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I am submitting herewith a dissertation written by John Robert Harp entitled "Incorporation of host fatty acids promotes tolerance to membrane stressors by modifying the lipid content of *Enterococcus faecalis*." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Elizabeth M. Fozo, Major Professor

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Incorporation of host fatty acids promotes tolerance to membrane stressors by modifying the lipid content of *Enterococcus faecalis*

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

John Robert Harp
December 2018

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DEDICATION

For Mom and Dad,

Who set me up to succeed.

And showed me the way.

ACKNOWLEDGEMENTS

"Life before Death.
Strength before Weakness.
Journey before Destination."
-Brandon Sanderson, The Way of Kings

The process of getting a PhD is rough and was part of the reason I was so reticent to pursue one during my first foray in graduate school. However, the decision to return to graduate school to pursue a PhD was one of the best I have made. This grueling, rewarding, frustrating and exhilarating experience would have required way more activation energy if I did not have a great support network all around me. First, I would like to thank my advisor, Dr. Elizabeth Fozo. Liz has been one of the best mentors I've ever had. Her energy, passion, and desire to be a great principle investigator, manager, and teacher has helped me find it in myself to be all of those things and more. Next, I would like to thank my committee members, Dr. Tessa Calhoun, Dr. Todd Reynolds, and Dr. Erik Zinser. Your commitment to my PhD, through your advice and encouragement has made me a better and more thoughtful scientist. I also need to thank Elizabeth McPherson who has been a guiding light during my time as a graduate teaching assistant. Elizabeth's thoughts and advice regarding pedagogical approaches and life have been valuable and I will definitely be taking those lessons with me into my future endeavors. Further, I would like to thank Rachelle Allen, Misty Jones, and Dr. John Dunlap, whom each have helped me when I needed the little things done right. To all of my lab mates, friends, and fellow grad students who have shared in this journey, it has been a real pleasure: Jia Wen, Holly Saito, Chelsi Cassilly, Bikash Bogati, Rachel Steele, Will Brewer, Eric Tague, Bob Tams, Tian Chen, Kyle Bonifer, Andy Wagner, Shawn Walker, Thomas Nipper, Oana Stoiculescu…thank you.

To my parents who have been a stable force in my life and to whom I owe just about everything. My ability to climb to the pinnacle of academic achievement is due to your support and belief in me. I will be forever grateful. To my wife, Marie. Wow – here we are. Thank you for everything that you do for me. You make me want to wake up each day with the drive to make our lives better. Your passion, creativity, and love have made me a better person and husband. I cannot wait to see where our future takes us.

ABSTRACT

Enterococcus faecalis is a Gram-positive commensal bacterium that resides in the human gastrointestinal tract. Unfortunately, *E. faecalis* can also cause infections in humans and is notoriously difficult to treat due to drug resistance. One treatment that is used to treat enterococcal infections is the cell membrane targeting lipopeptide antibiotic, daptomycin. However, daptomycin resistant strains of *E. faecalis* have been isolated. Studies aimed at understanding these resistant strains show that mutations in genes associated with membrane homeostasis are involved. *E faecalis* can also incorporate exogenous fatty acids from environments in which it thrives, bile (GI tract) and serum (wounds), which cause increased physiological tolerance to daptomycin. The host fatty acids, oleic acid (C18:1 *cis* 9) and linoleic acid (C18:2 *cis* 9, 12), that are prevalent in serum and bile, are the major factors that contribute to this induction of daptomycin tolerance. Within this work, I determined that the *cis* bond at the 9th carbon of oleic acid is critical for increased tolerance. Moreover, I found that when the carboxyl group of oleic acid or linoleic acid was replaced with an amide group, tolerance was lost. To assess if increased tolerance induced by these host fatty acids was a consequence of a membrane stress response, I examined a strain of *E. faecalis* which lacks the response regulator of the LiaFSR three-component system and concluded that host fatty acid induced tolerance was not mediated by LiaFSR. Finally, I investigated whether or not supplementation with host fatty acids was altering the membrane phospholipid composition leading to increased tolerance. After mass spectrometry analysis, I discovered alterations in the composition of the major phospholipids in *E. faecalis.* To test these alterations, I deleted genes responsible for production of lysyl-phosphatidylglycerol (*mprF2)* and cardiolipin (*cls1* and *cls2)*. After supplementation with oleic acid and linoleic acid, I still observed increased tolerance to daptomycin. However, long term exposure to daptomycin resulted in no recovery even after supplementation with host fatty acids. These data suggest that oleic acid and linoleic acid can induce lipid alterations, but alteration in the composition of L-PG and CL are not responsible for acute daptomycin tolerance.

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CHAPTER I: Introduction

Enterococci

Enterococci are an ancient genus of bacteria, believed to have originated when animals underwent terrestrialization around 425 million years ago (1). First observed and characterized in 1899 (2, 3), a species of enterococci (then known as *Micrococcus zymogenes*) was isolated from a case of acute endocarditis and septicemia. The observations in these studies became the basis for understanding enterococci pathogenesis. To date, there have been over 50 species of enterococci isolated of varying genome sizes (from 2.3 Mb to 5.3 Mb). Enterococci are low GC, Gram-positive diplococci, that naturally reside in the gastrointestinal (GI) tracts of humans (comprise <0.1% of the total consortia) and other animals as well as insects (4–6). Further, these organisms can be isolated from diverse environments. The intrinsic hardiness of these organisms allows them to aid in the production of fermented foods and dairy products and survive in environmental niches such as plants, soil, and water (7). However, a more thorough understanding of the characteristics that allow enterococci to colonize diverse environments is of significant interest. In this chapter, I will specifically highlight the current information about enterococci physiology and processes involved in governing the success of this organism as a human pathogen.

Enterococci in the clinic

Enterococci began to emerge as a significant hospital pathogen in the 1970's and 1980's, and as demonstrated by the increased reporting of enterococci nosocomial isolates, are now considered a serious threat to human health (8, 9). Although enterococci naturally reside in the GI tract and genital tract (and to a lesser extent in the oral cavity), in an immunodeficient host, the bacteria can translocate the mucous membrane to cause systemic infection (10), colonize in-dwelling medical devices, infect surgical wounds, and cause endocarditis (11, 12). These infection outcomes typically arise after antibiotic treatment, where the intestinal microbial community is significantly altered (13–15) such that the density of bacterial flora is greatly reduced. However, after discontinued use of antibiotics, specific genera of bacteria, including enterococcus begin to increase (16). As a consequence of the increased numbers of these specific bacteria, enterococci can enter the bloodstream and cause downstream health effects. More recent epidemiological

investigations, however, suggests that the primary method for patient to patient spread occurs via the hands of healthcare workers since identical strains of enterococci have been found on patients and healthcare worker hands (17). This spread is attributed to the ability of enterococci to survive on fomites for long periods of time (18). This strategy of spreading bacteria becomes particularly important when dealing with nosocomial pathogens such as vancomycin-resistant enterococcus (VRE). If a patient carries VRE, other patients in the hospital are 40% more likely to be colonized by VRE (19).

Enterococcus faecium and *Enterococcus faecalis* are the two most prevalent species of enterococcus that cause disease in a clinical setting (20). There are significant differences between these two species, but they share common features related to intrinsic antibiotic tolerance and resistance mechanisms (21). Both species are tolerant to β-lactam and aminoglycoside antibiotics meaning that their growth can be inhibited by clinical doses of these drugs, but much higher doses are required for killing. The tolerance mechanism to β-lactams are shared across all enterococci species examined to date and involve the production of low affinity penicillin binding proteins (PBPs), which prevent β-lactams from covalently binding to PBPs thus allowing cell wall synthesis even when exposed to drug (22). Importantly, these species are not only broadly tolerant to the β-lactam class of antibiotics, but specifically resistant to penicillin, ampicillin, and cephalosporins. Along with high tolerance to β-lactams, the enterococci are tolerant to aminoglycosides due to a proposed method of exclusion (23, 24). The enterococci species found in the clinic can also be genetically resistant to clindamycin and vancomycin, and many are multi-drug resistant making treatment a challenge.

Enterococci are also efficient at horizontally acquiring and sharing antibiotic resistance by using mobile genetic elements, such as conjugative transposons, pathogenicity islands, and plasmids (25). These mobile genetic elements can carry resistance genes and be shared with other clinically relevant bacterial isolates, which was the case for methicillin resistant *Staphylococcus aureus* acquiring vancomycin resistance from a transposon element (Tn*1546*) on a conjugative plasmid (26). With as much as 25% of the *E. faecalis* genome consisting of foreign DNA or mobile genetic elements, there are a considerable number of genes that have been discovered to promote survival in diverse conditions (27). Some of these genes help enterococci survive in hostile

conditions, such as the production of proteins for oxidative stress and cation homeostasis. Further, pathogenicity islands likely derived from integrated plasmids, confer the presence of aggregation substance (believed to be important for pathogenicity and conjugation (28)), cytolysin and vancomycin resistance (29).

While horizontal gene acquisition is important for resistance to antibiotics, resistance to daptomycin is linked to gene mutations. Daptomycin is a calcium-dependent lipopeptide antibiotic used to combat multi-drug resistant enterococci. Although the action mechanism is still unclear, daptomycin inserts a 10-carbon fatty acid tail into the Gram-positive membrane, in association with phosphatidylglycerol, then oligomerizes with other daptomycin monomers to destabilize the membrane and cause cell death (30–34). Despite this apparently non-specific cell membrane targeting mechanism, daptomycin resistant strains have been isolated in the clinic (35, 36).

Daptomycin resistance

Daptomycin is a cyclic lipopeptide antibiotic and natural product of the soil actinomyces, *Streptomyces roseosporus*. Isolated from Mount Ararat in Turkey in the 1980's, daptomycin was first approved by the Food and Drug Administration (FDA) for clinical use in 2003 to treat complicated skin and soft tissue infections, infective endocarditis, and bacteremia associated with these diseases in *Staphylococcus aureus* (37, 38). Early *in vitro* experiments using the Gram-positive bacterium, *S. aureus,* suggested that resistance to daptomycin was rare and that spontaneous resistance rates were low (39). However, subsequent work with *S. aureus* demonstrated that serial passage with daptomycin resulted in mutations in certain genes (*mprF*, *rpoB*, *yycG* and others)(40) and that these genetic mutations could also be found in daptomycin resistant *S. aureus* clinical isolates (41, 42).

Due to the multidrug resistance of enterococci clinical isolates, clinicians have been using daptomycin to treat enterococcal infections (36). In 2011, Arias et al. characterized a clinical pair of daptomycin-susceptible and daptomycin-resistant *E. faecalis* strains from a patient with fatal bacteremia. Deep-genome sequencing of these strains, showed the presence of several mutations, including lipid II interacting antibiotic component F (*liaF* of the LiaFSR three component system – discussed in more detail below), cardiolipin

synthase (*cls* – OG1RF_RS01975 "*cls1"*), and glycerophosphoryl diester phosphodiesterase (*gdpD*). Moreover, serial passaging of *E. faecalis* in the presence of increasing concentrations of daptomycin (*in vitro* evolution) demonstrated similar mutations to the clinical pair (35). Interestingly and not surprisingly, given the proposed mechanism of action of daptomycin, these mutations are in genes that encode for proteins involved in membrane homeostasis.

Membrane fatty acid biosynthesis

The cell envelope (consisting of cell wall and cell membrane) is a vital structure that allows bacteria to survival in a wide variety of environments. Moreover, it protects the cell from harmful or changing environmental conditions (temperature, pH, salinity, etc.), but allows the passive diffusion of important nutrients into the cell and waste products out of the cell. The cell membrane, in particular, is dynamic because there are many vital processes occurring at this region which can dictate the success of an organism. The membrane must change as a result of environmental alterations in order to maintain proper viscosity, such that passive permeability of hydrophobic molecules, active transport, and protein-protein interactions can function appropriately (43). It has been observed that during changing temperatures, organisms such as *Escherichia coli* (44) *and Bacillus subtilis* (45), have the ability to alter the ratio of saturated (carbon tail with no double bonds) and unsaturated (carbon tail with at least one double bond) fatty acids to help maintain proper membrane flexibility and fluidity (46). *E. coli* can also perform post-synthetic modifications of unsaturated fatty acids to generate cyclopropane fatty acids, which protect the organism from acid shock (47). Additionally, *Streptococcus mutans* can increase the abundance of unsaturated fatty acids when it is exposed to a low pH environment, which in turn is linked to its ability to cause disease (48). It is clear that bacteria alter their fatty acid content to adjust the biophysical properties of their membranes in response to changing environment.

De novo synthesis of fatty acids differs in bacteria (fatty acid biosynthesis type II – FAS II) as compared to eukaryotes (fatty acid biosynthesis type I – FAS I). FAS I, which occurs in the eukaryotic cytoplasm, employs a single multiprotein complex to complete fatty acid synthesis. For bacteria, however, a set of conserved genes encode individual

proteins for each step in fatty acid synthesis. Succinctly, in FAS II, the acyl-carrier protein (ACP) is responsible for carrying each fatty acid intermediate throughout fatty acid biosynthesis ending in a fatty acid product typically between 16 to 18 carbons in length (Summarized in Figure 1.1) (43, 49). First, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (AccABCD). The malonyl group is then transferred to ACP by malonyl transacylase (FabD) to form Malonyl-ACP. Malonyl-ACP condenses with another acetyl-CoA by β-ketoacyl-ACP synthase III (FabH) to form β-ketoacyl-ACP. This in turn is reduced by β-ketoacyl-ACP reductase (FabG) to form β-hydroxy-acyl-ACP. β-hydroxy-acyl-ACP dehydratase (FabA/FabZ or FabN in enterococcus) will then convert β-hydroxy-acyl-ACP to *trans*-2-enoyl-ACP. Finally, enoyl-ACP reductase (FabI/FabK/FabL/FabV – isoforms depend upon the species) acts on *trans*-2-enoyl-ACP, thus completing the cycle and resulting in an acyl-ACP two carbons longer. Subsequent condensation reactions of 2-carbon units facilitated by β-ketoacyl-ACP synthase II (FabF in enterococci and other species or FabB) drive the elongation of the fatty acid until the chain length reaches 16-18 carbons. Depending on the requirement for saturated or unsaturated fatty acids (typically driven by temperature), the *trans*-2-enoyl-ACP can be isomerized to form *cis-2*-decanoyl-ACP and elongated by FabF/FabO (50) (Figure 1.2). Once the fatty acid has been synthesized, it can be transferred to the membrane for attachment to a polar head group to form a glycerolipid (51, 52).

Membrane phospholipids and incorporation of fatty acids

The formation of phospholipids and the ability to modulate phospholipid composition is vitally important for cell homeostasis. The majority of bacterial species examined to date produce the following major phospholipids: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) (53), but can also form phosphorous-free membrane lipids (e.g. sulfolipids and hopanoids) (54). In Gram-positive bacteria, the major glycerophospholipids are typically PG and CL, but the relative abundance of these phospholipids is species dependent as well as growth phase and growth condition dependent (Reviewed in 54, 55–58).

The most common proteins involved in the biosynthesis of membrane phospholipids in Gram-positive bacteria are PlsX, PlsY, and PlsC (51). After several rounds of

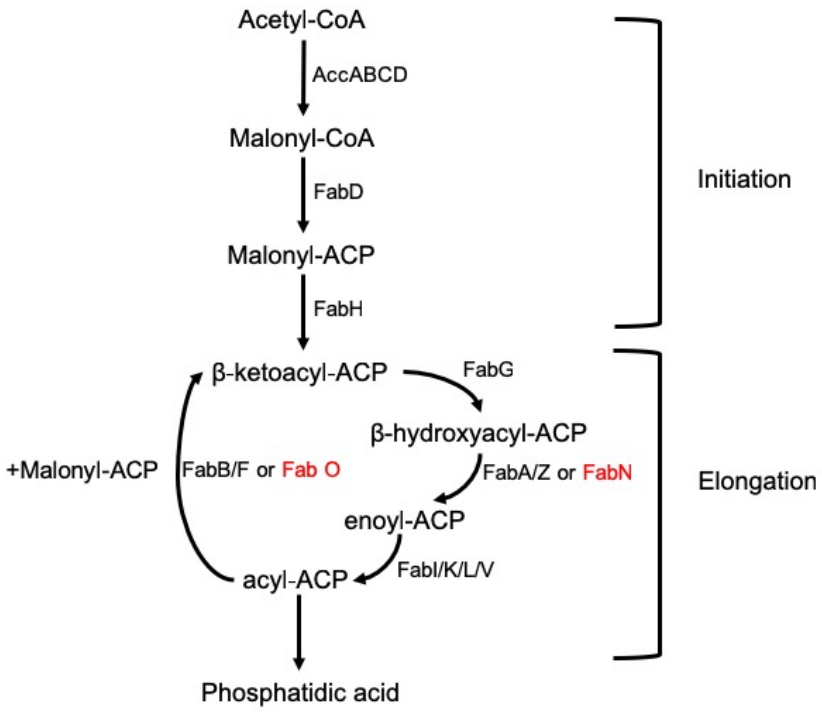

Figure 1.1. The fatty acid biosynthetic pathway and formation of phosphatidic acid. Acetyl-CoA carboxylase (Acc) consists of four subunits and initiates fatty acid synthesis. Acyl carrier protein (ACP) carries each fatty acid intermediate to the next step in the pathway. Fatty acid biosynthesis (Fab) enzymes are shown next to arrows which designate the next product in the pathway. Enzymes in red are found in *Enterococcus faecalis.*

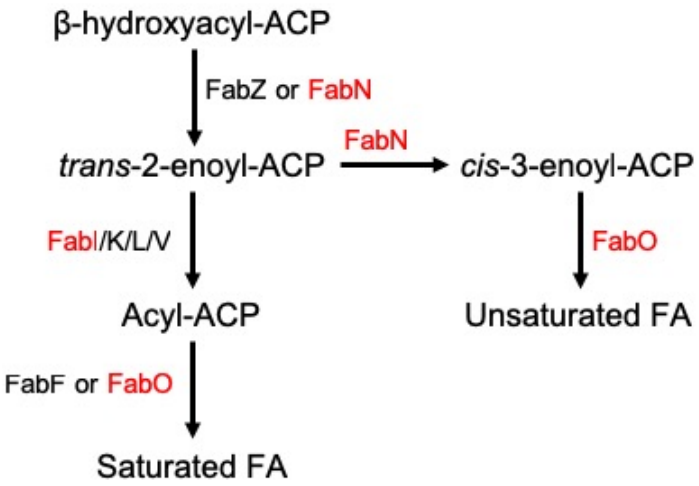

Figure 1.2. Saturated or unsaturated fatty acid (FA) synthesis pathway.

Fatty acid biosynthetic (Fab) enzymes involved in the synthesis of saturated or unsaturated fatty acids are next to arrows which designate the next product in fatty acid biosynthesis. Enzymes in red are those used by *Enterococcus faecalis.*

elongation, long chain acyl-ACP becomes a poor substrate for the elongation condensing enzymes of FASII, but an ideal substrate for the acyltransferase system. First, the acyl-ACP is phosphorylated by PlsX to form acyl-PO_4. The acyl-PO_4 can then be used by PlsY to acylate the sn-1 position of glycerol-3-phosphate to form lysophosphatidic acid. Further, the sn-2 position can then be acylated by PlsC to form phosphatidic acid, which is the central precursor in the formation of membrane phospholipids. In brief, cytidine diphosphate-diacylglycerol (CDP-DAG) synthase (CdsA) condenses phosphatidic acid and cytidine triphosphate (CTP) to form CDP-DAG. CDP-DAG can then be used in two sequential reactions (absent in enterococcus) to first result in phosphatidylserine and then form phosphatidylethanolamine. Alternatively, CDP-DAG can serve as a precursor for phosphatidylglycerol which can then result in cardiolipin (43) (See Fig 1.3).

An alternative mechanism involved in the biosynthesis of phospholipid precursors in Gram-positive bacteria relies on the incorporation of exogenous fatty acids using a series of fatty acid kinase (Fak) proteins (59). In this system, elucidated in *S. aureus,* FakB1 or FakB2 bind saturated or unsaturated fatty acids, respectively that are thought to have “flipped” into the membrane, which then allows FakA to phosphorylate the fatty acid bound by FakB. Depending on the length of the fatty acid, this phosphorylated fatty acid can then be used by the PlsX/Y/C system or undergo elongation and then incorporation.

Phosphatidylglycerol

One of the most abundant glycerophospholipids in Gram-positive bacterial membranes is the anionic lipid phosphatidylglycerol (PG). This glycerophospholipid is formed after two sequential reactions. First, there is a condensation reaction between glycerol-3-phosphate (G3P) and cytidine diphosphate-diacylglycerol (CDP-DAG) which is catalyzed by phosphatidylglycerolphosphate synthase (PgsA) to form PG phosphate (PGP). Next, phosphatidylglycerol phosphate phosphatase (PgpP) dephosphorylates PGP to form PG.

Overall, PG contains two acyl chains that are esterified to a glycerol that is then bound to a headgroup, which gives this phospholipid a net negative charge. PG has a variety of functions in the membrane depending on the bacteria in question, but it has been demonstrated via X-ray crystallography to bind transmembrane proteins and is thought to help stabilize those proteins (e.g. cytochrome *c* oxidase) (60, 61). Moreover, the

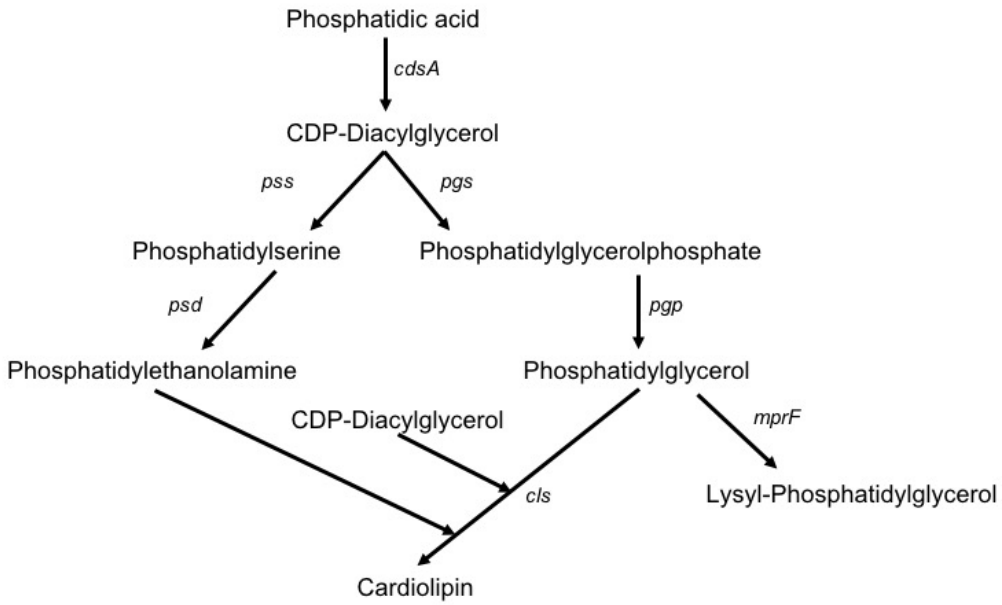

Figure 1.3. Phospholipid synthesis pathway in bacteria.

Phosphatidic acid produced from fatty acid synthesis is converted to cytosine diphosphate (CDP)- diacylglycerol (DAG) and is the central precursor. Phosphatidylethanolamine is a major phospholipid in most bacteria and is produced in two steps. Phosphatidylglycerol is another major phospholipid and also produced in two steps. Lysinylation of phosphatidylglycerol produces lysyl-phosphatidylglycerol. Cardiolipin is produced using several methods 1) condensation of two phosphatidylglycerol, 2) CDP-DAG and phosphatidylglycerol, 3) phosphatidylethanolamine and phosphatidylglycerol. *Enterococcus faecalis* lacks the enzymes to make phosphatidylserine and phosphatidylethanolamine.

headgroup of PG can be modified by the transfer of an amino acid, such as alanine, arginine, and lysine (62). Aminoacylation of PG in the membrane of bacteria is a mechanism that species of bacteria employ to repel positively charged antimicrobial peptides (e.g. cationic antimicrobial peptides) (63). Further, one particular aminoacylation that has been implicated in daptomycin resistance is lysyl-phosphatidylglycerol (64).

Lysyl-phosphatidylglycerol

Aminoacylated phosphatidylglycerols are commonly found in Gram-positive cytoplasmic membranes. The addition of lysine to PG is one way that bacteria can modulate the charge of the membrane, shifting PG from a net negative to a net positive charge. The process by which lysine is added to PG is facilitated by multiple peptide resistance factor *F* (MprF) which has been shown to transfer lysine from Lys-tRNA^{Lys} to the distal hydroxyl group of the glycerol on PG (65). Further, it is believed that MprF synthesizes L-PG in the inner leaflet and that the N-terminal hydrophobic domain of MprF can transfer L-PG from the inner leaflet to the outer leaflet (66). Studies have shown a clear link between increasing lysyl-PG and resistance to killing by antimicrobial peptides (67) and other cationic peptides such as daptomycin in *S. aureus.*

MprF has been found in several Gram-positive bacteria. In *E. faecalis*, there are two putative *mprF* genes (*mprF1* – OG1RF_RS00150 and *mprF2* – OG1RF_RS03930) (68). Conversely, in *S. aureus* (69) *and Listeria monocytogenes* (70) there is only one *mprF* gene. To assess the function of *mprF1 and mprF2* in *E. faecalis*, each gene was deleted and under the growth conditions examined, it was shown that loss of *mprF2* abolished lysyl-PG production, while loss of *mprF1* was no different from the parental strain. Further, resistance to cationic antimicrobial peptides was attributed to *mprF2* (68).

Cardiolipin

Cardiolipin is a an anionic (carrying two negative charges) glycerophospholipid that makes up a portion of the bacterial cell membrane. This glycerophospholipid is unusual from the other lipids discussed above due to the presence of two diacylphosphatidyl molecules linked by a central glycerol group. As a consequence, the presence of a small head group relative to the hydrophobic tails, gives this lipid a peculiar and vital function

for cellular processes, such as the stabilization of membrane proteins and formation of membrane domains. Specifically, cardiolipin can act as a flexible linker that fills gaps at protein interfaces resulting in the stabilization of individual subunits of oligomeric proteins (71). Moreover, given the propensity for this lipid to form domains in the membrane, it can act as a proton trap when in proximity to oxidative phosphorylation complexes (72) and other respiratory complexes (73). Additionally, given its shape, domains of cardiolipin can form at regions of higher curvature and aid in bacterial division (74).

Cardiolipin domains in several model organisms have been shown to reside at the cell septa and cell poles (75). It is thought that the intrinsic-curvature (small head group relative to large hydrophobic tails) promotes the self-organization of this lipid to these regions (76). Additionally, in *Bacillus subtilis* it was observed that the phospholipid synthase responsible for generating cardiolipin was found primarily at the septa (77). Interestingly, the number of genes responsible for cardiolipin synthesis varies by bacterial species, and the expression of these genes appears to correlate with physiological state (increase when approaching stationary phase, nutrient depravation or osmotic stress (78)).

Cardiolipin is synthesized by bacteria using three currently known mechanisms: 1) the condensation of two PG molecules via a phospholipase D superfamily type cardiolipin synthase (PLD – Cls most commonly found throughout Gram-positive and -negative bacteria), 2) the condensation of CDP-DAG and PG via CDP alcohol phosphatidyltransferase type cardiolipin synthase (CAP-Cls discovered in *Streptomyces coelicolor* and most Actinobacter (79)), and 3) the condensation of PG and phosphatidylethanolamine (PE) via PLD-Cls (discovered in *E. coli* (80)). Interestingly, the number of cardiolipin synthase genes depends on the bacterial species and the reason for this is not known. In *E. faecalis*, there are two predicted cardiolipin synthase (*cls)* genes, similar to *S. aureus* (81). Alternatively, *B. subtilis* (82) and *E. coli* (80) have three cardiolipin synthase genes. Despite the number of *cls* genes in each organism, there does appear to be specific regulation of the genes such that they are expressed at relevant times. This mechanism was particularly important in *S. aureus* and the organism's ability to respond to osmotic stress (55). In these data it was found that *cls2* was a housekeeping gene and that *cls1* responded to high-salt concentration.

Cell envelope function and stress response

An ability to respond efficiently to environmental changes or niche competition is a key requirement for bacterial cell survival. This requires sensitive and constant monitoring of the environment, such that an appropriate response can be initiated and achieved. The interface between the environment and the inner cell is known as the cell envelope. For most bacterial species, immediately outside of the cytoplasmic membrane is a layer of peptidoglycan, consisting of sugar chains crosslinked with peptide bridges, which make up a flexible yet strong cell wall. In Gram-positive bacteria, this layer tends to be thicker on average and is exposed directly to the environment. Conversely, in Gram-negative bacteria, this layer is often thinner and is located between an inner and outer membrane. The peptidoglycan layer is an active region as it must undergo biosynthesis, assembly, maturation, disassembly, and recycling to allow for the maintenance of cell shape and cell division (83). Overall, the cell wall portion of the cell envelope provides protection from the environment and also offsets the turgor pressure from within the cell. However, many of the stress response elements that a cell uses to respond to environmental changes are found at the cell membrane where lipid modifications and the embedded proteins play a specialized role in responding to environmental cues.

Modifying the composition of the cell membrane (as outlined above) is critical for survival during environmental stress. Beyond the architecture of the lipid constituents, the regulatory mechanisms controlling stress responses are vast. In short, there are numerous two or three component systems (TCS) and extracytoplasmic function (ECF) sigma factors that can be induced in the presence of environmental stresses. In Gram-positive bacteria, these two systems are functionally similar because they each contain a membrane-anchored sensor (histidine kinase or anti-sigma factor, respectively) and a cytoplasmic transcriptional regulator (response regulator or ECF sigma factor, respectively) (84). Under homeostatic conditions, these two stress response mechanisms are similar in that the transcriptional regulator is kept inactive until the regulator is turned on by stress and subsequently involved in inducing gene expression. However, the mechanisms by which the membrane sensor and the regulator interact with each other is different.

In a TCS, there is a transfer of a phosphoryl group from the transmembrane sensor to the response regulator. This response regulator can then act on its cognate genetic sequence to induce a transcriptional response to mitigate damage caused by the stress. Alternatively, the ECF sigma factor is normally bound to the anti-sigma factor. Upon stress stimulation, the anti-sigma factor is released either by conformational change or by anti-sigma factor proteolysis. The free sigma factor can then recruit RNA polymerase to initiate transcription at the designated promoter to result in transcription of the appropriate stress response genes.

A particularly important three component system associated with cell envelope stress responses is the LiaFSR (lipid II cycle interfering antibiotic sensor and response regulator) system. In this well conserved system found across the *Firmicutes* group, LiaS is the transmembrane sensor histidine kinase, LiaR is the cytoplasmic response regulator, and LiaF acts as a strong inhibitor of LiaR-dependent activity (85). Work using *Bacillus subtilis* has shown that LiaSR responds to cell wall antibiotics, such as vancomycin, bacitracin, and other cationic antimicrobial peptides as well as cell membrane targeting antibiotics like daptomycin (86, 87). Moreover, the orchestrated activity of this three-component system in *Streptococcus mutans* showed transcription induction of membrane protein synthesis, peptidoglycan biosynthesis, and other systems involved in cell envelope homeostasis (88). In enterococci, a single amino acid deletion in LiaF results in daptomycin resistance (89–91). Conversely, the loss of LiaR results in daptomycin hypersusceptibility in enterococcal species (92, 93).

Fatty acid supplementation provides protection against the membrane stressors, sodium dodecyl sulfate and daptomycin

Bacteria within a host have access to a rich milieu of fatty acids from complex sources, like bile (gastrointestinal tract) and serum (wounds). Further, some bacteria will incorporate these fatty acids from host fluids (94, 95). Yet, the mechanism of how exogenous fatty acids are brought into bacterial cells is not completely understood. in Gram-negative bacteria, there is a transport mechanism which relies on FadL to facilitate entry of long chain fatty acids into the periplasmic space and then FadD to bring the fatty acid through the inner membrane where it can be acted upon by downstream enzymes

(96, 97). Gram-positive bacteria, on the other hand, do not have a known transport system for fatty acids and it is thought that these bacteria can act upon exogenous fatty acids after they have flipped into the lipid bilayer using a pH gradient (59). Despite the uncertainty of the fatty acid uptake mechanism in Gram-positive bacteria, accessing the fatty acids in bile and sera does occur for a variety of species (94, 95).

We have published data showing that *E. faecalis* can take in and incorporate exogenous fatty acids. Moreover, incorporating exogenous fatty acids can impact physiology, but the effect a supplied fatty acid can have on the cell appears to be dependent on the type of fatty acid provided. In general, supplementing *E. faecalis* with either saturated fatty acids or unsaturated fatty acids results in a membrane dominated by the supplemented fatty acid, displacing a membrane composition which has a saturated:unsaturated fatty acid ratio of 1 (98, 99). Interestingly, *E. faecalis* incorporates exogenous fatty acids at the detriment of its physiology because supplementing with specific, individual fatty acids can have disparate effects on generation time as well as cell morphology (99). Saturated fatty acids ($C_{12:0}$-$C_{20:0}$) tend to result in improper division, except for arachidic acid ($C_{20:0}$). Further, unsaturated fatty acids can result in more rounded cellular morphology (particularly palmitoleic acid – $C_{16:1cis9}$). Surprisingly, supplementation with specific fatty acids can impact sensitivity to membrane damaging agents.

