

**Perturbing fatty acid metabolism in *Enterococcus faecalis* disrupts
responses to exogenous fatty acids and the antibiotic daptomycin**

**A Dissertation Presented for the
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Degree
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DEDICATION

For my parents, who made me feel I could do anything.

For my friends, who encouraged me along the journey.

For my advisor, who drew the roadmap.

For my husband, who has been there for me every step of the way.

With love, this dissertation is dedicated to each of you.

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ABSTRACT

Enterococcus faecalis is an opportunistic pathogen. In the host, it is exposed to fatty acids which impact cellular physiology and induce tolerance to the antibiotic daptomycin. To determine the requirements for induction of daptomycin tolerance, I examined the impacts of blocking *de novo* fatty acid synthesis or protein synthesis and removing cell wall. I observed that removal of the cell wall induced daptomycin tolerance, indicating that peptidoglycan is necessary for daptomycin to function. As specific exogenous fatty acids induce protection against daptomycin in *E. faecalis*, I also opted to examine whether incorporation of these free fatty acids was necessary to induce drug tolerance. To do this, I deleted the genes predicted to encode fatty acid binding proteins (FakBs). I found that combined deletion of specific *fakB* genes caused altered physiology, including distorted morphology, increased levels of free fatty acids, and altered growth kinetics in the presence of exogenous fatty acids. This led me to investigate whether a thioesterase, TesS, which cleaves fatty acids from acyl-ACP, was causing a buildup of free fatty acids within the *fakB* deletion strains. Deletion of *tesS* in the *fakB* deletion strains reverted free fatty acid levels to wild-type levels. Additionally upon deletion of *tesS*, I was able to delete all four *fakB* homologs, generating a quintuple deletion strain. I followed up on the physiology of this strain and found that the growth kinetics and free fatty acid levels associated with deletion of the *fakB* genes were reverted to the wild-type phenotype. Further, I observed that the quintuple deletion strain could not grow in the presence of a *de novo* fatty acid biosynthesis inhibitor, even when supplemented with oleic acid. These data indicated that the quintuple deletion strain had a reduced ability to incorporate exogenous fatty acids. Additionally, deletion of *fakB* genes led to increased basal daptomycin tolerance, yet exogenous fatty acids were still able to induce daptomycin protection. These data indicated that the *fakB* genes play a role in normal cellular physiology, and that incorporation of fatty acids into membrane phospholipids is likely not the driver for fatty acid-mediated daptomycin tolerance.

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CHAPTER 1: INTRODUCTION

This article was written by Rachel D. Johnston with editorial commentary from Elizabeth M. Fozo. It has not been published anywhere, nor will it be before the final version is submitted at the estimated time of defense, so a publication statement was not included.

Enterococci

Enterococcus is a genus of Gram-positive, facultatively anaerobic bacterium. These organisms were first described as “entérocoque” due to their ovoid shape and their isolation source: an intestinal smear [1]. The organism was first named *Micrococcus zymogenes* by Hastings and Maccullum [2], who described their isolate (from a patient with acute, fatal endocarditis) as a very small Gram-positive microorganism which grows in pairs or short chains. They thus placed it in the genus *Micrococcus*, although they noted that their isolate was much smaller than the already-characterized *Streptococcus pneumoniae* (described in the literature of the time as *Micrococcus lanceolatus*) [2, 3]. As they found that the isolate was able to ferment lactose and gelatin, and because it was so different from *Streptococcus pneumoniae* and *Streptococcus pyogenes*, they gave it the genus of *zymogenes* [2]. A few years later, in 1906, Andrewes and Horder, in an attempt to classify 300 isolates of putative *Streptococci* strains, termed the organism *Streptococcus faecalis*, as the microorganism was typically found in the human intestinal tract [4]. This nomenclature, with multiple variates and classification strategies, was then used in the literature to describe small, Gram-positive diplococci or short chains, typically originating from the mammalian gastrointestinal tract. However, it was noted that there were several physiological differences among members of the *Streptococcus* genus: the enterococci of the group had marked tolerance for diverse environmental conditions, being able to grow at both high and low temperatures (45°C and 10°C), high salt (6.5%), high pH (9.6), and in media containing methylene blue (0.1%), as well as being able to survive at 60°C for 30 minutes, while other members of the *Streptococcus* genus were not so universally tolerant in these conditions [5]. Furthermore, the enterococci members of the *Streptococcus* genus were consistently noted to grow in the shape of a diplococcus [1, 2], forming chains only under stressful growth conditions (notably on supplementation

of the media with bile or in old cultures [6, 7]. Immunological evidence also surfaced, indicating different groupings within the *Streptococcus* genus: antisera generated for different species of the *Streptococcus* genus and subsequently used for precipitin tests against *Streptococcus* species revealed four distinct groups [7], of which the enterococci were group D [5]. Eventually, with the development of tools for elucidating genetic differences, further evidence arose separating enterococci from the other members of the *Streptococcus* genus. In 1984, Schleifer and Balz found, via DNA hybridization studies, that *Streptococcus faecalis* and *Streptococcus faecium* were separate species (they shared only 18% DNA homology), yet these two species had <9% DNA homology when compared to other streptococci [8]. While this was not direct sequencing data but hybridization data, it led to the proposal that *S. faecalis* and *S. faecium* be transferred to the genus *Enterococcus* [8]. The 1984 version of Bergey's manual supported these transfers into the newly minted genus *Enterococcus*.

While they normally colonize the gastrointestinal tract of mammals, enterococci are able cross the divide between commensal interactions and pathogenic interactions with their hosts, as demonstrated by their varying isolation sources (often from fecal matter, but also from blood or tissue in infected individuals). Enterococcal isolates are often described as “hardy” as they able to survive for months in culture with no nutrients added [2, 4], and can also survive from weeks to months on hospital fabrics and plastic [9]. As they are able to persist under such nutrient-limited conditions, it is little wonder that enterococci commonly cause healthcare-acquired infections.

Treatment options for enterococcal infections

A major problem when trying to treat an infection caused by *E. faecalis* (or *E. faecium*) is their resistance or high tolerance to a variety of antibiotics. As a whole, this genus has reduced susceptibility to β -lactams including ampicillin and penicillin, and is resistant to aminoglycosides and clindamycin [10, 11]. When other treatment options fail, severe *E. faecalis* infections are often treated with vancomycin, a cell-wall targeting antibiotic. However, in response to utilization of vancomycin as a treatment of enterococcal

infections, vancomycin resistance has increased in enterococcal populations. As enterococci have the ability to pass and receive genes through bacterial conjugation [12-16], the upsurge of vancomycin resistance is alarming. Of note, vancomycin resistance loci, predominantly mediated by the *vanA* gene cluster [17], have been found on plasmids and conjugative transposons which can integrate into both bacterial chromosomes and plasmids which are shared between bacterial cells [18-20]. The potential for horizontal spread of vancomycin resistance among enterococcal populations or to other bacterial species is of pressing concern, as these infections are difficult to treat, and result in an estimated cost of more than \$342 million annually [21, 22].

Vancomycin resistant enterococcal (VRE) infections have been treated in the clinic using daptomycin. However, with increases in daptomycin usage for the treatment of VRE infections, resistance to daptomycin has also increased [10, 23]. This rise of antibiotic resistance in enterococcal populations hampers treatment of infections, increases the risk of horizontal transfer of genes encoding resistance factors to other species, and increases national healthcare-associated costs. With limited treatment options for VRE infections, and with the difficulty with bringing new antimicrobials to market, it is critical to elucidate how microbes such as *Enterococcus* evade death by daptomycin treatment. Thus, the mechanism of this antibiotic must be examined.

Proposed mechanisms of daptomycin function

Daptomycin (originally A21978C), which was originally isolated from *Streptomyces roseosporus* found in the soil on the slopes of Mount Ararat, is a cyclic lipopeptide consisting of 13 amino acids with a fatty acid tail (usually of 10 carbons) [24]. Three primary modes of action for this drug have been proposed since its discovery: inhibition of peptidoglycan (PG) biosynthesis; inhibition of lipoteichoic acid (LTA) biosynthesis; and disruption of membrane potential. While an abundance of data points towards each of these three mechanisms, the specific details remain unclear.

The target of daptomycin was originally thought to be peptidoglycan (PG), as daptomycin inhibited the incorporation of alanine into PG, (93% inhibition at 100 µg/mL),

but incorporation of labelled DNA, RNA, lipid, and protein precursors was impacted to a lesser extent (<25% at 100 µg/mL) [25]. Adding daptomycin to *Bacillus megaterium* cultures also resulted in inhibition of biosynthesis of PG precursors, including lipid I (UDP-NAM-pentapeptide) and uridine diphosphate n-acetylglucosamine (UDP-NAG), lending support towards PG as daptomycin's target [26]. An investigation on the importance of presence of cell wall to daptomycin efficacy revealed that removal of the cell wall of *E. faecium* did not protect the cells against the drug [27].

Further study of daptomycin suggested that lipoteichoic acid (LTA) was a target, as LTA synthesis, which occurs in and near the membrane and cell wall, was inhibited by daptomycin [27, 28]. Some evidence supported this hypothesis: LTA precursors accumulated in cells following daptomycin exposure [29]. However, daptomycin did not appear to inhibit LTA synthesis more strongly than it inhibited other cellular processes, including lipid synthesis and RNA synthesis; thus, LTA synthesis was likely not the primary target [30].

Several lines of reasoning later pointed towards the membrane as the true target of daptomycin. First, daptomycin has an acyl tail, which, upon changing the length, alters toxicity [31]. As the acyl tail of daptomycin is hydrophobic, it was proposed that the tail mediated insertion of daptomycin into the bacterial membrane. Indeed, removal of the acyl tail resulted in abolishment of antimicrobial activity, while increasing the length of the fatty acid was found to increase toxicity against *Staphylococcus aureus* and *Streptococcus pyogenes* [31]. These data suggested that the acyl group plays a major role in the mechanism of action. Further, the structure of calcium-bound daptomycin revealed that daptomycin is similar to cationic antimicrobial peptides (CAMPs), many of which work by disrupting bacterial membranes resulting in depolarization [32]. This similarity, along with the fact that daptomycin requires calcium as a cofactor to function, led investigators to hypothesize that daptomycin had a CAMP-like activity. Experiments showed that indeed, daptomycin treatment dissipated membrane potential [33-37]. Furthermore, *in vitro* evidence showed that daptomycin was shown to interact with the membrane phospholipid phosphatidylglycerol and form oligomers in the membrane,

much like traditional CAMPs [38]. Thus, the leading hypothesis for years was that daptomycin inserts into the membrane in a calcium-dependent manner, oligomerizes, and forms a pore-like structure allowing ion leakage, membrane depolarization, and cell death.

Evidence tying the hypotheses for the above proposed targets together was found by studying daptomycin localization, physiological effects on cell shape, and binding activity. It was observed that exposure of cells to daptomycin caused the ordinarily rod-shaped *Bacillus subtilis* to bend and sometimes become swollen at one end [39]. In cells treated with sublethal doses of daptomycin for short (<1 h) time spans, the cell division protein DivIVA, which orchestrates localization of PG synthesis proteins in the membrane, was mislocalized, likely explaining the altered shape of cells treated with daptomycin [39]. These data indicated that treatment with daptomycin caused misplaced cell wall synthesis. Further data demonstrated that daptomycin binds the membrane at regions of increased fluidity (RIFs), causing misplacement of proteins critical for phospholipid synthesis and PG synthesis – thus targeting, in a potentially indirect manner, multiple vital cellular processes [40]. Daptomycin was also found to bind to the PG precursor lipid II, indicating PG biosynthesis as a primary target [41]. Importantly, lipid II also plays a role in the localization of proteins critical for cell division [42]. Taken together, these data suggest that while PG synthesis may be a primary target, the pleiotropic effects of daptomycin treatment described above may be explained by mislocalization of membrane proteins.

Cell wall synthesis and cell membrane organization

As the literature has consistently pointed towards daptomycin targeting different components of the cell envelope, primarily the cell membrane and cell wall, it is crucial to link these pathways together. Naturally, as components located outside the membrane, lipoteichoic acid and PG synthesis and anchoring are closely linked to the membrane. Lipoteichoic acid is a modification of the membrane lipid monoglucosyl-diacylglycerol (MGDAG; Figure 1.1). Type I LTA, which is the classically studied LTA and is the type

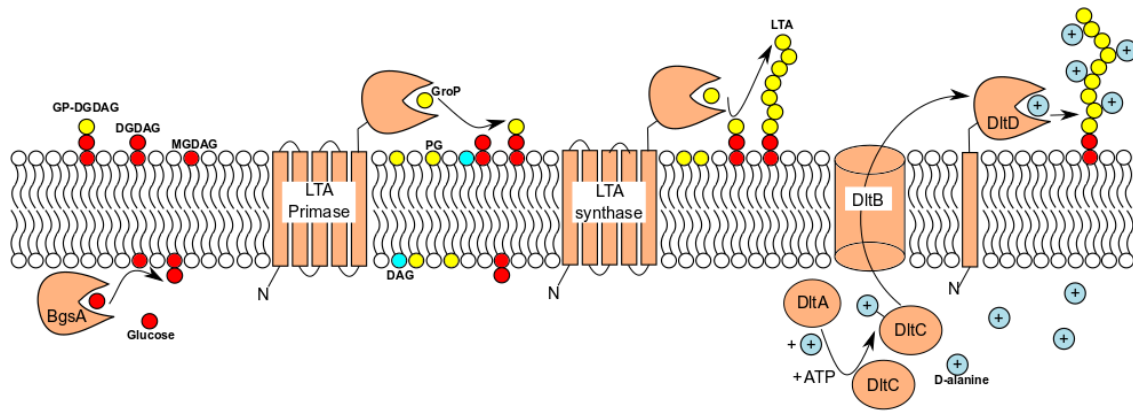


Figure 1.1. LTA biosynthesis.

In *E. faecalis*, the glycosyltransferase BgsA synthesizes diglycosyldiacylglycerols (DGDAGs) by adding a glucose to monoglycosyldiacylglycerol (MGDAG). In *B. subtilis*, glycerophospho-diglycodiacylglycerol (GP-DGDAG) is synthesized by addition of a glycerol-phosphate (GroP) group to DGDAG by an LTA primase, and lipoteichoic acid (LTA) is synthesized by successive addition of GroP groups to GP-DGDAG by an LTA synthase. LTA is then D-alanylated by enzymes encoded in the *dlt* operon.

observed in *E. faecalis*, is built upon a glycolipid backbone. Specifically, to build type I LTA, the enzyme BgsA adds a glucose to MGDAG, forming diglucosyl-diacylglycerol (DGDAG) [43, 44]. LTA primase then adds a glycerol-phosphate (GroP) group to the DGDAG, and the LTA molecule is extended by successive additions of GroP by LTA synthase [45]. The amino acid D-alanine is then added to the LTA molecule by genes encoded within the *dlt* operon [46]. Of note, many of these enzymes are located within the lipid membrane, and indeed, LTA itself is built upon DGDAG (Figure 1.1).

Lipoteichoic acid also has a role in membrane lipid turnover. In *S. aureus*, phosphatidylglycerol was confirmed as a GroP donor for LTA synthesis [47]. Loss of the GroP moiety from phosphatidylglycerol resulted in formation of diacylglycerol (DAG), which was used for phospholipid synthesis [47]. Thus, membrane makeup and LTA synthesis are closely linked. Furthermore, LTA synthesis has been linked with cell division through localization studies. It was observed that the proteins involved in LTA synthesis, including LTA synthase and the enzyme responsible for formation of the DGDAG glycolipid (named UgtP in *B. subtilis*), localized to the septum in *B. subtilis* cells [48]. Additionally, UgtP in *B. subtilis* was found to interact with FtsZ, a protein important for orchestrating the divisome in bacteria [49]. These data suggest that LTA synthesis, phospholipid synthesis and turnover, and coordination of cellular division are closely linked.

Peptidoglycan, like LTA, is partially synthesized in the bacterial membrane (Figure 1.2). In particular, once the peptide stem has been built, it is added to the lipid undecaprenol, and further steps, up until transpeptidation, are performed in the membrane. Of note, the lipid bound PG precursor, lipid II, has been implicated in localization of the cell division protein MreB in *B. subtilis*, which positions PG synthesis machinery [42]. Furthermore, MurG, the enzyme responsible for synthesizing lipid II, was found to specifically localize to the septum of *Mycobacterium smegmatis*, in correlation with sites of active cell wall synthesis [50]. In *B. subtilis*, these findings were corroborated with the discovery that MurG localizes to negatively curved regions of the membrane [51, 52]. Similarly, DivIVA, a protein involved in organizing cell division proteins, was found to

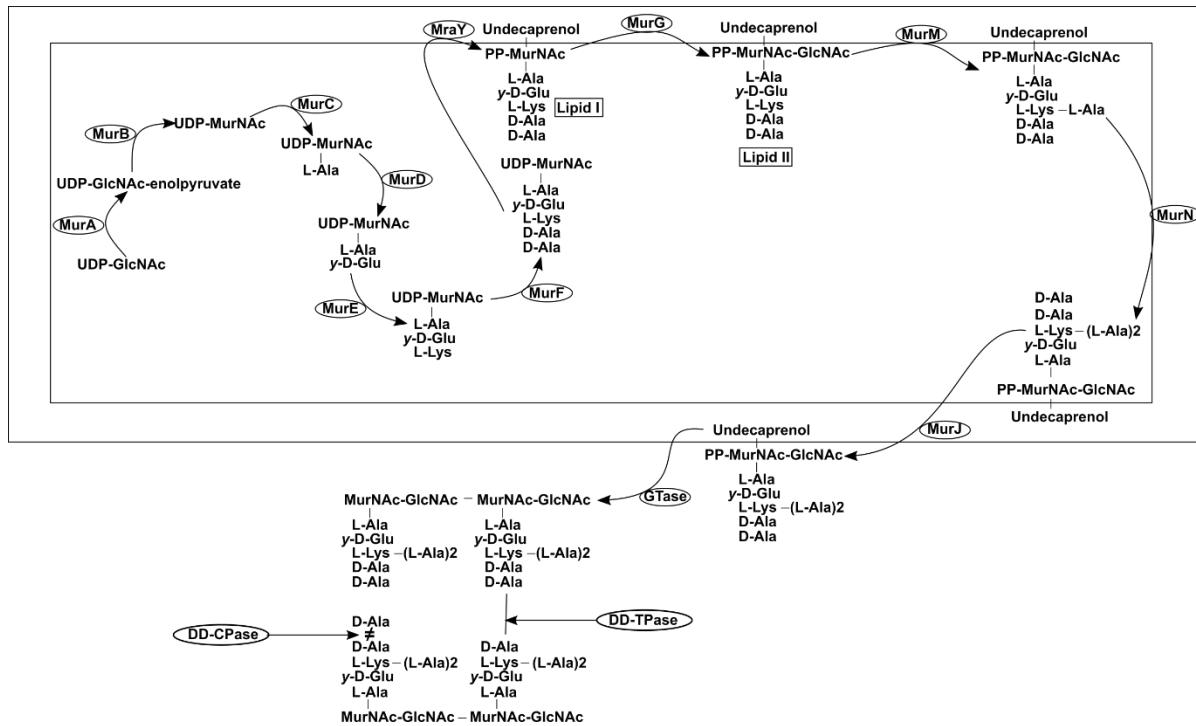


Figure 1.2. Peptidoglycan biosynthesis.

Shown is the pathway for Lys-type peptidoglycan synthesis. Note that this type of peptidoglycan is synthesized in *E. faecalis*, but other peptidoglycan forms are synthesized by other bacterial species. In this type of peptidoglycan synthesis, the stem peptide is built upon UDP-MurNAc by enzymes MurC, MurD, MurE, and MurF. This molecule is then attached to undecaprenol by MraY, forming the peptidoglycan (PG) precursor lipid I. Lipid II is then synthesized by addition of GlcNAc to the MurNAc moiety of lipid I by MurG. Following this reaction, in *E. faecalis*, other modifications are made to the peptide stem – namely two L-alanine groups are added to L-lysine by MurM and MurN, successively. The PG precursor is then flipped to the outer leaflet of the membrane by MurJ, and is subsequently crosslinked by glycosyltransferases (GTases). Crosslinking of the peptide chains is performed by DD-transpeptidases (DD-TPases), and trimming of the peptide stem is performed by DD-carboxypeptidases (CPases).

localize to negatively curved regions of the membrane [50, 53, 54]. Taken together, these data suggest that membrane organization and PG synthesis are intricately intertwined. Thus, while the target of daptomycin is unclear, it is logical that multiple effects of daptomycin treatment on bacterial physiology have been observed.

As direct studies on daptomycin mechanism of action have proven unsuccessful in affirming the specific target, studies have been performed to elucidate how bacterial cells resist treatment with daptomycin.

Daptomycin resistance

Over the course of treatment of bacterial infections with daptomycin, resistance (ability of the bacterium to grow in the presence of the drug) arises [23, 55-57]. In *E. faecalis*, several mechanisms contributing to daptomycin resistance have been discovered. The most prevalent contributor to daptomycin resistance is increased activation of the three-component regulatory system LiaFSR (lipid II-interacting antibiotic response regulator and sensor) [58-65], which is responsible for maintaining cell envelope homeostasis. Common mutations result in a loss-of-function in LiaF [58, 60-62, 65], which inhibits the activation of the regulatory component, LiaR. The downstream consequences of increased activation of LiaR are not fully understood; however, one known result is increased transcription of *liaX* [59, 66]. Interestingly, genetic evidence suggested that the presence of a functional LiaX protein is important for cell membrane organization – notably, a C-terminus truncation or full deletion of *liaX* caused altered anionic phospholipid arrangements [66]. Furthermore, a nonsense mutation resulting in a C-terminal truncation of *liaX* resulted in daptomycin nonsusceptibility in a clinical isolate [67], confirming the contribution of this protein to daptomycin efficacy. Notably, the role of LiaX in altering the response of cells to daptomycin is likely two-fold: this protein has two domains; the C-terminal domain negatively regulates LiaSR, while the N-terminal domain can be released from the cell and can bind to extracellular daptomycin [66]. Thus, a C-terminal truncation of *liaX* would likely result in increased activation of LiaR, while the N-terminus could potentially sequester daptomycin.

While the LiaFSR system is thought to mediate phospholipid distribution in response to membrane stress via LiaX [66], specific mutations (often paired with the daptomycin resistance driving mutations in the LiaFSR locus) in phospholipid metabolism genes can also contribute to daptomycin resistance in *E. faecalis*. These include mutations in cardiolipin (CL) synthase (*cls*), which catalyzes the condensation of two phosphatidylglycerol (PtdGro) molecules to form CL, and glycerophosphoryl diester phosphodiesterase (*gdpD*), which is likely involved in hydrolyzing phospholipids [68-70]. Introduction of the altered *cls* alleles into the daptomycin-susceptible laboratory strain *E. faecalis* OG1RF conferred daptomycin resistance to this bacterium [68]. Further, a directed evolution experiment in *E. faecalis* lacking a functional LiaFSR system revealed that mutations in another two-component system, YxdJK, which is associated with sensing and responding to antimicrobial compounds, arose in response to increasing concentrations of daptomycin [71]. Additionally, mutations in the *E. faecalis* homolog of FakA (efFakA; a protein involved in phosphorylation of free fatty acids originally described in *S. aureus*) [72], also arose in response to increasing concentrations of daptomycin [71]. The variants containing mutations in efFakA had decreased colony size and altered cell morphology [71].

Of note, other daptomycin resistant strains implicated altered envelope physiology, potentially mediated by increased net surface positivity, increased cell wall thickness, or membrane fluidity [58, 73, 74]. Cell charge can be modulated by altered production of lysyl-phosphatidylglycerol (lysyl-PtdGro). To generate lysyl-PtdGro, MprF (multiple peptide resistance factor) adds lysyl groups to phosphatidylglycerol (PtdGro) and flips the newly formed lysyl-PtdGro to the outer leaflet of the membrane [75]. In *S. aureus*, gain-of-function mutations in *mprF* are associated with daptomycin resistance [74, 76]. Cell surface charge can also be altered through alanylation of LTA. Increased transcription of the *dlt* operon, which encodes genes responsible for alanylation of LTA has been linked to daptomycin resistance in *S. aureus* [77, 78]. Interestingly, gain-of-function mutations in *mprF* and modified regulation of the *dlt* operon cause daptomycin resistance [74, 76]. However, while increased lysyl-PtdGro production and d-alanylation

of lipoteichoic acid alter the charge of cells, in *E. faecalis*, daptomycin protection was not correlated with gross changes in global surface charge or membrane fluidity [79]. Thus, while physiological changes in cells resistant or tolerant to daptomycin have been demonstrated in the literature, a unifying trend has not been observed.

Altered lipid content is also correlated to both daptomycin resistance. Namely, diglycosyldiacylglycerols (DGDAGs) are increased in resistant strains of enterococcus, while PtdGro is decreased in the same strains [80, 81]. The phospholipid composition of daptomycin-resistant *S. aureus*, like *E. faecalis*, significantly differs from its susceptible parental isolate; PtdGro is substantially decreased in the membrane, while glycolipid abundance is increased, possibly indicating a common means of tolerating daptomycin in both species [80, 81]. However, it is unknown whether these specific changes drive daptomycin tolerance or are a downstream effect of mutations conferring daptomycin resistance.

Daptomycin tolerance and persistence

Interestingly, many studies on the killing mechanism of daptomycin using high drug concentrations resulted in biphasic killing of cells [27, 35, 82-87], a known sign of persistence. In the 1940s, it was demonstrated by Hobby and Bigger that treatment of bacterial cells with high concentrations of the bactericidal antibiotic penicillin revealed two subpopulations of cells: one population of cells that is rapidly killed and a second population of cells (persister cells) which is able to survive [88, 89]. The persister cell population is genetically identical to the population which is rapidly killed and is unable to grow in the presence of the drug [55], yet when the drug is removed from the media, growth is again possible [90-92]. Persister cells are thought to arise from induction of stress responses [93-99] or by varying expression of the toxin portion of toxin/antitoxin systems [100-105]. It has been observed that the toxin portion of toxin-antitoxin systems can lead to metabolic dormancy, which is hypothesized to cause bacterial persistence [105]. Addition of nutrients to the growth media can resuscitate dormant cells [106], potentially leading again to the ability of an antibiotic to kill the cells. Indeed, this has been

demonstrated with daptomycin: the biphasic killing associated with treatment of *S. aureus* cells was eliminated by addition of glucose to the media following daptomycin treatment [86]. Interestingly, supplementation with specific fatty acids can lead to the opposite effect.

Fatty acids trigger daptomycin tolerance

Daptomycin tolerance (*i.e.*, ability to survive high dosages of the drug without an increase in the MIC [55]) can be induced by addition of fatty acids to the growth media [35, 79, 82, 87, 107]. Note that in the host environment, *E. faecalis* is exposed to bile in the gastrointestinal tract (commensal state) and sera in tissues (pathogenic state). Studies have established that exposure to these host fluids, which are rich in fatty acids, changes membrane composition and confers tolerance to daptomycin [35, 82]. Furthermore, exposure to the specific fatty acids, oleic acid (C_{18:1 cis9}) or linoleic acid (C_{18:2 cis9,12}), both of which are found in bile and serum, alters membrane composition and induces tolerance to daptomycin [35, 79, 82, 87, 107]. Notably, this induction of protection is not dependent on induction of the LiaFSR response, as oleic acid was protective in a strain deleted for *liaR* [82]. Addition of oleic acid was also protective in a daptomycin resistant clinical isolate of *E. faecalis* [82], as well as in strains lacking the genes necessary for synthesis of cardiolipin and lysyl-PtdGro [107]. However, protection against daptomycin by oleic acid was not due to direct interactions between the fatty acid and daptomycin [82]. Taken together, these data indicated that addition of fatty acids was protecting through an induction of altered regulation or activity of key players involved in daptomycin resistance or by sequestering daptomycin. Thus, the mechanism for fatty acid-induced daptomycin tolerance remains unclear. One area that has not been fully explored in *E. faecalis* is how fatty acids are used.

The use of free fatty acids by bacteria

In the context of living in a human host, bacteria are exposed to a multitude of nutrients, including fatty acids, which are present in bile and serum. Of note, bacteria have multiple

uses for free fatty acids. Free fatty acids may be used as a carbon source for bacterial growth (reviewed in [108]), or can be used in phospholipid synthesis. To be used as a carbon source, the fatty acid (acyl) must first be “activated” by the addition of coenzyme A (CoA). Two carbons are removed from the carboxylic end of the acyl chain, forming acetyl-CoA and a shortened (by two carbons) acyl-CoA. The resulting acyl-CoA can be consecutively shortened to produce more acetyl-CoA, which can be used in the citrate cycle for energy. This process, called β -oxidation, does not occur in all organisms however. Specifically, *Enterococcus faecalis* does not have the genes required for β -oxidation and fatty acids cannot be used as sole carbon sources [109, 110]. Therefore, the induction of daptomycin tolerance by exposure to free fatty acids in this organism is not due to the breakdown of fatty acid supplements.

While free fatty acids are not involved in generating energy via β -oxidation for *E. faecalis*, they can be used in phospholipid synthesis, as supplementation of *E. faecalis* cultures with fatty acids alters the lipid profile [107] and specific exogenous fatty acids can support growth of *E. faecalis* when *de novo* fatty acid biosynthesis is inhibited [87]. This is likely accomplished by uptake of free fatty acids through a fatty acid kinase (Fak) system (Figure 1.3).

The Fak system was first described in *Staphylococcus aureus* [72]. Free fatty acids (which are thought to passively associate with the cell membrane [111]) are bound by a fatty acid binding protein (FakB), and then phosphorylated (“activated”) by the enzyme FakA using ATP as the phosphate donor [72]. The resulting acyl-phosphates are then used in the phospholipid biosynthetic cycle *via* conversion to acyl-ACP by PlsX, or incorporation into the SN-1 position of glycerol-3-phosphate (G3P) by PlsY, generating lysophosphatidic acid [72]. If converted into acyl-ACP, the fatty acid can then enter into the fatty acid biosynthetic cycle (FasII), or can be incorporated onto the SN-2 position of lysophosphatidic acid by PlsC to generate phosphatidic acid, the precursor to membrane lipids [72]. Importantly, individual FakB homologs do not bind all fatty acids equally: the two individual FakB proteins of *S. aureus* were demonstrated to have specific binding affinities for different fatty acids [72]. FakB1 of *S. aureus* (saFakB1) bound with higher

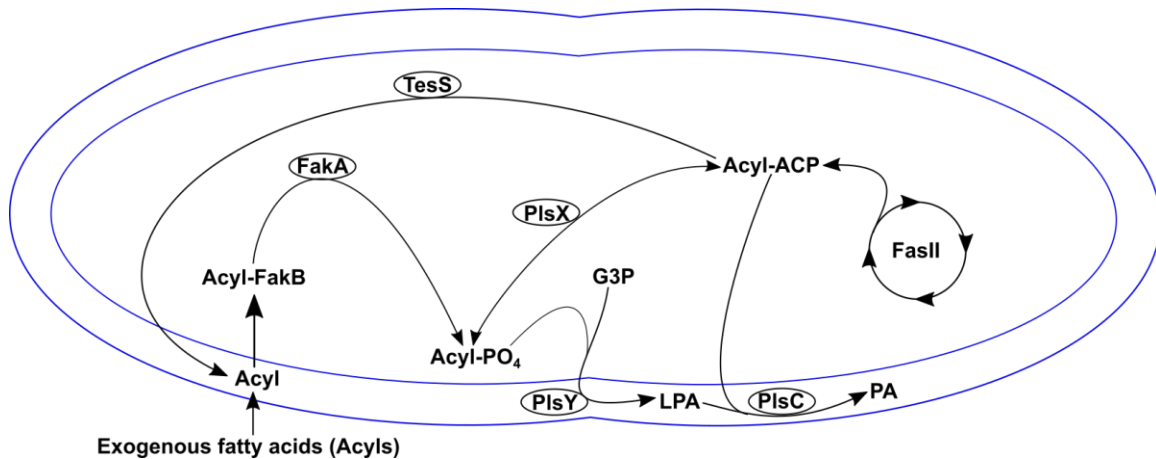


Figure 1.3. The proposed use of free fatty acids by *E. faecalis* based on homology to *S. aureus* and *S. pneumoniae*.

Free fatty acids, whether exogenous or produced by cleavage of a fatty acid (acyl) from acyl-ACP by TesS, are bound by a FakB protein. The FakA enzyme phosphorylates the acyl, forming acyl-phosphate (Acyl-PO₄). Acyl-phosphates are incorporated onto the SN-1 position of glycerol-3-phosphate by PlsY to form lysophosphatidic acid (LPA). Acyl-phosphates can also be bound to the acyl carrier protein (ACP) through the action of PlsX – this process is reversible. Acyl-ACP, generated through the *de novo* fatty acid synthesis cycle (FasII), is used as the acyl donor to form phosphatidic acid, the precursor to membrane phospholipids. The acyl group can be cleaved from acyl-ACP to form a free fatty acid.

affinity to saturated fatty acids, while saFakB2 bound with higher affinity to unsaturated fatty acids [72]. The same specificities were observed in two FakB homologs of *Streptococcus pneumoniae* (spFakB1 was specific for saturated fatty acids and spFakB2 was specific for monounsaturated fatty acids), but a third FakB (spFakB3) had specificity for polyunsaturated fatty acids [112]. Note that acyl-ACP, generated by FasII or by PlsX activity on acyl-phosphates, can be cleaved by TesS to release ACP and a free fatty acid; these free fatty acids could then be used as described above [113]. However, this was proposed to be a minor pathway, as PlsX had the ability to interconvert acyl-phosphates and acyl-ACP [113].

Interestingly, studies demonstrated that only specific fatty acids could induce protection against daptomycin in *E. faecalis*: oleic acid (C_{18:1 cis9}) and linoleic acid (C_{18:2 cis9,12}) [35, 87]. Additionally, individual fatty acids caused different physiological responses in the cells. Namely, supplementation with free fatty acids caused different effects on morphology (indicative of altered cell wall placement) depending on the fatty acid supplement, as well as differences in generation time [87]. This indicates that individual fatty acids play differing roles in the cellular physiology of *E. faecalis*.

Summary

Here I have investigated the contribution of cell wall and the *fakB* genes of *E. faecalis* on cellular physiology and daptomycin tolerance. This work began by examining how bacterial physiology impacts daptomycin tolerance. Given the variety of data suggesting that daptomycin targets the bacterial envelope and that daptomycin treatment reveals two subpopulations of cells (one of those subpopulations likely being persister cells), in Chapter 2 I investigated the role of cell wall, ATP levels, fatty acid biosynthesis, and protein synthesis on daptomycin efficacy. Upon finding that removal of PG greatly increases daptomycin tolerance, yet treatment with protein synthesis and fatty acid biosynthesis inhibitors also induces tolerance, I then investigated the role of exogenous fatty acid incorporation into phospholipids on daptomycin tolerance. Thus, in Chapter 3, I generated several *fakB* deletion strains. Unexpectedly, I noted that the specific deletion

strain $\Delta fakB1,2,5$ had hyperseptation compared to wild-type *E. faecalis*. Additionally, this strain grew slowly in liquid media containing fatty acid supplements or ethanol. These data, combined with the fact that I was initially unable to delete all four *fakB* homologs, led me to investigate whether the *tesS* homolog of *E. faecalis* played a role in the essentiality of the Fak system. I found that deletion of *tesS* enabled deletion of all of the *fakB* genes and the $\Delta fakB1,2,4,5/\Delta tesS$ deletion strain was able to grow in free fatty acids without the negative growth phenotypes associated with lower order deletion strains. Having characterized the Fak system of *E. faecalis*, in Chapter 4 I investigated the distribution of the *E. faecalis* FakB homologs within a network of fatty acid-binding domains (DegV). Within this network analysis, I observed that among the strains within the network, the majority were Gram-positive bacterial organisms. Furthermore, I found evidence that DegV domains cluster according to the isolation source(s) of the strains from which they originated. Within this chapter, I also investigated the role of incorporation of the *fak* genes on daptomycin tolerance. I observed that strains deleted for the *fak* genes had an increased basal tolerance to daptomycin. Further, addition of free fatty acids within these deletion strains still induced daptomycin protection, indicating that incorporation of free fatty acids may not be the key to inducing daptomycin tolerance. Finally, in chapter 5, I discuss my findings in the context of how global changes to cell wall placement, membrane makeup, and membrane organization alter daptomycin tolerance.

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**CHAPTER 2: REMOVAL OF PEPTIDOGLYCAN AND INHIBITION OF
ACTIVE CELLULAR PROCESSES LEADS TO DAPTOMYCIN
TOLERANCE IN *ENTEROCOCCUS FAECALIS***

Publication Note

A version of this chapter was originally published by Rachel D. Johnston, Brittini M. Woodall, Johnathan Harrison, Shawn R. Campagna, and Elizabeth M. Fozo:

Johnston RD, Woodall BM, Harrison J, Campagna SR, Fozo EM. Removal of peptidoglycan and inhibition of active cellular processes leads to daptomycin tolerance in *Enterococcus faecalis*. *PLoS One*. 2021 Jul 23;16(7).

Experiments were conducted by Rachel Johnston, with the exception of: mass spectrometry analysis of cellular metabolites which was completed by Brittini Woodall and the sodium dodecyl sulfate cell survival assay was performed by Johnathan Harrison.

Abstract

Daptomycin is a cyclic lipopeptide antibiotic used in the clinic for treatment of severe enterococcal infections. Recent reports indicate that daptomycin targets active cellular processes, specifically, peptidoglycan biosynthesis. Within, we examined the efficacy of daptomycin against *Enterococcus faecalis* under a range of environmental growth conditions including inhibitors that target active cellular processes. Daptomycin was far less effective against cells in late stationary phase compared to cells in exponential phase, and this was independent of cellular ATP levels. Further, the addition of either the *de novo* protein synthesis inhibitor chloramphenicol or the fatty acid biosynthesis inhibitor cerulenin induced survival against daptomycin far better than controls. Alterations in metabolites associated with peptidoglycan synthesis correlated with protection against daptomycin. This was further supported as removal of peptidoglycan induced physiological daptomycin tolerance. Additionally, a synergistic relation between daptomycin and fosfomycin, an inhibitor of the first committed step peptidoglycan synthesis, was observed, as well as an additive effect when daptomycin was combined with ampicillin, which targets crosslinking of peptidoglycan strands. Removal of the peptidoglycan of *Enterococcus faecium*, *Staphylococcus aureus*, and *Bacillus subtilis* also resulted in significant protection against daptomycin in comparison to whole cells with intact cell walls. Based on these observations, we conclude that bacterial growth phase and metabolic activity, as well as the presence/absence of peptidoglycan are major contributors to the efficacy of daptomycin.

Introduction

As therapeutic strategies have been developed for treating bacterial infections over the past century, there has been a subsequent rise in antibiotic resistant bacteria. Among the CDC's Resistance Threats in the United States are Gram-positive, vancomycin-resistant enterococci (VRE) [1]. VRE are of particular concern because of the lack of successful treatment strategies available for these pathogens. In the clinic, the Gram-positive targeting lipopeptide daptomycin has been used to treat VRE when other strategies fail. However, due to an abundance of contradictory data, an air of mystery has surrounded the mechanism of action of daptomycin since its discovery in the 1980s. Over the course of its characterization, three main modes of action have been proposed: inhibition of peptidoglycan synthesis, inhibition of lipoteichoic acid (LTA) biosynthesis, and disruption of membrane potential.

Peptidoglycan was proposed as the target of daptomycin, as treatment of both *Staphylococcus aureus* and *Bacillus megaterium* with the antibiotic resulted in a failure to incorporate L-alanine and L-lysine into peptidoglycan in either species [2], and cellular levels of lipid II precursors, needed components for peptidoglycan formation, were increased [3]. Gross cell wall changes were also correlated in some studies with daptomycin resistance and daptomycin treatment in *S. aureus* [4, 5], *Enterococcus faecalis* [5-8], and *Enterococcus faecium* [8], though this was not true in all cases [9]. However, peptidoglycan was dismissed as a target for daptomycin as removal of the cell wall (generating protoplasts) of *E. faecium* did not afford the cells protection against the drug [10]. Recent evidence in *S. aureus*, though, supports a model for daptomycin targeting peptidoglycan biosynthesis, as daptomycin was shown to bind to lipid II in the presence of the membrane lipid phosphatidylglycerol, and other lipid II binding molecules blocked daptomycin from interacting with lipid II [11].

Older data initially pointed towards LTA as a potential target, as daptomycin was found to inhibit LTA biosynthesis in *S. aureus* and *E. faecium* [10, 12], and LTA precursors within the cell were found to decrease over time following exposure to daptomycin [13].

However, LTA biosynthesis as the target was later abandoned, as daptomycin inhibited not only the production of LTA, but also that of lipids and RNA [14].

Given that daptomycin contains an acyl tail, many speculated that the cell membrane was the true target of the drug. *In vitro* studies using giant unilamellar vesicles demonstrated that daptomycin formed oligomers [15], caused protrusions within vesicles [16], and led to aggregation of lipid bound to daptomycin on the outer surface of the vesicle [16]. Several *in vivo* studies further found that daptomycin interacted with phosphatidylglycerol [17, 18], localized to the cell membrane [18], formed pores in the membrane [19], and dissipated membrane potential [19-23]. Importantly, many of the supportive *in vivo* studies for a membrane target of daptomycin were conducted using drug concentrations well above (~7–10x) the minimal inhibitory concentration (MIC) of the bacterium being tested or at late time points [17, 20, 22, 23].

