass strains, we generated Illumina sequence data from genomic DNA isolated from parent and pass strains. Draft genome assemblies were generated for the parent strains (see Table A.1 in the supplemental material), and sequence reads obtained for pass strains were mapped to the draft parent assemblies to identify sequence variations. Because a complete

reference genome is available for the vancomycin-resistant *E. faecalis* strain Efs807 (also called V583) [98, 104], Efs807 parent and pass sequence reads were mapped to the existing GenBank reference sequence (see Table A.2 in the supplemental material). For all genome comparisons, candidate mutations identified from read assemblies were confirmed to be true mutations by independent Sanger sequencing. Data are summarized in Table 2.2 (see also Table A.3 in the supplemental material). To facilitate comparisons with the laboratory model strains *C. difficile* 630 [32, 103], *E. faecalis* V583 [98, 104], and *E. faecium* DO [105], NCBI BLAST was used to identify orthologues of genes of interest in those strains (Table 2.2; see also Table A.3 and Table A.4 in the supplemental material).

2.5.2 Mutations associated with reduced surotomycin susceptibility in *C. difficile*

Mutations in *cls* (encoding cardiolipin synthase), *fabK* (encoding enoyl-ACP reductase II, where ACP is acyl carrier protein), *ftsH2* (encoding ATP-dependent membrane-bound metalloprotease), and a gene encoding a PadR family transcriptional regulator were detected among the *C. difficile* surotomycin pass strains (Table 2.2).

Cdi2179 Pass. The Cdi2179 pass strain has a surotomycin MIC of 8 μg/ml, a 16-fold increase over that of the parent (Table 2.1). A G-to-A transition was detected in a gene coding for a predicted cardiolipin synthase (Cls; TIGR04265 bac_cardiolipin family; E value, $2.72e^{-117}$). This mutation results in a D79N substitution in the Cls protein. Cls proteins synthesize cardiolipin (bisphosphatidylglycerol), a phospholipid that is enriched in septal and polar regions of Gram-negative and Gram-positive bacterial cells [106-108]. As expected for a Cls protein, two catalytic

Table 2.2. Mutations occurring in surotomycin serial passage strains[a]

Bacterium and strain	Description of gene	Nucleotide variation in gene (frequency of variation in assembly)[b]	Amino acid change in predicted protein	Best BLASTP hit in reference genomes
C. difficile				
Cdi2179 pass	**Cardiolipin synthase**	**G235A (599/603)**	**D79N**	**CD3404 Cls**
Cdi2989 pass	Enoyl-ACP reductase	G434T (849/850)	G145V	CD1180 FabK
	Cell division protein FtsH	G1387T (868/868)	E463Stop	CD3559 FtsH2
Cdi2994 pass	Transcriptional regulator PadR	G17T (102/102)	R6I	CD1345
E. faecium				
Efm14 pass	**HD family hydrolase**	**29-39del**	**E10fs**	**HMPREF0351_11908; EF2470**
	Aminopeptidase S	C1065A (826/828)	F355L	HMPREF0351_10886
	ATP-dependent nuclease subunit A	C2283G (776/777)	H761Q	HMPREF0351_11280 AddA; EF1113 RexA
	Alpha/beta family hydrolase	C582T (788/789)	Silent	HMPREF0351_11299; EF1536
	Hypothetical protein	A165G (828/830)	Silent	HMPREF0351_11264
	Intergenic region between two glycosyl transferase genes	C→G (631/631)	NA[d]	HMPREF0351_10908-HMPREF0351_10909
	Intergenic region upstream of catabolite control protein A	T→A (443/444)	NA	HMPREF0351_12002 CcpA
Efm277 pass	**Cardiolipin synthase**	**G632T (345/350)**	**R211L**	**HMPREF0351_11068 Cls; EF0631 Cls**
	HD family hydrolase	**159delA (132/136)**	**A53fs**	**HMPREF0351_11908; EF2470**
	Ribosomal large subunit methyltransferase A	**G801T (160/160)**	**K267N**	**HMPREF0351_12412 RrmA; EF2666 RrmA**
	RNA polymerase, beta subunit	**T2789G (661/670)**	**V930G**	**HMPREF0351_12666 RpoB; EF3238 RpoB**
	Lead, cadmium, zinc, mercury transporting ATPase	C552G (199/203)	F184L	HMPREF0351_11880 CopB; EF0875
	Sucrose-6-phosphate hydrolase	C700A (260/273)	P234T	Not present in *E. faecium* DO; EFA0069 ScrB-2

	Intergenic region upstream of putative phage repressor	GA→AG (105/105)	NA	Not present in *E. faecium* DO; Efm408 EFUG_02666
E. faecalis				
Efs201 pass	**Sensor histidine kinase LiaS**	**G330A (1115/1117)**	**M110I**	**EF2912[c]**
	Fe-S cluster binding subunit	G509T (849/868)	G170V	EF1109
Efs807 pass	EF0797 hypothetical protein	T155C (715/718)	L52P	EF0797
	EF1027 MprF2	**G85A (485/486)**	**A29T**	**EF1027 MprF2**
	EF1797 hypothetical protein (DrmA)	**449delA (565/608)**	**N150fs**	**EF1797 DrmA**
	Intergenic region downstream of EF1367	31 bp del	NA	EF1367

[a]Boldface indicates that the gene or pathway has been associated with daptomycin resistance in *Enterococcus faecalis*, *Enterococcus faecium*, and/or *Staphylococcus aureus*. See the text and Data Set S1A in the supplemental material for extended analysis.
[b]Shown in parentheses are raw sequence variation data from pass strain read assemblies, indicating the number of mapped reads with indicated sequence variation/total coverage at that position. del, deletion.
[c]The Efs201 LiaS protein is N-terminally truncated relative to the V583 LiaS protein. See Data Set S2 in the supplemental material for the Efs201 LiaS sequence.
[d]NA, not applicable.

HKD (phospholipase D) motifs are present in the Cdi2179 Cls, as are two predicted N-terminal transmembrane helices (see Figure A.1 in the supplemental material). The D79N substitution in the Cdi2179 pass strain Cls occurs outside transmembrane helix 2, between the helix and HKD motif 1. This substitution may result in altered synthesis and/or localization of cardiolipin in the *C. difficile* membrane. No *C. difficile* strains currently deposited in GenBank possess the D79N substitution in Cls.

Mutations in *cls* have been detected in daptomycin-resistant *E. faecalis*, *E. faecium*, and *S. aureus* strains (see Table A.3 in the supplemental material) [51, 56, 62, 67, 68, 109, 110]. The fact that a *cls* mutation arose in Cdi2179 during surotomycin exposure suggests that commonalities exist in the way the two drugs interact with cell surfaces and/or in the ways that cells cope with stresses imposed by these drugs.

Cdi2989 Pass. The Cdi2989 pass strain has a surotomycin MIC of 8 μg/ml, a 16-fold increase over that of the parent. The Cdi2989 pass strain possesses two G-to-T transversions. The first occurs in a gene coding for a predicted enoyl-ACP reductase II (TIGR03151 enACPred_II family; E value, $1.3e^{-142}$), resulting in a G145V substitution in the protein. The second is a nonsense mutation occurring in a gene coding for the membrane-bound metalloprotease FtsH (TIGR01241 FtsH_fam; E value, 0).

The enoyl-ACP reductase proteins FabK and FabI catalyze the final step in the bacterial type II fatty acid elongation cycle [111, 112]. FabI is highly conserved among many bacteria; however, some bacteria lack FabI and instead possess FabK [113, 114]. FabK and FabI are distinct in sequence [112], and the enoyl-ACP reductase identified in the Cdi2989 pass strain belongs to the

FabK family. *C. difficile* does not possess *fabI* (see Table A.4 in the supplemental material); therefore, *fabK* is likely to be an essential gene in *C. difficile*. The *fabK* gene is significantly upregulated (4-fold) in *C. difficile* 630 during growth with subinhibitory amoxicillin [115].

FabK utilizes flavin mononucleotide (FMN) as a cofactor and NADH as an electron donor [113] and is similar in sequence to members of the 2-nitropropane dioxygenase-like enzyme family [112]. Using the 2-nitropropane dioxygenase-like proteins as references, we used conserved domain analysis to map FMN-binding residues and a catalytic site to the Cdi2989 FabK sequence (see Figure A.1 in the supplemental material). The G145V substitution in the Cdi2989 pass strain occurs near the putative catalytic site of the enzyme (at position H143). The G145V substitution could alter FabK activity and membrane fatty acid composition in the Cdi2989 pass strain.

The second mutation in the Cdi2989 pass strain occurs in a gene annotated as *ftsH2* in *C. difficile* 630 (Table 2.2; see also Table A.3 in the supplemental material). The *ftsH2* gene was reported to be significantly upregulated (1.5-fold) in *C. difficile* 630 cells during growth with subinhibitory levels of either amoxicillin or metronidazole [115]. FtsH is essential for *Escherichia coli*, in which it is important for turnover of misfolded membrane proteins, among other functions [116]. Less is known about its function in Gram-positive bacteria. In *Bacillus subtilis* and *Lactobacillus plantarum*, *ftsH* is nonessential, but inactivation has a number of effects on the cell, including a reduced ability to cope with extracellular stresses [117, 118] and filamentous growth and a sporulation defect in *B. subtilis* [118]. In *B. subtilis*, deletion of *ftsH* leads to induction of the σ^{w} regulon, which is involved in extracytoplasmic stress response [119].

For Cdi2989 FtsH2, two N-terminal transmembrane helices, an AAA domain (ATPase family associated with various cellular activities; E value, $1.5e^{-42}$), and a peptidase domain (peptidase_M41; E value, $5.7e^{-77}$) are predicted (see Fig. S1 in the supplemental material). The protease active-site motif HEXXH [116] is present. The *ftsH2* mutation in the Cdi2989 pass strain generates a premature stop codon and a 194-amino-acid C-terminal truncation of the FtsH2 protein. The premature stop codon occurs within the peptidase domain of FtsH2, after the HEXXH motif. This inactivating mutation could lead to pleiotropic effects on *C. difficile*.

Three observations link FtsH to mechanisms associated with reduced daptomycin (and now, surotomycin) susceptibility. First, FtsH is localized to the division septum in *B. subtilis* [120]. Second, YvlB is among the proteins produced in elevated amounts in a *B. subtilis ftsH* mutant [119]; frameshift mutations in the *yvlB* homologue in *E. faecalis*, EF1753, are present in daptomycin-resistant *E. faecalis* strains [56, 110]. Finally, *L. plantarum ftsH* mutant and overexpression strains have an altered surface charge relative to that of the wild type [117]. All of these observations suggest that FtsH has roles in division septum architecture, surface charge modulation, and/or phospholipid metabolism in Gram-positive bacteria.

Since the Cdi2989 parent strain was a clinical isolate [84], it is possible that mutations identified in the Cdi2989 pass strain are actually reversion mutations that occurred as a result of *in vitro* growth (i.e., they could be unrelated to surotomycin exposure). The *fabK* mutation in the Cdi2989 pass strain is unlikely to be a reversion, since *C. difficile* strains currently deposited in GenBank, like the Cdi2989 parent strain, possess G145 in orthologues of FabK. Similarly, truncated FtsH2 proteins are not encoded by *C. difficile* GenBank strains.

Cdi2994 Pass. The Cdi2994 pass strain has a surotomycin MIC of 4 μg/ml, an 8-fold increase over that of the parent. The Cdi2994 pass strain possesses a G-to-T transversion in a gene coding for a predicted PadR family transcriptional regulator (Pfam E value, $2.1e^{-20}$), resulting in an R6I substitution in the protein. To distinguish this protein from the three other PadR family transcriptional regulators encoded by *C. difficile*, we have named this protein SurR. The mutation in *surR* does not appear to be a reversion, since *C. difficile* strains currently deposited in GenBank possess R6 in orthologues of SurR.

PadR-like transcriptional regulators are widespread in bacteria, but few have been experimentally characterized (reviewed by Fibriansah et al. [121]). These proteins possess an N-terminal winged helix-turn-helix (wHTH) DNA binding domain and a C-terminal domain of variable size that participates in dimerization. A model PadR family transcriptional regulator is *Lactococcus lactis* LmrR [122]. LmrR represses expression of *lmrCD* genes, which are cotranscribed with *lmrR* [123, 124] and encode an ATP-binding cassette (ABC) transport system that confers multidrug resistance [123-125]. Mutations in *lmrR* can lead to constitutive *lmrCD* expression and multidrug resistance [125]. Crystal structures are available for LmrR [122] and for two *Bacillus cereus* PadR-like regulator proteins, which also regulate proximally borne genes with likely roles in drug transport [121]. Cdi2994 SurR is shown in Fig. S1 in the supplemental material with the predicted wHTH DNA binding domain mapped. The R6I substitution in the Cdi2994 pass strain occurs just within the wHTH domain. This position was identified as being important for protein dimerization in *B. cereus* PadR-like regulators [121]. The R6I substitution in SurR may lead to derepression of genes in the SurR regulon.

Based on bioinformatic analysis, we hypothesize that SurR regulates the expression of itself and two downstream genes, as well as a predicted diacylglycerol glucosyltransferase gene that is divergently transcribed from *surR*. The organization of this region is identical in Cdi2994 and *C. difficile* 630. In *C. difficile* 630, CD1345 encodes SurR, CD1346 encodes a predicted membrane protein possessing three central transmembrane helices (RhaT family; E value, $2.04e^{-3}$), and CD1347 encodes a predicted membrane protein with a single N-terminal transmembrane helix. No intergenic regions are present between these genes, suggesting that they are cotranscribed. The *surR* gene is divergently transcribed from CD1344, which encodes a predicted diacylglycerol glucosyltransferase (PRK13609; E value, $9.67e^{-49}$). Because *C. difficile* 630 produces a number of glycolipids [126], this provides a potential link between SurR and membrane structure and biogenesis. Global gene expression comparisons between the Cdi2994 parent and pass strains as well as specific investigation of the CD1344 gene would be of interest for future studies.

2.5.3 Enterococci with reduced surotomycin susceptibilities

Each of the four enterococcal surotomycin pass strains possesses a mutation in at least one gene previously associated with daptomycin resistance (Table 2.2; see also Table A.3 in the supplemental material). The locations of amino acid substitutions in enterococcal proteins are shown in Figures A.2 to A.5 in the supplemental material. Because Efs807 (V583) has been used previously for the selection of daptomycin-resistant derivatives [56], we discuss this model strain in depth.

Efs807 Pass. The Efs807 pass strain has a surotomycin MIC of 4 μg/ml, a 4-fold increase over that of the parent. Three nonsynonymous mutations and a 31-bp deletion in an intergenic region

were identified for this strain. Mutations occur in *mprF2* and *drmA* (EF1797), each of which has been associated with daptomycin resistance (see Table A.3 in the supplemental material) [56, 59, 62, 110].

EF1797 encodes a predicted membrane protein for which a biochemical function is unknown. In previous work [56], three daptomycin-resistant derivatives of Efs807 were generated by serial passage, and each was found to possess mutations in EF1797. The Efs807 surotomycin pass strain possesses a predicted frameshift (N150fs) in EF1797 (Table 2.2). An N150fs was recently reported for an *E. faecalis* daptomycin-adapted strain [110]. Collectively, mutations identified in EF1797 from daptomycin and surotomycin studies localize a region of interest in the protein: the QKNKNL sequence occurring between the predicted transmembrane helices 4 and 5 (see Figure A.2 in the supplemental material). As a result of the frameshift mutation in the Efs807 surotomycin pass strain, the amino acid sequence occurring between predicted TM helices 4 and 5 is QKIKTF. EF1797 has a limited phylogenetic distribution [56] and does not occur in *C. difficile* (see Table A.3 in the supplemental material).

MprF in *S. aureus* catalyzes the addition of lysine to phosphatidylglycerol moieties, thereby mitigating the negative charge of the outer surface of the cytoplasmic membrane [63]. Mutations in *mprF* are associated with daptomycin resistance emergence in *S. aureus* [59, 62, 127], are sufficient for daptomycin resistance [128], and confer gain of function [128, 129]. Enterococcal MprF2 has an expanded substrate range compared to *S. aureus* MprF, producing lysine-, alanine-, and arginine-modified phosphatidylglycerol moieties [130, 131]. The A29T substitution in the Efs807 pass strain occurs within the first predicted transmembrane helix of MprF2 (see Figure A.2 in the supplemental material). In *S. aureus*, the N-terminal transmembrane helices of MprF are

required for translocation of the modified lipid from the inner to outer membrane leaflet [132]. The A29T substitution in *E. faecalis* MprF2 could impact phospholipid flipping. MprF is not encoded by *C. difficile* (see Table A.3 in the supplemental material).

The other two mutations occurring in the Efs807 pass strain are in the EF0797 open reading frame (ORF) and in the intergenic region downstream of the EF1367 ORF. EF0797 encodes a predicted hypothetical protein in *E. faecalis* V583. Conserved domain analysis did not yield clues to its function. Analysis with Psortb failed to resolve a predicted cellular location for the EF0797 protein; however, one N-terminal transmembrane helix is predicted by TMHMM (see Figure A.2 in the supplemental material). The amino acid substitution (L52P) in the Efs807 pass strain occurs in a predicted extracellular region. The EF0797 orthologue in *E. faecalis* OG1RF is upregulated in cells treated with 1.25× MIC levels of the antibiotics ampicillin, bacitracin, cephalothin, or vancomycin, and deletion of EF0797 led to an increase in virulence in a *Galleria mellonella* infection model [133]. EF0797 may be involved in stress response to cell wall-active antimicrobials. EF0797 is not encoded by *C. difficile* (see Table A.3 in the supplemental material).