When *E. faecalis* is grown in the presence of bile or serum, the composition of the membrane is altered with different native fatty acids as well as host specific fatty acids (98). Exposing bile or serum supplemented *E. faecalis* to membrane damaging agents like sodium dodecyl sulfate (SDS) or daptomycin, resulted in increased cell survival as compared to controls. When individual fatty acids were supplemented (as determined by those fatty acids that appeared after bile or serum supplementation), only the host associated unsaturated fatty acids, oleic acid and linoleic acid, improved tolerance to both SDS and daptomycin. Conversely, native unsaturated fatty acids, like *cis*-vaccenic acid, or native saturated fatty acids, like stearic acid, did not increase tolerance to SDS or daptomycin.

Summary

In the following dissertation work, I have investigated how host fatty acids increase tolerance to membrane stressors in the Gram-positive bacterium, *E. faecalis*. This work began by following the observation that supplementing *E. faecalis* with the host fluids, bile and serum, provided specific exogenous fatty acids that could improve survivability (protective) to sodium dodecyl sulfate, high bile, and the antibiotic daptomycin. However, the mechanism for how host fatty acids increase tolerance to membrane stressors remained unknown. In the subsequent chapters of this dissertation, I present work performed to address this question. In chapter 2, I examined the properties of the protective fatty acids, linoleic acid and oleic acid that induce membrane stress tolerance. In chapter 3, I addressed whether induction of daptomycin tolerance by fatty acids is dependent upon the LiaFSR three component system. In chapter 4, I assessed the role of specific phospholipid alterations in daptomycin tolerance. Finally, in chapter 5, I discuss the implications of my findings in regard to *E. faecalis*, daptomycin tolerance, and membrane stress responses for future work.

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CHAPTER II: Analogs of protective fatty acids do not promote daptomycin tolerance

Future Publication Note

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

Analogs of protective fatty acids do not promote tolerance to daptomycin. 2018/2019. John R. Harp, Eric D. Tague, William T. Brewer, Brittni M. Woodall, Katarina A. Jones, Shawn R. Campagna, and Elizabeth M. Fozo

Experiments were conducted by John Harp, with the exception of: GC-FAME which was completed by Microbial ID; cell growth for GC-FAME with help from Will Brewer, and Elizabeth Fozo; cell growth curves with help from Will Brewer; untargeted mass spectrometry analysis completed by Eric Tague, Brittany Woodall, and Katarina Jones with the assistance of Shawn Campagna. John Harp and Elizabeth Fozo wrote the manuscript.

Abstract

Enterococcus faecalis is a commensal bacterium and hospital acquired pathogen that is resistant to many antibiotics. Daptomycin is a lipopeptide antibiotic that is currently being used to treat drug resistant enterococcal infections. Previous data has shown that supplementation with host fluids can increase tolerance to daptomycin. Further experimentation with host fluid constituents showed that oleic acid and linoleic acid increased tolerance to daptomycin. In this study, we test certain properties of oleic acid and linoleic acid and show that analogs of these protective fatty acids can impact the induction of daptomycin tolerance. We found that changing the double bond in oleic acid from *cis* to *trans* (elaidic acid), resulted in a loss of daptomycin tolerance. Further, fatty acid analogs containing an amide-group could be found in the membrane after supplementation but could not increase daptomycin tolerance. These results indicate that bond orientation and the carboxyl-group are important for host fatty acid induced daptomycin tolerance.

Introduction

Enterococcus faecalis is a Gram-positive commensal bacterium capable of causing clinical complications in immunocompromised individuals (1). Unfortunately, this organism is inherently resistant to many therapeutics leaving few options for treatment. One treatment used by clinicians to eliminate drug resistant enterococci is the membrane targeting antibiotic daptomycin. Daptomycin is a lipopeptide antibiotic that associates with phosphatidylglycerol, oligomerizes with other daptomycin monomers to destabilize the membrane and causes cell death (2–5). Despite initial evidence that daptomycin resistance was rare in clinical trials (6), resistant strains have been isolated from the clinic and studied (7). Moreover, *in vitro* evolution experiments also showed a capacity for enterococci to develop resistance to daptomycin (8). Genetic analysis of daptomycin resistant clinical isolate and an *in vitro* evolved strain showed that genes responsible for cell membrane homeostasis were contributing (9, 10).

Genetic alterations are not the only mechanism for increasing daptomycin tolerance. When *E. faecalis* is grown in the presence of bile or serum, *E. faecalis* can incorporate fatty acids from these sources resulting in increased daptomycin tolerance (11, 12). It is thought that exogenous fatty acids flip into the Gram-positive membrane using a passive proton mediated process. Work in *Staphylococcus aureus* showed that fatty acid binding protein B (FakB) binds to the fatty acid, which subsequently allows fatty acid kinase (FakA) to phosphorylate the exogenous fatty acid (13, 14). After phosphorylation, the activated fatty acid can be used in phospholipid synthesis. *E. faecalis* incorporation of exogenous fatty acids is believed to occur in a similar fashion (13).

Not surprisingly, incorporation of exogenously supplied fatty acids into the membrane of *E. faecalis* leads to an altered membrane phospholipid composition (unpublished observations) and physiological changes, dependent upon the fatty acid provided (12). We have also shown that supplementation of an individual fatty acid to *E. faecalis* can impact generation time and dramatically alter cellular morphology (12). Further, we observed that supplementation with both host fatty acids, oleic acid ($C_{18:1\ cis\text{-}9}$) or linoleic acid ($C_{18:2\ cis\text{-}9,12}$), contributed to increased daptomycin tolerance but addition of either saturated fatty acids or *cis*-vaccenic acid ($C_{18:1\ cis\text{-}11}$ – unsaturated fatty acid produced by

E. faecalis), did not increase daptomycin tolerance (12). These data implied that specific properties of oleic acid and linoleic acid are contributing to daptomycin tolerance.

In the present study, we use analogs of oleic acid and linoleic acid (Supplemental Table 2.1) to determine which specific properties of these fatty acids induce daptomycin tolerance. We found that bond orientation is important, that increasing the degree of unsaturation does not increase tolerance, and that the carboxyl group in oleic acid and linoleic acid is critical for fatty acid mediated daptomycin tolerance.

Materials and Methods

Bacterial growth conditions. *E. faecalis* OG1RF was grown statically in brain heart infusion medium (BHI; BD Difco) at 37°C. To examine the effects of fatty acids and indicated analogs on growth, overnight cultures were diluted into fresh BHI medium containing fatty acid supplement to an optical density at 600 nm (OD_{600}) of 0.01 and allowed to grow until stationary phase. For short-term supplementation experiments, overnight cultures were diluted into fresh BHI medium as described above and grown until an OD_{600} of ~0.25. Fatty acid supplements or analogs were then added at concentrations indicated in the text and incubated at 37°C for 30 minutes (12). All fatty acids and chemicals were purchased from Sigma-Aldrich unless noted otherwise.

GC-FAME preparation and analysis. Cells were grown to log phase using the short-term supplementation strategy as described above. After exposure to exogenous fatty acids or analogs, 15 mL aliquots of cells were washed twice with 10 mL of 1X phosphate buffered saline (PBS), pelleted, and stored at -80°C prior to shipment to Microbial ID, Inc. (Newark, DE). Cells were then subjected to saponification with a sodium hydroxide-methanol mixture, a methylation step, and hexane extraction prior to GC-FAME analysis (15).

Membrane challenge assays. Cells were grown in BHI medium until exponential phase (OD_{600} of ~ 0.25) and supplemented with either ethanol (final concentration of 0.1%) or dimethyl sulfoxide (DMSO; final concentration of 0.2%) as solvent controls, 20 μg mL^{-1} oleic acid ($C_{18:1\ cis\text{-}9}$), 20 μg mL^{-1} elaidic acid ($C_{18:1\ trans\text{-}9}$), 20 μg mL^{-1} oleamide ($C_{18:1\ cis\text{-}9}$ with amide group), 20 μg mL^{-1} oleyl sulfate ($C_{18:1\ cis\text{-}9}$ with sulfate group), 10 μg mL^{-1} linoleic acid ($C_{18:2\ cis\text{-}9,\ 12}$), 10 μg mL^{-1} linoleamide ($C_{18:1\ cis\text{-}9,12}$ with amide group), or 10 μg mL^{-1}

linolenic acid ($C_{18:3\ cis\text{-}9,\ 12,\ 15}$) for 30 minutes (12). 10 mL of cells were harvested, washed twice with 10 mL of 1X PBS, then resuspended in BHI containing 1.5mM $CaCl_2$ and treated with 15 μg mL^{-1} of daptomycin. Serial dilutions were plated onto BHI agar at 0, 15, 30, and 60 minutes after exposure to daptomycin. The log ratio of survivors over time was calculated for three biological replicates and shown are the averages and standard deviations for each experiment.

Phospholipid Extraction for Mass Spectrometry. Cells that received short-term fatty acid supplementation were washed twice with 1X PBS and then resuspended in 1 mL of 1X PBS containing 100μg mL^{-1} of lysozyme. Cells were incubated at 37°C for 20 minutes and then transferred to a plastic screw top microfuge tube containing 0.5g of ≤106μm glass beads. Cells were subsequently homogenized using a mini-bead beater (Biospec Products, Bartlesville, OK) for two, one-minute intervals. Using a modification to the Folch et al. (16) and Bligh and Dyer (17) methods, homogenized cells were transferred from the microfuge tube to a 15 mL polypropylene conical containing 2:1 (v/v) chloroform:methanol. After vortexing gently, the conical tubes were centrifuged for 5 minutes at 2739xG. The organic and inorganic phases were collected, leaving behind any debris and transferred to a new 15mL polypropylene conical containing 1.5 mL of 0.9% NaCl. After vortexing gently, the conical tubes were centrifuged for 5 minutes at 2739xG. Finally, the lower organic phase, containing extracted lipids, was collected and transferred to a glass screw top and evaporated with nitrogen gas.

Ultra-performance liquid chromatography-high resolution mass spectrometry. An untargeted lipid analysis was performed on cells supplemented with the above-mentioned fatty acids using a Dionex UPLC (Thermo Scientific, Waltham, MA) and an Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA) in the method of Tague et al (submitted). Briefly, separation was preformed using a HILC column allowing for separation based on the head group's interaction with the stationary phase. Both mobile phases consisted of 10 mM ammonium formate adjusted to pH 3.0. Mobile phase A was 100% water, and mobile phase B was 97:3 acetonitrile: water. A gradient from 0% A to 52% A was used over 25 minutes followed by 10 minutes of re-equilibration. The elutant was analyzed using electrospray ionization and data was collected in negative and positive mode with a scan range from 100-1500 *m/z*. All ion

fragmentation at 35 eV was used as a qualitative confirmation of lipid fragments. Phospholipid standards (Avanti Polar Lipids, Inc. Alabaster, AL), were run to verify retention time (RT) and gain exact *m/z* for each lipid class. The phosphatidylglycerol standard had two octadecanoic acid tails (PG 36:0), and the lysyl-phosphatidylglycerol standard had two oleic acids tails (L-PG 36:2). All other lipids within each head group have the same ionization efficiency and RT as the corresponding standard so an exact standard for each individual phospholipid detected was not necessary.

Full Scan *.raw* files generated by the mass spectrometer were converted to *.mzML* files by MS convert (18, 19). MAVEN software (20) was used to integrate areas under the curve for all compounds detected based on a mass window of ± 5 ppm, exact *m/z* and known RT. Heat map visualizations were created in R using an in house created script by averaging values for each treatment and comparing them to the appropriate controls. Ratios were then $\log_2$ transformed and assigned a color based on degree of change. An orange color indicates the specific lipid is more abundant in the fatty acid supplemented cells compared to the control. A blue color represents the lipid detected is less abundant compared to the control. Five biological replicates for each supplement were analyzed and significance was determined using a two-tailed unpaired Student's *t*-test.

Statistical analysis. Differences in the membrane phospholipid or fatty acid content between growth conditions as well as differences in log ratio of survivors over time were determined using a two-tailed, unpaired Student's *t* test.

Results

Appropriate bond orientation of unsaturated fatty acids is required for the induction of daptomycin tolerance. We previously showed that growing *E. faecalis* OG1RF in the presence of specific host fatty acids (oleic acid and linoleic acid) increased tolerance to the antibiotic daptomycin (11, 12). However, the reason why these specific fatty acids, and not *cis*-vaccenic acid, stearic acid, or palmitic acid, increased daptomycin tolerance had yet to be elucidated. To better determine the unique properties of oleic acid and linoleic acid that induced protection, we used a series of oleic acid and linoleic acid analogs.

As both oleic acid and linoleic acid are 18-carbon containing unsaturated fatty acids with *cis* bonds, we were interested to determine if bond orientation (*cis* versus *trans*) was critical to our observations. Previous data showed that supplementing *E. faecalis* OG1RF with oleic acid ($C_{18:1cis\text{-}9}$) does not impact generation time as compared to solvent controls (11, 12) and similarly, elaidic acid ($C_{18:1trans\text{-}9}$), did not impact generation time (Table 2.1). To conclude if elaidic acid was present in the membrane of OG1RF, we conducted gas chromatography of fatty acid methyl esters (GC-FAME) (see Materials and Methods). As seen in Table 2.2, elaidic acid was abundantly present, with 67% of the membrane fatty acid composition containing this fatty acid. As a consequence, the percent totals of all other fatty acids, including palmitic acid and *cis*-vaccenic acid were reduced.

Both growth analysis and membrane analysis of fatty acids indicated that supplementation with elaidic acid ($C_{18:1\ trans\ 9}$) was similar to oleic acid ($C_{18:1\ cis\ 9}$) (11, 12). To assess whether or not a *cis* bond at the 9th position of a C_{18} acyl tail was critical for daptomycin tolerance, we supplemented OG1RF with oleic acid or elaidic acid. As shown in Figure 2.1, supplementation with elaidic acid did not induce daptomycin tolerance and was similar to the solvent control. Thus, changing the *cis* bond at the 9th position (oleic acid) to a *trans* bond at the 9th position (elaidic acid) does not induce daptomycin tolerance. These data suggest that the *cis* bond is critical for stress survival against daptomycin.

Supplementation with linolenic acid impacts growth kinetics, does not increase daptomycin tolerance. Our previous findings indicated that linoleic acid ($C_{18:2\ cis\ 9,\ 12}$, two double bonds) may induce better protection from daptomycin than oleic acid ($C_{18:1\ cis\ 9}$, one double bond) (11). These data made us question if increasing the number of *cis* bonds could further promote fatty acid induced tolerance. First, we tested how *E. faecalis* responded to supplementation with linolenic acid ($C_{18:3\ cis\ 9,12,15}$). We found that the generation time of OG1RF with linolenic acid was statistically greater than solvent control (Table 2.1), but similar to linoleic acid ($C_{18:2cis\text{-}9,12}$) (11). Surprisingly, short-term supplementation with linolenic acid was not detected in the membrane when using GC-FAME analysis (see Discussion). However, this is likely due to insufficient detection sensitivity during analysis.

Table 2.1. Generation times for *E. faecalis* OG1RF after supplementation.

Supplement[a]	Concentration	Generation time (min)
Ethanol	0.1% final	36.9 ± 0.3
DMSO	0.2% final	37.0 ± 0.7
Oleic acid	20 μg mL^{-1}	47.0 ± 1.1
Elaidic acid	20 μg mL^{-1}	48.9 ± 0.8
Oleamide	20 μg mL^{-1}	40.0 ± 1.6
Oleyl sulfate	20 μg mL^{-1}	37.9 ± 0.2
Linoleic acid	10 μg mL^{-1}	88.2 ± 8.3
Linolenic acid	10 μg mL^{-1}	78.2 ± 5.0
Linoleamide	10 μg mL^{-1}	850.1 ± 190

[a]BHI medium was used for all cultures.

Table 2.2. *E. faecalis* OG1RF membrane fatty acid composition after short-term supplementation with host fatty acid analogs.

	% of total membrane content (Avg ± SD)[a]						
	Ethanol	DMSO	$C_{18:1}$ *trans* 9 Elaidic acid	$C_{18:1}$ *cis* 9 Oleamide	$C_{18:1}$ *cis* 9 Oleyl sulfate	$C_{18:2}$ *cis* 9,12,15 Linolenic acid	$C_{18:2}$ *cis* 9,12 Linoleamide
Concentration	0.1% final	0.2% final	20 μg mL^{-1}	20 μg mL^{-1}	20 μg mL^{-1}	10 μg mL^{-1}	10 μg mL^{-1}
Fatty acid							
$C_{12:0}$	1.8 ± 0.3	1.7 ± 0.1	0.4 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.8 ± 0.1
$C_{14:0}$	4.8 ± 0.1	4.6 ± 0.5	2.1 ± 0.3	3.3 ± 0.1	3.9 ± 0.1	4.2 ± 0.1	3.4 ± 0.2
$C_{16:0}$ N Alcohol	ND	ND	ND	ND	1.7 ± 0.1	ND	ND
$C_{16:1}$ *cis* 9	7.2 ± 0.1	6.9 ± 0.5	2.1 ± 0.3	5.0 ± 0.1	6.3 ± 0.2	6.9 ± 0.2	5.6 ± 0.4
$C_{16:0}$	37.8 ± 0.4	38.5 ± 0.3	13.1 ± 1.5	25.0 ± 0.3	36.0 ± 0.2	34.9 ± 0.1	28.1 ± 0.5
$C_{17:1}$ *cis* 10	ND	ND	ND	ND	2.5 ± 0.1	0.2 ± 0.3	ND
$C_{18:2}$ *cis* 9,12	ND	ND	ND	ND	0.3 ± 0.2	0.6 ± 0.04	29.1 ± 0.7
$C_{18:1}$ *cis* 9	1.7 ± 0.2	2.1 ± 0.7	0.5 ± 0.1	34.8 ± 0.9	1.2 ± 0.3	ND	ND
$C_{18:1}$ *trans* 9	ND	ND	66.8 ± 4.0	ND	ND	ND	ND
$C_{18:1}$ *cis* 11	38.3 ± 0.5	37.2 ± 0.4	12.2 ± 1.2	23.2 ± 0.3	34.1 ± 0.6	43.2 ± 0.1	27.1 ± 0.5
$C_{18:0}$	5.0 ± 0.1	5.7 ± 0.6	2.1 ± 0.3	5.5 ± 0.2	6.6 ± 0.5	5.7 ± 0.4	4.0 ± 0.4
$C_{17:0\ 2OH}$	2.0 ± 0.2	2.1 ± 0.5	ND	0.8 ± 0.1	2.2 ± 0.2	1.5 ± 0.1	1.3 ± 0.1
$C_{19:0\ cyclo\ 11}$	ND	0.6 ± 0.1	ND	0.4 ± 0.04	0.8 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
$C_{20:1}$ *cis* 13	ND	ND	ND	ND	0.6 ± 0.02	0.9 ± 0.1	ND
[b]Other	1.3 ± 0.6	0.4 ± 0.1	0.6 ± 0.2	0.4 ± 0.02	2.8 ± 1.0	ND	ND
Sat/Unsat	1.1 ± 0.02	1.1 ± 0.03	0.2 ± 0.4	0.6 ± 0.01	1.1 ± 0.7	0.9 ± 1.0	0.6 ± 0.9
[c]C_{10} - C_{17} / C_{18} - C_{20}	1.2 ± 0.01	1.2 ± 0.1	0.2 ± 0.4	0.6 ± 0.01	1.2 ± 0.4	1.0 ± 1.3	0.6 ± 0.7

[a]Membrane content was determined using GC-FAME analysis by Microbial ID, Inc.; numbers represent average ± standard deviation from three independent cultures. ND indicates that fatty acid was not detected.
[b]Other indicates fatty acids that comprised <1% of the total membrane content.
[c]Fatty acid length ratio includes both saturated and unsaturated fatty acid.

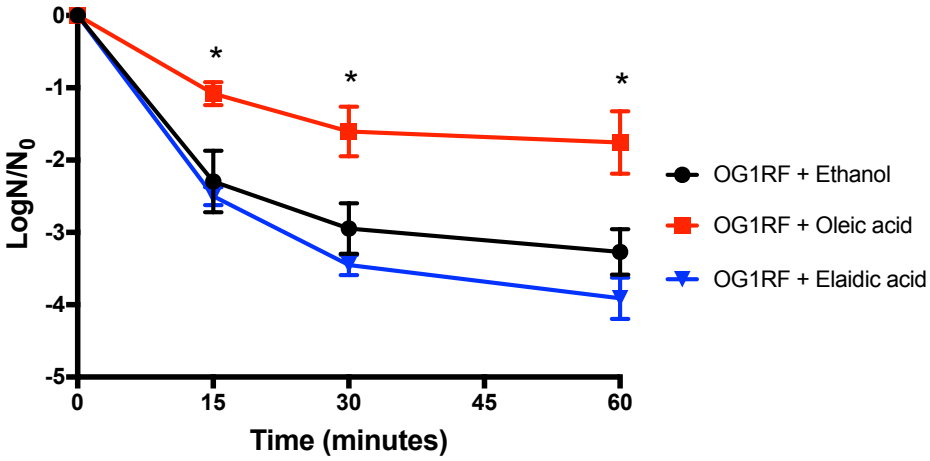

Figure 2.1. Short-term fatty acid supplementation with elaidic acid ($C_{18:1\ trans\ 9}$) fails to protect *E. faecalis* OG1RF from daptomycin challenge.

OG1RF was grown to mid log phase and then the culture was split and supplemented (see Materials and Methods). Oleic acid supplementation of OG1RF increased the number of survivors versus the solvent control at all time points (P = 0.008) while elaidic acid did not. Shown are the average ± standard deviation for n = 3.

Given the impact on growth, we examined whether short-term supplementation with the fatty acid could induce daptomycin tolerance in OG1RF. As shown In Figure 2.2, linolenic acid did not induce tolerance. Thus, simply increasing the number of *cis* bonds in a fatty acid is not sufficient to alter sensitivity to daptomycin.

Supplementation with fatty acids containing an amide group did not increase daptomycin tolerance. Studies have shown that the phosphorylation of exogenous fatty acids is important for incorporation into phospholipid synthesis (13). Further, fatty acids lacking a carboxyl-group cannot be phosphorylated by the fatty acid kinase system (21). To test if the carboxyl-group of linoleic acid and oleic acid is required for host fatty acid induced daptomycin tolerance, we tested several fatty acids which harbor an amide-group (oleamide or linoleamide) or a sulfate-group (oleyl sulfate) instead of a carboxyl-group.

Supplementing OG1RF with fatty acids lacking a carboxyl-group had variable effects on generation time (Table 2.1). Oleamide ($C_{18:1}$*cis*-9 with amide group) and oleyl sulfate ($C_{18:1}$*cis*-9 with sulfate group) had no impact on generation time. Conversely, the generation time after linoleamide ($C_{18:2}$*cis*-9,12 with an amide group) supplementation was ~22 times longer than the solvent control and ~10 times longer than linoleic acid. These alterations (or lack thereof) in generation times, however, were not necessarily reflective of their presence in the membrane via GC-FAME. After short-term supplementation with oleamide and linoleamide, we found that about 35% and 30% (reported as $C_{18:1\ cis\ 9}$ or $C_{18:2\ cis\ 9,12}$ respectively) of the membrane contained these fatty acids. However, oleyl sulfate was not detected via GC-FAME because this fatty acid cannot be esterified due to the lack of carbonyl moiety. Interestingly, after oleyl sulfate supplementation we did notice the presence of unique fatty acids, such as cetyl alcohol (C_{16} N alcohol) and heptadecenoic acid ($C_{17:1\ cis\ 10}$).

To test if fatty acid incorporation onto phospholipid head groups is necessary for increased daptomycin tolerance, we challenged cells supplemented with oleamide, linoleamide, or oleyl sulfate against daptomycin (Figure 2.3). As seen in Figure 2.3A, supplementation with oleamide did not increase tolerance to daptomycin and is similar to the dimethyl sulfoxide (DMSO) solvent control. Similarly, linoleamide (Figure 2.3B) did not increase tolerance to daptomycin. Surprisingly, supplementation with oleyl sulfate

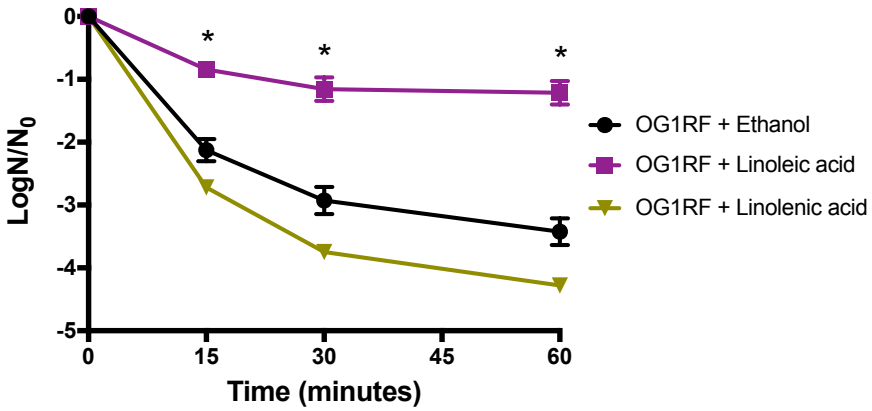

Figure 2.2. Short-term fatty acid supplementation with linolenic acid fails to protect *E. faecalis* OG1RF from daptomycin challenge.

OG1RF was grown to mid log phase and then the culture was split and supplemented (see Materials and Methods). Linoleic acid supplementation of OG1RF increased the number of survivors versus the solvent control at all time points (P = 0.0004) while linolenic acid supplementation of OG1RF did not. Shown are the average ± standard deviation for n = 3.

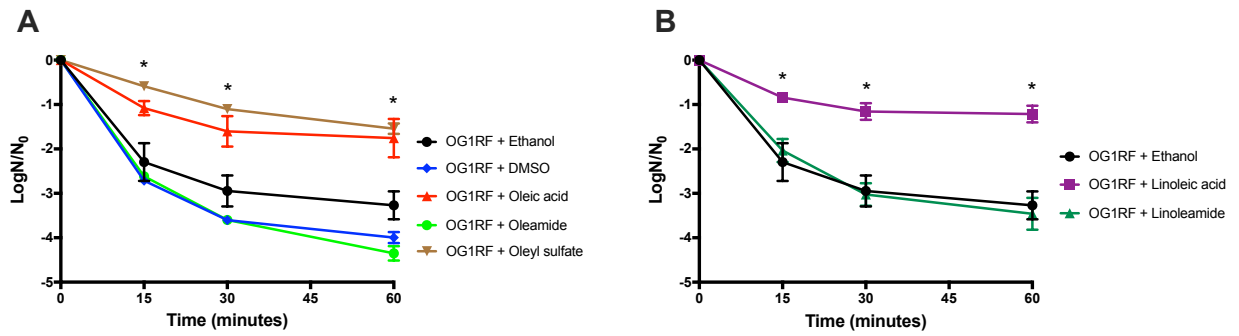

Figure 2.3. Short-term supplementation with fatty acids without a carboxyl group do not protect OG1RF from daptomycin challenge.

OG1RF was grown to mid log phase and then the culture was split and supplemented (see Materials and Methods). (A) Oleic acid supplementation of OG1RF increased the number of survivors versus the ethanol solvent control at all time points (P = 0.008) as did oleyl sulfate (P = 0.0024). Oleamide supplementation did not increase the number of survivors versus the DMSO solvent control. (B) Linoleic acid supplementation of OG1RF increased the number of survivors versus the ethanol solvent control at all time points (P = 0.0004) while linoleamide did not. Shown are the average ± standard deviation for n =3.

significantly increased ($P \leq 0.0024$) tolerance to daptomycin as compared to the ethanol control.

Taken together, short-term supplementation with fatty acids that lack a carboxyl-group are found in the membrane as determined by GC-FAME. Further, it appears that the carboxyl-group is important for increased tolerance to daptomycin. However, oleyl sulfate appears to be inducing a mechanism of tolerance, possibly unrelated to our previous observations (see Discussion).

Fatty acid analogs can be found free in the membrane. We observed that ~30% of the membrane fatty acid composition contained oleamide and linoleamide after short-term supplementation (Table 2.2). These data were surprising, since previous studies suggested that a fatty acid without a carboxyl group should not be phosphorylated by FakA (21), which is needed for incorporating exogenous fatty acids into phospholipid synthesis (13). Given that we observed the presence of oleamide and linoleamide in the GC-FAME results, we wanted to confirm whether these fatty acids were associated with phospholipid head groups, free within the membrane, or both. To discern these possibilities, we used an untargeted mass spectrometry method (see Materials and Methods). Collectively, our analysis detected 19 phosphatidylglycerol (PG) species, 18 lysyl-phosphatidylglycerol (L-PG) species, and the presence of each of the supplemented fatty acids (oleic acid, elaidic acid, linoleic acid, linolenic acid, oleamide, linoleamide, and oleyl sulfate) in our cell extract samples.

When cells were given oleic acid, the amount of oleic acid as a free fatty acid in the membrane was significantly higher than the solvent control (66 – fold increase) (Figure 2.4). As a whole, all detected L-PG species were increased over the solvent control. Conversely, most PG species were decreased (exception PG 38:1). Similarly, when cells were supplemented with linoleic acid, there was an increase in free linoleic acid (92,000 – fold), along with an increase in all L-PG species; with major increases in L-PG which could contain linoleic acid tails (34:3, 34:4, 36:3, 36:4). These data show that host fatty acids can be found free in the membrane as well as in membrane phospholipids, such as L-PG.

To test if changing the *cis* bond to a *trans* bond on a C18 acyl tail impacted localization

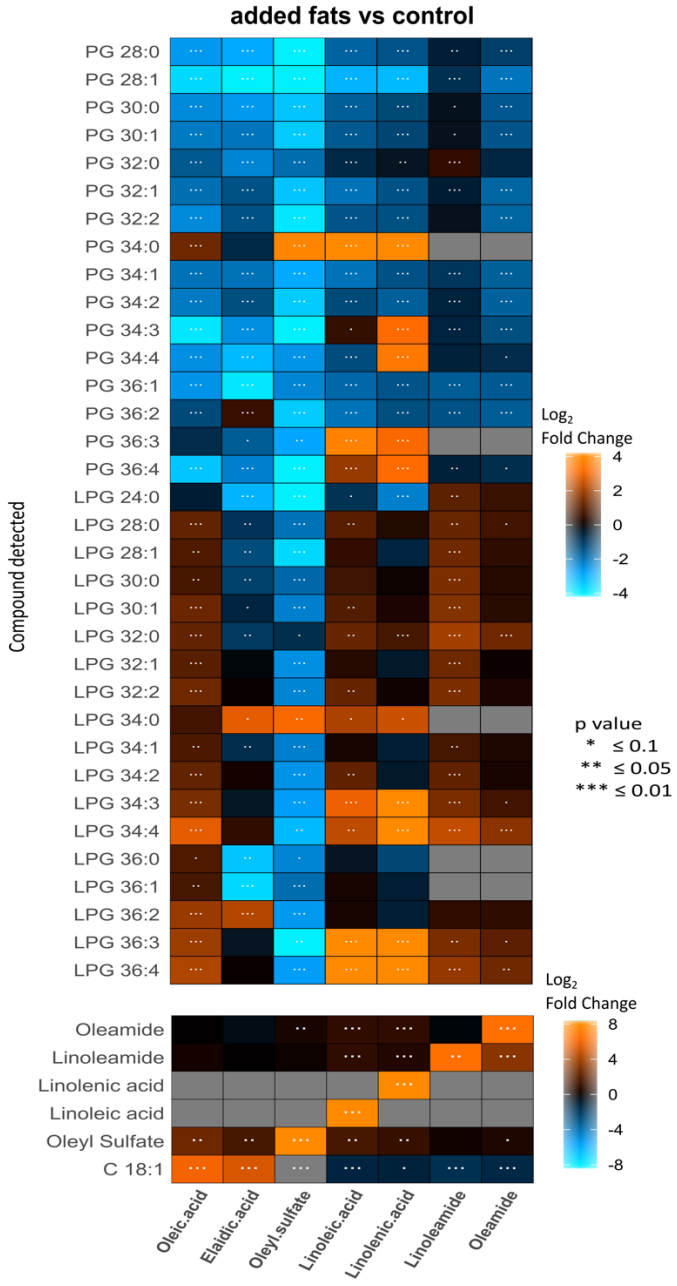

Figure 2.4. Untargeted mass spectrometry of phospholipids and free fatty acids after supplementation of *E. faecalis* OG1RF with host fatty acids and their analogs. Columns represent the supplement and rows represent the species detected (Top) phospholipids and (Bottom) free fatty acids. 20 μg mL^{-1} of oleic acid analogs (elaidic acid, oleyl sulfate, and oleamide) and 10 μg mL^{-1} of linoleic acid analogs (linolenic acid and linoleamide) were supplemented to n = 5 biological replicates. Shown is a heatmap of fold changes relative to solvent controls.

of the fatty acid, we supplemented OG1RF with elaidic acid and analyzed the cell membrane extract. We found that elaidic acid was free in the membrane (39.8 –fold) and was also likely incorporated onto several phospholipid species (L-PG 36:2 and PG 36:2).

