It’s All in the Mix: Physiological Effects of Fatty Acid Supplementation on *Enterococcus faecalis*

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Dedication

To my wife who sacrificed her own research to allow mine and to my children who I hope never to stop asking questions.

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Abstract

Enterococcus faecalis is a Gram-positive bacterium that normally exists as an intestinal commensal in humans. Additionally, it can survive in the environment for extended periods and cause infections in immunocompromised hosts, making *E. faecalis* a leading cause of nosocomial infections. Previous work noted that specific fatty acids found in serum contribute to tolerance of membrane damaging agents, including the drug daptomycin. However, not all fatty acids found in serum were able to induce such protection. Herein, we measured a wide array of physiological responses after supplementation with combinations of protective (i.e., induced daptomycin tolerance) and non-protective fatty acids. When supplementing cells with either non-protective fatty acid, palmitic acid or stearic acid, there was a significant increase in generation time and severely distorted morphology. Both physiological defects were rescued when these cultures were supplemented with one of the protective fatty acids identified in serum, oleic acid or linoleic acid. Similarly, membrane fluidity decreased with growth in either palmitic or stearic acid alone but returned to basal levels when supplemented in combination with a protective fatty acid. While cell envelope charge has been associated with tolerance to daptomycin in other Gram-positive bacteria, we concluded that it does not correlate with the fatty acid induced protection we observed. Further, daptomycin tolerance could be induced when cultures were supplemented with non-protective fatty acids in combination with a protective fatty acid. Beyond physiology, we measured changes in transcription after protective fatty acid supplementation, noting upregulation in a gene encoding for a glycoside hydrolase as well as the known daptomycin genetic resistance gene *gdpD*. Combined, we conclude that a single protective fatty acid is able to alleviate negative growth defects and protect cells from daptomycin.

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Chapter I: Introduction

Enterococci

Enterococcus species are a group of mammalian associated bacteria that persist in external environments [1]. These species serve as indicators of fecal contamination of both water systems and meats, but are also responsible for the fermentation of food products [2]. Both *Enterococcus faecalis* and *Enterococcus faecium* are leading causes of nosocomial infections around the world mainly due to their hardiness and ability to survive inside and outside of the body for extended periods of time [3]. Enterococci are considered opportunistic pathogens that live in the intestinal tracts of healthy individuals and upon niche expansion may cause endocarditis, wound infections and septicemia in immunocompromised individuals or patients that have received broad antimicrobial therapy. *Enterococci* species have both intrinsic and acquired resistance to β-lactams, fluoroquinolones and glycopeptide antibiotics [4]. Both *E. faecalis* and *E. faecium* possess many antibiotic defense mechanisms, including drug modification, target modification, and drug efflux pumps, some of which are intrinsic to most isolates [5]. Beyond genetic resistance, *E. faecalis* has increased tolerance to membrane damaging agents when grown in host components [1, 6]. More needs to be understood about the mechanisms used for persistence in external environments as well as resistance and tolerance to antimicrobial therapies. In this chapter I will explain what is known of the physiology of *E. faecalis* that enables it to be a leading worldwide nosocomial pathogen.

Fatty Acid Biosynthesis in *E. faecalis*

The selectivity of the cell membrane is due in part to the amphipathic nature of the primary component, phospholipids. The hydrophilic glycerophosphate head interacts with both the interior cytosol as well as the exterior environment, blocking charged and large molecules from entering the cell. Fatty acid tails attached to the glycerophosphate head are hydrophobic, creating a suitable environment for the localization of membrane proteins via hydrophobic residue interaction. There are two methods *E. faecalis* employs to create fatty acids suitable for phospholipid synthesis, *de* n*ovo* fatty acid biosynthesis and incorporation of exogenous fatty acids.

E. faecalis performs type 2 fatty acid biosynthesis (FASII) (Fig 1.1) to create both saturated and unsaturated fatty acids from the base molecule acetyl-CoA. Acetyl-CoA

carboxyltransferase (AccAD) transfers a carboxyl group to acetyl-CoA, resulting in malonyl-CoA. The free carboxyl is derived from biotin by biotin carboxylase carrier protein (AccB) and biotin carboxylase (AccC). The malonyl moiety of malonyl-CoA is transferred to the acyl carrier protein (ACP) by malonyl CoA-ACP transaclyase (FabD), making malonyl-ACP. Acyl-ACP and malonyl-ACP are then condensed to form β-ketoacyl-ACP by β-ketoacyl-ACP synthase III (FabH). This is reduced by 3-Oxoacyl-ACP-reductase (FabG) and NADPH producing β-hydroxyacyl-ACP and $NADP^+$. For saturated fatty acid synthesis, 3-hydroxyacyl-ACP-dehydratase (FabZ) dehydrates β-hydroxyacyl-ACP and forms trans-2-enoylacyl-ACP. The final reduction step by enoyl-ACP-reductase (FabI) results in the formation of an acyl-ACP two carbons longer than the starting molecule. Interestingly, FabI in *E. faecalis* is similar to FabB in *Escherichia coli* [7]. Also note that *E. faecalis* has both FabI and FabK, an enoyl-ACP- reductase found in *E. coli*, present in the genome, but FabK appears non-functional [8]. Elongation proceeds by the repeated condensation of acyl-ACP or malonyl-ACP with the acyl-ACP by β-ketoacyl-ACP synthase II (FabF). Elongation generally proceeds until the fatty acid measures 16 to 18 carbons in length.

Unsaturated fatty acid synthesis differs at the dehydration step of β-hydroxyacyl-ACP. Instead, 3-hydroxyacyl-ACP-dehydratase (FabN) is capable of an isomerization reaction, resulting in *cis*-2-enoylacyl-ACP. This molecule is unable to be reduced by FabI but can be condensed with acyl-ACP or malonyl-ACP by FabF, leading to elongation and the synthesis of unsaturated fatty acids, acyl chains containing carbon – carbon double bonds. The mechanism for selection of unsaturated fatty acid synthesis is currently unknown. However, in *E. coli*, data can explain how the UFAs may preferentially be incorporated into lipid headgroup precursors as the temperature decreases: similar system may exist in *E. faecalis*. in *E. coli*, *cis*-vaccenic acid ($C_{18:1\ cis11}$) and palmitic acid (C_{18}) compete for the first position in the phospholipid backbone [9]. At lower temperatures the activity of FabB is increased due to an unknown mechanism [10]. FabB and FabF are responsible for the elongation of cis-3-decanoyl-ACP into cis-vaccenoyl-ACP, ultimately resulting in cis-vaccenic acid. With a higher proportion of cis-vaccenic acid to palmitic acid available, cis-vaccenic acid preferentially

populates the first position of the phospholipid backbone, increasing membrane fluidity at lower temperatures [9].

Exogenous fatty acid incorporation

Most bacteria are able to transport free fatty acids from the environment into the cell, which expands the possible carbon sources in a given environment as well as reducing the cost and time of synthesizing acyl chains for phospholipids. Gram-negative bacteria such as *E. coli* use long-chain fatty acid transport protein (FadL) to bind long chain fatty acids at the outer membrane [11]. Once bound to FadL, acyl-CoA synthetase (FadD) forms an acyl-CoA using the free fatty acid. The acyl-CoA can be used for β-oxidation or phosphatidic acid synthesis if the correct acyl chain length is present, generally 14-16 carbons in length but is species dependent [12, 13]. If the environment is rich in unsaturated fatty acids, *E. faecalis* could grow without de novo fatty acid synthesis. This provides an energy advantage if fatty acids chains do not need to be synthesized by the organism, as well as a protective advantage if oleic acid or linoleic acid are available.

Gram-positive bacteria use a different system that begins with fatty acid binding protein (FakB), which as the name implies, binds to a free fatty acid and transports it to the cytosol. There it is phosphorylated by fatty acid kinase (FakA) and ready for degradation by the β-oxidation pathway or phospholipid synthesis. Bacteria such as *B. subtilis* can degrade the fatty acid for energy, but not *E. faecalis,* therefore I will focus on incorporation of free fatty acids onto phospholipid head groups. Once phosphorylated by FakA, two options take place for association onto a head group. The acyl-PO_4 can be used by PlsY for addition onto the sn-1 position of G3P (glyceraldehyde 3-phosphate), producing lysophosphatidic acid. The second option is for PlsX to create acyl-ACP that can then be added to the sn-2 position of lysophosphatidic acid, creating phosphatidic acid.

Interestingly, species belonging to the order Lactobacillale can repress de novo fatty acid synthesis while using exogenous fatty acids as a source for acyl chains [14]. Work from multiple groups has shown that the presence of exogenous fatty acids in growth medium lead to lower amounts of *fab* transcripts and Acp proteins that are necessary for *de novo* fatty acid synthesis in *E. faecalis* (Saito, unpublished data) [15].

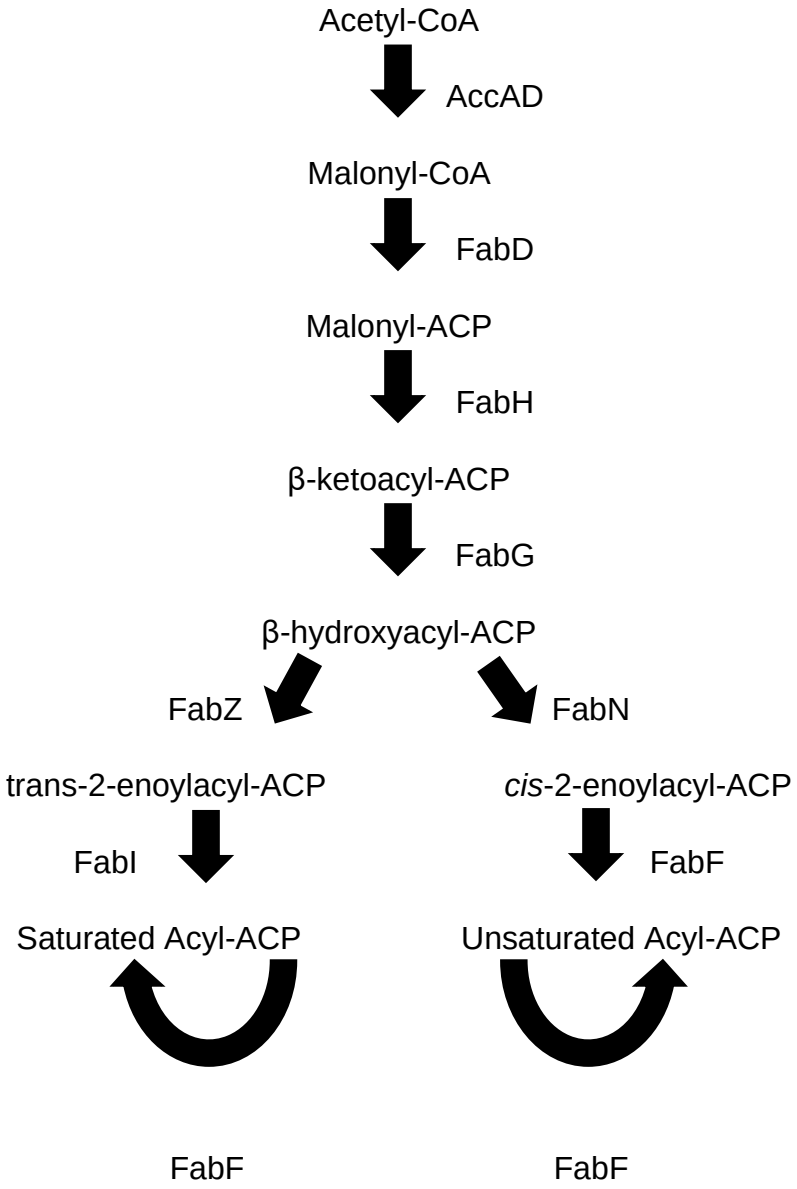

Figure 1.1: Type 2 fatty acid biosynthesis in Enterococcus faecalis.