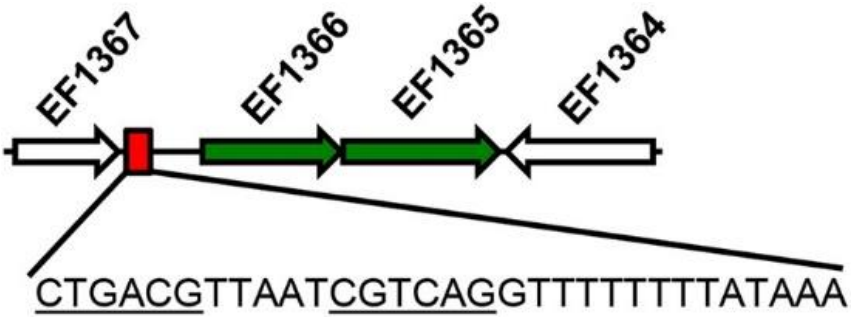

Figure 2.1. Deleted region in Efs807 (V583) pass strain. A region of the *E. faecalis* V583 genome encompassing ORFs EF1364 to EF1367 is shown. The figure is not drawn to scale. As described in the text, EF1367 encodes a predicted cold shock protein. EF1366 and EF1365 encode two membrane proteins of unknown function. The red box indicates where the 31-bp deletion in the Efs807 pass strain occurs. The deleted sequence is shown. The underlined sequence could base pair in mRNA to form a stem-loop structure.

A 31-bp deletion occurs in the Efs807 pass genome, downstream of the EF1367 gene (Figure 2.1). EF1367 encodes a cold shock domain-containing protein [134]. RNAfold analysis of the deleted 31-bp sequence revealed that the sequence is likely a Rho-independent terminator for EF1367. Deletion of the EF1367 transcription terminator may result in increased expression of the downstream genes EF1366 and EF1365, which are likely cotranscribed (16-bp intergenic region between the two genes). The EF1366 protein possesses six predicted transmembrane helices and a C-terminal YdcF-like domain (E value, $8.86e^{-40}$). Conserved domain analysis indicates that this protein may be involved in cell wall synthesis. The EF1365 protein possesses eight predicted transmembrane helices, and its function is unknown.

2.5.4 Summary and implications

Surotomycin, like daptomycin, dissipates the membrane potential of *S. aureus* and *C. difficile* [8, 88]. A recently proposed model for daptomycin's mechanism of action [19] posits that at sub-MIC levels and in the presence of phosphatidylglycerol and calcium, daptomycin inserts into the outer leaflet of the cytoplasmic membrane and aggregates. This process alters the local curvature of the outer leaflet, to which the cell responds by making compensatory changes in the inner leaflet, potentially including increased cardiolipin content. Cell division proteins and other proteins responsive to membrane ultrastructure changes are recruited to these sites, resulting in aberrant septation. At supra-MIC daptomycin levels, many of these sites are generated, and the cell is not able to mitigate the stress imposed on the membrane. This leads to dissipation of the membrane potential, among other deleterious effects, and ultimately cell death. Additional mechanistic

experiments with surotomycin and *C. difficile* will be required to determine whether surotomycin has similar phospholipid dependencies and impacts on membrane ultrastructure.

Daptomycin resistance is an active area of research. Most studies have focused on *S. aureus*, *B. subtilis*, *E. faecium*, and *E. faecalis*. Many different adaptive paths to resistance have been discovered for these species, and a few mechanistic models for resistance have been proposed and are well supported by experimental data (reviewed recently by Humphries et al. [55]). Collectively, many of these data indicate that modulation of membrane phospholipid content and localization (especially relative to the division septum and other sites of membrane curvature), cell surface charge, and the transcriptional networks responding to cell surface stress are each involved in daptomycin resistance. For enterococci, a recently proposed model by Tran et al. [70] centers on the LiaFSR signal transduction system and its regulon. In *B. subtilis*, this system perceives stress imposed by cell wall-active antimicrobials, resulting in differential expression of genes in the LiaR regulon [135-137]. Mutations in *liaFSR* genes are frequently detected in daptomycin-resistant enterococci [51, 69, 110, 138], and the LiaFSR system is activated and protective of *B. subtilis* in the presence of daptomycin [57]. In the study by Tran et al., a *liaF* mutation in *E. faecalis* led to redistribution of cardiolipin and diversion of daptomycin away from the division septum, resulting in low-level daptomycin resistance [70]. The underlying mechanism for this is likely dysregulation of as-yet-unknown genes in the LiaR regulon of *E. faecalis*. Additional mutations in *cls* and *gdpD* (encoding glycerophosphoryl diester phosphodiesterase) further increased the daptomycin MIC and altered the membrane phospholipid content of the cell [70]. In this study, we detected a *liaS* mutation in an *E. faecalis* strain with reduced surotomycin susceptibility (Table 2.2).

C. difficile possesses unique attributes that distinguish it from the species described above. First, the membrane structure of *C. difficile* appears to be distinct. *C. difficile* 630, like other clostridia [139], possesses membrane phospholipids with plasmalogen (having an ether, instead of ester, linkage at the sn-1 position of glycerol) fatty acid tails [126]. *C. difficile* 630 produces phosphatidylglycerol, cardiolipin, and monohexosyldiradylglycerol lipids of both plasmalogen and di- or tetra-acyl forms [126]. The relative ratios of these lipids are likely to impact *C. difficile* membrane fluidity, and the ratios may change in response to fluctuations in growth environment or in times of stress [126]. Whether these lipids are differentially distributed in the *C. difficile* membrane is unknown. The genes required for clostridial plasmalogen biosynthesis have not been identified [139]. Overall, the literature on *C. difficile* membrane structure and composition is very limited [126, 140]. While relevant for our surotomycin discussion, this also highlights a critical gap in knowledge about *C. difficile* physiology that is relevant to future drug development.

Other attributes that distinguish *C. difficile* from daptomycin resistance model species are that *C. difficile* lacks MprF and LiaF (see Table A.4 in the supplemental material). The absence of MprF suggests that *C. difficile* does not produce amino-acid-modified phospholipids, although this cannot be formally excluded using existing literature. Of note, an unusual amino-containing glycolipid has been identified in a study involving *C. difficile* 630, which the authors suggested could counterbalance the negative charges of phosphatidylglycerol and cardiolipin [126]. The absence of LiaF suggests that information on cell surface stress in *C. difficile* is transduced to the transcriptional machinery of the cell by a distinct mechanism. Proteins with identity to LiaSR, VraSR, and YycFG, each of interest for daptomycin resistance in other species [55], were

identified in *C. difficile* 630 (see Table A.4 in the supplemental material) but were not altered in the surotomycin passage strains studied here.

For each of the three *C. difficile* strain pairs studied here, mutations arose in genes with probable or potential roles in *C. difficile* membrane structure and biogenesis (*cls*, *fabK*, and *surR*). Allelic replacement studies will ultimately be required to confirm whether the mutations detected are responsible for surotomycin MIC increases.

For the enterococci studied here, each surotomycin pass strain possessed at least one mutation that also occurs in daptomycin-resistant strains (Table 2.2), and the pass strains have elevated daptomycin MICs (Table 2.1). This suggests that enterococcal strains with elevated daptomycin MICs could be cross-resistant to surotomycin, allowing for their outgrowth in the GI tract during surotomycin therapy for CDI. Alternatively, surotomycin therapy for CDI could select for daptomycin resistance-conferring mutations in GI tract enterococci. These possibilities should be evaluated with further *in vitro* and *in vivo* studies. We note also that isolates with high (128- to 256-fold) increases in daptomycin MICs emerged in daptomycin serial passage experiments with the vancomycin-resistant *Enterococcus* (VRE) species strain Efs807 [56]. Here, a relatively modest (4-fold) increase in surotomycin MIC was observed for the same parent strain over a similar period of passage. These differing results may be related to the oxygen status of *E. faecalis* in the serial passage experiments. Surotomycin passage experiments were performed under anaerobic conditions [84], while daptomycin passage experiments were performed under oxygenated conditions [56]. Interestingly, a recent metabolomic study found that *E. faecalis* membrane fatty acid content varies with oxygen status [141]. Further investigation of

enterococcal membrane physiology under various oxygen tensions could be informative for surotomycin and daptomycin studies.

2.6 Acknowledgments

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CHAPTER 3

STREPTOCOCCUS MITIS AND *S. ORALIS* LACK A REQUIREMENT FOR CDSA, THE ENZYME REQUIRED FOR SYNTHESIS OF MAJOR MEMBRANE PHOSPHOLIPIDS IN BACTERIA

3.1 Author contribution

This work was published with Luke Joyce (LJ), Ziqiang Guan (ZG), Ronda Akins (RA), and Kelli Palmer (KP) in *Antimicrobial Agents and Chemotherapy* in 2017 [142]. Hannah Adams (HA), LJ, and KP designed the experiments. HA and LJ performed the experiments. KP performed the lipid extractions and ZG performed the lipidomic analyses. HA and KP prepared the manuscript. Republication of the manuscript for a PhD dissertation is allowed per the ASM Journals Statement of Author's Rights. Copyright © American Society for Microbiology, Antimicrobial Agents and Chemotherapy, volume 61, 2017, doi: 10.1128/AAC.02552-16.

3.2 Abstract

Synthesis and integrity of the cytoplasmic membrane is fundamental to cellular life. Experimental evolution studies have hinted at unique physiology in the Gram-positive bacteria *Streptococcus mitis* and *S. oralis*. These organisms commonly cause bacteremia and infectious endocarditis (IE) but are rarely investigated in mechanistic studies of physiology and evolution. Unlike other Gram-positive pathogens, high-level (MIC ≥ 256 μg/mL) daptomycin resistance rapidly emerges in *S. mitis* and *S. oralis* after a single drug exposure. In this study, we find that inactivating mutations in *cdsA* are associated with high-level daptomycin resistance in *S. mitis* and *S. oralis* IE isolates. This is surprising given that *cdsA* is an essential gene for life in commonly studied model

organisms. CdsA encodes the enzyme responsible for the synthesis of cytidine diphosphate-diacylglycerol, a key intermediate for the biosynthesis of all major phospholipids in prokaryotes and most anionic phospholipids in eukaryotes. Lipidomic analysis by liquid chromatography/mass spectrometry (LC/MS) showed that daptomycin-resistant strains have an accumulation of phosphatidic acid and completely lack phosphatidylglycerol and cardiolipin, two major anionic phospholipids in wild-type strains, confirming the loss-of-function of CdsA in the daptomycin-resistant strains. To our knowledge, these daptomycin-resistant streptococci represent the first model organisms whose viability is CdsA-independent. The distinct membrane compositions resulting from the inactivation of *cdsA* not only provide novel insights into the mechanisms of daptomycin resistance, but also offer unique opportunities to study the physiological functions of major anionic phospholipids in bacteria.

3.3 Introduction

Streptococcus mitis and *S. oralis* are human oral colonizers, opportunistic pathogens, and species of the viridans group streptococci (VGS). VGS are associated with ~23% of Gram-positive bacteremia in immunocompromised patients [38, 143] and ~17% of infective endocarditis (IE) cases [39]. The *mitis* group VGS are difficult to accurately speciate in clinical settings [46, 48]. However, retrospective molecular studies have determined that *S. mitis* and *S. oralis* are major causative agents of VGS bacteremia and IE [73, 144-146]. Although unquestionably significant for human health, very little is known about the physiology and virulence of *S. mitis* and *S. oralis*. This is in part due to their close phylogenetic relationship with *S. pneumoniae* [147-152], to which *S. mitis* and *S. oralis* are often compared, but are rarely considered in their own right.

Recent research hints at unique physiology in *S. mitis* and *S. oralis* [75, 76, 153]. Daptomycin (DAP) is a cyclic lipopeptide antibiotic with potent activity against Gram-positive bacterial pathogens including vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) [154, 155]. Akins et al reported that high-level DAP resistance (defined as MIC ≥256 μg/mL) emerged in *S. mitis* and *S. oralis* IE isolates after a single DAP exposure in a simulated endocardial vegetation model [75]. High-level DAP resistance after a single drug exposure was also reported by García-de-la-Mària, et al. [76] for a collection of *mitis* group VGS. These results are surprising because DAP resistance in VRE, MRSA, and other model Gram-positive organisms typically emerges in a step-wise fashion by mutation accretion over days or weeks of DAP exposure [55, 156]. We infer that DAP resistance in *S. mitis* and *S. oralis* proceeds through a novel genetic mechanism compared to other Gram-positive bacteria.

Here, we use experimental evolution studies and lipidomics to show that one-step high-level DAP resistance in *S. mitis* and *S. oralis* is associated with loss of function mutations in *cdsA*, a gene for phospholipid biosynthesis that is essential for *Escherichia coli* [77], *Bacillus subtilis* [78], *Staphylococcus aureus* [79], better-studied streptococci including *S. pneumoniae* [80-83], and an organism with an engineered minimal genome [157]. We conclude that *S. mitis* and *S. oralis* possess unique physiology, allowing DAP resistance to emerge by a novel mechanism.

3.4 Materials and methods

3.4.1 Bacterial strains, media, and susceptibility testing

Bacterial strains used in this study are shown in Table 3.1. All testing was conducted in Mueller Hinton broth or agar supplemented with 50 μg/mL calcium and 12.5 μg/mL magnesium (CA-

SMHB and CA-SMHA, respectively) as required for DAP [158, 159], unless otherwise stated. Cultures were incubated at 37°C in a GasPak™ EZ Campy Container System (BD), unless otherwise stated. Etest (BioMérieux, Inc.) susceptibilities were carried out on tryptic soy agar (TSA) supplemented with 5% defibrinated horse blood (Remel) or on MHA.

3.4.2 Serial passage experiments with DAP

Parental strains (*S. mitis* 1643, *S. oralis* 1647, and *S. oralis* 1648) were cultured in 2 mL of CA-SMHB with DAP (2, 4, 8, and 16 µg/mL) overnight at 37°C. After 24 h of incubation, growth was observed in each inoculated well, regardless of the DAP concentration. Strains obtained from these cultures (referred to as HA0X; see Table 3.1) were confirmed to be DAP resistant and used for genome sequencing.

3.4.3 Genome sequence analysis

Genomic DNA from parental strains was isolated using Roche MagNA Pure per the manufacturer's instructions and sequenced with Illumina technology at GENEWIZ, Inc. Single end reads of 50 base pairs were obtained. Genomic DNA from resistant strains was isolated using a modified Qiagen Blood and Tissue DNeasy Kit protocol as previously described [160] and sequenced with Illumina technology at Molecular Research LP. Paired end, 2x300 bp reads were obtained. DAP resistance of the cultures was confirmed via Etest prior to genomic DNA extraction.

De novo draft genomes were assembled using CLC Genomics Workbench with default parameters (see Table B.1 in supplemental material) and annotated using Rapid Annotation using Subsystem Technology [97]. The taxonomic identification for the parental strains were confirmed using GyrB

typing [47]. Reads from the resistant strains were mapped to the draft parent genomes, and polymorphisms were detected using default parameters. Polymorphisms were manually curated. Polymorphisms occurring on contigs <500 bp, within 300 bp from a contig end, in rRNA or tRNA regions, or in polymorphic regions with sequence variation in both the parental *de novo* assembly and the resistant strain read mapping were removed from further analysis. This screening process generated a list of candidate mutations that were further curated by independent confirmation with Sanger sequencing. Primers were designed to amplify approximately 500 bp surrounding putative mutations (see Table B.2 in supplemental material). PCR with Taq polymerase (NEB) was used to amplify these regions from both parent and resistant strains. Products were sequenced at the Massachusetts General Hospital DNA Sequencing Core. Nucleotide sequences for wild-type and mutant loci of interest are in Text S1.

3.4.4 Bacterial growth curves

To quantify growth, brain heart infusion broth (BHI) was inoculated to an optical density at 600 nm (OD_{600}) of 0.05 from overnight cultures. The OD_{600} was monitored for parental and resistant strains every 60-75 min until cultures reached stationary phase. The experiments were performed independently three times. Revertant strains were monitored as above in 60 min intervals for two independent trials.

3.4.5 Reversion passage experiments

Resistant strains (*S. mitis* 1643-HA04, *S. oralis* 1647-HA06, and *S. oralis* 1648-HA08) were inoculated into 1 mL CA-SMHB in 1.5 mL Eppendorf tubes and cultured overnight. After each passage, 100 µL culture was used as inoculum for the next passage, 500 µL was used to generate

a frozen stock, and ~400 µL was spread on MHA for DAP Etest. Passaging was terminated when a zone of inhibition was observed on DAP Etest.

3.4.6 Spontaneous resistance incidence

Overnight cultures were used to inoculate BHI to an OD_{600} of 0.05. Cultures were incubated until mid-exponential phase, serially diluted, and 100 µL of culture or dilutions were plated on BHI agar, BHI agar with 50 µg/mL rifampin (n = 2 independent trials), CA-SMHA, and CA-SMHA with either 10 µg/mL DAP (n = 5 independent trials) or 128 µg/mL DAP (n = 3 independent trials). Plates were incubated for 20-24 h at 37°C in a chamber with a CO_2 gas pack prior to colony counting. For DAP tests, individual colonies were picked, and PCR and Sanger sequencing (Table B.2) were used to query the entire *cdsA* coding region as well as its putative promoter for mutations.