Upon supplementation with oleamide, a large amount of the fatty acid was found free in the membrane (99.3 – fold). Interestingly, we found minimal phospholipid incorporation of the fatty acid. It appeared that only L-PG 32:0, 34:4, and 36:4 were increased, and unlikely the result of oleamide incorporation.

Similarly, supplementation with linoleamide resulted in a higher abundance of free linoleamide in the membrane (103.8 – fold) as compared to the solvent control. Interestingly, there was an increase in several L-PG species such as L-PG 34:2, 34:3, 34:4 (highest fold change), 36:3, and 36:4, which is a result of linoleamide supplementation, but likely not direct phospholipid incorporation. There were also significantly lower levels of most detected PG classes after linoleamide addition, a reaction similar to supplementation with linoleic acid.

Unlike oleamide and linoleamide, oleyl sulfate possesses a sulfate instead of a carboxyl group. The lipid profile for this treatment resulted in large decreases in all L-PG and PG. Unlike GC-FAME, it was possible to measure free oleyl sulfate and there was a large increase in the relative amounts detected in the membrane (28,334 – fold). Despite the increased presence of this fatty acid free in the membrane, it did not appear to be present in the phospholipid profile.

Discussion

Supplementation of *E. faecalis* OG1RF with the host fatty acids, oleic acid and linoleic acid, can increase tolerance to daptomycin (11, 12). In this study, we provide evidence that certain physical properties of oleic acid and linoleic acid confer protection against daptomycin. Specifically, we find that the *cis* bond in oleic acid as well as the carboxyl group for both oleic acid and linoleic acid are important for increased daptomycin tolerance.

The *cis* bond in oleic acid is important for daptomycin tolerance. This study revealed that fatty acid induced daptomycin tolerance can be eliminated by changing the double

bond of $C18^{\Delta 9}$ from *cis* (oleic acid) to *trans* (elaidic acid). Bacterial membranes typically consist of saturated and unsaturated fatty acids in the *sn1* and *sn2* positions, respectively (22), although, this specific localization of fatty acids is not always the case (23). The presence of *cis* unsaturated fatty acids on phospholipids is believed to be critical for a number of reasons, but two of the primary reasons include providing a thermodynamically favorable membrane fluidity for membrane homeostasis (24) and for proper enzymatic activity of membrane bound proteins (25). Contrary to *cis* unsaturated fatty acids, *trans* unsaturated fatty acids can also be found in cell systems, but data from computer simulations and membrane fluidity experiments showed that *trans* unsaturated fatty acids impact membranes in a way similar to saturated fatty acids (26, 27). Previous data shows that when *E. faecalis* OG1RF is supplemented with saturated fatty acids, the cells do not have increased tolerance to daptomycin (11, 12) and so it is no surprise that a membrane packed with *trans* unsaturated fatty acids, that is biophysically similar to a membrane packed with saturated fatty acids, results in no increased daptomycin tolerance. However, despite our observations, other studies of daptomycin resistant strains have shown that decreased cell membrane fluidity appeared to be a contributing factor to resistance (10).

***E. faecalis* OG1RF can incorporate polyunsaturated fatty acids but they may not induce daptomycin tolerance.** Uptake or synthesis of unsaturated fatty acids containing one or two double bonds is a common feature of many bacterial cells (albeit, OG1RF shows no evidence of *de novo* synthesis of polyunsaturated fatty acids (11, 28, 29)). However, a limited number of bacteria (mostly marine) can produce acyl chains consisting of up to four double bonds and biochemical investigations show that a very specific array of genes (*pfa)* are responsible for the production of these tails (30). Although OG1RF does not produce polyunsaturated fatty acids (no evidence for *pfa* genes using *pfaA* from *Photobacterium profundum* as bait in BLAST), it can incorporate linoleic acid into its membrane and evidence suggests that it protects again daptomycin better than oleic acid (11). From this evidence, we examined whether the polyunsaturated fatty acid, linolenic acid could induce tolerance. We found that supplementation with linolenic acid did not increase daptomycin tolerance. Further, we found no evidence of this fatty acid in the membrane of OG1RF via GC-FAME likely due to insufficient detection sensitivity during

GC-FAME analysis. However, analysis of individual phospholipids (Figure 2.4) showed that several species of L-PG and PG, which could theoretically harbor linolenic acid (34:3. 34:4 36:3, and 36:4), were increased at least 15-fold. These data suggest that it is indeed possible for linolenic acid to be incorporated, but we also see an abundance of free linolenic acid in the membrane. This could be a consequence of an inability of FakB to bind unsaturated fatty acids with three *cis* bonds due to steric hindrance (13). Or perhaps it is because OG1RF does not appear to contain the protein that could facilitates addition of polyunsaturated fatty acids onto phospholipids (1-acylglycerol-3-phosphate-*O*-acyltransferase - AGPAT) (31). Additional work should be done to identify if the species that increase after linolenic acid supplementation (L-PG 34:3, 34:4) do indeed harbor linolenic acid.

The carboxyl group on fatty acids is important for fatty acid induced daptomycin tolerance increases. The carboxyl group of fatty acids is an important molecular moiety necessary for attachment of the fatty acids to the glycerol backbone of glycerol-3-phosphate (32). Further, phosphorylation of the carboxyl group by FakA is an apparent requirement for incorporation of exogenous fatty acids into phospholipid synthesis (13). Our data show that when the carboxyl group of either oleic acid or linoleic acid is replaced with an amide group, there is a loss of daptomycin tolerance. These data suggest that daptomycin tolerance under our experimental conditions relies on the incorporation of these fatty acids into phospholipids.

Contrary to this argument is the observation that oleyl sulfate can increase tolerance to daptomycin after supplementation. This particular fatty acid does not contain a carboxyl group, so presumably it cannot be phosphorylated using the FakA mechanism. This appears to be the case because we do not see any change in the detected phospholipid species (Figure 2.4). Given that we find a large amount of this fatty acid free in the membrane (2,718-fold increase versus solvent control), it is possible that increased negative charge is preventing daptomycin from acting on the cell.

Free fatty acids in the membrane. Supplementation with exogenous fatty acids, results in those fatty acids flipping into the membrane, which can be brought into the cell and

potentially incorporated onto membrane phospholipids or their derivatives. However, *E. faecalis* does not have the capacity to degrade fatty acids, so their fate is tied into the activity of fatty acid biosynthesis or phospholipid synthesis. If the fatty acid is not a good substrate for these processes, or there is excess fatty acid, toxicity may be the result (23). This is clear in how certain fatty acids impact generation time (Table 2.1). Interestingly, in our data, we observed that free linoleic acid is found at high levels in the membrane (92,006-fold increased), and significantly impacts generation time (~88 min) but is still able to increase tolerance to daptomycin. Conversely, linolenic acid, which is also found free to a high degree (28,334-fold increase), can impact generation time (~78 min), but does not increase tolerance to daptomycin. These data suggest that just because a fatty acid is found free in the membrane to a high abundance, does not result in increased daptomycin tolerance. Further, these data suggest that not all free fatty acids are equal in the membrane after supplementation and that some are more toxic to growth than others (linoleamide). An analysis of toxic fatty acids and the abundance of the fatty acid being free in the membrane may offer intriguing insights (12).

Taken together, the data presented in this study aimed to identify the characteristics of oleic acid and linoleic acid that may contribute to induction of daptomycin tolerance. We observed that the *cis* bond at the 9^{th} position of oleic acid was conferring a protective advantage against daptomycin while a *trans* bond at that position did not. Additionally, we tested if incorporation was important for host fatty acid induced tolerance. In this experiment, we used fatty acids that lack a carboxyl group and instead have an amide group, which has been proposed to lose the ability to be incorporated in *S. aureus* (21). Indeed, we found that oleamide and linoleamide failed to increase tolerance to daptomycin. Interestingly, we found that these particular fatty acids could be found in the membrane as incorporated fatty acids, but also as free fatty acids. Further experiments should be conducted to understand this particular outcome.

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Appendix

Supplemental Table 2.1 Fatty acids used in experiments.

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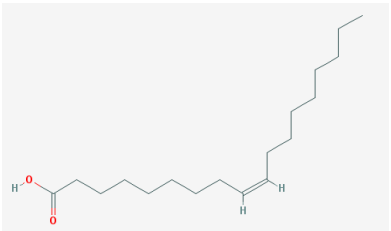	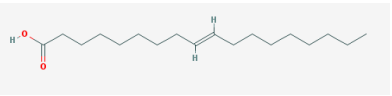
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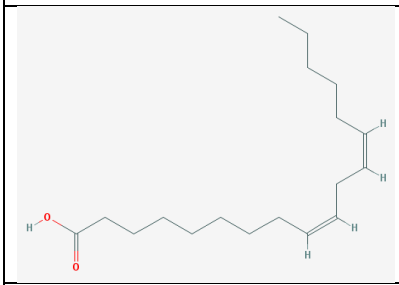
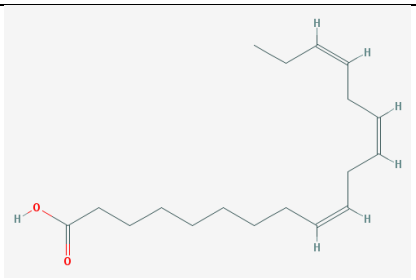
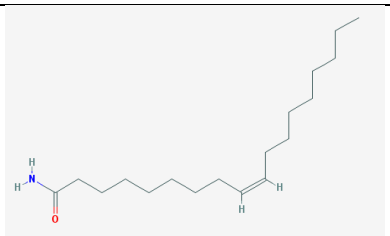
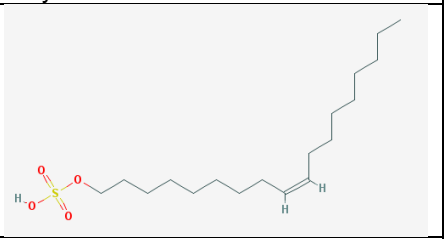
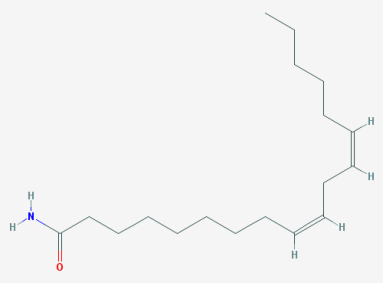
	
Oleamide	Oleyl sulfate
	
Linoleamide	
	Source: PubChem URL: https://pubchem.ncbi.nlm.nih.gov

CHAPTER III: Exogenous fatty acids protect *Enterococcus faecalis* from daptomycin induced membrane stress independent of the response regulator LiaR

Publication Note

A version of this chapter was originally published by John R. Harp, Holly E. Saito, Allen K. Bourdon, Jinetthe Reyes, Cesar A. Arias, Shawn R. Campagna and Elizabeth M. Fozo in Applied and Environmental Microbiology:

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Experiments were conducted by John Harp, with the exception of: GC-FAME which was completed by Microbial ID; cell growth for GC-FAME completed by Holly Saito; analysis of GC-FAME data by Holly Saito and Elizabeth Fozo; NMR analyses completed by Allen Bourdon with the assistance of Shawn Campagna. Jinetthe Reyes and Cesar Arias provided bacterial strains and manuscript feedback. John Harp and Elizabeth Fozo wrote the manuscript.

Abstract

Enterococcus faecalis is a commensal bacterium of the gastrointestinal tract that can cause nosocomial infections in immunocompromised humans. The hallmarks of this organism are its ability to survive in a variety of stressful habitats and, in particular, its ability to withstand membrane damage. One strategy used by *E. faecalis* to protect itself from membrane-damaging agents, including the antibiotic daptomycin, involves incorporation of exogenous fatty acids from bile or serum into the cell membrane. Additionally, the response regulator LiaR (a member of the LiaFSR [lipid II-interacting antibiotic response regulator and sensor] system associated with cell envelope stress responses) is required for the basal level of resistance *E. faecalis* has to daptomycin-induced membrane damage. This study aimed to determine if membrane fatty acid changes could provide protection against membrane stressors in a LiaR-deficient strain of *E. faecalis*. We noted that despite the loss of LiaR, the organism readily incorporated exogenous fatty acids into its membrane, and indeed growth in the presence of exogenous fatty acids increased the survival of LiaR-deficient cells when challenged with a variety of membrane stressors, including daptomycin. Combined, our results suggest that *E. faecalis* can utilize both LiaR-dependent and -independent mechanisms to protect itself from membrane damage.

Introduction

Enterococcus faecalis is a Gram-positive, facultative anaerobe that resides in the gastrointestinal tract of humans and many other mammalian species (1). Additionally, the organism is known to persist in the external environment for significant periods of time, demonstrating its ability to withstand a variety of changing conditions. Despite the commensal nature of *E. faecalis*, it is a significant contributor to nosocomial infections, including bloodstream, skin and soft tissue, and urinary tract infections, endocarditis, and meningitis in immunocompromised patients (2, 3). Eradication of *E. faecalis*, especially in regard to such infections, is challenging as the organism is inherently resistant to a variety of classes of antibiotics and has the ability to acquire additional resistance mechanisms via horizontal gene transfer (2, 4–6). Given this, enterococci are considered serious public health threats, and calls for new antibiotic therapies and surveillance are ongoing (7).

Although resistant to many antibiotics, infections caused by *E. faecalis* have successfully been treated with the antibiotic daptomycin. Daptomycin is naturally synthesized by *Streptomyces roseosporus* (8, 9) and is FDA approved for the treatment of skin and soft tissue infections caused by susceptible Gram-positive bacteria. The antibiotic targets the cell membranes of Gram-positive bacteria, leading to membrane depolarization and eventual cell death (10, 11). More detailed studies on the mechanism of daptomycin action suggest that the antibiotic inserts into bacterial cell membranes in a calcium-dependent manner, which then allows monomers of daptomycin to oligomerize in the outer leaflet and finally translocate to the inner leaflet, forming pore-like structures (12). This sequence of events leads to a loss of membrane homeostasis, including leakage of ions from the cytoplasm (13, 14). Despite the success of this antibiotic, daptomycin resistant strains of enterococci have been isolated during patient treatment (6, 15). Characterization of daptomycin resistant isolates by whole-genome sequencing indicates that resistance develops by chromosomal mutations in genes related to cell membrane and envelope homeostasis (16, 17) rather than by acquisition of horizontally acquired elements.

The ability to adapt and respond to environmental changes is essential for the survival of bacterial cells. Given that the cell envelope is constantly exposed to the environment, adaptive responses must be maintained or cell viability will be lost (18, 19). Across many

bacterial species, the regulatory process surrounding the cell envelope stress response consists of extracytoplasmic function (ECF) σ factors and two-component systems (TCS) (20–22). In the *Firmicutes* (low G+C Gram-positive bacteria), numerous two- and three-component systems respond to envelope damaging agents, including antimicrobial peptides and antibiotics (18, 23). One such example is the LiaFSR (lipid II-interacting antibiotic response regulator and sensor) system, which was first identified in *Bacillus subtilis* (24). In this system, LiaS is a membrane bound sensor histidine kinase, LiaR is the response regulator, and LiaF (25) is a membrane anchored negative regulator thought to affect the function of LiaS (25–27). LiaR was shown to regulate the expression of the *liaIHGFSR* locus, which, using an unknown mechanism, aids in the cellular response against cell envelope-targeting antibiotics and antimicrobial peptides (25).

Genomic analysis of a daptomycin-susceptible and -resistant clinical strain pair of *E. faecalis* revealed that a codon deletion in *liaF* was responsible for the resistance phenotype (6, 15, 28). It is thought that this mutation increased the expression of LiaSR, activating the damage response pathway and effectively abolishing the bactericidal activity of the antibiotic (28, 29). Moreover, deletion of *liaR*, encoding the response regulator of the system, can render both *E. faecalis* and *Enterococcus faecium* hypersusceptible to daptomycin, independent of the strain background (30, 31).

Our lab has recently discovered a previously unknown mechanism of environmentally induced tolerance to membrane-damaging agents (32). Specifically, we found that supplementing *E. faecalis* with bile or serum reduced susceptibility to high bile levels, sodium dodecyl sulfate (SDS), and daptomycin. Further analysis confirmed that *E. faecalis* was able to incorporate exogenous fatty acids from these supplements into its membrane, thus altering the fatty acid composition of the membrane. Supplementation with specific fatty acids, such as oleic acid, a dominant fatty acid found in bile and serum, confirmed that growth in the presence of fatty acids provided tolerance to these stressors (32).

Given these observations, we sought to address the hypothesis that the presence of exogenous fatty acids triggers a LiaFSR-mediated envelope stress response in *E. faecalis*, improving the organism's survival from membrane-damaging agents. Herein, however, we present data showing that supplementation of *E. faecalis* with exogenous

sources of fatty acids can reduce susceptibility to membrane stressors, including daptomycin, in the absence of *liaR*. These data suggest that the contribution of exogenous fatty acid incorporation to cell membrane protection is independent of the LiaFSR system.

Materials and Methods

Bacterial strains and growth conditions. *Enterococcus faecalis* strains OG1RF, OG1RFΔ*liaR* (31), OG1RFΔ*liaR::liaR* (31), and S613 and R712 (6, 15) were grown statically in brain heart infusion (BHI) medium at 37°C unless otherwise stated. Overnight cultures were used to inoculate medium to an optical density at 600 nm (OD_{600}) of 0.01. Cultures were supplemented as indicated in the text with bovine bile (Sigma-Aldrich), pooled human serum (ICN Biomedicals), fatty acids (Sigma-Aldrich), or the solvent control (ethanol).

GC-FAME preparation and analysis. Strains were grown as above with the supplements indicated in Table 3.1. At exponential phase (OD600 of ≈0.4), 10 to 12 ml of culture was centrifuged at 3,500 rpm for 10 min. Cell pellets were washed extensively twice with 1X phosphate-buffered saline (PBS). Pellets were subsequently stored at -80°C and shipped on dry ice to Microbial ID, Inc. (Newark, DE) for gas chromatography-fatty acid methyl ester (GC-FAME) analysis. Cells underwent saponification using a sodium hydroxide-methanol mixture and a hexane extraction before GC-FAME analysis as previously described (33). Results show averages and standard deviations for three independent cultures.

Membrane challenge assays. Cells were harvested at mid-log phase (OD_{600} of ≈0.4), washed with 1X PBS, centrifuged, and resuspended in the appropriate challenge medium, as performed previously (32). For antibiotic treatment, cells were resuspended in BHI medium containing 100mM $CaCl_2$ and either 10 μg/ml or 40 μg/ml daptomycin as indicated in the text. For bile treatment, cells were resuspended in an equivalent volume of 20% bovine bile. For SDS treatment, cells were resuspended in an equivalent volume of fresh BHI containing 0.05% SDS. Serial dilutions were plated onto BHI agar at 0, 15, 30, and 60 min after resuspension in the challenge medium. The log ratio of survivors

over time was calculated for three biological replicates; the averages and standard deviations for each experiment are shown.

Statistical analysis. Comparisons between the growth conditions, membrane content, and log ratio of survivors were determined using two-tailed, unpaired Student's *t* tests as indicated in the text.

^{1}H NMR analyses. Stock solutions at 1.23 mM, 50 mM, and 100 mM of daptomycin (1.0 mg, 617 nmol in 500 μl methanol-d_4), oleic acid (7.0 mg, 24.8 μmol in 500 μl methanol-d_4), and calcium chloride (5.5 mg, 49.6 μmol in 500 μl methanol-d_4), respectively, were prepared. The ^{1}H NMR spectra were recorded on a VNMRS 500MHz NMR spectrometer (Varian NMR Systems, Palo Alto, CA). The proton nuclear magnetic resonance (^{1}H NMR) experiments consisted of 128 scans using PRESAT solvent suppression peak selection. To examine a potential interaction between daptomycin and oleic acid, we looked at the spectra of mixtures containing either an equivalent concentration of daptomycin and oleic acid (1:1, daptomycin/oleic acid) or a mixture containing an excess of oleic acid (1:5, daptomycin/oleic acid). Each mixture was homogenized by vortexing, and the resulting solution was allowed to incubate at room temperature for 30 min. Following incubation, the ^{1}H NMR experiment was repeated.

To discover if calcium could induce an interaction between daptomycin and oleic acid, we also determined the spectra when calcium chloride was added. For all experiments, we used an overall molar ratio of 5:4 (calcium/daptomycin). The mixture was vortexed and allowed to incubate at room temperature for 30 min prior to the ^{1}H NMR experiment to determine the baseline spectra of a calcium-daptomycin complex. For those experiments examining how this mixture may interact with oleic acid, the fatty acid was added following the incubation of calcium chloride and daptomycin. Oleic acid was added into the mixture at either a 1:1 or 1:5 molar ratio, the mixture was homogenized by vortexing and incubated at room temperature for 30 min, and then the ^{1}H NMR experiment was repeated.

Results

Incorporation of exogenous fatty acids is similar in the presence or absence of *liaR* in *E. faecalis*. Previously, we demonstrated that growth in the presence of fatty acid

sources impacted the generation time of *E. faecalis* OG1RF (32). Most notably, growth in the presence of saturated fatty acids significantly increased the generation time of OG1RF compared to that in unsupplemented cultures. The organism also readily incorporated exogenous fatty acids into its membrane, even if those fatty acids negatively impacted growth. As the LiaFSR system has been shown to be important to the cell membrane stress response in enterococci, we wondered whether it contributed to our past observations. Thus, we examined the growth rates and membrane fatty acid contents of the parental OG1RF (control), the Δ*liaR*, and the genetically complemented Δ*liaR::liaR* strains (31) in the presence and absence of exogenous fatty acid sources. It is important to note that *E. faecalis* does not possess genes for β-oxidation; therefore, the organism either incorporates exogenous fatty acids into its membrane or, in the case of exogenous short-chain fatty acids, potentially elongates such fatty acids (34, 35).

In general, the growth rates and the membrane contents were similar for the three strains grown in BHI with a few notable differences. The generation times for all in unsupplemented medium was approximately 30 min (Table 3.1), which was increased to about 40 min when the strains were grown in the presence of ethanol (solvent control; final concentration of 0.2%). As shown in Table 3.2, the dominant fatty acids for all strains grown in BHI were *cis*-vaccenic acid ($C_{18:1\ cis\ 11}$, approximately 40%) and palmitic acid ($C_{16:0}$, about 37%). While the overall membrane content was similar between the strains, we did note that the genetic complement (Δ*liaR::liaR*) had significantly more palmitoleic acid ($C_{16:1\ cis\ 9}$) ($P < 0.05$) and less stearic acid ($C_{18:0}$) ($P < 0.05$) than the wildtype and Δ*liaR* strains. However, these differences did not influence the overall saturated/unsaturated ratio, which was close to 1 for all three strains (Table 3.2).

As *E. faecalis* can readily cause wound infections and bacteremia, we examined both the growth rate and the membrane composition upon supplementation with 15% pooled human serum. The generation times were similar for the three strains with no statistical significance observed (Table 3.1). As with growth in unsupplemented medium, the dominant saturated fatty acid was palmitic acid ($C_{16:0}$) for cultures grown in the presence of serum. Although not the major saturated fatty acid, stearic acid ($C_{18:0}$) was approximately 2-fold higher ($P < 0.001$) in all strains in comparison to that for growth without serum. The greatest differences, however, were in the unsaturated fatty acid

Table 3.1. Exponential phase generation times of *liaR* and clinical strains in minutes.

	Medium constituent				
Strain	BHI[a]	Serum[b]	Bile[c]	$C_{18:1\ cis\ 9}$[d]	Ethanol[e]
WT	32.2±0.5	36.5±0.8	32.7±1.9	39.6±0.7	42.1±1.0
Δ*liaR*	28.7±1.3	36.2±3.4	33.2±3.4	35.2±3.3	38.0±4.1
Δ*liaR::liaR*	31.4±2.9	34.1±2.8	27.5±3.4	35.2±2.4	35.0±1.9
S613	29.7±2.7	31.0±3.4	48.1±3.5	27.1±1.5	31.1±2.6
R712	29.4±2.5	36.0±1.2	40.8±3.8	30.0±2.2	38.0±1.0

[a] BHI media was used in all cultures with supplements as indicated.
[b] Pooled human serum was supplemented to a final concentration of 15%.
[c] Bovine bile was supplemented to a final concentration of 0.2%.
[d] Oleic acid was added to a final concentration of 20 µg/m.
[e] Ethanol solvent control was added to a volume equivalent to the oleic acid supplement.

Table 3.2. Membrane analysis of wild-type and mutant strains during log phase growth.

	Percent of total membrane content for indicated strain and supplement (Avg ± SD)[a]											
Strain	WT	Δ*liaR*	Δ*liaR::liaR*	WT	Δ*liaR*	Δ*liaR::liaR*	WT	Δ*liaR*	Δ*liaR::liaR*	WT	Δ*liaR*	Δ*liaR::liaR*
Fatty Acid	BHI	BHI	BHI	Serum[b]	Serum[b]	Serum[b]	Bile[c]	Bile[c]	Bile[c]	$C_{18:1\ cis\ 9}$[d]	$C_{18:1\ cis\ 9}$[d]	$C_{18:1\ cis\ 9}$[d]
$C_{12:0}$	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	ND	0.3 ± 0.0	0.3 ± 0.0	ND	ND	0.2 ± 0.2	0.7 ± 0.1	1.1 ± 0.3	1.1 ± 0.1
$C_{14:0}$	4.6 ± 0.1	4.4 ± 0.0	6.4 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.9 ± 0.1	1.1 ± 0.0	1.0 ± 0.0	1.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.3	0.5 ±0.1
$C_{16:1}$	7.0 ± 0.1	6.5 ± 0.4	9.7 ± 0.1	4.9 ± 0.1	4.9 ± 0.1	5.1 ± 0.1	1.5 ± 0.5	1.5 ± 0.0	1.7 ± 0.1	1.8 ± 0.2	1.6 ± 0.1	1.2 ± 0.1
$C_{16:0}$	37.6 ± 0.6	37.4 ± 0.2	34.9 ± 0.3	35.9 ± 0.6	36.1 ± 0.4	37.2 ± 0.3	42.6 ± 0.1	42.6 ± 0.2	42.4 ± 0.2	3.4 ± 0.5	3.1 ± 0.7	1.7 ± 0.3
$C_{17:1}$	ND	1.5 ± 0.1	ND	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	1.7 ± 0.2	1.0 ± 0.0	1.7 ± 0.1	0.3 ± 0.3	0.4 ± 0.3	0.7 ± 0.2
$C_{17:0\ 2OH}$	5.2 ± 0.7	5.2 ± 0.8	5.1 ± 0.4	0.2 ± 0.3	0.6 ± 0.1	0.5 ± 0.0	0.3 ± 0.2	0.3 ± 0.0	0.3 ± 0.0	ND	ND	ND
$C_{18:1\ cis\ 9}$	0.7 ± 0.7	0.2 ± 0.4	ND	21.2 ± 0.3	20.3 ± 0.9	18.4 ± 0.2	42.3 ± 0.2	38.4 ± 1.8	40.8 ± 0.5	76.3 ± 1.0	68.5 ± 5.0	75.1 ± 0.7
$C_{18:1\ cis\ 11}$	39.0 ± 0.5	38.5 ± 1.4	38.9 ± 0.5	4.7 ± 0.1	4.9 ± 0.2	4.4 ± 0.2	3.7 ± 0.1	3.8 ± 0.1	3.6 ± 0.0	ND	ND	ND
$C_{18:0}$	4.7 ± 0.1	5.0 ± 0.2	2.9 ± 0.1	9.2 ± 0.2	9.1 ± 0.1	7.8 ± 0.1	2.8 ± 0.1	6.9 ± 0.8	3.0 ± 0.3	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.1
$C_{20:0}$	ND	ND	ND	ND	ND	ND	0.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	15.9 ± 0.7	23.7 ± 4.1	19.2 ± 0.9
$C_{18:2}$	ND	ND	ND	18.5 ± 0.5	18.1 ± 0.3	20.7 ± 0.2	ND	ND	ND	ND	ND	ND
Others[f]	0.2 ± 0.4	0.3 ± 0.4	1.2 ± 1.1	2.5 ± 0.4	2.5 ± 1.6	2.3 ± 0.5	3.5 ± 0.3	3.9 ± 0.9	4.3 ± 0.8	ND	ND	ND
Sat:Unsat	1.0 ± 0.6	1.0 ± 0.2	0.9 ± 0.5	0.9 ± 0.9	1.0 ± 0.4	1.0 ± 0.7	0.9 ±0.5	1.1 ± 0.5	1.0 ± 1.1	0.3 ± 1.0	0.4 ± 1.0	0.3 ± 1.4
C_{10}-C_{17}: C_{18}-C_{20}[g]	1.2 ± 0.03	1.3 ± 0.4	1.4 ± 0.03	0.8 ± 0.02	0.9 ± 0.03	0.9 ± 0.01	1.0 ± 0.005	1.0 ± 0.02	1.0 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01

[a] Membrane content determined by GC-FAME analysis by Microbial ID, Inc. Values represent average and standard deviations of three independent cultures. ND indicates fatty acid was not detected.
[b] Pooled human serum was supplemented to a final concentration of 15%.
[c] Bovine bile was supplemented to a final concentration of 0.2%.
[d] Oleic acid was added to a final concentration of 20 µg/mL.
[f] Others indicates fatty acids comprised <1% of the total membrane content.
[g] Total fatty acid length ratios including both saturated and unsaturated fatty acids.

profiles upon supplementation. For cells grown with serum, the dominant unsaturated fatty acids were the eukaryote-derived oleic acid ($C_{18:1\ cis\ 9}$) and linoleic acid ($C_{18:2\ cis\ 9,12}$), which together constituted 40% of the total membrane content. There was a concomitant decrease in the amount of *cis*-vaccenic acid from approximately 40% in unsupplemented medium to less than 5% of the total membrane content in the presence of serum. These alterations did not alter the saturated/unsaturated ratios compared with those of unsupplemented cultures (Table 3.2). The lengths of the fatty acid tails were significantly longer for cells grown with serum ($P < 0.001$) (Table 3.2) as indicated by the ratio C_{10}-C_{17}/C_{18}-C_{20}. These findings are consistent with the composition of fatty acids in serum (32) and indicative of their incorporation by *E. faecalis*.

As *E. faecalis* naturally inhabits the intestine, we wanted to examine the effects of physiological levels of bile (0.2% bovine bile) upon growth and membrane content, as it too is a source of fatty acids that can be utilized by the organism (32). Growth with bile did not alter the generation times of any of the strains in comparison to growth in the absence of bile (Table 3.1). However, bile supplementation did impact the membrane contents of all strains examined. In all cases, palmitic acid ($C_{16:0}$) remained the dominant saturated fatty acid and comprised approximately 42% of the membrane, which was a modest, but significant ($P < 0.005$), increase from growth in BHI alone. As was noted with serum supplementation, oleic acid ($C_{18:1\ cis\ 9}$) was the dominant unsaturated fatty acid, at approximately 40% of the total membrane content, and there was a concomitant reduction in the amount of *cis*-vaccenic acid ($C_{18:1\ cis\ 11}$). There was also an overall decrease in the total amounts of shorter-chain fatty acids, as indicated by the C_{10}-C_{17}/C_{18}-C_{20} ratios ($P < 0.005$) (Table 3.2) when all strains were grown with bile versus without bile. Despite these changes, the saturated/unsaturated fatty acid ratios were essentially unaltered among the wild-type, Δ*liaR*, and Δ*liaR::liaR* strains.

We previously noted that *E. faecalis* can tolerate high levels of oleic acid ($C_{18:1\ cis\ 9}$) in culture and that this single fatty acid comprises the majority of the membrane content when supplemented at a final concentration of 20 μg/ml (32); indeed, this finding holds true even in a strain in which *liaR* is absent (Tables 3.1 and 3.2). For OG1RF and its derivatives examined here, oleic acid comprised approximately 70% of the membrane (Table 3.2). Essentially, *cis*-vaccenic acid ($C_{18:1\ cis\ 11}$), the native unsaturated C_{18} fatty

acid, was replaced entirely in the membrane by oleic acid. Oleic acid supplementation also markedly influenced the membrane saturated fatty acid composition. For all strains grown with exogenous oleic acid, the dominant saturated fatty acid was arachidic acid ($C_{20:0}$) and not palmitic acid ($C_{16:0}$) (Table 3.2), which is the dominant fatty acid found in cells grown without this supplement. This presence of arachidic acid was surprising, as it was not detected in unsupplemented cultures (see Discussion). Overall, the contributions of oleic acid and arachidic acid led to a membrane composition dominated by long-chain fatty acids, far different from what was observed in unsupplemented cultures (Table 3.2).