Combining these lines of evidence together, a study in *B. subtilis* found that daptomycin associated with fluid regions of the membrane (regions of increased fluidity; RIFs) and rigidified these regions [24]. This rigidification, potentially occurring after binding of daptomycin to lipid II and its precursors [11], occluded peripheral membrane proteins involved in cell wall and phospholipid biosynthesis [24]. The binding of daptomycin to lipid II and its precursors, along with subsequent delocalization of cell wall and lipid synthesis proteins at the membrane would negatively impact these cellular processes, likely contributing to lethality.

Regardless of the specific target of daptomycin, the evidence from the studies summarized above supports that daptomycin targets active cellular processes. Many of the studies of daptomycin demonstrated that treatment with this drug causes biphasic killing of cells [10, 22, 25-29], a hallmark of persistence [30, 31]. As demonstrated by Hobby and Bigger, when bactericidal antibiotics are given at doses above the MIC, they lead to rapid killing of actively growing cells while a genetically identical subset of cells, called persister cells, survive [30, 31]. Persister cells can arise through varying mechanisms – including induction of metabolic dormancy through varying expression of the toxin component of toxin/antitoxin systems [32-37] and induction of stress responses

[38-44]. In each of these cases, the persister cells in question are not genetically resistant, as they do not grow during treatment with the drug [45]: rather, they are able to survive antibiotic pressure through differential expression of genes which can be stochastic [32, 46] or triggered by environmental changes [38-44]. On removal of the antibiotic from the media, these persister cells are then able to grow [47-49].

While some cells may be able to survive treatment with high dosages of drug, survival alone does not indicate true genetic resistance, which results in a higher MIC compared to susceptible cells, along with active growth in the presence of antibiotic [45]. Cells that can survive antibiotic treatment without an increase in the MIC compared to susceptible cells are deemed tolerant [45]. Given that there are subpopulations of cells that survive high daptomycin concentrations [10, 22, 25-29, 50], we examined the role of active cellular processes in daptomycin tolerance through comparison of survival of cells in exponential phase, which are metabolically active and capable of stochastic switching to persister cells [32, 46, 47] to cells in stationary phase, which have reduced metabolic activity as well as a large population of persisters [51, 52]. Comparison of exponential to stationary phase cells is of particular interest, as cells in stationary phase have increased peptidoglycan turnover [53] and thicker peptidoglycan (for *E. coli* [54]), a phenomenon also associated with daptomycin resistance and treatment [4-8]. We observed significantly increased daptomycin tolerance as monitored by the number of survivors following transient exposure to high dosage of the drug (7.5-10x MIC) within stationary phase cells. We also tested whether protein and fatty acid biosynthesis were necessary for survival under high concentrations of daptomycin and found that inhibition of these processes significantly increased survival on treatment with daptomycin. Additionally, we observed metabolic changes within stationary phase cells and cells in which protein or fatty acid biosynthesis were inhibited pointed towards altered peptidoglycan metabolism. We removed peptidoglycan from cells and found that these cells were significantly more tolerant to daptomycin treatment. Combined, we demonstrate that inhibition of active growth of *E. faecalis* serves to increase its ability to survive high doses of daptomycin, supporting a model for daptomycin targeting peptidoglycan biosynthesis.

Materials and Methods

Bacterial growth conditions

Bacterial strains used in the study are listed in Supplemental Table 2.1. *E. faecalis*, *E. faecium*, and *S. aureus* strains were grown statically in brain heart infusion (BHI) medium at 37°C unless indicated otherwise. *B. subtilis* cultures were grown in LB with shaking (250rpm) at 37°C. Overnight growth cultures were diluted to a final optical density at 600 nm (OD₆₀₀) of 0.01 from overnight cultures and harvested at either exponential phase (OD₆₀₀ ~ 0.3) or late stationary phase (24 h after dilution). For examining the effects of de novo protein biosynthesis or fatty acid biosynthesis, chloramphenicol (15 µg/mL) or cerulenin (10 µg/mL), respectively, were added to cultures upon reaching OD₆₀₀ ~ 0.225–0.250 (chloramphenicol) or OD₆₀₀ ~ 0.100–0.125 (cerulenin). Cultures were exposed to chloramphenicol for 30 min or cerulenin for 90 min to ensure cellular stasis, with stasis confirmed by determining colony forming units pre- and post-treatment (Supplemental Figures 2.1 and 2.2).

Minimal inhibitory concentration (MIC) determination

To perform MIC assays, cells were diluted to a final OD₆₀₀ of 0.01 from overnight cultures into 2 mL of media containing 1.5 mM CaCl₂ and daptomycin (0, 0.25, 0.5, 1, 2, 4, and 8 µg/mL), and grown for 24 hours prior to determine the minimal inhibitory concentration (Supplemental Table 2.2). Minimum of $n = 3$ for all experiments.

Cell survival assays

For all daptomycin and SDS challenge assays, cellular survival was determined via enumeration of colony forming units. Daptomycin challenge concentrations were chosen to reflect ~7.5–10X the MIC of the respective organisms (Supplemental Table 2.2). Cells were harvested via centrifugation at 2739xg for 10 min. Where indicated, the cells were washed with either isotonic buffer (20% sucrose, 0.145M NaCl, 50mM Tris-HCl) [55] or 1x phosphate buffered saline solution (PBS) [25] before resuspension in challenge medium. Daptomycin challenge cultures were set up as follows: for *E. faecalis* and *E.*

faecium, daptomycin was added at 30 µg/mL to cells suspended in BHI with 1.5mM calcium chloride or isotonic buffer containing 1.5mM calcium chloride [50]. For *S. aureus* and *B. subtilis* cultures, daptomycin was added at 5 µg/mL. Serial dilutions were plated onto BHI agar (*E. faecalis*, *E. faecium*, and *S. aureus*) or LB agar (*B. subtilis*) at time zero (immediately prior to daptomycin or SDS addition) and at the indicated time points post-treatment. Colony forming units were enumerated after 18–24h of incubation at 37°C. Sodium dodecyl sulfate challenge medium consisted of BHI containing 20% sucrose as an osmoprotective agent and 0.05% SDS. Colony forming units at all time points were normalized to the colony forming units at time zero (before addition of daptomycin or SDS to the culture). Minimum of $n = 3$ for all experiments. Raw CFU data are in Supplemental Table 2.3.

ATP depletion and tracking

ATP levels were determined as indicated in the text using the BacTiterGlo kit (Promega). For ATP depletion assays, *E. faecalis* cells were grown to an OD₆₀₀ of ~0.25. Sodium arsenate dibasic heptahydrate (arsenate; Sigma-Aldrich) was added as indicated in the text to a final concentration of 10 mM, or an equivalent volume of water (solvent control) was added to the cells [56, 57]. The cells were incubated for 30 min at 37°C, harvested by centrifugation at 2739xg, washed twice with 1x PBS, then resuspended in BHI prior to use for ATP determination. Colony forming units were determined via plating to conclude arsenate treatment did not result in cell death (Supplemental Figure 2.3). Relative light unit values (RLUs) yielded by the BacTiterGlo kit (Promega) were normalized to colony forming units taken at the time the ATP assay was performed. Minimum of $n = 3$ for all experiments.

Daptomycin synergy

To determine synergy between daptomycin and other antibiotics, a microtiter plate checkerboard assay was performed [58-60]. Overnight cultures were diluted back as above into BHI containing 1.5 mM CaCl₂ and aliquoted to microplates. Daptomycin (0,

0.25, 0.5, 1, 2, 4, and 8 µg/mL) was added with the concentration increasing along the vertical axis, while the secondary drug was increased along the horizontal axis. The secondary drugs examined were fosfomycin (0, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512 µg/mL), D-cycloserine (0, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 µg/mL) or ampicillin (0, 0.015625, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 µg/mL). One row and one column of each plate contained media alone (BHI + 1.5 mM CaCl₂) to provide a no-growth control. For all experiments, $n = 3$. The fractional inhibitory concentration (FIC) of each drug (drug A and B, respectively) was calculated as follows [60]:

$$\text{Equation 1: FIC}_A = \frac{\text{MIC}_{\text{combined drug A/B}}}{\text{MIC}_{\text{drug A}}}$$

$$\text{Equation 2: FIC}_B = \frac{\text{MIC}_{\text{combined drug B/A}}}{\text{MIC}_{\text{drug B}}}$$

The fractional inhibitory concentration index (FICI) was calculated as follows [60]:

$$\text{Equation 3: FICI} = \text{FIC}_A + \text{FIC}_B$$

Synergy, additivity, and no interaction are defined as follows: FICI of ≤ 0.5 is defined as synergistic, FICI between 0.5 and 1 is additive, and FICI between 1 and 4 is defined as no interaction [60, 61].

Cell wall removal/protoplast generation

E. faecalis and *E. faecium* were grown in 50 mL BHI to the appropriate growth phase. A whole cell control (4 mL) aliquot and a protoplast (40 mL) aliquot were harvested from the original culture by centrifugation at 2739 $\times$ *g* for 10 min, washed once with isotonic buffer (20% sucrose, 0.145 M NaCl, 50 mM Tris-HCl), then resuspended in 5 mL isotonic buffer [55]. Lysozyme (final concentration of 1 mg/mL) [62] or an equivalent volume of water was added to the appropriate cell mixture. These mixtures were incubated at 37°C for 60 min. Following mock or lysozyme treatment, cells were harvested by centrifugation as

above, washed with isotonic buffer, and resuspended in 4 mL isotonic buffer prior to ATP determination (Supplemental Figure 2.5) or were resuspended in 4 mL isotonic buffer containing 1.5 mM CaCl₂ prior to use for cell survival assays (note that protoplasts were concentrated 10x while mock-treated cells were not). For *S. aureus*, cells were treated as above except that lysostaphin was added at 5 µg/mL and DNase was added at 16 µg/mL [55]. *B. subtilis* was grown in 100 mL LB to the appropriate growth phase. A whole cell control (4 mL) aliquot and protoplast (80 mL) aliquot were harvested from the original culture by centrifugation as above and resuspended in 4 mL osmoprotective LB (LB supplemented with 20% sucrose, 50 mM Tris-HCl and 0.145 M NaCl). Lysozyme (final concentration of 100 µg/mL) was added to the cells and incubated at 37°C with shaking (250 RPM) for 30 min. Cells were harvested as above, washed with osmoprotective LB and resuspended in an equivalent volume osmoprotective LB containing 1.5 mM CaCl₂ prior to treatment with daptomycin. Protoplasts and whole cells for each strain were confirmed by Gram-stain.

Metabolomics

Cell harvest and extraction (stationary-phase cells, chloramphenicol- and cerulenin-treated cultures and their respective controls)

Cells were grown as stated above and cell harvest and extraction was adapted from previous literature [63]. Cell culture volumes of 1 mL were harvested by filtration using 0.4 µm membrane filters (Fisher 09-300-71). Dilution plating of the original cell culture was performed at the time of harvest for colony forming unit enumeration. After harvest, the filters were washed with 8 mL of 1x PBS. To begin extraction, filters were immediately placed into respectively labeled petri dishes containing 1.3 mL of prechilled (4°C) extraction solvent (40:40:20 HPLC grade methanol, acetonitrile, water with 0.1% formic acid). Filters were then placed at -20°C for 20 min. Following incubation at -20°C, the filters were washed with 400 µL of extraction solvent, and the solvent was then transferred to respectively labeled 2 mL centrifuge tubes and subjected to centrifugation at 16xg. After centrifugation, the supernatant was transferred to a labeled centrifuge tube and the

remaining pellet was resuspended in 200 μ L of extraction solvent, placed at 4°C for an additional 20 min, subjected to centrifugation as above, and supernatant was added to the above. The samples were then subjected to drying under N₂. Dried samples were resuspended in 300 μ L HPLC water and stored in an autosampler vial at 4°C until analysis.

Cell harvest and extraction (whole cells and protoplasts):

As a very low number of protoplasts survived the process of filtration, we elected to harvest cell pellets for whole cell and protoplast cultures (generated as above) prior to extraction. Colony forming units were enumerated at the time of harvest and cell culture volumes of 2 mL for protoplasts (double the volume for whole cells to increase the number of viable cells used for analysis) and 1 mL for whole cells were harvested via centrifugation (15 min 16xg) and the supernatant was removed. After harvest, pellets were flash frozen and stored at -80°C prior to extraction. To begin extraction, samples were thawed at 4°C for 30 min. Thawed samples were subjected to the addition of prechilled extraction solvent (0.65 mL) and were shaken at 60 RPM. Afterwards, an additional 0.65 mL of prechilled extraction solvent was added to the samples and the samples were vortexed. Samples containing extraction solvent and cell pellets were chilled for 20 min at -20°C. Samples were then centrifuged for 5 min (16xg) and the supernatant was then transferred to new centrifuge tube. An additional 200 μ L of extraction solvent was added to cell pellet in the first tube and vortexed. After vortexing the samples were placed at -20°C for an additional 20 min. Samples were centrifuged for an additional 5 min (16xg) and the resulting supernatant was removed and placed in the respective tube for drying under N₂. After drying, samples were resuspended in 300 μ L HPLC water and placed into the autosampler at 4°C until analysis.

Liquid chromatography mass spectrometry analysis:

For liquid chromatography mass spectrometry analysis, 10 μ L of sample was injected onto a Synergi 2.5 μ M Hydro-RP, 100 Å, 200 x 2.00 mm liquid chromatography column

(Phenomenex, Torrance CA) held at 25°C. The data acquisition parameters adapted from Rabinowitz [64] included the operation of the mass spectrometer in full scan mode. The eluent was ionized via electron spray ionization (ESI) in negative mode coupled to a Thermo Scientific Exactive Plus Orbitrap mass spectrometer with the following parameters: spray voltage 3 kV, nitrogen sheath gas 10 (arbitrary units), capillary temperature 320°C, automatic gain control (AGC) target set to 3×10^6 ions. The mass spectrometer was operated at 140,000 resolution with a scan window from 85-800 m/z from 0-9 min and 110-1000 m/z from 9-25 min. Mobile phase A consisted of 97:3 water methanol 10mM tributylamine, and 15mM acetic acid while solvent B consisted of 100% methanol. Solvent gradient was 0 to 5 min: 0% B, 5-13 min: 20% B, 13-15.5 min: 55% B 15.5-19 min 95% B and 19 to 25 min 0% B with a flow rate of 200 μ L/min.

Data processing:

Raw data files generated by Xcalibur were converted to .mzML format using the MS convert from ProteoWizard. EI MAVEN was utilized to integrate areas under the curve for known metabolite masses (selected based on our validated standard library of 1080 metabolites) matched with retention time with a mass error of less than 5 ppm. Base background signal was removed, and integrated peak areas were normalized by the ratio of colony forming units of experimental versus control group for each paired control and treatment replicate. Heatmaps were constructed by determining the ratio of normalized, log transformed intensities of experimental values over control values. A paired Student's T-test was utilized to determine significance. PLSDA plots were constructed utilizing Metaboanalyst (metaboanalyst.ca) software. $n = 5$ replicates were performed for all metabolomics experiments with the exception of removal of one replicate from the chloramphenicol vs. ethanol experiment due to technical error, resulting in $n = 4$ replicates in that experiment.

Statistical analysis

All statistical analyses presented within the text, except where noted, were performed using two-tailed Welch's T-tests, using $\alpha = 0.05$. For cell survival assays, data were only compared within (not between) individual time points. For each described assay, a minimum of $n = 3$ biological replicates were used. *P*-values for cell survival assays and ATP assays are reported in Supplemental Table 2.4. In figures, *P*-values are represented in ranges as follows: ^{ns} $P > 0.05$; * $P = 0.05$ – 0.01 ; ** $P = 0.01$ – 0.001 ; *** $P \leq 0.001$.

Results

The sensitivity of E. faecalis to daptomycin is dependent upon its growth phase

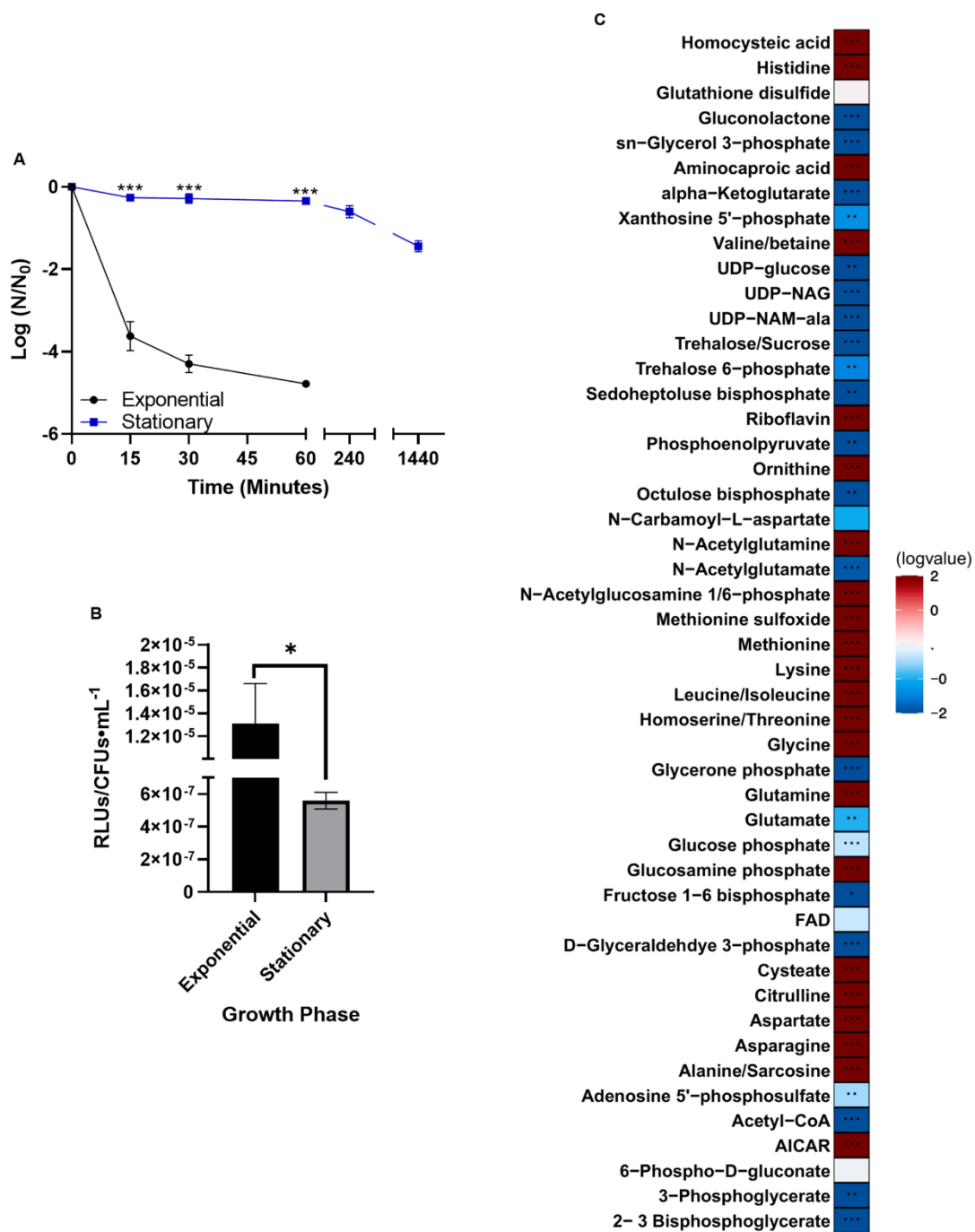
During exponential phase, cells are in a state of active growth and division. However, upon reaching stationary phase, metabolic activity is altered, resulting in physiological changes including thickening of the cell wall [54], reduced protein production [65, 66], and often, an increase in tolerance to antibiotics which target active cellular processes [26, 33]. To determine whether *E. faecalis* OG1RF stationary phase cells have increased tolerance, *i.e.*, increased survival, to daptomycin when compared to exponential phase cells, we examined sensitivity to daptomycin using cell survival assays (via enumeration of colony forming units over time; see Materials and methods). For these experiments, daptomycin concentration was ~ 7.5 – $10\times$ MIC (Supplemental Table 2.2).

Stationary phase cultures survived far better when exposed to the drug compared to cells in exponential phase (Figure 2.1A; $P < 0.001$ after 15 minutes of daptomycin treatment). We noted rapid killing of exponential phase cells within the first 15 min of daptomycin exposure, with a reduction in killing rate over the first hour of treatment. At four hours post-exposure, cells in exponential phase were below the limits of detection, while 3.74% of the cells in stationary phase were still present even after 24 hours of daptomycin exposure (Figure 2.1A and Supplemental Table 2.3).

Cells in exponential phase generally have higher ATP levels than do their stationary-phase counterparts, likely in order to support the active processes involved in cell division [67]. Further, as reduced ATP levels have been implied in the formation of antibiotic

Figure 2.1. Cells in stationary phase show increased daptomycin tolerance and an altered metabolic profile.

(A) Cells were treated with 30 µg/mL daptomycin as described in Materials and Methods. Shown are the average log ratios of colony forming units $\pm$ standard deviations for $n = 3$ replicates. *** $P < 0.001$. Note, cell survival for exponential phase cultures was below the limits of detection at 4 hours and beyond. (B) ATP levels of cells were measured using the Promega BacTiterGlo kit. Shown are the average relative light units (RLUs) normalized to colony forming units $\pm$ standard deviations for $n = 3$ replicates. * P 0.05–0.01; exact P -values are found in Supplemental Table 2.4. (C) Metabolites of cells were extracted and detected via mass spectrometry as outlined in Materials and methods. Represented are the log-transformed fold changes of normalized stationary phase cell metabolites to normalized exponential phase cell metabolites. Normalization was performed by dividing raw metabolite data by the ratio of colony forming units of stationary versus exponential phase cells. $n = 5$ replicates.



tolerant cells [46, 56, 68], we measured ATP in both exponential and stationary phase OG1RF cultures. As shown in Figure 2.1B, stationary phase OG1RF cells had approximately 22-fold less ATP than their exponential phase counterparts when normalized to colony forming units ($P = 0.024$; Supplemental Table 2.4).

As noted, metabolic state is altered when cells are in stationary versus exponential phase [69-72]. To confirm an altered metabolic profile, we performed an untargeted metabolomic approach (Figure 2.1C). Cells were grown as described above and used for metabolic analyses: note that metabolic peak values were normalized to colony forming units (see Materials and Methods) to ensure appropriate comparisons. Of interest, we noted compounds with mass/charge ratios corresponding to peptidoglycan precursors, UDP-NAG (UDP-N-acetylglucosamine) and UDP-NAM-Ala (UDP-N-acetylglucosamine-alanine) were reduced, suggesting that peptidoglycan synthesis was reduced. We also observed alterations in many mass/charge ratios corresponding to amino acids, indicative of altered rates of protein degradation and synthesis [73], unsurprising for cells in stationary phase.

To determine whether the increased survival of cells in late stationary phase was due to reduced ATP content, ATP depletion [56, 57] was performed using sodium arsenate. Importantly, treatment with sodium arsenate did not affect cell survival (Supplemental Figure 2.3 and Supplemental Table 2.3), but did reduce ATP levels in exponential phase cells (Figure 2.2B; $P = 0.001$). Cells treated with arsenate, however, survived daptomycin treatment in a similar fashion to the solvent control (Figure 2.2A and Supplemental Table 2.3; $P = 0.769$ after 15 minutes of daptomycin exposure). Thus, reduced ATP levels did not correlate with an increase in daptomycin tolerance.

Inhibition of active cellular processes protects OG1RF from daptomycin

Survival of a tolerant population has been attributed to a variety of mechanisms, including a reduction in cellular growth [47]. As stationary phase OG1RF cells survived daptomycin treatment far better than exponential cells, we hypothesized that reduced cellular growth could induce daptomycin tolerance. We artificially induced growth stasis through inhibition

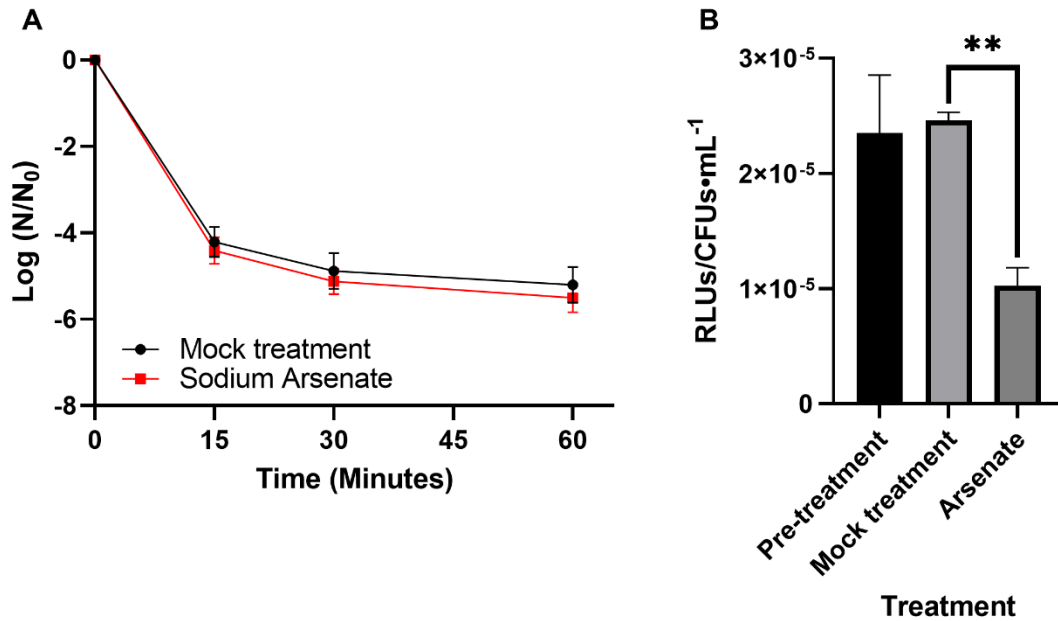


Figure 2.2. ATP depletion does not induce daptomycin tolerance.

(A) Cells grown to exponential phase were treated with 10mM sodium arsenate or mock-treated for 30 min, then subjected to 30 μ g/mL daptomycin. Shown are the average log ratios of colony forming units $\pm$ standard deviations for $n = 3$ replicates; exact P -values are found in Supplemental Table 2.4. (B) ATP levels of pre-treated, mock-treated or arsenate-treated cells (30 min) were measured using the Promega BacTiterGlo kit. Shown are the average relative light units (RLUs) normalized to colony forming units $\pm$ standard deviations for $n = 3$ replicates. * $P \leq 0.05$; exact P -values are found in Supplemental Table 2.4.

of either *de novo* protein biosynthesis *via* addition of chloramphenicol or *de novo* fatty acid biosynthesis *via* addition of cerulenin. Inhibition of cellular growth was used as a proxy for inhibition of either process (Supplemental Figures 2.1 and 2.2). Upon addition of chloramphenicol, cellular growth, as observed by colony forming units, was halted (Supplemental Figure 2.1) but ATP levels were comparable to solvent control-treated cultures (Figure 2.3B; $P = 0.59$). Yet, chloramphenicol treatment induced protection from daptomycin, particularly during the first 15 min of treatment (Figure 2.3A and Supplemental Table 2.3; 1.8 log fold change; $P = 0.002$ after 15 minutes of daptomycin exposure). This protective effect of pre-treatment with chloramphenicol was not as great as the protection observed in cells treated with daptomycin during late stationary phase, which, when compared with their exponential phase counterparts, had a 4.1 log fold increase in survivors during the first 15 min of treatment (Figure 2.1A).

We hypothesized that protection induced by chloramphenicol may be due to alteration in the metabolic profile upon the inhibition of cellular growth. Using metabolomics, we detected numerous species with altered ratios when examining chloramphenicol versus mock-treated cultures (normalized to colony forming units, please see Materials and Methods, Figure 2.3C). In the chloramphenicol-treated cultures, we did note statistically significant decreases in mass/charge ratios corresponding to UDP-NAG and UDP-NAM-Ala, a cytoplasmic precursor to the membrane-bound peptidoglycan precursors lipid I and lipid II, relative to solvent control-treated cultures (Figure 2.3C), along with statistically significant alterations of levels of mass/charge ratios corresponding to different amino acids, including glutamine, unsurprising given that chloramphenicol inhibits protein synthesis.

We also examined the effects of cellular growth stasis on daptomycin sensitivity by inhibiting membrane fatty acid biosynthesis *via* cerulenin treatment. Upon treatment of cells with cerulenin, cell growth was halted as observed *via* colony forming units (Supplemental Figure 2.2) and there was a 1.1 log fold protection induced relative to the solvent control (Figure 2.4A and Supplemental Table 2.3; $P = 0.022$ after 15 minutes of

Figure 2.3. Chloramphenicol-treated cell have increased tolerance to daptomycin and an altered metabolic profile.

(A) Cells were grown to an OD₆₀₀ of ~0.250 and treated for 30 min with chloramphenicol or solvent control, washed with 1xPBS, and then exposed to 30 µg/mL daptomycin. Shown are the average log ratios of colony forming units ± standard deviations for $n = 3$ replicates. * $P = 0.05$ – 0.01 ; ** $P = 0.01$ – 0.001 ; *** $P \leq 0.001$; exact P -values are found in Supplemental Table 2.4. (B) ATP levels of pre-treated, solvent-treated or chloramphenicol-treated cells were measured using the Promega BacTiterGlo kit. Shown are the average relative light units (RLUs) normalized to colony forming units ± standard deviations for $n = 3$ replicates. ^{ns} = $P > 0.05$; exact P -values are found in Supplemental Table 2.4 (C) Metabolites of cells were extracted and detected *via* mass spectrometry as outlined in Materials and Methods. Represented are the log-transformed fold changes of normalized chloramphenicol-treated cell metabolites to normalized solvent control-treated cell metabolites. Normalization was performed by dividing raw metabolite data by the ratio of colony forming units of chloramphenicol-treated versus solvent control-treated cells. $n = 4$ replicates.

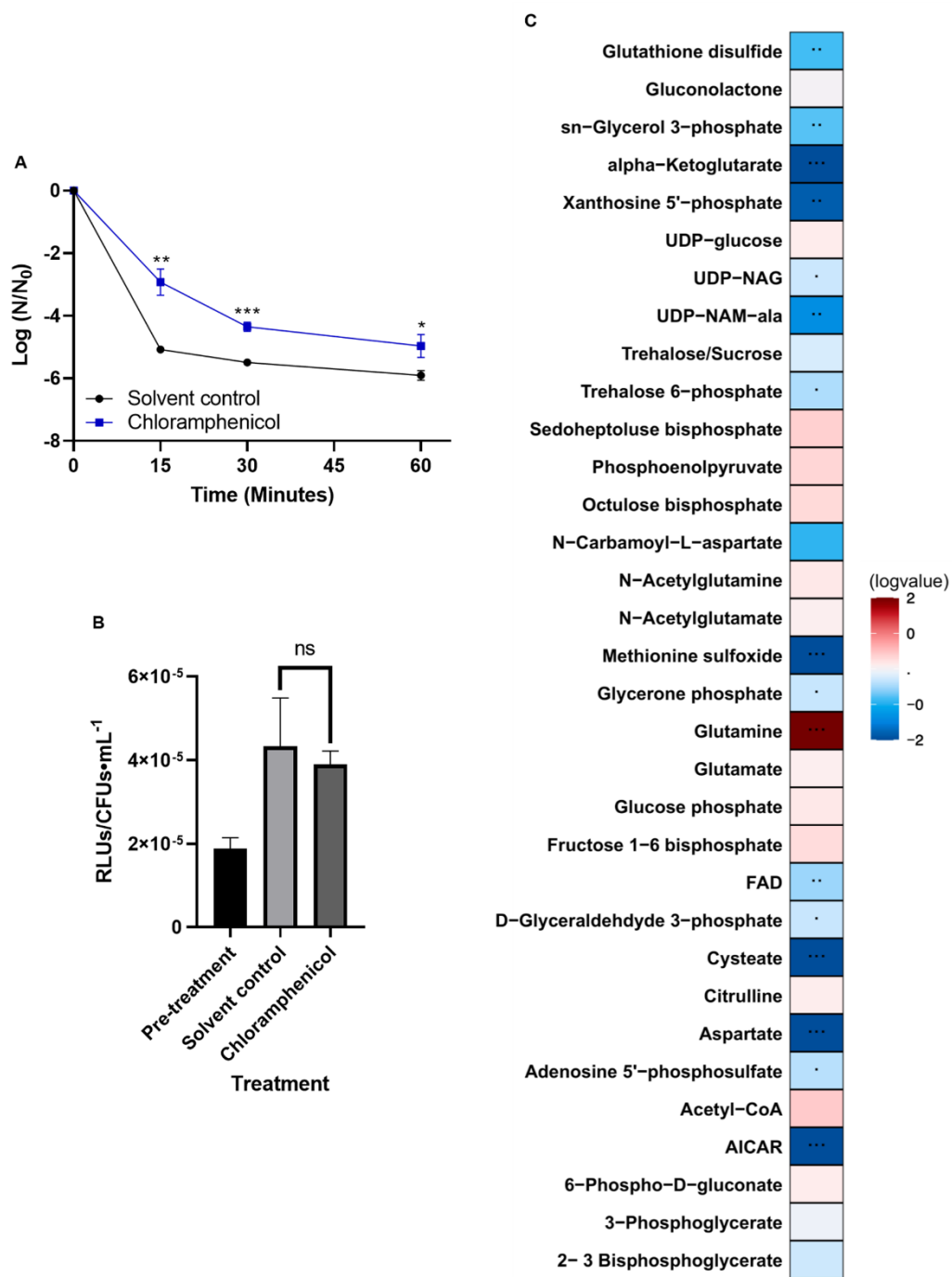
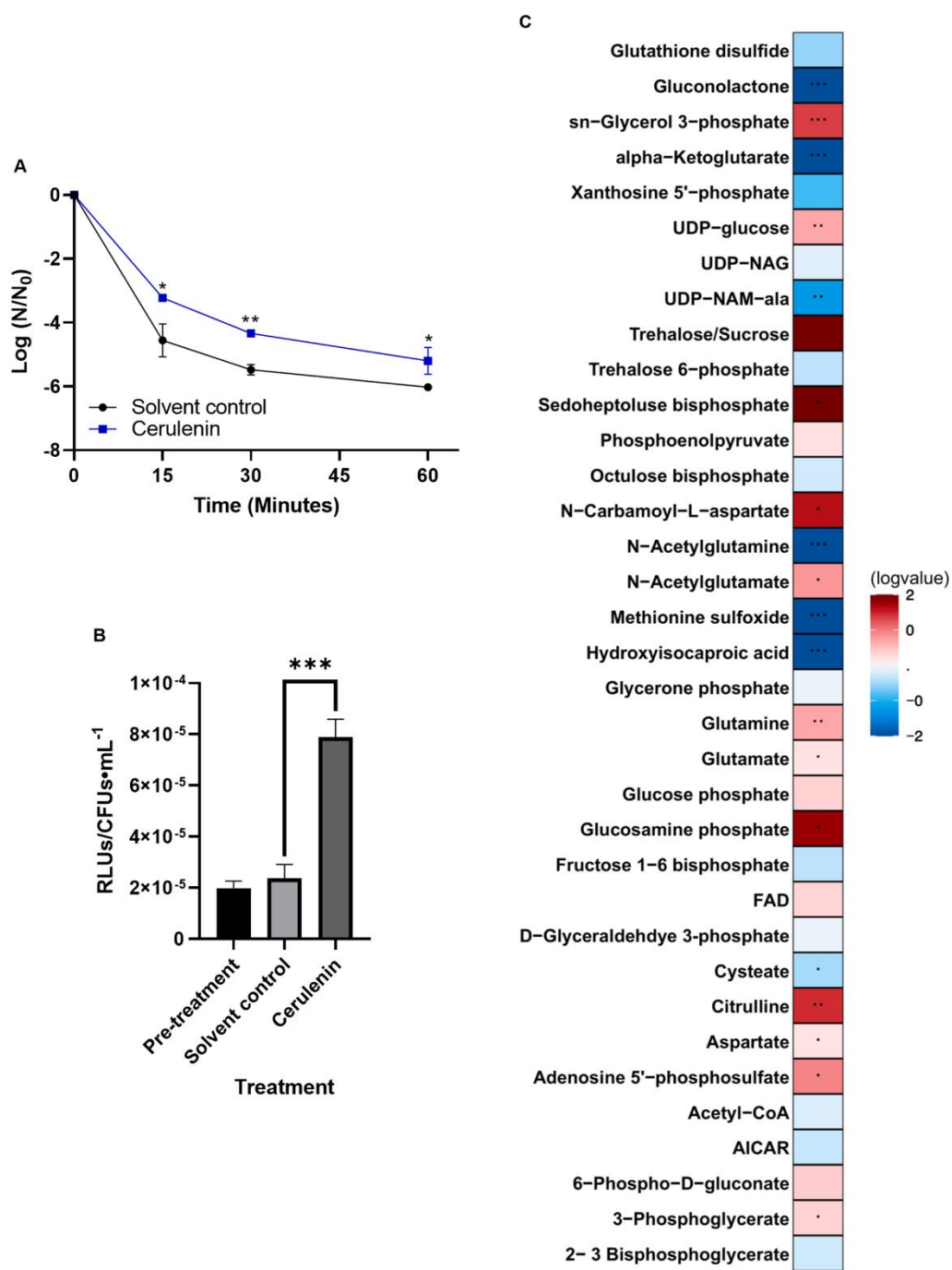


Figure 2.4. Cerulenin-treated cells have increased tolerance to daptomycin and an altered metabolic profile.

(A) Cells were grown to early exponential phase, treated with cerulenin or solvent control, washed with 1x PBS, and then subjected to treatment with 30 $\mu\text{g/mL}$ daptomycin. Shown are the average log ratios of survivors $\pm$ standard deviations for $n = 3$ replicates. * $P = 0.05\text{--}0.01$; ** $P = 0.01\text{--}0.001$; exact P -values are found in Supplemental Table 2.4. (B) ATP levels of pre-treated, solvent-treated and cerulenin-treated cells were measured using the Promega BacTiterGlo kit. Shown are the average relative luminescence units (RLUs) normalized to colony forming units $\pm$ standard deviations for $n = 3$ replicates. *** $P \leq 0.001$; exact P -values are found in Supplemental Table 2.4. (C) Metabolites of cells were extracted and detected *via* mass spectrometry as outlined in Materials and Methods. Represented are the log-transformed fold changes of normalized cerulenin-treated cell metabolites to normalized solvent control-treated cell metabolites. Normalization was performed by dividing raw metabolite data by the ratio of colony forming units of cerulenin-treated versus solvent control-treated cells. $n = 5$ replicates.



daptomycin treatment). However, unlike treatment with chloramphenicol, treatment with cerulenin resulted in increased ATP levels (Figure 2.4B; $P < 0.001$).

Given these findings, we examined the metabolic profile of cerulenin-treated cells versus their solvent controls, in order to identify potential metabolic signatures conserved between chloramphenicol and cerulenin treatment (Figure 2.4C). We again observed a decrease in the mass/charge ratio corresponding to UDP-NAM-Ala, but in this case, an insignificant decrease in detected UDP-NAG. Unique to cerulenin-treated cultures, we also observed a buildup of the mass/charge ratio corresponding to sn-Glycerol-3-phosphate (G3P), the precursor to membrane phospholipids – this is likely because the cerulenin-treated cultures were unable to synthesize fatty acids which are placed on G3P to form lysophosphatidic acid [74].

Interestingly, for all of our experimental treatments (stationary phase, chloramphenicol-treated, and cerulenin-treated cells), we noted decreases in the mass/charge ratio corresponding to UDP-NAM-Ala and alpha-ketoglutarate, a key player in the citrate cycle and a source of glutamine and glutamate, along with an increase in the amino acid glutamine. Importantly, glutamine is not only used in protein synthesis but is also converted to D-glutamate for building peptidoglycan. These changes in metabolite levels indicate a dysregulation of cell wall synthesis which we investigated further below.

Removal of peptidoglycan protects OG1RF from daptomycin

Despite evidence from multiple studies implicating the cell wall, specifically peptidoglycan, as the major target of daptomycin [2, 3], additional data supported that membrane targeting and subsequent pore formation were critical for activity of the drug [17-20, 22, 23]. Given these discrepancies and our own above findings, in particular the significant decrease in the mass/charge ratio corresponding to UDP-NAM-Ala in stationary phase and chloramphenicol- and cerulenin-treated cells, we examined the role of peptidoglycan in the daptomycin sensitivity of OG1RF. We divided exponential cultures such that a portion was mock-treated (remained as whole cells) and the rest were treated to remove peptidoglycan (protoplasts; see Materials and Methods).