3.4.7 Lipodomic analysis

A single colony was picked into 50 mL Todd Hewitt broth (THB) and incubated at 37°C with 5% CO_2 overnight. The 50 mL cultures were added to pre-warmed 250 mL THB and incubated until an OD_{600nm} of ~0.6 was obtained. 500 µL was removed for DAP Etest on 5% Horse blood TSA plates. The remaining culture was pelleted at 10,000 rpm and 4°C. Cell pellets were stored at -80°C prior to lipid extraction by the Bligh and Dyer method [161]. Normal phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 µm, 25 cm x 2.1 mm (Sigma-Aldrich) as published previously [162, 163]. Briefly, mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v); mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide

(600:340:50:5, v/v); mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The LC eluent (with a total flow rate of 300 mL/min) was introduced into the ESI source of a high resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). Instrumental settings for negative ion ESI and MS/MS analysis of lipid species were as follows: IS= -4500 V; CUR= 20 psi; GSI= 20 psi; DP= -55 V; and FP= -150 V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software (Sciex, Framingham, MA).

3.4.8 Accession numbers

Illumina sequence reads generated in this study have been deposited in the Sequence Read Archive under the accession number PRJNA354070.

3.5 Results

3.5.1 Emergence of high-level DAP resistance in *S. mitis* and *S. oralis*

The IE clinical isolates *S. mitis* strain 1643 and *S. oralis* strains 1647 and 1648 achieved high-level DAP resistance after a single DAP exposure in both simulated endocardial vegetation and bacteremia models [75]. We performed serial passage experiments to determine whether DAP resistance would similarly emerge under standard laboratory culture conditions. Parental strains

were cultured with DAP (2, 4, 8, and 16 μg/mL). After 24 h of incubation, growth was observed in each inoculated well, regardless of the DAP concentration. Resistant strains obtained from these cultures are referred to as *S. mitis* 1643-HA04 (derived from parental strain 1643), *S. oralis* 1647-HA06 (derived from 1647) and *S. oralis* 1648-HA08 (derived from 1648) (Table 3.1). We conclude that high-level DAP resistance emerges in *S. mitis* and *S. oralis* IE isolates after a single DAP exposure, regardless of the incubation or environmental conditions.

Table 3.1. DAP MIC and CdsA of *S. mitis* and *S.* oralis strains used in this study

	Strain derivation[a]	DAP MIC (μg/mL)[b]	CdsA change[c]
S. mitis 1643	Wild-type IE isolate	0.75	(Q31)
S. mitis 1643-HA04	DAP resistant	>256	*31
S. mitis 1643-HA14	DAP revertant	0.75	W31
S. oralis 1647	Wild-type IE isolate	1	(G246)
S. oralis 1647-HA06	DAP resistant	>256	C246
S. oralis 1647-HA16	DAP revertant	1.5	S246
S. oralis 1648	Wild-type IE isolate	<0.5	(D249)
S. oralis 1648-HA08	DAP resistant	>256	N249
S. oralis 1648-HA18	DAP revertant	<0.5	D249

[a]DAP resistant strains obtained after wild-type strains were exposed to DAP. DAP revertants were obtained by passage of DAP resistant strains in drug-free medium.
[b]MIC values were determined by Etest on MHA plates.
[c]Wild-type amino acid shown in parentheses.

DAP resistance confers a modest growth defect on *S. mitis* and *S. oralis*. In standard laboratory medium, the resistant strains grew with generation times ≥ 20% longer than parental strains (see Figure B.1 in supplemental material).

3.5.2 Mutations in *cdsA* occur in DAP resistant strains

We used genome sequencing to identify mutations in the DAP resistant strains (Table 3.2). Non-synonymous mutations in *cdsA* occurred in all three strain pairs. CdsA catalyzes the conversion of

phosphatidic acid (PA) into cytidine diphosphate-diacylglycerol (CDP-DAG), which is the precursor for synthesis of the major membrane phospholipids, including phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylethanolamine (PE), and phosphatidylserine (PS) (Figure 3.1).

Table 3.2. Mutations occurring in DAP resistant strains

Strain	Description of gene	Nucleotide variation in gene[a]	Amino acid change	Locus ID[b]
S. mitis 1643-HA04				
	Hypothetical	G531T (98)	S177R	SK608_0120 (100, 100)
	Serine/threonine kinase	A526C (90)	K176Q	smi_1622 (45, 29)
	Ribosomal S5p alanine acyltransferase	G502T (98)	H168N	TZ92_01497 (100, 99)
	Phosphatidate cytidylyltransferase (CdsA)[c]	**G91A (99)**	**Q31Stop**	**smi_1854 (100, 81)**
S. oralis 1647-HA06				
	Intergenic region 13	C to T (99)	N/A	9 bp between SOR_1153-1154
	Intergenic region 17	A to C (99)	N/A	47 bp upstream of SOR_0277
	Phosphatidate cytidylyltransferase (CdsA)	**C736A (97)**	**G246C**	**SOR_1730 (100, 96)**
S. oralis 1648-HA08				
	ppGpp synthetase	A2075C (96)	V692G	SOR_1513 (100, 99)
	Helicase PriA	T77G (93)	E26A	SOR_1544 (100, 96)
	Ribosomal SSU methyltransferase	C454A (96)	V152F	SOR_1542 (100, 95)
	Phosphatidate cytidylyltransferase (CdsA)	**C745T (97)**	**D249N**	**SOR_1730 (100, 96)**

[a]The frequency of the mutation in the read assembly is shown in parentheses.
[b]Loci were identified using protein BLAST. Query coverage and percent amino acid identity are shown in parentheses. References used were *S. oralis* Uo5 (SOR_) [166], *S. mitis* B6 (smi_) [147], *S. mitis* SK608 (SK608_) [167], and *S. oralis* SK141 (TZ92_) [168].
[c]Bold type indicates that the mutation reverted after serial passage without DAP.

We analyzed the *cdsA* mutations to predict their impacts on CdsA function. *S. mitis* 1643-HA04 possesses a G91A transition that generates a premature stop codon after the 31st amino acid in the polypeptide chain. We predict that this mutation results in a complete loss of CdsA function in *S. mitis* 1643-HA04. *S. oralis* 1648-HA08 possesses a C745T transition that alters the 249th residue from aspartic acid to asparagine. A recently determined crystal structure of CdsA from *Thermotoga maritima* (TmCdsA) identified residues necessary for TmCdsA function [164]. D249 of the streptococcal CdsA aligns to an active site residue (D246) in TmCdsA which was shown to be required for CdsA activity [164]. Finally, *S. oralis* 1647-HA06 possesses a C736A transversion that converts the 246th residue from a glycine to a cysteine. Analysis of putative secondary structures for the peptide chain using the garnier tool in EMBOSS [165] reveals that G246 in 1647-HA06 occurs in a bend in the peptide (data not shown). Altering the flexible glycine residue to a rigid cysteine residue may prevent the polypeptide chain from folding properly. In summary, *cdsA* mutations in the DAP-resistant strains likely result in CdsA proteins that have loss of function by

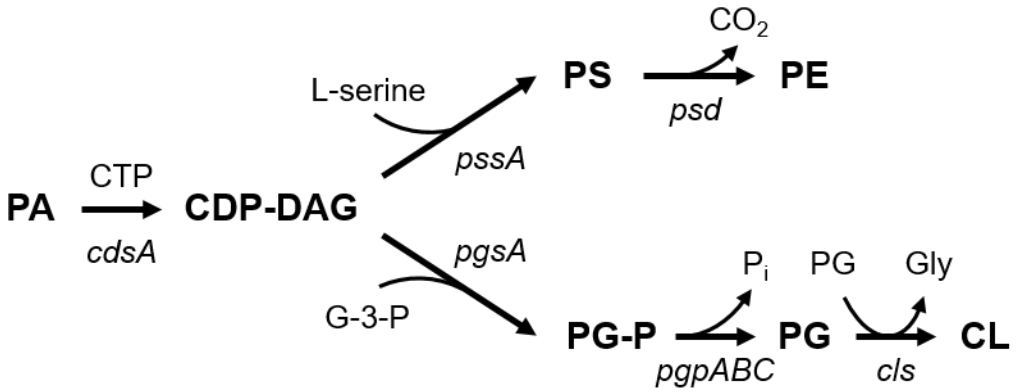

Figure 3.1. Biosynthetic pathway of the major anionic phospholipids in bacteria. Abbreviations: PA, phosphatidic acid; CDP-DAG, cytidine diphosphate diacylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; G-3-P, glycerol-3-phosphate; PG-P, phosphatidylglycerol-3-P; PG, phosphatidylglycerol; CL, cardiolipin.

altering an active site (*S. oralis* 1648-HA08), preventing proper protein folding (*S. oralis* 1647-HA06), or by preventing synthesis of the full polypeptide (*S. mitis* 1643-HA04).

3.5.3 Lipidomic analysis confirms CdsA loss of function in DAP-resistant *S. mitis* and *S. oralis*

To assess the functional consequences of *cdsA* mutations on the synthesis of phospholipids in *S. mitis* and *S. oralis*, we carried out lipidomic analysis. The total lipids from DAP-susceptible and DAP-resistant strains of S. *mitis* and *S. oralis* were subjected to normal phase LC-ESI/MS using a silica column for lipid separation. As shown by the total negative ion chromatograms (Figures 3.2, 3.3, and B.2 in supplemental material), PG and CL are the two major anionic phospholipids detected in all three DAP-susceptible strains. They appear at the retention time windows of ~12.5-13.5 min and 13.5-14.5 min, respectively. In contrast, PG and CL are completely absent in three

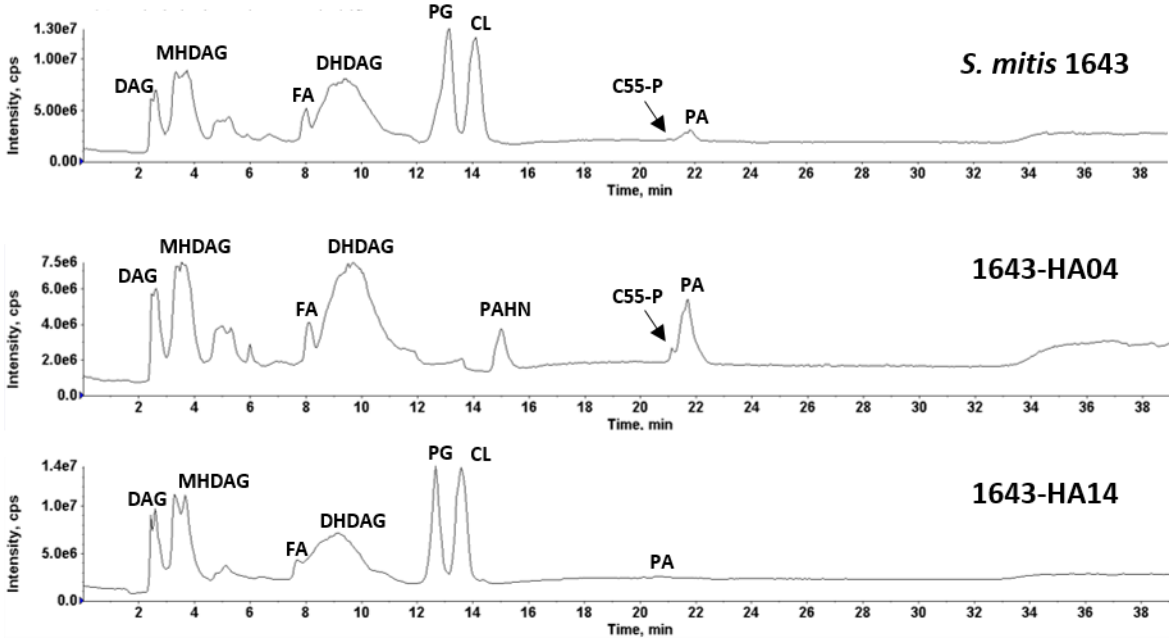

Figure 3.2. Normal phase LC-ESI/MS analysis of the total lipid extracts of DAP-susceptible and DAP-resistant strains of *S. mitis*. PG and CL are major lipids found in *S. mitis* 1643 (A). PG and CL are absent in the DAP^R derivative 1643-HA04 (B). After passage without selection, DAP susceptibility was restored, as were PG and CL levels (C).

DAP-resistant strains. The results confirm *cdsA* inactivation in the DAP-resistant strains as well as the essential role of *cdsA* for PG and CL synthesis in S. *mitis* and *S. oralis* under the conditions tested.

Other species identified in both DAP-susceptible and DAP-resistant strains are diacylglycerol (DAG), monohexosyldiacylglycerol (MHDAG), fatty acid (FA), dihexosyldiacylglycerol (DHDAG), undecaprenyl phosphate (C55-P), and PA. The levels of PA are consistently increased in all DAP-resistant strains (Figures 3.2 and 3.3). PA is a substrate of CdsA for the synthesis of CDP-DAG (Figure 3.1), thus its accumulation is an expected consequence of the inactivation of *cdsA*. The acyl compositions of PA in the DAP-sensitive strains are slightly different from those in the DAP-resistant strains (see Figure B.3 in supplemental material). For reference, the total lipid content of *S. mitis* 1643 and its derivatives is displayed in Table B.3 in the supplemental material.

An unknown species, appearing at 14.8-15.3 min, is significantly elevated in the DAP-resistant *S. mitis* 1643-HA04. Exact mass measurement and collision-induced dissociation (CID) tandem MS identified this unknown lipid as phosphatidyl-N-acetylhexosamine (PAHN), a PA-derived glycolipid (see Figure B.4 in supplemental material). A small amount of PAHN was detected in the DAP-sensitive *S. mitis* 1643-RA03 strain, but was not detected in any of the *S. oralis* strains.

Phosphatidylcholines (PC) were detected by positive ion ESI/MS in all strains used in this study. Shown in Figure B.3 are representative mass spectra of PCs in *S. mitis* 1643 and 1643-HA04. The level of PC is lower (3-10 fold) in the DAP-resistant strains compared to the DAP-sensitive strains (see Figure B.5 in supplemental material).

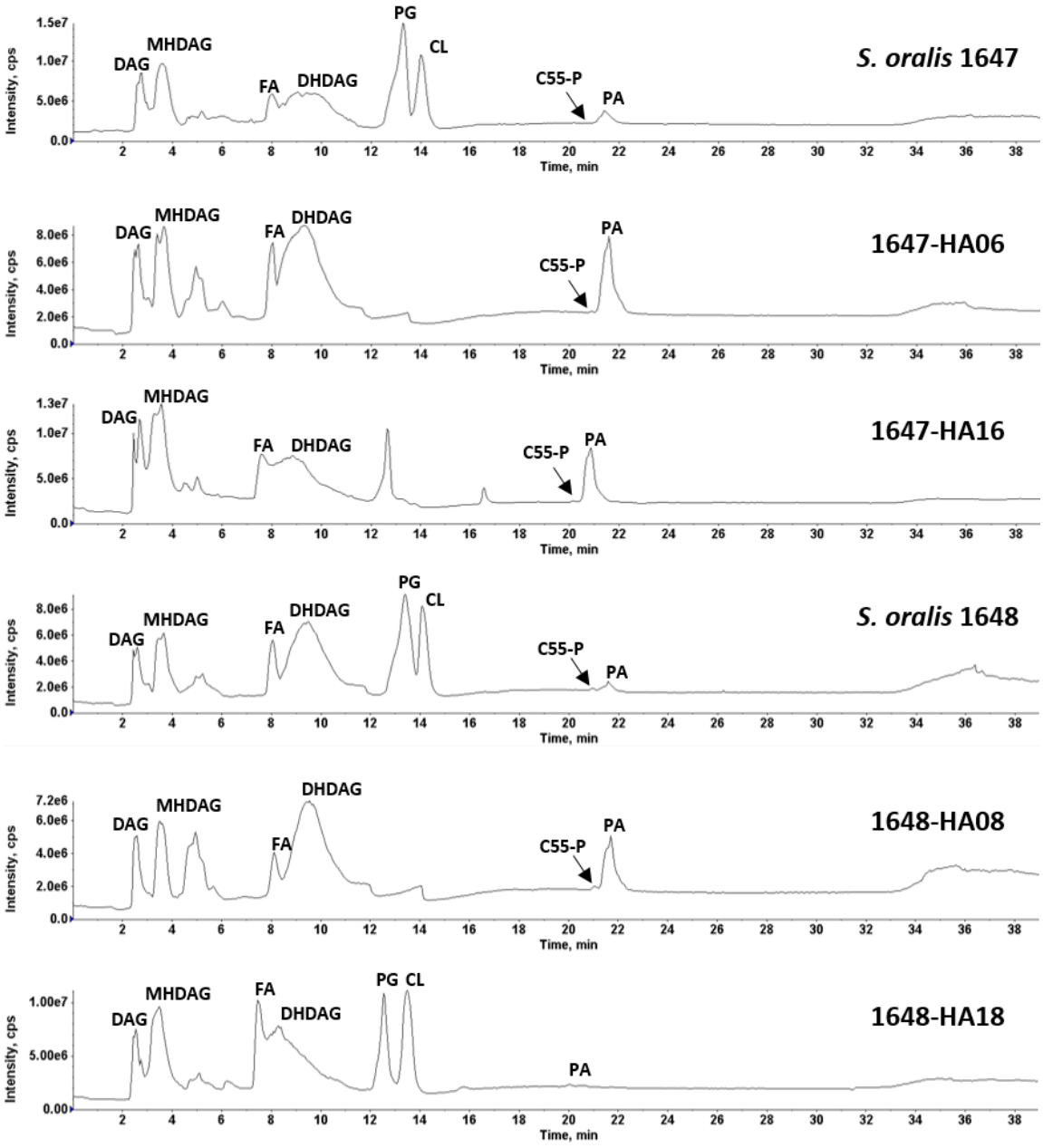

Figure 3.3. Normal phase LC-ESI/MS analysis of the total lipid extracts of DAP-susceptible and DAP-resistant strains of *S. oralis*. PG and CL are major lipids found in DAP-susceptible *S. oralis* 1647 (A) and 1648 (D). PG and CL are missing from DAP-resistant derivatives 1647-HA06 (B) and 1648-HA08 (E). Upon restoration of DAP-susceptibility in 1647-HA16 (C) and 1648-HA18 (F) PG levels return to normal. CL levels are restored in 1648-HA18, but not 1647-HA16.