A deletion experiment showed that FASII repression is achieved by FabT in *Streptococcus pneumoniae*, which binds to DNA and represses transcription of the *fab* locus when bound by a long chain acyl chain [16]. A similar phenotype was observed in *E. faecalis* after deletion of either *fabT* or *apcB* in *E. faecalis* [15]. Interestingly, repression by FabT in *E. faecalis* did not seem as strong as in *S. pneumoniae*, possibly necessitating the repeated function of AcpB [15].

Phospholipid Synthesis

Synthesis of phospholipids begins with the formation of the glycerophosphate (G3P) head group by the reduction of the glycolysis intermediate dihydroxyacetone phosphate (DHAP) by sn-glycero-3-phosphate synthesis (GpsA). Gram-negative bacteria regulate GpsA activity by the product, G3P, binding to GpsA. This ensures a steady supply of G3P for lipid synthesis [13]. Unlike *E. coli*, *Bacillus subtilis* lacks tight regulation on GpsA likely due to amount of G3P required to synthesize the cell wall component lipoteichoic acid, which contains anywhere from 14 to 33 G3P units [17]. This lack of regulation is likely present across Gram-positive bacteria, including *E. faecalis*, for the same reason as in *B. subtilis*, but this is not known. *E. coli* is able to transport G3P into the cell from the environment via GlpT using a phosphate antiport active transport system. *E. faecalis* strain V583 has low protein sequence similarity to a major facilitator transporter protein NP_814175. This low relation may imply that *E. faecalis* does not import G3P, relying on glycerol metabolism to synthesize G3P from dihydroxyacetone phosphate (Brewer, unpublished observation); however, this remains to be validated experimentally.

To add the acyl moieties formed by FASII, the cell requires a family of acyltransferases. Gram-positive organisms use the proteins PlsX, PlsY, and PlsC to populate both acyl positions, sn-1 and sn-2, of phosphatidic acid. PlsX converts acyl-ACP from FASII to acyl-phosphate (acyl-PO_4), which donates its acyl moiety to sn-1 of G3P via PlsY to generate lysophosphatidic acid. PlsC then populates sn-2 using an acyl moiety from an acyl-ACP to convert lysophosphatidic acid to phosphatidic acid, a precursor to most common bacterial phospholipids. For *E. faecalis*, those include phosphatidylglycerol (PG), amino modified PG, and cardiolipin (CL).

Phosphatidylglycerol

To generate PG from phosphatidic acid, cytidine diphosphate diaglycerol (CDP-DAG) is synthesized by the addition of cytidine phosphate by phosphatidate cytidylyltransferase (CdsA) and subsequent elimination of pyrophosphate (Fig 1.2) [18]. PgsA adds glycerol phosphate to CDP-DAG after to removal of cytidine monophosphate. PG is formed by the removal of a phosphate by phosphatidylglycerol phosphate phosphatase (PgpP). PG is the primary phospholipid present in the membrane of *E. faecalis* and represents the middle of the road as a two-acyl tailed anionic lipid (Harp, unpublished data). While plain, PG can be modified to contain a charged amino group or combined into an even more anionic four-acyl tailed lipid.

Lysyl-Phosphatidylglycerol

Amino-modified PG phospholipids are often found in the membrane of Gram-positive bacteria, modulating the charge of the cell. The most common amino-PG in *E. faecalis* is lysyl-PG (LPG), imbuing a positive charge to the cell. In *E. faecalis*, peptide resistance factor 2 (MprF2) catalyzes the lysinylation of PG to form LPG. To date, conditions have not been described where MprF1 is functional in *E, faecalis* [19]. After LPG formation, MprF then moves LPG from the inner leaflet to the outer leaflet by a flipping mechanism [20]. Increasing cell charge via an MprF gain of function mutation in *S. aureus,* only containing one *mprf* gene, has been implicated in defense from cationic antimicrobial peptides (CAMPs) via repulsion [21]. Other work has proposed that a large increase of LPG will protect *S. aureus* from daptomycin by repulsion [22].

Cardiolipin

CL is a less common phospholipid found in *E. faecalis* that is simply a combination of two PG phospholipids, mediated by cardiolipin synthase (Cls). A trio of glycerol molecules are attached to two diacylphosphatidyl moieties, imparting a unique structure, aiding in the support of curved cell membrane regions such as septa and poles. A vital division protein, DivIVA, localizes in microdomains rich in CL, indicating the role of proper phospholipid localization to proper cell division [23]. Two *cls* synthase genes have been identified in *E. faecalis* [24], but

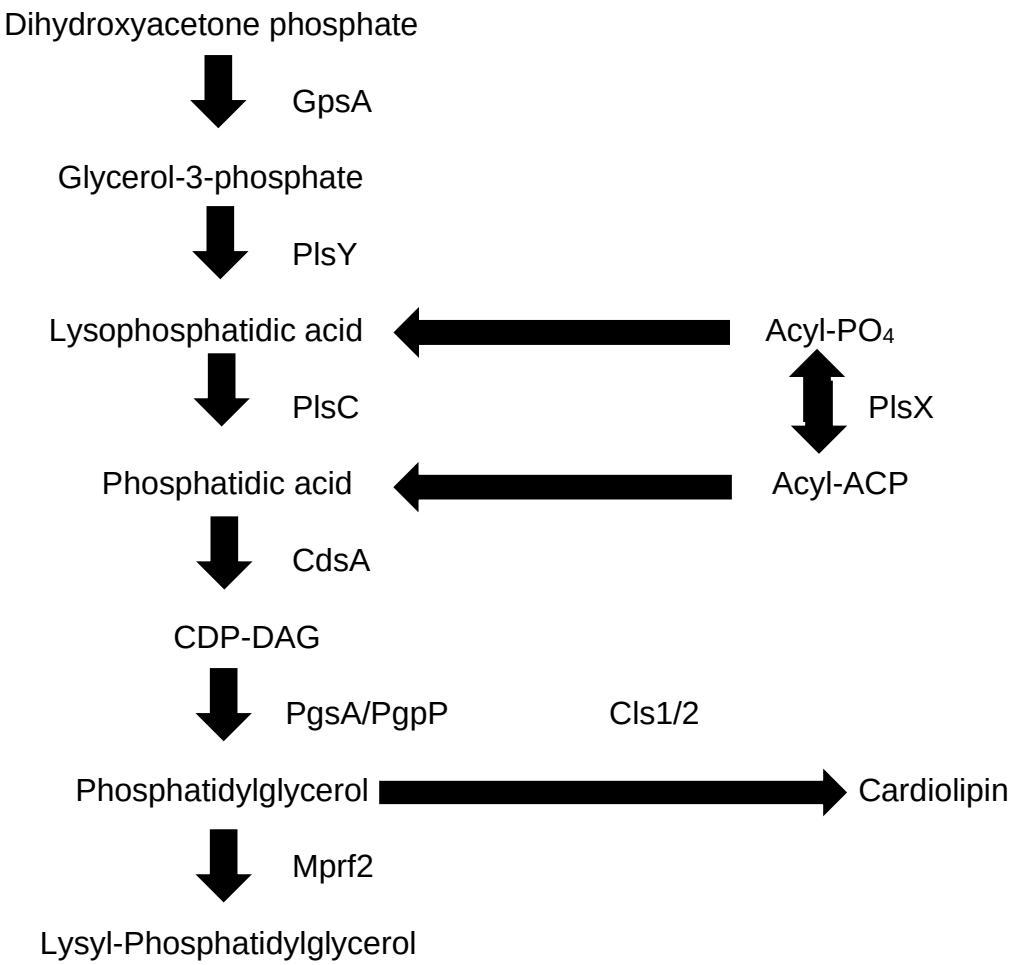

Figure 1.2: Phospholipid biosynthesis in *Enterococcus faecalis*.

deletion of both leads to mild diminishment of CL in the membrane, implying a yet-to-be determined additional synthesis mechanism (Harp et al, in preparation).

Membrane Adaptation

The cell envelope, including both the cellular membrane and cell wall, is responsible for protection of bacteria from environmental damage including temperature, pH, osmolarity, and so forth. At the same time, the envelope must be flexible in terms of composition and structure to adapt to the current environment. Specifically, for the cellular membrane component, a common and simple method of adaptation is to change the ratio of saturated fatty acids (fatty acids with no carbon to carbon double bonds) and unsaturated fatty acids (fatty acids with at least one carbon to carbon double bond). The higher the percentage of saturated fatty acids within a membrane, the more rigid, and less likely to destabilize at high temperatures [25]. A membrane dominated by unsaturated fatty acids remains in the liquid-crystalline state at lower temperatures, allowing vital cell functions to persist [25].

The Gram-positive bacterium *Bacillus subtilis* uses a lipid desaturase (Des) to increase membrane fluidity when cold shock is sensed [26]. DesK is a membrane bound protein that senses membrane fluidity and phosphorylates DesR when the membrane is too tightly packed [27]. Once DesR is phosphorylated, it transcriptionally activates *des,* which then creates a cis bond at the 5^{th} carbon on the acyl tail of saturated fatty acids [27]. Desaturation of the cell membrane in low temperatures maintains active transport functioning, and therefore proton dependent ATP generation continues [26]. In organisms lacking a desaturase mechanism, such as *E. coli*, increasing SFA:UFA occurs during biosynthesis, with temperature sensing systems determining the predominate fatty acid to synthesize [10].

Daptomycin

As noted above, *Enterococci* species carry resistance mechanisms to a plethora of drug classes [4]. An increasing prevalence in Vancomycin Resistant Enterococci (VRE), particularly for *E. faecium* infections, has caused a corresponding increase in burden for patients and healthcare systems in European countries [28]. *E. faecium* is less prevalent in the Americas, replaced by *E. faecalis* which is intrinsically resistant to a

common drug linezolid thanks to a multitude of drug efflux pumps. Linezolid binds to the 50s ribosomal subunit, halting translation of proteins, showing bacteriostatic activity in *Enterococci* infections [29]. Increased resistance to vancomycin and linezolid in the United States has led to reliance upon daptomycin to treat *E. faecalis* infections.