Upon treatment with daptomycin, when compared with whole-cell counterparts, we note that the protoplasts had at least 1.8 log-fold more colony forming units at every time point (Figure 2.5A and Supplemental Table 2.3; $P < 0.001$ after 15 minutes of exposure to daptomycin). As removal of peptidoglycan could stimulate a variety of responses, including possible stress-response regulons, we examined survival when cells were treated with SDS, an anionic detergent that disrupts membranes and acts as a non-specific stressor which activates efflux pumps and *rpoS* stress responses [75]. However, removal of peptidoglycan did not induce any protection from SDS (Supplemental Figure 2.4; $P = 0.222$ after 15 minutes of daptomycin treatment) or significant differences in ATP levels (Supplemental Figure 2.5). We also performed metabolomics analyses on protoplast cells compared to whole cells (Supplemental Figure 2.6), and observed increases in many metabolites, including UDP-NAG, however, the variation between biological replicates was very high, resulting in insignificant results for most detected metabolites.

Daptomycin activity is enhanced with specific peptidoglycan synthesis targeting antibiotics

Our above data suggests that the presence of peptidoglycan renders OG1RF sensitive to daptomycin. As there are a variety of different antibiotics that target unique, specific enzymatic processes in the synthesis of peptidoglycan, we performed a checkerboard assay to better conclude what daptomycin may specifically target. A checkerboard assay measures the effectiveness of two separate antibiotics when combined versus when given independently to an organism to quantitatively conclude whether the drugs are additive or synergistic [58-60]. We examined D-cycloserine, which blocks the interconversion of L-alanine and D-alanine, as D-alanine is found within the peptide chains of peptidoglycan; fosfomycin, which targets the first committed step of synthesis, namely condensation of enolpyruvate with UDP-N-acetylglucosamine by the enzyme MurA; and ampicillin, which inhibits the transpeptidation reaction that crosslinks two peptidoglycan molecules.

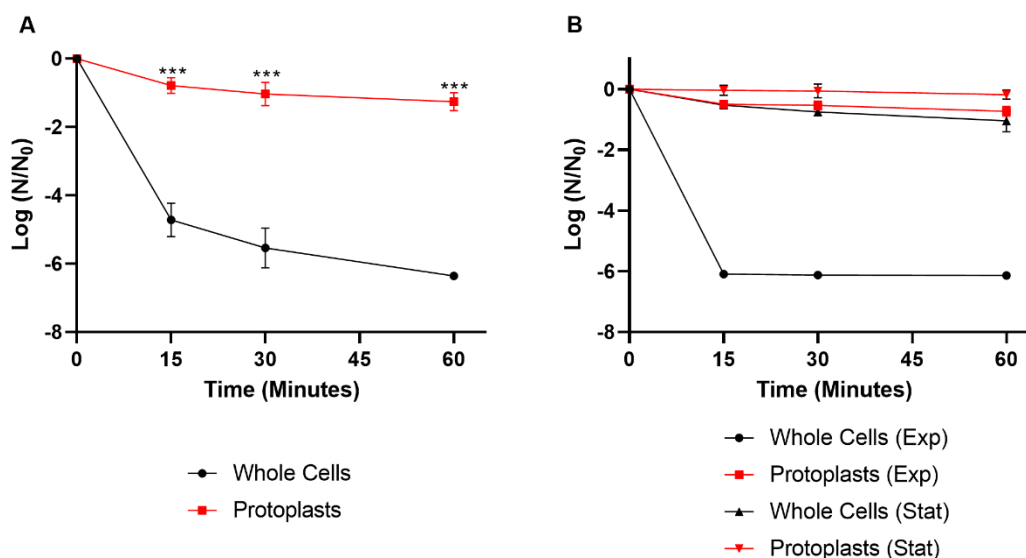


Figure 2.5. Removal of peptidoglycan increases tolerance to daptomycin in *E. faecalis*.

(A) Cells were grown to mid-logarithmic phase, split, and treated with either lysozyme to generate protoplasts or solvent control (whole cells) and then treated with 30 $\mu\text{g/mL}$ daptomycin. Shown are the average log ratios of colony forming units $\pm$ standard deviations for $n = 3$ replicates. *** $P \leq 0.001$; exact P -values are found in Supplemental Table 2.4. (B) Cells were grown to mid-logarithmic phase (Exp) or to late stationary phase (24 hour cultures; Stat) and treated with either lysozyme to generate protoplasts or solvent control (whole cells) and then treated with 30 $\mu\text{g/mL}$ daptomycin. Shown are the average log ratios of colony forming units $\pm$ standard deviations for $n = 3$ replicates.

We noted that there was no interaction between D-cycloserine and daptomycin, potentially due to the cellular growth conditions (Table 2.1, see discussion). Fosfomycin, in agreement with previous studies in *E. faecium* and *S. aureus* [76-79], was synergistic with daptomycin, suggesting that daptomycin impacts early peptidoglycan synthesis steps in *E. faecalis* (see discussion). Additionally, we observed that ampicillin was additive when combined with daptomycin (Table 2.1).

Peptidoglycan removal enhances stationary phase cell survival against daptomycin

Combining our above data, we found that stationary phase cells were far more tolerant to daptomycin when compared to their exponential-phase cell counterparts (Figures 2.1A and 2.5B), and that removal of peptidoglycan also results in high tolerance (Figure 2.5 and Supplemental Table 2.3). Given that peptidoglycan turnover is increased in stationary phase relative to exponential phase in *E. faecalis* [53], and that in stationary phase *E. coli*, peptidoglycan is thicker and comprises a higher percentage of the total dry weight compared to exponential phase cells [54], we decided to probe whether removal of the cell wall of stationary phase cells altered their daptomycin tolerance. We found that there was a small, but significant difference between survival of stationary phase whole cells versus stationary phase protoplasts (Figure 2.5B and Supplemental Table 2.3; $P = 0.023$ at all time points).

Removal of peptidoglycan protects multiple Gram-positive bacteria from killing by daptomycin

As removal of peptidoglycan in *E. faecalis* altered daptomycin tolerance, we examined whether its removal in other Gram-positive species results in increased daptomycin survival. Thus, we compared survival of whole cells and protoplasts of *E. faecium*, *S. aureus*, and *B. subtilis* upon treatment with 7.5–10x MIC (Supplemental Table 2.2). We found that protoplasts of *E. faecium* had a 2.1 log-fold survival (or more) over whole cell counterparts (Figure 2.6A; $P < 0.001$ at all time points). Similarly, removal of the

Table 2.1. Combination of peptidoglycan synthesis-targeting antibiotics with daptomycin on *E. faecalis*.

Combination (antibiotic A – antibiotic B)	Antibiotic A MIC (µg/mL)	Antibiotic B MIC (µg/mL)	Antibiotic B MIC (µg/mL) at Dap MIC of 0.5 µg/mL	FICI	Effect
Dap – FOF	4	32–128	8–32	0.25–0.375	Synergistic
Dap – DCS	2	128	128	1.25	No interaction
Dap – AMP	4	0.5	0.25	.625	Additive

Represented are the ranges of individual antibiotic MICs along with the range of the MICs of antibiotic B combined with daptomycin at 0.5 µg/mL. $n = 3$ for each experiment. $FICI \leq 0.5$: synergistic, $FICI = 0.5–1$: additive; $FICI = 1–4$: no interaction. DAP; daptomycin, FOF; Fosfomicin, DCS; D-cycloserine, AMP; ampicillin.

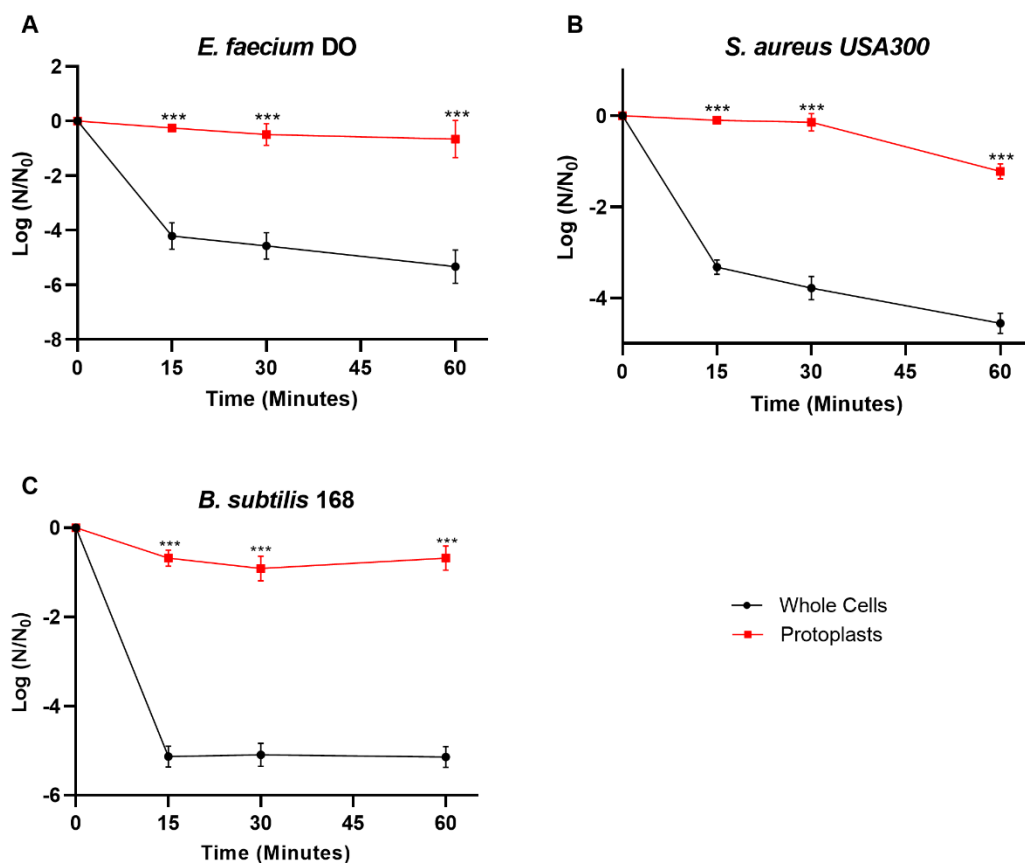


Figure 2.6. Removal of peptidoglycan increases tolerance to daptomycin in *E. faecium*, *S. aureus*, and *B. subtilis*.

(A) Treatment of either *E. faecium* whole cells or protoplasts (see Materials and Methods) with 30 μ g/mL daptomycin. Shown are the average log ratios of survivors $\pm$ standard deviations for $n = 3$ replicates. *** $P \leq 0.001$; exact P -values are found in Supplemental Table 2.4. (B) *S. aureus* whole cells or protoplasts (see Materials and Methods) treated with 5 μ g/mL daptomycin. Shown are the average log ratios of survivors $\pm$ standard deviations for $n = 3$ replicates. *** $P \leq 0.001$; exact P -values are found in Supplemental Table 2.4. (C) *B. subtilis* whole cells or protoplasts (see Materials or Methods) treated with 5 μ g/mL daptomycin. Shown are the average log ratios of survivors $\pm$ standard deviations for $n = 3$ replicates. *** $P \leq 0.001$; exact P -values are found in Supplemental Table 2.4.

peptidoglycan of *S. aureus* protoplasts had a minimum 3.4 log-fold increase in survivors (Figure 2.6B; $P < 0.001$ at all time points) while *B. subtilis* protoplasts had a 2.5 log fold increase (or more) in survivors compared to whole cell controls (Figure 2.6C; $P < 0.001$ at all time points). This demonstrates that across these bacterial species, removal of peptidoglycan negates most killing by daptomycin.

Discussion

While in the literature there have been multiple proposed targets for daptomycin, several lines of evidence support peptidoglycan biosynthesis being its major target [2-8, 11]. Here we demonstrate that daptomycin tolerance, *i.e.*, increased survival following treatment above the MIC, is dependent on several factors: growth phase, the ability of cells to synthesize proteins and fatty acids, and the presence/absence of peptidoglycan. Combined, these data support that peptidoglycan, which is intricately linked to membrane biosynthesis and requires protein biosynthesis, is necessary for full daptomycin efficacy.

E. faecalis stationary-phase cells were protected to a high degree against daptomycin relative to their exponential-phase counterparts (Figure 2.1A). However, similar levels of protection were induced by peptidoglycan removal in exponential phase compared to stationary phase whole cells (Figures 2.1B and 2.5B, Supplemental Table 2.3). This is possibly due to cell wall changes which occur in bacteria during stationary phase, including increases in wall thickness [54] and increases in peptidoglycan recycling [80] as well as decreased peptidoglycan synthesis [81]. Additionally, this could be due to a reduction in wall turnover in stationary phase cells compared to exponential phase cells [82] or due to increased degradation of MurA, which catalyzes the first committed step of peptidoglycan synthesis by transferring enolpyruvate from phosphoenolpyruvate to UDP-NAG [83], in stationary phase cells [84]. In our data, we noted that in stationary phase cells, phosphoenolpyruvate was decreased relative to exponential phase cells (Figure 2.1C), potentially indicating altered MurA activity.

Given these data and previous studies, why would addition of chloramphenicol, a *de novo* protein synthesis inhibitor, or cerulenin, an inhibitor of fatty acid biosynthesis, induce

daptomycin protection? The production of peptidoglycan is needed for cellular replication and expansion of the population size. Inhibiting protein biosynthesis, as shown *via* a halt in colony forming units (Supplemental Figure 2.1), prevents cellular division. However, in *E. faecalis* (formerly *Streptococcus faecalis*), previous work indicated that upon treatment with chloramphenicol, cellular division stopped while peptidoglycan synthesis still occurred, though at a lower rate, resulting in thickening of the cell wall [85]. Thus, perhaps the effectiveness of daptomycin may be altered because of a reduction in peptidoglycan synthesis. Similarly, previous studies have shown that addition of the fatty acid biosynthesis inhibitor cerulenin to exponentially growing *E. faecalis* reduces but does not abrogate peptidoglycan synthesis [86], and can lead to cell expansion and altered/misplaced cell wall biosynthesis [87]. The protective effect we observe on treating exponentially growing cultures with chloramphenicol or cerulenin could possibly also be explained by a dilution of targets caused by increased and misplaced wall synthesis [85-87].

Our metabolomics data also suggests that decreased peptidoglycan biosynthesis could be the cause of the protective effects we see, as the mass/charge ratio corresponding to UDP-NAM-Ala is decreased in stationary phase cells, cells treated with cerulenin, and cells treated with chloramphenicol (Figures 2.1C, 2.3C, and 2.4C). As noted, we also observed a significant decrease in the mass/charge ratio corresponding to alpha-ketoglutarate in stationary phase cells and upon treatment with chloramphenicol and cerulenin, as well as a significant increase in the mass/charge ratio corresponding to glutamine under these conditions. Fascinatingly, both of these metabolites can be linked back to peptidoglycan synthesis, as alpha-ketoglutarate and glutamine are suppliers of glutamate, which is an amino acid incorporated into the amino acid side-chains of peptidoglycan. It should be noted here that in order to confirm these significant metabolites among the experimental groups, a significant analysis microarray (SAM) was performed to calculate potential rates of false discovery and identify those metabolites that are statistically significant based on two different analyses (heatmap and SAM). The results of these experiments also provide some insights as to why treatment with high

levels of daptomycin result in “biphasic” killing of multiple bacterial species [10, 22, 25-29]. Those cells that survive high daptomycin do so likely because they are not actively synthesizing and turning over peptidoglycan [11, 24]. For both chloramphenicol-treated and cerulenin-treated cultures, we also noted a significant decrease in the mass/charge ratio corresponding to methionine sulfoxide, a precursor to methionine. However, due to the lack of corresponding increases of mass/charge ratios of methionine sulfoxide in stationary phase cells, we cannot confirm that this decrease is directly responsible for the increases in daptomycin tolerance we observe under these conditions.

Further evidence to support peptidoglycan as a target for daptomycin comes from the observed positive effects of combining daptomycin with other cell-wall targeting drugs (Table 2.1). The synergistic effects between fosfomycin and daptomycin could potentially be explained by an upregulation of MurA caused by exposure to daptomycin [88, 89]. In turn, fosfomycin tightly binds to MurA, thus blocking the first committed step of peptidoglycan biosynthesis [83]. Similarly, daptomycin was found in *S. aureus* to increase the expression of PBP1 [90], the target of ampicillin, thus potentially explaining the observed additive effect between daptomycin and ampicillin which is in line with synergy observed during time-kill experiments combining β -lactam antibiotics with daptomycin [91]. We did not observe an interaction between daptomycin and D-cycloserine: this could be because D-cycloserine simply “freezes” the pools of L-ala and D-ala by binding to alanine racemase. Note that its effect on peptidoglycan synthesis depends on the current state of the pools of L-ala and D-ala [92] and given that our experiments were carried out in rich media, it is possible that the pool of D-ala was great enough to prevent either a synergistic or additive effect between daptomycin and D-cycloserine.

Interestingly, a recent study gave evidence that pre-incubation of *B. subtilis* with teixobactin, a lipid II and lipid III targeting antibiotic [78], protected cells against daptomycin, rather than acting synergistically with it [11]. As teixobactin shares a target with daptomycin, a pre-treatment with (and subsequent washing away of, as performed by Grein *et al.*, 2020) teixobactin could reduce the number of targets for daptomycin to effectively bind to, leading to protection against daptomycin [11].

While the data presented above support peptidoglycan is a target for daptomycin, it was originally dismissed, as daptomycin still killed *E. faecium* cells which had their cell walls removed [10]. Though our observations agree with published literature that protoplasted cells can be subject to killing by daptomycin [10], our inclusion of a whole cell control provides a fuller picture. We found that some killing of protoplasted cells occurred, however, when protoplasts were compared to the whole cell control, there was a minimum of a 1.8 log-fold increase in survival against daptomycin, independent of the bacterial species being tested (Figures 2.5 and 2.6).

While we have demonstrated here that bacterial growth state and that the presence or absence of peptidoglycan are of major importance for daptomycin efficacy regardless of bacterial species, it is possible and indeed probable that treatment of cells with lysozyme and with different antibiotics may alter the cellular membrane, in particular phosphatidylglycerol amounts and localization, reducing daptomycin binding. This is critical as daptomycin has been shown to interact with lipid II and phosphatidylglycerol [11, 17, 18]. Further investigation is needed to determine the bacterial membrane composition under these conditions in order to assess whether specific changes to the bacterial membrane are responsible for altered daptomycin efficacy.

Within this work, we have demonstrated that intact peptidoglycan is required for full daptomycin efficacy, and that dysregulation of cellular metabolism can result in significant increases in daptomycin tolerance. Our results aid in informing of possible effective treatment combinations in the clinic and new therapeutic approaches.

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Appendix

Supplemental Table 2.1. List of bacterial strains used in this study.

Bacterial strain	Reference
<i>Enterococcus faecalis</i> OG1RF	Lab stock.
<i>Enterococcus faecium</i> DO (TX0016)	Generous gift of Barbara Murray.
<i>Staphylococcus aureus</i> USA300	From ATCC.
<i>Bacillus subtilis</i> sub. <i>subtilis</i> 168	Lab stock.

Supplemental Table 2.2. Minimal inhibitory concentrations of daptomycin for the bacterium used in this study.

Bacterial strain	MIC range (µg/mL)
<i>Enterococcus faecalis</i> OG1RF7	2-4
<i>Enterococcus faecium</i> DO (TX0016)	4
<i>Staphylococcus aureus</i> USA300	0.5-1
<i>Bacillus subtilis sub. subtilis</i> 168	0.5

Minimal inhibitory concentrations (MICs) were performed using broth dilution technique. The ranges observed are reported above. Brain heart infusion + 1.5 mM CaCl₂ was used for *Enterococcus* and *Staphylococcus* strains grown statically. LB + 1.5 mM CaCl₂ was used for *B. subtilis* grown shaking (250 RPM). *n* = 3 for all experiments.

Supplemental Table 2.3. Colony forming units per milliliter (CFU/mL) taken during cell survival assays within this study.

Variable	Corresponding figure in text	Time	CFU/mL (replicate 1)	CFU/mL (replicate 2)	CFU/mL (replicate 3)
Exponential	Figure 2.1A	0 minutes	2.54×10^8	2.71×10^8	2.92×10^8
		15 minutes	6.50×10^4	1.40×10^5	3.00×10^4
		30 minutes	1.81×10^4	1.71×10^4	8.40×10^3
		60 minutes	4.80×10^3	4.10×10^3	4.60×10^3
		240 minutes	1.00×10^2	2.30×10^2	2.30×10^2
		24 hours	Below limit of detection	Below limit of detection	Below limit of detection
Stationary	Figure 2.1A	0 minutes	1.27×10^9	1.49×10^9	1.42×10^9
		15 minutes	8.70×10^8	7.00×10^8	7.20×10^8
		30 minutes	8.70×10^8	7.80×10^8	5.70×10^8
		60 minutes	6.20×10^8	5.50×10^8	7.30×10^8
		240 minutes	4.70×10^8	3.00×10^8	3.00×10^8
		24 hours	6.40×10^7	5.00×10^7	4.00×10^7
Water	Figure 2.2A	0 minutes	3.50×10^8	5.00×10^8	3.80×10^8
		15 minutes	3.20×10^4	5.20×10^4	9.20×10^3
		30 minutes	1.07×10^4	7.80×10^3	1.78×10^3
		60 minutes	5.90×10^3	2.90×10^3	9.60×10^2
Arsenate	Figure 2.2A	0 minutes	1.78×10^8	1.81×10^8	2.08×10^8
		15 minutes	9.90×10^3	1.13×10^4	3.60×10^3
		30 minutes	2.21×10^3	1.83×10^3	7.20×10^2
		60 minutes	8.30×10^2	9.40×10^2	2.70×10^2
Solvent control	Figure 2.3A	0 minutes	2.12×10^8	2.97×10^8	2.40×10^8
		15 minutes	1.61×10^3	2.70×10^3	2.01×10^3
		30 minutes	8.90×10^2	8.80×10^2	6.50×10^2
		60 minutes	4.00×10^2	3.10×10^2	2.40×10^2
Chloramphenicol	Figure 2.3A	0 minutes	1.54×10^8	8.90×10^7	8.90×10^7
		15 minutes	6.90×10^4	1.05×10^5	2.80×10^5
		30 minutes	4.80×10^3	5.50×10^3	4.20×10^3
		60 minutes	6.60×10^2	1.21×10^3	1.96×10^3
Solvent control	Figure 2.4A	0 minutes	1.15×10^8	1.26×10^8	1.52×10^8
		15 minutes	7.30×10^3	5.90×10^3	1.09×10^3
		30 minutes	4.70×10^2	5.20×10^2	3.30×10^2
		60 minutes	9.00×10^1	1.10×10^2	1.90×10^2
Cerulein	Figure 2.4A	0 minutes	8.50×10^7	7.90×10^7	1.02×10^8
		15 minutes	6.30×10^4	4.60×10^4	5.10×10^4
		30 minutes	5.20×10^3	2.80×10^3	4.70×10^3
		60 minutes	6.10×10^2	1.80×10^2	1.61×10^3

Supplemental Table 2.3 Continued

Variable	Corresponding figure in text	Time	CFU/mL (replicate 1)	CFU/mL (replicate 2)	CFU/mL (replicate 3)
Whole cells	Figure 2.5A	0 minutes	3.15×10^8	3.25×10^8	2.53×10^8
		15 minutes	4.10×10^3	2.60×10^3	1.72×10^4
		30 minutes	1.00×10^3	2.40×10^2	2.65×10^3
		60 minutes	1.20×10^2	1.20×10^2	1.50×10^2
Protoplasts	Figure 2.5A	0 minutes	3.20×10^6	9.30×10^5	4.40×10^6
		15 minutes	9.00×10^5	9.10×10^4	7.00×10^5
		30 minutes	6.90×10^5	4.20×10^4	3.70×10^5
		60 minutes	3.40×10^5	3.09×10^4	2.09×10^5
Whole Cells (Exp)	Figure 2.5B	0 minutes	4.50×10^8	4.70×10^8	3.90×10^8
		15 minutes	4.40×10^2	2.90×10^2	3.40×10^2
		30 minutes	3.20×10^2	3.20×10^2	3.50×10^2
		60 minutes	3.30×10^2	3.10×10^2	3.10×10^2
Protoplasts (Exp)	Figure 2.5B	0 minutes	9.60×10^5	3.40×10^6	9.00×10^5
		15 minutes	3.20×10^5	1.12×10^6	2.60×10^5
		30 minutes	3.90×10^5	7.10×10^5	2.70×10^5
		60 minutes	2.45×10^5	4.30×10^5	1.82×10^5
Whole Cells (Stat)	Figure 2.5B	0 minutes	9.20×10^8	1.25×10^9	1.25×10^9
		15 minutes	2.56×10^8	3.60×10^8	4.20×10^8
		30 minutes	1.81×10^8	1.79×10^8	2.53×10^8
		60 minutes	3.20×10^7	1.63×10^8	2.01×10^8
Protoplasts (Stat)	Figure 2.5B	0 minutes	2.80×10^8	5.40×10^8	2.50×10^8
		15 minutes	3.00×10^8	3.15×10^8	3.00×10^8
		30 minutes	3.50×10^8	3.14×10^8	2.75×10^8
		60 minutes	2.11×10^8	2.40×10^8	2.15×10^8
Whole Cells [<i>E. faecium</i>]	Figure 2.6A	0 minutes	1.21×10^8	2.40×10^8	3.10×10^7
		15 minutes	6.30×10^3	5.40×10^3	6.40×10^3
		30 minutes	2.81×10^3	2.25×10^3	2.71×10^3
		60 minutes	2.00×10^2	6.40×10^2	7.20×10^2
Protoplasts [<i>E. faecium</i>]	Figure 2.6A	0 minutes	4.80×10^6	9.70×10^5	6.90×10^5
		15 minutes	2.08×10^6	6.00×10^5	4.50×10^5
		30 minutes	5.30×10^5	5.30×10^5	3.70×10^5
		60 minutes	1.70×10^5	5.40×10^5	3.70×10^5
Whole Cells [<i>B. subtilis</i>]	Figure 2.6B	0 minutes	1.82×10^7	1.76×10^7	3.20×10^7
		15 minutes	2.10×10^2	1.50×10^2	1.30×10^2
		30 minutes	2.20×10^2	1.90×10^2	1.30×10^2
		60 minutes	2.30×10^2	1.20×10^2	1.40×10^2

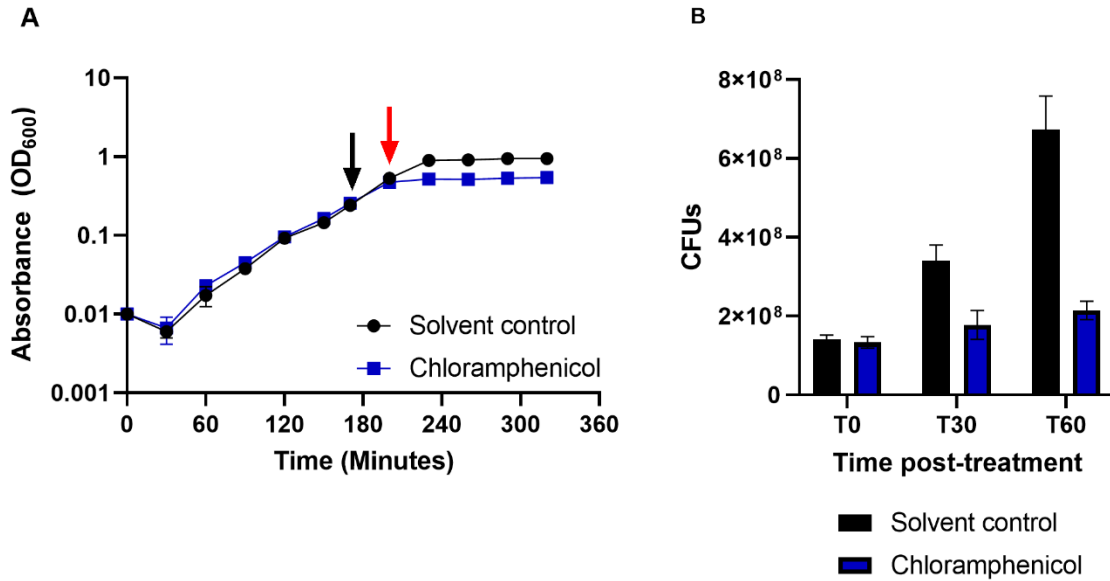
Supplemental Table 2.3 Continued

Variable	Corresponding figure in text	Time	CFU/mL (replicate 1)	CFU/mL (replicate 2)	CFU/mL (replicate 3)
Protoplasts [<i>B. subtilis</i>]	Figure 2.6B	0 minutes	2.90×10^5	7.00×10^5	5.40×10^5
		15 minutes	6.50×10^4	9.30×10^4	1.64×10^5
		30 minutes	3.10×10^4	4.90×10^4	1.32×10^5
		60 minutes	4.60×10^4	9.40×10^4	2.30×10^5
Whole Cells [<i>S. aureus</i>]	Figure 2.6C	0 minutes	4.70×10^6	2.81×10^7	8.10×10^6
		15 minutes	2.74×10^3	1.64×10^4	2.52×10^3
		30 minutes	1.17×10^3	2.36×10^3	1.70×10^3
		60 minutes	2.30×10^2	5.10×10^2	1.90×10^2
Protoplasts [<i>S. aureus</i>]	Figure 2.6C	0 minutes	1.39×10^7	2.28×10^7	1.50×10^7
		15 minutes	1.22×10^7	1.45×10^7	1.36×10^7
		30 minutes	1.52×10^7	1.04×10^7	1.12×10^7
		60 minutes	1.17×10^6	9.10×10^5	9.80×10^5
Mock Treatment	Supplemental Figure 2.3	0 minutes	3.08×10^8	3.40×10^8	3.06×10^8
		15 minutes	2.90×10^8	3.70×10^8	3.00×10^8
		30 minutes	5.30×10^8	3.90×10^8	5.50×10^8
		60 minutes	6.20×10^8	5.80×10^8	6.40×10^8
Sodium Arsenate	Supplemental Figure 2.3	0 minutes	2.06×10^8	2.20×10^8	1.95×10^8
		15 minutes	2.17×10^8	2.07×10^8	2.17×10^8
		30 minutes	3.00×10^8	3.00×10^8	2.97×10^8
		60 minutes	2.98×10^8	2.90×10^8	3.20×10^8
Whole Cells	Supplemental Figure 2.4	0 minutes	3.13×10^8	2.76×10^8	3.30×10^8
		15 minutes	3.61×10^5	3.70×10^5	1.07×10^5
		30 minutes	3.26×10^5	3.13×10^5	1.09×10^5
		60 minutes	2.72×10^5	2.32×10^5	8.50×10^4
Protoplasts	Supplemental Figure 2.4	0 minutes	7.30×10^6	1.01×10^6	1.31×10^6
		15 minutes	2.07×10^3	2.80×10^2	3.60×10^2
		30 minutes	1.75×10^3	4.70×10^2	3.30×10^2
		60 minutes	1.35×10^3	3.70×10^2	4.00×10^2

Supplemental Table 2.4. *P*-values determined within this study.

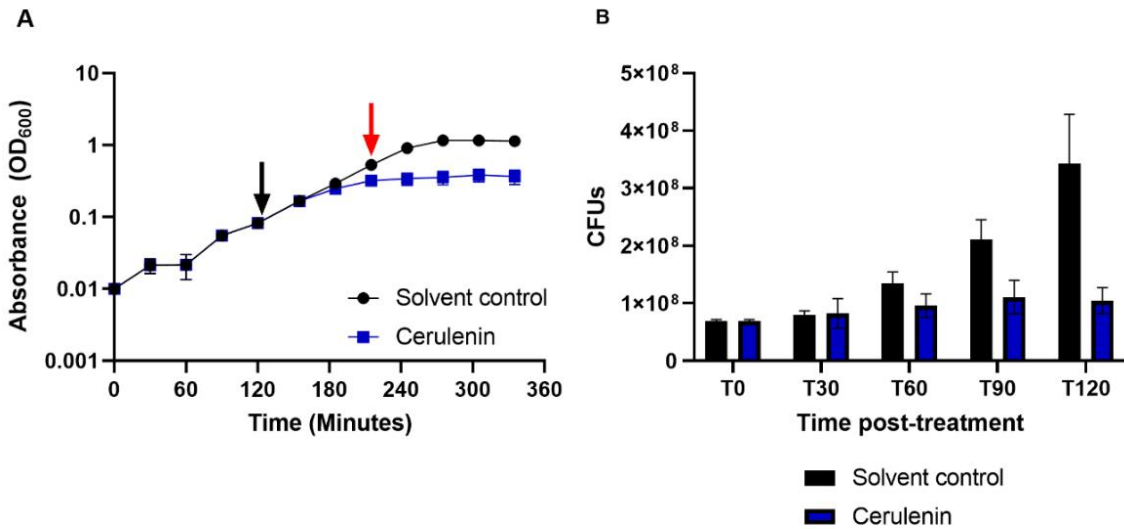
Cell survival assays				
Variable 1	Variable 2	Corresponding figure in text	Time (min)	P-value
Exponential	Stationary	Figure 2.1A	15	<0.001
			30	<0.001
			60	<0.001
Mock treatment	Sodium arsenate	Figure 2.2A	15	0.769
			30	0.769
			60	0.769
Solvent control	Chloramphenicol	Figure 2.3A	15	0.002
			30	0.001
			60	0.015
Solvent control	Cerulenin	Figure 2.4A	15	0.022
			30	0.002
			60	0.030
Whole Cells	Protoplasts	Figure 2.5A	15	<0.001
			30	<0.001
			60	<0.001
Whole Cells (Stat)	Protoplasts (Stat)	Figure 2.5B	15	0.023
			30	0.023
			60	0.023
Whole Cells [<i>E. faecium</i>]	Protoplasts [<i>E. faecium</i>]	Figure 2.6A	15	<0.001
			30	<0.001
			60	<0.001
Whole Cells [<i>B. subtilis</i>]	Protoplasts [<i>B. subtilis</i>]	Figure 2.6B	15	<0.001
			30	<0.001
			60	<0.001
Whole Cells [<i>S. aureus</i>]	Protoplasts [<i>S. aureus</i>]	Figure 2.6C	15	<0.001
			30	<0.001
			60	<0.001
Mock Treatment	Sodium Arsenate	Supplemental Figure 2.3	15	0.884
			30	0.884
			60	0.104
Whole Cells	Protoplasts	Supplemental Figure 2.4	15	0.222
			30	0.233
			60	0.233
ATP Determination Assays				
Variable 1	Variable 2	Corresponding figure in text	P-value	
Exponential	Stationary	Figure 2.1B	0.0246	
Mock treatment	Arsenate	Figure 2.2B	0.0011	
Solvent control	Chloramphenicol	Figure 2.3B	0.5948	
Solvent control	Cerulenin	Figure 2.4B	<0.001	
Whole Cells	Protoplasts	Supplemental Figure 2.5	0.2675	

Two-tailed Welch's *t*-tests were used to generate *P*-values. In the text, $\alpha = 0.05$ was used to determine significance.



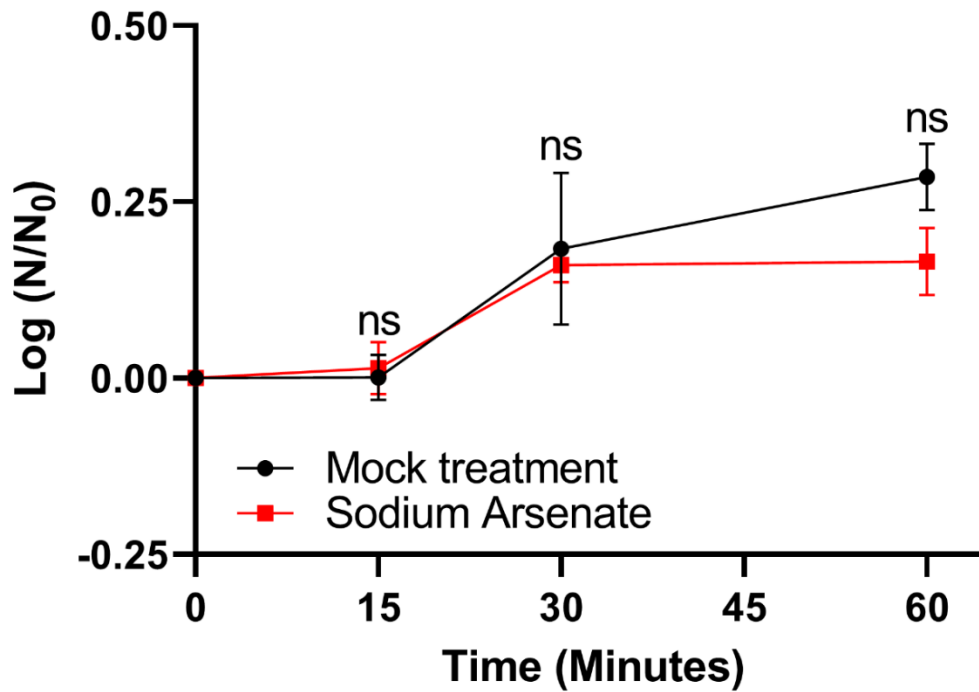
Supplemental Figure 2.1. Addition of chloramphenicol halts cellular division.

(A) Optical density of cells over time. Shown are the average optical densities at 600 nm $\pm$ standard deviations for $n = 3$ replicates. Black arrow indicates addition of chloramphenicol or solvent control at mid-logarithmic phase (at 175 min into the growth curve; OD₆₀₀ $\sim$ 0.250). Red arrow indicates when cells were harvested and processed prior to a subsequent cell survival assay (30 min post-chloramphenicol/solvent control treatment). (B) The number of colony-forming units as determined by dilution plating of the cultures in S1A. T0 is just prior to the addition of chloramphenicol or solvent control into the cultures, and T30 and T60 are 30 and 60 min after addition of chloramphenicol or solvent control into the cultures, respectively. $n = 3$.



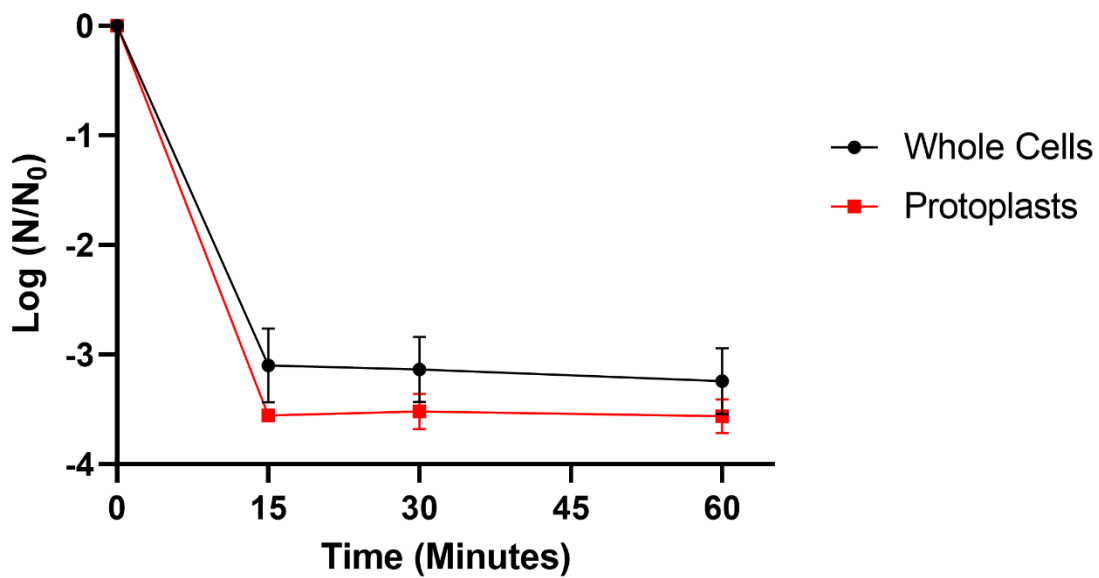
Supplemental Figure 2.2. Addition of cerulenin halts cellular division.

(A) Optical density of cells over time. Shown are the average optical densities at 600 nm $\pm$ standard deviations for $n = 3$ replicates. Black arrow indicates addition of cerulenin or solvent control at early-logarithmic phase (at 125 min into the growth curve; OD₆₀₀ ~ 0.125). Red arrow indicates when cells were harvested and processed prior to a subsequent cell survival assay (90 min post-cerulenin/solvent control treatment). (B) The number of colony-forming units as determined by dilution plating of the cultures in S2A. T0 is just prior to the addition of cerulenin or solvent control into the cultures. T30, T60, T90, and T120 are 30, 60, 90, and 120 min after addition of cerulenin or solvent control into the cultures, respectively. $n = 3$.