3.5.4 Mutations in *cdsA* revert concurrently with phenotypic reversion to DAP susceptibility

Resistant strains were passaged in CA-SMHB until DAP susceptibility was restored. *S. mitis* 1643-HA04 began showing a zone of inhibition, indicating reversion, after the 4th passage, while *S. oralis* 1647-HA06 and *S. oralis* 1648-HA08 reverted after the 12th passage. Revertant populations recovered from the 5th overnight for *S. mitis* 1643-HA04 and the 13th overnight for *S. oralis* 1647-HA06 and *S. oralis* 1648-HA08 were referred to as *S. mitis* 1643-HA14, *S. oralis* 1647-HA16, and *S. oralis* 1648-HA18, respectively. These populations have MIC values identical or comparable to the original susceptible parental strains (Table 3.1) and growth rates similar to the parental strains (see Figure B.1 in supplemental material).

Mutations depicted in Table 3.2 were queried in the revertant populations using PCR and Sanger sequencing. All mutations present in the DAP resistant strains were also present in the revertant populations, except for the *cdsA* mutations. Analysis of *cdsA* showed that the premature stop codon in *S. mitis* 1643-HA04 was replaced with a tryptophan codon in *S. mitis* 1643-HA14, allowing for proper read-through of the coding region; the C246 of *S. oralis* 1647-HA06 was replaced with a serine in *S. oralis* 1647-HA16, restoring flexibility to the region; and the active site of *S. oralis* 1648-HA08 was restored in *S. oralis* 1648-HA18, as the mutation reverted to wild type. Table 3.1 displays the DAP MIC values for the susceptible, resistant, and revertant populations of each lineage, as well as the corresponding amino acid changes in CdsA. Our data indicate that restoration of CdsA function results in DAP susceptibility.

To confirm CdsA functionality in our revertants, we subjected them to lipidomic analyses as described above. All three revertant strains synthesize PG, indicating the synthesis of CDP-DAG

and therefore confirming the restored function of CdsA (Figures 3.2 and 3.3). Interestingly, 1647-HA16 does not possess any CL, and continues to have a small accumulation of PA (Figure 3.3). It is possible that 1647-HA16 acquired a loss-of-function mutation in a cardiolipin synthase gene over the course of the reversion passage, thereby preventing formation of CL. Alternatively, and not mutually exclusive, 1647-HA16 might possess only a partial restoration of CdsA function, resulting in an accumulation of PA. That 1647-HA16 is DAP sensitive indicates that PG, and not CL, is required for DAP susceptibility in *S. oralis*.

3.5.5 Spontaneous DAP resistance is always associated with *cdsA* mutation

To assess the frequency and diversity of *cdsA* mutations, we plated *S. mitis* and *S. oralis* parental strains on DAP-containing agar, and screened colonies for *cdsA* mutation. In addition, we compared the frequency of spontaneous DAP resistance to that of spontaneous rifampin resistance, a commonly used antibiotic for mutation frequency studies. *S. oralis* 1648 was not analyzed due to its pre-existing rifampin resistance [75]. Spontaneous DAP resistance arose at an average frequency of 1×10^{-6} for both strains on 10 µg/mL DAP and at an average frequency of 7×10^{-7} for both strains on 128 µg/mL DAP. Rifampin resistance arose at frequencies of 1×10^{-7} for *S. mitis* 1643 and 8×10^{-9} for *S. oralis* 1647. Of colonies arising on the 10 µg/mL and 128 µg/mL DAP plates that were screened (n=15 and 14, respectively), all possessed non-synonymous mutations in *cdsA*. In two colonies, two separate polymorphisms were found. Polymorphisms were observed that resulted in amino acid substitutions (n=20; 15 unique), premature stop codons (n=6; 4 unique), large scale (>200 bp) deletions (n=2), or frameshifts due to small insertions or deletions (n=3). The two large deletions (268 bp and 252 bp) encompassed approximately the same region,

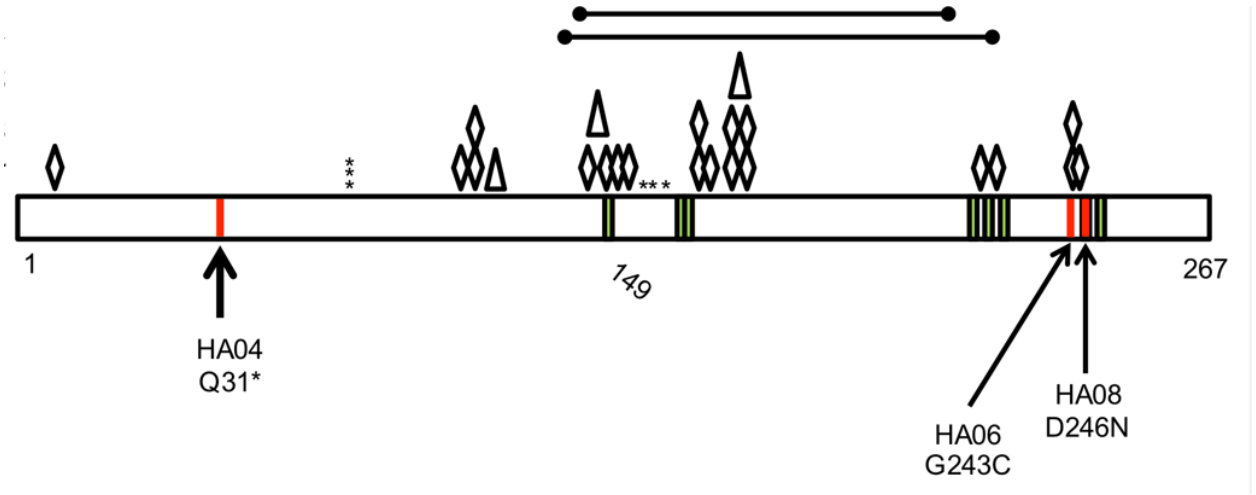

Figure 3.4. Summary of CdsA alterations identified in *S. mitis* and *S. oralis* after DAP selection. CdsA is depicted as a black rectangle, with amino acid positions indicated below. Green stripes represent active sites identified previously in *Thermotoga maritima* (36). Red stripes represent the original mutations identified in the broth-derived DAP-resistant strains. Asterisks depict premature stop codons, diamonds depict amino acid substitutions, triangles depict a nucleotide coding region deletion event of 1-2 bp, and the large lines above depict large areas of deletion identified in spontaneous resistance studies on DAP agar. HA04; *S. mitis* 1643-HA04. HA06; *S. oralis* 1647-HA06; HA08; *S. oralis* 1648-HA08.

but only one of the deletions resulted in a frame shift. Figure 3.4 and Table B.4 catalog all mutations detected in *cdsA* in this study. We conclude that diverse *cdsA* mutants rapidly emerge in *S. mitis* and *S. oralis* populations under DAP selection.

3.6 Discussion

CdsA catalyzes the synthesis of CDP-DAG, a key intermediate in phospholipid biosynthesis in all cells. CDP-DAG is the source of the phosphatidyl group for all major phospholipids in prokaryotes and for most anionic phospholipids in eukaryotes. Historical efforts to elucidate the biochemical pathways for membrane phospholipid biosynthesis used chemical mutagenesis to isolate *E. coli* mutants with reduced CDP-DAG synthase activity [169, 170]. Those mutants were observed to accumulate PA, but were not complete loss of function mutants due to the continued presence of

PG, CL, and PE in the *E. coli* membrane [169, 170]. More recently, *E. coli* partial loss of function *cdsA* mutants were found to accumulate PA and have decreased vancomycin susceptibility, presumably by altering outer membrane structure and access of the drug to the periplasm [171]. Efforts to define the essential gene cohorts of *E. coli*, *B. subtilis*, *S. pneumoniae*, and other bacteria have identified *cdsA* as being essential [77-83]. Crucially, efforts to define the minimum cohort of genes required to sustain cellular life also identified *cdsA* as being essential [157]. In contrast to these observations, *S. mitis* 1643-HA04, *S. oralis* 1647-HA06, and *S. oralis* 1648-HA08 lack CdsA function, as confirmed by lipidomics analysis. Furthermore, lipidomics results demonstrate that a CdsA-independent pathway for anionic phospholipid biosynthesis does not exist or is not active in these bacteria under the conditions tested here.

Why is *cdsA* essential in other bacteria, but not in *S. mitis* and *S. oralis*? Presumably, *cdsA* is essential in other bacteria because the lipids synthesized from the CdsA product, CDP-DAG, are essential for cytoplasmic membrane integrity, fluidity, and/or the localization of key proteins for cellular maintenance and replication. Alternatively, the substrate of CdsA, phosphatidic acid, could be toxic at high levels. Potential physiological mechanisms for growth of *S. mitis* and *S. oralis cdsA* mutants include altered cell wall structure and/or altered distribution or perhaps a lack of necessity for key membrane proteins involved in cell division. These mechanisms would be expected to be absent from model bacteria for which *cdsA* is essential. Unfortunately, little is known about the physiology and genetics of *S. mitis* and *S. oralis*. Characterization of peptidoglycan structure, membrane proteomics, and the transcriptomes of wild-type and *cdsA* mutants will be informative to understand the mechanism of survival of *S. mitis* and *S. oralis cdsA* mutants. In addition, studies which assess the impact of altered membrane structure and altered

growth rates on the virulence and *in vivo* physiology of DAP-resistant *S. mitis* and *S. oralis* are warranted.

PC is a relatively rare membrane phospholipid in the bacterial domain, with only ~15% of bacteria encoding the necessary biosynthetic pathway [172]. Eukaryotes synthesize PC using the Kennedy pathway [173]. A homologous pathway has been discovered in the bacterium *Treponema denticola* [174, 175]. The first two genes in the pathway are *licA* and *licC*. BioCyc is a pathway and genome database which predicts metabolic networks for various organisms [176]. *S. mitis* and *S. oralis* are predicted to possess homologs of the first two genes in the Kennedy pathway; however, the third gene has yet to be elucidated. *S. pneumoniae* uses *licA* and *licC* to incorporate choline into the cell wall to aid in adhesion to human epithelial cells [177]. It is possible that the *cdsA* mutants repurpose the *lic* pathway for PC synthesis under certain conditions. Other Gram-positive model organisms such as *E. faecalis*, *S. aureus*, and *B. subtilis* do not encode *licA* and *licC*. This is further evidence of *S. mitis* and *S. oralis* possessing unique physiological characteristics that allow them to tolerate CdsA loss of function.

Studies in other Gram-positive bacteria have associated a wide range of mutations with reduced DAP susceptibility. A common feature identified by some of these studies is a bacterial cell membrane with reduced PG content. Mutations in *mprF* were shown to increase lysyl-PG content in DAP non-susceptible MRSA, concomitantly reducing the PG content of the membrane [178]. In *B. subtilis*, an evolved DAP non-susceptible strain possessed reduced function mutations in *pgsA*, which is responsible for the synthesis of PG [54]. The evolved *B. subtilis* strain possessed PG levels >5-fold lower than its susceptible parental strain and a DAP MIC value nearly 30-fold

higher. Here, we have identified a novel mechanism by which *S. mitis* and *S. oralis* purge their membranes of PG and, as previously shown, DAP MIC values increase up to 512-fold higher than DAP-sensitive parental strains [75, 153].

Several models for DAP mechanism of action against Gram-positive bacteria have been proposed [55, 156]. These mechanisms generally implicate membrane pore formation by DAP or recruitment of DAP to sites of membrane curvature. More recently, a revised mechanism of DAP action was proposed for the model Gram-positive bacterium *Bacillus subtilis* [21]. In this model, DAP's lipid tail interacts with domains of increased membrane fluidity, allowing DAP to oligomerize at those sites. This causes rapid delocalization of membrane proteins essential for peptidoglycan biosynthesis (MurG) and lipid biosynthesis (PlsX), which are also associated with the membrane fluid domains. The displacement of MurG interrupts peptidoglycan biosynthesis, ultimately leading to cell lysis. DAP-resistant *S. mitis* and *S. oralis* may synthesize a membrane for which DAP has low affinity, and loss of CdsA activity is required for synthesis of this membrane. Our data indicate that PG is a critical phospholipid associated with DAP susceptibility. Alternatively, *S. mitis* and *S. oralis* may not organize essential membrane proteins in membrane fluid domains. The biophysical mechanism for DAP resistance in these strains remains to be determined. It will be interesting to determine whether *S. mitis* and *S. oralis cdsA* mutants emerge in humans treated with DAP, and whether these *cdsA* mutations are as permissive for *S. mitis* and *S. oralis* outgrowth in human *in vivo* conditions as they are in *in vitro* conditions.

3.7 Acknowledgments

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CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

4.1 Mutations associated with reduced surotomycin susceptibility in *Clostridium difficile* and *Enterococcus* species

4.1.1 Overview

C. difficile is an important nosocomial pathogen that represents a heavy burden to the healthcare industry. SUR has been used to treat CDAD in phase 3 clinical trials, and has been shown to be more effective than current treatment regimens such as vancomycin [9, 10]. While resistance to SUR is rare, it has been shown in a laboratory setting [84]. Due to similar structures, SUR is thought to have a MOA similar to that of DAP, however, very few studies have been done on SUR MOA [8, 23, 24, 88]. Continuing the work of Mascio et al [84], we used genomics and bioinformatics analyses to identify mutations associated with reduced SUR susceptibility in *C. difficile*, *E. faecalis*, and *E. faecium* (Chapter 2). To date, this is the first study on the exact mechanisms of resistance to SUR.

4.1.2 Summary of conclusions

Using Illumina sequencing technology, the full genome of SUR non-susceptible strains was compared to each respective SUR susceptible parental strain. This analysis identified several polymorphisms that were found only in the SUR non-susceptible derivatives. In the three *C. difficile* strain pairs, mutations were identified in *cls*, *fabK*, *ftsH2*, and a gene encoding for a PadR-like transcriptional regulator. *cls* has been implicated in DAP resistance in the enterococci [51,

56]. It is predicted that the other mutations have an effect on membrane composition or cell membrane stress-response systems. Analysis of *E. faecalis* and *E. faecium* strain pairs also identified mutations in genes previously associated with DAP resistance. These data suggest that SUR has a MOA similar to DAP.

4.1.3 Future work

This work was strictly a mutation identification study using bioinformatics and genomics. In order to conclusively link these mutations to SUR reduced susceptibility, genetic experiments must be performed. By cloning alleles from resistant strains into a susceptible background, the effect of each mutation alone and in combination on the SUR MIC can be tested. Importantly, the consensus on the MOA of DAP has evolved since the publication of this research [21], making additional studies on the effect of SUR on bacterial growth necessary.

4.2 *Streptococcus mitis* and *S. oralis* lack a requirement for *cdsA*, the enzyme required for synthesis of major membrane phospholipids in bacteria

4.2.1 Overview

DAP is a promising antibiotic for the effective treatment of Gram-positive pathogens, however, the development of resistance to DAP is of grave concern. Many different genes have been linked to DAP resistance in model organisms such as *B. subtilis*, *S. aureus*, and *Enterococcus* species [12, 51, 56, 62, 65]. Understanding these mechanisms of resistance is an important step in elucidating the exact MOA of DAP. Recently, studies have investigated the effect of DAP on two commensal species of VGS, *S. mitis* and *S. oralis* [75, 76]. In my research, I evolved high-level (>256 µg/mL)

DAP-resistant strains of *S. mitis* (n=1) and *S. oralis* (n=2) after a single overnight exposure. Using whole genome sequencing and bioinformatics analyses, I have shown that DAP resistance in these strains is directly linked to mutations in *cdsA*.

4.2.2 Summary of conclusions

Overnight exposure of *S. mitis* and *S. oralis* to low concentrations of DAP (2-4 µg/mL) led to the evolution of strains resistant to concentrations >256 µg/mL [75, 76]. The development of high-level DAP resistance after a single drug exposure is a phenomenon that has not been reported in *B. subtilis*, *S. aureus*, or *Enterococcus* species. In order to better understand DAP resistance in the VGS, I sequenced the entire genome of three DAP-resistant derivatives of *S. mitis* and *S. oralis*. Bioinformatic analysis revealed mutations in *cdsA*, which encodes for phosphatidate cytidylyltransferase (CdsA). CdsA is responsible for the conversion of PA to CDP-DAG, which is then used as the precursor for all major anionic phospholipids in bacteria. In order to determine the extent to which *cdsA* is mutated under DAP selection, we cultured *S. mitis* and *S. oralis* on DAP-containing agar plates. Of the 29 colonies screened for *cdsA* polymorphisms, all contained non-synonymous mutations. The mutations identified in *cdsA* revert concurrently with phenotypic reversion to DAP susceptibility. These data taken together indicate that mutations in *cdsA* are necessary and sufficient for DAP resistance in *S. mitis* and *S. oralis*.

Analysis of the polymorphisms in *cdsA* predicted that these were loss-of-function mutations. LC/MS analysis of the lipid content of each of the three DAP-resistant strains confirmed this prediction, as resistant strains had membranes composed of PA and devoid of PG and CL. The wild-type membrane composition was restored in the DAP-susceptible revertant strains. CdsA is

considered to be essential for bacterial survival [77-82], and this research is the first to identify strains capable of surviving CdsA loss-of-function. It is currently unknown how *S. mitis* and *S. oralis* survive the absence of PG and CL in their membrane, but this data suggests they possess a unique physiology not previously seen in model organisms.