Daptomycin is a cyclic lipopeptide effective at treating Gram-positive infections. The drug was isolated from the soil bacterium *Streptomyces roseosporus* in the 1980s and approved for use by the FDA in 2003 [30, 31]. While the exact mechanism is not known, daptomycin is thought to interact with negatively charged regions of the cell membrane in a calcium dependent manner and disrupt synthesis of membrane and wall components [32, 33]. It is one of few Gram-positive targeting antibiotics that is safe for systemic use. Other common drugs such as vancomycin and linezolid have serious side effects such as permanent vision or hearing loss.

Genetic resistance to daptomycin is not well understood but has been noted in *B. subtilis, S. aureus, E. faecium, and E. faecalis*. There are several mechanisms proposed for resistance, especially as different genetic markers are associated with the differing bacterial species. Some theories support a mechanism where a more positive cellular charge will lead to repulsion of the positively charged daptomycin-calcium complex [20]. Other proposed mechanisms involved the diversion of the target phosphatidylglycerol away from division sites, allowing cell reproduction to continue [20].

Sequencing of a clinical isolate pair before and after daptomycin treatment revealed multiple genetic alterations arose throughout therapy including mutations in cardiolipin synthase 1(*cls*), glycerophosphoryl diester phosphodiesterase (*gdpD*) and lipid II interacting antibiotic component (*liaF*) [34]. As mentioned above, Cls synthesizes the anionic cardiolipin from two PG molecules and GdpD is a membrane bound protein thought to assist in glycerol metabolism and phospholipid biosynthesis [34]. Mutations in *cls1* and *gdpD* both contribute to an altered membrane phospholipid composition. LiaF is responsible for repressing the membrane bound sensor histidine kinase LiaS, which phosphorylates the response regulator Lia*R*. LiaR then activates transcription of *liaIHGFSR* [35]. Studies with *B. subtilis* demonstrated that under normal conditions, LiaI

localizes to the membrane and LiaH is present in the cytosol, but during stress LiaH moves to the membrane with LiaI to perform an unknown function related to cell membrane homeostasis [36]. Complementation work In *E. faecalis* revealed that mutation of *liaF* increased the MIC of daptomycin, but mutation of *gdpD* had no effect on MIC, requiring both mutations for a synergistic effect [34]. Work with *cls* uncovered that the mutation in the resistant clinical isolate likely causes an increase in biochemical activity of the protein, and they speculated a higher percent of cardiolipin in the membrane, but no experimental evidence has been provided. All of these mutations must occur for maximum protection from daptomycin, foreshadowing the complicated effects of daptomycin killing.

Physiological tolerance to daptomycin *in E. faecalis*

Work by Saito first noted that the growth environment for *E. faecalis* could induce a physiological tolerance to daptomycin. Growth in serum or bile induced a daptomycin tolerant phenotype, but the causative agent was unknown [1]. Further investigation found the protective components in human fluids are in fact exogenous fatty acids [1]. After supplementation with 15% human serum, they noticed a rise of palmitic acid (C16:0), stearic acid ($C_{18:0}$), oleic acid ($_{C18:1\ cis9}$) and linoleic acid ($C_{18:2\ cis\ 9,12}$) [1]. Further, supplementing a single fatty acid led to the majority of the membrane compromising of the supplemented fatty acid [1, 6, 37]. While all mentioned fatty acids were readily incorporated, not all induced protection from daptomycin, introducing the question of what makes a fatty acid protective [1, 6]. Palmitic acid and stearic acid, though found in serum and increased in the membrane upon serum supplementation, did not protect from daptomycin and also introduced growth defects if provided independently to cultures [1, 6]. Conversely, the fatty acids oleic acid and linoleic acid provided protection from daptomycin but had no effect on growth [1, 6].

Given that genetic resistance to daptomycin is associated with signaling via *liaFSR*, perhaps fatty acids were signaling via this system, leading to the increased tolerance. Using a mutant strain lacking the stress response regulator *liaR*, the same membrane stress assays were performed, challenging cells with daptomycin after supplementation with fatty acids found in human serum [37]. When challenged with

daptomycin, Δ*liaR* strain showed increased survival when supplemented with a protective fatty acid [37]. This provides evidence for a tolerant mechanism separate from the stress response system LiaIHGFSR.

Further work by Harp investigated the roles of phospholipid synthesis proteins Cls and MprF2, responsible for production of cardiolipin and Lysyl-PG respectively. Interestingly, protective fatty acids are still able to increase daptomycin tolerance in mutants lacking MprF2 (Harp, Unpublished). Lysyl-PG was undetectable in the membrane of Δ*mprF2* cells, confirming that a simple charge repulsion is not responsible for the daptomycin tolerant phenotype (Harp, Unpublished data). A recently hypothesized model claims that cardiolipin prevents daptomycin from flipping into the inner leaflet and acts as a poor target for daptomycin [32]. Supporting data for this hypothesis includes daptomycin challenge of a mutant lacking cls2 and cls1 leading to lower basal survival than wild type cells (Harp, Unpublished data). After protective fatty acid supplementation, like with all other tested mutants, there was increased survival from daptomycin challenge (Harp, Unpublished). Once again it was shown that the daptomycin tolerant phenotype is not related to any known genetic resistance mechanisms in *E. faecalis* or closely related bacteria.

Given the lack of congruency between genetic mutations in clinical resistance and the induction of physiological tolerance by eukaryotic fatty acids, perhaps altered biophysical properties are responsible for this tolerance. It has already been shown up to this point that saturated fatty acids found in the membrane do not provide protection from daptomycin, and if supplemented alone will cause growth defects [6]. Growth defects range from longer generation time and growth stasis to deformed cell morphology at the individual cell level [1, 6]. Unsaturated fatty acids oleic acid and linoleic acid have little to no effect on growth and provide multiple logs of improved survival after challenge with daptomycin [1, 6]. Single fatty acid supplementation dominates the membrane composition as determined by gas chromatography of fatty acid methyl esters (GC-FAME) [1]. This data paired with observations of lowered fab transcription indicate that the supplied fatty acids are being incorporated into the cell membrane either as free fatty acids or phospholipids (Saito, Unpublished).

Research addressed in this work

Given the abundance of data showing the physiological effects that single fatty acids have on *E. faecalis,* we find it curious that a combination such as human serum, which contains fatty acids that are protective from daptomycin as well as deleterious to cell health, have no negative consequences. Chapter II is dedicated to characterizing the physiological effects of various fatty acid combinations, containing both protective and non-protective fatty acids. After all the mentioned past work, it was clear that the observed tolerance was induced by fatty acids, but it is not known if oleic acid and linoleic acid cause changes in membrane physiology or induce a transcriptional change. In chapter III I attempt to use transcriptomics in an effort to better understand the reaction of the cell leading to a daptomycin tolerant phenotype.

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Chapter 2: Protective Fatty Acids in Mixture Dominate the Tolerant Phenotype of *Enterococcus faecalis*

Future Publication Note

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

Protective Fatty Acids in Mixture Dominate the Tolerant Phenotype of *Enterococcus faecalis* William Brewer, Johnathan Harrison, Holly Saito, and Elizabeth Fozo

Microscopy, anisotropy, and daptomycin challenge assays were done by William Brewer. Growth curves and cell charge assays were done by William Brewer and Johnathan Harrison.

Abstract

Enterococcus faecalis is a Gram-positive bacterium that normally exists as an intestinal commensal in humans. Additionally, it can survive in the environment for extended periods and cause infections in immunocompromised hosts, making *E. faecalis* a leading cause of nosocomial infections. Previous work noted that specific fatty acids found in serum contribute to tolerance of membrane damaging agents, including the drug daptomycin. However, not all fatty acids found in serum were able to induce such protection. Herein, we measured a wide array of physiological responses after supplementation with combinations of protective (i.e., induced daptomycin tolerance) and non-protective fatty acids. When supplementing cells with either non-protective fatty acid, palmitic acid or stearic acid, there was a significant increase in generation time and severely distorted morphology. Both physiological defects were rescued when these cultures were supplemented with one of the protective fatty acids identified in serum, oleic acid or linoleic acid. Similarly, membrane fluidity decreased with growth in either palmitic or stearic acid alone but returned to basal levels when supplemented in combination with a protective fatty acid. While cell envelope charge has been associated with tolerance to daptomycin in other Gram-positive bacteria, we concluded that it does not correlate with the fatty acid induced protection we observed. Further, daptomycin tolerance could be induced when cultures were supplemented with non-protective fatty acids in combination with a protective fatty acid. Combined, we conclude that a single protective fatty acid is able to alleviate negative growth defects and protect cells from daptomycin.

Importance

With an increasing prevalence of antibiotic resistance in the clinic, we strive to understand more about microbial defensive mechanisms. An interesting tolerance physiology was discovered in *Enterococcus faecalis* that is not genetic in nature, but regardless results in the increased survival of bacterial populations after treatment with the antibiotic daptomycin. This unknown tolerance mechanism likely synergizes with antibiotic resistance in the clinic. Given the tolerance phenotype is induced by

incorporation of fatty acids present in the host, it can be assumed that all infections by this organism require a higher dose of antibiotic. The mixture of fatty acids in human fluids is quite diverse, with little understanding how combinations of fatty acids interplay with the tolerance phenotype we observe. It is crucial to understand the effects of fatty acid combinations on *E. faecalis* physiology if we are to suppress the tolerance physiology in the clinic.

Introduction

Enterococcus faecalis is a Gram-positive bacterium known primarily as a commensal in the mammalian intestine. In immunocompromised individuals, however, it can cause a variety of complications if it colonizes outside the intestine: these include surgical wound and urinary tract infections, endocarditis, and bacteremia. *E. faecalis* is also resilient to a variety of stressors allowing it to survive outside the body for extended periods of time which likely increases transfer to patients in the hospital setting [1]. Along with being resilient to environmental stressors such as heat, acid, and osmotic shock [2], *E. faecalis* is resistant to different classes of antibiotics, complicating treatment strategies [3].