Combined, these results suggest that induction of the LiaFSR response is not required for *E. faecalis* to incorporate exogenous fatty acids. Furthermore, the membrane content of the Δ*liaR* strain is not markedly altered from that of the parental strain under the conditions examined.

Supplementation of growth medium with fatty acids can protect an OG1RF Δ*liaR* strain from membrane stress. Although the above results suggest that *liaR* is not needed for incorporation of exogenous fatty acids by *E. faecalis*, we decided to determine whether the lack of *liaR* impacted the ability of exogenous fatty acids to protect from membrane-damaging agents.

To assess the impact of LiaR and the LiaFSR system on membrane stress responses, *E. faecalis* was grown in the presence or absence of fatty acid sources and then exposed to 20% bovine bile. Figure 3.1A shows that all strains were susceptible to 20% bovine bile and that the Δ*liaR* strain was by far the most sensitive at all time points analyzed (15, 30, and 60 min). When strains were supplemented with 0.2% bile prior to challenge (providing a source of exogenous fatty acids), we observed an increase in survival for all strains. Importantly, supplementation of the medium with low levels of bile improved the survival of the deletion strain to the levels observed for wild-type OG1RF (Figure 3.1A).

One of the main exogenous fatty acids incorporated into the membranes of these strains upon bile or serum supplementation was oleic acid ($C_{18:1\ cis\ 9}$) (Table 3.2). Our previous investigations demonstrated that supplementation solely with this fatty acid protected wild-type *E. faecalis* from membrane stress (32). We sought to determine if

supplementation with oleic acid alone could protect the Δ*liaR* strain from bile-induced stress. We examined this phenomenon by comparing cultures supplemented with 20 μg/ml oleic acid to those without oleic acid prior to challenge with 20% bovine bile. The addition of oleic acid to the growth medium did provide tolerance to this membrane stress (Figure 3.1B). Nonetheless, the overall survival for all strains was best when they were supplemented with 0.2% bile than with 20 μg/ml oleic acid. As we observed with bile supplementation, growth with oleic acid was able to protect the Δ*liaR* strain from bile at a level equivalent to that observed in the wild-type or the complemented strains; thus, the inherent sensitivity of the mutant strain could be overcome.

Given the improved survival of *liaR*-deficient *E. faecalis* when grown in medium supplemented with bile or oleic acid, we subsequently wanted to determine if such supplementation could protect from a different source of membrane damage. We grew strains in the presence of 0.2% bovine bile or 20 μg/ml oleic acid to exponential phase and then challenged the cells with 0.05% SDS (Figure 3.2A and 3.2B, respectively). Similar to the results for the 20% bile challenge, the cellular viability for all strains was increased in the presence of 0.05% SDS when cultures were supplemented with 0.2% bile or 20 μg/ml oleic acid. In the case of SDS treatment, however, the presence or absence of *liaR* had no impact on survival compared to that of wild-type or complemented strains, although modification of the membrane composition did indeed rescue all strains from SDS damage.

Sensitivity to daptomycin of *liaR*-deficient *E. faecalis* is decreased upon supplementation with exogenous sources of fatty acids. To test whether the reduced daptomycin susceptibility mediated by exogenous sources of fatty acids (32) occurred through activation of the LiaFSR response, we examined daptomycin sensitivity in *E. faecalis* OG1RFΔ*liaR* (31) grown in the presence or absence of fatty acid sources. The parental, deletion, and genetically complemented strains were grown to mid-log phase (OD_{600} of ≈0.4) in the presence of either 0.2% bile, 15% pooled human serum, or 20 μg/ml oleic acid and then exposed the cells to 10 μg/ml daptomycin. Figure 3.3 shows that the *liaR* deletion mutant was extremely susceptible to this concentration of daptomycin compared to the wild-type or Δ*liaR::liaR* strain. Survival against daptomycin challenge

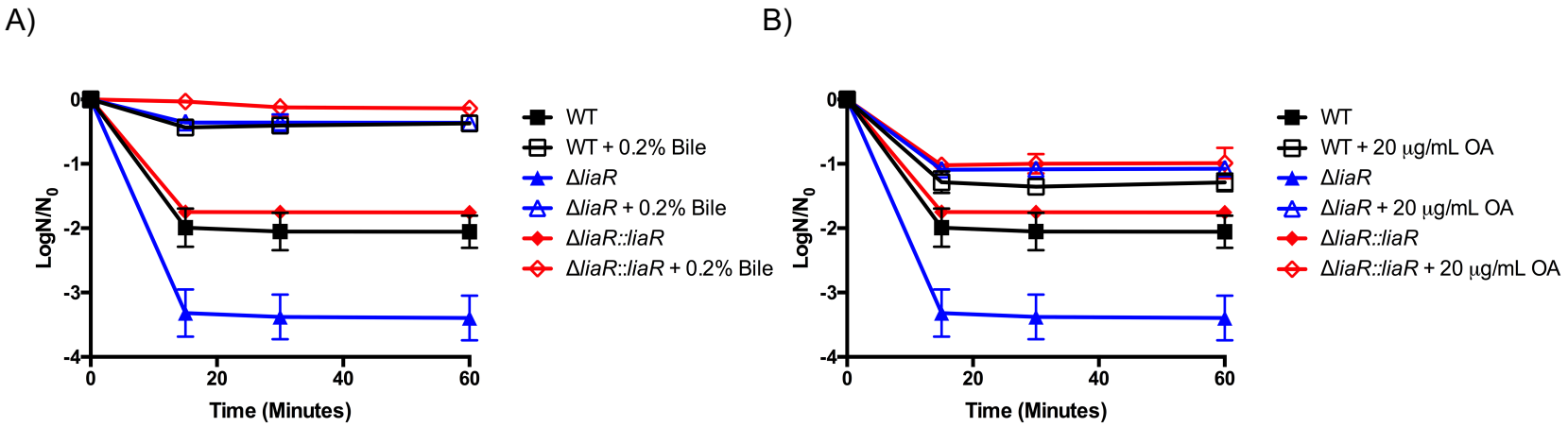

Figure 3.1. Fatty acid supplementation protects *liaR*-deficient *Enterococcus faecalis* from high bile challenge. Shown are the averages ± standard deviations for *n* = 3. (A) Bile supplementation and challenge with 20% bile. All strains supplemented with 0.2% bile had statistically increased numbers of survivors versus those of their unsupplemented counterparts at all time points analyzed ($P \leq 0.001$). (B) Oleic acid (OA) supplementation and challenge with 20% bile. All strains supplemented with 20 μg/ml OA had statistically increased numbers of survivors versus those of their unsupplemented counterparts at all time points analyzed ($P \leq 0.023$). WT, wild type.

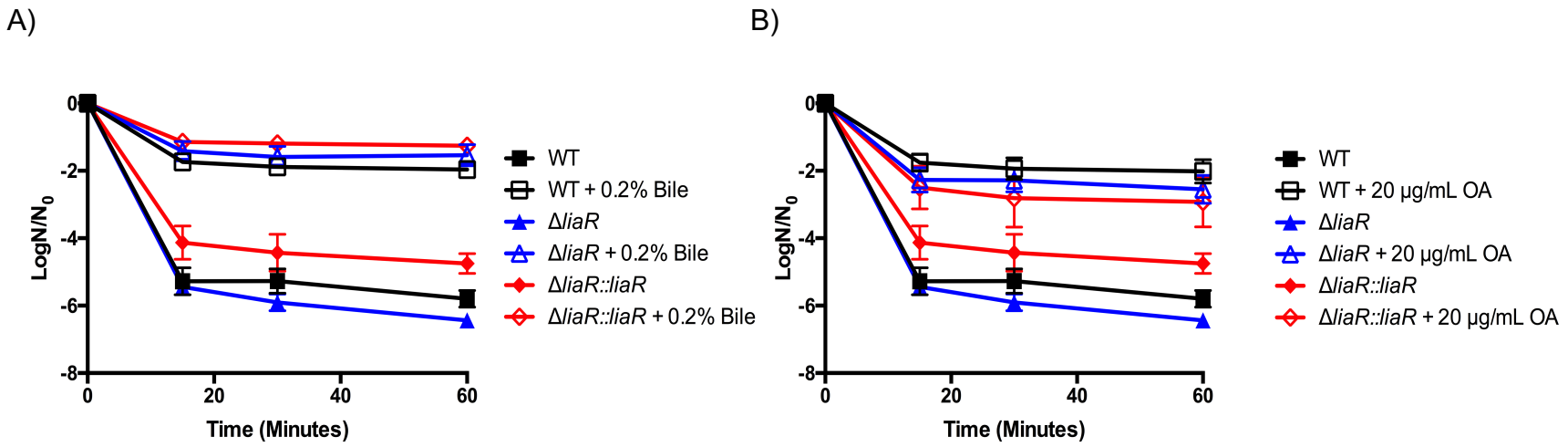

Figure 3.2. Fatty acid supplementation protects *liaR*-deficient *Enterococcus faecalis* from sodium dodecyl sulfate challenge.

Shown are the averages ± standard deviations for $n = 3$. (A) Bile supplementation and challenge with 0.05% sodium dodecyl sulfate (SDS). All strains supplemented with 0.2% bile had statistically increased numbers of survivors versus those of their unsupplemented counterparts at all time points analyzed ($P \leq 0.0001$). (B) Oleic acid (OA) supplementation and challenge with 0.05% SDS. All strains supplemented with 20 µg/ml OA had statistically increased numbers of survivors versus those of their unsupplemented counterparts at all time points analyzed ($P < 0.05$).

was significantly improved in the Δ*liaR* strain by supplementation with either bile, serum, or oleic acid. However, supplementation of the deletion strain did not yield as many survivors as supplementation of the wild-type or genetically complemented strains. Taken together, these data suggest that exogenous fatty acids can reduce daptomycin susceptibility using a mechanism independent of the LiaFSR response, but modification of the fatty acid membrane composition does not completely overcome the need for *liaR*.

Increased tolerance to daptomycin is not due to interaction with free fatty acids. Our data support the notion that the Δ*liaR* strain can incorporate exogenous fatty acids to a level similar to that of the wild-type strain, leading to better survival against membrane-damaging agents, including the antibiotic daptomycin. As daptomycin is known to insert into membranes and has a fatty acid tail (decanoic acid [$C_{10:0}$]) within its structure, we wanted to verify that our observations were not due to an interaction between daptomycin and free fatty acids. Additionally, since studies have demonstrated that the presence of calcium can alter the structure of daptomycin (13) and that the activity of the antibiotic is dependent upon calcium (36), we wanted to examine if calcium could potentially mediate an interaction between free fatty acids and daptomycin. To do this, we employed proton nuclear magnetic resonance (^{1}H NMR) to observe the interactions of daptomycin, calcium, and oleic acid.

Line broadening was observed in the spectrum containing calcium and daptomycin, which can be attributed to daptomycin aggregation, as previously reported (37). While the presence of calcium did impact the spectra of daptomycin (see Supplemental Figure 3.1 and 3.2 in the supplemental material), we noted no additional line broadening or chemical shifts in the spectra if oleic acid was added (Figure 3.4).

Thus, these data show that the lack of interaction between daptomycin and oleic acid indicates a role for altered cellular membranes and physiology in enhancing tolerance to daptomycin.

Clinically isolated *E. faecalis* strains can incorporate exogenous fatty acids. Given the breadth of diversity of *E. faecalis* isolates (34), we wanted to ensure that our observations were not limited to OG1RF. Thus, we expanded our studies to include a

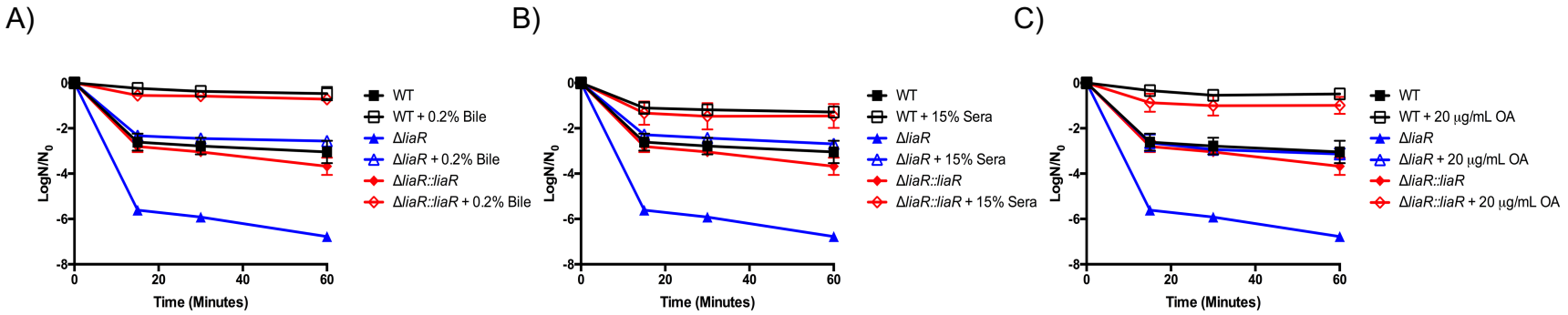

Figure 3.3. Fatty acid sources reduce daptomycin susceptibility in *liaR* deficient *Enterococcus faecalis*.
Shown are the averages ± standard deviations for *n*=3. (A) Bile supplementation and challenge with 10 μg/ml daptomycin. All strains supplemented with 0.2% bile had statistically increased numbers of survivors versus those of their unsupplemented counterparts at all time points analyzed ($P \leq 0.002$). (B) Serum supplementation and challenge with 10 μg/ml daptomycin. All strains supplemented with 15% sera had statistically increased numbers of survivors versus those of their unsupplemented counterparts at all time points analyzed ($P \leq 0.011$). (C) Oleic acid (OA) supplementation and challenge with 10 μg/ml daptomycin. All strains supplemented with 20 μg/ml OA had a statistically increased number of survivors versus those of their unsupplemented counterparts at all time points analyzed ($P \leq 0.002$).

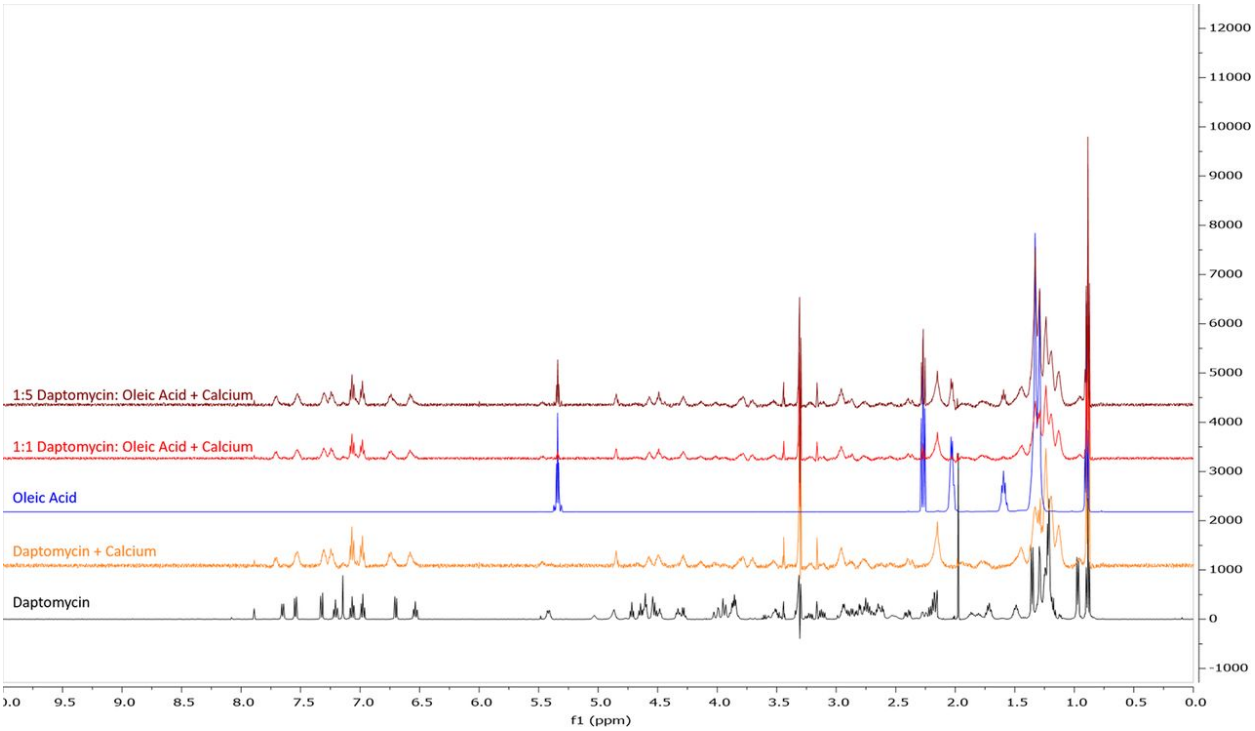

Figure 3.4. The addition of calcium does not direct an interaction between daptomycin and oleic acid.

Shown is a superimposed image of five individual ^{1}H NMR spectra, between 0.0 to 10.0 ppm. The spectra are organized as follows, from top to bottom, a 1:5 mixture of daptomycin/oleic acid plus excess calcium (maroon), a 1:1 mixture of daptomycin/oleic acid plus excess calcium (red), 50 mM solution of oleic acid (blue), 1.2 mM daptomycin plus excess calcium (orange), and 1.23 mM daptomycin solution (black). All solutions were made using methanol-d_4, and spectra were generated using a VNMRS 500 MHz instrument. Spectra were superimposed using MestReNova software.

clinical strain pair of daptomycin-susceptible and -resistant *E. faecalis* that were obtained from the bloodstream of a patient before and after daptomycin therapy (6). *E. faecalis* S613 is daptomycin susceptible (MIC of 0.5 to 1 μg/ml), and R712 is a daptomycin-resistant derivative of S613 (MIC of 8 μg/ml) (15, 28). Previous analyses of these strains showed that the sole contributing factor for their differences in daptomycin susceptibility was a mutation in the negative regulator *liaF* (28). Given that these strains are true clinical isolates, we sought to examine their abilities to both incorporate exogenous fatty acids and respond to membrane stressors.

Similar to OG1RF and its derivatives that were examined (Table 3.2), both clinical isolates had membranes dominated by palmitic acid ($C_{16:0}$) and *cis*-vaccenic acid ($C_{18:1\ cis\ 11}$) when grown in BHI (Table 3.3). However, the clinical isolates had significantly larger amounts of *cis*-vaccenic acid ($P < 0.001$) than OG1RF and its derivatives (5 to 10% increase) and, consequently, statistically lower levels of palmitic acid ($P < 0.005$); this was particularly true for R712. Despite these differences, the saturated/unsaturated ratio was not significantly different from that for OG1RF or its derivatives examined here.

Upon supplementation with 15% serum, the clinical strains did not show major changes in generation times (Table 3.1). Additionally, their membrane contents, while altered from growth in unsupplemented medium, were similar to each other's and to those of OG1RF. We again noted that the proportion of stearic acid ($C_{18:0}$), while not dominant, did double for both strains when grown in serum, and we saw similar, if not higher, increases in OG1RF as well (Table 3.2 and Supplemental Table 3.1). The same decrease in *cis*-vaccenic acid ($C_{18:1\ cis\ 11}$), and concomitant increases in oleic acid ($C_{18:1\ cis\ 9}$) and linoleic acid ($C_{18:2\ cis\ 9,\ 12}$) observed in the OG1RF-derived strains were also present in the clinical isolates supplemented with serum.

When supplemented with 0.2% bile, the clinical strains showed increases in generation times that were not observed in OG1RF or its derivatives (Table 3.1). However, this was statistically significant only for S613 ($P < 0.001$). The overall trends in membrane incorporation remained constant between the clinical isolates and OG1RF derivatives. In particular, we observed high levels of palmitic acid ($C_{16:0}$) and also increases in stearic acid ($C_{18:0}$) (Table 3.3 and Supplemental Table 3.2) for the clinical isolates that were similar to those observed with the OG1RF derivatives (Table 3.2).

Table 3.3. Membrane fatty acid analysis of clinical isolates during log phase growth.

	Percent of total membrane content for indicated strain and supplement (Avg ± SD)[a]							
Strain	S613	R712	S613	R712	S613	R712	S613	R712
Fatty Acid	BHI	BHI	Serum[b]	Serum[b]	Bile[c]	Bile[c]	$C_{18:1\ cis\ 9}$[d]	$C_{18:1\ cis\ 9}$[d]
$C_{12:0}$	0.6 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.2	0.3 ± 0.3	0.9 ± 0.0	1.1 ± 0.0
$C_{14:0}$	4.8 ± 0.1	3.7 ± 0.1	3.6 ± 0.1	2.4 ± 0.0	1.3 ± 0.1	1.6 ± 0.0	0.4 ± 0.1	0.6 ± 0.0
$C_{16:1}$	7.9 ± 0.2	7.8 ± 0.2	5.0 ± 0.2	4.7 ± 0.0	1.1 ± 0.1	1.7 ± 0.1	1.0 ± 0.2	1.4 ± 0.1
$C_{16:0}$	33.2 ± 0.2	28.2 ± 0.6	37.4 ± 0.6	36.6 ± 0.9	43.4 ± 0.7	43.0 ± 0.9	1.5 ± 0.2	1.9 ± 0.1
$C_{17:0\ 2OH}$	4.8 ± 0.2	6.1 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	ND	ND	ND	ND
$C_{18:1\ cis\ 9}$	ND	ND	15.8 ± 0.8	18.6 ± 0.4	32.0 ± 1.6	38.1 ± 1.7	56.1 ± 3.4	57.8 ± 2.9
$C_{18:1\ cis\ 11}$	44.1 ± 0.3	49.1 ± 0.2	7.5 ± 0.4	4.8 ± 0.2	3.1 ± 0.2	3.9 ± 0.2	0.5 ± 0.8	0.5 ± 0.8
$C_{18:0}$	3.4 ± 0.2	3.4 ± 0.2	7.4 ± 0.1	7.3 ± 0.1	10.8 ± 1.7	6.2 ± 1.4	0.5 ± 0.0	0.4 ± 0.0
$C_{20:0}$	ND	ND	0.1 ± 0.1	ND	2.6 ± 0.4	0.4 ± 0.4	38.6 ± 4.6	36.4 ± 3.6
$C_{18:2}$	ND	ND	18.9 ± 1.2	21.3 ± 1.4	ND	ND	ND	ND
$C_{20:4}$	ND	ND	1.1 ± 0.1	1.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	ND	ND
Others[f]	0.6 ± 1.0	0.7 ± 1.1	2.4 ± 1.4	2.3 ± 1.6	5.1 ± 0.7	4.4 ± 0.8	0.4 ± 0.1	ND
Sat:Unsat	0.8 ± 0.5	0.6 ± 0.8	1.0 ± 0.3	0.9 ± 0.5	1.6 ± 1.4	1.2 ± 1.1	0.7 ± 1.1	0.7 ± 1.0
C_{10}-C_{17}: C_{18}-C_{20}[g]	1.1 ± 0.01	0.9 ± 0.01	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.3	1.0 ± 0.04	0.04 ± 0.0	0.05 ± 0.0

[a] Membrane content determined by GC-FAME analysis by Microbial ID, Inc. Values represent average and standard deviations of three independent cultures. ND indicates fatty acid was not detected.
[b] Pooled human serum was supplemented to a final concentration of 15%.
[c] Bovine bile was supplemented to a final concentration of 0.2%.
[d] Oleic acid was added to a final concentration of 20 µg/mL.
[f] Others indicates fatty acids comprised <1% of the total membrane content.
[g] Total fatty acid length ratios including both saturated and unsaturated fatty acids.

The most dramatic difference between the clinical isolates and the OG1RF-derived strains can be seen in the membrane content of cultures supplemented with oleic acid ($C_{18:1\ cis\ 9}$). For the clinical strains, the membrane consisted of 57% oleic acid (Table 3.3), whereas for the OG1RF derivatives it was closer to 70% (Table 3.2). Again, for the clinical isolates, the dominant saturated fatty acid was $C_{20:0}$ (arachidic acid), which made up more than 36% of the total membrane content. Interestingly, for OG1RF and its derivatives, as well as for the clinical isolates, this fatty acid was detected at significant levels only upon supplementation with oleic acid ($C_{18:1\ cis\ 9}$). It should be noted that the levels of arachidic acid in the clinical isolates (Table 3.3) were nearly double those observed for OG1RF and its derivatives (Table 3.2).

In summary, similar to OG1RF and its derivatives, the clinical isolates S613 and R712 readily incorporate exogenous sources of fatty acids into their membranes. Thus, despite genetic differences between the strains, the ability to incorporate fatty acids appears to be consistent.

Specific fatty acid sources can alter sensitivity to membrane stress agents in clinical isolates. Our analysis of the membrane content of clinical isolates upon supplementation with fatty acid sources demonstrated that OG1RF is not unique in its ability to incorporate exogenous fatty acids (Table 3.3). Given these data, we sought to understand how these clinical isolates responded to membrane stress after exogenous fatty acid supplementation using the experimental design outlined before for OG1RF and its derivatives.

We exposed the clinical strain pair, S613 and R712, to high levels of bovine bile (20%) when grown in the presence or absence of 0.2% bile or 20 μg/ml oleic acid. We noted increased survival across all time points assessed (15, 30, and 60 min) when cultures were supplemented with low levels of bile (Figure 3.5A). Surprisingly, supplementation with 20 μg/ml oleic acid was unable to protect either clinical isolate from the high bile challenge (Figure 3.5B), in stark contrast to what we observed with OG1RF and its derivatives (Figure 3.1B).

Given these findings, we attempted to determine if supplementing this clinical pair with exogenous fatty acids could alter daptomycin susceptibility. We first supplemented

these strains with 0.2% bile until exponential phase and then exposed S613 or R712 to 10 μg/ml or 40 μg/ml daptomycin, respectively. The rationale for this range of concentrations is the intrinsic daptomycin resistance of R712 (15). Interestingly, supplementation with 0.2% bile caused a significant increase in the ratio of S613 survivors for the entire time course when the strain was exposed to daptomycin ($P < 0.001$). Conversely, R712 had a moderate increase in survivors only after 60 min of exposure ($P = 0.005$) (Figure 3.6A). When the clinical isolates were supplemented with 15% pooled human serum, a result similar to that observed with 0.2% bile was documented for S613 (Figure 3.6B). However, R712 did not appear to benefit from the fatty acids in serum.

Given that growth in both bile and serum reduced the daptomycin sensitivity of S613, we wanted to investigate whether oleic acid alone also altered daptomycin susceptibility, as was observed for the OG1RF derivatives. As shown in Figure 3.6C, supplementation with oleic acid greatly reduced the sensitivity to daptomycin in S613 compared to that in unsupplemented cultures. We again examined the resistant isolate R712 under the same conditions and noted that growth in the presence of oleic acid decreased daptomycin susceptibility at a concentration of 40 μg/ml (Figure 3.6C). This effect was far greater than that observed by supplementation with bile or serum. Taken together, these data suggest that exogenous sources of fatty acids can indeed be taken up and incorporated and subsequently alter the susceptibility of *E. faecalis* clinical strains to membrane-damaging agents.

Discussion

Our previous data showed that *E. faecalis* OG1RF is able to incorporate exogenous fatty acids, which provide increased tolerance to membrane stressors such as bile, SDS, and daptomycin (32). These observations provided us with insights into how *E. faecalis* can utilize exogenous fatty acids from the host to reduce sensitivity to membrane stressors or membrane-damaging antibiotics. Moreover, the data described here suggest that increased tolerance to membrane stress was not a result of exogenous fatty acids activating the LiaFSR system. Using a clinical strain pair of *E. faecalis* clinical isolates, we also demonstrated that the ability to incorporate exogenous fatty acids, as well as the ability of such fatty acids to induce protection against membrane damage, is not limited

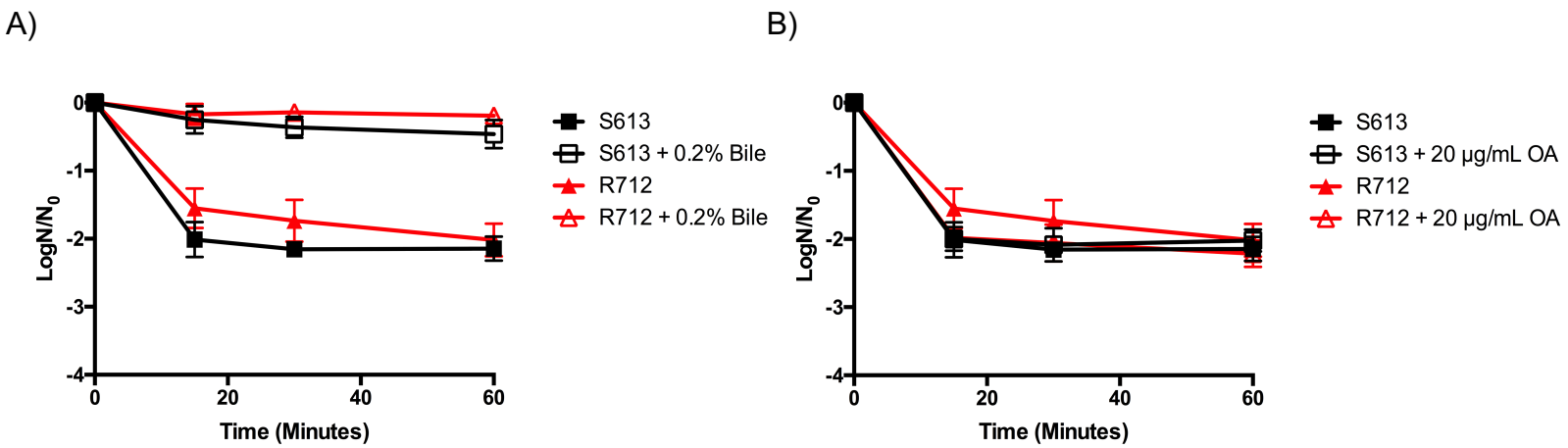

Figure 3.5. Fatty acid supplementation shows variable protection in daptomycin-sensitive (S613) and daptomycin-resistant (R712) clinical pair isolates versus high bile challenge.

Shown are the averages ± standard deviations for *n* = 3. (A) Bile supplementation and challenge with 20% bovine bile. All strains supplemented with 0.2% bile had statistically increased numbers of survivors versus those of their unsupplemented counterparts at all analyzed time points ($P \leq 0.001$). (B) Oleic acid (OA) supplementation and challenge with 20% bovine bile. All strains supplemented with 20 µg/ml OA versus those of their unsupplemented counterparts were not statistically different at all time points analyzed (*P* value > 0.05).

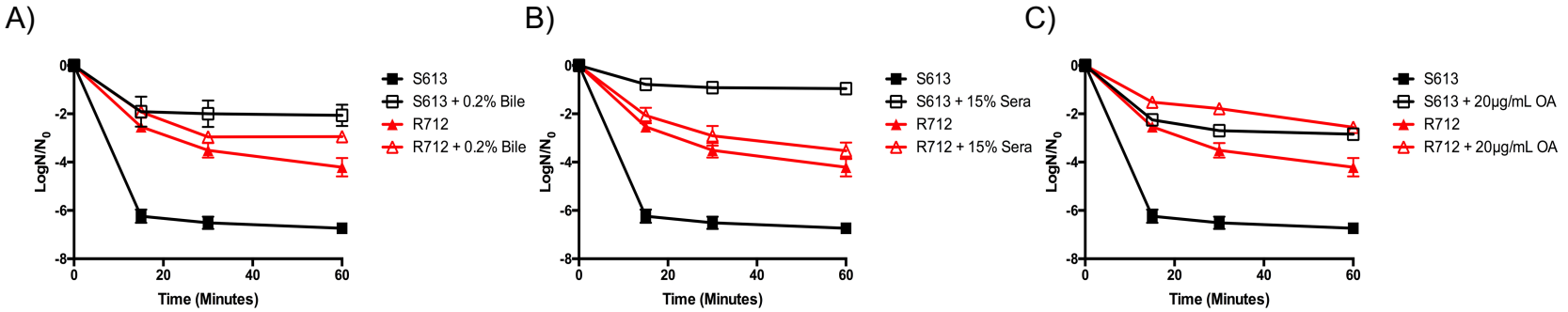

Figure 3.6. Fatty acid sources demonstrate variable protection from daptomycin in daptomycin-sensitive (S613) and daptomycin-resistant (R712) clinical pair isolates.

Shown are the averages ± standard deviations for *n* = 3. (A) Bile-supplemented S613 challenged with 10 µg/ml daptomycin or R712 challenged with 40 µg/ml daptomycin. S613 supplemented with 0.2% bile had statistically increased numbers of survivors versus those of its unsupplemented counterparts at all time points analyzed ($P \leq 0.037$). (B) Serum supplementation and challenge with 10 µg/ml or 40 µg/ml daptomycin. S613 supplemented with 15% sera had a statistically increased number of survivors versus that of its unsupplemented counterpart at all time points analyzed (P values ≤ 0.0001), while the R712 supplemented cultures were not significantly different ($P > 0.05$). (C) Oleic acid (OA) supplementation and challenge with 10 µg/ml or 40 µg/ml daptomycin. All strains supplemented with 20 µg/ml OA had statistically increased numbers of survivors versus those of their unsupplemented counterparts at all time points analyzed ($P \leq 0.002$).

to laboratory strains of *E. faecalis* such as OG1RF.