4.2.3 Future work

This research has shown that DAP-resistant *S. mitis* and *S. oralis* possess an altered membrane composition. It would be interesting to study the effects of this alteration on virulence and fitness in the polymicrobial environment of the human body. In addition, the exact way in which *S. mitis* and *S. oralis* cope with this altered membrane would be of great interest. Transposon mutagenesis coupled with whole-genome sequencing (Tn-seq) could elucidate this mechanism. Using the *cdsA*$_{null}$ derivatives as a background for transposon mutant library generation, Tn-seq could identify conditionally-essential genes which might contribute to the ability of these strains to grow with a membrane devoid of PG and CL. In addition, it would be interesting to determine whether or not other mutations that lead to the same altered membrane composition would affect DAP resistance. For instance, would loss-of-function mutations in the PG synthesis gene *pgsA* result in the same DAP-resistant phenotype? If the MOA of DAP relies solely on the presence of PG in the membrane, it stands to reason that this genetic background would also be DAP-resistant. These studies would contribute to the better understanding of the exact MOA of DAP and how bacteria are able to evolve resistance.

APPENDIX A

SUPPLEMENTAL MATERIAL FOR CHAPTER 2

Table A.1. *De novo* genome assemblies using CLC Genomics Workbench (single end reads only).

Strain name	# reads (151 bases/read)	# contigs	Min contig size (bp)	Max contig size (bp)	Average contig size	Contig N_{50} (bp)	# genes annotated by RAST	Genome size
Cdi2179[b]	17,786,604	861	128	263,413	5,211	94,116	4192	4.49 Mb
Cdi2989[b]	16,000,099	702	121	236,376	6,206	76,852	4004	4.36 Mb
Cdi2994[a]	16,169,705	379	148	271,320	11,593	97,218	4144	4.39 Mb
Efs201[b]	18,575,124	603	123	140,203	5,054	40,197	2915	3.05 Mb
Efm14[b]	18,354,871	493	127	170,021	5,946	57,717	3058	2.93 Mb
Efm277	21,077,510	926	112	148,413	3,457	36,818	3281	3.20 Mb

[a]R1 reads were used
[b]R2 reads were used

Table A.2. Efs807 reads aligned to Genbank V583 reference sequences.

Strain	Total reads[a]	Chr.[b]	Chr. Coverage[c]	pTEF1[b]	pTEF1 coverage[c]	pTEF2[b]	pTEF2 coverage[c]	pTEF3[b]	pTEF3 coverage[c]	Non-assembled
Parent	17,244,147	16,208,107	44-2018 (716)	308,424	84-1621 (655)	172,904	29-1063 (422)	115,165	136-1679 (900)	439,682
Pass	19,135,853	17,836,074	0-2431 (783)	321,309	69-1798	176,634	28-1153 (426)	105,861	111-1522 (813)	696,103

[a]Total single end reads generated by Illumina sequencing, after demultiplexing. Each read is 151 nucleotides long.
[b]Number of reads aligned to reference sequence (chromosome, pTEF1, pTEF2, or pTEF3). Chr, chromosome.
[c]Range of fold coverage is shown; average fold coverage is in parentheses.

Table A.3. Expanded bioinformatics analysis of candidate surotomycin resistance genes.

Strain	Gene annotation	Gene name	Protein change in pass strain	Best BLASTP hit to *C. difficile* 630[a]	Best BLASTP hit to *E. faecium* DO[a]	Best BLASTP hit to *E. faecalis* V583[a]
Cdi2179	**Cardiolipin synthase[b]**	*cls*	D79N	CD630_34040 (100, 100, 0); CD630_01920 (94, 41, $1e^{-120}$)	HMPREF0351_12025 (96, 42, $6e^{-134}$); HMPREF0351_11068 (96, 38, $1e^{-116}$)	EF1608 (98, 39, $2e^{-116}$); EF0631 (96, 40, $8e^{-112}$)
Cdi2989	Enoyl-[ACP] reductase	*fabK*	G145V	CD630_11800 (100, 100, 0)	No hit meeting e-value of $\leq 10^{-3}$	EF2883 (98, 61, $1e^{-119}$)
Cdi2989	Cell division protein FtsH	*ftsH2*	E463Stop	CD630_35590 (100, 100, 0)	HMPREF0351_12545 (97, 51, 0)	EF0265 (81, 58, 0)
Cdi2994	Transcriptional regulator PadR		R6I	CD630_13450 (100, 100, $1e^{-76}$)	HMPREF0351_10440 (92, 40, $3e^{-21}$)	EF1297 (86, 40, $9e^{-18}$)
Efm14	**HD family hydrolase**		E10fs	CD630_14600 (78, 43, $6e^{-37}$)	HMPREF0351_11908 (100, 99, $2e^{-123}$)	EF2470 (98, 84, $2e^{-105}$)
Efm14	Aminopeptidase S		F355L	CD630_35950 (99, 45, $9e^{-130}$)	HMPREF0351_10886 (100, 97, 0)	*EF2204; has frameshift mutation in V583
Efm14	ATP-dependent nuclease subunit A	*addA/rexA*	H761Q	CD630_10410 (98, 33, 0)	HMPREF0351_11280 (100, 98, 0)	EF1113 (99, 56, 0)
Efm14	Alpha/beta family hydrolase		(Synonymous mutation)	CD630_08660 (96, 26, $8e^{-24}$)	HMPREF0351_11299 (100, 96, 0)	EF1536 (99, 66, $5e^{-160}$)
Efm14	Hypothetical protein		(Synonymous mutation)	No hit meeting e-value of $\leq 10^{-3}$	HMPREF0351_11264 (100, 88, $3e^{-68}$)	No hit meeting e-value of $\leq 10^{-3}$
Efm277	**Cardiolipin synthase**	*cls*	R211L	CD630_34040 (98, 38, $1e^{-116}$); CD630_01920 (98, 36, $4e^{-105}$)	HMPREF0351_11068 (100, 100, 0); HMPREF0351_12025 (100, 42, $4e^{-134}$)	EF0631 (100, 70, 0); EF1608 (99, 48, $2e^{-163}$)
Efm277	**HD family hydrolase**		A53fs	CD630_14600 (78, 43, $6e^{-36}$)	HMPREF0351_11908 (100, 99, $6e^{-124}$)	EF2470 (98, 83, $4e^{-105}$)

Efm277	**Ribosomal large subunit methyltransferase A**	*rrmA*	K267N	No hit meeting e-value of $\leq 10^{-3}$	HMPREF0351_12412 (100, 100, 0)	EF2666 (97, 59, $2e^{-126}$)
Efm277	**RNA polymerase, beta subunit**	*rpoB*	V930G	CD630_00660 (99, 63, 0)	HMPREF0351_12666 (100, 99, 0)	EF3238 (99, 95, 0)
Efm277	Lead, cadmium, zinc, mercury transporting ATPase	*copB*	F184L	CD630_21150 (91, 36, $7e^{-123}$)	HMPREF0351_11880 (100, 99, 0)	EF0875 (99, 69, 0)
Efm277	Sucrose-6-phosphate hydrolase	*scrB*	P234T	CD630_18050 (96, 35, $3e^{-86}$)	HMPREF0351_11800 (81, 24, $2e^{-7}$)	EFA0069 (100, 79, 0)
Efs201	**Sensor histidine kinase LiaS**	*liaS*	M110I	CD630_12700 (74, 27, $5e^{-12}$)	HMPREF0351_10937 (96, 74, $5e^{-154}$)	EF2912 (100, 100, 0)
Efs201	Fe-S cluster binding subunit		G170V	No hit meeting e-value of $\leq 10^{-3}$	No hit meeting e-value of $\leq 10^{-3}$	EF1109 (100, 99, 0)
Efs807	EF0797 hypothetical protein		L52P	No hit meeting e-value of $\leq 10^{-3}$	No hit meeting e-value of $\leq 10^{-3}$	EF0797 (100, 100, 0)
Efs807	**EF1027 MprF2**	*mprF2*	A29T	No hit meeting e-value of $\leq 10^{-3}$	HMPREF0351_11082 (100, 62, 0); HMPREF0351_12586 (98, 34, $2e^{-157}$)	EF1027 (100, 100, 0); EF0031 (99, 34, $7e^{-168}$)
Efs807	**EF1797 hypothetical protein (DrmA)**	*drmA*	N150fs	No hit meeting e-value of $\leq 10^{-3}$	HMPREF0351_10297 (94, 46, $1e^{-50}$); HMPREF0351_10318 (95, 43, $1e^{-47}$)	EF1797 (100, 100, $9e^{-156}$)

[a]For each hit, values shown in parentheses are: % query coverage, % amino acid sequence identity, expectation value.
[b]Boldface indicates mutations which have previously been associated with daptomycin resistance. See text for discussion.

Table A.4. Identifying additional proteins of interest in *Clostridium difficile* 630 using BLASTP.

Protein name	Query species and strain	Query identifier	Best hit to *C. difficile* 630	Query Coverage, % identity, e-value
FabI	*Bacillus subtilis* 168	AIY92455.1	CD1182 FabG	97%, 27%, $2e^{-13}$
LiaF	*Enterococcus faecalis* V583	NP_816531.1	No hit with e-value of $\leq 10^{-3}$	No hit with e-value of $\leq 10^{-3}$
LiaS	*Enterococcus faecalis* V583	NP_816530.1	CD1270	61%, 27%, $1e^{-17}$
LiaR	*Enterococcus faecalis* V583	NP_816529.1	CD1269	97%, 41%, $3e^{-45}$
VraS	*Staphylococcus aureus* N315	Q99SZ7.1	CD1270	56%, 26%, $5e^{-13}$
VraR	*Staphylococcus aureus* N315	Q7A4R9.1	CD1269	98%, 37%, $3e^{-40}$
YycG	*Staphylococcus aureus* COL	YP_184931.1	CD2637	69%, 31%, $3e^{-60}$
YycF	*Staphylococcus aureus* COL	AAW37407.1	CD1624	99%, 43%, $6e^{-65}$

Table A.5. Primers used.

Primer name	Sequence
Clostridium difficile	
Cdi2179 Cls For	GGAGGGAATTTTATGTTTGATA
Cdi2179 Cls Rev	GTCTATTATTTCAGTTCCGA
Cdi2989 FtsH2 For	GCTACATACTCTAATCTATCCA
Cdi2989 FtsH2 Rev	TACCATGAAGGAGGGCATGC
Cdi2989 FabK For	CCTACTTGAACTGCACTGGC
Cdi2989 FabK Rev	ATAGCAGCAGGAAACGCACC
Cdi2994 PadR For	GCAGGACCTTCATTAGATTC
Cdi2994 PadR Rev	GCAGAACATAGTACTGTGTT
Enterococci	
Efm14 HD Hydrolase For	GCGAGCAGATAACTATAGTA
Efm14 HD Hydrolase Rev	GCAGAACATAGTACTGTGTT
Efm14 αβ Hydrolase For	GGCCAGAGCGCAAAGACTA
Efm14 αβ Hydrolase Rev	GCCGAACAAGGCGAAGATG
Efm14 Aminopeptidase For	GCTCAAGCCCAATCTCCA
Efm14 Aminopeptidase Rev	GCCGAACAAGGCGAAGATG
Efm14 Hypothetical For	CCTCAAGAAGTTCGTGCAC
Efm14 Hypothetical Rev	CCTCGAACCAGTCAGTTTTC
Efm14 ATP Nuclease For	CGGGATATCTAGAGTATGTG
Efm14 ATP Nuclease Rev	CCCGTGTTTCAGGATCCAT
Efm277 Ribosomal MTase For	CGGTGTCAGTCACTGCGA
Efm277 Ribosomal MTase Rev	CGTGTAGTAGCCAAGTTTG
Efm277 RNAPol Subunit β For	CTCGTGAAGCTGGAGATGA
Efm277 RNAPol Subunit β Rev	CGCATCGCTGGCCATACC
Efm277 Metal Transporter For	CCATCTTTCGTCAAGCGTT
Efm277 Metal Transporter Rev	GGCTCGTATTGATCTTAGCA
Efm277 Cls For	CGGCCAGCAAGTCTTACAT
Efm277 Cls Rev	GCGGCACCGAAAATCCGTA
Efm277 HD Hydrolase For	GGCGTGAAGACGAAGAATA
Efm277 HD Hydrolase Rev	CCGACAAATCGGTGATCTTT
Efm277 Putative For	GGCCTGATTCTCTTGGCGC
Efm277 Putative Rev	CCAATCCAGCTCCTCGATT
Efm277 Sucrose Hydrolase For	CGCATACGCTTCAAATCCAT
Efm277 Sucrose Hydrolase Rev	GCGATTGTTGGAGCACAA
Efs201 Lia For	CCGATCGGATTTCAAGACGC
Efs201 Lia Rev	CGTTTTCTGCTTCGCCTACG

Efs201 YkgF For	AACCGTGCCCTCCAATATGG
Efs201 YkgF Rev	TGGGAGAAACGAGAAGGTGC
Efs807 EF0797 For	GTACGAGTGGTATTGATGGTT
Efs807 EF0797 Rev	CAACAGGGATTCTCTTATCCA
Efs807 MprF2 For	CCGATAACCATCATCAATAACA
Efs807 MprF2 Rev	CGATTGCTGATGCCATTCCT
Efs807 DrmA For	CAGTGCCAGCATTGGTAGAT
Efs807 DrmA Rev	CTCTTCAGCGTAGACACTTC
Efs807 EF1367 IG For	GCTGTGCCACGAATGATTTC
Efs807 EF1367 IG Rev	CCTCAAGCAACTAACGTTACT

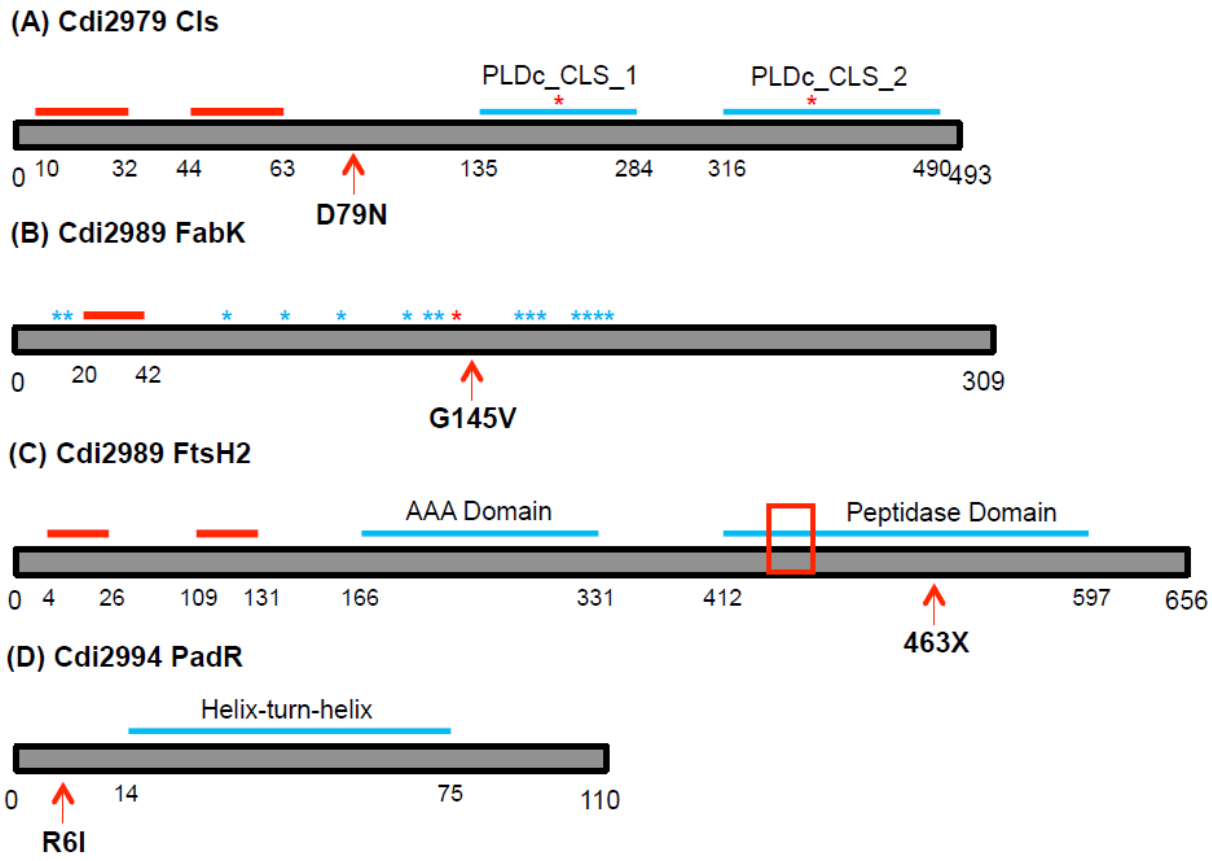

Figure A.1. *C. difficile* proteins of interest. Proteins of interest in the *C. difficile* strains Cdi2979, Cid2989, and Cdi2994 are depicted. NCBI Conserved Domain analysis identified putative catalytic sites (red asterisks) and putative co-factor binding sites (blue asterisks). Conserved protein domains of interest are shown. Transmembrane regions were predicted using TMHMM v2.0 and are shown as red bars above the protein. Amino acid substitutions occurring in pass strains are indicated by red arrows. (A) Cls. Red asterisks indicate putative catalytic residues at positions 228 and 411. (B) FabK. Blue asterisks represent FMN binding sites at residues 18, 19, 70, 94, 115, 136, 140-141, 167-169, and 188-191. A single catalytic site was indicated at residue 143. (C) FtsH2. The HEXXH catalytic site is indicated by a red box. (D) SurR.