To combat drug resistant enterococcal infections, clinicians have utilized the antibiotic daptomycin. This drug is thought to insert into phosphatidylglycerol portions of the cell membrane in a calcium dependent manner leading to ion leakage and cell death [4]. Despite its success, clinical resistance to daptomycin occurs for enterococcal infections [5]. Further, studies have also shown that the growth environment of *E. faecalis* can induce a physiological tolerance to this antibiotic. Specifically, growth in the presence of bile or serum, mimicking its lifestyle as a commensal and pathogen respectively, led to protection from daptomycin, and this tolerance was induced by specific fatty acids found within bile or serum [6]. This priming also protected *E. faecalis* from high concentrations of human bile and sodium dodecyl sulfate (SDS), demonstrating that protection was not specific to daptomycin. Further, this protection was not due to the selection of genetic mutants but rather due to altered cellular physiology [6, 7]

Fatty acid analysis of *E. faecalis* cells after serum supplementation showed an increase in oleic acid ($C_{18:1\ cis9}$), linoleic acid ($C_{18:2\ cis\text{-}9,12}$), palmitic acid ($C_{16:0}$), and

stearic acid ($C_{18:0}$) [6, 8]. The increase in these four species led to a decreased proportion of other native fatty acids, most notably *cis*-vaccenic acid ($C_{18:1\ cis11}$). Further analysis showed that supplementation only with oleic acid or linoleic acid provided protection from membrane stress agents [6, 9]. Despite stearic acid and palmitic acid being produced and found natively in the membrane of *E. faecalis*, supplying either one exogenously did not induce tolerance [6, 9].

Not only do stearic acid and palmitic acid fail to induce daptomycin tolerance, they also caused considerable defects to cellular physiology when supplemented exogenously to cultures [6, 9]. Specifically, generation time was increased significantly from control cultures, and the morphology of these cells was greatly perturbed. Importantly though, protection and physiology were found not to be exclusive of one another, as the addition of linoleic acid to cultures induced daptomycin tolerance and also increased generation time [6, 9]. Based on the observed differences in daptomycin induced protection of each fatty acid, we chose to define any exogenously supplied fatty acid that induces daptomycin tolerance as a protective fatty acid, and all other fatty acids as non-protective fatty acids.

It is interesting to note that human serum is effective at conferring daptomycin tolerance to *E. faecalis* while containing the non-protective fatty acids stearic and palmitic acid that can cause severe physiological defects [6, 8]. We hypothesized that the eukaryotic fatty acids oleic acid and linoleic acid drive a membrane protective response even in the presence of non-protective fatty acids. Within this work we show the supplementation of protective and non-protective fatty acids in combination induce tolerance from daptomycin in *E. faecalis* without observable negative physiological effects.

Materials and Methods

Bacterial Growth Conditions. *E. faecalis* OG1RF was grown statically at 37°C in brain heart infusion (BHI) for all experimental conditions. Overnight cultures were diluted to OD_{600nm} 0.01 before experimentation. For long-term supplementation, fatty acids were added at the time of dilution. For short-term supplementation, fatty acids were added to cultures during exponential phase, OD_{600nm} 0.25 [9]. Unless otherwise noted, cells were harvested at OD_{600nm} 0.3 for long-term supplementation and 30 minutes after fatty acid

addition for short-term supplementation. Growth was monitored by OD_{600} and all fatty acids (Sigma) were supplemented to a final concentration of 5 µg ml^{-1}: oleic acid ($C_{18:1\ cis9}$)(17.7µM), linoleic acid ($C_{18:2\ cis\text{-}9,12}$) (17.82 µM), palmitic acid ($C_{16:0}$) (19.49 µM), and stearic acid ($C_{18:0}$) (17.58 µM). Human serum (MP biomedicals) was supplemented at a final concentration of 15%.

Gas Chromatography of Fatty Acid Methyl Esters (GC-FAME). Cells were grown using both long-term and short-term supplementation methods described above. Cultures (15mls) were harvested at 2739g for 10 minutes, washed extensively in 1x phosphate buffered saline (PBS) twice, pelleted and stored at -80°C. GC-FAME was performed by Microbial ID (Newark, DE) using methods described previously [10].

Scanning electron microscopy. Bacterial cultures (25mls) were grown using the long-term supplementation methods described above. The cells were harvested at 2739g for 10 minutes, washed in 1x PBS and resuspended in 500 µl 3% glutaraldehyde for one hour in order to fix the cells. Cells were washed three times in 10 mls sterile water, decanted and resuspended in the remaining water. An aliquot (20µl) of each sample was fixed to a 5 x 5 mm silicon chip with poly-lysine then dehydrated using a series of ethanol washes (25, 50, 70, 95, and 100%) for ten minutes each. After ethanol washes, the cells were placed in a LADD critical point dryer for three cycles of 10 minutes. The dried samples were then coated with iridium using a sputter coater and visualized using a Zeiss Auriga 40 at the Center for Advanced Microscopy and Imaging at the University of Tennessee at a kEV of 5.0. Biological duplicates were performed for each growth condition and a minimum of 10 fields were imaged for each sample.

Determination of cellular charge. The overall charge of the bacterial cells was determined using a previously described method [11] with modifications. Bacterial cultures were grown using the short-term supplementation methods described above. Cells were washed twice with PBS and resuspended to a final OD_{600nm} of 3.0 in 3.0 mL 20mM 3-(N-morpholino)propanesulfonic acid (MOPS) with 1 mg ml^{-1} cytochrome C and incubated at room temperature for 10 minutes. The samples were centrifuged at 2739g for 10 minutes, the supernatant was extracted and measured at OD_{530nm}. The concentration of cytochrome C measured was based on a standard curve of known

cytochrome C concentrations, and the percent unbound cytochrome C was calculated for *n*=3 biological replicates. A higher percentage of unbound cytochrome C represents a more positively charged population of cells.

Protoplast generation. Protoplast generation was based on [12] with modifications. Cultures of OG1RF (10ml) were grown as described above for short-term supplementation conditions. Cultures were harvested via centrifugation (2739g), washed with 1x PBS and resuspended in half (5mls) the original volume with isotonic buffer (20% sucrose, 0.145M NaCl, 50mM Tris-HCl). Samples were incubated with 1 mg ml^{-1} lysozyme for 60 minutes at 37°C. Removal of cell wall was verified by Gram staining.

Anisotropy. Protoplasts were generated using the method above. Protoplasts in isotonic solution were incubated with 2nM 1,6 diphenyl 1,3,5 hexatriene (DPH) at 37°C for 30 minutes. An Agilent Technologies Cary Eclipse fluorescent spectrophotometer was used to measure the r value with an excitation wavelength of 350 nm emission wavelength of 428 nm. The observed biological range of values for anisotropy using DPH is 01. to 0.3 with a lower number meaning the membrane is more fluid [13].

Cerulenin Rescue assay. Cells were grown as described above and diluted back into medium containing cerulenin at 5 μg ml^{-1} and either ethanol at 0.1% final concentration or the indicated fatty acid(s) at 5 μg ml^{-1}. As a control, cells were also diluted back into medium lacking the inhibitor or fatty acids and indicated as no treatment.

Daptomycin challenge assay. Cultures were grown and harvested using short-term supplementation described above, with the exception of those grown in the presence of serum, which was via the long-term supplementation method. Immediately after harvest, cells were spun at 2,739 g, decanted, and washed with 1x phosphate buffer solution (PBS) twice. Cells were resuspended in an equivalent volume BHI with 10 mM calcium chloride. Cultures were then treated with 30 μg ml^{-1} of daptomycin. After addition of daptomycin, cells were incubated at 37°C; aliquots were removed at 15, 30, and 60 minutes and serially diluted using 0.9% sodium chloride (NaCl). The dilution series was plated on BHI agar plates, grown for 16-20 hours at 37°C, and colony

forming units were enumerated. The log number of survivors was plotted against time for n=3 biological replicates.

Results

Protective fatty acids rescue E. faecalis growth from non-protective fatty acids. Previous work has shown that the addition of single fatty acids to *E. faecalis* OG1RF cultures can impact cellular growth, morphology and induce daptomycin tolerance [6, 9]. However, these effects were dependent solely on the fatty acid supplied. If supplied independently, both palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) caused an increase in generation time, led to altered cell morphology, and neither could induce tolerance to daptomycin. Yet, these two fatty acids are found at increased levels in cellular membranes when supplemented with either bile or serum, and growth in bile and serum can induce daptomycin tolerance. On the contrary, oleic acid ($C_{18:1\ cis\ 9}$) and linoleic acid ($C_{18:2\ cis9,12}$) are also found in bile, serum, and can induce daptomycin tolerance independently. We wanted to examine if the negative consequences of exogenously supplied palmitic or stearic acid on the physiology of OG1RF would be alleviated if oleic acid or linoleic acid were also provided.

Palmitic acid supplementation alone caused an early stasis period (Fig. 2.1) and only reached stationary phase after selection of a suppressor mutant population [4, 7]. However, supplementation with either oleic acid or linoleic acid prevented this early stasis and growth resembled that of solvent control cultures (Fig. 2.1). Given how well the additional oleic acid or linoleic could ameliorate the negative consequences of palmitic acid on growth, we investigated whether oleic acid could restore growth to cultures that entered a palmitic acid-induced stasis. We supplied palmitic acid at dilution, and once stasis was established, oleic acid was added. By supplementing oleic acid as late as 90 minutes post-stasis, the culture was able to resume growth, (Fig. 2.1). Re-dilution of these rescued cells indicated that oleic acid did not select for a genetic mutant isolate, as these cells were unable to grow in the presence of palmitic acid when challenged again.