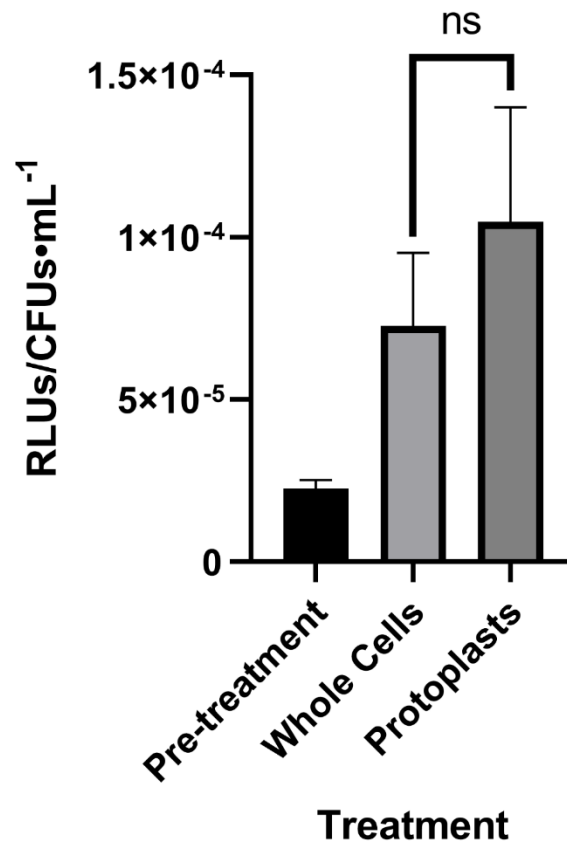
Supplemental Figure 2.3. Cells treated with sodium arsenate are still viable.

E. faecalis OG1RF cells were grown in BHI until OD₆₀₀ reached ~0.250. The cultures were split and treated with 10mM arsenate or mock-treated with water for 30 minutes. Following treatment, the cells were washed twice to remove arsenate and resuspended in BHI prior to enumerating colony forming units. Represented are the log ratio of colony forming units $\pm$ standard deviations over time. ^{ns} $P > 0.05$. $n = 3$.



Supplemental Figure 2.4. Protoplasts are not protected against sodium dodecyl sulfate.

E. faecalis OG1RF cells were grown to mid-logarithmic phase ($OD_{600} \sim 0.3\text{--}0.4$), split and treated with either lysozyme to generate protoplasts or solvent control to leave cell whole. Subsequently washed cells were subjected to killing by 0.05% SDS. Shown are the average log ratios of survivors $\pm$ standard deviations for $n = 3$ replicates. No significant differences were observed. Exact P -values are reported in Supplemental Table 2.4.

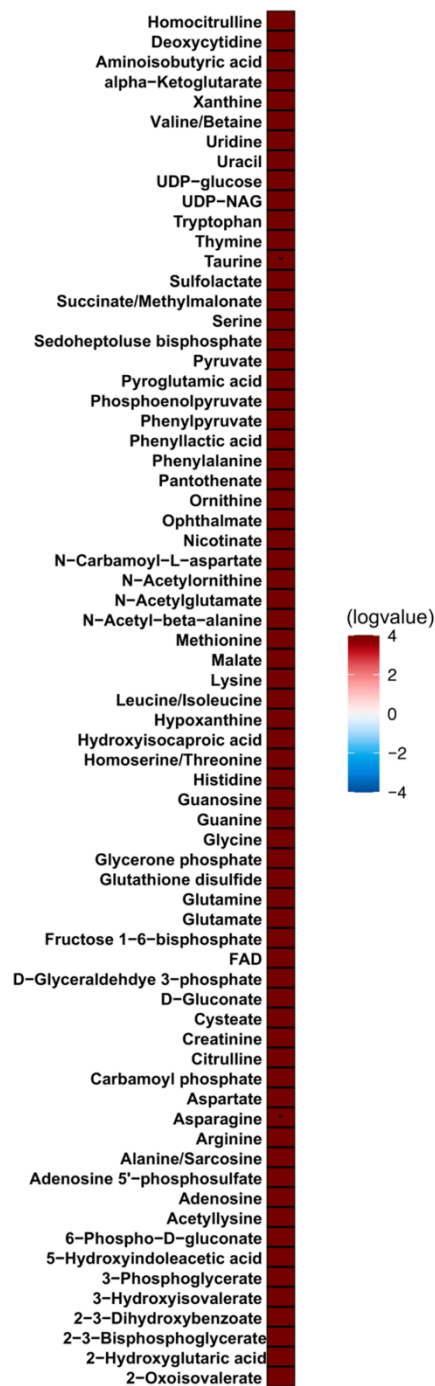


Supplemental Figure 2.5. ATP levels of *E. faecalis* OG1RF whole cells and protoplasts normalized to colony forming units.

Cells were grown to mid-logarithmic phase ($OD_{600} \sim 0.3\text{--}0.4$) and treated with either lysozyme to generate protoplasts or solvent control to leave cells whole. ATP levels of pre-treatment cells and whole cells or protoplasts were measured using the Promega BacTiterGlo kit and normalized to colony forming units. Shown are the average relative light units (RLUs) normalized to colony forming units $\pm$ standard deviations for $n = 3$ replicates. ** $P = 0.01\text{--}0.001$; exact P -values are reported in Supplemental Table 2.4.

Supplemental Figure 2.6. Removal of peptidoglycan results in a buildup of several metabolites.

Exponentially growing ($OD_{600} \sim 0.3$) *E. faecalis* cells were grown to mid-logarithmic phase ($OD_{600} \sim 0.3\text{--}0.4$), split and treated with either lysozyme to generate protoplasts or solvent control to leave cell whole as described in Materials and Methods. Metabolites of the cells were extracted and detected via mass spectrometry as outlined in Materials and Methods. Represented are the log-transformed fold changes of normalized whole cell metabolites to normalized protoplast metabolites. Normalization was performed dividing raw metabolite data by the ratio of colony forming units of protoplast versus whole cells. $n = 5$ replicates.



CHAPTER 3: AN EXPANDED ROLE OF THE FAK SYSTEM AND THE THIOESTERASE TESS IN *ENTEROCOCCUS FAECALIS*

Future Publication Note

A version of this chapter will eventually be submitted for publication.

Johnston RD, Woodall BM, King, A, Getty T, Campagna SR, and Fozo EM. An expanded role for the Fak system and TesS in *Enterococcus faecalis*. 2022/2023.

Experiments were completed by Rachel Johnston, with the following exceptions: Transmission electron microscopy was performed by Ainsley King. Troy Getty performed the growth assay of wild-type *E. faecalis* and deletion strains in 2.5 µg/mL palmitic acid. Mass spectrometry experiments were performed by Brittini Woodall. GC-FAME was completed by Microbial ID.

Abstract

Enterococcus faecalis is a Gram-positive organism which commensally resides in the human gastrointestinal tract, yet can cause infections in other bodily tissues. Although these environments are rich in fatty acids, *E. faecalis* cannot use exogenous fatty acids as carbon sources. It does, however, possess homologs to the Fak (fatty acid kinase) pathway which enables organisms to utilize exogenous fatty acids in phospholipid synthesis. Herein, we examined the role of these homologs in lipid synthesis. Further, we demonstrate that upon deletion of three of the four *fakB* homologs ($\Delta fakB_{1,2,5}$) of *E. faecalis*, several phenotypes arise, including accumulation of free fatty acids in the membrane, an altered lipid profile, and severe growth issues. Moreover, we were unable to delete all four *fakB* homologs without first deleting *tesS*, a gene which encodes a thioesterase that liberates fatty acids from the acyl carrier protein. Upon deletion of all of the *fakB* homologs and *tesS* (generating a quintuple deletion strain), growth was restored. Taken together, these data indicate that the Fak system and TesS work together to generate acyl-phosphates necessary for generating phospholipids. Additionally, we found that deletion of the *fakB* homologs along with *tesS* resulted in inability of a supplied fatty acid, oleic acid, to rescue growth inhibition by the *de novo* fatty acid biosynthesis inhibitor cerulenin. This indicated that incorporation of exogenous fatty acids was reduced in the quintuple deletion strain. This strain, however, was able to grow uninhibited in fatty acids toxic to wild-type *E. faecalis*, indicating that incorporation of these fatty acids may mediate their toxicity. Taken together, these data indicate that the FakB and TesS proteins of *E. faecalis* play a role in fatty acid homeostasis.

Introduction

Many bacterial species have a range of interactions with their hosts. These bacteria can adapt to different host environments and may, in the same host, occupy both a commensal niche and a pathogenic niche. One such organism, *Enterococcus faecalis*, commensally colonizes the human gastrointestinal tract, yet upon entering wounds or other tissues, causes infection. These two different environments, where *E. faecalis* is exposed to bile (gastrointestinal tract; commensal state) or serum (host tissues; pathogenic state), impact the physiology of *E. faecalis* given the presence of different nutrient constituents, including long-chain fatty acids, found within these host fluids [1]. Long-chain exogenous fatty acids can be used by bacteria to generate energy, as signaling molecules, or for membrane modification or synthesis. Gram-negative organisms such as *Escherichia coli* can transport fatty acids (acyls), attach them to coenzyme A (CoA) and begin the process of energy generation [2]. The acyl-CoA can then be used in β -oxidation, (*i.e.*, fatty acid catabolism), eventually forming acetyl-CoA, which can then enter the citric acid cycle for generating energy [2]. Acyl-CoA can also be transferred to glycerol-3-phosphate (G3P) to form phospholipid precursors [3].

While exogenous fatty acids can be used as a carbon source in some bacteria, they can also act as signaling molecules which alter fatty acid metabolism. This has been demonstrated in one such species, *Bacillus megaterium*, which can convert toxic unsaturated fatty acids to epoxides, rendering them non-toxic [4]. In this system, unsaturated fatty acids act as signaling molecules by binding to the repressor protein Bm3R1, enabling the transcription of *cyP102* [4] which codes for an enzyme capable of completing the epoxidation reaction [5]. One other organism which demonstrates the ability use fatty acids as signaling molecules is *E. coli*. Although *E. coli* can use free fatty acids as a carbon source, growth in media containing fatty acids with a carbon chain length of 12 or greater induced expression of genes involved in fatty acid degradation [6]. For many other bacterial species, exogenous long-chain fatty acids block *de novo* fatty acid biosynthesis [3, 7-9]. In *E. faecalis*, fatty acids repress *de novo* fatty acid biosynthesis through binding an auxiliary acyl carrier protein, AcpB [10]. The resulting acyl-AcpB then

interacts with FabT, repressing fatty acid gene expression [10]. Deletion of *acpB* resulted in continued *de novo* fatty acid synthesis even in the presence of supplied fatty acids [10]. Thus, exogenous fatty acids are important for regulation of *de novo* fatty acid synthesis.

Exogenously supplied long-chain fatty acids can also be used for forming phospholipids (Figure 3.1). In organisms such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, β -oxidation does not occur; however, in these organisms, exogenous fatty acids are bound by a FakB protein and are activated via phosphorylation by a FakA protein [11, 12]. The phosphorylated fatty acid can then be incorporated onto glycerol-3-phosphate by the enzyme PlsY or can be converted to acyl-ACP by the enzyme PlsX [12]. Acyl-ACP can enter the *de novo* fatty acid synthesis cycle or can be used as an acyl donor to form phospholipid precursors [12]. In *S. aureus*, there are two proteins which initially bind exogenous fatty acids prior to their activation: saFakB1 (for *S. aureus* FakB1), which specifically binds to saturated fatty acids, and saFakB2, which binds to unsaturated fatty acids [12]. Similarly, *S. pneumoniae* possesses spFakB1 (for *S. pneumoniae* FakB1) and spFakB2, which follow the specificity of *S. aureus*, along with an additional protein, spFakB3, which binds polyunsaturated fatty acids [11]. While their specificities remain unclear, we and others have noted that there are four FakB homologs in *E. faecalis* [13].

Regardless of whether formed from exogenous or internally produced fatty acids, phosphorylated acyl chains (acyl-phosphates) are critical for phospholipid biosynthesis (Figure 3.1). Specifically, PlsY uses acyl-phosphates, generated either from PlsX (using acyl-ACP) or from the Fak system, along with G3P to generate lysophosphatidic acid (Reviewed in [14]). Additionally, while the Fak system phosphorylates free fatty acids from the external environment for lipid synthesis, free fatty acids can also be generated internally by cleavage of an acyl from acyl-ACP by the thioesterase TesS in *S. pneumoniae* [15]. The newly generated free fatty acid can then be used as a substrate for the Fak system to generate acyl-phosphates [15]. However, given that deletion of the *fak* genes in *S. aureus* and *S. pneumoniae* was not lethal and did not impact the growth of the cells, this route is thought to be a minor pathway in this organism.

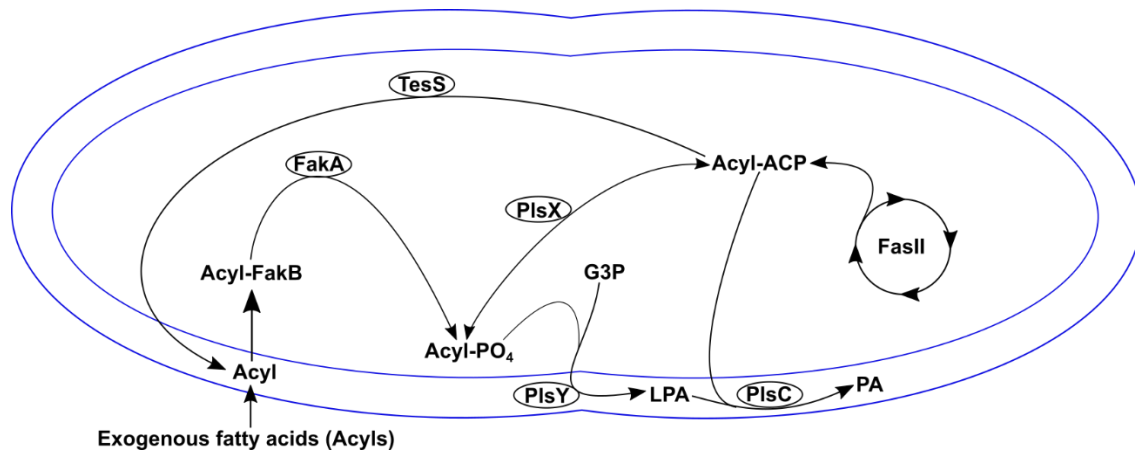


Figure 3.1. Synthesis and use of free fatty acids in *E. faecalis*.

Free fatty acids, whether exogenously supplied or produced by cleavage of a fatty acid (acyl) from acyl-ACP by TesS, are bound by a FakB protein. The FakA enzyme phosphorylates the acyl, forming acyl-phosphate (Acyl-PO₄). Acyl-phosphates are incorporated onto the SN-1 position of glycerol-3-phosphate by PlsY to form lysophosphatidic acid (LPA). Acyl-phosphates can also be bound to the acyl carrier protein (ACP) through the action of PlsX. Acyl-ACP, generated through the *de novo* fatty acid synthesis cycle (FasII), is used as the acyl donor to form phosphatidic acid, the precursor to membrane phospholipids. The acyl group can be cleaved from acyl-ACP by TesS to form a free fatty acid.

Whether being metabolized, functioning as signaling molecules, or being funneled into the fatty acid biosynthesis pathway and/or membrane phospholipids, exogenous long-chain fatty acids have a variety of effects on bacterial physiology. In *S. aureus*, exposure to specific free fatty acids impacts membrane composition [16]. Furthermore, exposure of *S. aureus* to specific “toxic” fatty acids (*i.e.*, toxic to growth for this species), such as palmitoleic acid (C_{16:1cis9}), resulted in membrane depolarization and the release of proteins into the growth medium [17]. In *E. faecalis*, the addition of specific saturated fatty acids impacts the gross morphology of the cells [18]. Additionally, some exogenously-supplied fatty acids severely impacted the generation time of *E. faecalis*, and in some cases caused premature growth stasis [1, 18]. Importantly, several fatty acids examined had positive effects on enterococcal physiology: for example, the addition of oleic acid (C_{18:1cis9}), which is prevalent in host fluids, yet not synthesized by *E. faecalis*, enabled cells to overcome inhibition of membrane biosynthesis by cerulenin [18].

Given the clear evidence that exogenous fatty acids alter the physiology of *E. faecalis*, and that *E. faecalis* cannot use these fatty acids for energy *via* β -oxidation, we examined the role of the FakA/FakB system in enterococcal physiology. Unexpectedly, we discovered a requirement for the Fak system in media lacking exogenous fatty acids. Specifically, we were unable to delete all four *fakB* genes in *E. faecalis*. Combined deletion of specific *fakB* genes resulted in altered growth in specific fatty acids; in particular, the Δ *fakB1,2,5* strain had a longer generation time when unsaturated exogenous fatty acids were present in its growth media compared to a wild-type strain, and in unsupplemented medium had hyperseptation and alterations to its fatty acid profile.

However, we were able to delete all four *fakB* genes when combined with deletion of *tesS*. We confirmed that this quintuple deletion strain (Δ *fakB1,2,4,5*/ Δ *tesS*) was dependent upon *de novo* fatty acid biosynthesis for membrane formation, as the addition of exogenous fatty acids could not overcome inhibition by cerulenin. Furthermore, the negative growth phenotypes observed in Δ *fakB1,2,5* were rescued in the quintuple deletion strain. Finally, while the addition of specific fatty acids inhibited growth of the wild-type strain, these same fatty acids had no effect on growth of the quintuple deletion

strain, suggesting that incorporation of these fatty acids into phospholipids is responsible for their toxicity.

Materials and Methods

Identification of FakA and FakB homologs

FakA and FakB homologs were identified using NCBI Basic Local Alignment Search Tool for proteins (BLASTP). Briefly, each individual *S. aureus* (SAUSA300_0733 and SAUSA300_1318 [12]) and *S. pneumoniae* FakB protein (Sp1557, Sp1112, and Sp0742 [11]) was used as a query for BLASTP (protein-protein BLAST algorithm) against *E. faecalis* OG1RF using the non-redundant protein sequences (nr) database. Percent identity and percent similarity from each BLAST result were reported from the resulting alignments.

FakB protein modelling and multiple sequence alignment

The identified FakB homologs of *E. faecalis* (OG1RF_RS00080, OG1RF_RS07200, OG1RF_RS05020, and OG1RF_RS06660) along with saFakB1 were modelled using Protein Homology/analogy Recognition Engine V 2.0 (Phyre²; [19]). Briefly, the amino acid sequence for each *E. faecalis* FakB homolog was entered into the server and modelled using intensive mode. A multiple sequence alignment of the FakB homologs of *E. faecalis* along with the FakB proteins of *S. aureus* and *S. pneumoniae* were aligned using Clustal Omega [20, 21]. The resulting alignment was annotated by highlighting residues important for FakB function noted by [22].

Bacterial growth conditions

Enterococcus faecalis strains (listed in Supplemental Table 3.1) were grown statically in brain heart infusion medium (BHI; BD Difco) at 37°C. Overnight cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.01 prior to performing assays. For the determination of generation times, fatty acids were supplemented at the following concentrations: oleic acid (C_{18:1 cis9}) at 20 µg/mL, linoleic acid (C_{18:2 cis9,12}) at 10 µg/mL,

stearic acid (C_{18:0}) at 20 µg/mL, myristic acid (C_{14:0}) at 5 µg/mL, and palmitic acid (C_{16:0}) at 5 µg/mL or 2.5 µg/mL as indicated in the text. For cerulenin outgrowth assays, cerulenin was supplemented at 5 µg/mL and oleic acid (C_{18:1 cis9}) was supplemented at 5 µg/mL as previously described [18]. For all experiments utilizing fatty acids, a solvent control was also included, whereby an equal volume of ethanol was added to the control culture. All fatty acids and reagents were purchased from Millipore-Sigma unless otherwise noted. Counterselection was performed on M9 minimal medium containing yeast extract and glucose (M9MMYEG) agar plates (final concentrations 1X M9 salts, 0.25% yeast extract, 150 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.5% glucose, and 10 mM *p*-Cl-phenylalanine (*p*-Cl-Phe; Sigma-Aldrich or Alfa Aesar). *Escherichia coli* cultures were grown in LB medium at 37°C with shaking. Antibiotics were used at the following concentrations when needed: erythromycin, 10 µg/mL (*E. faecalis*) or 100 µg/mL (*E. coli*); spectinomycin, 1,000 µg/mL; fusidic acid, 25 µg/mL; rifampicin, 250 µg/mL; chloramphenicol, 25 µg/mL. All media components, unless otherwise noted, were purchased from ThermoFisher.

Generation of deletion strains

The strains and plasmids used in this study are listed in Supplemental Table 3.1, and the sequences for oligonucleotides used are given in Supplemental Table 3.2. Deletion strains were generated following the protocol of Kristich *et al.*, 2007 [23]. Briefly, to delete the *fakB* genes and *tesS*, ~1 kb upstream (designated as piece 1) and ~1 kb downstream (designated as piece 2) of the respective gene was amplified from *E. faecalis* OG1RF genomic DNA using primers with complementary overlaps with the vector (pCJK47) and the neighboring piece. The vector, pCJK47, was amplified with complementary overlaps to both piece 1 and piece 2. The amplified inserts and vectors were assembled using NEB Gibson Assembly Master Mix. The resulting assembly was transformed into *E. coli* EC1000. The resulting plasmids, pRDJ1, pRDJ2, pRDJ3, pRDJ4, and pRDJ5, used to generate $\Delta fakB1$, $\Delta fakB2$, $\Delta fakB4$, $\Delta fakB5$, and $\Delta tesS$ respectively, were transformed into a conjugative donor strain of *E. faecalis*, CK111/pCF10-101. The resulting

CK111/pCF10-101 derivatives were then mixed with an OG1RF recipient at a ratio of 1 part donor to 9 parts recipient. The mixture was plated on BHI and mating was allowed to proceed for 5 h at 37°C. To select for transconjugant recipients, the resulting mixture was plated on BHI agar containing rifampicin, fusidic acid, erythromycin, and X-gal. To select for donors, the resulting mixture was plated on BHI agar containing spectinomycin and erythromycin. Blue colonies from the recipient plates were then re-isolated on the same medium. The isolates were grown in BHI for 18-24 h, passaged again into fresh BHI, and plated on M9MMYEG. White colonies were isolated onto BHI, tested for erythromycin sensitivity by patching isolates onto BHI containing erythromycin, and screened for deletion of the respective gene.

Phospholipid extraction and thin layer chromatography (TLC)

Briefly, 5 mL cultures were grown to mid-logarithmic phase in BHI. Following growth, the cells were harvested by centrifugation (2379 $\times$ g for 10 min) and washed twice with 1X PBS to remove the growth media. The cells were pelleted and resuspended in 200 μ L 4:1 ethanol:water, then boiled at 99°C for 15 min, with vortexing midway through. The cells were then pelleted at 16,200 $\times$ g for 3 min and the supernatant was collected into an autosampler vial. This process was repeated on the pellets an additional two times with 100 μ L 4:1 ethanol:water. Following the final harvest, the supernatant collected in the autosampler vials was allowed to air dry. After drying, the phospholipid extracts were stored at -20°C until used for TLC. Just prior to performing TLC, the phospholipid extracts were resuspended in a mixture of 1:1 chloroform:methanol.

To perform TLC, a chamber was washed three times with 1:1 chloroform:methanol. The solvent system, 40:35:30:2 of chloroform:triethylamine:ethanol:water, was made fresh, added to the TLC chamber, and allowed to equilibrate for a minimum of 45 min. Meanwhile, samples were loaded onto a Silica gel 60 F₂₅₄ plate (Millipore). The plate was then placed in the TLC chamber and was allowed to run for ~45 min or until the solvent front reached within 1.5-2 cm of the top of the plate. The TLC plate was removed from the chamber and was allowed to dry. The dried plate was then rapidly dipped into

primuline (Sigma-Aldrich) solution (50 mg/mL in 80:20 acetone:water) and allowed to dry prior to visualization using a handheld UV lamp. Band intensities were quantified using the gel analysis tool in ImageJ [24].

Ultra-High Performance Liquid Chromatography High Resolution Mass Spectrometry (UHPLC-HRMS)

To analyze the lipidome of the wild type strain (OG1RF) and the $\Delta fakB1,2,5$ mutant, cells were grown as indicated above to an OD₆₀₀ of ~0.3 for harvest. At harvest, OD₆₀₀ measurements were documented and 5 mL of cell culture was centrifuged (2739xg for 10 min) forming a cell pellet. Following centrifugation, cell pellets were resuspended and washed with 15 mL 1X phosphate buffered saline (1XPBS) and stored at -80°C prior to lipid extraction. Lipids were extracted according to the protocol listed in Woodall *et al.*, 2021 [25, 26].

Lipidome analysis was performed in the manner of Woodall *et al.*, 2021 [26]. In brief, an UltiMate 3000 ultra-high performance liquid chromatography system (UHPLC, Dionex, Sunnyvale, CA) was used to inject 10 µl of sample onto a CORTECS C18 column (90 Å, 2.7µm, 2.1mm x 150mm; Waters,) controlled at 40°C. Mobile phase A was 60:40 acetonitrile:water with 10 mM ammonium formate as a buffer and 0.1% formic acid while mobile phase B consisted of 90:10 2-propanol:acetonitrile with 10 mM ammonium formate as a buffer and 0.1% formic acid. Eluent was introduced to the mass spectrometer via an electrospray ionization (ESI) source, and mass analysis was performed using an Exactive Plus (Thermo Scientific, Waltham, MA) mass spectrometer operated in dual polarity mode. Ions of respective DAG and MGDG species were detected in positive mode while free fat, PG, L-PG, and CL were detected in negative mode. Masses were detected in full scan mode within a scan range of 100-1500 *m/z*, operated at a resolution of 140,000, with an automatic gain control target of (AGC) of 3x10⁶ ions, and a maximum IT time of 100 ms. Full scan data was complemented with all ion fragmentation data at a resolution of 35,000 utilizing 35 eV collisional energy. The mass spectrometer was calibrated every 24 hours.

Statistical analysis and data processing also followed same procedure as stated in Woodall *et al.*, 2021 [26]. Raw files from Xcalibur were converted to .mzMLformat via MS convert (ProteoWizard) and then imported into EI MAVEN where area under the curve for mass to charge (m/z) values matching retention time of an in-house curated lipid library were integrated with a mass error of less than 5 ppm. Ion intensities from integration and retention time data was analyzed in Excel and data visualizations were performed in R studio.

Gas chromatography-fatty acid methyl ester (GC-FAME) analysis

GC-FAME analysis was performed as previously described [1]. Briefly, *E. faecalis* OG1RF wild-type, $\Delta tesS$, $\Delta fakB1,2,5$ and $\Delta fakB1,2,4,5/\Delta tesS$ were grown as indicated until mid-log phase ($OD_{600} = 0.3-0.4$). Aliquots (10 mL) were harvested. Cell pellets were washed with 1XPBS twice to remove the media. The cell pellets were then stored at -80°C prior to gas chromatography fatty acid methyl ester analysis (GC-FAME), which was performed by Microbial ID, Inc (Newark, DE).

Transmission electron microscopy

Transmission electron microscopy sample preparation and analysis was performed as follows. Briefly, OG1RF wild-type (100 mL) and $\Delta fakB1,2,5$ (150 mL) cultures were grown in BHI as described above until mid-logarithmic phase ($OD_{600} = 0.35-0.4$) and harvested by centrifugation (2379 $\times g$ for 10 min). Cell pellets were washed twice with 1X PBS to remove the growth media, pelleted (2379 $\times g$ for 10 min) and resuspended in 1.5 mL 1X PBS containing 3% glutaraldehyde (Electron Microscopy Sciences). Primary fixation was allowed to occur at room temperature for two h. Following primary fixation, the cells were washed with 1XPBS three times, then pelleted (2379 $\times g$ for 10 min) and resuspended in 1.5 mL 1XPBS and stored overnight at 4°C . The following day, the cells were washed once with 1XPBS and resuspended in 600 μL 1XPBS containing 2% osmium tetroxide. Secondary fixation was performed at room temperature for a minimum of 2 h. Following secondary fixation, the cells were washed three times with water, pelleted (16,200 $\times g$ for

5 min) and dehydrated with a graded series of ethanol (15 min incubations each with 1.5 mL ethanol at 25%, 50%, and 70% ethanol). The partially dehydrated cells were stored overnight in 70% ethanol at 4°C. The following day, dehydration was completed as above, using 1mL of 95%, 100%, and 100% ethanol. Cells were stored overnight at 4°C in 100% ethanol. Following dehydration with ethanol, the cells were pelleted (16,200xg for 5 min) and incubated for 15 min with 100% acetone (Electron Microscopy Sciences) twice. The cells were then mixed with 3:1 acetone:resin (Embed 812, Electron Microscopy Sciences) and incubated for a minimum of 2 h at room temperature. This step was repeated with 2:1 acetone:resin and 1:1 acetone:resin for 1 h each. The samples were incubated overnight in a vacuum chamber to remove air bubbles and acetone. The following day, cells were pelleted (16,200xg for 5 min) and transferred to 1 mL 100% resin and incubated for 3 h at room temperature. This treatment was repeated with a 1 h incubation, followed by centrifugation and resuspension in fresh 100% resin. The cells were then transferred to Beem® capsules and pelleted (16,200xg for 5 min), then incubated at 68°C for a minimum of 24 h.

Embedded samples were sectioned and placed onto a copper grid. Grids were stained with UranylLess (Electron Microscopy Sciences) for 30 min, washed with water, then stained with lead citrate for an additional 30 min and washed with water prior to imaging on the JEOL JEM 1400-Flash at the Advanced Microscopy and Imaging Center at the University of Tennessee Knoxville campus.

Results

Identification of Staphylococcus aureus FakA and FakB2 homologs

Previous work has demonstrated that growth in exogenous fatty acids altered the membrane composition, affecting both fatty acid tails and polar head group composition of *E. faecalis* OG1RF [1, 18, 26-29]. We wanted to test if *E. faecalis* OG1RF utilizes the FakA/B system as described in *Staphylococcus aureus* [12] and *Streptococcus pneumoniae* [11] for incorporation of fatty acids into membrane phospholipids. In both *S. aureus* and *S. pneumoniae*, FakB1 specifically binds saturated fatty acids and FakB2

binds monounsaturated fatty acids [11, 12]. However, in *S. pneumoniae*, a third FakB protein (FakB3) binds to polyunsaturated fatty acids [11]. Following fatty acid binding, a FakA enzyme phosphorylates the fatty acid, which can then be utilized in the fatty acid biosynthetic pathway or to form phospholipids (Figure 3.1; [12]). Cuypers *et al.*, 2019 noted four possible FakB homologs in *E. faecalis* V583 [13]. Herein, we used BLAST to identify homologs of the Fak proteins in *E. faecalis* OG1RF. We identified a single homolog of FakA (OG1RF_RS12165) and four homologs of the FakB proteins of *S. aureus* and *S. pneumoniae* (OG1RF_RS00080, OG1RF_RS07200, OG1RF_RS05020, and OG1RF_RS06660).

Given the specificity of the FakB proteins for different fatty acid types, we compared each predicted OG1RF homolog to those of *S. aureus* and *S. pneumoniae* in an attempt to predict the specificity of the enterococcal homologs (Table 3.1). However, the protein identities and positive substitutions did not lead to any clear conclusions as to potential specificity. For example, OG1RF_RS05020 has 28% and 51% identity and similarity to saFakB1, but also has 29% and 51% identity and similarity to saFakB2. Predicted protein structures (Phyre²; [19]) also did not lead to specificity hypotheses for the *E. faecalis* FakB proteins (Supplemental Figure 3.1), and known residues important for fatty acid binding are conserved in each of the *E. faecalis* FakB homologs (Supplemental Figure 3.2). However, given that OG1RF_RS06660 shared the highest percent identity and positive substitutions with saFakB1 and spFakB1, we named it efFakB1 (for *E. faecalis* FakB1). Similarly, we named OG1RF_RS07200 as efFakB2, as it shared the highest percent identity and positive substitutions with saFakB2 and spFakB2. The remaining two proteins, OG1RF_RS00080 and OG1RF_RS05020, neither of which shared significant homology with spFakB3, we named as efFakB4 and efFakB5, respectively.

Combined deletion of fakB genes impacts growth in specific fatty acids

In previous studies, it was noted that the addition of fatty acids present in bile and serum impact the growth of *E. faecalis*. Specifically, the polyunsaturated fatty acid linoleic acid (C_{18:2 cis9,12}), the monounsaturated fatty acid palmitoleic acid (C_{16:1 cis9}), and saturated fatty

Table 3.1. Percent identity and positive substitutions of *Enterococcus faecalis* FakB proteins to *Staphylococcus aureus* and *Streptococcus pneumoniae* FakB proteins as determined by NCBI BLAST.

<i>E. faecalis</i> FakB loci and proteins	Percent identity	Percent positive substitutions
<i>S. aureus</i> FakB1 (saturated specificity)		
OG1RF_RS06660 (FakB1)	40%	60%
OG1RF_RS05020 (FakB5)	28%	51%
OG1RF_RS07200 (FakB2)	26%	51%
OG1RF_RS00080 (FakB4)	26%	50%
<i>S. aureus</i> FakB2 (unsaturated specificity)		
OG1RF_RS07200 (FakB2)	37%	58%
OG1RF_RS06660 (FakB1)	33%	55%
OG1RF_RS00080 (FakB4)	31%	53%
OG1RF_RS05020 (FakB5)	29%	51%
<i>S. pneumoniae</i> FakB1 (saturated specificity)		
OG1RF_RS06660 (FakB1)	41%	61%
OG1RF_RS05020 (FakB5)	27%	49%
OG1RF_RS00080 (FakB4)	27%	48%
<i>S. pneumoniae</i> FakB2 (monounsaturated specificity)		
OG1RF_RS07200 (FakB2)	54%	73%
OG1RF_RS06660 (FakB1)	27%	50%
OG1RF_RS00080 (FakB4)	26%	49%
<i>S. pneumoniae</i> FakB3 (polyunsaturated specificity)		
OG1RF_RS07200 (FakB2)	20%	46%

acids palmitic acid (C_{16:0}), and stearic acid (C_{18:0}) caused increased generation time of OG1RF relative to growth in media supplemented with the solvent control (ethanol) [18]. We decided to investigate whether deletion of the *fakB* genes alters growth in these specific fatty acids, as this could give insights into the specificity for the four predicted FakB proteins. Given the possible redundancy of the protein functions, we generated single, double, and triple *fakB* deletion strains. However, when we attempted to make a quadruple *fakB* deletion strain we were unable to do so unless *tesS* was also deleted (see below).

When we grew the triple deletion strain $\Delta fakB1,2,4$ in rich media (BHI) containing solvent control, we found there was an approximate 8 min increase in generation time relative to wild-type (Table 3.2; Supplemental Figure 3.3). Note that there are no fatty acids detected in BHI at the levels of detection via GC-FAME [1]. When medium was supplemented with linoleic acid (polyunsaturated; C_{18:2 cis9,12}), $\Delta fakB1,2,4$ had a 20-min higher generation time compared to the wild-type strain (Table 3.3: $\Delta fakB1,2,4$, 86 min; wild-type, 66 min; $P = 0.04$). Similarly, supplementation of $\Delta fakB1,2,4$ with palmitoleic acid (C_{16:1 cis9}) or oleic acid (C_{18:1 cis9}) resulted in a generation time approximately 20 and 30 min slower, respectively, than that of the wild-type strain in the same fatty acid (Table 3.3). No difference was observed in generation time for growth in saturated fatty acids C_{16:0} and C_{18:0} (data not shown).

Growth of $\Delta fakB1,4,5$ in unsupplemented media or in media containing the fatty acid solvent control was similar to the wild-type strain (Table 3.2, Supplemental Figure 3.3). However, when supplied with stearic acid (C_{18:0}), $\Delta fakB1,4,5$ had a generation time nearly double that of wild-type (Table 3.4: 185.9 min for $\Delta fakB1,4,5$ compared to 82 min for wild-type; $P = 0.03$). Interestingly, we observed no difference in growth in palmitic acid (C_{16:0}) or any unsaturated fatty acids examined (data not shown).

To pinpoint which specific combinations of *fakB* deletions were responsible for these phenotypes, we grew related single and double gene deletion strains in the same media and found that the combined deletion of *fakB1* and *fakB2* ($\Delta fakB1,2$) had slower growth in both solvent control and in media supplemented with unsaturated fatty acids, as

Table 3.2. Generation times of *E. faecalis* strains grown in unsupplemented media and in the fatty acid solvent control.

P-values for each deletion strain compared to wild-type OG1RF calculated using Welch's T-test. White cells indicate no difference and red indicates a significant increase in generation time of the given strain compared to wild-type OG1RF. *n* = 3 replicates.

Media ^a Supplement and Concentration ^b	Strain	Generation Time (Minutes)	P-values against OG1RF
No supplement	OG1RF	32.3 ± 0.5	NA
	$\Delta tesS$	31.8 ± 1.6	0.75
	$\Delta fakB1,2,5$	36.3 ± 1.2	0.03
	$\Delta fakB1,2,5/\Delta tesS$	36.2 ± 1.0	0.02
	$\Delta fakB1,2,4,5/\Delta tesS$	31.2 ± 0.4	0.09
Ethanol ^c	OG1RF	37.6 ± 0.5	NA
	$\Delta fakB1$	39.1 ± 0.8	0.10
	$\Delta fakB2$	40.7 ± 0.7	0.01
	$\Delta fakB4$	38.6 ± 1.2	0.40
	$\Delta fakB5$	39.9 ± 0.4	P < 0.01
	$\Delta fakB1,2$	42.4 ± 0.0	P < 0.01
	$\Delta fakB1,5$	37.3 ± 2.8	0.88
	$\Delta fakB2,4$	38.1 ± 0.8	0.49
	$\Delta fakB4,5$	31.2 ± 5.4	0.23
	$\Delta fakB1,2,4$	45.5 ± 0.8	P < 0.01
	$\Delta fakB1,2,5$	46.2 ± 1.4	P < 0.01
	$\Delta fakB1,4,5$	36.9 ± 2.4	0.72
	$\Delta tesS$	38.5 ± 0.3	0.12
	$\Delta fakB21,2,5/\Delta tesS$	46.5 ± 0.7	P < 0.01
	$\Delta fakB1,2,4,5/\Delta tesS$	39.5 ± 1.0	0.09

^aBrain heart infusion (BHI)

^bEthanol: 0.1%

^cEthanol is the solvent control for fatty acid supplements

Table 3.3. Generation times of *E. faecalis* strains in unsaturated fatty acids.

P-values for each deletion strain compared to wild-type OG1RF calculated using Welch's T-test. White cells indicate no difference, blue indicates a significant decrease in generation time, and red indicates a significant increase in generation time of the given strain compared to wild-type OG1RF. *n* = 3 replicates, with the exception of OG1RF wild-type, which was *n* = 6 replicates.

Media ^a Supplement and Concentration ^b	Strain	Generation Time (Minutes)	<i>P</i> -values against OG1RF
C _{18:2} <i>cis</i> _{9,12} (linoleic acid)	OG1RF	66.0 ± 15.2	NA
	$\Delta fakB1$	57.4 ± 5.4	0.27
	$\Delta fakB2$	49.7 ± 11.1	0.06
	$\Delta fakB4$	58.3 ± 13.3	0.35
	$\Delta fakB1,2$	86.4 ± 5.7	0.05
	$\Delta fakB2,4$	51.9 ± 5.4	0.09
	$\Delta fakB1,2,4$	86.0 ± 5.8	0.04
	$\Delta fakB1,2,5$	135.3 ± 9.6	<i>P</i> < 0.01
	$\Delta tesS$	81.4 ± 8.9	0.15
	$\Delta fakB1,2,5/\Delta tesS$	118.6 ± 2.4	<i>P</i> < 0.01
	$\Delta fakB1,2,4,5/\Delta tesS$	75.7 ± 1.4	0.22
C _{16:1} <i>cis</i> ₉ (palmitoleic acid)	OG1RF	41.3 ± 0.5	NA
	$\Delta fakB1$	42.9 ± 4.92	0.69
	$\Delta fakB2$	38.6 ± 2.2	0.22
	$\Delta fakB4$	40.2 ± 1.6	0.44
	$\Delta fakB1,2$	55.2 ± 3.9	0.04
	$\Delta fakB2,4$	42.6 ± 3.7	0.68
	$\Delta fakB1,2,4$	62.9 ± 4.6	0.02

^aBrain heart infusion (BHI)

^bLinoleic acid: 10 µg/mL; Palmitoleic acid: 10 µg/mL; Oleic acid: 20 µg/mL

Table 3.3 Continued

Media ^a Supplement and Concentration ^b	Strain	Generation Time (Minutes)	<i>P</i> -values against OG1RF
C _{18:1} <i>cis</i> 9 (oleic acid)	OG1RF	37.9 ± 0.6	NA
	$\Delta fakB1$	35.5 ± 1.5	0.14
	$\Delta fakB2$	35.3 ± 0.7	0.01
	$\Delta fakB4$	36.4 ± 1.7	0.35
	$\Delta fakB1,2$	58.2 ± 3.0	0.008
	$\Delta fakB2,4$	35.9 ± 0.9	0.07
	$\Delta fakB1,2,4$	67.1 ± 4.9	0.01
	$\Delta fakB1,2,5$	92.6 ± 4.9	P < 0.01
	$\Delta tesS$	37.5 ± 1.1	0.71
	$\Delta fakB1,2,5/\Delta tesS$	85.8 ± 2.7	P < 0.01
	$\Delta fakB1,2,4,5/\Delta tesS$	37.9 ± 8.6	1.0

^aBrain heart infusion (BHI)^bLinoleic acid: 10 µg/mL; Palmitoleic acid: 10 µg/mL; Oleic acid: 20 µg/mL

Table 3.4. Generation times of *E. faecalis* strains in saturated fatty acids.