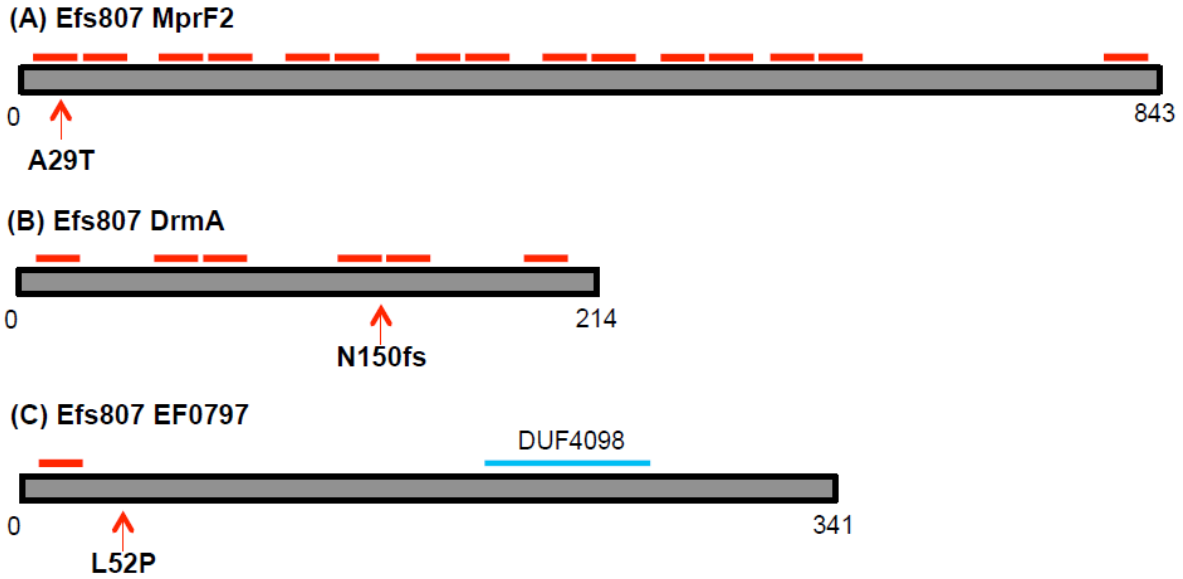

Figure A.2. Efs807 (V583) proteins of interest. The three V583 proteins from Table 2.2 are depicted. Conserved domains were identified using Pfam v27.0 and NCBI Conserved domains. TMHMM v2.0 was used to predict transmembrane helices and are represented by red bars above the protein. Amino acid substitutions occurring in pass strains are indicated by red arrows. (A) MprF2. Transmembrane helices occur at amino acid positions 13-35; 50-72; 93-115; 130-152; 165-182; 202-224; 236-258; 273-295; 324-346; 361-383; 390-407; 412-426; 447-466; 486-505; 818-837. (B) DrmA. Transmembrane helices occur at amino acid positions 7-26; 54-76; 83-105; 125-147; 154-176; 186-205. (C) EF0797. Transmembrane helix occurs from amino acids 5-27. DUF, domain of unknown function.

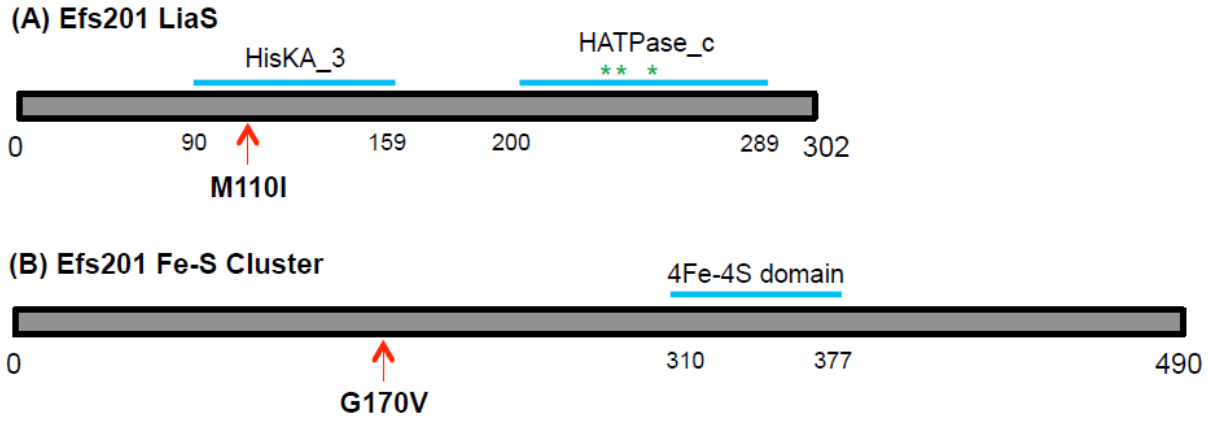

Figure A.3. *E. faecalis* 201 proteins of interest. NCBI Conserved Domain analysis was used to identify conserved protein domains (represented as blue bars) and putative catalytic sites and important residues. Amino acid changes occurring in pass strains are indicated by red arrows. (A) LiaS. Green asterisks represent the G-X-G motif of the HATPase_c domain on residues 239, 241, and 255. (B) Fe-S cluster protein.

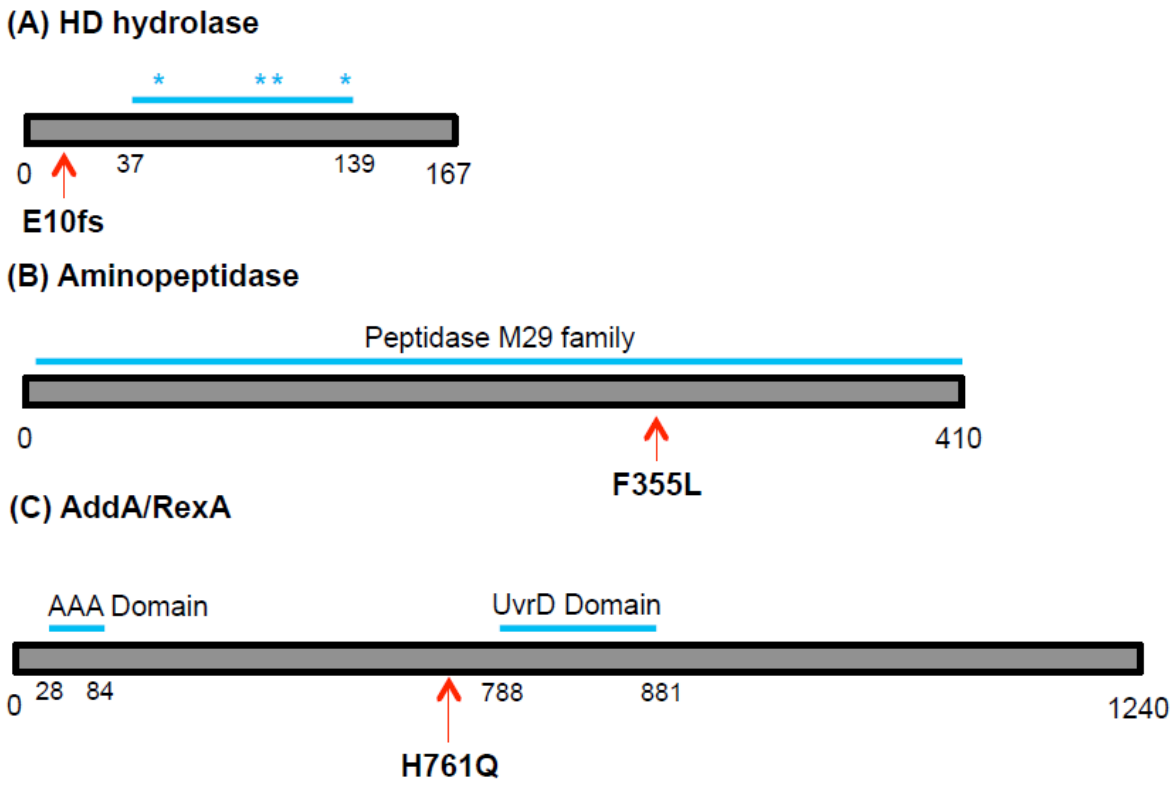

Figure A.4. *E. faecium* 14 proteins of interest. NCBI Conserved Domain analysis identified putative co-factor binding sites. Conserved domains are indicated by blue bars above the protein. Amino acid changes occurring in pass strains are indicated by red arrows. (A) HD family hydrolase. Blue asterisks represent Zn2+ binding sites at residues 41, 70, 71, and 137. (B) Aminopeptidase PepS. (C) AddA/RexA.

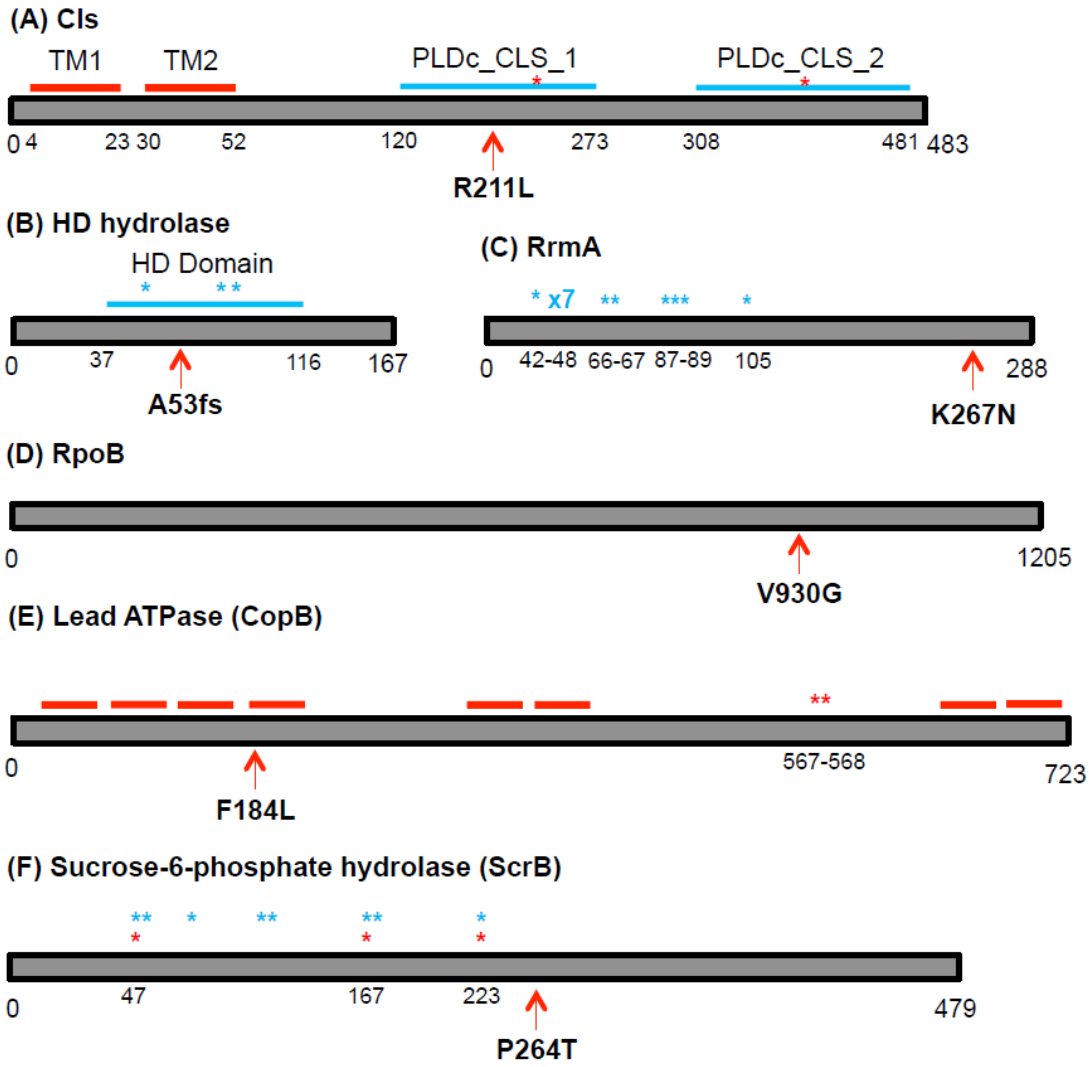

Figure A.5. *E. faecium* 277 proteins of interest. NCBI Conserved Domain analysis identified conserved domains as well as putative catalytic residues and co-factor/substrate binding sites. TMHMM v2.0 was used to predict transmembrane domains, which are depicted as red bars above the protein. Amino acid changes occurring in pass strains are indicated by red arrows. (A) Cls. Red asterisks indicate putative catalytic sites (residues 217 and 401). (B) HD family hydrolase. Blue asterisks represent Zn2+ binding sites at residues 41, 70, and 71. (C) RrmA. Blue asterisks represent *S*-adenosyl-methionine binding sites. The x7 indicates seven binding sites in close proximity. (D) RpoB. Many protein interaction sites surround the V930G substitution; the interaction sites are centered roughly around residues 830 and 990. The interaction sites, while not depicted, may be affected by the substitution. (E) Lead ATPase CopB. Red asterisks indicate putative catalytic residues. Transmembrane helices occur at residues 83-102; 117-136; 148-170; 180-197; 332-354; 364-386; 672-694; 698-720. (F) Sucrose-6-phosphate hydrolase ScrB. Red asterisks represent putative catalytic residues (positions indicated on figure), while blue asterisks represent substrate binding sites at residues 46, 47, 63, 106, 107, 166, 167, and 223.

APPENDIX B

SUPPLEMENTAL MATERIAL FOR CHAPTER 3

Table B.1. De novo assembly data for parental strains.

Strain	# reads	# contigs	Min contig size	Max contig size	Average contig size	Contig N50 (bp)	Genome Size (Mbp)
1643	12,173,871	119	211	253,142	15,805	66,153	1,880,744
1647	14,577,054	158	202	224,156	12,380	53,890	1,956,052
1648	14,503,619	199	205	153,663	9,413	48,229	1,873,268

Table B.2. Primers used in this study.

Primer Name	Sequence
1643_IntGen_19_F	CCAGTGTAACCTTTACTGCA
1643_IntGen_19_R	CGAGCTGACTGAAGCAGAC
1643_CellWall_F	GCCTGACTTAACTAATCTGG
1643_CellWall_R	CCACGAAGTCGAATCGTTAC
1643_Hypothetical_F	GGAGATTTAGAGGGGTGTC
1643_Hypothetical_R	CGGAACTATATCCAATCTGTC
1643_SerThr_Kinase_F	GCGTCAGATAAGTGAAGTGG
1643_SerThr_Kinase_R	CCCATCCACGAAGAAAACAT
1643_IntGen_32_F	GTGCCGTCAGTTTTGACTTG
1643_IntGen_32_R	GGACTACTGTAGCTGACAC
1643_Ribo_S5p_F	GCCAGATGGCGTCAAAGT
1643_Ribo_S5p_R	GGCTAGTGATGTCGGAGAG
1643_cdsA_F	GCAATTGGGAAAGCGGCA
1643_cdsA_R	CCGTACGAGTGGTGAGTTG
1647_IntGen_13_F	GCTACAAAAGCGCAGGCTTG
1647_IntGen_13_R	GGCTTACTATGGCTTTGAC
1647_DAG_Kinase_F	CGGCTCCTGCCGCCATGT
1647_DAG_Kinase_R	GCCTACATTGGGGAACTTTTC
1647_IntGen_17_F	CGAAATCAGATAGACTTGTCC
1647_IntGen_17_R	GATTTGGCTCTTTTACCATAC
1647_cdsA_F	GGGCCCCATACCAATGGC
1647_cdsA_R	GCGCAGCCTACCTGACAG
1647_Ribo_Mtase_F	CCAGCAGTCGCCTCATAG
1647_Ribo_Mtase_R	GCCCTAAACACTGTGCCA
1647_ppGpp_Synthetase_F	GGACTATGCTACCGAACG
1647_ppGpp_Synthetase_R	CTCCTCCGCCAGATAGTT
1648_cdsA_F	GAGGAAGGATTCGAATGGT
1648_cdsA_R	GGCTCCGAGAGTTTCTCC
1648_IntGen_27_F	GCTCACCAATCCCAAACAC
1648_IntGen_27_R	GGAACGCGTTCTCAGAAAG
1648_ppGpp_Synthetase_F	CCTCTGGGCCAACACCG
1648_ppGpp_Synthetase_R	GCAATCCAGTGCCGGGTG
1648_Ribo_SSU_Mtase_F	CCAGTTGACTAGACTCGTC
1648_Ribo_SSU_Mtase_R	CGAAGACAGAGATAAGCTAG
1648_Helicase_F	GGAAGCATGGCTTTTAGGATG
1648_Helicase_R	CGTGAAGCGGTTCGTCTC
Full_cdsA_F	GGTTAGCAATCCAATCATTC
Full_cdsA_R	CCATCGCAATCGCCGTTTTG
CdsA_promoter_F	GCCTCCTAGAGGAAGGAT
CdsA_promoter_R	GGGACGGTATACTAGGTTC
CdsA_Sequence_1	GCCTTGCAGGAAGCTATTG

Table B.3. Major lipid species in wild-type, *cdsA* mutant, and revertant *S. mitis* detected by negative ion ESI/MS.