For all strains examined in this study, incorporation of exogenous fatty acids was conserved and fairly consistent across the genetic backgrounds. One interesting distinction, however, was the increased levels of arachidic acid ($C_{20:0}$) in the membranes of the clinical isolate strains S613 and R712 compared to those of the OG1RF strains upon supplementation with oleic acid (Tables 3.2 and 3.3). The observation of arachidic acid in any of the strains was surprising: none of the strains produced detectable levels of this fatty acid when grown without supplementation. How then does supplementation with oleic acid lead to arachidic acid in the membrane? If *E. faecalis* were to elongate oleic acid, one would expect to see $C_{20:1}$ *cis* $_{11}$ (38) and not arachidic acid ($C_{20:0}$). It is possible that the cell is producing longer fatty acids through its *de novo* fatty acid biosynthetic pathway. The length of fatty acid tails during *de novo* biosynthesis is controlled via competition between the fatty acid acyltransferase and the fatty acid condensation (elongation) enzyme (39). Perhaps oleic acid supplementation directly or indirectly impacts the activity of one or both enzymes, leading to the observed increased fatty acid tail length. It is possible that the clinical isolates are more sensitive to these enzymatic changes, which might explain why we observe higher levels in these strains. Ongoing studies are geared to determine the source of this fatty acid.

Although the membrane composition of OG1RFΔ*liaR* is similar to that of the wild-type strain, the deletion strain is far more sensitive to 20% bile and daptomycin (Figure 3.1 and 3.3). These data support the critical role of the LiaR-mediated membrane stress responses seen in other bacterial species (24, 27). However, supplementation with specific sources of exogenous fatty acids can increase survival of the Δ*liaR* strain when challenged with 20% bile or daptomycin (Figure 3.1A and C and Figure 3.3A and C). Thus, while *liaR* is required for the basal level of tolerance to high bile, the cell can circumvent this need if exogenous fatty acid sources are provided (Figure 3.1). In the case of daptomycin challenge, protection induced by fatty acids is independent of LiaFSR, but *liaR* is absolutely required for optimal membrane responses (Figure 3.3). These observations indicate that there are different cellular responses, depending on the type of membrane damage. The combined data also support previous findings that the host-

derived fatty acid, oleic acid, can reduce membrane damage and even have a role in cell growth and survival (32, 40).

Surprisingly, we did not observe increased sensitivity of the Δ*liaR* strain to SDS compared to that of the wildtype (Figure 3.2). This suggests that *liaR* is dispensable for the basal level of tolerance to SDS and again suggests that *E. faecalis* responds to different membrane stressors in unique ways. Previous work has shown that *E. faecalis* has an altered transcriptional response when exposed to bile versus SDS (41). It is likely that other components within bile, such as bile salts, may contribute to these altered responses, but further analysis is needed.

Another interesting aspect of our study is that the supplementation of exogenous fatty acids to clinical isolates of *E. faecalis* may impact their tolerance to membrane damage (Figure 3.5 and 3.6). However, the ability of fatty acids to induce protection in the clinical isolates was not necessarily consistent with that in the OG1RF derivatives. For example, growth in oleic acid was unable to protect either R712 or S613 from high bile damage (Figure 3.5B), unlike what we observed for the derivatives of OG1RF (Figure 3.1B). It is not clear what differences may contribute to this observation. One possibility is differences in the amounts of arachidic acid ($C_{20:0}$) between the strains (Tables 3.2 and 3.3). For the clinical isolates, this fatty acid comprised >35% of the membrane content when the culture was supplemented with oleic acid, nearly double what was seen in the OG1RF derivatives This is reflected in the reduced ratio of C_{10}-C_{17}/C_{18}-C_{20} fatty acids. This alteration might impact the expression or activity of membrane proteins (for example, efflux pumps) that may contribute to the overall sensitivity or resistance of the strains. More work is needed to elucidate the mechanism contributing to these observations. However, growth in oleic acid protects both S613 and R712 from daptomycin-induced damage (Figure 3.6C), mirroring what is seen in OG1RF derivatives. This indicates that damage induced by daptomycin and bile is not equivalent and that there are genetic or physiological differences between enterococcal strains in how they handle membrane-damaging agents.

Overall, our results show that exogenous fatty acids impact membrane composition and the ability to survive a variety of membrane stressors. However, the mechanism by which fatty acids confer this protection is unclear. An altered membrane fatty acid profile

would likely impact the level, distribution, and potential activity of membrane-associated proteins that may contribute to survival. Additionally, it is not clear what other metabolic processes may be impacted by shifting from *de novo* fatty acid biosynthesis for the generation of membranes to the use of exogenous fatty acids. These observations and the underlying mechanism(s) of fatty acid-induced membrane protection are critically important for understanding the host-pathogen interaction and bacterial response to antimicrobial peptides due to the abundance of free fatty acids. *E. faecalis* is a commensal organism that has access to fatty acids located in bile and serum and is naturally tolerant to these compounds. In the human host, *E. faecalis* from the gut may enter different compartments and alter their membranes in order to succeed in a hostile environment. Fatty acids in serum and tissues might help the bacterium survive membrane stressors driven by the innate immune system (i.e., antimicrobial peptides).

A growing number of studies are demonstrating that the microbes within the host, both commensal organisms and pathogens, are capable of utilizing host metabolites, including fatty acids. Utilization of these sources significantly impacts the microbes, leading to altered physiology, gene expression, and possibly virulence (42). These studies, in conjunction with our previous findings, demonstrate that host fatty acids can induce protection from membrane stressors, including antibiotics. It is worth noting, however, that measurements of MICs of antimicrobials are not often performed in the presence of host fatty acid sources (43). It is worth considering further how the host environment may lead to an altered sensitivity to such damaging agents and to take into account the host environment when such analyses are performed.

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Appendix

Supplemental materials and methods

Proton Nuclear Magnetic Resonance (^{1}H NMR) Profile

1.23 mM Daptomycin: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.65 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.54 (dt, *J* = 7.9, 1.0 Hz, 1H), 7.32 (dd, *J* = 8.2, 0.9 Hz, 1H), 7.21 (ddd, *J* = 8.4, 6.9, 1.4 Hz, 1H), 7.15 (s, 1H), 7.07 (ddd, *J* = 8.1, 7.0, 1.1 Hz, 1H), 6.98 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 6.70 (dd, *J* = 8.5, 1.1 Hz, 1H), 6.54 (ddd, *J* = 8.1, 6.9, 1.2 Hz, 1H), 5.45 – 5.39 (m, 1H), 4.72 (t, *J* = 6.7 Hz, 1H), 4.64 (t, *J* = 6.7 Hz, 1H), 4.63 – 4.58 (m, 2H), 4.54 (d, *J* = 3.4 Hz, 1H), 4.52 (d, *J* = 7.6 Hz, 1H), 4.48 (t, *J* = 5.4 Hz, 1H), 4.36 – 4.30 (m, 1H), 4.28 (t, *J* = 7.1 Hz, 1H), 3.99 (d, *J* = 4.3 Hz, 1H), 3.94 (d, *J* = 11.7 Hz, 2H), 3.86 (qd, *J* = 11.0, 9.9, 4.2 Hz, 3H), 3.51 (q, *J* = 7.0, 4.6 Hz, 1H), 3.24 (dd, *J* = 14.3, 7.8 Hz, 1H), 3.12 (dd, *J* = 14.3, 7.3 Hz, 1H), 2.98 – 2.79 (m, 5H), 2.74 (dt, *J* = 15.9, 7.5 Hz, 3H), 2.69 – 2.58 (m, 3H), 2.40 (dd, *J* = 15.6, 6.5 Hz, 1H), 2.30 – 2.12 (m, 5H), 1.97 (d, *J* = 0.7 Hz, 1H), 1.91 – 1.76 (m, 2H), 1.72 (q, *J* = 7.6 Hz, 2H), 1.50 (q, *J* = 7.6 Hz, 2H), 1.38 – 1.27 (m, 9H), 1.27 – 1.14 (m, 16H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.89 (t, *J* = 7.0 Hz, 4H).

50 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 5.34 (ddd, *J* = 5.7, 4.4, 1.1 Hz, 2H), 2.27 (t, *J* = 7.4 Hz, 2H), 2.03 (q, *J* = 5.8 Hz, 4H), 1.59 (p, *J* = 7.3, 7.3 Hz, 2H), 1.39 – 1.24 (m, 20H), 0.89 (t, *J* = 6.9 Hz, 3H).

1.20 mM Daptomycin and 1.20 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.66 (d, *J* = 8.1 Hz, 1H), 7.55 (d, *J* = 7.9 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.21 (t, *J* = 8.0 Hz, 1H), 7.14 (s, 1H), 7.07 (t, *J* = 7.5 Hz, 1H), 6.98 (t, *J* = 7.5 Hz, 1H), 6.70 (d, *J* = 8.4 Hz, 1H), 6.54 (t, *J* = 7.6 Hz, 1H), 5.46 – 5.39 (m, 1H), 5.34 (t, *J* = 4.9 Hz, 1H), 4.70 (t, *J* = 6.7 Hz, 1H), 4.67 – 4.52 (m, 4H), 4.47 (s, 1H), 4.37 – 4.26 (m, 2H), 4.06 – 3.97 (m, 1H), 3.94 (d, *J* = 15.3 Hz, 2H), 3.88 (d, *J* = 16.6 Hz, 3H), 3.52 (s, 2H), 3.44 (p, *J* = 1.6 Hz, 2H), 3.16 (p, *J* = 1.7 Hz, 2H), 3.12 (dd, *J* = 14.4, 7.3 Hz, 1H), 2.97 – 2.91 (m, 2H), 2.89 – 2.66 (m, 4H), 2.66 – 2.58 (m, 2H), 2.39 (dd, *J* = 15.5, 6.6 Hz, 1H), 2.33 – 2.13 (m, 6H), 2.06 – 1.99 (m, 2H), 1.92 – 1.78 (m, 1H), 1.72 (q, *J* = 7.5 Hz, 2H), 1.65 – 1.56 (m, 1H),

1.52 – 1.45 (m, 2H), 1.38 – 1.27 (m, 20H), 1.23 (dd, *J* = 16.8, 5.5 Hz, 15H), 0.98 (d, *J* = 6.9 Hz, 4H), 0.89 (td, *J* = 6.9, 5.0 Hz, 5H).

1.10 mM Daptomycin and 5.50 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.66 (d, *J* = 8.1 Hz, 1H), 7.55 (d, *J* = 7.9 Hz, 1H), 7.32 (d, *J* = 8.1 Hz, 1H), 7.24 – 7.18 (t, *J* = 8.0 Hz, 1H), 7.14 (s, 1H), 7.07 (t, *J* = 7.5 Hz, 1H), 6.98 (t, *J* = 7.5 Hz 1H), 6.70 (d, *J* = 8.1 Hz, 1H), 6.54 (t, *J* = 7.6 Hz, 1H), 5.46 – 5.39 (m, 1H), 5.34 (ddd, *J* = 5.6, 4.4, 1.1 Hz, 10H), 4.71 (m, 1H), 4.62 (t, *J* = 6.4 Hz, 1H), 4.54 (s, 2H), 4.47 (s, 1H), 4.37 – 4.26 (m, 1H), 4.02 (d, *J* = 16.2 Hz, 1H), 3.94 (d, *J* = 15.0 Hz, 2H), 3.85 (d, *J* = 15.8 Hz, 3H), 3.52 (s, 2H), 3.44 (p, *J* = 1.7 Hz, 2H), 3.16 (p, *J* = 1.6 Hz, 2H), 2.94 (s, 3H), 2.89 – 2.67 (m, 9H), 2.63 (d, *J* = 15.3 Hz, 2H), 2.40 (dd, *J* = 15.5, 6.4 Hz, 1H), 2.25 (t, *J* = 7.5 Hz, 10H), 2.18 (t, *J* = 7.6 Hz, 3H), 2.07 – 1.99 (m, 20H), 1.76 – 1.69 (m, 2H), 1.64 – 1.55 (m, 10H), 1.49 (s, 3H), 1.38 – 1.27 (m, 100H), 1.27 – 1.19 (m, 12H), 0.98 (d, *J* = 6.9 Hz, 4H), 0.89 (td, *J* = 6.9, 5.3 Hz, 18H).

1.21 mM Daptomycin and 1.64 mM Calcium Chloride:^{1}H NMR (500 MHz, Methanol-d_4) δ 7.65 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.53 (s, 1H), 7.30 (s, 1H), 7.24 (s, 1H), 7.15 (s, 1H), 7.07 (t, J = 7.6 Hz, 1H), 6.98 (t, J = 7.5 Hz, 1H), 6.75 (d, J = 10.6 Hz, 1H), 6.58 (s, 1H), 5.45 – 5.39 (m, 1H), 4.72 (t, *J* = 6.7 Hz, 1H), 4.64 (t, *J* = 6.7 Hz, 1H), 4.63 – 4.58 (m, 2H), 4.54 (d, *J* = 3.4 Hz, 1H), 4.52 (d, *J* = 7.6 Hz, 1H), 4.49 (s, 1H), 4.36 – 4.30 (m, 1H), 4.29 (s, 1H), 4.02 (s, 1H), 3.94 (m, 2H), 3.86 (m, 3H), 3.53 (m, 1H), 3.31 (dd, J = 7.3, 2.2 Hz, 1H), 3.12 (m, 1H), 2.96-2.84 (s, 5H), 2.77 (s, 3H), 2.69 – 2.58 (m, 3H), 2.38 (d, J = 18.0 Hz, 1H), 2.30 – 2.12 (m, 5H), 1.95 (s, 1H), 1.77 (s, 2H), 1.72 (m, 2H), 1.45 (s, 2H), 1.33 (m, 9H), 1.29 – 1.13 (m, 16H), 0.95 (s, 3H), 0.89 (t, J = 7.1 Hz, 4H).

1.20 mM Daptomycin, 1.60 mM Calcium Chloride, and 1.20 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.71 (s, 1H), 7.53 (s, 1H), 7.30 (s, 1H), 7.24 (s, 1H), 7.07 (t, *J* = 7.6 Hz, 1H), 6.98 (t, *J* = 7.5 Hz, 1H), 6.74 (s, 1H), 6.58 (s, 1H), 5.46 – 5.39 (m, 1H), 5.34 (m, 1H), 4.70 (t, *J* = 6.7 Hz, 1H), 4.67 – 4.52 (m, 4H), 4.49 (s, 1H), 4.29 (s, 2H), 4.01 – 3.92 (m, 3H), 3.79 (s, 3H), 3.54 – 3.12 (m, 7H), 2.96 (s, 2H), 2.87 – 2.80 (m, 4H), 2.66 – 2.58 (m, 2H), 2.38 (d, *J* = 18.2 Hz, 1H), 2.34 – 2.13 (m, 6H), 2.06 – 1.97 (m, 2H),

1.94 – 1.78 (m, 1H), 1.77 (s, 2H), 1.60 – 1.45 (m, 3H), 1.35 – 1.25 (m, 20H), 1.24 (s, 15H), 1.19 (s, 2H), 1.13 (s, 2H), 0.89 (t, *J* = 7.1 Hz, 4H).

1.05 mM Daptomycin, 1.45 mM Calcium Chloride, and 5.8 mM Oleic Acid: ^{1}H NMR (500 MHz, Methanol-d_4) δ 7.70 (s, 1H), 7.53 (s, 1H), 7.30 (s, 1H), 7.24 (s, 1H), 7.14 (s, 1H), 7.07 (t, *J* = 7.6 Hz, 1H), 6.98 (t, *J* = 7.5 Hz, 1H), 6.74 (s, 1H), 6.58 (s, 1H), 5.34 (ddd, *J* = 5.7, 4.5, 1.1 Hz, 10H), 4.71 (m, 1H), 4.63 (t, *J* = 6.4 Hz, 1H), 4.54 (m, 2H), 4.48 (s, 1H), 4.29 (s, 1H), 4.02 (d, *J* = 16.5 Hz, 1H), 3.79 – 3.40 (m, 7H), 3.21 (td, *J* = 5.0, 2.4 Hz, 2H), 2.96 (s, 3H), 2.87 – 2.62 (m, 11H), 2.40 (s, 1H), 2.27 – 2.11 (m, 10H), 2.03 – 1.95 (m, 20H), 1.77 (s, 2H), 1.63 – 1.56 (m, 10H), 1.45 (s, 2H), 1.36 – 1.26 (m, 100H), 1.24 (s, 15H), 1.19 (s, 3H), 1.13 (s, 2H), 0.95 (s, 4H), 0.89 (td, *J* = 7.0, 4.8 Hz, 15H).

Supplemental Table 3.1. Membrane analysis of wild type and mutant strains during log phase growth.

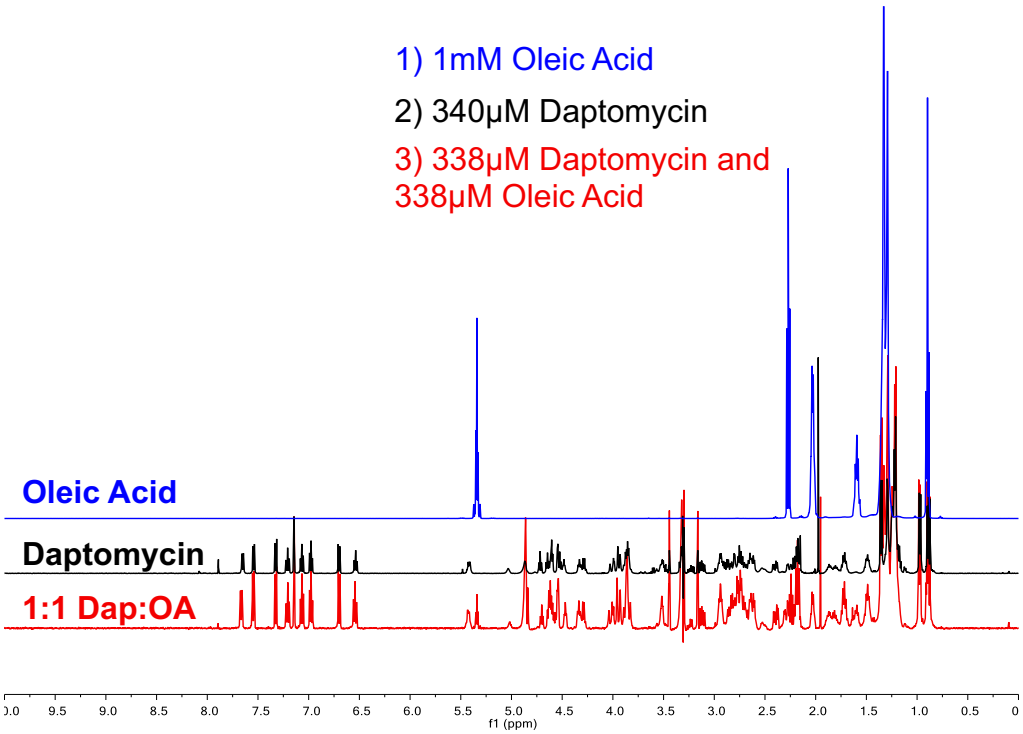
	Percent of total membrane content for indicated strain and supplement (Avg ± SD)[a]		
Strain	WT	Δ*liaR*	Δ*liaR::liaR*
Fatty Acid	Ethanol[e]	Ethanol[e]	Ethanol[e]
$C_{12:0}$	1.4 ± 1.1	1.3 ± 0.9	0.9 ± 0.1
$C_{14:0}$	4.6 ± 0.4	4.2 ± 0.2	6.4 ± 0.1
$C_{16:1}$	5.7 ± 0.3	5.5 ± 0.8	8.7 ± 0.1
$C_{16:0}$	39.8 ± 0.5	42.7 ± 1.8	37.5 ± 0.1
$C_{17:1}$	ND	ND	ND
$C_{17:0\ 2OH}$	7.5 ± 1.0	5.3 ± 3.1	5.9 ± 0.3
$C_{18:1\ cis\ 9}$	0.3 ± 0.6	0.7 ± 1.3	0.6 ± 0.5
$C_{18:1\ cis\ 11}$	34.0 ± 1.0	32.6 ± 1.1	35.7 ± 0.1
$C_{18:0}$	5.6 ± 0.1	6.9 ± 0.8	3.2 ± 0.1
$C_{20:0}$	ND	ND	ND
$C_{18:2}$	ND	ND	ND
Others[f]	0.7 ± 0.3	0.4 ± 0.4	0.7 ± 0.2
Sat:Unsat	1.3 ± 1.0	1.4 ± 1.1	1.1 ± 0.6
C_{10}-C_{17}:C_{18}-C_{20}[g]	1.5 ± 0.3	1.5 ± 0.03	1.5 ± 0.1

[a] Membrane content determined by GC-FAME analysis by Microbial ID, Inc. Values represent average and standard deviations of three independent cultures. ND indicates fatty acid was not detected.
[e] Ethanol solvent control was added to a final concentration of 0.2%.
[g] Total fatty acid length ratios including both saturated and unsaturated fatty acids.

Supplemental Table 3.2. Membrane fatty acid analysis of clinical isolates during log phase growth.

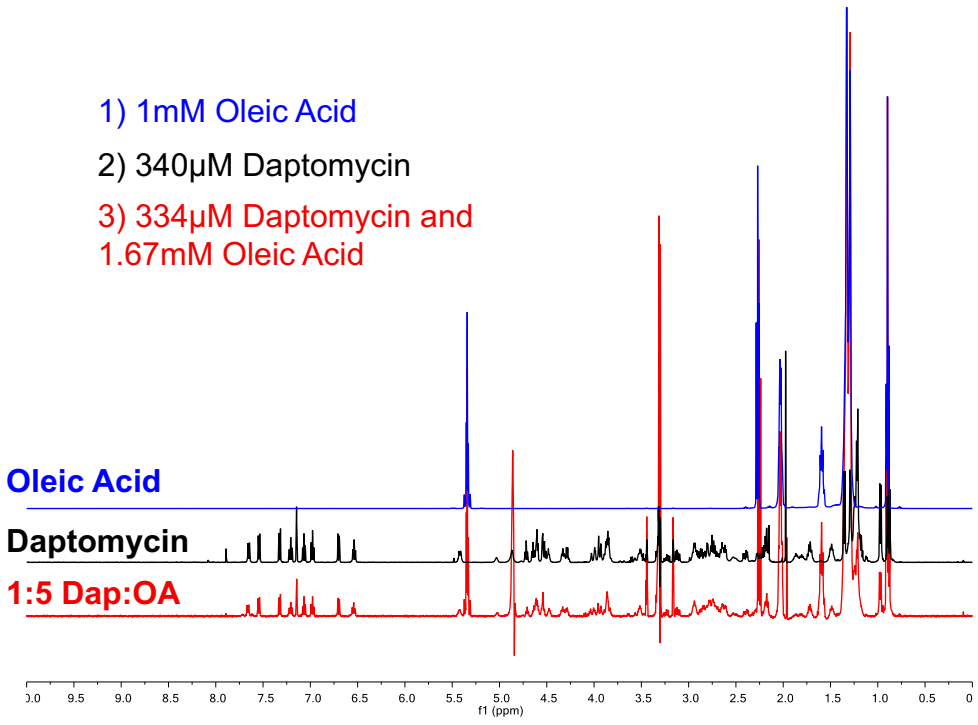
	Percent of total membrane content for indicated strain and supplement (Avg ± SD)[a]	
Strain	S613	R712
Fatty Acid	Ethanol[e]	Ethanol[e]
$C_{12:0}$	1.0 ± 0.0	0.7 ± 0.1
$C_{14:0}$	5.5 ± 0.1	3.8 ± 0.2
$C_{16:1}$	8.0 ± 0.1	7.8 ± 0.4
$C_{16:0}$	36.4 ± 0.2	29.3 ± 1.0
$C_{17:0\ 2OH}$	4.4 ± 0.3	4.5 ± 3.9
$C_{18:1\ cis\ 9}$	0.9 ± 0.1	ND
$C_{18:1\ cis\ 11}$	39.1 ± 0.4	50.0 ± 2.1
$C_{18:0}$	3.8 ± 0.1	3.5 ± 0.1
$C_{20:0}$	ND	ND
$C_{18:2}$	ND	ND
$C_{20:4}$	ND	ND
Others[f]	0.4 ± 0.7	ND
Sat:Unsat	1.0 ± 0.4	0.6 ± 0.6
C_{10}-C_{17}:C_{18}-C_{20}[g]	1.3 ± 0.02	0.9 ± 0.08

[a] Membrane content determined by GC-FAME analysis by Microbial ID, Inc. Values represent average and standard deviations of three independent cultures. ND indicates fatty acid was not detected.
[e] Ethanol solvent control was added at a final concentration of 0.2%.
[g] Total fatty acid length ratios including both saturated and unsaturated fatty acids.



Supplemental Figure 3.1. Mixing 1:1 daptomycin and oleic acid reveals no observable interaction.

Visual comparison of three individual ^{1}H NMR spectra, between 0.0 to 10.0 ppm, shows no interaction between daptomycin and oleic acid. The top spectra (blue) represents a 1mM solution of oleic acid, the middle spectra (black) represents a 340μM solution of daptomycin, and the bottom spectra (red) represents a 1:1 mixture of daptomycin:oleic acid. All solutions were made using methanol-d*4*, and spectra were generated using a VNMRS 500 MHz instrument. Spectra were superimposed using MestReNova software.



Supplemental Figure 3.2. Excess oleic acid reveals no observable interaction when mixed with daptomycin.

The overlap of three individual ^{1}H NMR spectra, between 0.0 to 10.0 ppm, shows no spatial interaction with daptomycin in the presence of excess oleic acid. The top spectra (blue) represents a 1mM solution of oleic acid, the middle spectra (black) represents a 340μM solution of daptomycin, and the bottom spectra (red) represents a 1:5 mixture of daptomycin:oleic acid. All solutions were made using methanol-d*4*, and spectra were generated using a VNMRS 500 MHz instrument. Spectra were superimposed using MestReNova software.

CHAPTER IV: Host associated fatty acids induce global lipid composition alterations in *Enterococcus faecalis*

Future Publication Note

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

Host associated fatty acids induce global lipid composition alterations in *Enterococcus faecalis*. 2018/2019. John R. Harp, Eric D. Tague, William T. Brewer, Brittni M. Woodall, Katarina A. Jones, Shawn R. Campagna, and Elizabeth M. Fozo

Experiments were conducted by John Harp, with the exception of: GC-FAME which was completed by Microbial ID; cell growth for GC-FAME with help from Will Brewer, and Elizabeth Fozo; targeted mass spectrometry analysis completed by Eric Tague and Brittany Woodall with the assistance of Shawn Campagna. John Harp and Elizabeth Fozo wrote the manuscript.

Abstract

Enterococcus faecalis can incorporate exogenous host fatty acids, which increases tolerance to membrane stressors like daptomycin, but the mechanism explaining this observation is unknown. Using quantitative targeted mass spectrometry, we found that the abundance of targeted lysyl-phosphatidylglycerol (L-PG) species increases and the abundance of targeted phosphatidylglycerol and cardiolipin species decreases after supplementing *E. faecalis* with the host fatty acids, oleic acid and linoleic acid. Deletion of the *mprF2* gene*,* resulted in a loss of L-PG, however addition of oleic acid or linoleic acid still increased daptomycin tolerance. Further, deletion of *cls1* and *cls2*, reduced the amount of cardiolipin produced by *E. faecalis,* but did not eliminate production completely. While, a Δ*cls1*/Δ*cls2* deletion strain was more sensitive to daptomycin, supplementation with host fatty acids could still induce tolerance. These data suggest that exposure to the host fatty acids, oleic acid and linoleic acid, cause changes to the phospholipid profile of *E. faecalis,* but that *mprF2*, *cls1*, and *cls2* are not involved in host fatty acid mediated daptomycin tolerance.

Introduction

Enterococcus faecalis is a Gram-positive commensal in the gastrointestinal tract of humans, but can colonize in-dwelling medical devices, infect wounds, and cause septicemia if its niche expands due to antibiotic treatment or the compromised immunostatus of the host (1). Unfortunately, *E. faecalis* is inherently resistant to many therapeutics leaving few options for treatment. One treatment that is used to combat multi-drug resistant enterococci is the calcium-dependent lipopeptide antibiotic, daptomycin. Although the specific details of the action mechanism are not validated, daptomycin inserts a 10-carbon containing fatty acid tail into the Gram-positive bacterial membrane in association with phosphatidylglycerol, then oligomerizes with other daptomycin monomers to destabilize the membrane and cause cell death (2–5). Even with use only for multidrug resistant cases, daptomycin resistant strains have been isolated in the clinic (6). Sequencing of these resistant strains, along with *in vitro* evolved daptomycin resistant strains, showed the presence of several genetic mutations, including within *liaF* (of the LiaFSR three component system), *cls* (cardiolipin synthase), and *gdpD* (glycerophosphoryl diester phosphodiesterase) genes (6, 7).

Our lab discovered that supplementing *E. faecalis* with host sources of exogenous fatty acids, such as bile (exposed to during commensal state) or serum (pathogenic state), increased the tolerance to general membrane stress agents as well as daptomycin (8). Moreover, this tolerance occurred only upon supplementing *E. faecalis* OG1RF with host-derived oleic acid ($C_{18:1\ cis\ 9}$) or linoleic acid ($C_{18:2\ cis\ 9,\ 12}$) (9). As LiaFSR responses have been implicated in tolerance to daptomycin, we hypothesized that exogenous fatty acids triggered a LiaFSR mediated stress response (10). Using a *liaR* deficient strain of *E. faecalis* OG1RF*,* we found that exogenous fatty acids still provided increased protection against general membrane stressors or daptomycin (11). Consequently, induction of LiaFSR was not involved in increasing tolerance to daptomycin after supplementation.

An alternative hypothesis to explain the observed tolerance to membrane stressors after host fatty acid supplementation was that the membrane phospholipid composition was altered. This is supported by several observations demonstrating that changes in phospholipid composition (12), localization of cardiolipin (13) and mutations in the gene that encodes cardiolipin synthase (14) can contribute to daptomycin resistance in

enterococci. Further, increased lysinylation of phosphatidylglycerol has been attributed to cationic antimicrobial peptide resistance (15, 16).

Given these observations, we hypothesized that supplementation with host fatty acids alters the phospholipid composition, thus resulting in increased membrane stress tolerance. Herein, we present data showing that supplementation of *E. faecalis* with host fatty acids alters the phospholipid profile, specifically by increasing the proportion of lysyl-phosphatidylglycerol (L-PG) species and decreasing the proportion of phosphatidylglycerol (PG) species and cardiolipin (CL) species. To test if alterations to L-PG or CL were specifically contributing, we deleted the genes responsible for production of these phospholipids, *mprF2* and *cls1/cls2* respectively. We found that strains lacking *mprF2*, *cls1*, *cls2, cls1/cls2, and cls1/cls2*/*mprF2* still had increased tolerance to daptomycin even though their membrane phospholipid content was altered from the parental strain. These data suggest that supplementation with the host fatty acids, oleic acid and linoleic acid, induce lipid alterations, but changes in specific L-PG or CL species are not responsible for induced daptomycin tolerance.

Materials and Methods

Bacterial growth conditions. *E. faecalis* strains were grown statically in brain heart infusion medium (BHI; BD Difco) at 37°C. For the determination of growth rate and generation time, overnight cultures were diluted into fresh BHI medium to an optical density at 600 nm (OD_{600}) of 0.01 with supplements added to the final concentration as indicated in the text and allowed to grow until stationary phase. For all other fatty acid exposure experiments, overnight cultures were diluted into fresh BHI medium as described above and grown until an OD_{600} of ~0.25. Fatty acid supplements were then added at concentrations indicated in the text and incubated at 37°C for 30 minutes (9). All fatty acids and chemicals were purchased from Sigma-Aldrich unless noted otherwise. *Escherichia coli* strains were grown in BHI medium at 37°C with shaking. Counter-selection was performed on MM9YEG agar plates (Final concentration: 1X M9 salts, 0.25% yeast extract, 250μg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 0.5% glucose) containing 10mM *p*-Cl-phenylalanine (*p*-Cl-Phe). Antibiotics were used at the following concentrations when needed: erythromycin, 10μg mL^{-1} (*E. faecalis*)

or 100μg mL^{-1} (*E. coli*); spectinomycin, 1000μg mL^{-1}; fusidic acid, 25 μg mL^{-1}; rifampicin, 250 μg mL^{-1}.