P-values for each deletion strain compared to wild-type OG1RF calculated using Welch's T-test. White cells indicate no difference, blue indicates a significant decrease in generation time, and red indicates a significant increase in generation time of the given strain compared to wild-type OG1RF. *n* = 3 replicates, with the exception of OG1RF wild-type, which was *n* = 6 replicates.

Media ^a Supplement and Concentration ^b	Strain	Generation Time (Minutes)	P-values against OG1RF
C _{18:0} (Stearic acid) ^{b1}	OG1RF	82.0 ± 12.5	NA
	$\Delta fakB1$	107.3 ± 16.0	0.137
	$\Delta fakB4$	84.8 ± 5.1	0.68
	$\Delta fakB5$	107.0 ± 8.3	0.02
	$\Delta fakB1,5$	162.0 ± 10.8	P < 0.01
	$\Delta fakB4,5$	94.4 ± 13.7	0.34
	$\Delta fakB1,2,5$	60.3 ± 6.8	0.02
	$\Delta fakB1,4,5$	185.9 ± 28.1	0.03
	$\Delta tesS$	63.0 ± 7.2	0.04
	$\Delta fakB1,2,5/\Delta tesS$	62.3 ± 11.2	0.11
	$\Delta fakB1,2,4,5/\Delta tesS$	37.9 ± 1.4	P < 0.01
C _{16:0} (Palmitic acid) ^{b1}	$\Delta fakB1,2,4,5/\Delta tesS$	43.8 ± 0.9	NA ^c
C _{14:0} (Myristic acid)	$\Delta fakB1,2,4,5/\Delta tesS$	44.6 ± 1.3	NA ^c
C _{16:0} (Palmitic acid) ^{b2}	$\Delta fakB1,2,5$	120.2 ± 10.6	NA ^c
	$\Delta fakB1,2,5/\Delta tesS$	115.7 ± 16.1	NA ^c
	$\Delta fakB1,2,4,5/\Delta tesS$	40.8 ± 1.7	NA ^c

^aBrain heart infusion (BHI)

^{b1}Stearic acid: 20 µg/mL; Palmitic acid: 5 µg/mL; Myristic acid: 5 µg/mL

^{b2}Palmitic acid: 2.5 µg/mL

^cThe fatty acids at these concentrations caused wild-type *E. faecalis* to reach an early growth stasis followed by the outgrowth of a suppressor, as previously reported [1]. This resulted in inability to calculate a generation time; thus P-values were not calculated.

observed in the $\Delta fakB1,2,4$ strain (Tables 3.2 and 3.3, Supplemental Figure 3.3). Additionally, $\Delta fakB1,5$ had an increased generation time in stearic acid ($C_{18:0}$), as observed in the $\Delta fakB1,4,5$ strain (Table 3.4). Taken together, these phenotypes indicate a possible function for FakB1 as a generalist fatty acid binding protein, with potential roles for FakB2 and FakB5 in specific uptake of either unsaturated or saturated fatty acids, respectively.

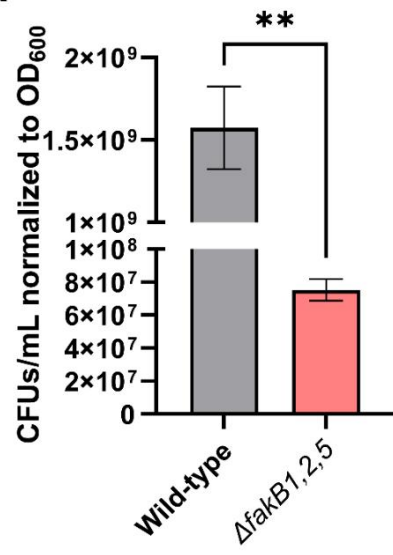
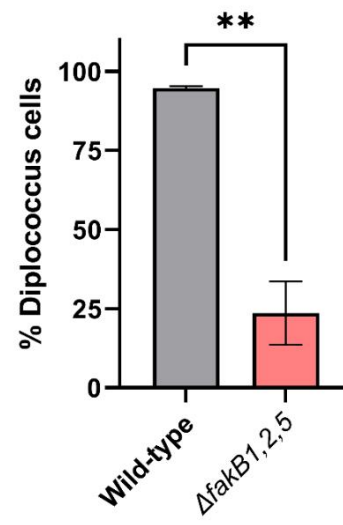
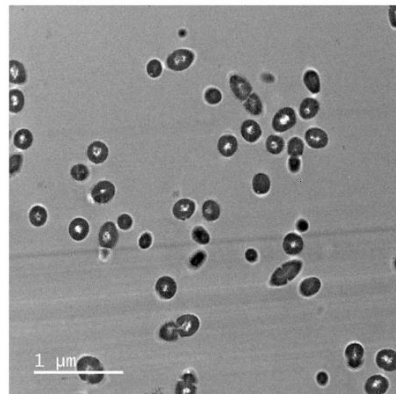
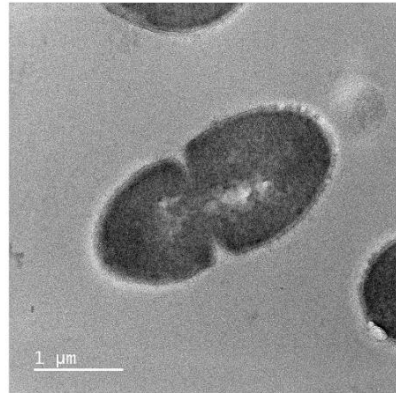
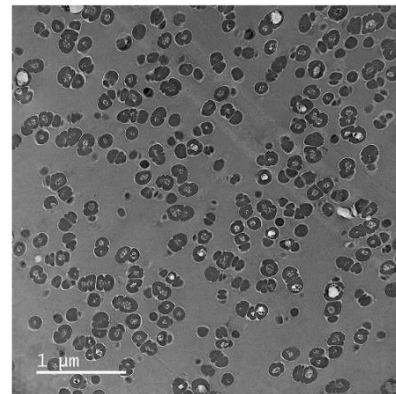
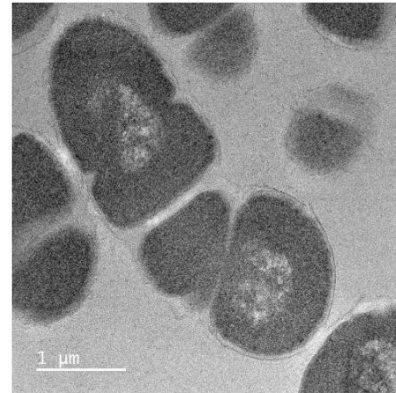
Combined deletion of *fakB1*, *fakB2*, and *fakB5* leads to growth defects

Given that $\Delta fakB1,2$ and $\Delta fakB1,5$ grew poorly in media supplemented with unsaturated or saturated fatty acids, respectively (Tables 3.3 and 3.4), we examined a strain deleted for *fakB1*, *fakB2*, and *fakB5* ($\Delta fakB1,2,5$). This strain had a significantly increased generation time compared to the wild-type strain in the presence of the solvent control, a phenotype observed in $\Delta fakB1,2,4$ but not $\Delta fakB1,4,5$ (Table 3.2). The phenotype did not initially appear to be caused by a generalized growth defect, as growth in BHI was equivalent to the wild-type strain (Table 3.2). Yet, we noted that for a given OD₆₀₀ value, the $\Delta fakB1,2,5$ strain had approximately 10-fold fewer CFUs relative to wild-type when grown in BHI media lacking any additional supplements (Figure 3.2A; $P = 0.001$). Given these combined data, along with the observation that cells in overnight cultures tended to clump towards the bottom of the tube (data not shown), we examined the cellular morphology using transmission electron microscopy (TEM). As shown in Figures 3.2B and 3.2C, wild-type OG1RF possesses an elongated diplococcus morphology when grown in rich media. However, $\Delta fakB1,2,5$ cells were very irregular in shape, size, with clear hyperseptation relative to the wild-type control (Figures 3.2B and 3.2D). Indeed, only a small percentage of the $\Delta fakB1,2,5$ cells were diplococci (Figure 3.2B; $P = 0.009$). Note that due to an error in image gathering, the scale bars may not be accurate in the TEM images.

Growth of $\Delta fakB1,2,5$ in media supplemented with unsaturated fatty acids also resulted in increased generation time. Comparison of the growth of $\Delta fakB1,2,5$ and $\Delta fakB1,2,4$ in media with linoleic acid revealed that $\Delta fakB1,2,5$ had an approximately 50

Figure 3.2. $\Delta fakB1,2,5$ has reduced CFUs/mL at exponential phase compared to wild-type *E. faecalis*.

(A) Wild-type *E. faecalis* and $\Delta fakB1,2,5$ were grown in plain BHI until mid-logarithmic phase ($OD_{600} \sim 0.3$), and colony forming units (CFUs) were enumerated. Shown are the average CFUs/mL normalized to $OD_{600} \pm$ standard deviations for $n = 3$ replicates. (B) Transmission electron microscopy was performed on wild-type *E. faecalis* and $\Delta fakB1,2,5$ grown to mid-logarithmic phase. Shown are the average percentages of diplococci cells $\pm$ standard deviations for a total of $n = 100$ cells measured across $n = 3$ images. Representative wild-type (C) and $\Delta fakB1,2,5$ (D) electron microscopy images are shown. Note that the scale bars may not be accurate. ** $P = 0.01$ – 0.001 .

A**B****C****Wild-type****D** **Δ fakB1,2,5**

min increase in generation time relative to $\Delta fakB1,2,4$ (Table 3.3; $P < 0.01$). Further, growth of $\Delta fakB1,2,5$ and $\Delta fakB1,2,4$ in oleic acid revealed that $\Delta fakB1,2,5$ had an approximately 25 min increase in generation time relative to $\Delta fakB1,2,4$ (Table 3.3; $P < 0.01$).

When compared to wild-type, the generation time of $\Delta fakB1,2,5$ in stearic acid did not significantly increase (in fact, generation time decreased; Table 3.4). Additionally, we were surprised to observe that when grown in stearic acid, the generation time of $\Delta fakB1,2,5$ was significantly lower than that of $\Delta fakB1,5$ in stearic acid (Table 3.4; $P = 0.02$). This is corroborated by the data where we observed that the generation time of $\Delta fakB1,2,5$ was significantly lower than that of $\Delta fakB1,4,5$ (Table 3.4; $P = 0.02$). As deletion of *fakB2* rescued the growth effects observed in $\Delta fakB1,5$, it seems likely that an overlap in protein specificities may cause substrate competition dynamics, which drive the increased generation time phenotype stearic acid observed in $\Delta fakB1,5$, as well as its subsequent rescue in the quintuple deletion strain (see Discussion).

Combined deletion of fakB1, fakB2, and fakB5 leads to altered cellular morphology and accumulation of free fatty acids in the membrane

Interestingly, similar morphological issues as seen in $\Delta fakB1,2,5$ were observed on supplementing wild-type cells with the saturated fatty acids stearic acid (C_{18:0}) or palmitic acid (C_{16:0}) or myristic acid (C_{14:0}) [18]. Further, data suggests that when supplemented with fatty acids, those specific supplied fatty acids accumulate as free fatty acids within the membrane of OG1RF [26, 29]. Given the morphological similarities of $\Delta fakB1,2,5$ to that of wild-type OG1RF grown in saturated fatty acids [18], we hypothesized that the phenotypes observed were due to the accumulation of free fatty acids in the cells. We thus performed TLC to separate free fatty acids from the major phospholipids. As lipid extracts are not easily quantifiable, we normalized the free fatty acid signal to that of the major lipid phosphatidylglycerol (PG) [26, 29, 30] and found that $\Delta fakB1,2,5$ had increased levels of free fatty acids relative to wild-type (Figure 3.3A; $P < 0.001$). As TLC could not separate different free fatty acids from one another, to determine which specific

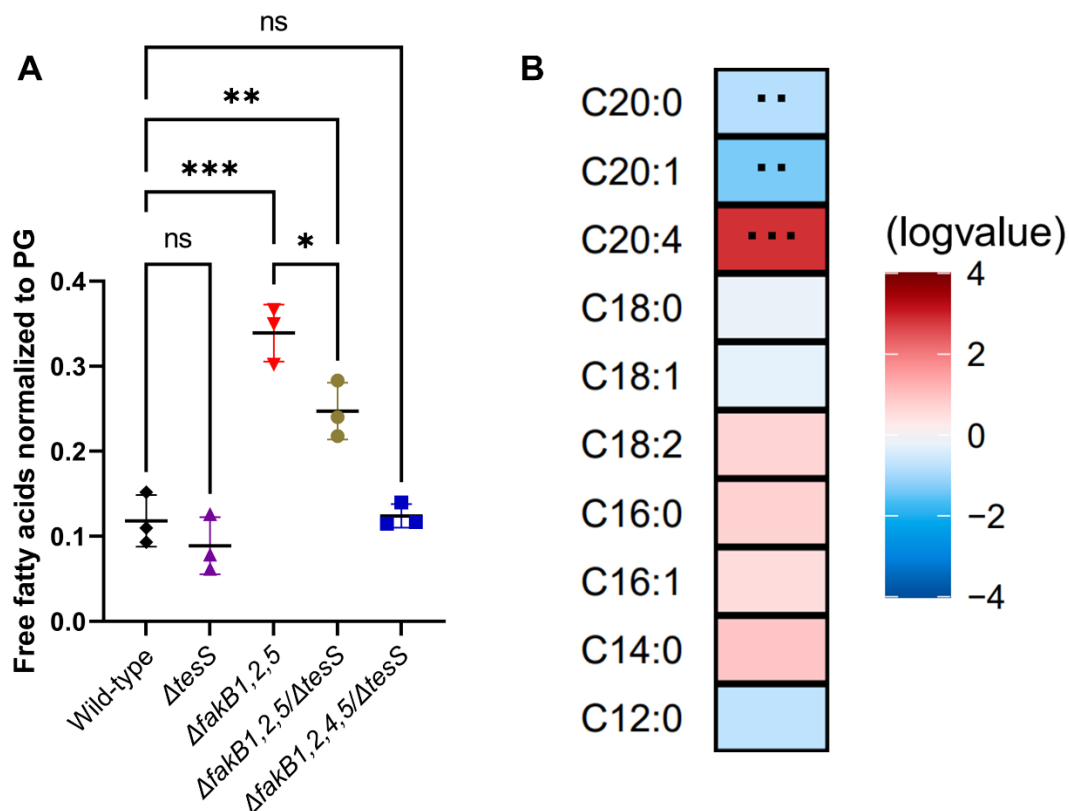


Figure 3.3. Deletion of *fakB* genes leads to accumulation of free fatty acids.

(A) Thin layer chromatography (TLC) was performed on lipids extracted from indicated strains grown in unsupplemented BHI (see Materials and Methods). Depicted are the levels of free fatty acids normalized to phosphatidylglycerol (PG) detected by TLC. One way ANOVA and Tukey's multiple comparisons tests were used to determine the differences between means for $n = 3$ replicates. $*P = 0.05-0.01$; $**P = 0.01-0.001$; $***P \leq 0.001$; $^{ns} P > 0.05$ (B) Mass spectrometry lipid analysis as described in Materials and Methods was used to determine relative levels of free fatty acids in $\Delta fakB1,2,5$ compared to wild-type OG1RF. Depicted are the log-transformed fold changes of free fatty acids in $\Delta fakB1,2,5$ compared to wild-type OG1RF for $n = 5$ replicates. Data represented is normalized to OD₆₀₀ at time of harvest. $*P = 0.05-0.01$; $**P = 0.01-0.001$; $***P \leq 0.001$.

free fatty acids were increased in $\Delta fakB1,2,5$, we performed an ultra-high performance liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) analysis on the lipids of wild-type *E. faecalis* and the $\Delta fakB1,2,5$ strain (Figure 3.3B). We observed that in $\Delta fakB1,2,5$ C_{14:0} was increased relative to wild-type by ~2-fold, C_{16:0} was increased by ~1.6-fold relative to wild-type, and that C_{20:0} and C_{20:1} were decreased by 2.5-fold and 1.7-fold, respectively (Figure 3.3B). We also observed an increase a mass corresponding to the *m/z* ratio of C_{20:4}, a lipid which OG1RF is not known to produce. However, a lipid standard was not used to confirm the presence of C_{20:4}.

To validate the UHPLC-HRMS data, we performed a gas chromatography fatty acid methyl ester (GC-FAME) analysis of the lipids present within select deletion strains. Of note, the fatty acids present in the cell globally, regardless of being attached to phospholipid head groups, as detected by GC-FAME, were also altered in the $\Delta fakB1,2,5$ deletion strain (Table 3.5). Most notably, we observed an ~7% increase in palmitic acid (C_{16:0}) in the $\Delta fakB1,2,5$ strain ($P = 0.002$), combined with an ~14% decrease in *cis*-vaccenic acid (C_{18:1 cis11}; $P < 0.001$) and a ~1% decrease in stearic acid (C_{18:0}; $P = 0.002$; Table 3.5; Supplemental Table 3.3). These data matched the overall trends observed *via* UHPLC-HRMS (Figure 3.3B). Interestingly, we also observed that $\Delta fakB1,2,5$ had a significant increase in the ratio of saturated fatty acids in its membrane: while wild-type had a nearly 1:1 ratio of saturated to unsaturated fatty acids, $\Delta fakB1,2,5$ had a 2:1 ratio of saturated to unsaturated fatty acids in its membrane (Table 3.5, Supplemental Table 3.3). These data indicate that combined deletion of *fakB1*, *fakB2*, and *fakB5* alters lipid metabolism even in the absence of supplied fatty acids.

Deletion of tesS rescues negative growth effects of the fakB deletion strains and reduces accumulation of free fatty acids

Given the above combined data, including the increase in free fatty acids in $\Delta fakB1,2,5$ relative to wild-type, we hypothesized that the activity of TesS may be contributing to these observations. TesS is a thioesterase that will cleave fatty acids from the acyl carrier protein; normally, these fatty acids are bound and phosphorylated *via* the Fak system

Table 3.5. GC-FAME analysis of strains during log phase growth in BHI.

Shown are the averages $\pm$ standard deviations for $n = 3$ replicates. ND indicates that the fatty acid indicated was not detected for the given strain.

Fatty acid	% of total membrane content for indicated strain (avg $\pm$ SD)			
	Wild-type	$\Delta tesS$	$\Delta fakB1,2,5$	$\Delta fakB1,2,4,5/\Delta tesS$ (quintuple)
C _{12:0}	0.9 $\pm$ 0.0	0.9 $\pm$ 0.1	0.9 $\pm$ 0.1	0.1 $\pm$ 0.2
C _{14:0}	4.3 $\pm$ 0.1	5.5 $\pm$ 0.2	5.4 $\pm$ 0.1	5.5 $\pm$ 0.0
C _{16:1}	5.7 $\pm$ 0.0	6.7 $\pm$ 0.24	4.8 $\pm$ 0.1	6.6 $\pm$ 0.1
C _{16:0}	41.4 $\pm$ 0.7	38.8 $\pm$ 0.1	48.4 $\pm$ 0.1	37.2 $\pm$ 0.7
C _{17:1} <i>cis10</i>	1.0 $\pm$ 0.8	0.9 $\pm$ 0.8	3.1 $\pm$ 0.1	2.3 $\pm$ 0.2
C _{17:0} 2OH	4.4 $\pm$ 0.1	4.6 $\pm$ 0.3	5.8 $\pm$ 0.1	6.1 $\pm$ 0.2
C _{18:1} <i>cis11</i>	37.0 $\pm$ 0.5	36.3 $\pm$ 0.3	22.7 $\pm$ 0.4	37.0 $\pm$ 0.5
C _{18:0}	4.8 $\pm$ 0.1	4.8 $\pm$ 0.2	3.6 $\pm$ 0.2	3.5 $\pm$ 0.0
C _{19:0} cyclo11	0.6 $\pm$ 0.0	0.7 $\pm$ 0.1	1.6 $\pm$ 0.1	1.4 $\pm$ 0.2
Unknown	ND	0.3 $\pm$ 0.6	1.9 $\pm$ 0.6	0.2 $\pm$ 0.4
Others ^a	ND	0.3 $\pm$ 0.4	0.9 $\pm$ 0.2	0.1 $\pm$ 0.1
Saturated/ Unsaturated ratio	1.3 $\pm$ 0.0	1.3 $\pm$ 0.0	2.1 $\pm$ 0.1	1.2 $\pm$ 0.0

^aOthers are fatty acids which comprised <1% of the total membrane content.

(Figure 3.1) [15]. Loss of multiple *fakB* genes in OG1RF could result in free fatty acid build up due to continued activity of TesS. Thus, we deleted *tesS* in the $\Delta fakB1,2,5$ strain. After deleting *tesS*, we were able to delete all of the *fakB* genes, generating the $\Delta fakB1,2,4,5/\Delta tesS$ strain (referred to as the “quintuple deletion strain”).

Notably, some of the lipid changes observed in $\Delta fakB1,2,5$, were reversed in the quintuple deletion strain (Figure 3.3, Table 3.5). Indeed, with regards to palmitic acid ($C_{16:0}$), the quintuple deletion strain had a significant reduction relative to wild-type ($P = 0.002$), while palmitoleic acid ($C_{16:1}$) was significantly increased in the quintuple deletion strain relative to wild-type ($P = 0.007$; Table 3.5 and Supplemental Table 3.3). However, stearic acid ($C_{18:0}$) was reduced in the quintuple deletion strain relative to wild-type ($P = 0.002$; Table 3.5 and Supplemental Table 3.3), similar to what was observed for $\Delta fakB1,2,5$. These trends indicate that the Fak system is necessary for *de novo* fatty acid biosynthesis and phospholipid production when TesS is active. However, on deletion of *tesS*, the cells are able to return closer to a wild-type membrane makeup.

Having observed the restoration of free fatty acid levels in the quintuple deletion strain in the absence of fatty acids, we next determined its growth phenotypes in media supplemented with specific fatty acids. We observed that the quintuple deletion strain had generation times in unsaturated fatty acids similar to wild-type (Table 3.3).

Wild-type *E. faecalis*, as well as other bacterial strains, can overcome inhibition by cerulenin, an inhibitor of *de novo* fatty acid biosynthesis, if supplied with specific fatty acid(s) in its growth media [7, 18]. This is likely because the Fak system can utilize the supplied fatty acids to generate the acyl-phosphates required for lipid synthesis (Figure 3.1). To confirm that the quintuple deletion strain ($\Delta fakB1,2,4,5/\Delta tesS$) could no longer utilize exogenous fatty acids to support lipid synthesis, we examined its growth when treated with cerulenin, an inhibitor of *de novo* fatty acid biosynthesis (and hence membrane synthesis). Like the wild-type strain, growth was inhibited by cerulenin (Figure 3.4A). However, whereas the addition of oleic acid could prevent cerulenin-induced growth inhibition of wild-type OG1RF, it could not for the quintuple deletion strain (Figure 3.4A).

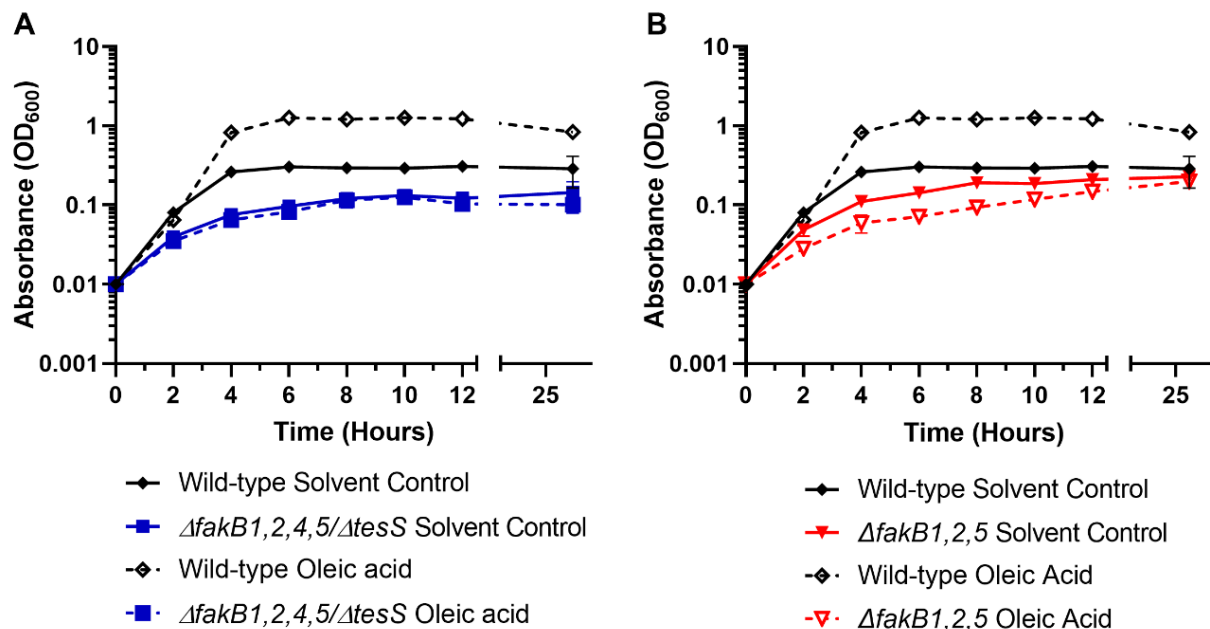


Figure 3.4. Oleic acid does not rescue growth inhibition by cerulenin in the $\Delta fakB1,2,4,5/tesS$ and $\Delta fakB1,2,5$ deletion strains.

Wild-type *E. faecalis* OG1RF, (A) $\Delta fakB1,2,4,5/\Delta tesS$, and (B) $\Delta fakB1,2,5$ were grown in BHI containing 5 $\mu\text{g/mL}$ cerulenin. The cultures were supplemented with 5 $\mu\text{g/mL}$ oleic acid or an equivalent volume of solvent (see Materials and Methods for full details). Represented are average OD₆₀₀ values $\pm$ standard deviations for $n = 3$ replicates.

This indicated that the presence of the *fakB* and *tesS* genes are required for utilization of exogenous fatty acids to generate phospholipid precursors.

Combined deletion of tesS and the fakB genes rescues growth in saturated fatty acids

The saturated fatty acids myristic acid (C_{14:0}) and palmitic acid (C_{16:0}) negatively impact cellular growth and result in severely distorted morphology of *E. faecalis* [18]. Indeed, growth in either fatty acid when supplemented at 5 µg/ml occurs only upon outgrowth of a genetic suppressor (mutant) cell [18]. As the quintuple deletion strain cannot incorporate free fatty acids into phospholipids (Figure 3.4), we examined whether incorporation of free fatty acids is responsible for these negative effects. We thus grew the quintuple deletion strain in media containing either palmitic acid or myristic acid, and observed that in each of these fatty acids, it grew with a generation time of ~44 min, while the other strains were inhibited (Table 3.4, Figure 3.5). Deletion of *tesS* alone was not enough to allow the cells to overcome growth inhibition by myristic acid or palmitic acid (Figure 3.5).

To determine whether the *fakB* genes or *tesS* were primarily responsible for the ability of the quintuple deletion strain to grow unimpeded in myristic acid and palmitic acid, we grew these strains in lower concentrations of palmitic acid. We noted that when grown in 2.5 µg/mL palmitic acid (half the concentration used for Figure 3.5), the $\Delta fakB_{1,2,5}$ and $\Delta fakB_{1,2,5}/\Delta tesS$ strains were able to continue growing (Table 3.4 and Figure 3.6). Generation times of $\Delta fakB_{1,2,5}$ and $\Delta fakB_{1,2,5}/\Delta tesS$ were higher relative to the quintuple deletion strain in 2.5 µg/mL palmitic acid (Table 3.4 and Figure 3.6). Under this condition, the generation time of $\Delta fakB_{1,2,5}$ was 120 min, while the quintuple deletion strain had a generation time of 44 min ($P < 0.01$). Further, the $\Delta fakB_{1,2,5}/\Delta tesS$ deletion strain was approximately 75 min longer than that of the quintuple deletion strain ($P = 0.02$; Table 3.4 and Figure 3.6). These results suggested that the toxicity of palmitic acid in wild-type OG1RF is due to its incorporation into the phospholipid biosynthesis cycle of *E. faecalis*; reduced incorporation due to deletion of the *fakB* genes resulted in ability to grow in these fats.

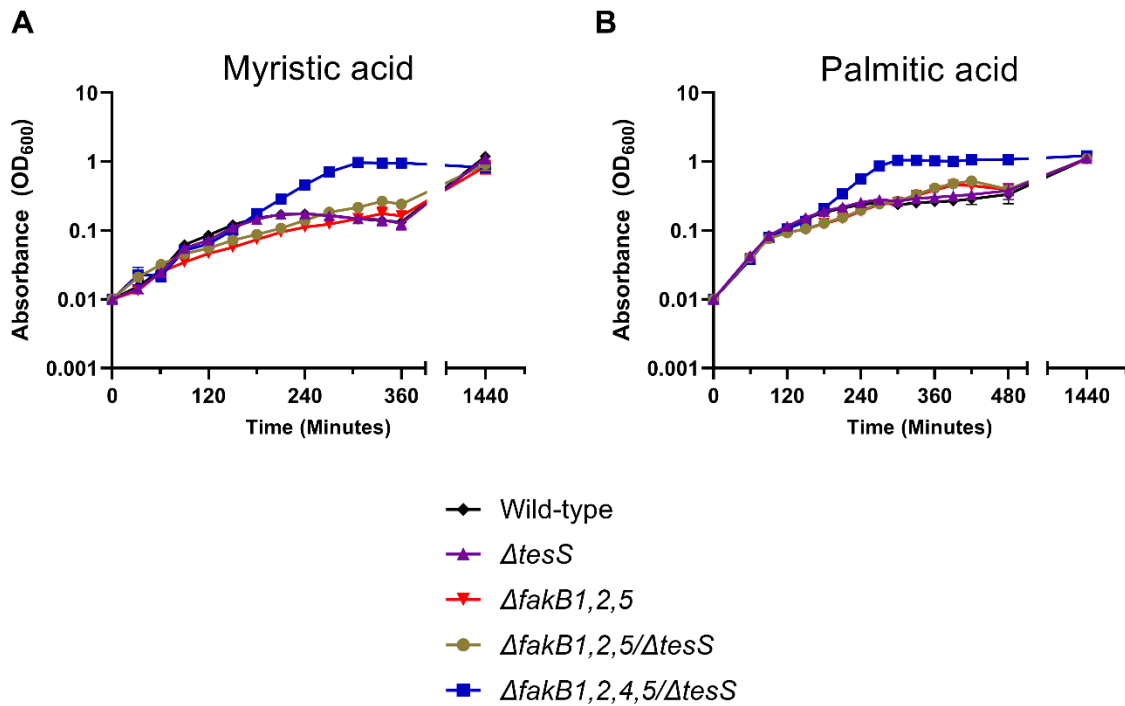


Figure 3.5. The quintuple deletion strain grows unimpeded in the presence of toxic fatty acids.

Indicated strains were grown in media supplemented with (A) 5 $\mu\text{g/mL}$ myristic acid or (B) 5 $\mu\text{g/mL}$ palmitic acid. Shown are the average OD_{600} values over time $\pm$ standard deviations for $n = 3$ replicates.

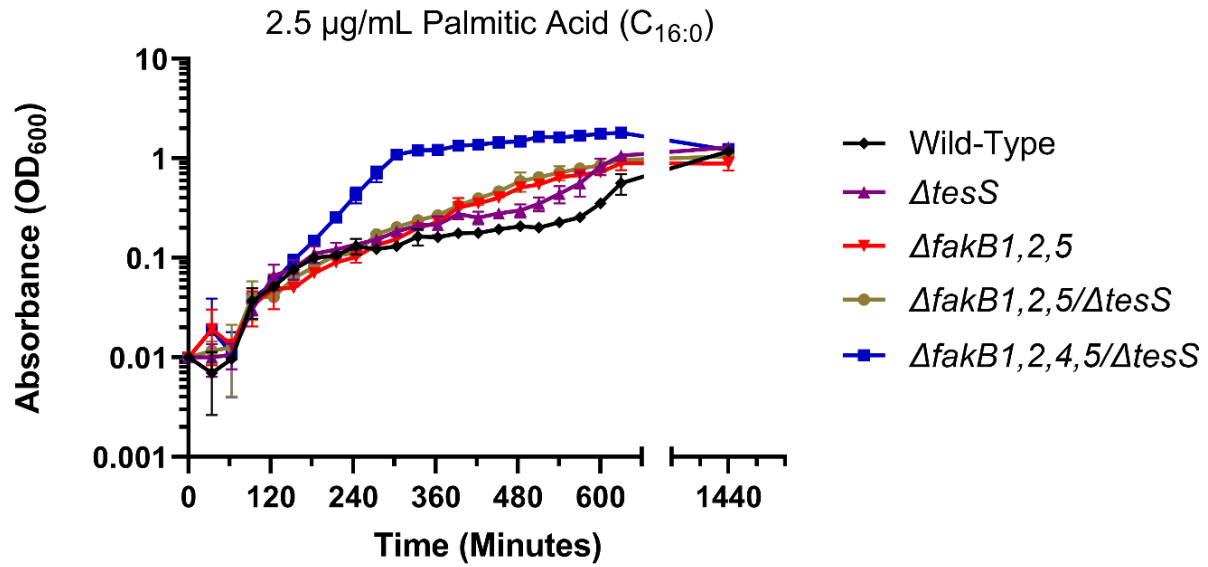


Figure 3.6. Deletion of *fakB1*, *fakB2*, and *fakB5* drive ability of the quintuple deletion strain to grow in palmitic acid.

Indicated strains were grown in media supplemented with 2.5 $\mu\text{g/mL}$ palmitic acid. Shown are the average OD_{600} values over time $\pm$ standard deviations for $n = 3$ replicates.

Discussion

While previous literature has indicated that the primary role for the Fak system is scavenging fatty acids from the environment, we report that in *E. faecalis*, the Fak system is necessary for phospholipid synthesis and proper cellular division. Given that the *fak* genes were deleted from *S. aureus* and *S. pneumoniae* with ease [11, 12], indicating a nonessential role in cell growth, we were surprised when we were unable to do so in *E. faecalis*. Indeed, other researchers attempted to delete the *fakA* homolog in *E. faecalis*, but could only truncate it. The resulting strain carrying truncated *fakA* had altered morphology with multiple septation events [31]. These data indicate that the Fak system plays an essential role in enterococcal physiology. Interestingly, *E. faecalis* has four homologs of the FakBs in *S. aureus* and *S. pneumoniae* – this is possibly due to the need to specifically bind fatty acids or to allow for better regulation under diverse environmental conditions. The ability to bind and incorporate different fatty acids may also explain why different fatty acids are toxic to different Gram-positive organisms. For instance, palmitic acid is toxic to *E. faecalis* [18] but not to *S. aureus* [32] at the concentrations used. Another example is that in *Streptococcus pyogenes*, lauric acid is toxic [33], while this fatty acid is not toxic to *E. faecalis* at the concentrations used [18]. The fatty acid binding specificities of the FakB proteins in these strains may explain the differential effects of fatty acid supplementation.

Given that the *S. pneumoniae* FakB proteins can bind to saturated, monounsaturated, and polyunsaturated fatty acids, it is interesting that enterococci typically have four FakB homologs. Our findings indicated that the FakBs of *E. faecalis* have specific fatty acid binding functions, as well as potential differences in protein expression, stability, or activity. Here, deletion of *fakB1* was necessary, combined with deletion of either *fakB2* (resulting in increased generation time in unsaturated fatty acids) or *fakB5* (resulting in increased generation times in saturated fatty acids) to elicit marked phenotypes in media supplemented with free fatty acids (Tables 3.3 and 3.4). This is interesting, as it indicates that efFakB1 may have a generalist fatty acid binding function which is heretofore unreported in the literature. We also observed that when *fakB4* was

deleted in combination with other *fakBs*, there was no phenotype under the growth conditions tested, yet we were unable to delete *fakB4* in the $\Delta fakB1,2,5$ background. This indicates that while phenotypes were not observed on deletion of *fakB4*, efFakB4 has a function. Notably, the models generated by Phyre² [19] did not reveal any predicted differences in protein folding or in the shape of the fatty acid binding pocket (Supplemental Figure 3.1). Additionally, residues important for fatty acid binding [22] were conserved in each of the efFakB protein sequences (Supplemental Figure 3.2). Given this, it is possible that the expression, activity, or stability may be different for efFakB4 (likely lower given our phenotypic evidence) or FakB1 (likely higher given our phenotypic evidence). Due to our lack of data on the efFakB proteins, additional detailed biochemical analyses are warranted to measure the binding specificities, activity, stability, and expression of FakB4 and FakB1.

While we noted that deletion of *fakB1* alone had no measurable impacts, deletion of *fakB2* and *fakB5* each had an effect when combined with deletion of *fakB1*. This suggested that efFakB1 plays a role as a generalist fatty acid binding protein. These effects were seemingly driven by the presence of free fatty acids, which, whether present due to activity or expression of TesS, or due to supplementation in the media, likely have pleiotropic effects on the cell. Free fatty acids have been demonstrated to impact membrane permeability [34], membrane protein function [35, 36], and membrane fluidity [27]. Furthermore, free fatty acids, in both *E. faecalis* and *S. pneumoniae*, repress *de novo* fatty acid biosynthesis [8-10]. Given these findings, it is possible that some of the effects observed in our triple deletion strain background upon supplementation are due to a combination of repressed *de novo* fatty acid biosynthesis and reduced ability to efficiently phosphorylate fatty acids.

While it might be expected that generation time differences would be observed in media supplemented with fatty acids, the altered morphology and reduced CFUs of $\Delta fakB1,2,5$ compared to wild-type OG1RF in BHI lacking fatty acid supplements were unexpected, as deletion of the *fakB* genes of *S. aureus* or *S. pneumoniae* did not alter normal cellular physiology [11, 12]. Given the gross morphological differences observed

for the $\Delta fakB1,2,5$ strain, we suspected that these phenotypes were caused by increased free fatty acids in the membrane, which is similar to what we observed when supplementing wild-type cultures with fatty acids [18, 26, 29]. An accumulation of free fatty acids, negative growth phenotypes, and altered morphology have not been previously noted on deletion of the *fak* genes of *S. aureus* or *S. pneumoniae* [11, 12]. This is in contrast to published literature, which indicated that truncation of the *fakA* gene of *E. faecalis* led to morphological issues [31]. We therefore hypothesize that in *S. aureus* and *S. pneumoniae*, the thioesterase TesS plays an auxiliary role, while in *E. faecalis*, this protein may play a more prominent role in phospholipid synthesis. Expression and protein activity data are needed to further test this hypothesis.

While we initially noted that fatty acids increased generation times significantly in select mutants, we also noted that additional deletions decreased generation times. It was not surprising, given our initial data for $\Delta fakB1,2$ and $\Delta fakB1,5$, to see major increases in generation time upon exposure of $\Delta fakB1,2,5$ to unsaturated exogenous fatty acids. However, we were surprised to note that $\Delta fakB1,2,5$ had a decreased generation time in stearic acid relative to wild-type levels (Table 3.4). Further, we observed a rescue of generation time in unsaturated fatty acids for the quintuple deletion strain (Table 3.3). One potential reason for this observation is that supplementation of specific deletion strains with fatty acids might overload the system when there is only one FakB remaining, which has ability to bind the fatty acid supplement. In this instance, deletion of all of the *fakB* genes which have binding specificity for that supplement would ameliorate the negative growth phenotype observed in a lower-order or different combination deletion strain. As we noted that the generation time of $\Delta fakB1,5$ in stearic acid was rescued in $\Delta fakB1,2,5$, this leads us to the hypothesis that FakB2 may be able bind to the saturated fatty acid stearic acid, even if weakly. Alternatively, it is possible that $\Delta fakB1,2,5$ in this case was unable to bind to stearic acid; thus stearic acid was not incorporated into membrane phospholipids and because it was not incorporated, did not have a negative impact on generation time.

Given that $\Delta fakB1,2,5$ had increased levels of free fatty acids (Figure 3.3), we initially anticipated that deletion of *tesS* would rescue many of the phenotypes we previously noted in $\Delta fakB1,2,5$, especially accumulation of free fatty acids. However, we observed that $\Delta fakB1,2,5/\Delta tesS$ had many of the same peculiarities as $\Delta fakB1,2,5$. Specifically, this deletion strain had increased levels of free fatty acids relative to wild-type (Figure 3.3) and similar generation times to $\Delta fakB1,2,5$ when grown in media supplemented with fatty acids (Tables 3.3 and 3.4). These data indicated that while deletion of *tesS* was necessary to partially reduce free fatty acid levels, deletion of all of the *fakB* genes was necessary to complete this reversion, indicating again that retaining a FakB with at least partial binding specificity for a supplement can be detrimental. However, our analysis of free fatty acids using thin layer chromatography relied upon normalizing levels of free fatty acids to phosphatidylglycerol. If phosphatidylglycerol levels are different between wild-type and deletion strains, this would impact our data analysis. Thus, mass spectrometry data will be required to determine whether free fatty acid levels in $\Delta fakB1,2,5/\Delta tesS$ are truly increased relative to free fatty acid levels in wild-type *E. faecalis*.