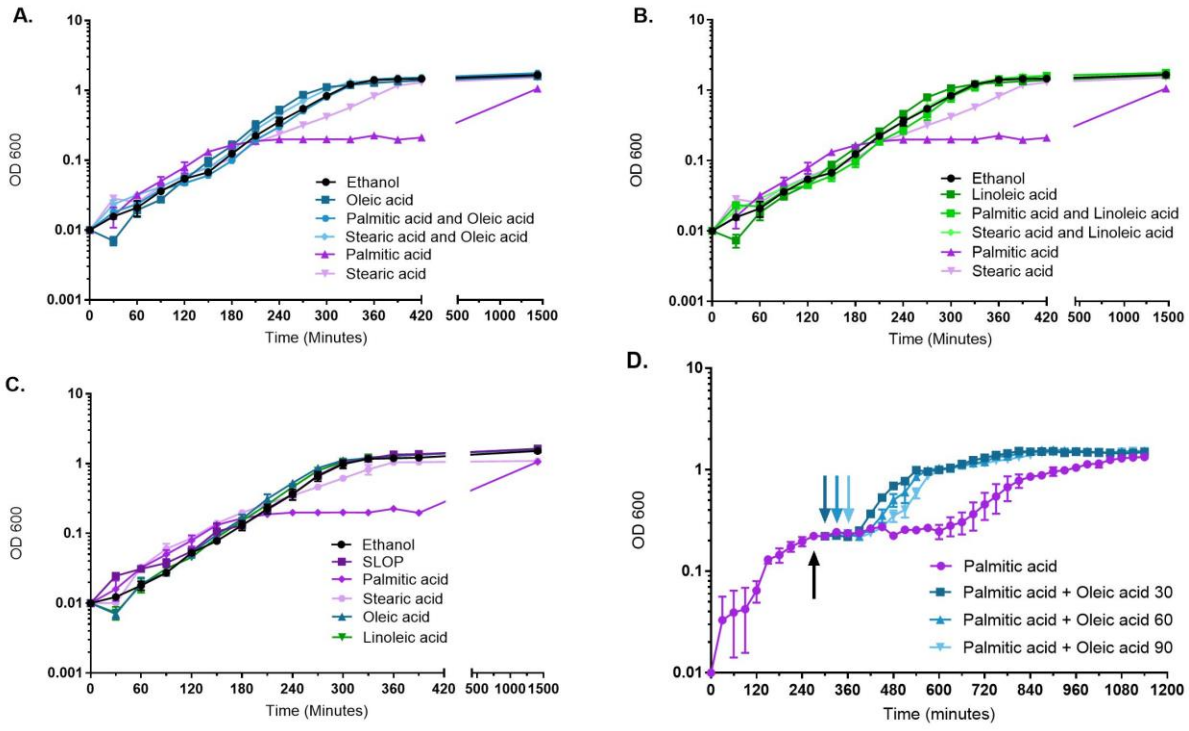

Figure 2.1: Growth of OG1RF in fatty acid mixtures. Addition of a protective fatty acid relieves growth defects of either palmitic acid (C16:0) or stearic acid (C18:0) on OG1RF. All fatty acids were added as a final concentration of 5 µg ml^{-1}, ethanol (solvent control) was added at an equal volume. (A) Addition of Oleic acid ($C_{18:1\ cis\ 9}$); (B) addition of linoleic acid ($C_{18:2\ cis\ 9,\ 12}$). (C) Addition of SLOP, mixture stearic acid, palmitic acid, oleic acid, and linoleic acid). (D) Palmitic acid was added at the time of dilution: stasis indicated by the black arrow. Oleic acid addition (indicated in arrows) at 30, 60, and 90 min post stasis. N=3 for all experiments.

Supplementation with stearic acid, while not as severe in terms of growth effects as palmitic acid, increased generation time of OG1RF (Table 2.1). However, supplementation with linoleic acid mixture restored growth kinetics to that of control cultures (Fig. 2.1, Table 2.1). Supplementation with oleic acid mixtures lowered the generation time from the non-protective fatty acid alone but did not completely restore to control levels (Fig. 2.1, Table 2.1). Generation time of palmitic acid supplemented cells is not calculable due to early stasis, but combination supplementation with palmitic acid and a protective fatty acid prevents that stasis (Figure 2.1).

As noted, OG1RF cultures supplemented with serum have increased amounts of stearic, linoleic, oleic, and palmitic acids in their membranes [6, 8]. To mimic this, we simulated this membrane profile by adding a mixture containing 5 μg ml^{-1} each of these fatty acids to the culture (referred to as SLOP: stearic acid, linoleic acid, oleic acid, and palmitic acid). This mixture, however, had no obvious impacts on growth dynamics as cultures supplemented with SLOP resembled that of solvent control (Fig. 2.1C, Table 2.1). These different growth conditions indicate that the presence of a protective fatty acid is sufficient to rescue cultures from the negative growth effects caused by non-protective fatty acids.

Supplementation with unsaturated fatty acids has been previously shown to rescue cells from fatty acid biosynthesis inhibition by cerulenin [6]. To see if fatty acids mixtures could rescue from cerulenin, we supplemented cultures with single fatty acids and combinations. Similar to their effects on growth, fatty acid mixtures predominantly induce physiological responses like a protective fatty acid alone. Both oleic acid and linoleic acid rescue growth of OG1RF from cerulenin as a single fatty acid supplement and in combination with a non-protective fatty acid (Figure 2.3). Oleic acid combinations mimic the rescue seen by oleic acid alone, greatly increasing growth from solvent control and saturated fatty acid supplementation (P value <0.001). Further, linoleic acid combinations are similar to linoleic acid alone, rescuing from cerulenin unlike solvent control and saturated fatty acid supplements (P value <0.001). This indicates that fatty acid synthesis inhibition is bypassed by incorporating unsaturated exogenous fatty acids.

Protective fatty acids prevent negative morphological effects from saturated fatty acids. It was previously noted that addition of palmitic acid and stearic acid caused severe distortion to cellular morphology, despite their presence in bile and serum [6, 9]. Given the ability of oleic acid and linoleic acid to suppress negative growth effects, we hypothesized that their addition would also help restore morphology of OG1RF cells grown in either palmitic or stearic acids to that of control cultures.

We found that the protective fatty acids induced a truncated morphology (Fig. S2.1), but otherwise the morphology resembled that of control cultures, as noted previously. Cells supplemented with palmitic acid were severely distorted in their shape, oftentimes with little or no distinction from one cell to the next. Unlike the characteristic diplococci shape, we observed what appeared to be curved cells branching off from non-polar ends (Fig 2.3). Cells supplemented with palmitic acid in conjunction with oleic acid or linoleic acid however, had a morphology like control cells, with apparent normal septum placement.

Stearic acid supplementation also led to apparent misplacement of septa and improper cellular division, similar to palmitic acid supplemented cells. The cells also appeared “wrinkled” which may be due to cell wall defects (see discussion). However, when given in combination with oleic or linoleic acids, cellular morphology is restored to that of control cells.

Given these results and the above-mentioned growth kinetics, it was not surprising to note that cells supplemented with the mixture SLOP also had a morphology that resembled control cultures. Combined, the addition of either oleic or linoleic acids can prevent negative cellular morphology associated with supplementation of the saturated, non-protective fatty acids palmitic and stearic acid.

Fatty acid supplementation causes a varied response to cell envelope charge. In *Staphylococcus aureus*, an increase in positively charged lysl-phosphatidylglyecerol (LPG) has been associated with a reduction in ion leakage caused by cationic antimicrobial peptides[14]. A study with *Bacillus subtilis* noted than increase in positively charged LPG or a decrease in anionic phosphatidylglycerol (PG) led to increased resistance to daptomycin [15]. Given the morphological alterations noted above, we

Table 2.1 Generation time of OG1RF in BHI and fatty acid supplements.

Medium Constituent	Generation Time (minutes)
Ethanol[a]	39.69 ± 0.54
$C_{18:1\ cis9}$[b]	34.91 ± 1.03
$C_{18:2\ cis\text{-}9,12}$[b]	37.07 ± 0.97
$C_{16:0}$[b]	N/A
$C_{18:0}$[b]	69.34 ± 3.41
$C_{18:1\ cis9}$ $C_{16:0}$[c]	43.35 ± 0.67
$C_{18:1\ cis9}$ $C_{18:0}$[c]	43.61 ± 0.85
$C_{18:2\ cis\text{-}9,12}$ $C_{16:0}$[c]	40.17 ± 0.51
$C_{18:2\ cis\text{-}9,12}$ $C_{18:0}$[c]	39.34 ± 0.84
SLOP[c]	37.87 ± 1.05
Human Serum[d]	41.32 ± 0.97

[a]Ethanol solvent control was added to a final concentration of 0.2%

[b]Single fatty acid supplemented to a final concentration of 5 µg ml^{-1}

[c]Combination of fatty acids supplemented to a final concentration of 5 µg ml^{-1} each

[d]Human serum supplemented to a final concentration of 15%

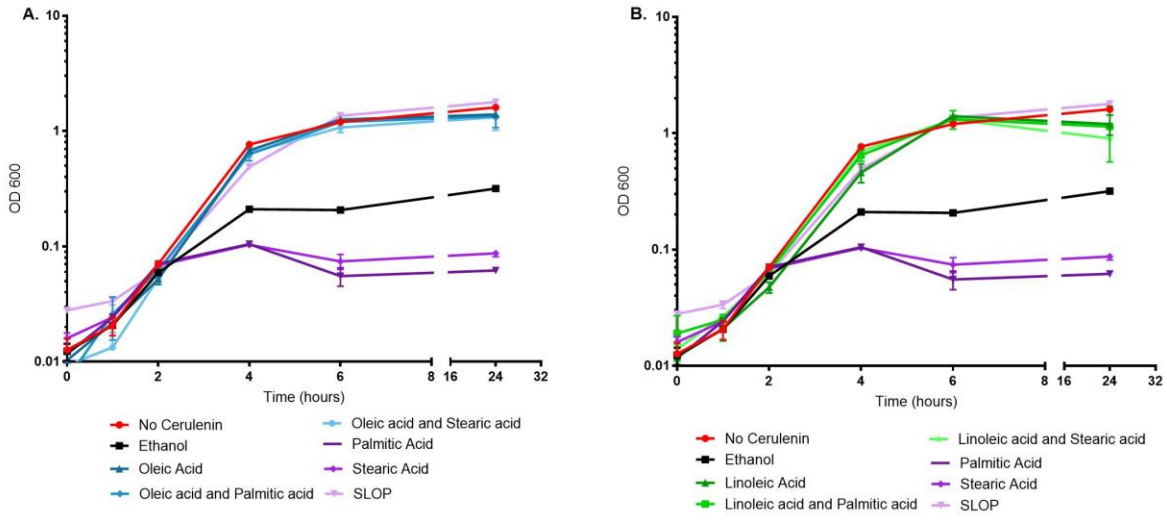

Figure 2.2: Cerulenin challenge of OG1RF. OG1RF supplemented with indicated fatty acid(s) and 5 µg ml^{-1} cerulenin. A) shows oleic acid combinations and B) shows linoleic acid combinations. n=3.