Generation of bacterial deletion strains. The strains generated in this study are listed in Supplemental Table 4.1 and the sequence of all nucleotide primers used in this study are in Supplemental Table 4.2. Generation of deletion strains of *E. faecalis* OG1RF was through the method of Kristich *et al*. (17). To delete the *mprF2* gene and two predicted cardiolipin synthase genes, ~1000bp flanking regions of OG1RF_RS03930 (*mprF2*), OG1RF_RS01975 (*cls1*) or OG1RF_RS06840 (*cls2*) were amplified from *E. faecalis* OG1RF genomic DNA. The two products for each gene region were then spliced together using the external primers (18). Primers containing complementary overlaps with the spliced gene regions were used to amplify pCJK47 (17). The amplified inserts and vectors were assembled using NEB Gibson Assembly Master Mix. *E. coli* strain EC1000 was then transformed and colonies selected for erythromycin resistance. Once verified, pMPRF2 (to generate ΔOG1RF_RS03930 – *mprF2*), pJRH1 (to generate ΔOG1RF_RS01975 – *cls1*) or pJRH2 (to generate ΔOG1RF_RS06840 – *cls2*) was transformed into an *E. faecalis* conjugative donor strain (CK111/pCF10-101). After clonal selection, conjugative donors containing pMPRF2, pJRH1 or pJRH2 were mixed with an OG1RF recipient at a ratio of 1 part donor to 9 parts recipient. After conjugation, cells were placed on recipient (rifampicin, fusidic acid, erythromycin and X-gal) or donor (spectinomycin and erythromycin) selection media. Blue colonies from the recipient plates were then re-isolated on the same selective medium. Confirmed colonies were then grown to stationary phase in BHI in the absence of selection, diluted, and then isolated on MM9YEG. White colonies were then tested for erythromycin sensitivity and sequenced for verification.

GC-FAME preparation and analysis. Cells were grown to log phase using the experimental strategies outlined in bacterial growth conditions. 15 mL aliquots of cells were washed twice with 10 mL of 1X phosphate buffered saline (PBS), pelleted, and stored at -80°C prior to shipment to Microbial ID, Inc. (Newark, DE). Cells were then subjected to saponification with a sodium hydroxide-methanol mixture, a methylation step, and hexane extraction prior to GC-FAME analysis (19).

Membrane challenge assays. Cells were diluted to an OD_{600} of 0.01 in BHI medium, incubated until exponential phase (OD_{600} of ~ 0.225-0.25), and then supplemented with

either solvent control, 20 μg mL^{-1} oleic acid ($C_{18:1\ cis\text{-}9}$) or 10 μg mL^{-1} linoleic acid ($C_{18:2\ cis\text{-}9,\ 12}$) for 30 minutes (9). 10 mL of cells were harvested and washed twice with 10 mL of 1X PBS and then resuspended in BHI (for SDS challenge) or BHI containing 1.5mM $CaCl_2$ (for daptomycin challenge) and treated with either a final concentration of 0.05% SDS or 15 μg mL^{-1} of daptomycin. Serial dilutions were plated onto BHI agar at 0, 15, 30, and 60 minutes after exposure to the indicated membrane stressor. Additionally, prolonged exposure to SDS or daptomycin was performed, with aliquots of cells plated at 0, 1, 2, 4, and 18 hours post addition of membrane stressor. The log ratio of survivors over time was calculated for three biological replicates and shown are the averages and standard deviations for each experiment.

Phospholipid Extraction for Mass Spectrometry. Total lipids were isolated using a modification to the Folch *et al*. (20) and Bligh and Dyer (21) methods. Briefly, cells were grown and supplemented with fatty acids using the short-term supplementation method indicated above, washed twice with 1X PBS, and then resuspended in 1 mL of 1X PBS containing 100μg mL^{-1} of lysozyme. Cells were incubated at 37°C for 20 minutes and then the lysozyme treated cells were transferred to a plastic screw top microfuge tube containing 0.5g of ≤106μm glass beads. Cells were subsequently homogenized using a mini-bead beater (Biospec Products, Bartlesville, OK) for two, one-minute intervals. Homogenized cells were transferred from the microfuge tube to a 15 mL polypropylene conical containing 2:1 (v/v) chloroform:methanol. After vortexing gently, the conical tubes were centrifuged for 5 minutes at 2739xG. The organic and inorganic phases were collected, leaving behind any debris and transferred to a new 15mL polypropylene conical containing 1.5 mL of 0.9% NaCl. After vortexing gently, the conical tubes were centrifuged for 5 minutes at 2739xG. Finally, the lower organic phase, containing extracted lipids, was collected and transferred to a glass screw top and evaporated using nitrogen gas.

Ultra-Performance Liquid Chromatography High Resolution Mass Spectrometry (UPLC-HRMS). Lipid identification was performed in the manner of Tague *et al* (submitted). Lipid extracts (see above) were evaporated using nitrogen gas then suspended in 240 μL of 1:1 methanol: chloroform and 60μL of internal standard (IS) before being transferred to autosampler vials. Samples were stored in the autosampler at 4°C prior to analysis. An UltiMate 3000 ultra performance liquid chromatography

system (UPLC, Dionex, Sunnyvale, CA) was used to inject 10µL of sample onto a Kinetex HILIC column (100 Å, 2.6µm, 150mm x 2.1mm; Phenomenex, Torrance, CA) controlled at 35°C. Mobile phase A was 100% water with 5 mM ammonium acetate adjusted to pH 5.8, and mobile phase B was 97:3 acetonitrile: water with 5mM ammonium acetate adjusted to pH 5.8. The gradient started at 97%B for 1 min, decreased to 95%B from 1.0-1.2 min, held constant at 95%B from 1.2-4.0 min, decreased to 90%B from 4.0-4.2 minutes, held constant at 90% from 4.2-7.6 min, decreased to 70%B from 7.6-8.1 min, held constant at 70%B from 8.1-10.9 min, decreased to 50%B from 10.9-11.0 min, and held constant at 50%B from 11.0-18.0 min. The flow rate for the separation was held constant at 200µL/min. The column was re-equilibrated for 12 minutes at 500µL/min.

Eluent was introduced to the mass spectrometer via an electrospray ionization (ESI) source, with the following parameters: sheath gas 30 (arbitrary units), aux gas 8 (arbitrary units), sweep gas 3 (arbitrary units), spray voltage 3 kV, capillary temperature 300°C.

Mass analysis was performed using a Q Exactive Plus (Thermo Scientific, Waltham, MA) mass spectrometer operated in parallel reaction mode (PRM). All ions were targeted in negative mode at a resolution of 140,000, automatic gain control (AGC) of $3x10^6$ ions, maximum IT time was 100 ms, isolation window was 0.6 *m/z,* and the normalized collision energy was 35 eV. The mass spectrometer was calibrated every 24 hours with a negative mode calibration solution.

UPLC-HRMS Standards and Calibration curves. Quantification of lipid species was performed in the manner of Tague *et al* (submitted). All standards were purchased from Avanti Polar Lipids, Inc, and diluted in 9:1 methanol: chloroform for calibration curves. A standard from each of three classes of phospholipids was run to determine retention time and fragmentation. A non-natural internal standard (IS) was incorporated into the standards and biological samples at a final concentration of 8.3µM. Details of each standard are showed in Supplemental Table 4.4. The dehydrated glycerol-3-phosphate ion (m/z 152.9958) was used for quantification of all cardiolipin (CL) and phosphatidylglycerol (PG) compounds and a lysine fragment (*m/z* 145.0945) was used for lysyl-phosphatidylglycerol (L-PG) species. CL was targeted as the $[M+2]^{2-}$while PG and L-PG was targeted as $[M-1]^{1-}$. Standards were run in replicates of 6, before and after biological samples, ranging in concentration from 1nM to 50 uM, and the lower limit of

detection was 500nM for the CL and PGs and 50nM for L-PGs. Calibration plots were made by a log transformation of the ratio integration of the analyte to the internal standard against the log of ratio of the concentration of the analyte to the internal standard.

UPLC-HRMS Targeted Compounds. A list of potential targeted lipids was created from previous FAME data showing which tail lengths are present in the biological system (8, 9, 11). With this targeted list (Supplemental Table 4.3), we were able to quantify the lipids based on the common head group fragment, and subsequently extract the relative abundance of acyl tails from the PRM data. This allowed for an additional level of confirmation for the assigned peaks as well as being able to determine which tails are incorporated into each phospholipid. PG *m/z* were scanned from 0-4 minutes, CL *m/z* from 0-8 minutes and L-PG *m/z* 8-15 minutes. Xcalibur software from Thermo Scientific was used integrate areas under curve within a 5ppm widow of the exact fragment mass. Concentrations of all compounds within each head group class were calculated based on the calibration plot for the group's external standard. Quantitated values were normalized to the optical density (OD_{600} nm) of the culture at the time of extraction. If values were below the limit of quantitation for more than three of five biological replicates, that phospholipid species was eliminated from the analysis and not accounted for in the averages of the phospholipid species.

Statistical analysis. Differences in the membrane fatty acid content between growth conditions as well as differences in log ratio of survivors over time were determined using a two-tailed, unpaired Student's *t* test.

Results

Supplementation with host fatty acids increases abundance of lysyl-phosphatidylglycerol in the membrane of *E. faecalis* OG1RF. We previously showed that *E. faecalis* OG1RF can incorporate exogenous fatty acids from host fluids like bile and sera (8, 9). Additionally, short-term supplementation with oleic acid or linoleic acid, which are found in bile and sera, protected OG1RF from daptomycin (9). One possible explanation for these data is that incorporation of host fatty acids altered the phospholipid composition, leading to daptomycin tolerance (12, 13). To test this, we first isolated total lipids from *E. faecalis* OG1RF after short-term supplementation with either oleic acid,

linoleic acid, or solvent control and analyzed the composition of the cell membrane using targeted mass spectrometry (see Materials and Methods). We specifically quantified species of phosphatidylglycerol (PG), cardiolipin (CL) and lysyl-phosphatidylglycerol (L-PG), the three major lipid classes identified in previous studies (12, 22, 23) and targeted individual lipid species based upon known fatty acid tail content (see Supplemental Table 4.1) (8, 9, 11). Additionally, given the challenges of quantification of the CL class of species (24, 22, 25–27), we employed internal and external calibration curves.

Following supplementation with oleic acid or linoleic acid, there was an overall reduction in the abundance of targeted PG species (from 57.4% to 22.1% in oleic acid and 20.4% in linoleic acid) and an increase in the abundance of targeted L-PG species (from 17.1% to 73.3% for both fatty acid supplements). Moreover, the abundance of CL was lower after supplementation with oleic acid or linoleic acid (from 25.5% to 4.7% in oleic acid and 6.4% in linoleic acid). Consequently, these trends were driven by specific species (Table 4.1). In the solvent control samples, the dominant PG species was PG 34:1 (22.3 μM) followed by PG 32:1 (5.32 μM) and PG 36:2 (5.34 μM). These species were also the most dominant in the oleic acid and linoleic acid (exception PG 36:2) supplemented cells. The primary cardiolipin species was CL 72:0 and after supplementation with oleic acid and linoleic acid, we saw a reduction in this species from 14.1 μM to 1.02 μM and 3.23 μM respectively. Surprisingly, we observed that every targeted L-PG species was increased after supplementation with oleic acid (73.3% of targeted L-PG) and linoleic acid (73.2% of targeted L-PG) relative to the solvent control (17.1% of targeted L-PG).

Given these findings, we hypothesized that increased tolerance after host fatty acid supplementation was due to increased L-PG. To test this hypothesis, we deleted *mprF2* (ΔOG1RF_RS03930 – *mprF2*), which is responsible for transferring a lysine from lysyl-tRNA to PG in *E. faecalis* (16).

Deletion of *mprF2* alters total membrane lipid content upon fatty acid supplementation. After supplementing parental OG1RF and Δ*mprF2* strains with oleic acid or linoleic acid, we observed no differences in generation time (Supplemental Table 4.5) or in the ability to incorporate exogenous fatty acids after short-term fatty acid

Table 4.1. *E. faecalis* OG1RF phospholipid composition during exponential-phase growth after short-term supplementation.

	μM concentrations of phospholipid species (Avg. ± SD)		
	OG1RF	OG1RF	OG1RF
Phospholipid	Ethanol[a]	Oleic acid[b]	Linoleic acid[c]
PG 32:0	1.83±0.62	0.96±0.31	0.97±0.24
PG 32:1	5.32±1.31	1.97±0.58	1.67±0.25
PG 34:0	2.29±0.69	0.93±0.20	0.91±0.15
PG 34:1	22.3±3.95	9.83±2.35	8.13±1.64
PG 34:2	2.17±0.55	1.14±0.32	1.11±0.11
PG 36:0			
PG 36:1	1.78±0.60		
PG 36:2	5.34±2.1	1.33±0.50	0.78±0.10
PG 36:3			
PG 36:4			
CL 56:0			
CL 64:0			
CL 64:1			
CL 64:2	0.74±0.19		
CL 66:0			
CL 66:1			
CL 68:0	0.87±0.10	0.74±0.20	
CL 68:1			
CL 70:0	1.42±0.42	1.65±0.30	1.02±0.41
CL 70:1			
CL 70:3			
CL 70:4			
CL 72:0	14.1±3.74	1.02±0.22	3.23±0.64
CL 72:1			
CL 72:2	1.13±0.13		
CL 72:3			
CL 72:4			
CL 72:8			
L-PG 32:0	0.54±0.10	3.11±0.90	3.47±0.61
L-PG 32:1	1.04±0.29	7.83±2.35	5.24±0.71
L-PG 34:0	0.74±0.14	2.95±1.00	3.66±0.67
L-PG 34:1	7.09±1.11	28.9±9.36	24.3±4.51
L-PG 34:2	0.72±0.23	2.79±0.99	5.61±0.98
L-PG 36:0		0.13±0.06	0.12±0.03
L-PG 36:1	0.54±0.13	1.08±0.42	1.26±0.27
L-PG 36:2	1.45±0.16	6.71±2.98	3.24±0.36
L-PG 36:3			1.10±0.17
L-PG 36:4	0.09±0.02	0.20±0.11	0.81±0.19
%PG	57.4	22	20.4
%CL	25.5	4.7	6.4
%L-PG	17.1	73.3	73.2

PG – phosphatidylglycerol, CL – cardiolipin, L-PG – lysyl-phosphatidylglycerol
BHI medium was used for all cultures
[a]Ethanol was used at a final concentration of 0.1%
[b]Oleic acid was used at a final concentration of 20 μg mL^{-1}
[c]Linoleic acid was used at a final concentration of 10 μg mL^{-1}

supplementation (Supplemental Table 4.6). To confirm that the Δ*mprF2* strain was null for L-PG, we again conducted targeted mass spectrometry on both the parental strain and the Δ*mprF2* strain. As expected, L-PG levels were below the limits of detection in Δ*mprF2* with the exception of L-PG 32:0 (Table 4.2). Overall, the parental strain and the Δ*mprF2* strain are dominated by the targeted PG species (85.8% of total and 90.2% of total, respectively), but that the Δ*mprF2* phospholipid profile has decreased levels of PG 32:1, 34:0, and 34:1 compared to the parental strain after ethanol supplementation. Targeted CL species were similar in these strains (7.9% of total and 9.8% of total, respectively) with CL 72:0 being the dominant cardiolipin species in both strains.

After supplementation with host fatty acids, we observed an overall decrease in all targeted PG and CL species in Δ*mprF2,* which was similar to the parental strain (except CL 70:3). After oleic acid supplementation, the targeted PG species in the Δ*mprF2* strain went from 90.2% of the total to 77.5% of the total, while the targeted CL species went from 9.8% of total to 27.5% of total. Similar trends were observed after linoleic supplementation of the Δ*mprF2* strain (see Discussion).

Short-term supplementation with oleic acid and linoleic acid increases tolerance to daptomycin but not SDS in a Δ*mprF2* strain. After confirming the loss of detectable levels of L-PG in the Δ*mprF2* strain, we wanted to conclude whether *mprF2,* and consequently L-PG, is responsible for the induction of membrane stress tolerance by host fatty acids. We first evaluated sensitivity to SDS and noted that overall basal tolerance to the detergent was greater than that in the parental strain (Supplemental Figure 4.1). However, supplementation with oleic acid (Supplemental Figure 4.1A) or linoleic acid (Supplemental Figure 4.1B) did not induce tolerance in either strain. Moreover, supplementation with linoleic acid seems to adversely impact the basal level tolerance to SDS ($P \leq 0.01$).

As supplementation with oleic acid or linoleic acid increased tolerance to daptomycin in OG1RF (9), we wanted to test if this observation would hold in the absence of L-PG. Interestingly, unlike what was noted for SDS sensitivity, the basal sensitivity to daptomycin was equivalent in the Δ*mprF2* strain and parental strain. Furthermore, supplementation of Δ*mprF2* with oleic acid (Figure 4.1A) or linoleic acid (Figure 4.1B) also

Table 4.2. *E. faecalis* OG1RF and *E. faecalis* Δ*mprF2* phospholipid composition during exponential-phase growth after short-term supplementation.

	μM concentrations of phospholipid species (Avg. ± SD)							
	OG1RF	OG1RF	Δ*mprF2*	Δ*mprF2*	OG1RF*	OG1RF*	Δ*mprF2**	Δ*mprF2**
Phospholipid	Ethanol	Oleic acid	Ethanol	Oleic acid	Ethanol	Linoleic acid	Ethanol	Linoleic acid
PG 32:0	0.99±0.49				3.08±0.33	0.61±0.05	2.03±0.90	
PG 32:1	5.28±1.76		3.80±2.97		26.8±3.31	1.18±0.12	14.1±7.44	
PG 34:0	4.57±2.80		2.07±0.77		7.00±1.29	0.63±0.06	4.06±2.19	
PG 34:1	125.7±39.5	9.85±13.0	77.4±73.7	12.1±7.19	388.0±65.8	19.8±4.69	201.7±93.1	2.93±0.79
PG 34:2	8.43±4.42	2.54±1.61	9.28±8.57	1.89±2.51	14.8±1.98	1.96±0.53	7.02±2.92	
PG 36:0								
PG 36:1					2.03±0.62		1.23±0.58	
PG 36:2					10.55±3.01	0.77±0.19	7.72±4.35	
PG 36:3								
PG 36:4								
CL 56:0								
CL 64:0	0.91±0.52							
CL 64:1								
CL 64:2			0.75±0.11		0.61±0.10			
CL 66:0	1.13±0.42				0.72±0.13			
CL 66:1	1.03±0.32	0.69±0.07	0.75±0.06	0.68±0.04	0.69±0.20			
CL 68:0	1.82±0.12				5.17±1.20	1.83±0.30	4.13±1.44	0.72±0.27
CL 68:1	1.74±1.50		1.57±0.36	0.97±0.31	1.06±0.10	0.70±0.14	1.03±0.43	
CL 70:0		4.11±0.70			6.51±1.34	1.95±0.30	5.16±1.63	0.78±0.24
CL 70:1								
CL 70:3			0.69±0.09	1.10±0.57	0.65±0.08			
CL 70:4	1.13±0.52		1.76±0.92	1.56±0.73				
CL 72:0	4.84±1.73	1.91±1.40	4.56±4.52	1.01±0.65	14.4±7.39	3.43±0.74	9.42±2.85	1.45±0.41
CL 72:1					0.64±0.16			
CL 72:2					1.36±0.64		0.87±0.18	
CL 72:3								
CL 72:4								
CL 72:8	0.76±0.11							
L-PG 32:0	0.47±0.13	1.26±0.25			2.20±0.13	5.42±1.12	0.21±0.18	0.73±1.33
L-PG 32:1	1.36±0.47	2.36±0.23			7.62±0.96	9.30±1.61		
L-PG 34:0	0.77±0.19	1.49±0.26			3.01±0.33	5.67±0.57		
L-PG 34:1	6.51±1.90	10.3±1.40			32.35±2.90	38.1±5.21		
L-PG 34:2	0.60±0.19	1.20±0.24			3.64±0.28	9.20±1.13		
L-PG 36:0					0.13±0.02	0.26±0.10		
L-PG 36:1	0.27±0.07	0.53±0.08			1.66±0.15	1.96±0.25		
L-PG 36:2	0.59±0.22	2.38±0.26			3.78±0.35	4.79±1.05		
L-PG 36:3					0.07±0.01	1.94±0.39		
L-PG 36:4					0.10±0.02	0.71±0.21		
%PG	85.8	32.1	90.2	77.5	84	22.6	91.9	44.3
%CL	7.9	17.4	9.8	27.5	5.9	7.1	8	44.6
%L-PG	6.3	50.5	0	0	10.1	70.3	0.1	11.1

PG – phosphatidylglycerol, CL – cardiolipin, L-PG – lysyl-phosphatidylglycerol
*Separate experiment
BHI medium was used for all cultures.
Ethanol was used at a final concentration of 0.1%.
Oleic acid was used at a final concentration of 20 μg mL^{-1}.
Linoleic acid was used at a final concentration of 10 μg mL^{-1}.

increased tolerance to daptomycin to a similar level as observed in the parental strain. These data suggest that the increased abundance of L-PG in the membrane was not responsible for increased tolerance to daptomycin.

Given these data, we hypothesized that CL may contribute to membrane stress tolerance since altered levels of CL have been attributed to daptomycin tolerance (12, 13). To test this, we deleted the two predicted genes responsible for cardiolipin synthesis, cardiolipin synthase 1 (ΔOG1RF_RS01975 - Δ*cls1*) and cardiolipin synthase 2 (ΔOG1RF_RS06840 - Δ*cls2*).

Supplementation with host fatty acids impacts growth of *E. faecalis* OG1RF lacking the two predicted cardiolipin synthase genes. We first wanted to assess how the loss of cardiolipin synthases might impact growth (Supplemental Table 4.5). The generation time in BHI and the solvent control, ethanol, was ~37 minutes for all strains. Upon supplementation with oleic acid, the parental and cardiolipin synthase deletion strains resulted in increased generation time (P = 0.0001). Further, the Δ*cls1/cls2* strain was modestly impaired after oleic acid supplementation (~52 minutes, P = 0.0019), relative to the oleic acid supplemented parental strain. The generation times after supplementing parental, Δ*cls1,* and Δ*cls2* strains with linoleic acid also greatly increased (~88 min, 78 min, and 86 min respectively) versus the solvent control, which is consistent with previous observations (8). However, the Δ*cls1/cls2* strain generation time was about three times greater after supplementation with linoleic acid (~253 min, P = 0.048) relative to the parental strain. Additionally, when *mprF2* was deleted from the Δ*cls1/cls2* strain generating a Δ*mprF2/cls1/cls2* mutant, we still observed a significant impact to generation time (~195 min, P = 0.0014) versus the linoleic acid supplemented parental strain.

Despite the impact to generation time in the Δ*cls1*/*cls2* and Δ*mprF2/cls1/cls2* strain after supplementation, these strains were not deficient in the uptake of exogenous fatty acid and the fatty acid composition is similar to previous observations with a wild-type strain of *E. faecalis* (Supplemental Table 4.6, 4.7, and 4.8) (9).

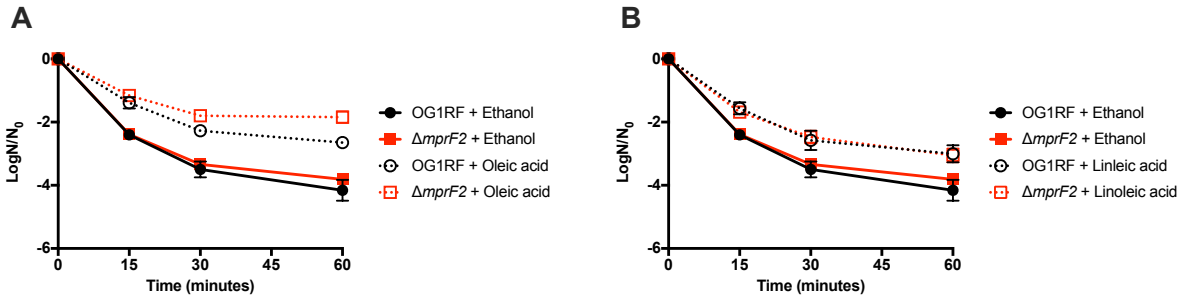


Figure 4.1. Host fatty acid supplementation protects *mprF2* deficient *Enterococcus faecalis* from daptomycin challenge.

OG1RF or OG1RFΔ*mprF2* was grown to mid log phase and then the culture was split and supplemented (see Materials and Methods). (A) Oleic acid supplementation and challenge with daptomycin. All strains supplemented with oleic acid had statistically increased numbers of survivors versus the solvent control at all time points analyzed (P = 0.002). (B) Linoleic acid supplementation and challenge with daptomycin. All strains supplemented with linoleic acid had statistically increased numbers of survivors versus the solvent control at all time points analyzed (P = 0.005). Shown are the averages ± standard deveiation for n = 3.

Deletion of the two predicted cardiolipin synthase genes results in a modified phospholipid profile after short-term supplementation with host fatty acids. To confirm a reduction in cardiolipin species in the Δ*cls1*/*cls2* strain, we again performed targeted mass spectrometry. Surprisingly, we found that the mutant strain still produced CL. However, the Δ*cls1*/*cls2* strain had a lower total percentage of CL species (15.4%) compared to the parental strain (25.5%), increased total percentage of PG (70.6% in the deletion versus 57.4% in the parent), and similar percent totals for L-PG species (14% versus 17.1% respectively) (Table 4.3).

Previous reports have shown that as a cell transitions from exponential phase into stationary phase, the abundance of cardiolipin increases in the membrane (28–30). Consequently, we examined the cell membranes of parent and Δ*cls1*/*cls2* strains (24-hour growth normalized to OD_{600}) to measure how the loss of OG1RF_RS01975 (Δ*cls1*) and ΔOG1RF_RS06840 (Δ*cls2*) impacted cardiolipin production at stationary phase. As shown in Table 4.4, percentage of total was reduced in the Δ*cls1*/*cls2* strain (45% in parental and 21.9% in deletion strain), supportive of a role for these genes in stationary phase production of CL. When we analyzed the targeted CL species in the Δ*cls1*/*cls2* strain, there was a loss of several species that were present in the parental strain. Importantly, these data suggest that there is another, unidentified mechanism to generate cardiolipin that exists in OG1RF (see Discussion).

In log phase cells, the parental and Δ*cls1*/*cls2* strains supplemented with oleic acid resulted in similar trends in CL and L-PG but were quite disparate with respect to PG levels. In the Δ*cls1*/*cls2* strain, we noted modest reductions in PG species (exception PG 36:2), a 5-fold decrease in CL72:0, 2-fold increases in CL70:0, and overall increases in targeted L-PG species when compared to solvent control. In the parental strain, we noted a 2-fold reduction in PG species, a 14-fold decrease in CL72:0, and an overall increase in targeted L-PG species.

Similar to oleic acid, we observed a disparity between the PG changes in parental and Δ*cls1*/*cls2* strains after linoleic acid supplementation. In the Δ*cls1*/*cls2* strain, we noted that some PG species levels stayed the same (PG 32:1, 34:0 34:1, 34:2), increased (PG 32:0), or decreased (PG 36:1, 36:2) when compared to the solvent control. Conversely, the parental strain showed 2-fold decreases in targeted PG species. In the

Table 4.3. *E. faecalis* OG1RF and OG1RFΔ*cls1/cls2* phospholipid composition during exponential-phase growth after short-term supplementation.

	μM concentrations of phospholipid species (Avg. ± SD)					
	OG1RF	OG1RF	OG1RF	Δ*cls1/cls2*	Δ*cls1/cls2*	Δ*cls1/cls2*
Phospholipid	Ethanol	Oleic acid	Linoleic acid	Ethanol	Oleic acid	Linoleic acid
PG 32:0	1.83±0.62	0.96±0.31	0.97±0.24	3.02±0.69	3.14±0.73	6.11±1.80
PG 32:1	5.32±1.31	1.97±0.58	1.67±0.25	8.69±1.54	5.16±1.10	8.65±2.86
PG 34:0	2.29±0.69	0.93±0.20	0.91±0.15	4.18±1.06	3.42±1.02	4.94±1.75
PG 34:1	22.3±3.95	9.83±2.35	8.13±1.64	45.4±11.8	35.2±9.57	42.7±13.4
PG 34:2	2.17±0.55	1.14±0.32	1.11±0.11	4.11±0.90	2.90±0.88	4.51±1.08
PG 36:0						
PG 36:1	1.78±0.60			2.90±0.77	0.66±0.13	1.27±0.41
PG 36:2	5.34±2.1	1.33±0.50	0.78±0.10	8.80±1.52	2.32±0.64	4.70±1.79
PG 36:3						
PG 36:4						
CL 56:0						
CL 64:0						
CL 64:1						
CL 64:2	0.74±0.19					
CL 66:0				0.88±0.37	1.13±0.33	0.89±0.55
CL 66:1						
CL 68:0	0.87±0.10	0.74±0.20		0.79±0.30	1.24±0.88	1.28±0.42
CL 68:1						
CL 70:0	1.42±0.42	1.65±0.30	1.02±0.41	1.97±0.28	4.00±0.97	3.85±0.77
CL 70:1						
CL 70:3						
CL 70:4						
CL 72:0	14.1±3.74	1.02±0.22	3.23±0.64	12.0±2.25	2.14±0.82	8.30±3.90
CL 72:1						
CL 72:2	1.13±0.13			1.19±0.32		
CL 72:3						
CL 72:4						
CL 72:8						0.86±0.36
L-PG 32:0	0.54±0.10	3.11±0.90	3.47±0.61	0.63±0.10	7.53±1.95	8.34±1.30
L-PG 32:1	1.04±0.29	7.83±2.35	5.24±0.71	1.27±0.54	16.8±4.47	11.7±2.37
L-PG 34:0	0.74±0.14	2.95±1.00	3.66±0.67	0.99±0.17	8.31±2.57	10.1±2.60
L-PG 34:1	7.09±1.11	28.9±9.36	24.3±4.51	8.70±2.00	78.4±21.8	56.2±10.4
L-PG 34:2	0.72±0.23	2.79±0.99	5.61±0.98	0.94±0.23	6.22±1.68	10.9±2.16
L-PG 36:0		0.13±0.06	0.12±0.03		0.38±0.16	0.30±0.03
L-PG 36:1	0.54±0.13	1.08±0.42	1.26±0.27	0.73±0.13	2.29±0.85	3.36±0.87
L-PG 36:2	1.45±0.16	6.71±2.98	3.24±0.36	1.81±0.34	11.9±4.39	9.66±1.60
L-PG 36:3			1.10±0.17		0.13±0.04	2.00±0.47
L-PG 36:4	0.09±0.02	0.20±0.11	0.81±0.19	0.13±0.03	0.82±0.44	1.66±0.11
%PG	57.4	22	20.4	70.6	27.2	36
%CL	25.5	4.7	6.4	15.4	4.4	7.5
%L-PG	17.1	73.3	73.2	14	68.4	56.5

PG – phosphatidylglycerol, CL – cardiolipin, L-PG – lysyl-phosphatidylglycerol
BHI medium was used for all cultures
Ethanol was used at a final concentration of 0.1%
Oleic acid was used at a final concentration of 20 μg mL^{-1}
Linoleic acid was used at a final concentration of 10 μg mL^{-1}

Table 4.4. *E. faecalis* OG1RF, Δ*cls1*/Δ*cls2*, Δ*cls1*, and Δ*cls2* phospholipid composition during stationary phase.