While it seems energetically inefficient for cells to make a thioesterase which cleaves endogenous fatty acids from the acyl carrier protein, one potential reason for this is that it could help the cell to produce acyl-phosphates. In Gram-positive organisms, acyl-phosphates can be generated in two ways. As discussed above, the Fak system can directly phosphorylate free fatty acids [12], or PlsX can convert acyl-ACP into acyl-phosphates (Figure 3.1) [3]. If PlsX functions poorly to convert acyl-ACP to acyl-phosphate, it would then be necessary for the cells to find other ways to generate acyl-phosphates. Thus, if PlsX activity is weak, if it is inadequately expressed, or if its stability is poor, having another means to produce free fatty acids for phosphorylation by the Fak system might be necessary to generate lysophosphatidic acid. Consequently, such a situation would likely necessitate that *TesS* play a more prominent role in phospholipid biosynthesis. Our data point therefore towards *TesS* having higher expression or activity and *PlsX* having lower expression or activity in *E. faecalis* relative to *S. aureus* and *S. pneumoniae*. This is further evidenced by the ability of the quintuple deletion strain

($\Delta fakB1,2,4,5/\Delta tesS$) to grow in the toxic fatty acids myristic acid and palmitic acid; it is likely that the toxicity of these fatty acids in wild-type OG1RF is due to their incorporation onto lysophosphatidic acid (Figure 3.1). In wild-type OG1RF, these fatty acids may never enter the *de novo* fatty acid synthesis cycle following conversion from acyl-phosphates to acyl-ACP; instead they may be incorporated as-is onto the SN-1 position of glycerol-3-phosphate by PlsY. This could cause significant alterations to the lipid makeup of the cells and could have an effect on membrane motion, as it was previously found that increased tail length increases ability of the lipid bilayer to bend [37]. Furthermore, if exogenous fatty acids are typically incorporated at the SN-1 position while endogenous fatty acids are typically incorporated at the SN-2 position, this could lead to lipid asymmetry. Studies on lipid asymmetry demonstrated that a bilayer consisting of phospholipids with asymmetric tails has reduced ability to stretch [37, 38]. Alteration of the biophysical properties of the lipid bilayer might thus partially explain the reason that specific fatty acids are toxic when incorporated into membrane phospholipids.

However, while these data may point towards an inefficient PlsX, it is also possible that *E. faecalis* has a highly efficient Fak system. As *E. faecalis* is commensal to the mammalian intestine, it is exposed to numerous fatty acids present in bile. To utilize the plentiful fatty acid resources around it, *E. faecalis* may have evolved to, with extreme efficiency, incorporate exogenous fatty acids into its membrane using the Fak system. In an environment where fatty acids are plentiful, TesS may not be extremely useful, except perhaps to ensure that endogenous fatty acids are incorporated onto the correct position of a phospholipid head group. However, *E. faecalis* can survive for long periods in nutrient-limiting environments [39]. If the Fak system were extremely efficient, in environments where exogenous fatty acids are not abundant, it may be beneficial for *E. faecalis* to also have a high-functioning TesS. Our data point towards an imbalance, relative to other studied organisms, in the fatty acid metabolism of *E. faecalis*, where the Fak system, along with TesS, play a vital role in membrane homeostasis.

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Appendix

Supplemental Table 3.1. Bacterial strains and plasmids used in this study.

Strain	Relevant genotype or description	Source
<i>Enterococcus faecalis</i> OG1RF	Laboratory strain Rif ^R , Fus ^R	Lab stock
<i>Escherichia coli</i> EC1000	Cloning strain for <i>repA</i> -dependent plasmids	G. Dunny, University of Minnesota
<i>E. faecalis</i> CK111/pCJ10-101	Conjugative donor strain; harbors non-transferable pCJ10 derivative plasmid Spec ^R	Kristich et al. (2007)
Plasmid		
pCJK47	Used for markerless exchange	Kristich et al. (2007)
pCIE	Used for overexpression in <i>E. faecalis</i>	K. Weaver, Weaver et al., (2017)
pRDJ1	pCJK47 derivative containing flanking regions of OG1RF_RS06660 (<i>fakB1</i>)	This work
pRDJ2	pCJK47 derivative containing flanking regions of OG1RF_RS07200 (<i>fakB2</i>)	This work
pRDJ3	pCJK47 derivative containing flanking regions of OG1RF_RS00080 (<i>fakB4</i>)	This work
pRDJ4	pCJK47 derivative containing flanking regions of OG1RF_RS05020 (<i>fakB5</i>)	This work
pRDJ5	pCJK47 derivative containing flanking regions of OG1RF_RS01405 (<i>tesS</i>)	This work

Supplemental Table 3.2. Oligonucleotides used in this study.

Name	Sequence	Use
EF1492	GTGATACACATGGAAGCAATTCGTCGTGTTGCCCGGGTA CCATGGCATGCTAAGCTTGATTTTCGTTT	Generate overhangs on pCJK47 for <i>fakB2</i>
EF1493	GAAAGAATGGATAACCAAATCAGAAGGTAGTCTAGAACT AGCGATTCTGAAATCACCATTAAAAAACTC	Generate overhangs on pCJK47 for <i>fakB2</i>
EF1494	GAGTTTTTTAAATGGTGATTTCAGAATCGCTAGTTCTAGA CTACCTTCTGATTGGTTATCCATTCTTTT	Generate overhangs on <i>fakB2</i> piece 1 insert
EF1495	CTTTTAAAGAAAGAGTGATAAATTTATGACAAACGCTATA CAGACTAAGAAATAACGAGGTTGGGTCTCAC	Generate overhangs on <i>fakB2</i> piece 1 insert
EF1496	GTGAGACCCAACCTCGTTATTTCTTAGTCTGTATAGCGTT TGTCATAAATTTATCACTCTTTCTTTAAAAG	Generate overhangs on <i>fakB2</i> piece 2 inserts
EF1497	GAACGAAAATCAAGCTTAGCATGCCATGGTACCCGGGCA ACACGACGAATTGCTTCCATGTGTATCAC	Generate overhangs on <i>fakB2</i> piece 2 insert
EF1564	CTGAGTCAGCGATGAATGCCACTTTATCCCCGGGTACCA TGGCATGCTAAGCTTGATTTTCGTTT	Generate overhangs on pCJK47 for <i>fakB4</i>
EF1512	GATTCCCACCCGTGCTGCTTTATCCATGTCTAGAACTAG CGATTCTGAAATCACCATTAAAAAACTC	Generate overhangs on pCJK47 for <i>fakB4</i>
EF1511	GAGTTTTTTAAATGGTGATTTCAGAATCGCTAGTTCTAGA CATGGATAAAGCAGCACGGGTGGGAATC	Generate overhangs on <i>fakB4</i> piece 1 insert
EF1565	CAAAAGAAAACACAGCAATACAACCTATTCCGGGACAAT TGCCCCCTTCATGTTTTCTTTATTTTAC	Generate overhangs on <i>fakB4</i> piece 1 insert
EF1566	GTAAAATAAAAGAAAACATGAAGGGGGCAATTGTCCCGG AATAGGTTGTATTGCTGTGTTTTCTTTTG	Generate overhangs on <i>fakB4</i> piece 2 insert
EF1567	GAACGAAAATCAAGCTTAGCATGCCATGGTACCCGGGGA TAAAGTGGCATTTCATCGCTGACTCAG	Generate overhangs on <i>fakB4</i> piece 2 insert
EF1526	GAAAGTATCAAATAAGATTAGAAGGAAAAAGAGCCCGGG TACCATGGCATGCTAAGCTTGATTTTCGTTT	Generate overhangs on pCJK47 for <i>fakB5</i>
EF1527	CATCAACGTAAGTAGTAATACGCCATAGCACTCTAGAACT AGCGATTCTGAAATCACCATTAAAAAACTC	Generate overhangs on pCJK47 for <i>fakB5</i>
EF1528	GAGTTTTTTAAATGGTGATTTCAGAATCGCTAGTTCTAGA GTGCTATGGCGTATTACTAGTTACGTTGATG	Generate overhangs on <i>fakB5</i> piece 1 insert
EF1529	CAAAAAAATTTGAGGGGATTTTTCATTTAATTAATCGTGC GATCTTTTCTTTATTCATGTGTCATCTCATC	Generate overhangs on <i>fakB5</i> piece 1 insert
EF1530	GATGAGATGACACATGAATAAAGAAAAGATCGCACGATT AATTAAATGAAAAATCCCCTCAAATTTTTTTG	Generate overhangs on <i>fakB5</i> piece 2 insert
EF1531	GAACGAAAATCAAGCTTAGCATGCCATGGTACCCGGGCT CTTTTTCCTTCTAATCTTATTTGATACTTTC	Generate overhangs on <i>fakB5</i> piece 2 insert
EF1536	GAAAAACGACAACACTTTGCAGAACGTATGCCCGGGTAC CATGGCATGCTAAGCTTGATTTTCGTTT	Generate overhangs on pCJK47 for <i>fakB1</i>
EF1537	GAACCGTTGCCACCACCTGAATCTGTTTGTCTAGAACTA GCGATTCTGAAATCACCATTAAAAAACTC	Generate overhangs on pCJK47 for <i>fakB1</i>
EF1538	GAGTTTTTTAAATGGTGATTTCAGAATCGCTAGTTCTAGA CAAACAGATTTCAGGTGGTGGCAACGGTTC	Generate overhangs on <i>fakB1</i> piece 1 insert

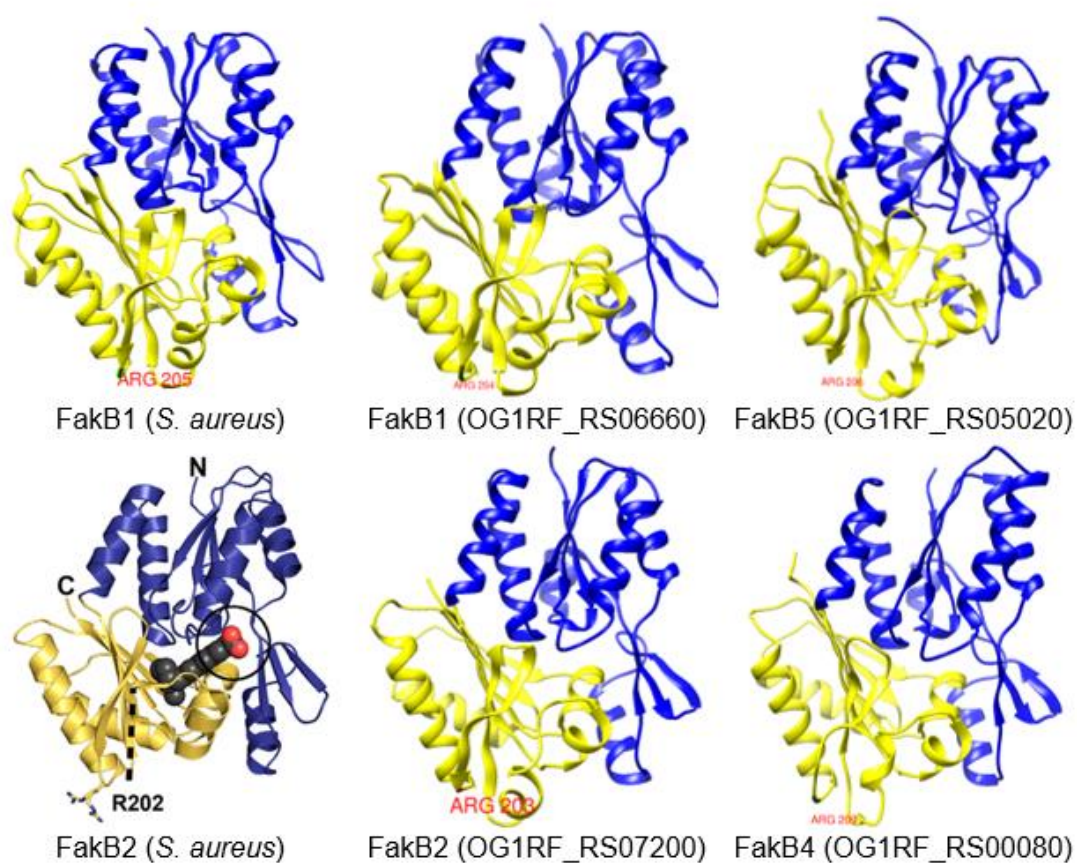
Supplemental Table 3.2 Continued

Name	Sequence	Use
EF1539	GAGACAGAAGTAGTTTACTTTTGTCTCATCCATTTTGTAACCTCGTTTCACTAATTTTC	Generate overhangs on <i>fakB1</i> piece 1 insert
EF1540	GAAAATTAGTGAAACGAGGTTACAAAATGGATGAGACAA AAGTAACTACTTCTGTCTC	Generate overhangs on <i>fakB1</i> piece 2 insert
EF1541	GAACGAAAATCAAGCTTAGCATGCCATGGTACCCGGGCA TACGTTCTGCAAAGTGTTGTCGTTTTTC	Generate overhangs on <i>fakB1</i> piece 2 insert
EF1877	CTTGAATTTGAGTTACGATGATTTGAATCCCCCGGGTAC CATGGCATGCTAAGCTTGATTTTCGTTT	Generate overhangs on pCJK47 for <i>tesS</i>
EF1878	CTTTCAGAAGTAAATCAAGCCTACAATGTCTCTAGAACTA GCGATTCTGAAATCACCATTAAAAAACTC	Generate overhangs on pCJK47 for <i>tesS</i>
EF1873	GAGTTTTTTTAAATGGTGATTTTCAGAATCGCTAGTTCTAGA GACATTGTAGGCTTGATTTACTTCTGAAAG	Generate overhangs on <i>tesS</i> piece 1 insert
EF1874	GACTTTCGACTTCTTGGCCATATTCTACCCACGAAATAA TCTCCTTTTTCCAATG	Generate overhangs on <i>tesS</i> piece 1 insert
EF1875	CATTGGAAAAAGGAGATTATTTTCGTGGGGTAGAATATGG CCAAGAAGTCGAAAGTC	Generate overhangs on <i>tesS</i> piece 2 insert
EF1876	GAACGAAAATCAAGCTTAGCATGCCATGGTACCCGGGG GATTCAAATCATCGTAACTCAAATTCAAG	Generate overhangs on <i>tesS</i> piece 2 insert

Supplemental Table 3.3. *P*-values for GC-FAME analysis.

Welch's T-test was used to compare the average % total membrane content of fatty acids detected by GC-FAME analysis. The strains compared are as "strain 1 vs. strain 2". Colors indicate whether there was an increase or a decrease of the indicated fatty acid in the strain 2 relative to strain 1. White indicates no difference, blue illustrates that strain 2 had a decrease in the indicated fatty acid, and red indicates that strain 2 had an increase in the indicated fatty acid relative to strain 1. ND means the specified fat was not detected in either of the indicated strains.

Fatty acid	Wild-type vs. Δ tess	Wild-type vs. Δ fakB1,2,5	Wild-type vs. Quintuple	Quintuple vs. Δ tess	Quintuple vs. Δ fakB1,2,5
C _{12:0}	P = 1.000	P = 0.553	P = 0.013	P = 0.007	P = 0.004
C _{14:0}	P = 0.002	P < 0.001	P < 0.001	P = 0.909	P = 0.087
C _{16:1}	P = 0.019	P = 0.003	P = 0.007	P = 0.566	P < 0.001
C _{16:0}	P = 0.018	P = 0.002	P = 0.002	P = 0.059	P < 0.001
C _{17:1} <i>cis</i> 10	P = 0.981	P = 0.044	P = 0.105	P = 0.099	P = 0.014
C _{17:0} 2OH	0.232	P < 0.001	0.003	0.002	P = 0.195
C _{18:1} <i>cis</i> 11	P = 0.128	P < 0.001	P = 0.894	P = 0.121	P < 0.001
C _{18:0}	P = 0.755	P = 0.002	P = 0.002	P = 0.005	P = 0.518
C _{19:0} cyclo11	P = 0.159	P < 0.001	P = 0.026	P = 0.026	P = 0.299
Unknown	P = 0.423	P = 0.031	P = 0.423	P = 0.791	0.020
Others: C _{15:1} <i>cis</i> 7	ND	P < 0.001	P = 0.423	P = 0.423	P = 0.011
Others: C _{17:1} <i>iso cis</i> 12	P = 0.423	ND	ND	P = 0.423	ND
Others: C _{18:1} <i>cis</i> 9	P = 0.423	P = 0.013	ND	P = 0.423	P = 0.013
Others: 11 methyl C _{18:1} <i>cis</i> 11	ND	ND	P = 0.423	P = 0.423	P = 0.423
Others: C _{20:1} <i>cis</i> 13	ND	ND	P = 0.423	P = 0.423	P = 0.423
Others: C _{20:2} <i>cis</i> 11,14	ND	ND	P = 0.423	P = 0.423	P = 0.423
Saturated: Unsaturated Ratio	P = 0.225	P < 0.01	P = 0.019	P = 0.014	P < 0.01



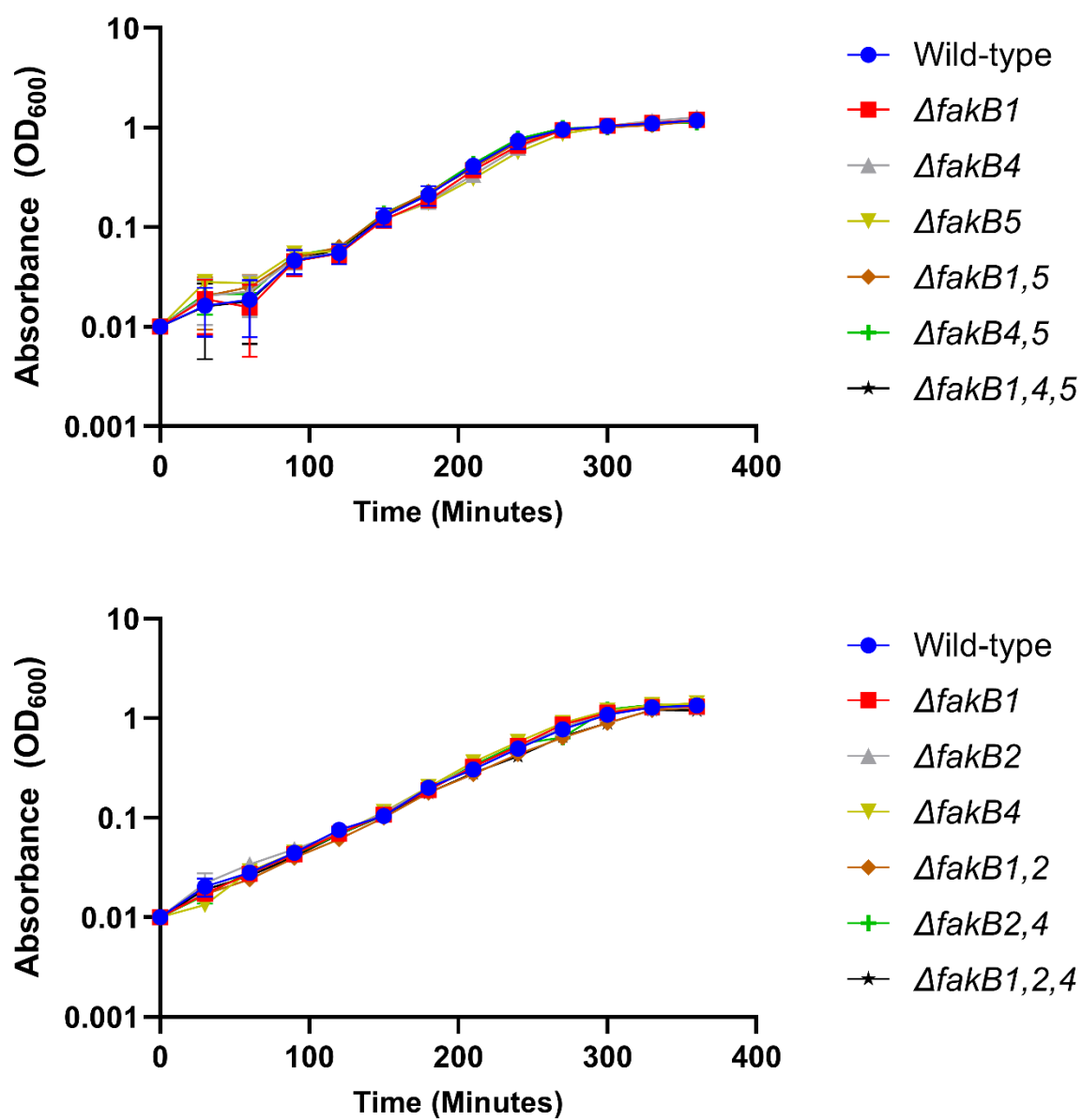
Supplemental Figure 3.1. Predicted structural models of FakB homologs generated by Phyre².

Phyre² models were generated for the FakB homologs of *E. faecalis* and FakB1 of *S. aureus*. The models are depicted alongside the published crystal structure of *S. aureus* FakB2 (Broussard 2016).

Supplemental Figure 3.2. Residues important for binding fatty acids are conserved in the *E. faecalis* FakB homologs.

Clustal Omega was used to align the protein sequences of saFakB1, saFakB2, spFakB1, spFakB2, spFakB3, efFakB1, efFakB2, efFakB3, and efFakB4. Residues necessary for Fak activity (Broussard 2016) are highlighted. Red: residues are conserved with saFakB2; Blue: residues are similar to saFakB2. Note that spFakB3 had two substitutions for strongly similar amino acid residues at Thr-61 His-266 of saFakB2.

ABJ55200.1_spFakB3	---MTWKIIADSGCDYRQLPTPAINTTFVSVPLTIQV-ADQVFVDDASLD--IDQMMETM	54
WP_002357950.1_effFakB5	MNKEKIALLDVSGTDVPEALVKQYGMVYL--PLQIIY-PEKTYTDKVDIT--PEEVYQRL	55
WP_002359214.1_effFakB4	---MNYQLVTDSCCDLPYTYLKENQVPFI--SMNIQI-DGKEYRDDLGETFDYQNFLTAI	54
ABD20605.1_saFakB1	---MKIAVMTDSTSYLSQDLIDKYNIQIA--PLSVTFDDGKNFTESNEIA--IEEFYNKM	53
ABJ55113.1_spFakB1	---MKLAVFTDSSAYLSAETLQREDLFVL--DIPVNI-DGEEYVEGINLS--AEFEYQKM	52
WP_002382324.1_effFakB1	---MKIAIVTDSTAYLPERIKDHPNLFVI--PIPVIL-DGKIYNEGIDIE--ADEYYALL	52
ABD22554.1_saFakB2	--MTKQIIIVTDSTSDLSKEYLEANNIHWI--PLSLTI-EGASYVDQVDIT--SEEFNNHI	53
ABJ55472.1_spFakB2	--MTKIKIVTDSSVTIEPELVKQLDITIV--PLSVMI-DNVVYSADADLKE--EGKFLQLM	53
WP_002357444.1_effFakB2	--MTNVKIVTDSSCTMEKSLRDELNIHMM--PLSIMV-DGVVYPDDHLP--GEKFMDDM	53
	. :..** . : : : : :	
ABJ55200.1_spFakB3	YATAEASKSACPSDDYLRAFE-----GAKNIFLVTTITGTLSSGSHNSAQLAKNIYLEDHP	109
WP_002357950.1_effFakB5	E--KEIPSTSLPDGATIQAFDKIKEAGYEVKVLAVTISSGLSGTYNVVRLLEGEQ----TE	109
WP_002359214.1_effFakB4	KN-GSMPTTSQVNVGRYNEFRFPVEQG-L-PVIYLAFFSSGLSGSYQSALQSVEMLKEEYD	112
ABD20605.1_saFakB1	ASSQTIPTTSQPAIGEWITKYEMLRDQGYTDIIIVICLSSSGISGSYQSSYQAGEM----VE	109
ABJ55113.1_spFakB1	AQASELPKTSQPSIAKDEILTSLEKEGYTHALGLFLSSSGISGFYQSIQYMVDD----YE	108
WP_002382324.1_effFakB1	NNSKEFPTTSQPALGEVLELYKSIAEQGYDTIISIHLSSSGISGFVHTLHGLTDE----IP	108
ABD22554.1_saFakB2	ENDED-VKTSQPAIGEFISAYEELGKDG-S-EIISIHLSSGLSGTYNTAYQASQM----VD	107
ABJ55472.1_spFakB2	QESKNLPKTSQPPFVGFAEIFEDLCKDGG-QILAIHMSHALSGTVEAARQGASL----ST	108
WP_002357444.1_effFakB2	ANAKALPKTSQPPIGEFVELYDRLEGDS-EVISIHMTKGLSGTVEAARQASNL----SS	108
	.: : * : : : : ** . .	
ABJ55200.1_spFakB3	DTKIHVIDSLSAGGEVDLLVEKLNLDLIDQGLSFEEVVEAITAYQEKTKLLFVLAKVDNLV	169
WP_002357950.1_effFakB5	GLDVFVLDTKNIGIGAGIQAIRAAELIETGLGWQELQKQKLTTEEVANAKVFNATLEYLQ	169
WP_002359214.1_effFakB4	NVEIHIIDTKAASLGQGMVLVREAIRLQTDGHSLEGVVAYLEEQKMKLHSWVTVDLKHLE	172
ABD20605.1_saFakB1	GVNVHAFDSKLAAMIEGCVLRAIEMVEEGYEPQIIDDLTNMRHTGAYLIVDDLKNLQ	169
ABJ55113.1_spFakB1	GLTIAFPDTLTISAPLGIMVESVFNWRDQDDFASIQDKLAIQISRTSAFIMVDDLHLV	168
WP_002382324.1_effFakB1	GVALYPYDSKITSMPMGHMVAAALDLTEEKASLEEIAKDLDIRDNTYAYLIVEDLNNLV	168
ABD22554.1_saFakB2	-ANVTVIDSKISISFGLGYQIQLHVELVKEGVSTSEIVKKNLHLRENKILFVVGQLNQLI	166
ABJ55472.1_spFakB2	-ADVIVVDSSFTDQALKEFQVVEAAKLAQEGKDMAILSHVEEVKNHTELYIGVSTLENLV	167
WP_002357444.1_effFakB2	-SKVTVIDSDFTDQGLSFQVIAAKLAQAGAGVPEILAEIERVKQNTKLYIGISTLDNLV	167
	: * : : : . . : : *	
ABJ55200.1_spFakB3	KNGRLSKSLIGTVVGLLNIRMVGKASETGTLELLQKARGSKKSQVQAAYDELVKAG---YAG	226
WP_002357950.1_effFakB5	KGGRIIGLVTSILGNALKLNPIISCNEEGIYYTVAKSRGRKSLDKTFELVTNFIGEA-PR	228
WP_002359214.1_effFakB4	RGGRIKTAALGGLMNKPIIRVDAAGKLASVGKTRGRNKSLOKIAQETIQGIVEP-MK	231
ABD20605.1_saFakB1	KSGRITGAQAWVGTLLKMKPVLKFEDG-KIIPEEKVRTKKRAIQTLKKVLDIVKDF-EE	227
ABJ55113.1_spFakB1	KGGRLSNGAAILGNLLSIKPILYFNDQGVIEVYEKVRTEKKATKRLEIIEIKETTAS--GQ	226
WP_002382324.1_effFakB1	RGGRLTNGAALIAGLLKIKPILTFEDG-KIVLFEKIRSTKKAFARAEEKIIGERNAGIEAP	227
ABD22554.1_saFakB2	KGGRIKTKGLIGNLMKIKPIGTLDG-RLELVHNAARTQNSSIQYLKKEIAEFIGDH-EI	224
ABJ55472.1_spFakB2	KGGRIGRVGTLLSSLLNIRVVMQMKDH-ELQPMVKGRGRTKTFKKWLDELITSLSER--AV	224
WP_002357444.1_effFakB2	KGGRISRTTGLLSNIFNMKVVMDFENT-ELIPVAKRGVKTFFNKWFDLKSSELSKIP-NV	225
	:.**.: . : : : . : * : . .	
ABJ55200.1_spFakB3	GRIVMAQRNNEKCCQQLSERIRETFPQ-ADIKILPSTGLCSFYAEEGLLMGYEID----	281
WP_002357950.1_effFakB5	FRLAVAHGAEEEEAKAMMERLKAAPFQAEIYFGTISPALVVHTGPGLLGVGIQLLND--	286
WP_002359214.1_effFakB4	QTLIIAYAGTKDDAEKVKELEIEIEV-NEILYPLGPTITSHTGIGCIAVFSFGEKRR-	289
ABD20605.1_saFakB1	VTLFVINGDHFEDGQALYKKLQDDCPSAYQVAYSEFGPVVAAHLGSGGLGLGYVGRKIRL	287
ABJ55113.1_spFakB1	YRVIVIHGNAPEKAEELRQHLLDFGLG-SDVSLATFGSVIGTHLGAGSIALGYIPVI---	282
WP_002382324.1_effFakB1	VKLYVIHANNRIVAEKEQAKLQKLYPN-AEIEIGHFGPVGITHLGEKAIGLAISAQ----	282
ABD22554.1_saFakB2	KSIGVAHANVIEYVDKLVKVFNEAFHV-NNYDINVTTPVISAHGTGQGAIGLVVLK----	279
ABJ55472.1_spFakB2	AEIGISYSGSDDWAKEMKESLQAY-VE-KPISVLETGSIQHTGTGENAWAILIRYHS---	279
WP_002357444.1_effFakB2	RQIGISHADGLELANGFKEGLQALFKD-MDIPVLHTNFPVIATHTKNAFAIMYYTD----	280
	: : . : : : : :	
ABJ55200.1_spFakB3	- 281	
WP_002357950.1_effFakB5	- 286	
WP_002359214.1_effFakB4	- 289	
ABD20605.1_saFakB1	T 288	
ABJ55113.1_spFakB1	- 282	
WP_002382324.1_effFakB1	- 282	
ABD22554.1_saFakB2	- 279	
ABJ55472.1_spFakB2	- 279	
WP_002357444.1_effFakB2	- 280	



Supplemental Figure 3.3 $\Delta fakB1,4,5$ and $\Delta fakB1,2,4$ do not have different growth kinetics from wild-type OG1RF in BHI media supplemented with ethanol (0.1%). Shown are the average optical density readings $\pm$ standard deviations for $n = 3$ replicates.

**CHAPTER 4: THE FATTY ACID KINASE SYSTEM OF *ENTEROCOCCUS*
FAECALIS CONTRIBUTES TO DAPTOMYCIN TOLERANCE**

Future Publication Note

A version of this chapter will eventually be submitted for publication.

Johnston RD, Aitolo G, and Fozo EM. The fatty acid kinase system of *Enterococcus faecalis* contributes to daptomycin tolerance. 2022/2023.

Experiments were completed by Rachel Johnston, with the exception of MIC determination for $\Delta fakB1,2,5$ in ampicillin and fosfomycin, which was performed by Georgina Aitolo.

Abstract

Fatty acids are an important nutrient for bacterial cells, as they can be used as carbon sources or for building the lipid membrane. As a commensal colonizer of the gastrointestinal tract, and as a pathogen of clinical import, *Enterococcus faecalis* is exposed to free fatty acids in its environment, yet cannot break them down to generate energy. However, previous evidence suggested that free fatty acids are incorporated into the membrane phospholipids of *E. faecalis*, indicating the presence of a pathway for fatty acid uptake. Here we performed a network analysis of DegV domains, which have been shown to bind fatty acids, and identified that *E. faecalis* has four distinctly-clustering DegV domain-containing proteins. Two of these proteins cluster with the FakB proteins of *S. aureus* and *S. pneumoniae*, which were previously found to bind different fatty acids, allowing for their phosphorylation by the enzyme FakA. The remaining FakB homologs of *E. faecalis* clustered separately from each other and from the FakB proteins of *S. aureus* and *S. pneumoniae*. Our network analysis showed that organisms which are isolated from different environmental sources cluster differently. This indicated that having different numbers and/or functions of fatty acid-binding proteins may be necessary depending on niche. As previous findings have shown that supplementation of *E. faecalis* with free fatty acids increases tolerance to the drug daptomycin, we investigated how the *fakB* homologs of *E. faecalis* impact daptomycin tolerance, and found that combined deletion of *fakB* genes results in increased basal daptomycin tolerance. Furthermore, we found that on supplementation of these strains with protective free fatty acids, daptomycin tolerance was still induced. Taken together, these findings indicate that the FakB homologs of *E. faecalis* decrease the ability of this organism to tolerate exposure to daptomycin.

Introduction

Enterococcus faecalis is a Gram-positive facultative anaerobe which is ordinarily a commensal colonizer of the gastrointestinal tracts of humans and other mammals [1]. However, this bacterium is also able to colonize other tissues within its host, causing infections which include bacterial endocarditis, infections of the urinary tract, and sepsis [1]. In recent years, infections by enterococcal strains resistant to other treatments have been combatted with the antibiotic daptomycin, which has been hypothesized in the literature to act by disrupting the cell membrane [2-5]. Several instances of daptomycin resistance have been reported in the clinic [6-8], and subsequent research has demonstrated that mutations within genes involved in cell membrane homeostasis and phospholipid metabolism are linked to daptomycin resistance in *E. faecalis* [9-17]. Further, exposure to the host fluids bile and serum, both of which are rich in fatty acids, changes the membrane composition of *E. faecalis* and confers tolerance *via* unknown mechanisms to membrane stressors, including daptomycin [18, 19]. Given that exposure to fatty acids drastically changes the response of *E. faecalis* to daptomycin, we wished to determine whether incorporation of fatty acids into membrane phospholipids is required for inducing daptomycin tolerance.

Incorporation of exogenous fatty acids into the membrane of Gram-positive organisms such as *Staphylococcus aureus* and *Streptococcus pneumoniae* is mediated by a fatty acid kinase system. In this system, FakB, a protein consisting of a sole fatty acid-binding DegV domain, binds to free fatty acids which are subsequently phosphorylated by FakA [20]. The resulting acyl-phosphates can be used as donors for acylation of glycerol-3-phosphate, or can be converted into acyl-ACP, which can be used to acylate lysophosphatidic acid or enter into the fatty acid biosynthesis cycle. In *E. faecalis*, there are 4 homologs to the characterized FakB proteins of *S. aureus* and *S. pneumoniae* ([20, 21], Chapter 3). Here, we performed a network analysis to determine potential fatty acid binding specificities of the FakB homologs of *E. faecalis*. We found that OG1RF_RS07200 (efFakB2) clustered with the FakB2 proteins of *S. aureus* and *S. pneumoniae*, while OG1RF_RS06660 (efFakB1) clustered with the FakB1 proteins of *S.*

aureus and *S. pneumoniae*. OG1RF_RSS00080 (efFakB4) and OG1RF_RS05020 (efFakB5) were demonstrated to cluster away from annotated FakB proteins. To determine whether the FakB homologs of *E. faecalis* play a role in fatty acid-mediated daptomycin tolerance, we tested the ability of several *fakB* deletion strains to tolerate daptomycin. Interestingly, on deletion of multiple *fakB* homologs of *E. faecalis*, we found that there was an increase in basal daptomycin tolerance. Furthermore, we observed that supplementation with fatty acids, which, in wild-type *E. faecalis*, protect from daptomycin killing, was still protective in strains deleted for *fakB* genes, indicating that incorporation of free fatty acids into phospholipids is not necessary for fatty acid-induced daptomycin tolerance.

Materials and Methods

Identification of E. faecalis FakB homologs and network analysis of DegV domain-containing proteins

EFI-EST (Enzyme Function Initiative's Enzyme Similarity Tool, which draws networks of proteins and clusters them based on amino acid sequence similarity [22]) was used to generate a representative node sequence similarity network (rep node SSN) for proteins within the DegV family (Pfam PF02645). As this family is large (containing 34,797 proteins at date of accession, (8/7/2021), we grouped sequences that shared $\geq 50\%$ identity over 80% of the sequence into cluster IDs (UniRef50). Each of these cluster IDs is a node within the rep node SSN. Additionally, as we wished to gain insight into fatty acid binding specificities rather than additional functions, we trimmed the input sequences to the domain boundaries prior to generating the rep node SSN. Note that the annotated FakB proteins of *S. aureus* and *S. pneumoniae* only contain one domain which makes up the majority ($>95\%$) of the proteins, yet there are other instances where DegV is one domain within a multidomain protein. Fragments (sequences which do not contain a start and/or a stop codon) were not used in the analysis. An initial (permissive) BLAST E-value of 10^{-5} was used to calculate the similarity between cluster IDs. On calculating the similarities, the edges between cluster IDs in the rep node SSN were calculated using an alignment

score of 55 (more stringent than initial E-value; corresponding to ~35% identity between nodes). The EFI-EST Color SSN tool was used to assign cluster numbers.

Network plot visualization

Networks were visualized and interpreted using Cytoscape v3.7.1 [23] and Gephi V0.9.2 [24]. Nodes were arranged with the OpenOrd layout algorithm. As the FakB proteins of *S. aureus* and *S. pneumoniae*, along with the *E. faecalis* FakB homologs were present in Cluster 1, we opted to analyze only this cluster. Cluster 1 originally contained 1,332 nodes, and 5,176 organisms were represented. However, as we wished to obtain accurate information on copy number, organisms which appeared within the network nodes were eliminated from further analysis if they represented >1 strain of that organism, or if they could not be identified as a single strain (for instance, if “marine sediment metagenome” or *Coproccoccus sp.* were listed as an organism, they would be eliminated). Thus, the resulting network contained 3,423 organisms, 1,010 nodes, and 7,515 edges. To visualize subclusters within cluster 1, network connectivity, calculated by EFI-EST, was used to color the network plot.

Using NCBI Taxonomy Browser and the Joint Genome Institution’s resources, each organism within the network was examined and manually curated for the type of organism (Gram stain, virus, or archaea), along with environmental origin, consisting of the following categories: animal gut, animal airways, animal oral cavity/throat, other animal sources, water sources, terrestrial sources, food sources, plant/algae sources, manmade object sources, multiple sources, atmospheric (air) sources, and other sources. Using GephiV0.9.2, these categories were used to color the network plot.

Bacterial growth conditions

Bacterial strains and plasmids used in this study are listed in Supplemental Table 4.1. *Enterococcus faecalis* was grown statically in brain heart infusion medium (BHI; BD Difco) at 37°C. Overnight cultures were diluted to an optical density at 600 nm (OD₆₀₀) of 0.01 prior to performing assays. Where indicated, cultures were supplemented with fatty acids

at the following concentrations: oleic acid (C_{18:1 cis9}) at 20 µg/mL; linoleic acid (C_{18:2 cis9,12}) at 10 µg/mL. Antibiotics were added as indicated in the text at the following concentrations: daptomycin: 30 or 40 µg/mL; ampicillin: 0, 1, 2, 4, 6, or 8 µg/mL; and Fosfomycin: 64, 128, 256, 512, 1024, and 2048 µg/mL.

Cell survival assays

Where indicated, cells were grown short-term in supplied fatty acids, where the fatty acid was added to cultures at early exponential phase (OD₆₀₀ = 0.225 to 0.250) and the cultures were grown an additional 30 minutes. Cells were then harvested by centrifugation (2379xg for 10 minutes), washed twice with 1X phosphate buffered saline (1X PBS), and resuspended in an equivalent volume of “challenge medium” consisting of BHI containing 1.5 mM CaCl₂ and 30 or 40 µg/mL daptomycin, as indicated in the text. Serial dilutions were plated onto BHI agar at 0, 15, 30, and 60 min after resuspension in the challenge medium.

Minimal inhibitory concentration determination

To determine minimal inhibitory concentration, the indicated strain was grown overnight in BHI, and in the morning, was diluted to an OD₆₀₀ of 0.01 in BHI containing daptomycin and 1.5 mM CaCl₂, ampicillin, or Fosfomycin. The cells were grown overnight at 37°C, and after 24 hours were examined for growth.