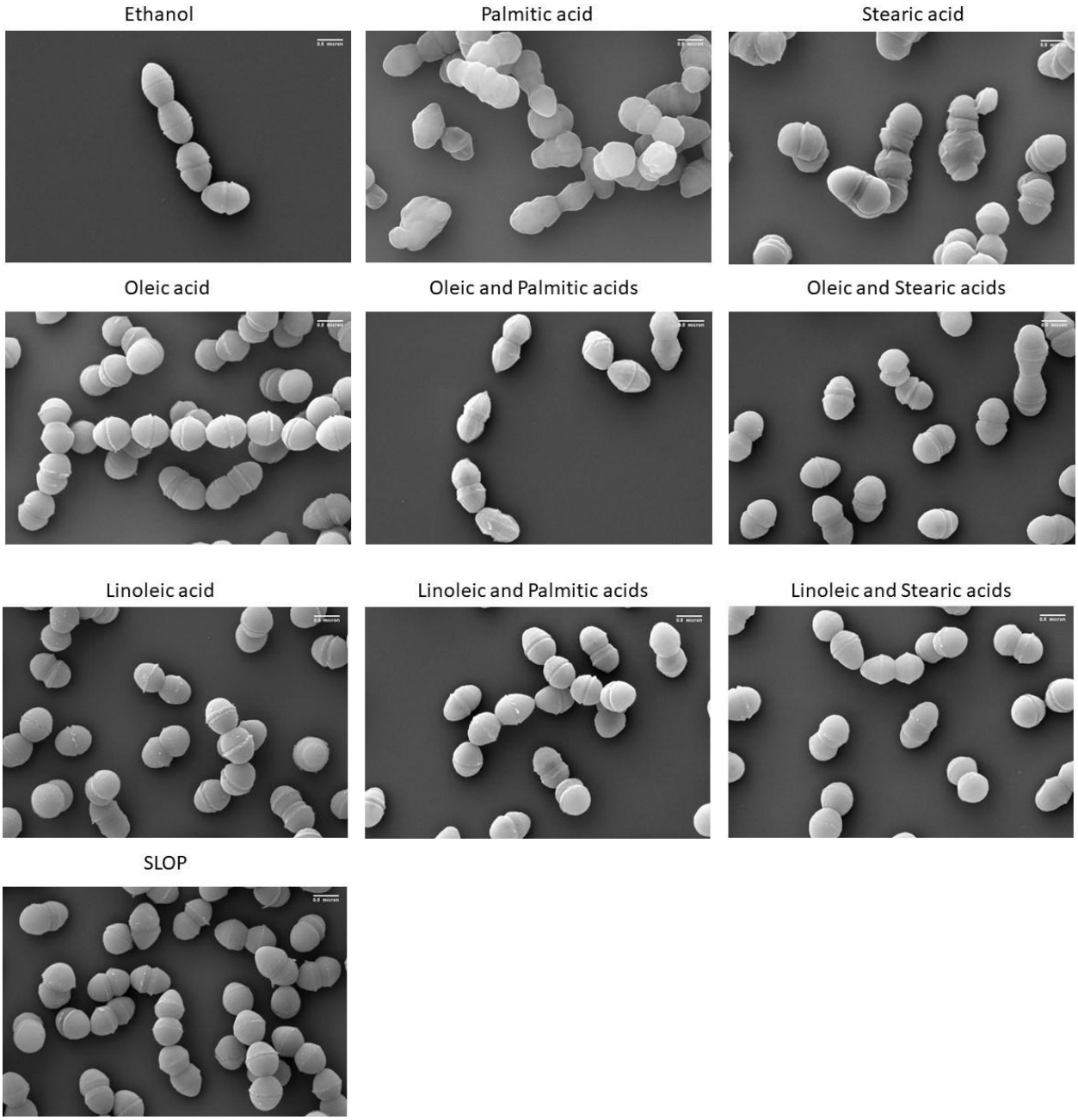

Figure 2.3: Scanning electron microscopy of OG1RF in mixed fatty acid supplements. Images of OG1RF in exponential phase after long-term supplementation with 5µg ml^{-1} fatty acids. Images taken at 45,000 magnification at KeV 5.0. Scale bar represents 0.5 µm. Sample image of n=2 biological replicates with 10 fields examined per replicate.

hypothesized that there may be overall changes to cellular envelope charges that were influencing cellular physiology and daptomycin protection/tolerance. Thus, we choose to measure overall cell envelope charge via interactions with the positively charged protein cytochrome C using previously described methods with modifications [11].

Surprisingly, not all conditions impacted cellular charge in comparison to control cultures (Fig 2.4). Addition of oleic acid, which is protective, caused no change, while linoleic acid caused a significant decrease in cell charge (P value < 0.005). Linoleic acid combinations are significantly different from linoleic acid alone (P value < 0.0001), contrary to previous trends. Both serum and SLOP supplementation caused a modest but significant increase in cell charge (P values < 0.005 and 0.0001 respectively). Palmitic acid, but not stearic acid alone, supplementation led to a significant increase in surface charge (P value < 0.0001). Thus, while cell charge did vary with fatty acid supplement, there was no obvious correlation between overall cell charge and protection from daptomycin.

Non-protective fatty acids alter membrane fluidity. The nature of the cellular membrane is to be flexible dependent upon the environment, altering membrane fluidity to maintain both a protective barrier and functioning membrane proteins. Fatty acid supplementation caused a significant change in proportion of fatty acid species incorporated in the membrane as reported previously [6, 9] These changes, particularly those that impact the ratio of saturated to unsaturated fatty acids, should also impact membrane fluidity, altering the ability of molecules to interact, which may impact the effectiveness of daptomycin [16]. We performed anisotropy using the dye DPH to determine membrane fluidity on protoplasts to ensure proper interaction of the dye with the cellular membrane.

Cells supplemented solely with the non-protective fatty acid palmitic or stearic acid gave a statistically significant higher r value, indicative of a more rigid membrane than control (Fig 2.5). Both stearic acid and palmitic acid supplemented cells were significantly different from all other conditions. This correlated well with the previously reported saturated: unsaturated fatty acid ratio of such cells [6]. However,

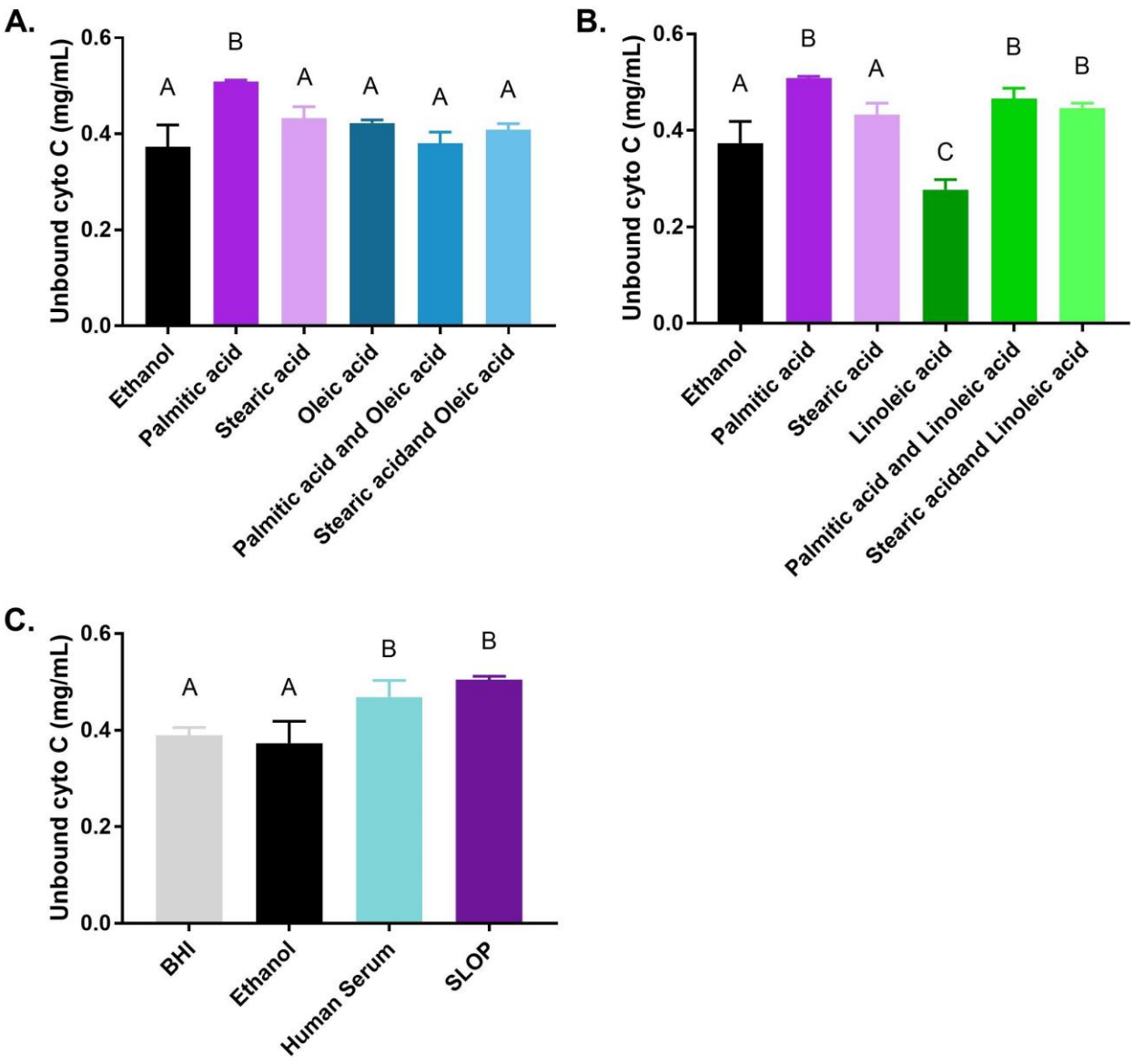

Figure 2.4: Cell charge of OG1RF after mixed fatty acid supplementation.
Alterations in cell envelope charge upon supplementation. Interaction of positively charged cytochrome C with OG1RF cells; y axis indicates a more positive cell charge. For all cases, cells supplemented with 5 ug ml^{-1} each fatty acid, 15% human serum, or equivalent volume of ethanol (solvent control) until exponential phase. A) Oleic acid ($C_{18:1cis9}$) combinations, B) linoleic acid ($C_{18:2cis9, 12}$) mixtures and C) human serum and SLOP. For all samples, n=3. Letter (A, B or C) above bar denotes groups of significance, with different letters representing a significant difference between groups, $P<0.05$.

supplementation with either oleic acid or linoleic acid did not alter fluidity from that of control cells. Combining the fatty acids, we noted that membrane fluidity returned to that of control cultures. Thus, while saturated fatty acids rigidify the overall membrane, growth with protective fatty acids appear to have no impacts (see discussion).

Protection from daptomycin by beneficial fatty acid supplements is not hindered by the presence of toxic fatty acids. Thus far our data is supportive of protective fatty acids driving cellular physiology when given with non-protective fatty acids. We hypothesized that protection from daptomycin by the fatty acid mixtures serum and/or bile is thus likely driven by oleic or linoleic acids despite the presence of non-protective fatty acids. Therefore, we examined the induction of daptomycin tolerance by our mixtures as previously described [9].

As noted previously, the addition of supplementation with either oleic acid or linoleic acid led to a 2-log increase in survival after daptomycin treatment compared to the solvent control (Fig 2.6). This was in contrast to supplementation with either palmitic acid or stearic acid, which had similar survival to the solvent control. However, addition of oleic acid to either palmitic acid or stearic acid resulted in increased survivors over solvent control, similar to protection by oleic acid alone. Supplementation with linoleic acid or a linoleic acid combination also led to a significant increase in survivors. However, protection induced by human serum was significantly greater than control, non-protective fatty acids, oleic acid, and oleic acid combination supplemented cells. The protection provided by SLOP was 4-fold over control and significantly better than all supplements except human serum. These findings reinforce the conclusion that host derived fatty acids are sufficient to provide protection from daptomycin. This data also indicates that a single protective fatty acid is both necessary and sufficient for increased survival when challenged with daptomycin in the presence of a non-protective fatty acid.