Lipid class	$[M - H]^-$	Molecular species [a]	WT	CdsA	Revertant
DAG	547.42[b]	DAG (26:0)	+	+	+
	573.43[b]	DAG (28:1)	+	+	+
	575.44[b]	DAG (28:0)	+	+	+
	601.46[b]	DAG (30:1)	+	+	+
	603.47[b]	DAG (30:0)	+	+	+
	627.47[b]	DAG (32:2)	+	+	+
	629.49[b]	DAG (32:1)	+	+	+
	657.52[b]	DAG (34:1)	+	+	+
FA	225.19	FA (14:1)	+	+	+
	227.2	FA(14:0)	+	+	+
	253.22	FA (16:1)	+	+	+
	255.23	FA (16:0)	+	+	+
	281.25	FA (18:1)	+	+	+
	283.26	FA (18:0)	+	+	+
MHDAG	709.46[b]	MHDAG (26:0)	+	+	+
	735.48[b]	MHDAG (28:1)	+	+	+
	737.49[b]	MHDAG (28:0)	+	+	+
	763.50[b]	MHDAG (30:1)	+	+	+
	765.51[b]	MHDAG (30:0)	+	+	+
	791.54[b]	MHDAG (32:1)	+	+	+
	819.57[b]	MHDAG (34:1)	+	+	+
DHDAG	871.52[b]	DHDAG (26:0)	+	+	+
	897.53[b]	DHDAG (28:1)	+	+	+
	899.54[b]	DHDAG (28:0)	+	+	+
	925.56[b]	DHDAG (30:1)	+	+	+
	927.57[b]	DHDAG (30:0)	+	+	+
	953.58[b]	DHDAG (32:1)	+	+	+
	981.62[b]	DHDAG (34:1)	+	+	+

PG	693.47	PG(14:0/16:0)	+	—	+
	719.49	PG(16:0/16:1)	+	—	+
	721.5	PG(16:0/16:0)	+	—	+
	773.53	PG(16:0/18:1)	+	—	+
	775.54	PG(18:0/18:1)	+	—	+
CL	1321.91	CL(62:1)	+	—	+
	1349.94	CL(64:1)	+	—	+
	1375.96	CL(66:2)	+	—	+
	1403.99	CL(68:2)	+	—	+
	1432.02	CL(70:2)	+	—	+
PAHN	848.53	PAHN(16:0/16:1)	+	+	+
	874.55	PAHN(16:1/18:1)	+	+	+
	876.56	PAHN(16:0/18:1)	+	+	+
	902.58	PAHN(18:1/18:1)	+	+	+
	904.6	PAHN(18:0/18:1)	+	+	+
PA	619.43	PA(14:0/16:0)	+	+	+
	645.45	PA(16:0/16:1)	+	+	+
	647.46	PA(16:0/16:0)	+	+	+
	673.48	PA(16:0/18:1)	+	+	+
	699.49	PA(18:1/18:1)	+	+	+
	701.50	PA(18:0/18:1)	+	+	+
PC	740.50[b]	PC(14:0/16:0)	+	+	+
	766.51[b]	PC(16:0/16:1)	+	+	+
	792.53[b]	PC(16:1/18:1)	+	+	+
	794.54[b]	PC(16:0/18:1)	+	+	+
	820.56[b]	PC(18:1/18:1)	+	+	+

[a]The individual or total numbers of acyl chain carbon atoms and double bonds are shown in parentheses.

[b]DAG, MHDAG, DHDAG, and PC values are for the $[M + Cl^-]^-$ ions.

Abbreviations: WT, *S. mitis* 1643; CdsA, *S. mitis* 1643-HA04; Revertant, *S. mitis* 1643-HA14; DAG, diacylglycerol; MHDAG, monohexosyldiacylglycerol; DHDAG, dihexosyldiacylglycerol; FA, fatty acid; CL, cardiolipin; PA, phosphatidic acid; PAHN, phosphatidyl N-acetylhexosamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine.

Table B.4. Mutations in *cdsA* identified in daptomycin spontaneous resistance experiments.

Strain ID	Nucleotide Change	Amino Acid Change
LJ35	G35A	A12E
LJ39	T264A	Y88*
LJ310	T264A	Y88*
LJ311	T264A	Y88*
LJ713	C351A	S117R
LJ72	G361T	G121C
LJ76	G361T	G121C
LJ313	T366del	F122fs
LJ510	G394-T645del	V132-S215 del
LJ72	G421A	A141T
LJ54	T428-T695del	F143-F232delfs
LJ34	T431ins	I144fs
LJ56	G445A	D149N
LJ514	C450G	S150R
LJ714	C455T	A152V
LJ76	C459G	Y153*
LJ52	A481T	R161*
LJ53	A499T	R167*
LJ31	C506T	S169F
LJ51	C506T	S169F
LJ712	G516C	K172N
LJ33	G527C	G176A
LJ37	G527C	G176A
LJ711	T531del	F177fs
LJ32	C551A	A184E
LJ59	C551A	A184E
LJ36	G679C	A227P
LJ512	C688T	R230C
LJ312	A731C	H244P
LJ314	A731C	H244P
LJ55	G734T	G245V

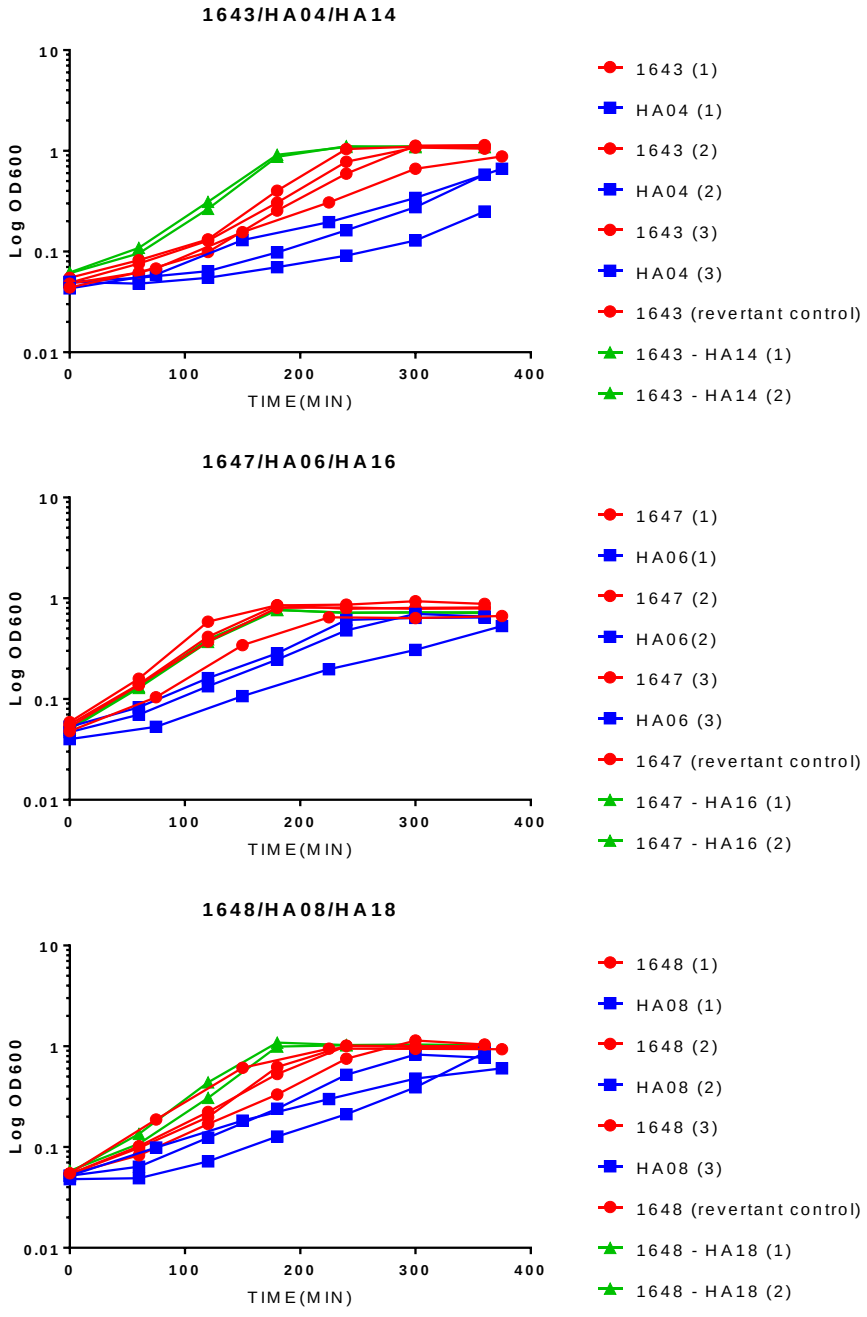

Figure B.1. Growth curves for parental strains, their DAP^{R} derivatives, and DAP^{S} revertants. *S. mitis* 1643, DAP^{R} derivative 1643-HA04, and DAP^{S} revertant 1643-HA14 are displayed top; *S. oralis* 1647, DAP^{R} derivative 1647-HA06, and DAP^{S} revertant 1647-HA16 are displayed middle; *S. oralis* 1648, DAP^{R} derivative 1648-HA08, and DAP^{S} revertant 1648-HA18 are shown bottom. Red lines represent parental strains, blue lines represent resistant strains, and green lines represent revertant strains. Cultures were grown overnight in BHI and diluted to an

OD_{600} of 0.05 in fresh BHI. OD_{600} readings were taken either every hour or every 75 minutes for six hours.

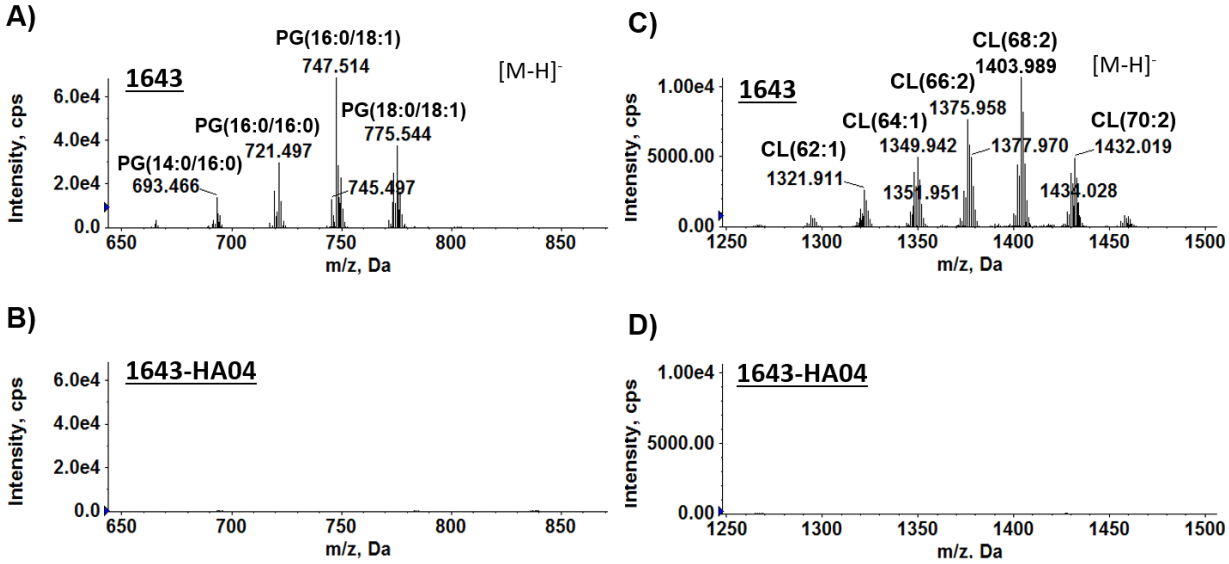

Figure B.2. Representative negative ion ESI mass spectra of PG and CL species in *S. mitis* 1643 and its derivative. PG and CL are detected normally by MS in the DAP-susceptible *S. mitis* 1643 (panels A and C), but are not detected in the DAP-resistant derivative 1643-HA04 (panels B and D).

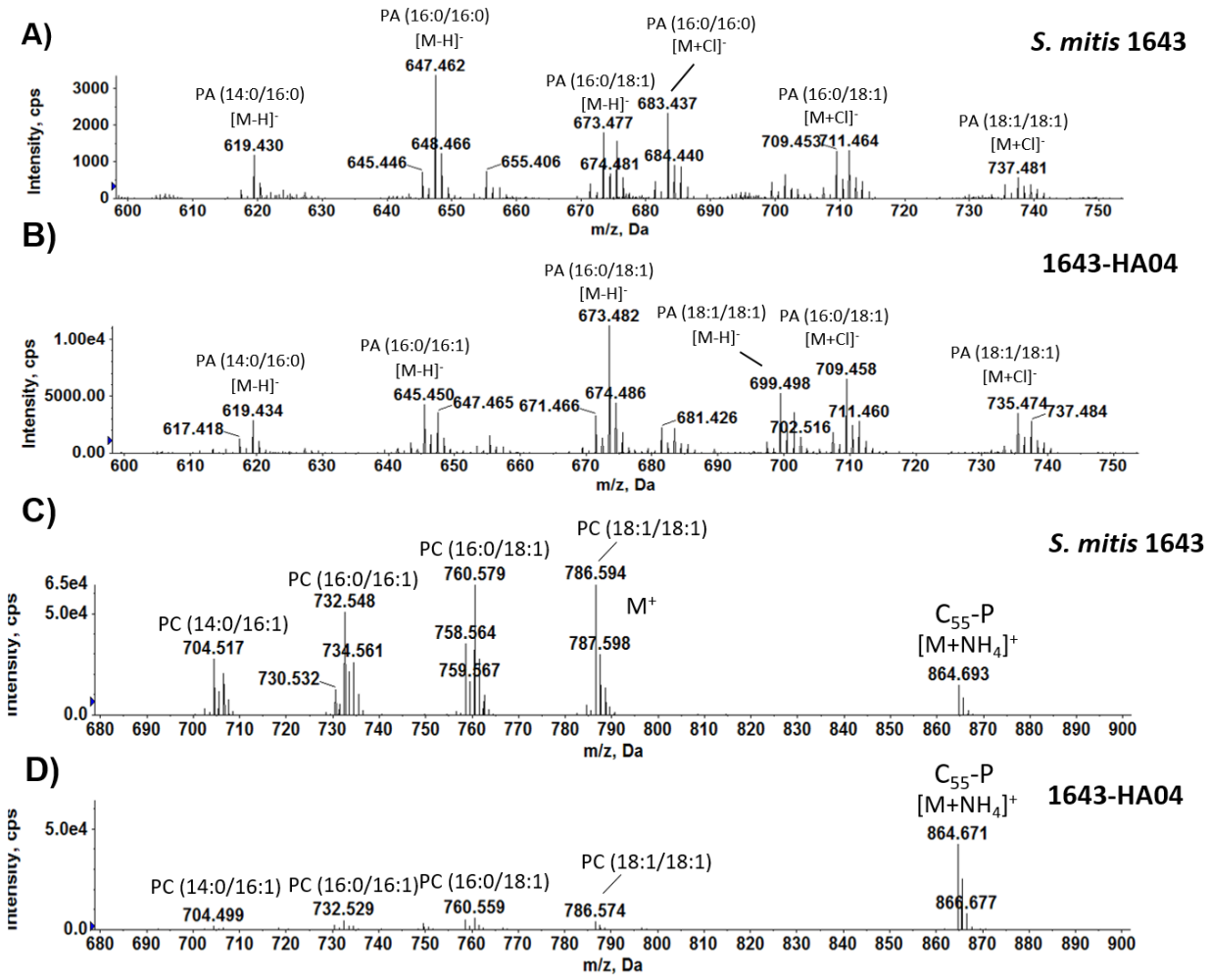

Figure B.3. Mass spectra of PAs, PCs, and undecaprenyl phosphate (C_{55}-P) in *S. mitis* 1643 and the DAP-resistant derivative 1643-HA04. The mass spectra shown are averaged from spectra acquired by negative ion ESI/MS during the 20.2-21.2 min window. Both deprotonated $[M-H]^-$ and chloride adduct $[M+Cl]^-$ ions of PA are detected. The level of PC is significantly lower in the DAP-resistant strain compared to the parental DAP-sensitive strain.