Results

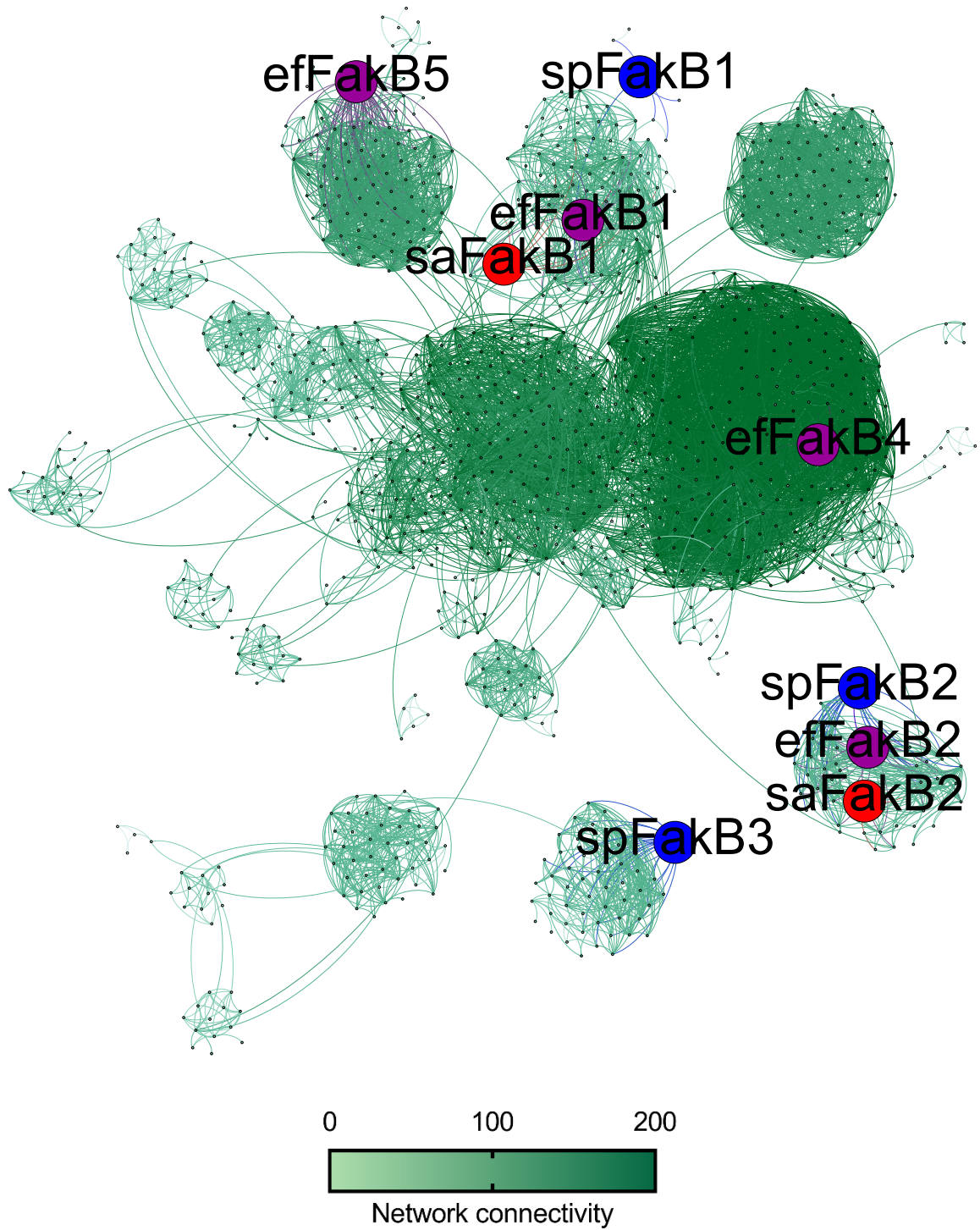
Network analysis of DegV domains reveals clustering of annotated FakB proteins

On generating a sequence similarity network (SSN) of the DegV domains (Pfam = PF02645) and annotating the locations of the FakB homologs of *E. faecalis*, along with those of *S. aureus* and *S. pneumoniae* (Figure 4.1), we found that OG1RF_RS06660 clustered with saFakB1 and spFakB1, which are specific for binding of saturated fatty acids [20, 21]. Further, we observed that OG1RF_RS07200 clustered with the saFakB2 and spFakB2, indicating its similarity with proteins specific for binding monounsaturated

Figure 4.1. *E. faecalis* FakB homologs cluster distinctly.

SSN of DegV protein domains (protein family = PF02645) generated by the EFI-EST as accessed on 8/7/2021. Each node is representative of a group of proteins sharing $\geq 50\%$ identity over 80% of the sequence (see Materials and Methods) to each other; although in many cases, they are from different organisms. Edges were drawn between nodes using an alignment score of 55, resulting in edges being drawn only between nodes where the DegV domains represented therein had $\geq 35\%$ identity. Node darkness demonstrates connectivity between nodes as calculated by EFI-EST. The darker the color, the higher the network connectivity. The specific nodes containing the DegV sequences of the FakB proteins of *S. aureus* and *S. pneumoniae* are highlighted in red and blue, respectively, while the nodes containing the FakB homologs of *E. faecalis* OG1RF are highlighted in purple.

● *Staphylococcus aureus* ● *Streptococcus pneumoniae* ● *Enterococcus faecalis*



fatty acids. We thus named OG1RF_RS06660 and OG1RF_RS07200 FakB1 and FakB2, respectively. We noted that none of the *E. faecalis* FakB homologs clustered with spFakB3. However, OG1RF_RS00080 was located in a highly connected portion of the network, yet did not cluster with annotated FakB proteins, thus we named it efFakB4. Similarly, OG1RF_RS05020 clustered alone in a separate branch of the network, thus we named it efFakB5.

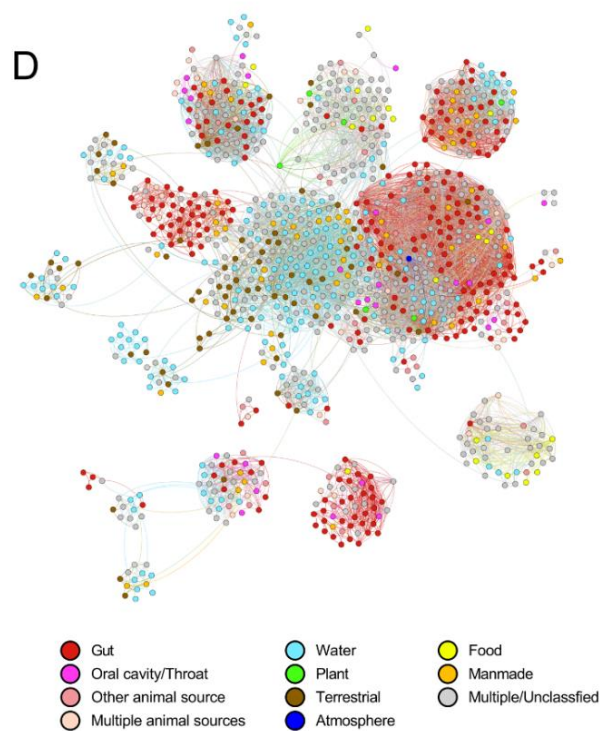
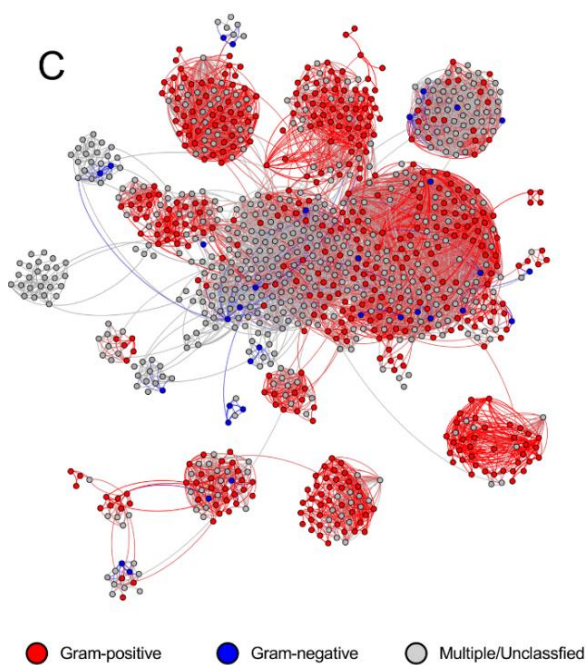
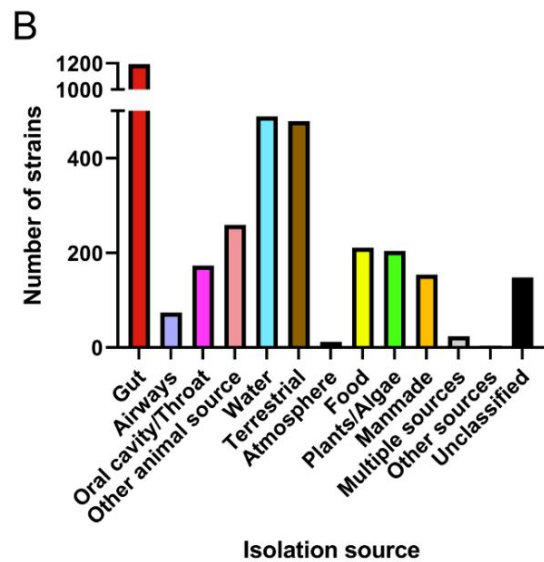
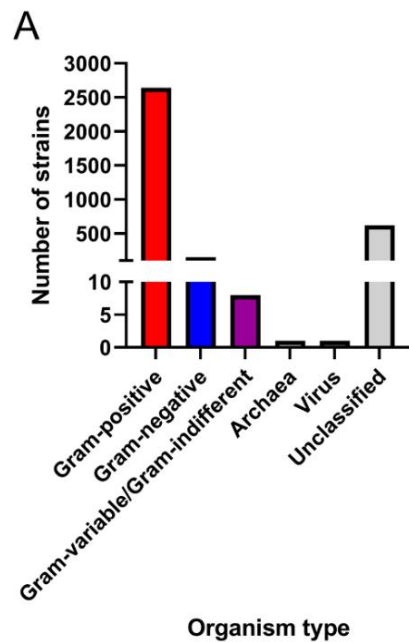
The network data indicate that OG1RF_RS06660 may be specific for binding of saturated fatty acids, while OG1RF_RS07200 may be responsible for binding of monounsaturated fatty acids. The specificity of efFakB2 for unsaturated fatty acids is supported by data demonstrating that combined deletion of *fakB2* and *fakB1* resulted in increased generation time in unsaturated fatty acids (Chapter 3; Table 3.3). While deletion *fakB1* or *fakB2* alone did not give this result (Chapter 3; data not shown), indicating that efFakB2 is the primary FakB necessary for binding unsaturated fatty acids. Peculiarly, deletion of *fakB1* alone did not result in growth defects in saturated fatty acids, yet combined deletion of *fakB1* with *fakB5* (note that deletion of *fakB5* had no observed effect) resulted in increased generation time in these fatty acids (Chapter 3; Table 3.4). These combined data indicate that efFakB5 may play a role in utilization of saturated fatty acids, while efFakB1 may play a more promiscuous role (Chapter 3). This is in contrast with previous literature reporting FakB1 homologs as primarily binding saturated fatty acids [20, 21].

Organisms with DegV domains are predominantly Gram-positive and cluster according to isolation source

Cluster 1 of the DegV network predominantly comprised Gram-positive bacteria (Figure 4.2A). However, some were Gram-negative bacteria, and there was one archaeote, *Methanomassilicoccales* archaeon RumEn M1, which was found in the rumen of a cow [25] (thus it was classified as a gut organism). The archaeote had only one appearance in cluster 1 of the network, indicating that it only had one DegV domain within the cluster. In addition, we observed a virus in our network plot, which was originally found inserted

Figure 4.2. Organisms which encode DegV domain proteins are predominantly Gram-positive, are predominantly found in the gut, and partially cluster in the DegV network according to their isolation source.

(A) Number of organisms in the DegV network classified as Gram-positive, Gram-negative, Gram-variable or Gram-indifferent, archaea, virus, or organisms which could not be classified. (B) Number of organisms classified according to isolation source. In both A and B, shown are the raw numbers of organisms belonging to each classification. (C) Clustering of Gram-positive and Gram-negative organisms in the DegV network. (D) Clustering of organisms isolated from different sources in the DegV network.



in the genome of *Streptococcus suis*, an emerging zoonotic pathogen [26]. The specific strain the virus was isolated from, *S. suis* P1/7, did not appear in our network analyses, although 6 other strains of *S. suis* did, each with 4 or 5 appearances in different nodes, indicating that they each had 4 or 5 different DegV domains.

To determine if specific fatty acid-binding domains are correlated with environment, we examined the isolation sources of the organisms within our analysis by manual annotation. Over one-third of the organisms within the network were from the gut (Figure 4.2B), although other animal sources, including the respiratory system (airways), and oral cavity and oropharynx (oral cavity/throat) were also noted (Figure 4.2B). Other isolation sources observed were various water sources (including ocean, aquariums, pools, lakes and ponds, wastewater, ground water, and hot springs) or various terrestrial sources (including soil, compost, and aquifers), as well as food sources, plants and algae, and manmade objects (such as spacecraft, airplanes, and bioreactors). Other sources were from fungi and dinoflagellates ($n = 4$ organisms).

Of note, several of the classifications had some overlap with others: for instance, aquifers, while made of permeable rock, are often filled with water, although some of the aquifers annotated as isolation sources in this network were used for oil storage. Additionally, while bioreactors are manmade objects, many of the bioreactors annotated as isolation sources within this study were used for the breakdown of wastewater sludge, although others were used for production of biofuels and breakdown of plant matter.

With these limitations in mind, we plotted both the organism classification (Figure 4.2C) and the isolation source (Figure 4.2D) onto the network plot. We observed that many of the nodes held only Gram-positive organisms (Figure 4.2C). However, towards the center of the network, we noted a grouping of nodes which contained more Gram-negative organisms or with multiple or no Gram-classification (Figure 4.2C). This area of the graph became even more distinct when we plotted isolation sources onto the network graph (Figure 4.2D). Here we observed that there was a large grouping of organisms predominantly isolated from water and terrestrial sources which corresponded with the grouping of Gram-negative and multiple/unclassified organisms (Figure 4.2C).

Different isolation sources are associated with different numbers of DegV nodes

As we observed that many of the subclusters in the network plot had organisms from mixed isolation sources, and because it was previously noted that different organisms have different numbers of FakBs [27], we wished to determine if there was a pattern with regards to the isolation source of an organism and the number of DegV domains it contains. Thus, we calculated the number of nodes each organism appeared in, and for each isolation source, calculated the percentage of organisms from that isolation source found in different numbers of nodes (Table 4.1). Interesting trends we observed were that organisms isolated from the gut were overall evenly split between appearing in one node and four nodes, indicating that the number of different DegV domains ranged between one and four. Interestingly, there was a heavy shift towards favoring three DegV domains in several other isolation sources, including animal airways, the oral cavity/throat, terrestrial sources, the atmosphere, and plants. However, in water sources, species with only one DegV domain dominated. Organisms isolated from food shifted towards having increased numbers of DegV domains, as ~19% of these organisms had five nodes, more than double the percentage of organisms from other isolation sources. Organisms isolated from fungal sources and dinoflagellate sources (Other sources) all appeared in four nodes, indicating the presence of 4 DegV domains.

Of note, *S. aureus* strains typically only had between one and two DegV domains within separate nodes (95% were found in two nodes, while the remaining 5% were found in one node; Supplemental Figure 4.1). This is in contrast to *S. pneumoniae* isolates, which appeared in three nodes (87% of isolates) or four nodes (13%), indicating the presence of 3-4 DegV domains within species (Supplemental Figure 4.1). However, *E. faecalis* had predominantly 4 DegV domains, as isolates appeared in four nodes (96% of isolates) or three nodes (4% of isolates; Supplemental Figure 4.1). These pathogens each have a different commensal colonization niche of mammals. *S. aureus* colonizes the skin and nasal cavity; *S. pneumoniae* colonizes the upper airway; and *E. faecalis*, named for its primary isolation source [28], colonizes the gut. While there were only 18 skin isolates analyzed within the DegV network, ~44% of these were found to appear in only two

Table 4.1. Number of nodes in which organisms from specific isolation sources appear.

Shown in percentages is the number of nodes that organisms from different isolation sources appeared in.

Organism isolation source	Number of organisms	% in one node	% in two nodes	% in three nodes	% in four nodes	% in five nodes	% in six+ nodes
Gut	1194	22.3	23.9	24.3	20.9	5.8	2.8
Airways	74	5.4	13.5	50.0	31.1	0.0	0.0
Oral cavity/Throat	173	16.8	14.5	38.2	25.4	3.5	1.7
Other animal sources	259	10.4	22.8	33.6	29.0	2.7	1.5
Water	488	32.4	19.9	24.0	9.8	8.8	5.1
Terrestrial	478	20.7	13.2	43.1	12.6	8.4	2.1
Atmosphere	12	16.7	8.3	50.0	16.7	8.3	0.0
Food	211	4.7	14.7	33.2	24.2	19.4	3.8
Plants/algae	204	8.8	18.6	44.1	22.1	4.9	1.5
Manmade	154	21.4	22.1	24.7	18.2	7.1	6.5
Multiple	24	4.2	16.7	16.7	58.3	4.2	0.0
Other	4	0.0	0.0	100	0.0	0.0	0.0
Unclassified	148	12.8	23.0	19.6	35.8	6.1	2.7

nodes, indicating that necessity for increased numbers fatty acid binding domains is reduced relative to organisms which colonize the gut and respiratory pathways. These data are indicative of potential differences in nutrient scavenging or regulation needs for organisms which reside in different environments.

Deletion of fakB genes increases basal daptomycin tolerance

As a commensal member of the gut microbiota, *E. faecalis* is likely exposed to high concentrations of and numerous fatty acids in its environment. Specifically, bile contains palmitic acid (C_{16:0}), stearic acid (C_{18:0}), oleic acid (C_{18:1 cis9}), and linoleic acid (C_{18:2 cis9,12}) [19]. Of note, exposure to these fatty acids alters overall physiology, including cell morphology and growth patterns of *E. faecalis* [29]. Further, specific fatty acids can confer the ability to survive in bactericidal concentrations of the antibiotic daptomycin [18, 19, 29-31]. Specifically, long-term exposure of *E. faecalis* to specific fatty acids, namely the unsaturated fatty acids found in bile and serum, oleic acid (C_{18:1 cis9}) and linoleic acid (C_{18:2 cis9,12}), conferred protection to membrane damaging agents, including the detergent sodium dodecyl sulfate (SDS) and the membrane depolarizing antibiotic daptomycin [19, 29]. We thus investigated whether the *fakB* homologs play a role in inducing tolerance to these agents in the presence of known protective fatty acids in *E. faecalis*. Specifically, we wished to address whether these beneficial fatty acids need to be incorporated onto phospholipid head groups *via* the Fak system to induce daptomycin tolerance. We first examined the *fakB* deletion strains for their basal sensitivity to the antibiotic without exogenous fatty acid supplementation. We found that the basal tolerance of $\Delta fakB1,2,4$ and $\Delta fakB1,4,5$ was increased relative to wild-type (Figure 4.3), indicating that together, the FakB proteins of *E. faecalis* contribute to the efficacy of daptomycin.

We previously observed that combined deletion of *fakB1*, *fakB2*, and *fakB5* (e.g., *fakB4* only) led to several phenotypic differences from wild-type, including hyperseptation of cells in unsupplemented medium and increased generation time in medium supplemented with unsaturated fatty acids (Chapter 3; Figure 3.3, Tables 3.3 and 3.4). Further, supplementation of $\Delta fakB1,2,5$ with fatty acids did not overcome inhibition of *de*

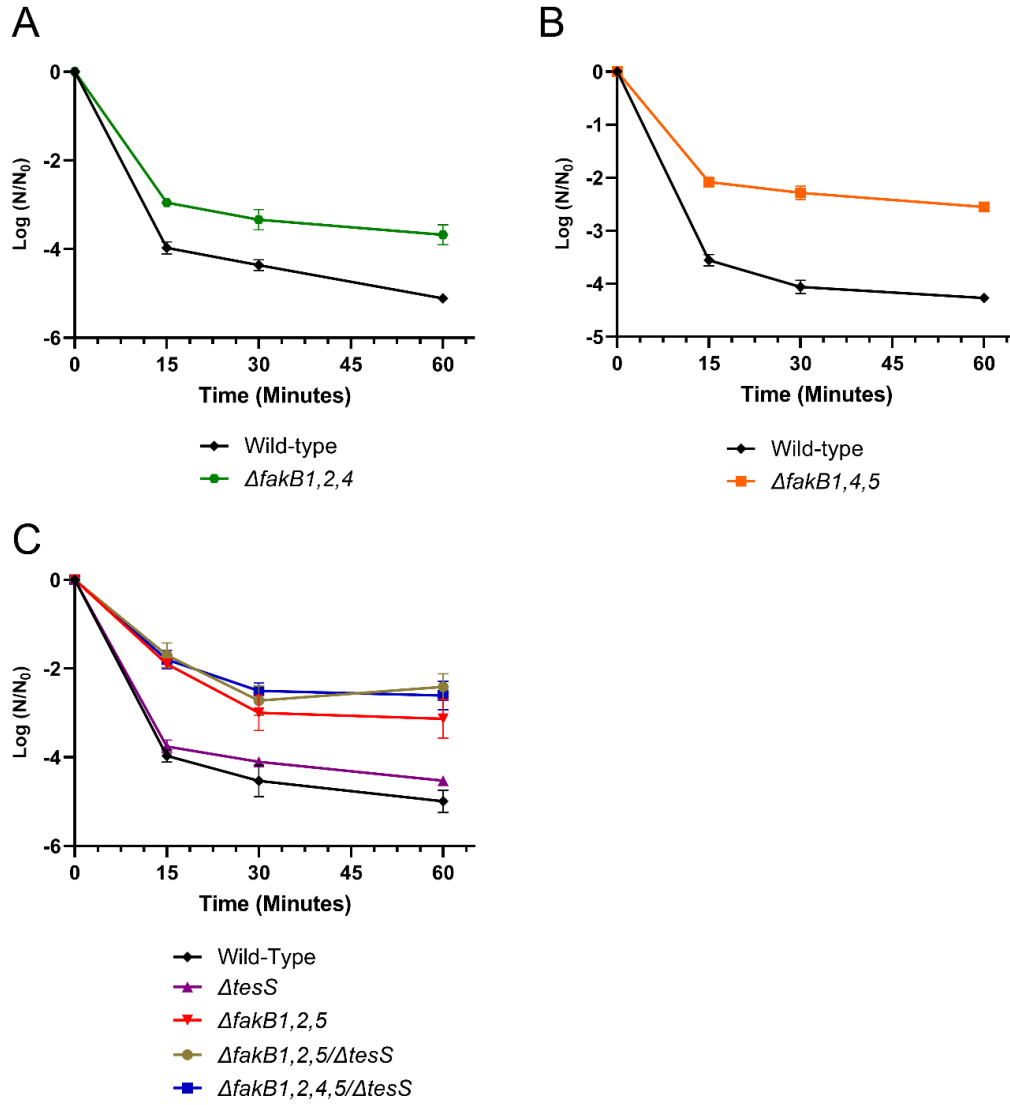


Figure 4.3. Deletion of *fakB* genes results in increased basal tolerance against daptomycin.

Wild-type *E. faecalis* OG1RF and (A) $\Delta fakB1,2,4$, (B) $\Delta fakB1,4,5$, and (C) $\Delta tesS$, $\Delta fakB1,2,5$, $\Delta fakB1,2,5/\Delta tesS$, and $\Delta fakB1,2,4,5/\Delta tesS$ were grown in BHI until mid-logarithmic phase, were harvested, washed, and challenged with daptomycin at 30 $\mu\text{g/mL}$. Depicted are the average log ratios of survivors over time $\pm$ standard deviations. $n = 3$ replicates for all experiments.

novo fatty acid biosynthesis by the drug cerulenin, (Chapter 3; Figure 3.4; [29]), suggesting that $\Delta fakB1,2,5$ could no longer incorporate protective fatty acids into its phospholipids. As cellular morphology is driven by cell wall placement, and $\Delta fakB1,2,5$ had gross differences in its morphology (Chapter 3; Figure 3.2), and because lipid II, a peptidoglycan precursor, has been indicated as a target of daptomycin [32], we wished to determine whether this strain had increased daptomycin tolerance. We thus challenged $\Delta fakB1,2,5$ (as above) with daptomycin, and observed a large increase in survivors relative to wild-type (Figure 4.3C).

Altered phospholipid composition and levels of free fatty acids in the cell were previously linked to ability to withstand daptomycin treatment [19, 31]. As we previously observed, $\Delta fakB1,2,5$ had increased levels of free fatty acids (note, normalized to phosphatidylglycerol) in the membrane (Chapter 3; Figure 3.3). Therefore, our observations suggest that the daptomycin tolerance observed in $\Delta fakB1,2,4$, $\Delta fakB1,4,5$, and $\Delta fakB1,2,5$ may be driven by an accumulation of free fatty acids in the membrane. Notably, free fatty acid levels were restored to wild-type levels in the quintuple deletion strain $\Delta fakB1,2,4,5/\Delta tesS$, which was deleted for the *fakB* genes along with a thioesterase thought to cleave fatty acids from acyl-ACP ([33]; Chapter 3; Figure 3.3). Given that the quintuple deletion strain had wild-type levels of free fatty acids normalized to phosphatidylglycerol, we examined whether the basal tolerance of the quintuple deletion strain was reverted to wild-type basal tolerance. We found, however that the quintuple deletion strain also had increased basal daptomycin tolerance relative to wild-type (Figure 4.3). This phenotype was not driven by deletion of *tesS*, as deletion of *tesS* did not increase basal tolerance. These data suggest that free fatty acids may not be the drivers for increasing basal daptomycin tolerance.

Exposure of fakB deletion strains to fatty acids still induces protection to daptomycin

While the *fakB* deletion strains displayed increased basal tolerance to daptomycin relative to wild-type *E. faecalis*, this observation did not disentangle whether incorporation of

exogenous fatty acids is required for induction of tolerance to daptomycin. Our previous data demonstrated that combined deletion of *fakB1*, *fakB2*, and *fakB5* resulted in a strain which was unable to utilize exogenous fatty acids to rescue growth inhibition by the *de novo* fatty acid biosynthesis inhibitor cerulenin (Chapter 3; Figure 3.4). These results indicated that incorporation of exogenous fatty acids into membrane phospholipids was greatly reduced. Thus, we exposed our deletion strains to protective fatty acids and assayed for daptomycin tolerance. Given that the deletion strains grew slowly relative to the wild-type strain in media supplemented with specific fatty acids, we used short-term exposure of fatty acids. Specifically, we exposed cultures to oleic acid or linoleic acid at log phase, grew an additional half hour, then challenged with daptomycin. We found that short-term exposure to oleic acid still induced protection against daptomycin in $\Delta fakB1,2,4$, $\Delta fakB1,4,5$ and $\Delta fakB1,2,4,5/\Delta tesS$ (Figure 4.4). However, for each of these strains, the fold protection was reduced by ~1 log relative to protection observed for the wild-type strain at every time point (Table 4.2). These trends were repeated on exposure of the strains to linoleic acid (Figure 4.5 and Table 4.2). However, linoleic acid was 3 logs more protective in wild-type compared to $\Delta fakB1,4,5$ (Table 4.2). Notably, exposure of $\Delta fakB1,2,5$ to these fatty acids did not induce protection; indeed, the solvent control in these experiments yielded no difference from exposure to the fatty acids oleic acid (Figure 4.4, Table 4.2) and linoleic acid (Figure 4.5, Table 4.2).

To test the protective role of fatty acids in $\Delta fakB1,2,5$, we used daptomycin at a higher (40 $\mu\text{g/mL}$) concentration (as has been used for resistant isolates in the past; [18]) to accommodate the high basal tolerance of this strain. We noted that exposure of $\Delta fakB1,2,5$ to a higher concentration of daptomycin caused a decrease in survivors relative to the starting population (Figure 4.6). Additionally, oleic acid was protective against daptomycin at all time points in these cases (Figure 4.6A), yet when $\Delta fakB1,2,5$ was treated with linoleic acid, a short-term exposure resulted in a significant difference between the solvent control and linoleic acid-treated cells at early time points but not at late time points (Figure 4.6B; $P < 0.05$ after 15 and 30 minutes of daptomycin exposure and $P > 0.05$ at the remaining time points).

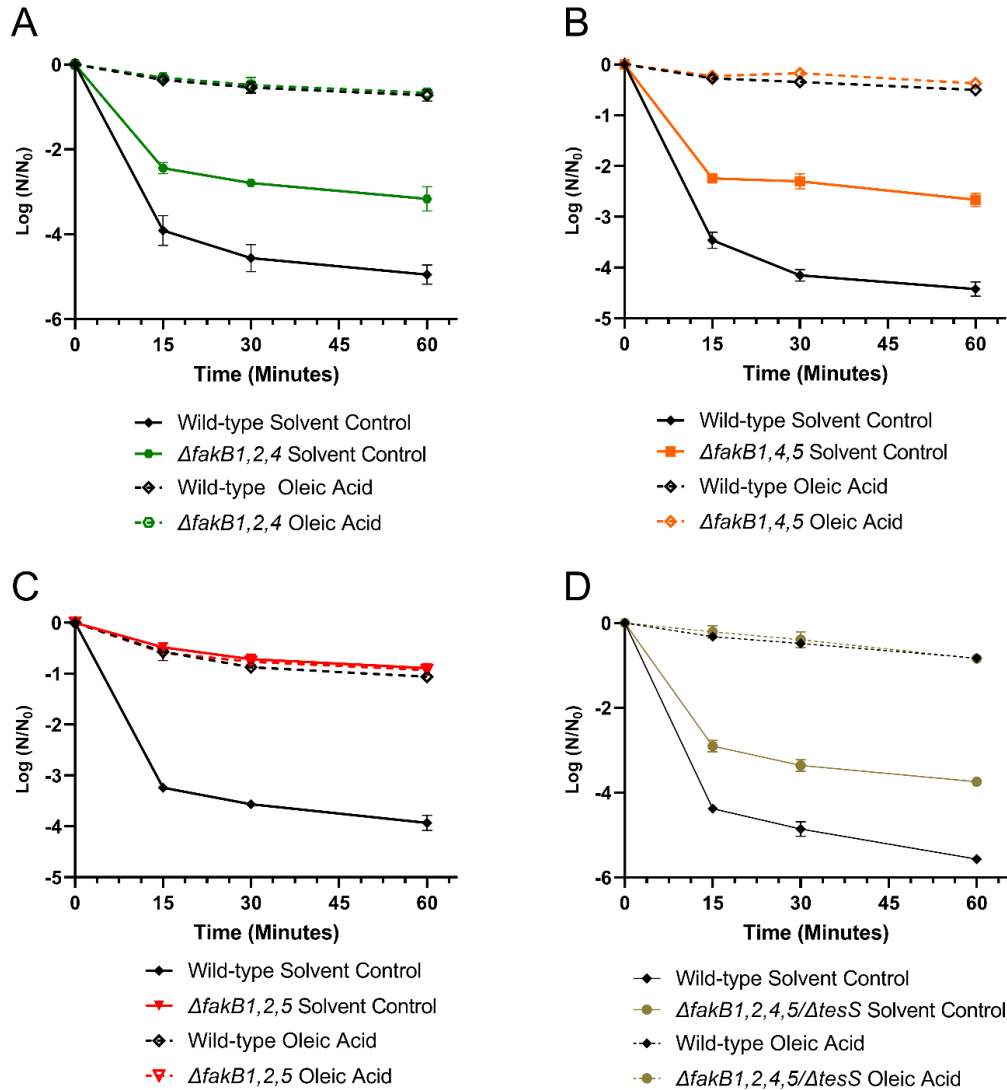


Figure 4.4. Addition of oleic acid protects against daptomycin in *fakB* deletion backgrounds.

Wild-type *E. faecalis* OG1RF and (A) $\Delta fakB1,2,4$, (B), $\Delta fakB1,4,5$ (C) $\Delta fakB1,2,5$, and (D) $\Delta fakB1,2,4,5/\Delta tesS$ were grown to exponential phase, then the solvent control or 20 $\mu\text{g/mL}$ oleic acid were added. The cells were then grown an additional 30 minutes prior to harvesting. After harvest, cells were washed and challenged with daptomycin at 30 $\mu\text{g/mL}$. Depicted are the log ratios of survivors over time. $n = 3$ replicates for all experiments.

Table 4.2. Log fold protection against daptomycin induced by addition of fatty acids.

Represented are the average log fold protection values $\pm$ standard deviations induced by addition of oleic acid or linoleic acid relative to the solvent control.

		Oleic acid		Linoleic acid	
Strain	Time (min)	Log fold protection	<i>P</i> -value against Wild-type	Log fold protection	<i>P</i> -value against Wild-type
30 µg/mL daptomycin					
Wild-type ^a	0	0 ± 0	NA	0 ± 0	NA
	15	3.4 ± 0.6	NA	3.5 ± 0.6	NA
	30	3.7 ± 0.7	NA	3.8 ± 0.8	NA
	60	3.9 ± 0.7	NA	4.1 ± 1.0	NA
<i>ΔfakB1,2,4</i>	0	0 ± 0	<i>P</i> = 1	0 ± 0	<i>P</i> = 1
	15	2.1 ± 0.2	<i>P</i> < 0.01	2.6 ± 0.1	<i>P</i> < 0.01
	30	2.3 ± 0.1	<i>P</i> < 0.01	3.0 ± 0.3	<i>P</i> = 0.03
	60	2.5 ± 0.3	<i>P</i> < 0.01	3.1 ± 0.2	<i>P</i> = 0.02
<i>ΔfakB1,4,5</i>	0	0 ± 0	<i>P</i> = 1	0 ± 0	<i>P</i> = 1
	15	2.0 ± 0.1	<i>P</i> < 0.01	0.8 ± 0.2	<i>P</i> < 0.01
	30	2.1 ± 0.1	<i>P</i> < 0.01	0.9 ± 0.1	<i>P</i> < 0.01
	60	2.3 ± 0.1	<i>P</i> < 0.01	0.8 ± 0.1	<i>P</i> < 0.01
<i>ΔfakB1,2,5</i>	0	0 ± 0	<i>P</i> = 1	0 ± 0	<i>P</i> = 1
	15	-0.1 ± 0.1	<i>P</i> < 0.01	0.0 ± 0.1	<i>P</i> < 0.01
	30	0.0 ± 0.1	<i>P</i> < 0.01	0.1 ± 0.1	<i>P</i> < 0.01
	60	0.0 ± 0.1	<i>P</i> < 0.01	0.1 ± 0.1	<i>P</i> < 0.01
<i>ΔfakB1,2,4,5/ΔtesS</i>	0	0 ± 0	<i>P</i> = 1	0 ± 0	<i>P</i> = 1
	15	2.3 ± 0.2	<i>P</i> < 0.01	2.7 ± 0.1	<i>P</i> < 0.01
	30	3.0 ± 0.1	<i>P</i> < 0.01	2.9 ± 0.2	<i>P</i> = 0.01
	60	2.9 ± 0.1	<i>P</i> < 0.01	2.9 ± 0.1	<i>P</i> < 0.01

^a $n=12$ for wild-type replicates; $n = 3$ for other deletion strains.

^bWild-type was not compared, as daptomycin concentrations tested were different.

Table 4.2 Continued

		Oleic acid		Linoleic acid	
Strain	Time (min)	Log fold protection	<i>P</i> -value against Wild-type	Log fold protection	<i>P</i> -value against Wild-type
40 µg/mL daptomycin ^b					
<i>ΔfakB1,2,5</i>	0	0.0 ± 0.0	NA	0.0 ± 0.0	NA
	15	0.6 ± 0.1	NA	1.3 ± 0.2	NA
	30	0.5 ± 0.1	NA	1.1 ± 0.2	NA
	60	0.7 ± 0.2	NA	0.5 ± 0.2	NA
	120	0.6 ± 0.3	NA	0.2 ± 0.3	NA
	240	0.8 ± 0.1	NA	0.1 ± 0.3	NA

^a*n*=12 for wild-type replicates; *n* = 3 for other deletion strains.

^bWild-type was not compared, as daptomycin concentrations tested were different.

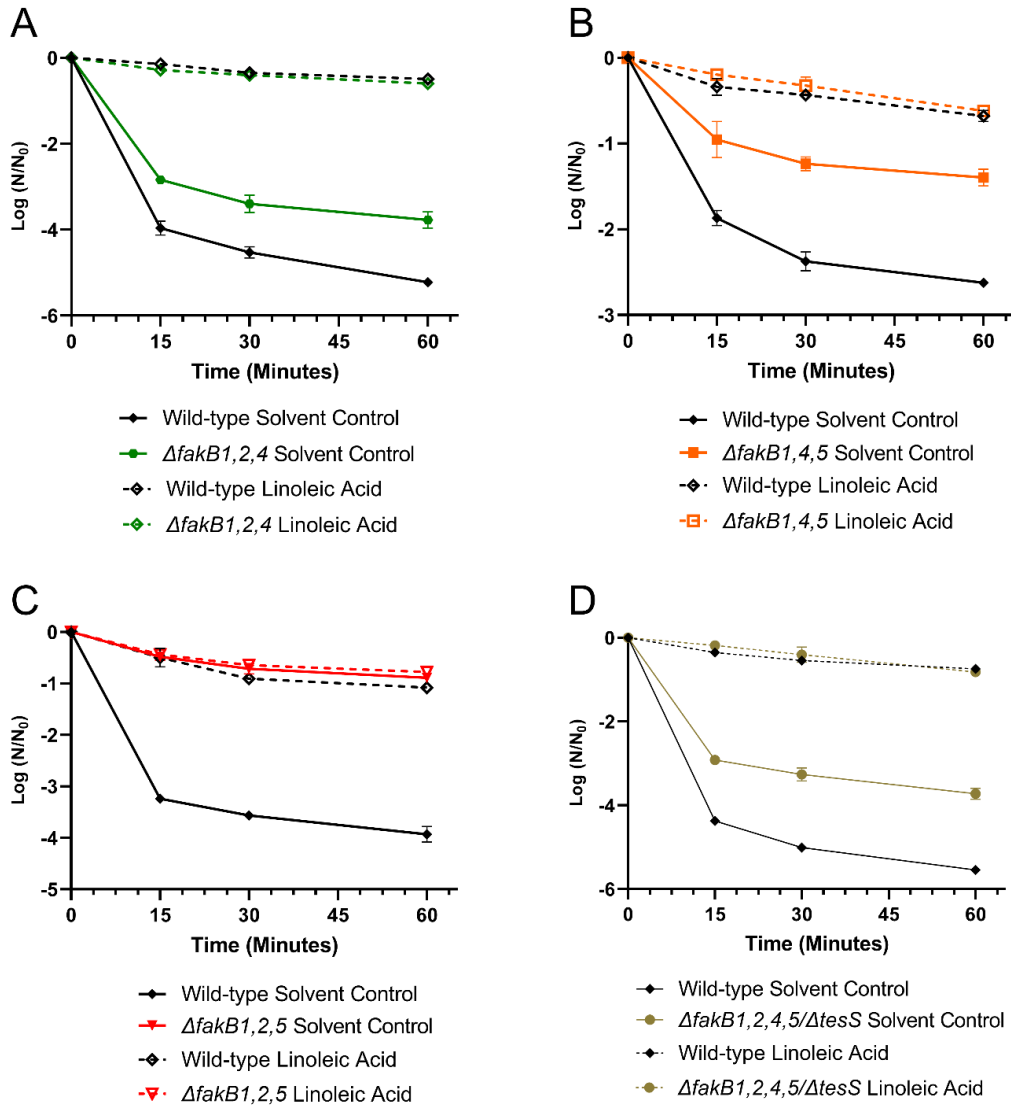


Figure 4.5. Addition of linoleic acid protects against daptomycin in *fakB* deletion backgrounds.

Wild-type *E. faecalis* OG1RF and (A) $\Delta fakB1,2,4$, (B), $\Delta fakB1,4,5$ (C) $\Delta fakB1,2,5$, and (D) $\Delta fakB1,2,4,5/\Delta tesS$ were grown to exponential phase, then the solvent control or 10 $\mu\text{g/mL}$ linoleic acid were added. The cells were then grown an additional 30 minutes prior to harvesting. After harvest, cells were washed and challenged with daptomycin at 30 $\mu\text{g/mL}$. Depicted are the log ratios of survivors over time. $n = 3$ replicates for all experiments.

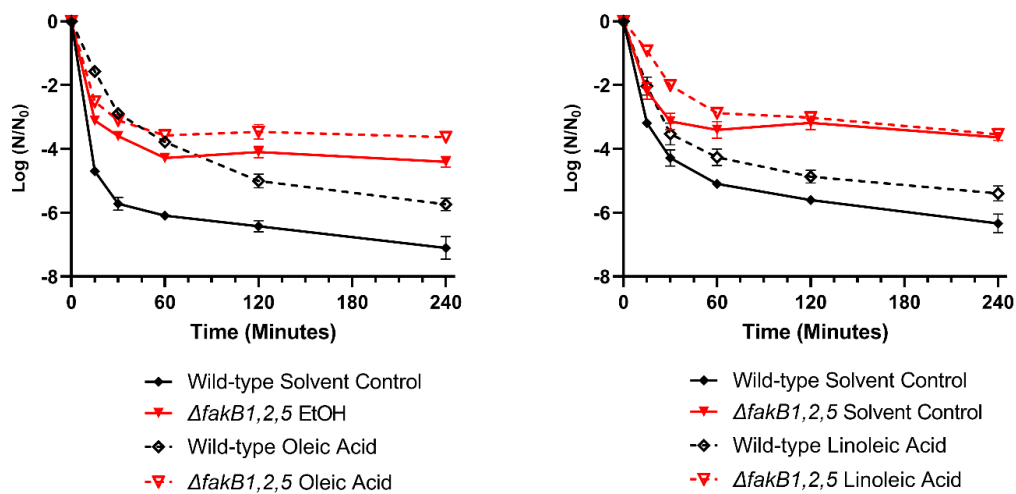


Figure 4.6. Oleic acid and linoleic acid protect $\Delta fakB1,2,5$ against daptomycin, but only at concentrations above that seen for other strains.

The solvent control and (A) 20 $\mu\text{g/mL}$ oleic acid or (B) 10 $\mu\text{g/mL}$ linoleic acid were added to exponential phase cultures of wild-type *E. faecalis* OG1RF and $\Delta fakB1,2,5$. The cells were then grown an additional 30 minutes prior to harvesting. After harvest, cells were washed and challenged with daptomycin at 30 $\mu\text{g/mL}$ (wild-type) or 40 $\mu\text{g/mL}$ ($\Delta fakB1,2,5$) daptomycin. Depicted are the log ratios of survivors over time. $n = 3$ replicates for all experiments.