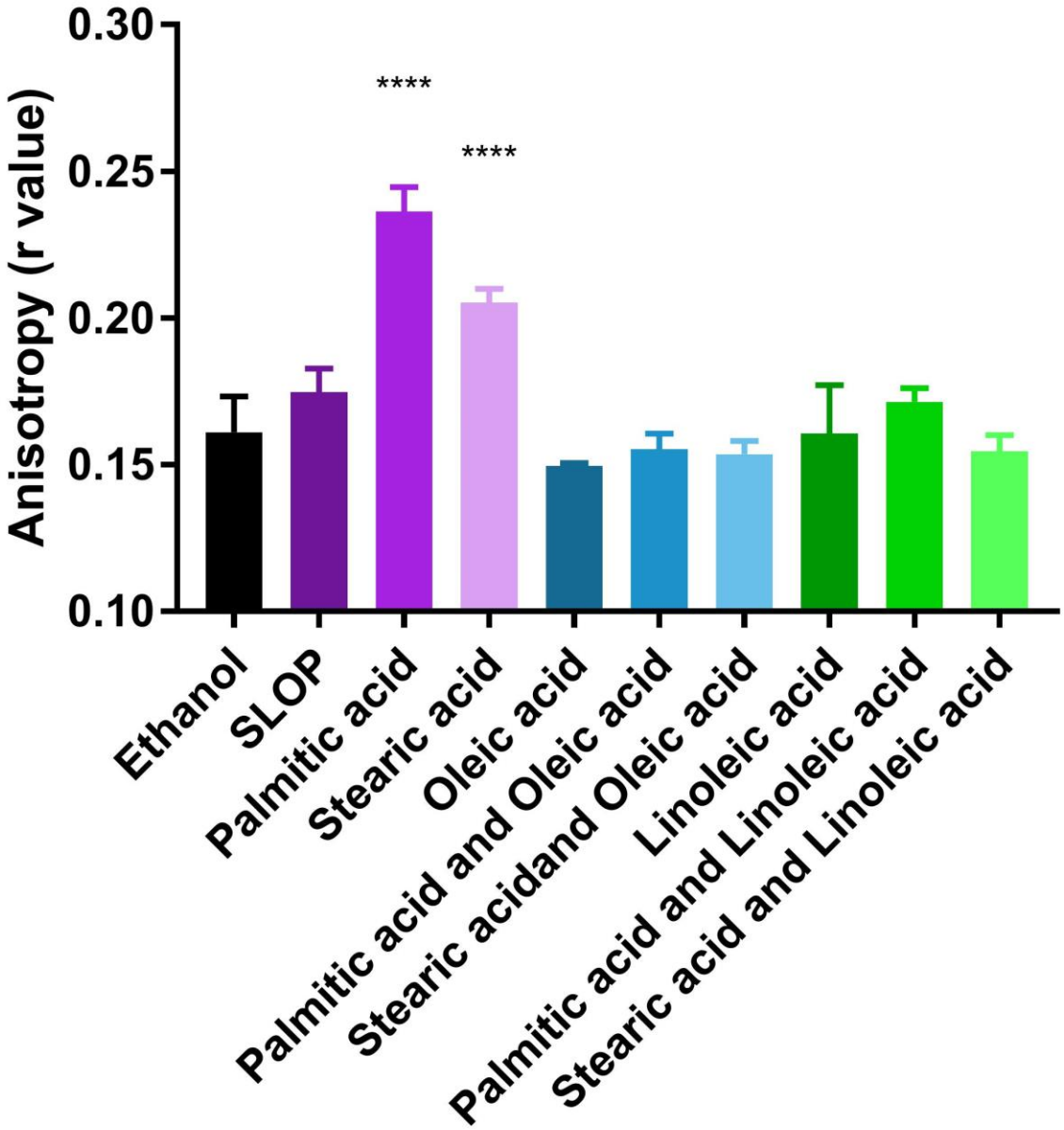

Figure 2.5: Membrane fluidity of OG1RF after short term fatty acid supplementation. Anisotropy was determined using DPH with excitation wavelength 350nm and emission wavelength 428nm. n=3. Asterisks indicate significance from ethanol (solvent control) as follows: **** P-value < 0.0001.

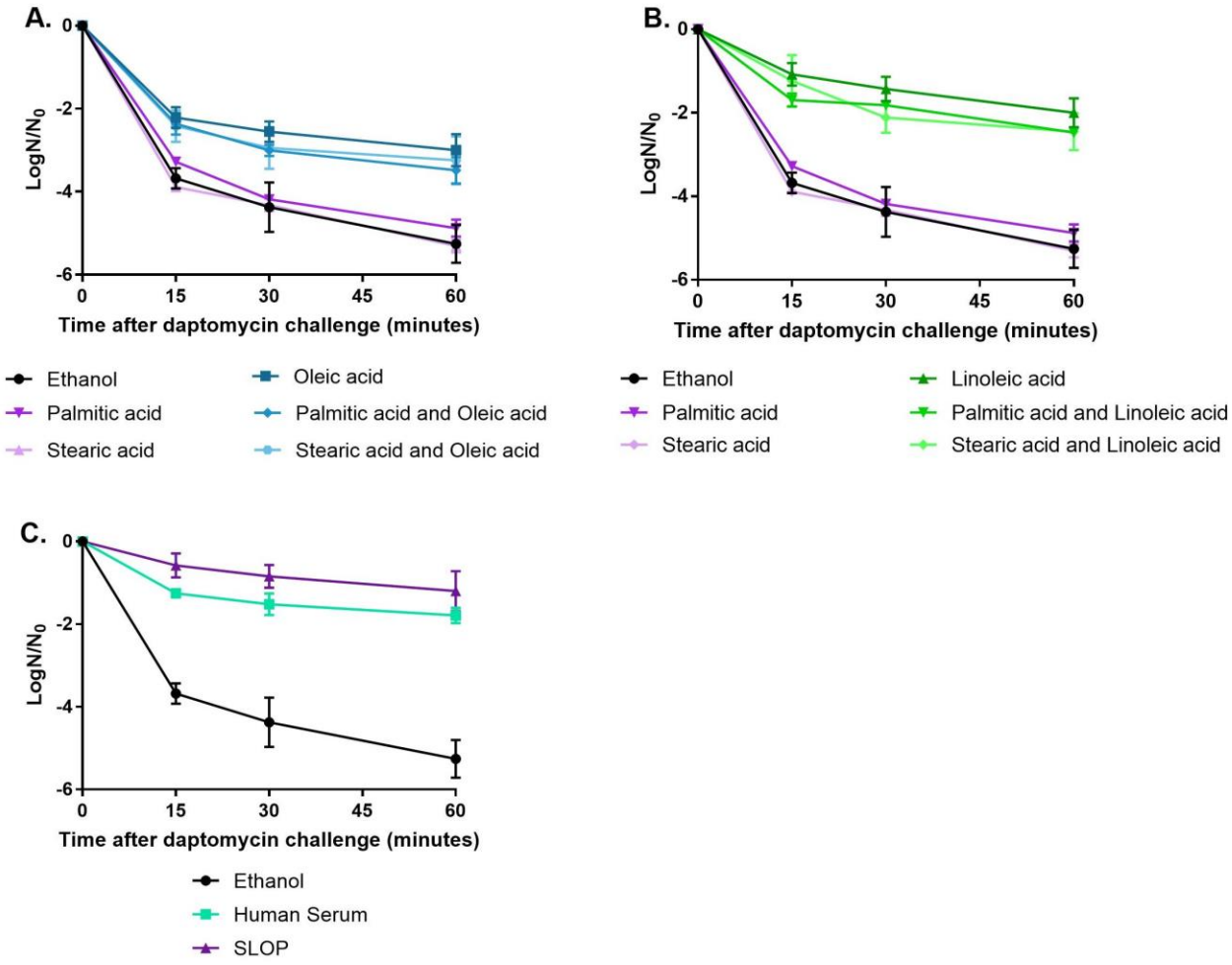

Figure 2.6: Daptomycin challenge of OG1RF after fatty acid mixture supplementation. Oleic or linoleic acid can induce daptomycin protection in the presence of non-protective fatty acids. Membrane challenge by 30 µg ml^{-1}daptomycin after short term supplementation with fatty acids. (A) Supplementation with oleic acid ($C_{18:1\ cis9}$) or mixtures containing oleic acid led to an increase in survivors for all time points ($P < 0.0001$). Cultures supplemented with oleic acid did not differ from cultures supplemented with oleic acid mixtures. (B) Supplementation with linoleic acid ($C_{18:2\ cis-9,12}$) or mixtures containing linoleic acid also led to an increase in survivors at all time points ($P < 0.0001$). Cultures supplemented with linoleic acid did not differ from cultures supplemented with linoleic acid mixtures. (C) Supplementation with either human serum (15%) or SLOP led to an increase in survivors at all time points ($P < 0.0001$).

Discussion

Previously, we had noted that growth of *E. faecalis* in either serum or bile resulted in increased proportions of palmitic acid (C_{16}), stearic acid (C_{18}), oleic acid ($C_{18:1\ cis9}$) and linoleic acid ($C_{18:2\ cis9,12)}$ and protection from daptomycin [6, 9]. However, supplementation with individual fatty acids resulted in two distinctive phenotypes: daptomycin protective versus non-protective. In this study, we examined the effects of these specific fatty acid mixtures on the physiology of *E. faecalis*. We found that a combination containing at least one protective fatty acid will induce protection from daptomycin and can prevent negative physiological effects that are observed after supplementing with a saturated, non-protective fatty acid. In particular, we noted that membrane fluidity was restored to control culture levels, which may be driving the overall improved health of these mixture cells. Changes in cellular charge did not correlate with restored cellular health and daptomycin tolerance, indicating that this feature may not be the main driver in *E. faecalis* for fatty-acid induced tolerance.

A major consequence we noted from supplementing cells with either palmitic acid or stearic acid was severe morphological deformities (Fig 2.3). Cells appeared with altered placement of septa and clear problems with proper cellular division. This morphology is reminiscent of *E. faecalis* with non-functioning DivIVA, which localizes to cell poles and septa to mediate proper cell division [17]. We clearly observe defective reproduction in cells supplemented with saturated fatty acids, possibly due to improper localization of DivIVA and consequently aberrant peptidoglycan synthesis [17]. As shown with growth, defective cell morphology is rescued by addition of either protective fatty acid, suggestive that these unsaturated fatty acids may help restore proper membrane protein localization and/or function

A recent investigation into the mechanism of daptomycin killing revealed that membrane fluid microdomains of *Bacillus subtilis* are disrupted, leading to delocalization of membrane associated machinery vital for cell division. [14]. Daptomycin is thought to decrease fluidity after insertion, so a more fluid membrane is theoretically less sensitive to the membrane disrupting effects of this proposed mechanism. While we do not observe increased fluidity after protective fatty acid supplementation, it is entirely

possible that we have encountered a technical lower limit while using fluorescent anisotropy. Another possibility is that membrane proteins compensate for the increased phospholipid fluidity. Regardless, more work must be done on interactions between daptomycin and cell membrane fluid microdomains.