	μM concentrations of phospholipid species (Avg. ± SD)			
Phospholipid	OG1RF	Δ*cls1*/Δ*cls2*	Δ*cls1*	Δ*cls2*
PG 32:0	4.50 ± 2.0	8.14 ± 3.3	4.76 ± 1.7	3.11 ± 1.4
PG 32:1	7.65 ± 1.3	13.86 ± 9.0	7.65 ± 3.5	4.78 ± 3.1
PG 34:0	2.71 ± 0.3	7.70 ± 4.6	3.42 ± 2.1	3.12 ± 0.7
PG 34:1	16.33 ± 7.1	43.25 ± 22.7	11.32 ± 6.8	15.10 ± 6.5
PG 34:2	6.95 ± 3.2	7.69 ± 4.9	3.13 ± 1.6	7.83 ± 4.4
PG 36:0				
PG 36:1	1.29 ± 0.4	2.77 ± 0.3	0.68 ± 0.1	0.65 ± 0.1
PG 36:2	2.12 ± 0.4	4.17 ± 1.1	1.79 ± 1.3	0.78 ± 0.1
CL 64:0	1.66 ± 0.4		0.98 ± 0.2	BLD
CL 64:1	1.39 ± 0.6		1.32 ± 0.2	0.64 ± 0.2
CL 64:2	2.52 ± 0.7		2.17 ± 0.2	1.08 ± 0.5
CL 66:0	3.61 ± 1.8	2.21 ± 0.4	2.08 ± 0.4	1.21 ± 0.3
CL 66:1	1.78 ± 0.3	0.61 ± 0.4	1.36 ± 0.5	0.82 ± 0.4
CL 68:0	3.74 ± 1.9	1.30 ± 0.4	1.21 ± 0.3	0.94 ± 0.3
CL 68:1	0.94 ± 0.3		0.72 ± 0.1	
CL 70:0	2.18 ± 0.8	1.38 ± 0.2	0.62 ± 0.1	
CL 70:3	0.76 ± 0.1			
CL 70:4				
CL 72:0	33.49 ± 29.3	26.97 ± 14.8	37.34 ± 26.7	53.23 ± 27.8
CL 72:1			1.11 ± 0.1	1.37 ± 0.7
CL 72:2	0.94 ± 0.3		0.90 ± 0.3	1.40 ± 0.7
CL 72:3				
CL 72:4			1.41 ± 3.1	
CL 72:8		1.16 ± 0.2		
L-PG 32:0	2.98 ± 0.4	3.52 ± 1.1	4.52 ± 1.2	3.85 ± 0.5
L-PG 32:1	4.13 ± 0.4	3.95 ± 1.0	3.95 ± 0.8	3.17 ± 0.5
L-PG 34:0	2.02 ± 0.3	3.23 ± 1.1	3.52 ± 0.8	3.49 ± 0.4
L-PG 34:1	7.15 ± 0.9	13.41 ± 4.5	7.37 ± 4.3	8.88 ± 1.2
L-PG 34:2	3.74 ± 0.7	3.32 ± 1.3	4.61 ± 1.5	4.75 ± 1.2
L-PG 36:0	0.24 ± 0.1	0.41 ± 0.2	0.33 ± 0.1	0.45 ± 0.1
L-PG 36:1	1.15 ± 0.1	1.81 ± 0.5	1.55 ± 0.4	1.60 ± 0.2
L-PG 36:2	1.55 ± 0.4	2.11 ± 0.5	2.60 ± 2.4	1.69 ± 0.4
L-PG 36:3	0.24 ± 0.03	0.15 ± 0.1	0.18 ± 0.1	0.30 ± 0.1
L-PG 36:4	0.11 ± 0.1	0.09 ± 0.1	0.08 ± 0.04	0.13 ± 0.1
%PG	35.3	57.2	29.1	28.4
%CL	45	21.9	45.4	48.8
%L-PG	19.8	20.9	25.5	22.8

PG – phosphatidylglycerol, CL – cardiolipin, L-PG – lysyl-phosphatidylglycerol
BHI medium was used for all cultures
Ethanol was used at a final concentration of 0.1%
Oleic acid was used at a final concentration of 20 μg mL^{-1}
Linoleic acid was used at a final concentration of 10 μg mL^{-1}

Δ*cls1*/*cls2* strain, the CL levels stayed consistent with the solvent control (exception CL72:0) and like the oleic acid supplemented samples, L-PG species increased after supplementation with linoleic acid. The parental strain showed decreases in CL and an overall increase in L-PG after linoleic acid supplementation.

Taken together, these data demonstrate that despite the presence of cardiolipin in the membrane of the Δ*cls1*/*cls2* strain, our targeted lipidomics analyses showed increased amounts of specific targeted PG and L-PG species after supplementation with oleic acid and linoleic acid.

Supplementation of strains lacking the predicted cardiolipin synthase genes provide no protection against SDS induced membrane stress. Despite the presence of cardiolipin in the Δ*cls1*/*cls2* strain, we observed an altered phospholipid profile compared to parental that could impair membrane stress tolerance. (8, 9). When the strains were challenged with SDS, we noted no difference in the single deletion strains (Supplemental Figure 4.2), but the Δ*cls1*/*cls2* strain was more sensitive than the parental strain*,* implying that basal tolerance to SDS requires these two gene products (Figure 4.2A*).* Nor did supplementation with oleic acid or linoleic acid alter this enhanced sensitivity to SDS (Supplemental Figure 4.3).

Given this heightened sensitivity of the Δ*cls1/cls2* to SDS, we extended the length of SDS challenge to 18 hours. As shown in Figure 4.2B, the parental strain did recover after 4 hours of treatment. Conversely, the Δ*cls1/cls2* strain was unable to recover from SDS challenge. This lack of protection in the Δ*cls1*/*cls2* mutant strain suggest that both predicted cardiolipin synthase enzymes are involved in extended basal sensitivity and that they are required for recovery from SDS challenge. Further, these data suggest that fatty acid supplementation does not facilitate recovery from SDS.

Host fatty acids can induce daptomycin tolerance in OG1RF strains lacking *cls1* and *cls2*. Given the increased basal sensitivity to SDS, we examined daptomycin sensitivity to Δ*cls1*/*cls2.* We first noted, that the Δ*cls1*/*cls2* strain had increased basal sensitivity to daptomycin (P = 0.002), while the Δ*mprF2/cls1*/*cls2* strain did not (Figure 4.3). Again, this is supportive of a role of *cls1* and *cls2* for membrane response, despite

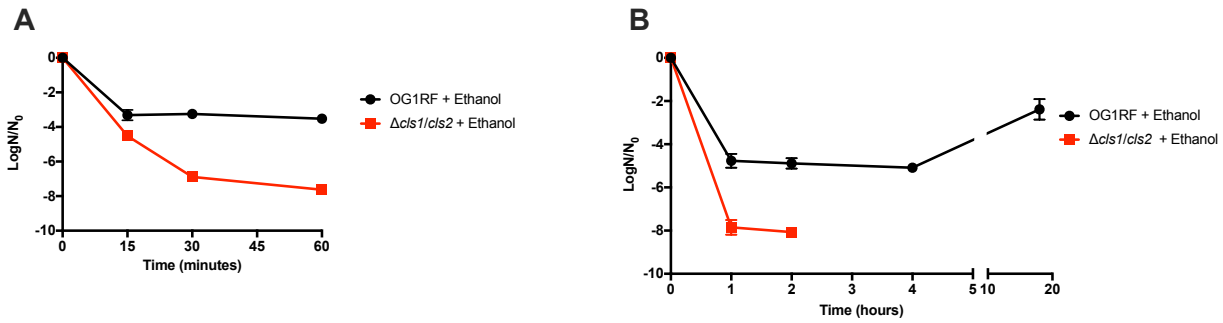


Figure 4.2. Cardiolipin synthase double deletion strain has increased sensitivity to SDS challenge.

(A) The Δ*cls1/cls2* strain has increased sensitivity as compared to the parental OG1RF strain. (B) Extended challenge with SDS results in no detectable cells in the Δ*cls1/cls2* strain after 2 hours. Shown are the average ± standard deviation for *n* = 3.

having detectable levels of CL (see Discussion). As shown in Figure 4.3, the Δ*cls1*/*cls2* strain demonstrated increased tolerance to daptomycin after supplementation with oleic acid (Figure 4.3A) or linoleic acid (Figure 4.3B) similar to the parental and single deletion strains (Supplemental Figure 4.4A and 4.4B). Despite oleic acid and linoleic acid inducing tolerance within an hour of daptomycin treatment, after 4 hours of daptomycin exposure, there were no detectable cells (Figure 4.4).

Combined, these data suggest that the two predicted cardiolipin synthases are critical for long term survival against daptomycin, but that supplementation with host fatty acids, which induce tolerance, is independent of *mprF2, cls1*, and *cls2*.

Discussion

Previous data from our lab showed that supplementation with specific host fatty acids, from host fluids, can increase tolerance to membrane stressors including daptomycin (8, 9, 11). As there have been notable links to phospholipid content and distribution in daptomycin protection, we examined if phospholipid alterations may contribute to daptomycin tolerance (12, 13). Here, we demonstrated that oleic acid and linoleic acid supplementation leads to alterations in the proportion of specific phospholipid species, particularly L-PG in the membrane of *E. faecalis*. Studies have shown that L-PG, and other amino-modifications to PG are protective from cationic antimicrobial peptides (31). It is likely that within fatty acid rich host fluids, *E. faecalis* has a membrane composition that is primed for tolerance to host defenses, contributing to its stability in the host environment. However, as shown in this study, formation of L-PG via MprF2 is not needed to induce tolerance to daptomycin by host fatty acids.

Other genetic data has implied a role for cardiolipin in daptomycin resistance, although it is not the primary reason for genetic resistance in *E. faecalis*. Genome sequencing of a daptomycin clinical strain pair demonstrated that a mutation in *cls* which encodes a cardiolipin biosynthetic enzyme is associated with resistance (6). However, removing a sensitive *cls* allele from one strain of *E. faecium* and exchanging it for a daptomycin resistant allele did not change daptomycin susceptibility (32). Conversely, in *E. faecalis,* expressing a mutated putative cardiolipin synthase allele in *trans* increased resistance to daptomycin in an otherwise daptomycin sensitive strain (7). Herein, we

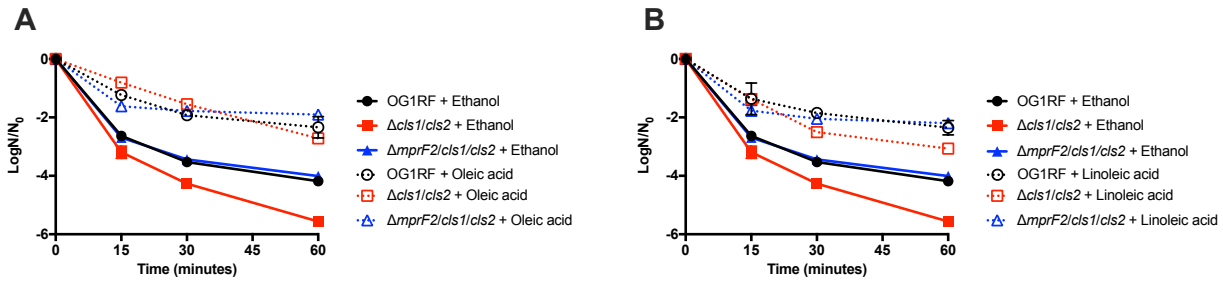


Figure 4.3. Short-term fatty acid supplementation with host fatty acids protects cardiolipin synthase double deletion mutants from daptomycin challenge.

(A) Oleic acid supplementation of Δ*cls1*/*cls2* and Δ*mprF2*/*cls1*/*cls2* had statistically increased numbers of survivors versus the solvent control at all time points (P <0.0001). (B) Linoleic acid supplementation of Δ*cls1*/*cls2* and Δ*mprF2*/*cls1*/*cls2* had statistically increased numbers of survivors versus the solvent control at all time points assessed (P = 0.0001). Shown are the average ± standard deviation for n = 3.

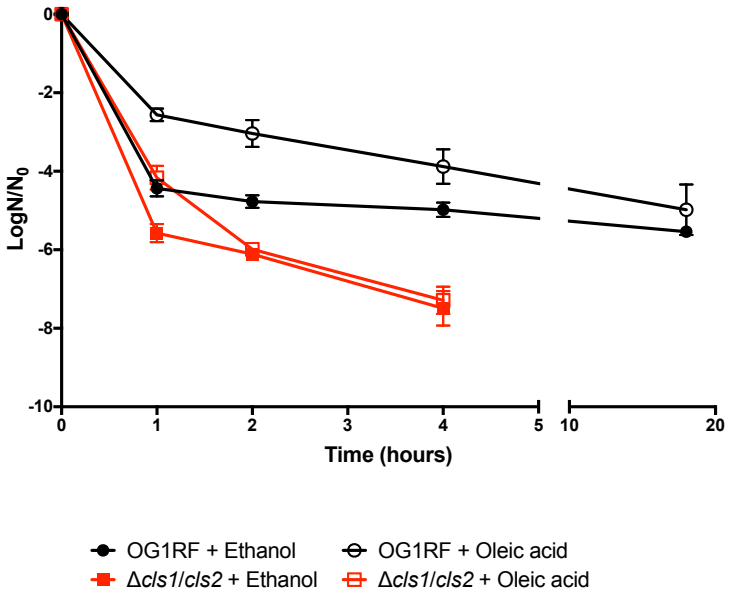


Figure 4.4. Short-term supplementation with oleic acid does not protect cardiolipin synthase double knock out strains from extended daptomycin challenge.

Oleic acid supplementation and challenge with daptomycin. OG1RF supplemented with oleic acid shows delayed kinetics of killing. The cardiolipin synthase double mutant succumbs to extended daptomycin exposure. Shown are the average ± standard deviation for *n* = 3

showed that deletion of two predicted cardiolipin synthases surprisingly only impaired total CL levels in stationary phase. These two genes were critical for basal sensitivity to the membrane damaging agents, SDS and daptomycin, but, fatty acids can induce tolerance to daptomycin. This was also seen in a strain lacking *mprF2*/*cls1*/*cls2.* Could this increased basal sensitivity be explained by the increased presence of phosphatidylglycerol in the membrane? Studies using liposomes and *Bacillus subtilis* have shown that daptomycin preferentially inserts into phosphatidylglycerol rich areas (33–35). Given this, after supplementation with oleic acid and linoleic acid, we still see relatively high amounts of PG even though the percent total of this phospholipid is reduced. Alternatively (or perhaps both), oleic acid and linoleic acid induced specific gene expression and as a consequence, increases tolerance. Studies in *Staphylococcus aureus* have shown that supplementation with oleic acid can indeed impact gene expression especially those genes under the control of the SaeRS two-component system (36). Further work is needed to determine if the presence of oleic acid or linoleic is inducing transcriptional changes in *E. faecalis.*

The results of this study concluded that tolerance was independent of *mprF2*, *cls1*, and *cls2*, however, several results were surprising. We chose in our analysis to measure quantitatively the major phospholipid species in *E. faecalis* OG1RF (PG, CL, and L-PG) using mass spectrometry and for CL species, we specifically targeted those specific phospholipid species that we believed would best represent the dominant fatty acid tails found in OG1RF in the presence or absence of host fatty acid supplementation (8, 9, 11). Consequently, we were unable to fully analyze the entire lipidome of OG1RF and there are phospholipid species that are not accounted for in our analysis (22, 37). However, the loss of L-PG does not cause an increase in targeted PG levels. These data infer that other phospholipid species may be accumulating. One possibility is that in the absence of L-PG, OG1RF is producing other amino-containing PG species. This is unlikely, however, given the evidence that *mprF2* can aminoacylate PG with other amino acids besides lysine (16). Alternatively, as L-PG is no longer formed, perhaps PG is now serving as a precursor of glycerophospho-diglucosyl-diacylglycerol or lipoteichoic acid constituents (38). Further analyses would be needed to determine these changes.

Another notable finding from this work is that despite deletion of the two predicted *cls* genes, the strain produced detectable levels of cardiolipin. Note that additional attempts to identify other *cls* genes using the characteristic HKD motif were unsuccessful (data not shown). Thus, there is an alternative mechanism for CL production in OG1RF. This result is not totally surprising given that CL is one of the major phospholipids in enterococcus. Moreover, studies have shown that cardiolipin plays a role in survival of *E. coli* as the cell enters stationary phase (28, 39), organizing the membrane, and cell division (40). Data have shown that species such as *Escherichia coli* have three Cls-isoenzymes that can produce CL. The purpose of having these three different enzymes to produce CL still remains unclear (41). However, there are several mechanisms that can make CL. One mechanism found in most bacteria, involves the condensation of two PG molecules. Another mechanism utilizes phosphatidylethanolamine (PE) and PG as substrates (42). Moreover, there is another cardiolipin synthesis mechanism that has been discovered in bacteria using a “eukaryotic-like” pathway where cytidine diphosphate-diacylglycerol (CDP-DAG) donates a phosphatidyl group to PG to form CL (43). As previously stated, given the number of mechanisms involved in the synthesis of CL, and a possible yet to be identified mechanisms, it is not surprising that we find CL in *E. faecalis* OG1RFΔ*cls1*/*cls2.* However, despite the presence of cardiolipin, we observed subsequent increased basal sensitivity to daptomycin.

Taken together, we found that after supplementation with host fatty acids, oleic acid and linoleic acid, the phospholipid profile changed. Despite an altered profile with the parental strain, deletion of *mprF2, cls1,* and *cls2* still resulted in increased tolerance during early exposure to daptomycin. However, extended exposure to daptomycin in the *cls* double deletion strain elucidates a possible role of these genes in our observations. Thus, host fatty acid induced daptomycin tolerance during acute exposure to membrane stressors is through a yet to be determined pathway.

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Appendix

Supplemental Table 4.1. Stains and plasmids used in this study.

Strain	**Relevant genotype or description**	**Source**
Enterococcus faecalis OG1RF	Lab strain	
Escherichia coli EC1000	Cloning strain for *repA*-dependent plasmids; *repA* on chromosome	
E. faecalis CK111/pCF10-101	Conjugative donor strain; *repA* on chromosome and harbors non-transferrable pCF10 derivative plasmid	Kristich *et al. (*2007) Plasmid 57(2) 131-144
Plasmids		
pCJK47	Used for markerless exchange; requires RepA in trans. Contains: $oriT_{pCF10}$, *lacZ*, and P-pheS.	Kristich *et al. (*2007) Plasmid 57(2) 131-144
pJRH1	pCJK47 derivative containing flanking regions of OG1RF_RS01975 (*cls1)*	This work
pJRH2	pCJK47 derivative containing flanking regions of OG1RF_RS06840 (*cls2)*	This work
pMprF2	pCJK47 derivative containing flanking regions of OG1RF_RS03930 (*mprF2*)	This work

Supplemental Table 4.2. Oligonucleotides used in this study.

Name	Sequence	Use
Oligonucleotides used for splicing by overlap extension (SOE)[1] to generate inserts		
EF1097	GTCCAAGGATTCTCACCATTGATGCAAGGCC	Isolate DNA upstream of *cls1* **(*cls1*A)**
EF1098	CTGGAGTCATTGTTGTACTATACCATCAATTACGGATTTGGAATATC	Isolate DNA upstream of *cls1* (*cls1*B)
EF1099	CGTAATTGATGGTATACGTACAACAATGACTCCAGAAGTTGTTCGTGAC	Isolate DNA downstream of *cls1* (*cls1*C)
EF1105	GACTGTCCATGGTATGTTGCACAGCTTCCATCG	Isolate DNA downstream of *cls1* **(*cls1*D)**
EF1103	CAAGACTGGTGACCAACGTCATCATCCATGCTAAAACGCTGG	Isolate DNA upstream of *cls2* (*cls2*C)
EF1108	CCCAAGCCCGGGCGATTGACCAGGACCACTTAAAACTCC	Isolate DNA upstream of *cls2* **(*cls2*D)**
EF1113	GGTCACGGATTCTCCAAACAAGGTAACC	Isolate DNA downstream of *cls2* **(*cls2*A)**
EF1102	CATGGATGATGACGTTGGTCACCAGTCTTGTAATCAATAAATCAGCAGTG	Isolate DNA downstream of *cls2* (*cls2*B)
Oligonucleotides used for Gibson assembly		
EF1449	CAATCACTAGTGAATTCGCGGCCGCCACGGCGATATCGG	Generate overhangs on pCJK47 for *cls1*
EF1450	CAATCGAATTCCCGCGGCCGCTCTAGAACTAGCGATTCTGAAATCAC	Generate overhangs on pCJK47 for *cls1*
EF1451	CAGAATCGCTAGTTCTAGAGCGGCCGCGGGAATTCGATTGT	Generate overhangs on *cls1* insert
EF1452	CATATGGATCCGATATCGCCGTGGCGGCCGCGAATTCACTAGTGATTGACTG	Generate overhangs on *cls1* insert
EF1453	GGGAATCACTAGTGAATTCGCGGCCGCACGGCGATATCGGATCC	Generate overhangs on pCJK47 for *cls2*
EF1454	CCGTGACCAATCGAATTCCCGCGGCCGCTCTAGAACTAGCGATTCTGAAATCAC	Generate overhangs on pCJK47 for *cls2*
EF1455	CAGAATCGCTAGTTCTAGAGCGGCCGCGGGAATTCGATTGG	Generate overhangs on *cls2* insert
EF1456	CATATGGATCCGATATCGCCGTGCGGCCGCGAATTCACTAGTGATTCCCAAG	Generate overhangs on *cls2* insert
EF1601	GACAAAACCCCTAATAATTCTTTTGCTTCATCCATGCCCGGGTACCATGGCATGC TAAGCTTGATTTTCGTTC	Generate overhangs on pCJK47 for *mprF2*
EF1602	CTTTTGATCAAAAACAGGATTTTCTCTAGAACTAGCGATTCTGAAATCACCATTT AAAAAACTC	Generate overhangs on pCJK47 for *mprF2*
EF1603	GAGTTTTTTAAATGGTGATTTCAGAATCGCTAGTTCTAGAGAAAATCCTGTTTTT GATCAAAAG	Generate overhangs on DNA upstream of *mprF2*
EF1604	CAATCCAGCTACTTTTAGAATAAAGTGTATAGCAACAACAATAATTGAGACCGCA ATAACAAAC	Generate overhangs on DNA upstream of *mprF2*
EF1605	GTTTGTTATTGCGGTCTCAATTATTGTTGTTGCTATACACTTTATTCTAAAAGTA GCTGGATTG	Generate overhangs on DNA downstream of *mprF2*
EF1606	GAACGAAAATCAAGCTTAGCATGCCATGGTACCCGGGCATGGATGAAGCAAAAGA ATTATTAGGGGTTTTGTC	Generate overhangs on DNA downstream of *mprF2*

Supplemental Table 4.3. List of targeted phospholipid species.

Phosphatidylglycerol	Cardiolipin	Lysyl-phosphatidylglycerol
PG 16:0	CL 56:0	L-PG 32:0
PG 32:0	CL 64:0	L-PG 32:1
PG 32:1	CL 64:1	L-PG 34:0
PG 34:0	CL 64:2	L-PG 34:1
PG 34:1	CL 66:0	L-PG 34:2
PG 34:2	CL 66:1	L-PG 36:0
PG 36:0	CL 68:0	L-PG 36:1
PG 36:1	CL 68:1	L-PG 36:2
PG 36:2	CL 70:0	L-PG 36:3
	CL 70:1	L-PG 36:4
	CL 70:3	
	CL 70:4	
	CL 72:0	
	CL 72:1	
	CL 72:2	
	CL 72:3	
	CL 72:4	
	CL 72:8	

Supplemental Table 4.4. Standards used for targeted mass spectrometry.

Compound Class	Tails	Abbreviation	Standard Type
Cardiolipin	4-$C_{18:1}$	CL (72:4)	External Standard
Cardiolipin	4-$C_{14:0}$	CL (54:0)	Internal standard
Phosphatidylglycerol	2-$C_{18:0}$	PG (36:0)	External Standard
Phosphatidylglycerol	2-$C_{8:0}$	PG (16:0)	Internal standard
Lysyl-phosphatidylglycerol	2-$C_{18:1}$	LPG (36:1)	External Standard

Supplemental Table 4.5. Exponential phase generation times.

	Generation times in medium constituent (min)		
Strain	Ethanol[a]	Oleic acid[b]	Linoleic acid[c]
OG1RF	37.0 ± 0.3	47.0 ± 1.1	88.2 ± 8.3
Δ*mprF2*	38.3 ± 1.0	36.2 ± 1.5	74.8 ± 6.9
Δ*cls1*	36.7 ± 1.5	47.2 ± 0.2	77.5 ± 1.1
Δ*cls2*	35.9 ± 1.0	50.1 ± 2.3	86.0. ± 6.8
Δ*cls1*/*cls2*	35.0 ± 0.9	52.2 ± 0.4	252.9 ± 98.5
Δ*mprF2*/*cls1*/*cls2*	38.8 ± 5.1	39.7 ± 3.5	195.5 ± 22.2

BHI medium was used for all cultures.
[a]Ethanol was used at a final concentration of 0.1%.
[b]Oleic acid was used at a final concentration of 20 μg mL^{-1}.
[c]Linoleic acid was used at a final concentration of 10 μg mL^{-1}.

Supplemental Table 4.6. *E. faecalis* OG1RF and Δ*mprF2* membrane fatty acid composition during exponential-phase growth.

	% of total membrane content (Avg. ± SD)					
	Ethanol[a]		Oleic acid[b] - $C_{18:1\ cis\ 9}$		Linoleic acid[c] - $C_{18:2\ cis\ 9,12}$	
Fatty acid	OG1RF	Δ*mprF2*	OG1RF	Δ*mprF2*	OG1RF	Δ*mprF2*
$C_{12:0}$	1.8 ± 0.3	1.2 ± 0.1	0.7 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.2
$C_{14:0}$	4.8 ± 0.1	4.6 ± 0.2	2.5 ± 0.2	4.2 ± 0.1	3.7 ± 0.1	4.3 ± 0.4
$C_{16:1\ cis\ 9}$	7.2 ± 0.1	6.6 ± 0.3	3.8 ± 0.3	5.7 ± 0.3	6.1 ± 0.04	5.9 ± 0.2
$C_{16:0}$	37.8 ± 0.4	38.6 ± 0.4	19.5 ± 0.6	31.1 ± 0.1	29.6 ± 0.5	31.0 ± 2.1
$C_{18:1\ cis\ 9}$	1.7 ± 0.2	ND	46.7 ± 1.7	11.1 ± 1.0	ND	ND
$C_{18:1\ cis\ 11}$	38.3 ± 0.5	39.6 ± 0.1	19.0 ± 0.6	29.9 ± 0.3	28.6 ± 0.5	26.6 ± 1.7
$C_{18:0}$	5.0 ± 0.1	5.5 ± 0.2	2.9 ± 0.2	3.8 ± 0.2	4.3 ± 0.1	4.2 ± 0.5
$C_{18:2\ cis\ 9,12}$	ND	ND	ND	ND	24.0 ± 0.9	23.4 ± 3.1
$C_{17:0\ 2OH}$	2.0 ± 0.2	2.8 ± 0.4	1.1 ± 0.1	1.8 ± 0.3	1.6 ± 0.1	1.9 ± 0.2
$C_{19:0\ cyclo\ 11}$	ND	0.8 ± 0.0	ND	0.8 ± 0.1	ND	0.7 ± 0.1
$C_{20:0}$	ND	ND	3.2 ± 0.3	10.0 ± 1.0	ND	ND
Other[d]	1.3 ± 0.6	0.4 ± 0.7	0.6 ± 0.2	0.5 ± 0.5	0.9 ± 0.3	0.7 ± 1.0
Sat/Unsat	1.1 ± 0.02	1.2 ± 0.04	0.4 ± 0.5	1.1 ± 0.1	0.7 ± 0.5	0.8 ± 0.6
C_{10} - C_{17} / C_{18} - C_{20}[e]	1.2 ± 0.01	1.2 ± 0.01	0.4 ± 0.4	0.8 ± 0.02	0.7 ± 0.5	0.8 ± 0.6

Membrane contents were determined using GC-FAME analysis by Microbial ID, Inc.; numbers represent average ± standard deviation from three independent cultures.; ND indicates that fatty acid was not detected.

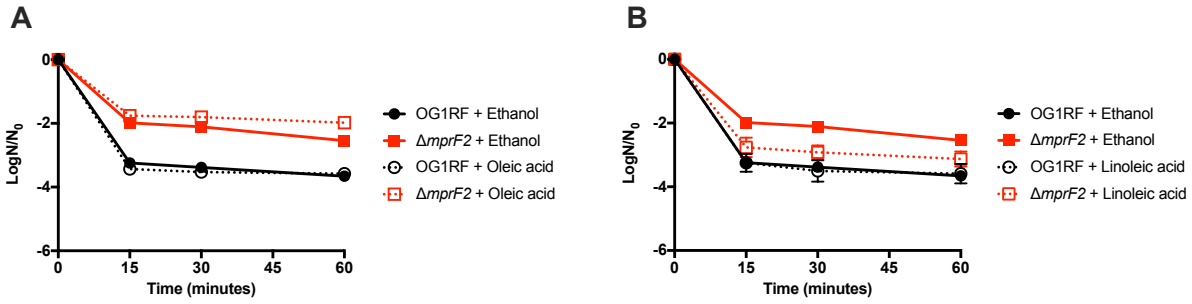
[a]Ethanol was added at a final concentration of 0.1%.

[b]Oleic acid was added at a final concentration of 20 μg mL^{-1}.

[c]Linoleic acid was added at a final concentration of 10 μg mL^{-1}.

[d]Other indicates fatty acids that comprised <1% of the total membrane content.

[e]Fatty acid length ratio includes both saturated and unsaturated fatty acid.



Supplemental Figure 4.1. Short-term fatty acid supplementation with host fatty acids does not protect OG1RFΔ*mprF2* from SDS challenge.

The Δ*mprF2* strain has decreased sensitivity as compared to the parental OG1RF strain. (A). Supplementation of Δ*mprF2* with oleic acid does not increase protection against SDS challenge. (B) Supplementation of Δ*mprF2* with linoleic acid does not increase protection against SDS challenge. Shown are the average ± standard deviation for *n* = 3.

Supplemental Table 4.7. *E. faecalis* OG1RF parental, Δ*cls1*, and Δ*cls2* membrane fatty acid composition during exponential-phase growth.

	% of total membrane content (Avg. ± SD)								
	Ethanol[a]			Oleic acid[b] - $C_{18:1\ cis\ 9}$			Linoleic acid[c] - $C_{18:2\ cis\ 9,12}$		
Fatty acid	OG1RF	Δ*cls1*	Δ*cls2*	OG1RF	Δ*cls1*	Δ*cls2*	OG1RF	Δ*cls1*	Δ*cls2*
$C_{12:0}$	1.8 ± 0.3	1.6 ± 0.1	1.7 ± 0.4	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.1 ± 0.04
$C_{14:0}$	4.8 ± 0.1	4.6 ± 0.04	4.4 ± 0.4	2.5 ± 0.2	2.4 ± 0.1	2.4 ± 0.02	3.7 ± 0.1	3.6 ± 0.1	3.5 ± 0.1
$C_{16:1\ cis\ 9}$	7.2 ± 0.1	6.7 ± 0.1	7.1 ± 0.4	3.8 ± 0.3	3.8 ± 0.1	4.0 ± 0.1	6.1 ± 0.04	5.9 ± 0.2	6.1 ± 0.1
$C_{16:0}$	37.8 ± 0.4	37.5 ± 0.3	36.0 ± 0.2	19.5 ± 0.6	18.6 ± 0.5	18.0 ± 0.3	29.6 ± 0.5	29.2 ± 0.2	29.0 ± 0.6
$C_{18:1\ cis\ 9}$	1.7 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	46.7 ± 1.7	47.1 ± 1.7	42.2 ± 6.0	ND	ND	ND
$C_{18:1\ cis\ 11}$	38.3 ± 0.5	39.2 ± 0.4	41.3 ± 0.9	19.0 ± 0.6	18.3 ± 0.4	19.0 ± 0.7	28.6 ± 0.5	28.4 ± 0.2	30.8 ± 0.9
$C_{18:0}$	5.0 ± 0.1	5.5 ± 0.1	4.6 ± 0.1	2.9 ± 0.2	2.8 ± 0.3	2.6 ± 0.1	4.3 ± 0.1	4.4 ± 0.3	4.0 ± 0.1
$C_{18:2\ cis\ 9,12}$	ND	ND	ND	ND	ND	ND	24.0 ± 0.9	28.4 ± 0.2	22.0 ± 1.4
$C_{17:0\ 2OH}$	2.0 ± 0.2	2.1 ± 0.3	1.9 ± 0.3	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.3	1.6 ± 0.1	1.8 ± 0.03	1.7 ± 0.1
$C_{19:0\ cyclo\ 9}$	ND	ND	ND	ND	ND	ND	ND	ND	ND
$C_{20:0}$	ND	ND	ND	3.2 ± 0.3	4.8 ± 1.2	9.3 ± 5.1	ND	ND	ND
Other[d]	1.3 ± 0.6	1.5 ± 0.1	1.5 ± 0.6	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.9 ± 0.3	0.7 ± 0.2	1.7 ± 0.8
Sat/Unsat	1.1 ± 0.02	1.1 ± 0.01	1.0 ± 0.03	0.4 ± 0.5	0.4 ± 1.0	0.5 ± 0.9	0.7 ± 0.5	0.7 ± 0.7	0.7 ± 0.4
C_{10} - C_{17} / C_{18} - C_{20}[e]	1.2 ± 0.01	1.1 ± 0.01	1.1 ± 0.05	0.4 ± 0.4	0.4 ± 0.3	0.4 ± 0.1	0.7 ± 0.5	0.7 ± 0.5	0.7 ± 0.3

Membrane contents were determined using GC-FAME analysis by Microbial ID, Inc.; numbers represent average ± standard deviation from three independent cultures.; ND indicates that fatty acid was not detected.

[a]Ethanol was added at a final concentration of 0.1%.

[b]Oleic acid was added at a final concentration of 20 μg mL^{-1}.

[c]Linoleic acid was added at a final concentration of 10 μg mL^{-1}.

[d]Other indicates fatty acids that comprised <1% of the total membrane content.

[e]Fatty acid length ratio includes both saturated and unsaturated fatty acid.

Supplemental Table 4.8. *E. faecalis* OG1RF parental, Δ*cls1/ cls2*, and Δ*mprF2/ cls1/ cls2* membrane fatty acid composition during exponential-phase growth.