Daptomycin and other cell wall-targeting antibiotics do not have a higher MIC against $\Delta fakB1,2,5$ relative to wild-type *E. faecalis*

As we observed that $\Delta fakB1,2,5$ had increased basal tolerance relative to wild-type *E. faecalis*, we tested the minimal inhibitory concentration (MIC) of daptomycin against $\Delta fakB1,2,5$. The $\Delta fakB1,2,5$ strain had the same daptomycin MIC as wild-type (Table 4.3). Of note, recent evidence suggests that daptomycin is a wall-targeting antibiotic [32], and we previously noted that removal of the cell wall of *E. faecalis* resulted in increased daptomycin tolerance ([34]; Chapter 2). Furthermore, $\Delta fakB1,2,5$ had hyperseptation relative to wild-type *E. faecalis* (Chapter 3; Figure 3.2). Thus, we tested the efficacy of the cell wall-targeting antibiotics ampicillin and fosfomycin against $\Delta fakB1,2,5$ (Table 4.3), and observed no difference. These data suggest that there is a difference between the killing kinetics observed when performing a cell survival assay (which is a short-term exposure at 7-10X the MIC of the antibiotic) and performing an MIC assay (a long-term exposure at ~1X the MIC of the antibiotic). These data indicate that while $\Delta fakB1,2,5$ is not resistant to daptomycin, as it does not have increased ability to grow in it or in other peptidoglycan-targeting drugs. However, it is able to better survive short-term exposure to high concentrations of daptomycin relative to wild-type.

Discussion

In this study, we identified that two out of four of the FakB homologs of *E. faecalis* clustered spatially with the characterized FakB proteins of *S. aureus* and *S. pneumoniae*, indicating that they may share similar functions. Notably, none of the *E. faecalis* FakB homologs clustered with FakB3 of *S. pneumoniae*, which is specific for binding of polyunsaturated fatty acids [21]. However, we observed distinct clustering of the remaining two FakB homologs of *E. faecalis* in other regions of the network. efFakB4 (OG1RF_RS00080) clustered in a densely connected portion of the network, which was predominantly populated with organisms isolated from the gut (Figure 4.1). This potentially indicates that this specific FakB homolog may play an important role in scavenging fatty acids abundant in the gut. It is possible that while deletion of the gene

Table 4.3. Minimal inhibitory concentrations of daptomycin, ampicillin, and fosfomycin for wild-type OG1RF and $\Delta fakB1,2,5$.

Antibiotic	Bacterial strain	MIC range ($\mu\text{g/mL}$)
Daptomycin	Wild-type	3-4
	$\Delta fakB1,2,5$	1-3
Ampicillin	Wild-type	2-6
	$\Delta fakB1,2,5$	2-4
Fosfomycin	Wild-type	512-1024
	$\Delta fakB1,2,5$	256-512

encoding this homolog did not cause specific phenotypes to arise on supplementation with long-chain fatty acids (Chapter 3; Tables 3.3 and 3.4), that it may be central for utilization of other resources, including short-chain fatty acids, which are produced by members of the gut microbiota [35]. The other FakB homolog (efFakB5), OG1RF_RS05020, clustered distinctly with a predominantly Gram-positive region of the network containing other organisms with a variety of isolation sources.

While there were numerous Gram-negative organisms annotated within cluster 1, with no distinct spatial organization within the network, we noted that some of these were in a “gray” area in terms of classification. For instance, the genus *Acidaminococcus*, while noted as being Gram-negative *via* staining, is more closely related to Gram-positive organisms [36]. Similarly, the genus *Dehalobacter*, which came up in the network analysis and was also annotated as being Gram-negative, is a member of the clostridia class, which is made up of predominantly Gram-positive organisms. *Paenibacillus* species annotated as being Gram-negative also appeared. This genus is known to stain in a Gram-variable manner [37] – thus it is of little surprise that several isolates were annotated as Gram-negative. Other Gram-negative organisms within the cluster analyzed included members of the well-known genera *Deinococcus*, *Thermus*, *Pseudomonas*, and *Fusobacterium*. An underwhelming number of the species annotated in cluster 1 were of Gram-negative origin, however this does not rule out that other clusters not examined contained higher ratios of Gram-negative to Gram-positive organisms.

Interestingly, we also observed an archaeote isolated from the gut of a cow [25]. Given that there was only one such example of an archaeote, this leads to speculation on whether there could have been a horizontal gene transfer event between another gut bacterium and *Methanomassiliicoccales* archaeon RumEn M1. However, the evidence for this from our analysis is poor, given that while the other organisms within this node were of gut origin, none were from the rumen of a cow. Similarly, one could speculate that the virus (Streptococcus phage phiZJ20091101-1) annotated within this study acquired the DegV domain from its *Streptococcus* host, yet that specific isolate was not found within our network.

One captivating observation that we made was that organisms partially clustered according to isolation source. Specifically, several distinct subclusters appeared to contain more isolates which were found in animal sources, while a large subcluster at the center of the network appeared to have more isolates from water sources. Of note, many of the nodes which had multiple or unclassified Gram-staining also appeared within water environments. Over 33% of the Gram-negative organisms annotated as a part of this study were from water sources, while over 28% of the unclassified organisms were also from water sources, potentially accounting for this observation. Many of the unclassified organisms were from metagenome analyses, where a genome of a strain was assembled, but not further classified experimentally.

We observed a large subcluster of organisms, which included efFakB4. Note that there was no specific phenotype different from wild-type associated with long-chain fatty acid supplementation upon deletion of *fakB4* (Chapter 3; Tables 3.3 and 3.4). However, the efFakB4 homologs within the network contained many isolates of animal gut origin (Figure 4.2). These observations lead to the question of whether there is a specific fatty-acid binding motif which is important for survival under different conditions. Further, our data highlight that some environments, such as the animal gut, may select for more copies of FakB homologs, potentially with different binding specificities. These observations call into question why specific strains may have more copies of genes encoding FakB proteins. We and others noted that *S. aureus* often has two copies of FakB, *S. pneumoniae* often has three, and *E. faecalis* primarily has four (Supplemental Figure 4.1) [27]. Given that *S. aureus* resides on the skin or in the nares, it is likely that it has little need to scavenge for fatty acids, as the fatty acids present are often antimicrobial in nature [38-41]. However, it may be beneficial for this organism to be able to bind and subsequently detoxify the fatty acids with which it comes into contact. Additionally, incorporation of specific fatty acids can be detrimental to the bacterium: uptake of linoleic acid (C_{18:2 cis9,12}) by *S. aureus* boosted the host immune response against it [42]. In the case of *S. pneumoniae*, which resides in the upper respiratory tract, exposure to fatty acids likely occurs on coming into contact with sputum, of which most of the dry weight

consists of fatty acids [43], including palmitic acid (C_{16:0}), stearic acid (C_{18:0}) and oleic acid (C_{18:1 *cis*9}) [44]. Notably, although *S. pneumoniae* has a FakB (spFakB3) specific for binding polyunsaturated fatty acids, this class of fatty acids was not detected in sputum samples [44]. However, sputum samples from patients with different conditions (tuberculosis and other diseases) had different compositions, indicating that *S. pneumoniae* could retain FakB3 for surviving under specific conditions [44]. One of these conditions could be to respond to fatty acids released as a part of inflammation, such as arachidonic acid (C_{20:4}).

As a resident of the mammalian gut, *E. faecalis* is likely exposed to an abundance of fatty acids. The fatty acids present in bile have varying effects on *E. faecalis* physiology, with alterations in morphology induced by saturated fatty acids [29] and increases in ability to tolerate daptomycin and membrane stressors induced by specific unsaturated fatty acids [18, 19, 29-31]. Prior to this work, we hypothesized that induction of protection against daptomycin by exogenous fatty acids was due to its incorporation onto phospholipid head groups. However we instead found that deletion of the *fakB* genes, which are required for incorporation of fatty acids onto phospholipids (Chapter 3; Figure 3.4) resulted in increased basal daptomycin tolerance. As we had previously observed that free fatty acids were increased in Δ *fakB*1,2,5 (Chapter 3; Figure 3.3), we next hypothesized that free fatty acids in this strain were responsible for inducing daptomycin tolerance (Figure 3.3). However, in our quintuple deletion strain, which had wild-type levels of free fatty acids normalized to phosphatidylglycerol (Chapter 3; Figure 3.3), the basal tolerance was not restored to wild-type levels. However, the quantification of free fatty acids performed by thin layer chromatography (Chapter 3; Figure 3.3) is not the most accurate way to quantify fatty acids, and our analysis of free fatty acids did not take into account that phospholipid levels may differ between strains. Therefore, to better understand whether the quintuple deletion strain has free fatty acid levels similar to that of wild-type, mass spectrometry analyses will need to be performed.

Another hypothesis for why deletion of the *fakB* genes would drive daptomycin tolerance up could be that deletion of the *fakB* genes alters cell wall placement due to

altered lipid metabolism. This is supported through the data demonstrating hyperseparation of $\Delta fakB1,2,5$ (Chapter 3; Figure 3.2). Peptidoglycan synthesis is closely tied to the membrane. Indeed, the peptidoglycan precursors lipid I and lipid II, and the enzymes which act upon them, MraY, MurG, MurM, MurN, and MurJ are membrane-bound. Thus, it is possible that alterations of membrane organization through altered lipid biosynthesis might drive mislocalization of the enzymes and lipid-bound precursors of peptidoglycan. Mislocalization of lipid II, a potential target of daptomycin; [32, 34]) might then drive increased basal daptomycin tolerance.

While basal daptomycin tolerance was increased in our deletion strains, fatty acid supplements still provided some protection against daptomycin in the *fakB* deletion strains (Figures 4.3–4.5), although not to the same extent as wild-type. These findings indicate that exogenous fatty acids cause other physiological changes which induce daptomycin tolerance. One known physiological effect of exogenous fatty acids on cells is altered cellular morphology [29]. Specifically, supplementation with palmitic acid (C_{16:0}), myristic acid (C_{14:0}), and stearic acid (C_{18:0}) resulted in aberrant placement of septa, while supplementation with palmitoleic acid (C_{16:1 cis9}) resulted in a decrease in cell length [29]. Additionally, supplementation of cells with exogenous fatty acids was demonstrated to increase the levels of free fatty acids within *E. faecalis* cells [31, 45]. In the $\Delta fakB1,2,5$ deletion strain, we noted both an increase in free fatty acids and multiple septations present in individual “cells” (Chapter 3; Figures 3.2 and 3.3). In this deletion strain, we observed that addition of exogenous fatty acids did not induce protection beyond its basal level of daptomycin tolerance until we increased the daptomycin concentration (Figures 4.4–4.6). This suggested that the physiological differences between these cells and wild-type *E. faecalis* may partially correspond with the changes induced by supplementation with protective fatty acids. These data indicate that supplementation with free fatty acids may induce daptomycin tolerance by altering the placement of cell division machinery, including peptidoglycan synthesis proteins.

Overall, our data suggests that the specificities of DegV domains may be predictable based on network clustering, as efFakB2 clustered with saFakB2 and spFakB2 and

deletion of *fakB2* in *E. faecalis* yielded an increased generation time in media supplemented with unsaturated fatty acids (Chapter 3; Table 3.3). However, discrepancies in observed phenotypes and fatty acid binding specificities may occur. Our previous data indicated that efFakB1 may be a generalist fatty acid binding protein, as deletion of the gene encoding this protein was necessary to elicit phenotypes in media supplemented with both saturated and unsaturated fatty acids (Chapter 3; Tables 3.3 and 3.3). The efFakB1 DegV domain, however, clustered with the FakBs of *S. aureus* and *S. pneumoniae* specific for binding saturated fatty acids [20, 21]. This discrepancy could potentially be explained if *fakB1* of *E. faecalis* is more highly expressed than the other *fakB* genes. However, biochemical data indicating fatty acid binding specificities, along with data elucidating transcription, translation, and efficiency of efFakB1 relative to the other efFakBs, are needed to explain these data.

Overall, our data suggest that specific fatty acid binding domains are important for organisms to survive and thrive in differing environments. Further, our data indicate that disturbing the phospholipid biosynthesis pathway has broad impacts on the cell, as cell wall placement, growth in the presence of fatty acids, and cell viability (Chapter 3; Figures 3.2, Tables 3.3 and 3.4) are impacted on deletion of the *fakB* genes. However, the specific impacts of deleting these genes and supplementation with exogenous fatty acids on daptomycin tolerance remains to be discovered.

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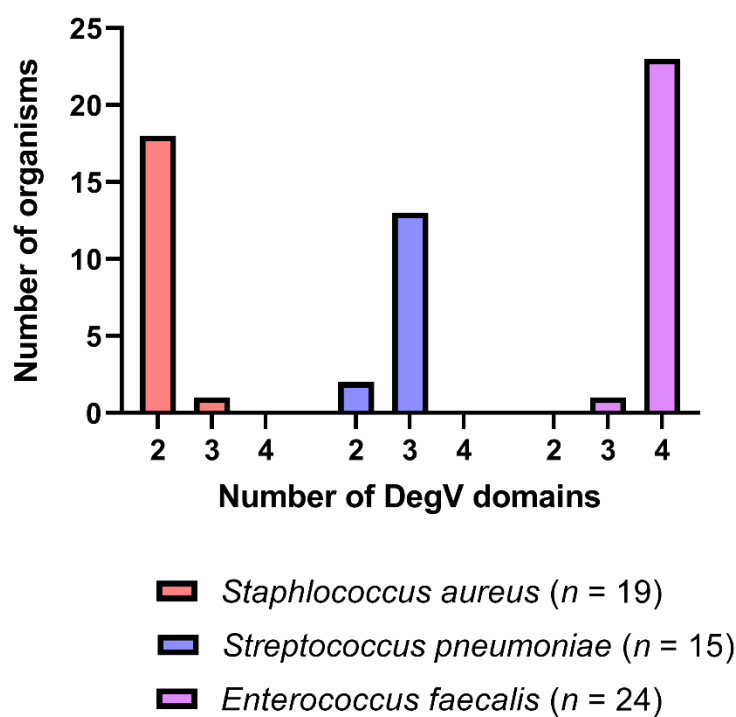
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Appendix

Supplemental Table 4.1. Bacterial strains and plasmids used in this study.

Strain	Relevant genotype or description	Source
<i>Enterococcus faecalis</i> OG1RF	Laboratory strain Rif ^R , Fus ^R	Lab stock
$\Delta fakB1,2,4$	<i>E. faecalis</i> OG1RF deleted for <i>fakB1</i> , <i>fakB2</i> , and <i>fakB4</i>	Chapter 3
$\Delta fakB1,4,5$	<i>E. faecalis</i> OG1RF deleted for <i>fakB1</i> , <i>fakB4</i> , and <i>fakB5</i>	Chapter 3
$\Delta fakB1,2,5$	<i>E. faecalis</i> OG1RF deleted for <i>fakB1</i> , <i>fakB2</i> , and <i>fakB5</i>	Chapter 3
$\Delta fakB1,2,5/\Delta tesS$	<i>E. faecalis</i> OG1RF deleted for <i>fakB1</i> , <i>fakB2</i> , <i>fakB5</i> , and <i>tesS</i>	Chapter 3
$\Delta fakB1,2,4,5/\Delta tesS$	<i>E. faecalis</i> OG1RF deleted for <i>fakB1</i> , <i>fakB2</i> , <i>fakB4</i> , <i>fakB5</i> , and <i>tesS</i>	Chapter 3



Supplemental Figure 4.1. DegV domain numbers of *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis*.

Represented are the number of isolates of *Staphylococcus aureus* (red), *Streptococcus pneumoniae* (blue), and *Enterococcus faecalis* (purple) which had two, three, or four DegV domains.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

This article was written by Rachel D. Johnston with editorial comments from Elizabeth M. Fozo. It has not been published anywhere, nor will it be before I turn in the final version at my ETD, so I did not include a publication statement.

Introduction

Daptomycin tolerance can be induced by supplementing *Enterococcus faecalis* cultures with specific exogenous fatty acids [1-5]. However, the mechanism underlying this phenomenon has not been fully elucidated. Previous work has ruled out links between fatty acid-induced daptomycin tolerance and membrane fluidity (*via* anisotropy) [1]. Furthermore, supplementation of *E. faecalis* cultures with protective fatty acids induced protection even in the absence of a functional LiaFSR system, which has been implicated in daptomycin resistance, indicating that fatty acid-induced protection was not dependent on LiaFSR [2]. Mass spectrometry data, combined with gas chromatography analyses, indicated that supplementation with fatty acids results in decreased phosphatidylglycerol and increased cardiolipin species [5, 6]. These data, combined with findings indicating phospholipid changes occurred in daptomycin resistant mutants [7, 8], led to the hypothesis that specific changes in lipid composition are responsible for the induction of daptomycin tolerance by free fatty acids. Specifically, cardiolipin synthase genes were mutated in daptomycin resistant isolates [9-11]. Interestingly however, while deletion of the specific genes required for synthesizing cardiolipin eliminated cardiolipin production, it did not eliminate the protection afforded by exposure to the protective fatty acids oleic acid (C_{18:1} *cis*9) or linoleic acid (C_{18:2} *cis*9,12) [5]. This indicates that changes in cardiolipin production induced by fatty acid exposure did not result in daptomycin protection [5]. The mechanism of daptomycin thus remained unclear.

Notably, daptomycin treatment results in a pattern of biphasic killing [2-4, 12-16], which is indicative of the presence of a subpopulation of persister cells. Persister cells, while genetically identical to the non-persister portion of the population [17], are killed slowly by the drug [18, 19] and can outgrow on removal of the drug from the growth medium [20-22]. Daptomycin persister cells can be awakened by the addition of glucose

to the media [16]. On awakening, these persisters were more efficiently killed by daptomycin [16]. Thus, we wanted to determine whether daptomycin tolerance could be induced by perturbation of active cell processes. Recent literature proposed that daptomycin directly interacts with a peptidoglycan (PG) precursor, lipid II [23]. Notably, fatty acids alter the morphology of *E. faecalis*, and cellular morphology is directly linked to placement of the cell wall. Thus, we tested how cell wall and incorporation of exogenous fatty acids into membrane lipids play a role in daptomycin efficacy.

Cell wall and daptomycin efficacy

In the literature, new evidence supports that daptomycin's primary mechanism of action is to bind to the lipid-bound peptidoglycan precursor, lipid II, impacting cell wall synthesis [23]. Our findings in Chapter 2 corroborate these data by demonstrating that daptomycin tolerance-inducing conditions lead to changes in peptidoglycan precursor levels ([24]; Figures 2.1, 2.3, and 2.4). Additionally, we found that removal of the cell wall led to daptomycin tolerance. Furthermore, when we combined other cell wall-targeting antibiotics with daptomycin, they acted in an additive or synergistic fashion ([24]; Table 2.1). These findings, along with the discovery that daptomycin strongly inhibited the incorporation of alanine into peptidoglycan and the formation of peptidoglycan precursors [25] and that daptomycin binds to lipid II [23], indicate that lipid II is a target of daptomycin. However, it remains unclear whether the daptomycin tolerance-inducing conditions elucidated in Chapter 2 (entry into stationary phase, blocked protein synthesis, and disruption of *de novo* fatty acid biosynthesis) alter the composition of the cell wall and whether cell wall composition plays a role in daptomycin tolerance. To probe this, it would be of major interest to isolate the cell wall of wild-type *E. faecalis* under daptomycin tolerance-inducing conditions as well as the cell wall of deletion strains tolerant to daptomycin (Chapter 4) to see if there is a clear pattern of changes inducing daptomycin tolerance. Preliminary evidence gathered through cell wall extraction and running the extract on a native polyacrylamide gel indicates that major shifts in cell wall composition occur under daptomycin tolerance inducing conditions (data not shown). However, the

specific chemical changes would need to be elucidated through compositional analysis, which is achievable through GC/MS [26]. If the changes are not chemical, as would be detected in the above assay, but rather a specific pattern of wall misplacement, it would be of interest to characterize localization of peptidoglycan synthesis proteins under daptomycin-tolerance inducing conditions. Given that daptomycin was demonstrated to interact with lipid II [23], localization of the enzyme responsible for synthesizing lipid II, MurG, is of particular interest.

Impact of cellular processes on membrane organization

While in Chapter 2 we demonstrated that peptidoglycan, fatty acid biosynthesis, and protein synthesis are important for daptomycin efficacy, the full impacts of these processes on membrane organization remain unclear. However, evidence in the literature suggests that membrane organization is critical for daptomycin function, as daptomycin localizes to and subsequently causes deterioration of regions of increased fluidity (RIFs) of the membrane, causing delocalization of several proteins [27, 28] and altered cell morphology [28]. Notably, lipid II, the putative target of daptomycin [23], has an undecaprenyl chain which is inserted into the membrane. This molecule has been observed to localize to regions of the membrane with sharp curvature [29]. Thus, it is crucial to elucidate how the processes indicated in daptomycin function, including peptidoglycan synthesis, protein synthesis, and *de novo* fatty acid biosynthesis impact membrane organization.

Treatment of cells with chloramphenicol, which blocks protein synthesis, alters membrane microdomain structure [30, 31], thus potentially explaining why treatment with this drug impacts daptomycin efficacy ([24]; Chapter 2; Figure 2.3). Further, treatment of *B. subtilis* cells with the drug cerulenin, which blocks *de novo* fatty acid biosynthesis, causes a down-regulation of flotillin [32] (*E. faecalis* homolog Accession: WP_002357808), a lipid raft-associated protein which is important for formation of membrane microdomains [33]. Flotillins of *B. subtilis* are critical for localization of peptidoglycan synthesis [34], potentially explaining older data which demonstrated that

treatment with cerulenin led to misplaced peptidoglycan synthesis [35]. These data suggest that multiple cellular processes impact membrane microdomains. However, it remains unclear whether alteration of protein synthesis, fatty acid biosynthesis, and peptidoglycan synthesis impact daptomycin efficacy by perturbing membrane microdomains.

Lipid II and membrane organization

As noted above, daptomycin interacts with lipid II [23], which in turn influences protein localization [36-38] and may facilitate bacterial cell division. Remarkably, while lipid II is a precursor to peptidoglycan, lipid II biosynthesis enzymes are conserved amongst and produced by bacterial species that lack cell wall, indicating that lipid II is important for processes other than peptidoglycan production [36]. For *Bacillus subtilis*, lipid II controls the membrane association of MreB, a cytoskeletal element which forms membrane-associated filaments that regulate cell wall synthesis and cell division [38]. Depletion of the enzyme responsible for generating lipid II, MurG, caused MreB to disassemble [38]. Lipid II is also responsible for the localization of MurJ, which flips lipid II from the inner membrane leaflet to the outer membrane leaflet [37]. Further, depletion of MurG (and thus lipid II) caused altered anionic lipid accumulation at the cell poles [39]. Moreover, sequestration of lipid II by nisin results in aberrant division site placement, with multiple sites of division and the formation of minicells [40]. These data suggest that localization of lipid II is critical for the recruitment of membrane-associated proteins, cell membrane organization, and localization of the divisome. Previous data from Saito *et al.* demonstrated that supplementation of *E. faecalis* cultures with free fatty acids impacted morphology [4], indicating mislocalization of cell division machinery. It is thus our hypothesis that exogenous free fatty acids impact localization of lipid II, resulting in daptomycin tolerance. However, further lipid II localization studies are needed to determine if there is a correlation between fatty acid supplementation, lipid II delocalization, and daptomycin tolerance.

Impact of fatty acid supplementation on cellular physiology

Fatty acids within the growth environment can have major impacts in bacterial physiology. In the Gram-negative organism *Escherichia coli*, polyunsaturated fatty acids increase membrane permeability [41]. Similarly, the polyunsaturated fatty acid linoleic acid (C_{18:2 cis9,12}), which induces daptomycin protection in *E. faecalis*, increased the membrane permeability of *Pseudomonas aeruginosa* [42]. *In vitro* evidence suggested that free fatty acids can also affect the activity of transmembrane proteins. Notably, in *Streptococcus sobrinus* and *S. mutans*, polyunsaturated fatty acids inhibited the activity of glucosyltransferase, an enzyme involved in glucan production [43, 44]. Furthermore, free fatty acids inhibit fatty acid biosynthesis in several organisms, including *E. faecalis* [45-48]. Given that free fatty acids can be utilized in phospholipid biosynthesis, it is to be expected that in *E. faecalis*, the protective fatty acids oleic acid (C_{18:1 cis9}) and linoleic acid (C_{18:2 cis9,12}), were found to induce global phospholipid composition changes [5, 6]. Specifically, supplementation of wild-type *E. faecalis* with these fatty acids resulted in increases in free fatty acids in the membrane as well as increases in cardiolipin [5, 6]. Yet further investigation revealed that deletion of the genes responsible for producing cardiolipin synthases did not eliminate the protection induced by fatty acid supplements, revealing that their incorporation into cardiolipin did not explain why these supplements are protective [5].

One major finding of the studies outlined in this dissertation is that free fatty acids induce protection against daptomycin independently of their incorporation into membrane phospholipids (Chapter 4; Figures 4.4 and 4.4, quintuple deletion strain). Given this, it seems that free fatty acids, rather than specific phospholipid changes induced by fatty acid supplementation, induce protection against daptomycin. Combined with the above data demonstrating that membrane organization is impacted by and influences several cellular processes, including orchestration of the divisome, it is unsurprising that supplementation of cells with free fatty acids results in altered cellular morphology [4] and membrane fluidity [1]. However, these studies were unable to directly correlate cellular morphology or membrane fluidity with daptomycin protection. Yet, given that incorporation

of free fatty acids is not necessary to induce daptomycin tolerance (Chapter 4), it is likely that free fatty acid supplements induce another physiological response leading to tolerance. Thus, a particularly enticing question which remains is whether supplementing cells with protective fatty acids stabilizes the membrane in such a way that daptomycin cannot interfere with membrane organization. Worth noting is the fact that while we previously observed that membrane fluidity was not correlated with daptomycin protection [1], this measurement represents a bulk measurement, and does not investigate how microdomains are impacted. Thus, it would be of interest to further investigate how addition of fatty acids to the growth media impacts membrane microdomains.

Impact of the Fak system on cellular physiology

While we observed that free fatty acids induce tolerance even when they are not incorporated into membrane phospholipids (Figures 4.4-4.6), we also observed that combined deletion of three or more *fakB* genes increases daptomycin tolerance (Figure 4.3). Given our findings that combined deletion of *fakB1*, *fakB2*, and *fakB5* ($\Delta fakB1,2,5$) resulted in several physiological changes, including altered cell size and morphology and accumulation of free fatty acids (Chapter 3), this was foreseeable. From these data, we hypothesize that the accumulation of free fatty acids in this strain impacts membrane organization and the biophysical properties of the membrane, thus leading to daptomycin mislocalization and consequently, decreased efficacy.

As previous research carried out in *S. aureus* and *S. pneumoniae* indicated that the Fak system was mainly useful for uptake of exogenous fatty acids [49, 50], the difficulty of deleting all four *fakB* homologs and our observation that the $\Delta fakB1,2,5$ deletion strain had altered phenotypes even in the absence of fatty acid supplements was initially puzzling. However, we found that deletion of the gene encoding a homolog of a thioesterase, *tesS*, demonstrated in *S. pneumoniae* to cleave the acyl group from acyl-ACP [51], enabled deletion of all four *fakB* homologs of *E. faecalis*. Examining our quintuple deletion strain ($\Delta fakB1,2,4,5/\Delta tesS$) revealed that phenotypes associated with supplementation of toxic free fatty acids were relieved (Table 3.4, Figures 3.5 and 3.6),

indicating that incorporation of free fatty acids facilitates their impact on cellular physiology. These data were interesting, as they highlighted a potential difference between the function of enterococcal PlsX and streptococcal or staphylococcal PlsX.

In the literature, PlsX has been clearly demonstrated to interconvert (in both directions) acyl-phosphate and acyl-ACP [45, 52]. However, if this conversion was equally efficient in both directions in *E. faecalis*, one might expect that the toxifying effects of supplementation with myristic acid (C_{14:0}) or palmitic acid (C_{16:0}) could be reduced if they were assimilated into the *de novo* fatty acid biosynthesis pathway through the action of PlsX converting the corresponding acyl-phosphate into acyl-ACP. Given that these fatty acids are toxic to *E. faecalis*, causing growth stasis (and eventual selection of suppressor strains which can grow in these fats) [4], it seems likely that these fatty acids, in wild-type *E. faecalis*, are converted to acyl-phosphates through the activity of the Fak system, and are then directly incorporated onto the SN-1 position of glycerol-3-phosphate (G3P), forming lysophosphatidic acid. The secondary addition of an endogenously produced fatty acid onto lysophosphatidic acid (forming phosphatidic acid) might then produce an asymmetric phospholipid. Asymmetric phospholipids (meaning an imbalance of different acyls on the SN-1 vs. the SN-2 position of the phospholipid head group) impact membrane biophysical properties, namely through decreasing the stretching capability of the membrane [53, 54].

These suppositions lead to the novel question of whether there is a specific difference between enterococcal PlsX and the PlsX homologs of other bacterial genera. PlsX has been studied most thoroughly in *B. subtilis*, where several amino acid residues have been identified as important for its function (Table 5.1). Of these residues, most are conserved in *Enterococcus* (Figure 5.1; conservation indicated by red letters, substitution with a similar amino acid indicated in blue letters). While substitution with a similar amino acid occurred in some cases, these substitutions were only observed in the sequences of *S. aureus* or *S. pneumoniae*, except in the cases of: Ile242, substituted in *E. faecalis* for methionine; Leu254, substituted in *E. faecalis* for valine; and Leu258, substituted in *E. faecalis* for methionine. The Ile242 residue in bsPlsX was found to be critical for

Table 5.1. PlsX residues of interest and their phenotypes on mutation.

Organism/reference	Residues (mutation) -- result	Important for
<i>Bacillus</i> /Jiang 2019	Thr253 (mutated to Ala)	Localizing to RIFs; membrane binding
<i>Bacillus</i> /Jiang 2019	Thr255 (mutated to Ala)	Localizing to RIFs; membrane binding
<i>Bacillus</i> /Jiang 2019	Lys257 (mutated to Ala)	Localizing to RIFs; membrane binding
<i>Bacillus</i> /Jiang 2019	Leu258 (mutated to Ser and Ala) – caused delocalization	Localizing to RIFs; membrane binding
<i>Bacillus</i> /Jiang 2019	Leu254 (mutated to Glu) – no effect; (mutated to Asp and Ala) – caused delocalization	Localizing to RIFs; membrane binding
<i>Bacillus</i> /Sastre 2020	Leu254 (mutated to Glu) – no protein in pellet/membrane	Localizing to membrane
<i>Bacillus</i> /Sastre 2020	Val262 (mutated to Glu) – no protein in pellet	Localizing to membrane
<i>Bacillus</i> /Sastre 2020	Leu258+Ala259 (mutated to Glu) – no protein in pellet	Localizing to membrane
<i>Bacillus</i> /Sastre 2020	Ile242+Phe243 (insertion of Ala-Glu between) – monomers/multimers form, compromised membrane localization, and abolished catalysis; (insertion of Glu-Glu-Leu between) – monomers/dimers form, mixed localization pattern, and catalysis still occurred	Dimerization/membrane localization/catalysis
<i>Bacillus</i> /Sastre 2020	Leu235 (mutated to Glu) – monomers/multimers instead of dimers, compromised membrane localization and catalysis abolished	Dimerization/membrane localization/catalysis
<i>Enterococcus</i> /Kim 2010	Proposed but not tested as catalytic triad: Ser279-Tyr278-Lys295	Catalytic triad; NADP binding
<i>Enterococcus</i> /Kim 2010	Proposed but not tested as catalytic triad: Ser107-Tyr136-Lys140	Catalytic triad; NADP binding

Table 5.1 Continued

Organism/reference	Residue, mutation(s), and result	Important for
<i>Bacillus</i> /Jiang 2020	Arg73 (mutated to Ala) – increased K_m for acyl-ACP	Catalytic activity; acyl-ACP binding
<i>Bacillus</i> /Jiang 2020	Arg120 (mutated to Ala) – increased K_m for acyl-ACP	Catalytic activity; acyl-ACP binding
<i>Bacillus</i> /Jiang 2020	Arg120+Arg73 (mutated both to Ala) -- activity undetectable	Catalytic activity
<i>Bacillus</i> /Jiang 2020	Asn229 (mutate to Ala) – K_{cat} decreased and increased K_m for phosphate and acyl-ACP; (mutate to Glu) -- recovery of catalytic activity	Catalytic activity; substrate binding
<i>Bacillus</i> /Jiang 2020	Lys184 (mutate to Ala) – K_{cat} decreased and increased K_m for phosphate; (mutate to Gln) – no recovery; (mutate to Arg) – activity recovered	Phosphate binding; stabilizing transition state
<i>Bacillus</i> /Jiang 2020	Glu181 (mutate to Ala) – increased K_m for phosphate	Phosphate binding; stabilizing transition state
<i>Bacillus</i> /Jiang 2020	Proposed but not tested: Val178	Stabilizing transition state

dimerization of PlsX [55]. This residue in *E. faecalis* therefore is unlikely to be the driver for a difference in function, as a crystal structure for *E. faecalis* V583 PlsX demonstrated that the protein formed dimers [56]. Interestingly, the other residue substitutions occurred in an α -helix, observed in *B. subtilis* to be responsible for localization of PlsX [57]. These differences are reflected in an overlay of Phyre2 models of the PlsX proteins, (Figure 5.2; red box shows a modelled loop corresponding to residues important for localization PlsX to the membrane).

In *Bacillus subtilis*, PlsX associates with RIFs in the membrane [27, 57]. The targeting of bsPlsX was observed to be driven by a 9-residue amphipathic α -helix (residues 253-261) [57]. Alteration of the interaction of this α -helix to decrease its interaction with the membrane (mutation of nonpolar residues to polar residues) or to increase its interaction with the membrane (mutation of polar residues to nonpolar residues) caused delocalization of PlsX from RIFs in the membrane [57]. This suggests that there is a narrow range of hydrophobicity required for localization of PlsX to RIFs. Given this tight specificity of PlsX for localization to specific regions of the membrane, it is probable that membrane alterations delocalize PlsX. Thus, it is tempting to speculate that increased levels of free fatty acids in the membrane lead to delocalization of PlsX. This would lead to an inability to convert acyl-phosphates (generated by the Fak system) to acyl-ACP. Thus, exogenous fatty acids would only be used for incorporation onto the SN-1 position of G3P, while endogenous fatty acids would be necessary for acylating the SN-2 position of G3P. This rewiring of phospholipid metabolism would then hypothetically induce phospholipid changes and lead to mislocalization of important cellular machinery within the membrane, thus potentially inducing daptomycin tolerance. Further testing through studies elucidating whether PlsX is delocalized on addition of free fatty acids or in the $\Delta fakB1,2,5$ deletion strain, which we noted had accumulation of free fatty acids (Chapter 3; Figure 3.3) will be critical for elucidating whether delocalization is occurring.

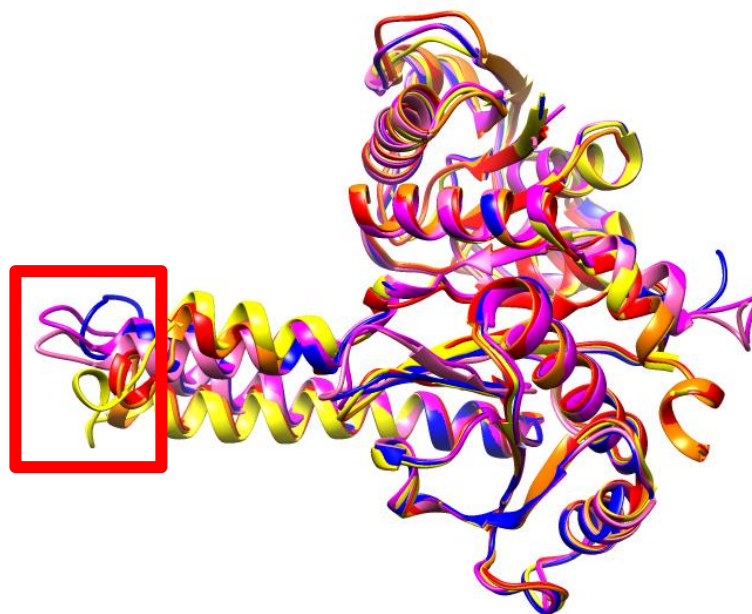


Figure 5.2. Phyre² models of PlsX of several organisms.

Models of the PlsX proteins of: *B. subtilis* str. 168 (red), *B. subtilis* PY79 (orange), *S. pneumoniae* D39 (Blue), *S. aureus* USA300_FPR3757 (Yellow), *E. faecalis* OG1RF (hot pink), and *E. faecalis* V583 (magenta) were modelled using Phyre² and overlaid using Chimera. The red box indicates the loop important for localization of PlsX to the membrane.

Importance of fatty acid binding across species

Fatty acids are important constituents of many environments. As previously noted, several species of fatty acids are present in bile as well as serum [3]. Additionally, fatty acids are present on the skin [58] in sputum [59, 60], in water sources [61, 62], and in the soil [63]. Given that fatty acids are present in these environments, it is logical to hypothesize that the organisms present within these environments have adapted to utilize the fatty acids around them. In Chapter 4, we performed a network analysis to determine how the FakB proteins of *E. faecalis* are related to the DegV (fatty acid binding) domains found in other organisms. Our findings indicated that the four FakB proteins of *E. faecalis* each have a different functionality, as they clustered to different areas of the network (Figure 4.1). Given that *E. faecalis* is commensal to the mammalian intestine, where it may be exposed to bile, which contains numerous fatty acids [3], we were not surprised that this was the case. Additionally, the network data matched our previous data (Table 3.1) which indicated that efFakB1 was most closely related to saFakB1 and spFakB1, while efFakB2 was most closely related to saFakB2 and spFakB2 (Figure 4.1). However, we noted that the two other FakB homologs of *E. faecalis* clustered with other regions of the network (Figure 4.1). Specifically, efFakB1 clustered in a region of the network which was dominated by Gram-positive organisms, yet there did not appear to be any pattern with regards to the isolation source in the clustering of the DegV domain associated with this protein (Figures 4.1 and 4.2). Interestingly however, FakB4 clustered in a region of the network which was dominated by animal isolates – especially isolates originating from the gut (Figures 4.1 and 4.2). This is indicative that the DegV domains within this region are important to binding free fatty acids within the gut. In our studies, we noted that deletion of *fakB4* did not correspond to any specific phenotypes, except in the quintuple deletion strain (Chapter 3). It is possible that the reason for this is because we did not examine the correct conditions to observe a phenotype. While bile, which contains numerous long-chain fatty acids (Saito 2014), is present in the gut, dietary fatty acid sources are present as well. In particular, medium-chain fatty acids, including caproic acid (C_{6:0}), caprylic acid (C_{8:0}), capric acid (C_{10:0}), and lauric acid (C_{12:0}), are constituents of

plant and dairy food products [64]. It remains possible that efFakB4 is specific for dietary fatty acids such as these, for microbially produced short-chain fatty acids which are abundant in the gut [65, 66], or for omega-3 dietary fatty acids.

Another trend we noted was that there was a higher abundance of Gram-positive organisms within the cluster of the network analyzed (Figure 4.2). There are two possible reasons for this observation. It is possible that in other clusters of the network, Gram-negative organisms dominated as they were more distantly related to the DegV domains of Gram-positive organisms. A second possibility could be that the organisms which lack a system for β -oxidation of fatty acids may be more likely to have DegV domains for binding fatty acids. In these organisms, while fatty acids cannot be used as carbon sources, they might be used for building the bacterial membrane. However, the distribution of organisms containing systems for β -oxidation across Gram-positive and Gram-negative genera has not been examined. Overall, our data however point towards specific trends linking isolation source with DegV relatedness.

Final thoughts

E. faecalis is a bacterium commensal to the mammalian gut, yet is able to colonize other tissues. In these environments, it is exposed to free fatty acids, which it can ordinarily incorporate into its membrane phospholipids. The work presented in this dissertation started by asking how cellular physiology impacts tolerance to daptomycin. On our way to answering this question, we discovered that the Fak system is important for normal cellular physiology in *E. faecalis*. Further, we determined that inactivation of this pathway not only increases basal daptomycin tolerance, but that free fatty acids, even when not incorporated into phospholipids, are still able to protect against the clinically important drug, daptomycin. This work further illuminates our understanding of the interplay between cell wall synthesis and the bacterial membrane, and has expanded our knowledge of how *E. faecalis* uses its resources to survive in its host.

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VITA

Rachel Johnston was born in the warm month of June in Pensacola, Florida. Over the course of her life, she has made a slow crawl northeastwards: from Pensacola FL (birth) to Troy AL (undergrad school), to Knoxville TN (grad school). With each new move there were new thermal horizons to explore for Rachel. In undergrad, she learned to double up on clothing in the winter. In Knoxville she learned how to triple up on her clothing in the winter. Now on to the next thermal challenge: Rachel will be pursuing a postdoc in Bethesda MD. Will she be able to successfully wear four layers of clothing in the wintertime? Only time will tell.