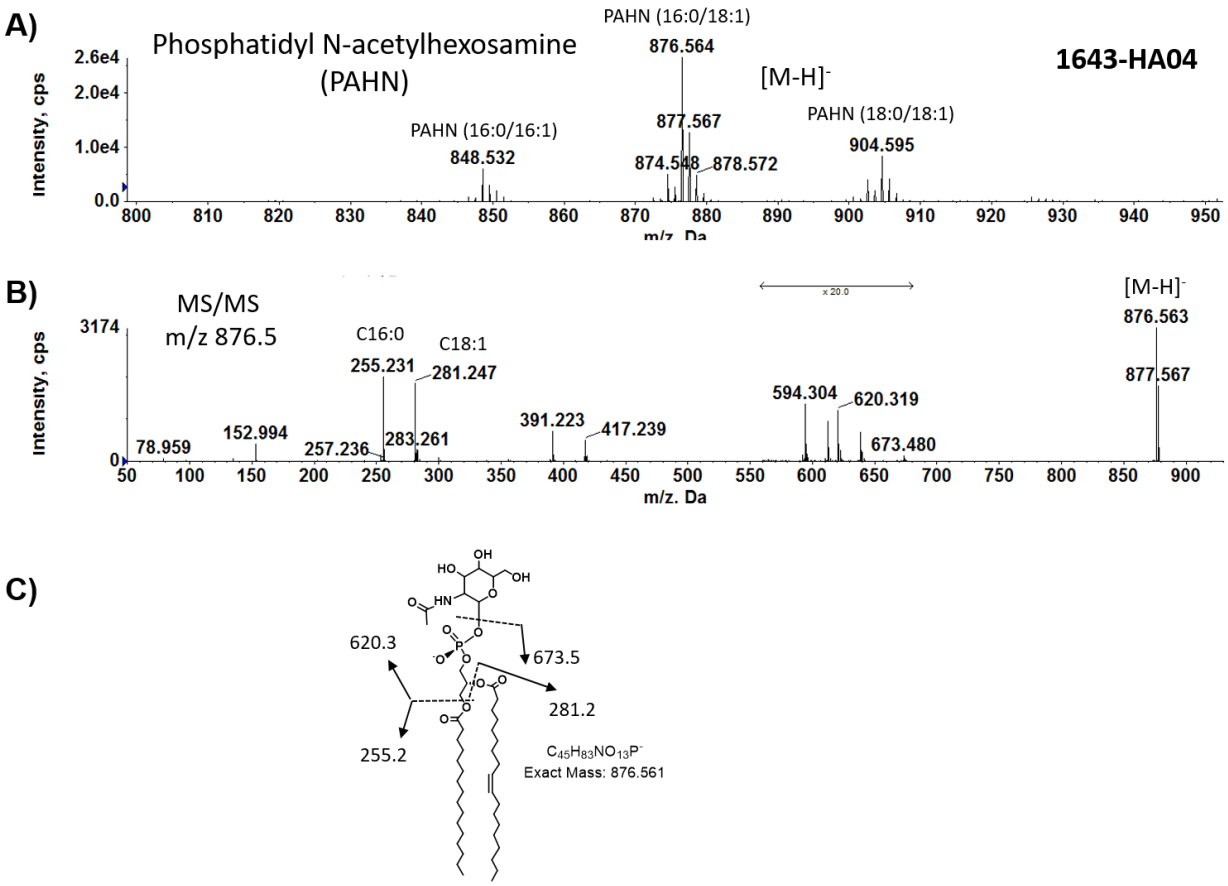

Figure B.4. Identification of phosphatidyl N-acetylhexosamine (PAHN) from strain 1643-HA04. Negative ion ESI mass spectrum showing the $[M-H]^-$ ions of the PAHN molecular species emerging at 14.8-15.3 min (A). Collision-induced dissociation (CID) MS/MS spectrum of the $[M-H]^-$ ion PAHN (16:0/18:1) at *m/z* 876.5 (B). The arrows indicating x20 reflect magnification of the product ion peaks in the corresponding region of the *m/z* values on the mass spectrum. Chemical structure of PAHN (16:0/18:1) and the fragmentation scheme for the observed product ions in B (C).

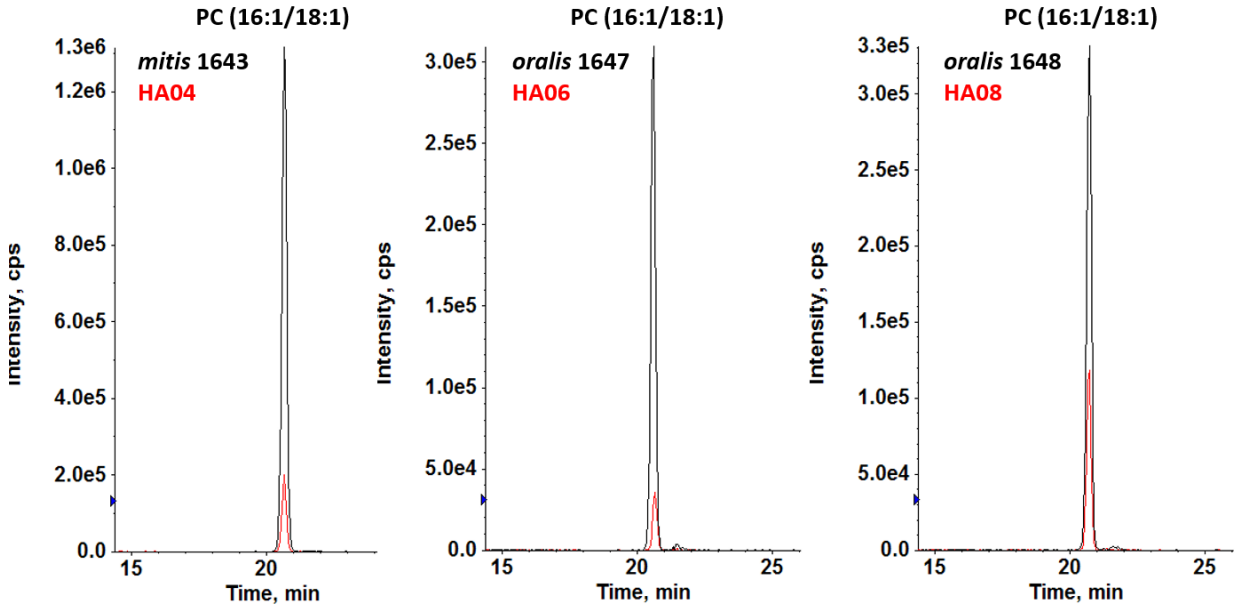

Figure B.5. Comparison of the level of PC (16:1/18:1) in the DAP-resistant and DAP-sensitive pair strains. Extracted ion chromatograms of *m/z* 758.5 for the M^+ ion of PC (16:1/18:1), a major PC species in *S. mitis* 1643 (black) and *S. mitis* 1643-HA04 (red).

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BIOGRAPHICAL SKETCH

Hannah Adams was born in Dallas to Terese and Harold Adams on May 29th, 1990. She graduated valedictorian from McKinney High School in 2008. She then went on to receive her BS in Biology and Neuroscience from The University of Texas at Dallas in 2012. After fast-tracking into the Molecular and Cell Biology Graduate Program, Hannah received her MS from UT Dallas in 2014. She then continued her doctoral research in microbiology. She was awarded the Eugene and Millicent Goldschmidt Graduate Student Award from the Texas Branch of the American Society of Microbiology in 2016.

CURRICULUM VITAE

Hannah Adams
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Education

2014 – Present	**Ph.D. Candidate**, Molecular and Cell Biology Department of Biological Sciences The University of Texas at Dallas Richardson, Texas PI: Dr. Kelli L. Palmer Expected July, 2017
2012 – 2014	**M.S. in Molecular and Cell Biology** Department of Biological Sciences The University of Texas at Dallas Richardson, Texas PI: Dr. Kelli L. Palmer
2008 – 2012	**B.S. in Biology and Neuroscience** School of Natural Science and Mathematics The University of Texas at Dallas Richardson, Texas

Teaching Experience

2017 Spring	**Instructor of Record, UTD, BIOL 2281 Introductory Biology Laboratory** Taught two sections of Introductory Biology Laboratory. Guided students through laboratory procedures. Assessed student learning through weekly reports, quizzes, and worksheets. The course had two summative assessments, a midterm and a final exam, which consisted of multiple choice questions, application problems, and practical skill demonstrations.
2017 Spring	**Teaching Assistant, UTD, BIOL 3V20, General Microbiology with Lab.**
2016 Summer	Instructor of record, Dr. Ernest Hannig.
2013 Summer	Prepared laboratory experiments for students and guided them through new microbial techniques. Answered questions regarding protocols and results of biochemical testing. Provided guidance when students were instructed to identify unknown bacterial species using the procedures taught in class.

2016 Fall **Teaching Assistant, UTD, BIOL 3301, Classical and Molecular Genetics.**
2013 Fall Instructors of record, Dr. Ernest Hannig and Dr. Dennis Miller.
2012 Fall Taught lecture material in supplemental workshops for students. I held two, one hour long workshops a week in which I would cover material taught in class. I also developed additional problems that were not found in the course book to help students better understand the material presented. I answered any questions students had via email as well as hosted exam review sessions prior to tests.

2014 Spring **Teaching Assistant, UTD, BIOL 3301, Classical and Molecular Genetics.**
2013 Spring Instructors of record, Dr. Jeff De Jong and Dr. Dennis Miller.
Hosted workshops for students to further supplement their learning as well as assisted in exam grading. Each week, I would hold two, one hour long workshops to reiterate concepts taught in class. I developed notes from scratch to better explain the material. I helped the students develop problem-solving skills to confidently work through problems they might encounter on the exam.

Research Experience

2013 Summer – Present **Research Assistant**
Department of Biological Sciences
The University of Texas at Dallas
Project: Novel restriction-modification systems in *Enterococcus faecium*
Project: Mechanisms of lipopeptide antibiotic resistance in Gram-positive bacteria.
Supervisor: Dr. Kelli L. Palmer

2013 Spring **Lab rotation**
Department of Biological Sciences
The University of Texas at Dallas
Supervisor: Dr. Kelli L. Palmer

2012 Fall **Lab rotation**
Department of Biological Sciences
The University of Texas at Dallas
Supervisor: Dr. Juan E. González

Educational Outreach

1. College Journey: Teaching for Tomorrow. July 18th, 2016.

   The Science and Engineering Education Center at The University of Texas at Dallas.

   One hundred 9th-12th grade girls were hosted at UTD for a week long camp centered around the theme "Access to Clean Water." I was responsible for designing a presentation and

interactive activity to teach the girls about waterborne pathogens and diseases. The lecture was given four times to groups of 25 girls each. The girls walked away with a better appreciation of the public water systems we are able to use in the United States and how they prevent serious diseases that some countries battle on a daily basis.

2. Girls, Inc. Eureka! Program. June 23rd, 2016.

   The University of Texas at Dallas partnered with Girls, Inc.

   A week long learning camp was hosted at UTD for middle school girls. I taught a two hour long Microbiology session to introduce the girls to microbes and their impact on humans. In addition, I arranged an activity in order to demonstrate how microbe-based diseases can spread amongst a population.

3. Comet Cosmetics Lipstick Challenge. May 18th, 2016.

   The Science and Engineering Education Center at The University of Texas at Dallas.

   UTD hosted 150 8th grade girls from Balch Springs Middle School to participate in a Lipstick Challenge. The students were tasked with incorporating chemistry and marketing into a craft activity where they created their own lipstick colors. I helped run a small group of girls through the activity and incorporate learning concepts.

Educational Training

1. Graduate Student Workshop: Fundamentals of excellent academic writing, April 14th, 2016 at The University of Texas at Dallas.
2. Graduate Teaching Assistant Workshop: How to deal with difficult students, April 8th, 2016 at The University of Texas at Dallas.
3. Graduate Teaching Assistant Workshop: Developing your teaching portfolio, March 8th, 2016 at The University of Texas at Dallas.
4. Graduate Teaching Assistant Workshop: How to implement undergraduate research opportunities, February 23rd, 2016 at The University of Texas at Dallas.
5. Graduate Teaching Assistant Workshop: Creating a teaching portfolio, February 17th, 2016 at The University of Texas at Dallas.
6. Graduate Teaching Assistant Workshop: Assessing learning outcomes, February 10th, 2016 at The University of Texas at Dallas.

Mentoring

1. Cristian Trejo, January 2014-May 2015

   Graduated with a B.S. in Biology in May 2015

Accepted into the Texas A&M Health Science Center College of Medicine

I taught him new techniques that were specific to a molecular biology lab and aided in his research for his Honors Thesis. I then helped him compile his data into a poster for scientific presentations. He presented his research three separate times, once at the Texas Branch ASM Fall meeting, once at the UT Systems LSAMP program, and once at the Exhibition of Excellence in Undergraduate Research here at UTD.

2. Rohit Badia, May 2015-present

   Junior at UTD in the Biology degree program

   Rohit joined the Palmer lab with no prior lab experience, so I helped introduce him to the basic concepts of microbiology and taught him many common molecular protocols. He is part of the CV Honors College at UTD and used his lab experience as extracurricular credit for the program. Despite receiving all the credits for laboratory work, Rohit continues to work in the lab due to his enjoyment of the research subject.

3. Luke Joyce, January 2016-present

   Graduated with a M.S. in Molecular and Cell Biology in May 2016

   Accepted into the MCB Ph.D. program at UTD in May of 2016

   While Luke had prior lab experience, he had not worked in a microbiology lab before, so I helped introduce him to all of the new techniques for working with bacteria. He came in to work on my second project concerning antibiotic resistance within the streptococci and will be second author on the paper. He now has been given his own project to build off the one he worked on with me.

Ethics Training

Completed the Responsible Conduct of Research Seminar Series provided by the Office of Research Compliance here at UTD. Training was received weekly, covering the following topics:

- Research Misconduct & Scientific Fraud
- Responsible Authorship, Publication and Peer Review
- Mentor/Mentee Responsibilities and Relationships
- Collaborative Research Including Responsibilities and Relationships with Industry
- Human & Animal Subjects in Research
- Conflicts of Interest
- Intellectual Property
- Data Management and Ownership
- Scientific Citizenship – Responsibility to Colleagues and the Community

Publications (reverse chronological order)

1. **Adams HM,** Joyce LR, Guan Z, Akins RL, Palmer KL. 2017. *Streptococcus mitis* and *S. oralis* lack a requirement for CdsA, the enzyme required for synthesis of major membrane phospholipids in bacteria. Antimicrobial Agents and Chemotherapy doi: 10.1128/AAC.02552-16 [Epub ahead of print].
2. **Adams HM**, Li X, Mascio C, Chesnel L, Palmer KL. 2015. Mutations associated with reduced surotomycin susceptibility in *Clostridium difficile* and *Enterococcus* species. Antimicrobial Agents and Chemotherapy **59**(7):4139-4147.
3. Huo W, **Adams HM**, Zhang MQ, Palmer KL. 2015. Genome modification in *Enterococcus faecalis* OG1RF assessed by bisulfite sequencing and single-molecule real-time sequencing. Journal of Bacteriology **197**(11):1939-1951.

Honors/ Awards

2017	American Society for Microbiology (ASM) Science Teaching Fellow
2017	Texas Branch ASM Eugene and Millicent Goldschmidt Graduate Student Award
2016	Student Travel Award for 2016 Wind River Conference, Colorado
2016	Graduate Teaching Certificate, The University of Texas at Dallas
2015	Sam Kaplan Award for Graduate Molecular Microbiology Poster Presentation, Third place, Texas Branch ASM Fall Meeting 2015, Sam Houston State University, Huntsville, Texas.

Presentations

Talks:

1. *Streptococcus mitis* and *S. oralis* mutate an 'essential' gene upon exposure to daptomycin
   Hannah Adams, Luke Joyce, Ziqiang Guan, Ronda Akins and Kelli L. Palmer.
   2017 ASBMB Experimental Biology Annual Conference (upcoming). Chicago, IL.
2. *Streptococcus mitis* and *S. oralis* mutate an 'essential' gene upon exposure to daptomycin
   Hannah Adams, Luke Joyce, Ziqiang Guan, Ronda Akins and Kelli L. Palmer.
   Eugene and Millicent Graduate Student Award Lecture
   2017 Texas Branch ASM Spring Meeting, New Braunfels, TX.

Posters:

1. One-step daptomycin resistance in viridans group Streptococci
   Hannah Adams, Luke Joyce, Ronda Akins and Kelli L. Palmer.
   2016 ASM Conference on Streptococcal Genetics. Washington, D.C.
2. One-step daptomycin resistance: a widespread concern or a unique phenomenon?
   Hannah Adams, Luke Joyce, Ronda Akins and Kelli L. Palmer.
   2016 Wind River Conference on Prokaryotic Biology. Estes Park, CO.
3. Clade-specific restriction-modification systems in *Enterococcus faecium.*
   Hannah Adams, Cristian Trejo, Wenwen Huo, and Kelli L. Palmer.
   2015 Texas Branch ASM Fall Meeting. Huntsville, TX.

4. Clade specific restriction-modification systems in *Enterococcus faecium*.
   Cristian Trejo, **Hannah Adams**, Wenwen Huo, and Kelli L. Palmer.
   2014 Exhibition of Excellence in Undergraduate Research. Richardson, TX.
5. Clade specific restriction-modification systems in *Enterococcus faecium*.
   Cristian Trejo, **Hannah Adams**, Wenwen Huo, and Kelli L. Palmer.
   2014 UT Systems LSAMP Program. Dallas, TX.
6. Clade specific restriction-modification systems in *Enterococcus faecium*.
   Cristian Trejo, **Hannah Adams**, Wenwen Huo, and Kelli L. Palmer
   2014 Texas Branch ASM Fall Meeting. Houston, TX.
7. Clade-specific restriction-modification systems in *Enterococcus faecium*.
   Hannah Adams, Cristian Trejo, Wenwen Huo, and Kelli L. Palmer.
   2014 Molecular Genetics of Bacteria and Phage Meeting. Madison, WI.
8. Genomic analysis of restriction-modification systems in *Enterococcus faecium*.
   Hannah Adams and Kelli L. Palmer.
   2013 Texas Branch and South Central Branch ASM Fall Meeting. New Orleans, LA.

Additional Meetings Attended

1. ASM Conference for Undergraduate Educators (upcoming). July 27-30, 2017. Denver, CO.
2. Molecular Genetics of Bacteria and Phage Meeting. August 4-8, 2015. Madison, WI.
3. 113th ASM General Meeting. May 18- 21, 2013. Denver, CO.

Other Achievements

1. Graduated Valedictorian of McKinney High School Class of 2008.
2. Was awarded a scholarship from the McKinney Education Foundation for my academic accomplishments.
3. Was awarded the Academic Excellence Scholarship throughout the entirety of my undergraduate career.
4. Graduated Magna Cum Laude within the School of Natural Science and Math.
5. Was awarded the EEF Scholarship for graduate students within the School of Natural and Science and Math for the 2012-2013 and 2013-2014 academic years.
6. Have successfully mentored undergraduates from the UTeach program at The University of Texas at Dallas.

Memberships

Member of the American Society of Biochemistry and Molecular Biology, ASBMB.
Member of the American Society of Microbiology, ASM.
Member of the American Society of Microbiology, Texas Branch, ASM.

References

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