	% of total membrane content (Avg. ± SD)								
	Ethanol[a]			Oleic acid[b] - $C_{18:1\ cis\ 9}$			Linoleic acid[c] - $C_{18:2\ cis\ 9,12}$		
Fatty acid	OG1RF	Δ*cls1*/Δ*cls2*	Δ*mprF2*/*cls1*/Δ*cls2*	OG1RF	Δ*cls1*/Δ*cls2*	Δ*mprF2*/*cls1*/Δ*cls2*	OG1RF	Δ*cls1*/Δ*cls2*	Δ*mprF2*/*cls1*/Δ*cls2*
$C_{12:0}$	1.8 ± 0.3	1.7 ± 0.2	1.3 ± 0.3	0.7 ± 0.1	0.6 ± 0.1	1.6 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	1.3 ± 0.2
$C_{14:0}$	4.8 ± 0.1	4.2 ± 0.1	4.4 ± 0.4	2.5 ± 0.2	1.6 ± 0.1	4.0 ± 0.2	3.7 ± 0.1	3.0 ± 0.1	3.5 ± 0.3
$C_{16:1\ cis\ 9}$	7.2 ± 0.1	6.5 ± 0.2	7.0 ± 0.6	3.8 ± 0.3	2.7 ± 0.2	5.7 ± 0.4	6.1 ± 0.04	5.4 ± 0.1	5.3 ± 0.3
$C_{16:0}$	37.8 ± 0.4	37.1 ± 0.3	37.3 ± 0.8	19.5 ± 0.6	14.7 ± 0.6	29.5 ± 0.1	29.6 ± 0.5	26.8 ± 0.4	29.0 ± 1.1
$C_{18:1\ cis\ 9}$	1.7 ± 0.2	1.3 ± 0.1	0.3 ± 0.5	46.7 ± 1.7	59.8 ± 2.1	11.5 ± 4.3	ND	ND	ND
$C_{18:1\ cis\ 11}$	38.3 ± 0.5	40.2 ± 0.6	41.9 ± 0.9	19.0 ± 0.6	14.8 ± 0.4	28.0 ± 0.4	28.6 ± 0.5	29.2 ± 0.8	27.0 ± 0.8
$C_{18:0}$	5.0 ± 0.1	5.4 ± 0.2	4.9 ± 0.3	2.9 ± 0.2	2.5 ± 0.1	3.6 ± 0.2	4.3 ± 0.1	4.0 ± 0.2	4.1 ± 0.1
$C_{18:2\ cis\ 9,12}$	ND	ND	ND	ND	ND	ND	24.0 ± 0.9	27.8 ± 0.9	26.1 ± 2.7
$C_{17:0\ 2OH}$	2.0 ± 0.2	1.9 ± 0.4	1.6 ± 1.4	1.1 ± 0.1	0.7 ± 0.1	1.3 ± 0.2	1.6 ± 0.1	1.8 ± 0.1	2.2 ± 0.2
$C_{19:0\ cyclo\ 11}$	ND	ND	1.0 ± 0.2	ND	ND	1.3 ± 0.1	ND	ND	1.2 ± 0.1
$C_{20:0}$	ND	ND	ND	3.2 ± 0.3	1.8 ± 1.6	13.0 ± 3.3	ND	ND	ND
Other[d]	1.3 ± 0.6	1.6 ± 0.3	0.3 ± 0.6	0.6 ± 0.2	0.8 ± 0.1	0.5 ± 0.4	0.9 ± 0.3	1.1 ± 0.2	0.5 ± 0.4
Sat/Unsat	1.1 ± 0.02	1.1 ± 0.01	1.0 ± 0.02	0.4 ± 0.5	0.3 ± 0.9	1.2 ± 0.2	0.7 ± 0.5	0.6 ± 0.5	0.7 ± 0.5
C_{10} - C_{17} / C_{18} - C_{20}[e]	1.2 ± 0.01	1.1 ± 0.02	1.1 ± 0.06	0.4 ± 0.4	0.3 ± 0.2	0.74 ± 0.03	0.7 ± 0.5	0.6 ± 0.4	0.7 ± 0.6

Membrane contents were determined using GC-FAME analysis by Microbial ID, Inc.; numbers represent average ± standard deviation from three independent cultures.; ND indicates that fatty acid was not detected.

[a]Ethanol was added at a final concentration of 0.1%.

[b]Oleic acid was added at a final concentration of 20 μg mL^{-1}.

[c]Linoleic acid was added at a final concentration of 10 μg mL^{-1}.

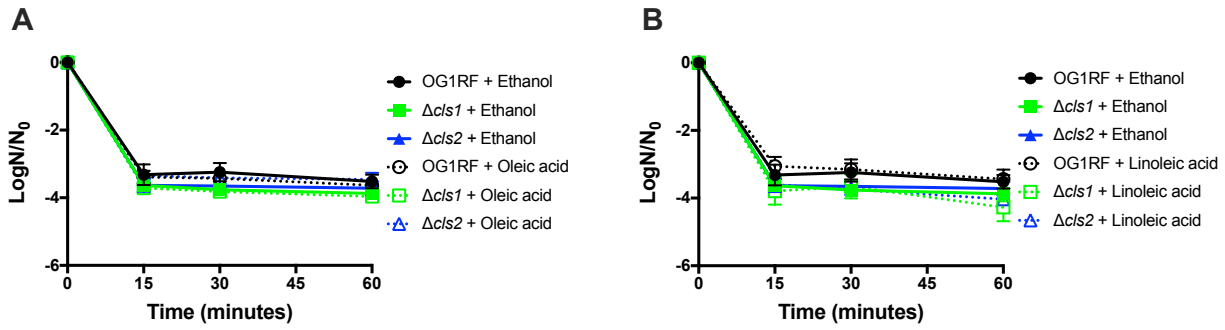
[d]Other indicates fatty acids that comprised <1% of the total membrane content.

[e]Fatty acid length ratio includes both saturated and unsaturated fatty acid.

Supplemental Table 4.9. *E. faecalis* OG1RF, Δ*cls1*, and Δ*cls2* phospholipid composition during exponential-phase growth after short-term supplementation.

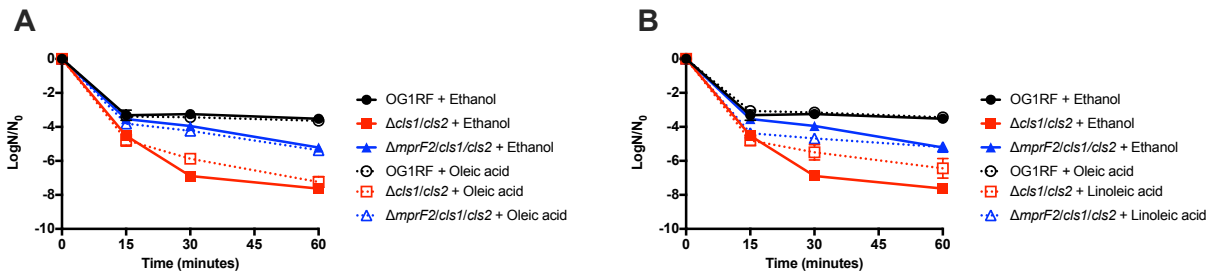
	μM concentrations of phospholipid species (Avg. ± SD)								
	Ethanol[a]			Oleic acid[b] - $C_{18:1\ cis\ 9}$			Linoleic acid[c] - $C_{18:2\ cis\ 9,12}$		
Phospholipid	OG1RF	Δ*cls1*	Δ*cls2*	OG1RF	Δ*cls1*	Δ*cls2*	OG1RF	Δ*cls1*	Δ*cls2*
PG 32:0	1.83±0.62	2.10±0.30	2.01±0.44	0.96±0.31	1.17±0.15	1.13±0.22	0.97±0.24	3.02±0.43	1.25±0.66
PG 32:1	5.32±1.31	6.28±0.34	7.33±2.07	1.97±0.58	2.13±0.25	2.83±0.44	1.67±0.25	4.77±1.08	2.36±0.46
PG 34:0	2.29±0.69	2.40±0.23	2.64±0.86	0.93±0.20	1.03±0.17	1.50±0.29	0.91±0.15	2.22±0.34	1.27±0.31
PG 34:1	22.3±3.95	25.5±2.82	27.7±10.3	9.83±2.35	13.0±2.12	18.7±3.81	8.13±1.64	19.0±2.44	10.7±1.52
PG 34:2	2.17±0.55	2.67±0.52	3.11±1.07	1.14±0.32	1.37±0.30	1.90±0.29	1.11±0.11	2.68±0.30	1.39±0.34
PG 36:0									
PG 36:1	1.78±0.60	1.49±0.20	2.09±0.38						
PG 36:2	5.34±2.1	5.03±1.60	7.63±1.01	1.33±0.50	1.37±0.58	2.34±0.62	0.78±0.10	1.59±0.35	1.06±0.28
PG 36:3									
PG 36:4									
CL 56:0									
CL 64:0									
CL 64:1									
CL 64:2	0.74±0.19								0.81±0.26
CL 66:0									
CL 66:1									
CL 68:0	0.87±0.10	0.78±0.36	1.06±0.42	0.74±0.20		1.00±0.55		0.97±0.27	0.80±0.09
CL 68:1									
CL 70:0	1.42±0.42	1.49±0.65	1.67±0.56	1.65±0.30	1.71±0.70	2.23±0.41	1.02±0.41	2.64±0.70	1.19±0.41
CL 70:1									
CL 70:3						1.04±0.35			
CL 70:4									
CL 72:0	14.1±3.74	15.1±7.29	14.7±3.46	1.02±0.22	0.82±0.15	2.04±0.68	3.23±0.64	4.74±0.76	4.35±0.85
CL 72:1			0.89±0.39						
CL 72:2	1.13±0.13	1.35±0.39	1.43±0.49						
CL 72:3									
CL 72:4									
CL 72:8									
L-PG 32:0	0.54±0.10	0.52±0.15	0.48±0.10	3.11±0.90	5.25±1.61	1.99±0.79	3.47±0.61	5.41±1.01	2.92±0.84
L-PG 32:1	1.04±0.29	0.94±0.44	1.08±0.25	7.83±2.35	14.1±4.85	5.88±1.99	5.24±0.71	8.04±1.63	4.70±1.12
L-PG 34:0	0.74±0.14	0.70±0.21	0.78±0.15	2.95±1.00	5.12±1.57	2.29±0.84	3.66±0.67	5.62±1.50	3.73±0.99
L-PG 34:1	7.09±1.11	7.24±2.80	7.73±1.09	28.9±9.36	52.7±19.2	25.6±8.41	24.3±4.51	33.0±4.40	26.1±5.30
L-PG 34:2	0.72±0.23	0.67±0.26	1.09±0.21	2.79±0.99	4.63±1.47	2.33±0.82	5.61±0.98	7.35±0.49	5.80±1.46
L-PG 36:0				0.13±0.06	0.27±0.07	0.12±0.04	0.12±0.03	0.18±0.05	0.10±0.04
L-PG 36:1	0.54±0.13	0.49±0.17	0.60±0.11	1.08±0.42	1.72±0.64	0.93±0.40	1.26±0.27	2.03±0.32	1.33±0.31
L-PG 36:2	1.45±0.16	1.12±0.35	1.66±0.29	6.71±2.98	10.3±4.22	6.07±2.67	3.24±0.36	4.79±0.63	3.83±1.18
L-PG 36:3					0.09±0.02		1.10±0.17	1.38±0.16	1.13±0.37
L-PG 36:4	0.09±0.02	0.10±0.01	0.11±0.01	0.20±0.11	0.51±0.29	0.16±0.06	0.81±0.19	1.11±0.14	0.65±0.16
%PG	57.4	59.9	61.2	22.1	17.1	35.5	22.4	30.1	23.9
%CL	25.5	24.6	23	4.6	2.2	7.9	6.4	7.6	9.5
%L-PG	17.1	15.5	15.8	73.3	80.7	56.6	73.2	62.3	66.6

PG – phosphatidylglycerol, CL – cardiolipin, L-PG – lysyl-phosphatidylglycerol
BHI medium was used for all cultures.
[a]Ethanol was used at a final concentration of 0.1%.
[b]Oleic acid was used at a final concentration of 20 μg mL^{-1}.
[c]Linoleic acid was used at a final concentration of 10 μg mL^{-1}.



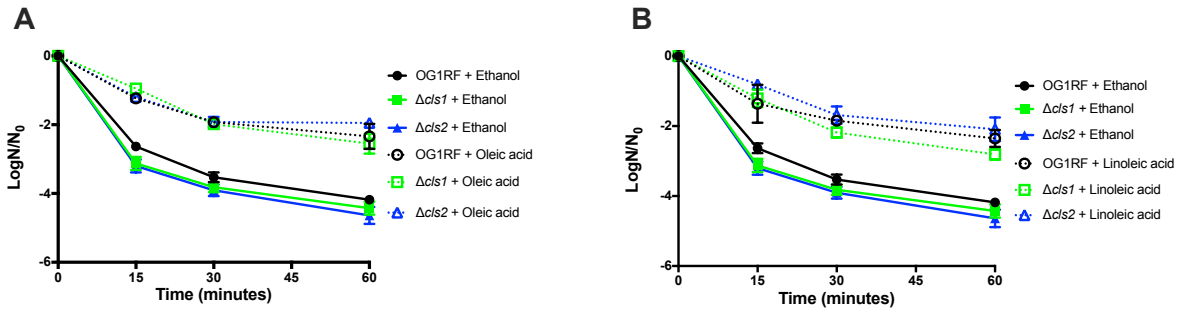
Supplemental Figure 4.2. Short-term fatty acid supplementation with host fatty acids does not protect OG1RFΔ*cls1* or Δ*cls2* from SDS challenge.

(A). Supplementation of Δ*cls1* or Δ*cls2* with oleic acid does not increase protection against SDS challenge. (B) Supplementation of Δ*cls1* or Δ*cls2* with linoleic acid does not increase protection against SDS challenge. Shown are the average ± standard deviation for *n* = 3.



Supplemental Figure 4.3. Increased sensitivity to SDS in the cardiolipin synthase double deletion strain.

(A) Oleic acid supplementation and challenge with SDS. The parental strain (OG1RF) supplemented with oleic acid was similar to the ethanol control. The cardiolipin synthase double mutant strains had increased sensitivity to SDS (P = 0.0006) and no increased tolerance after oleic acid supplementation. (B) Linoleic acid supplementation and challenge with SDS. Each strain supplemented with linoleic acid was similar to the solvent control. At time 60, the cardiolipin synthase mutant was more sensitive to SDS as compared to the parent strain (P = 0.002). Shown are the average ± standard deviation for n = 3.



Supplemental Figure 4.4. Short-term fatty acid supplementation with host fatty acids protects OG1RFΔ*cls1* or OG1RFΔ*cls2* from daptomycin challenge.

(A) Oleic acid supplementation of Δ*cls1* or Δ*cls2* statistically increased numbers of survivors versus the solvent control at all time points (P <0.0001). (B) Linoleic acid supplementation of Δ*cls1* or Δ*cls2* had statistically increased numbers of survivors versus the solvent control at all time points assessed (P = 0.0001). Shown are the average ± standard deviation for n = 3.

CHAPTER V: Conclusions and future direction

Previous data from our lab found that growth of *E. faecalis* with host fluids, such as bile (GI tract) or serum (wounds), provided fatty acids which can be incorporated into the membrane of *E. faecalis* and result in increased tolerance to membrane damaging agents (1, 2). Further work showed that *E. faecalis* responds uniquely to each fatty acid supplement and that of those examined only oleic acid and linoleic acid increase tolerance to membrane damaging agents (2). The studies presented in this dissertation were designed to elucidate why the eukaryotic host fatty acids, oleic acid and linoleic acid, increase tolerance to membrane damaging agents in the hospital-acquired pathogen *Enterococcus faecalis.*

Fatty acid analogs to understand fatty acid induced daptomycin tolerance

Oleic acid and linoleic acid are similar in that they contain fatty acid tails consisting of 18 carbons and are unsaturated with the bond(s) in the *cis* position. However, supplementing with fatty acids containing 18 carbons does not confer tolerance because stearic acid ($C_{18:0}$) and *cis*-vaccenic acid ($C_{18:1\ cis\ 11}$) do not rescue (1, 2). I designed experiments to better discern how the cell may respond "positively" to oleic acid or linoleic acid, whereas similar fatty acids do not promote tolerance. My experiments found that the *cis* bond was important for daptomycin tolerance but increasing the number of unsaturated *cis* bonds was not (Figure 2.1). The most critical finding from this analysis was that oleic acid and linoleic acid need the carboxyl group to induce tolerance, as was demonstrated by using analogs containing an amide group instead of a carboxyl group (Figure 2.3). Lipidomic analysis demonstrates that the loss of the carboxyl group prevents phospholipid incorporation and supports the hypothesis that incorporation into a head group is an important step in triggering tolerance (Figure 2.4). This is further supported by published work in *S. aureus* (3).

Fatty acid induced membrane stress responses

To assess whether or not protective fatty acids increase tolerance to membrane damaging agents by triggering a membrane stress response, I used a daptomycin hypersusceptible mutant strain of OG1RF that lacks *liaR* of the LiaFSR three component system (4). LiaFSR consists of a membrane bound sensor histidine kinase (LiaS), a

negative regulator (LiaF), and response regulator (LiaR) and has been shown to be important for responses to cell envelope targeting antibiotics and antimicrobial peptides (5) including daptomycin (6). By using a strain that is deficient in *liaR*, we discovered that supplementing with oleic acid increased tolerance to daptomycin (7) and suggests that the LiaFSR pathway is not involved in our observations. However, these data could not eliminate the possibility that another membrane sensing mechanism is involved in our observations.

To address the possibility that another membrane stress response system is involved, we collaborated with Dr. Chris Kristich at the Medical College of Wisconsin. The Kristich laboratory tested to see if two cell envelope stress sensing two component systems, CroSR and IreK, might be responding to the addition of oleic acid (data not shown). CroSR is normally involved in cell wall stress responses and was found to elicit cell wall repair upon stimulation. In the absence of CroR, cells are more sensitive to cell wall targeting antibiotics, such as vancomycin (8). Further, IreK has been shown to be involved in cell wall homeostasis (9). By assessing these two potential stress response pathways we could test the possibility that host fatty acids might be triggering a response. We found that they were not. However, these results again do not mean that supplementation with host fatty acids are not having an impact on other cell stress/two-component systems.

Perturbations in cell envelope homeostasis can trigger a stress response (10) and modifying the normal composition of acyl tails could indeed have an effect. Further, unsaturated fatty acids can impact the lateral pressure within the membrane bilayer, which can subsequently impact the activity of proteins embedded in the membrane (11). Also, the position of the double bond within the fatty acid tail can also impact the membrane. Data shows that a fatty acid with a double bond at the geometric center of the molecule has the lowest phase transition temperature (12), can increase the overall surface area of the membrane, cause thinning of the membrane, and cause the acyl chains to be more disordered (13). Each of these biophysical changes that occur with unsaturated fatty acids might be causing downstream effects that influence not only membrane signaling proteins, but overall cellular metabolic processes, which could then impact gene expression. Given this, rather than targeting each stress response

mechanisms specifically at first, I want to test how the transcriptome of OG1RF changes upon addition of host fatty acids. To test the hypothesis that protective host fatty acids are triggering a membrane stress response or influencing other changes, RNA sequencing will be conducted.

Unexplored phospholipid alterations could contribute to membrane stress tolerance.

We conducted targeted mass spectrometry to conclude if supplementation with oleic acid or linoleic acid alters the phospholipid profile, and thereby increases protection. We observed that supplementation with oleic acid and linoleic acid increased the amount of lysyl-phosphatidylglycerol (L-PG) and reduced the level of cardiolipin (CL) (Table 4.1). To conclude if these alterations contributed to host fatty acid induced tolerance, we deleted the predicted genes responsible for lysyl-phosphatidylglycerol synthesis (*mprF2*) and cardiolipin synthesis (*cls1* and *cls2*), which resulted in loss of L-PG and a reduction of CL respectively (Table 4.2 and Table 4.3). However, supplementation of these strains with oleic acid and linoleic acid still resulted in increased tolerance to daptomycin during the first 60 minutes of challenge (Figure 4.1 and Figure 4.3). These data suggested that supplementation with oleic acid or linoleic acid alter the levels of L-PG and CL, but the altered levels are not responsible for increased membrane tolerance during the early exposure to membrane stressors.

Previous studies have shown that there are a number of membrane changes that can impact daptomycin sensitivity. Studies using *E. faecalis* clinical isolates or evolved strains and liposome models have identified causes of reduced daptomycin sensitivity including: decreased amounts of PG, increased amounts of CL, altered localization of CL, deletion of LiaR of the LiaFSR stress response systems, and increased amounts of glycerophospho-diglycodiacylglycerol (GD-DGDAG) (14). As stated above, we have investigated changes to the phospholipid profile, but we have not investigated all of the phospholipid species that may be changing after supplementation. One species of phospholipid that may be increasing as a consequence of host fatty acid supplementation is GD-DGDAG (Figure 5.1). Analysis of a daptomycin resistant strain of *E. faecalis* found that there was nearly a two-fold increase in the amount of GD-DGDAG in the membrane

(14, 15). Following up on this particular lipid species could be important because it has been shown that glycolipids are involved in the synthesis of lipotechoic acids (LTA), which are important for cation homeostasis, antimicrobial peptide resistance, and biofilm formation (16–18). If the incorporation of host fatty acids promotes glycolipid production, this might increase LTA production, which could increase tolerance to membrane stressors. An approach to address this hypothesis could involve generating a Δ*bgsB* (a glycosyltransferase responsible for synthesizing monoglucosyldiacylglycerol) strain and testing if oleic acid or linoleic acid supplementation can still induce daptomycin tolerance.

Location of supplemented fatty acids in the phospholipid profile

An unresolved question we have had since the beginning of this work is where do host fatty acids go when they are incorporated into the membrane of *E. faecalis*? We have mass spectrometry data showing that free fatty acids can be found in the membrane, and we can also infer based on *m/z* that after supplementation with host fatty acids, we see an increase in L-PG (34:1, 34:2, or 36:2) species that could contain the supplemented fatty acid. However, ascertaining specific details regarding the localization of supplemented fatty acids within phospholipids has not been conducted with confidence. In bacteria, the current hypothesis is that exogenous fatty acids are incorporated into the *sn1* and *sn2* positions of glycerol-3-phosphate using fatty acid kinase and then shuttled through phospholipid synthesis (19) Further, there is very little evidence that bacteria can remodel their membrane phospholipids (20, 21). However, in the yeast model organism, *Saccharomyces cerevisiae*, it is known that a post-synthetic process can occur to replace one or both acyl chains in a phospholipid (22).

In an effort to specifically identify the localization of supplemented fatty acids, we used ^{14}C-containing oleic acid to understand where the supplemented fatty acid was going in the phospholipid profile. In this radiolabeled fatty acid tracking experiment using extracted phospholipids and one-dimensional thin layer chromatography (TLC), we observed a rapid increase in localization of this radiolabeled fatty acid into cardiolipin and then a subsequent localization to phosphatidylglycerol (Figure 5.2). These preliminary data were quite interesting and made me hypothesize that *E. faecalis* is exchanging the acyl tails on cardiolipin instead of adding the exogenous fatty acids onto newly

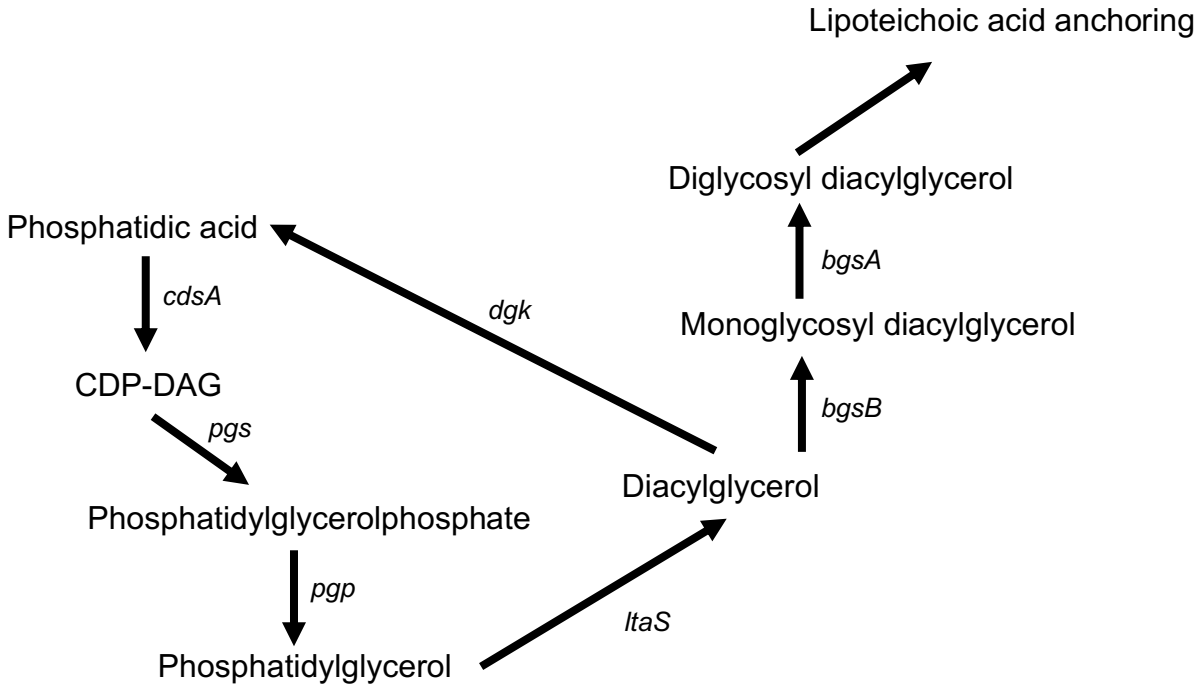


Figure 5.1 Phospholipid synthesis and lipoteichoic acid precursor synthesis in *E. faecalis* and the predicted enzymes involved.

After production of phosphatidylglycerol, enterococcus can synthesize precursors for lipoteichoic acid or recycle phosphatidic acid. *cdsA*, cytidine diphosphate synthase; *pgs,* phosphatidylglycerol-phosphate synthase; *pgp,* phosphatidylgycerol phosphate phosphatase; *ltaS,* lipoteichoic acid synthase; *bgsB* and *bgsA,* glycosyltransferase; *dgk,* diacylglycerol kinase.

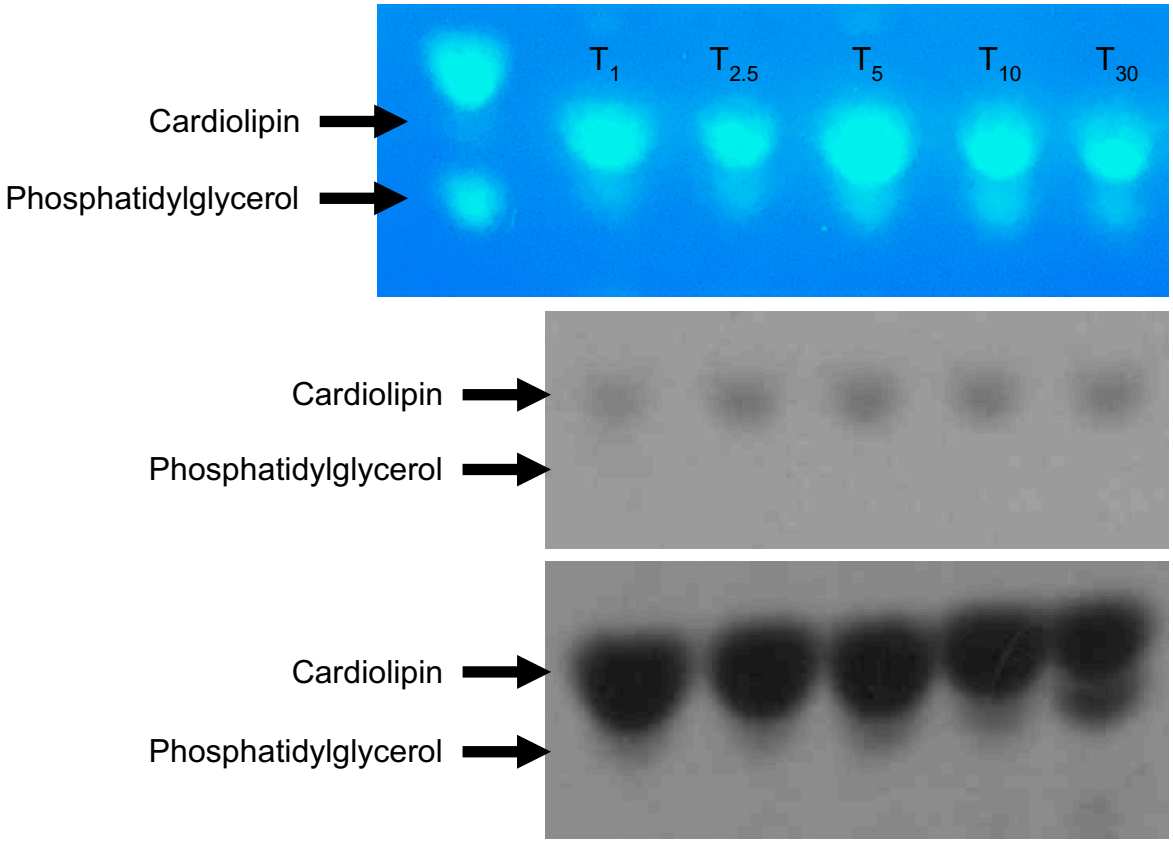


Figure 5.2 Extracted lipids from log phase *E. faecalis* OG1RF after supplementation with radiolabeled oleic acid shows quick incorporation into cardiolipin using thin layer chromatography.

(Top) Primulin stained lipids shows location of cardiolipin and phosphatidylglycerol standards and increased abundance of CL after supplementation with fatty acid. (Middle) 30 minutes exposure shows increasing CL over time. (Bottom) 8 hour exposure shows increasing localization of radiolabeled oleic acid in PG over time.

synthesized headgroups. Studies in eukaryotes show that CL has a fatty acyl composition enriched in unsaturated fatty acids and that transacylases are responsible for shuttling fatty acids between CL and other glycerolipids (23).

To conduct this experiment more convincingly and address my hypothesis, two-dimensional TLC should be used to clearly separate and identify the major phospholipid species in *E. faecalis* that could contain the radiolabeled fatty acid. If we confirm that radiolabeled signal first appears in CL then transitions to PG, we can conduct additional experiments. The primary experimental concern is how to identify whether or not the kinetics of fatty acid incorporation facilitate inclusion of the fatty acid into phospholipid synthesis by one minute (T_1 – Figure 5.2) or if the fatty acid is directly added to CL. If we indeed observe an initial increase of radiolabeled fatty acid in CL and then a subsequent transition into PG after 2-D TLC, we can generate conditional gene deletion strains that prevent incorporation of exogenous fatty acid into phospholipid synthesis. Work with *Bacillus subtilis* has demonstrated genes for PlsX, PlsY, and PlsC are essential (24). However, to overcome this, Paoletti *et al* leveraged an inducible promoter system that permitted the study of these genes. This study showed that removal of inducer led to *plsX* inhibition and the subsequent loss of both fatty acid and phospholipid synthesis. Generating an inducible system of *plsX* in *E. faecalis*, will allow us to detect if radiolabeled fatty acids require fatty acid/phospholipid synthesis or if radiolabeled fatty acids can be loaded directly onto CL. Depending on the outcome of this experiment, we can begin to analyze the genome of *E. faecalis* for proteins that could have putative acyl chain remodeling activity (22).

Final thoughts

From a physiological perspective, it seems reasonable to think that *E. faecalis* should be able to use the materials inside of a host given that they have been living inside of animals since animals underwent terrestrialization about 425 million years ago (25). Furthermore, the ability of enterococci to survive harsh environments, both in host niches and environmental niches, is a testament to its many years of success as a bacterium and perhaps more importantly, why it is a significant hospital pathogen. However, what is enlightening is the result of membrane stress tolerance after incorporating host fatty

acids. Recent studies assessing proper dosage with daptomycin in order to treat vancomycin resistant enterococci (VRE) have reported issues in treatment outcome (26). This is perhaps because bacterial sensitivity to drugs is often tested in conditions that do not consider the host environment. As illustrated in this dissertation work, growth in the presence of exogenous fatty acids from the host environment improves tolerance to daptomycin. Although the details regarding failure of dosing have not been fully elucidated, it is clear that basic MICs and drug susceptibility measurements of clinical isolates are not performed in the presence of host fluids which clearly can protect against daptomycin (27). The work conducted in this dissertation may help shine a light on how host fatty acids can improve tolerance to membrane damaging agents, provide evidence that the host must be considered when testing drug sensitivity, and expands on why *Enterococcus faecalis* is able to survive so well in both clinical and homeostatic conditions.

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VITA

John Harp was born on a sunny day in southern California. He was then driven across the country to grow up in the Garden State, New Jersey. After graduating from West Virginia University, he went to The University of Tennessee to acquire a MS in microbiology. He thought he would be content to work in industry after acquiring the MS. He was wrong. After several years, he returned to The University of Tennessee to push through and conquer a PhD. Now, he is graduating and will be returning to industry. Let's hope he is content; is there another degree a person can get that's above a PhD? No, no. John doesn't want to go there.