Other work has implied that daptomycin is susceptible to repulsion by positively charged cell envelopes and does not interact to form appropriate pores [14, 18]; our own observations (data not shown) suggest that growth supplementation can lead to an increase in lysl-phosphatidylglycerol (LPG) in cellular membranes, which would have an overall impact of reducing the negative charge of enterococcal cellular envelope. Mutants lacking MprF2 have greatly reduced LPG in the membrane and are more susceptible to cationic antimicrobial peptides (CAMPs) [19]. In the closely related organism, *Staphylococcus aureus*, the *dlt* operon is responsible for the D-alanylation of lipoteichoic acid, resulting in an increased cell wall charge. Upregulation of the *dlt* operon in *S. aureus* has been associated with tolerance to daptomycin [20]. However, under our conditions, there does not seem to be a correlation in overall cell charge and protection from daptomycin. Indeed, the highest cell charge, which would theoretically lead to greater tolerance to daptomycin, is induced by palmitic acid supplementation. Protective fatty acid supplementation caused a variety of cell charges, ranging from higher to lower than the solvent control. These data combined lead us to conclude that daptomycin repulsion is likely not how fatty acid supplementation induce protection.

As shown previously [6], growth in saturated fatty acids severely alters saturated: unsaturated fatty acids in the cell membrane. This alteration would likely lead to a more rigid membrane, and perhaps exacerbate the effects of daptomycin. Given the target and proposed mechanism of daptomycin, it is likely that the correlation observed between membrane fluidity and protection strongly indicates how exogenous fatty acids provide tolerance to the drug. To investigate this hypothesis, we looked at overall membrane fluidity using the fluorescent lipid dye DPH on OG1RF protoplasts. While we do see a reduction in membrane fluidity after supplementation with non-protective fatty acids, fluidity is not increased from solvent control after supplementation with protective fatty acids or protective mixtures. It should be noted that this method does not

distinguish lipid microdomains, but rather measures the overall or average membrane fluidity; regardless, the decreased overall fluidity observed may be influencing the proper interaction of daptomycin with the cellular membrane.

Herein we showed that oleic acid and linoleic acid are able to rescue from the negative physiological effects of non-protective fatty acids, but the exact mechanism by which they induce daptomycin tolerance is still unclear. Our combination of host derived, exogenous fatty acids induced the same protection as human serum, indicating that not only are protective fatty acids capable of inducing daptomycin tolerance in the presence of non-protective fatty acids, but oleic acid and linoleic acid are solely responsible for providing the protection observed after supplementation with human serum. In conclusion, we have described physiological effects that are restored by protective fatty acid combination supplementation and demonstrated that protective fatty acids induce tolerance to daptomycin in the presence of non-protective fatty acids.

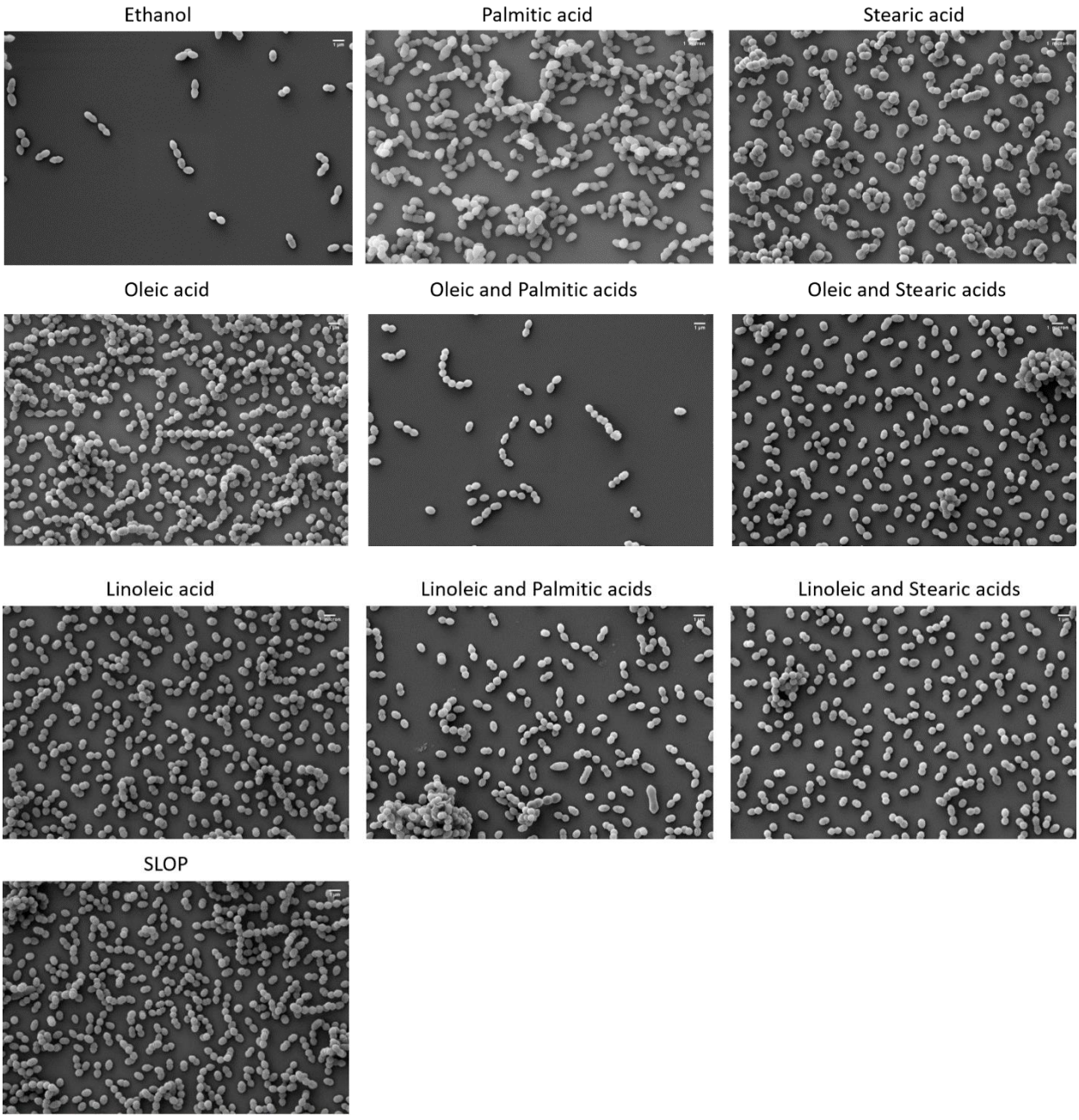

Figure 2.7: Large field view of OG1RF via scanning electron microscopy. Scanning Electron Microscopy of Enterococcus faecalis after long-term supplementation with fatty acids. Images taken at 10,000 magnification at KeV 5.0. Scale bar represents 1 micron.

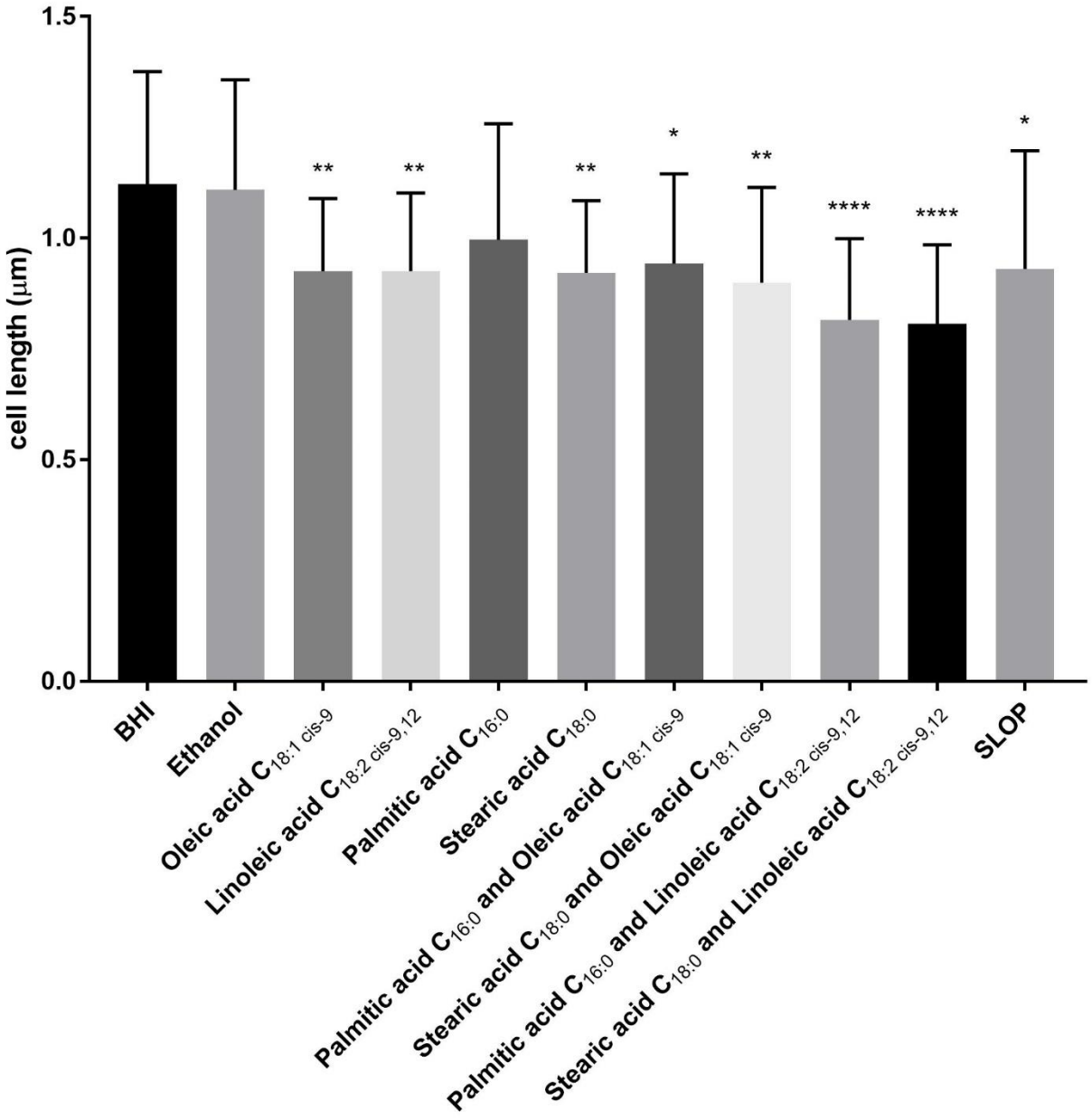

Figure 2.8: Average cell length of OG1RF after mixed fatty acid supplementation. Average cell length of 30 individuals per condition. Palmitic acid (C_{16}) does not induce change in cell length from solvent control. All other conditions shorten the cell length. High standard error is attributed to varying stages of reproduction that was captured for each sample.

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Chapter 3: Gene Expression in Response to Fatty Acid Supplementation

Future Publication Note

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

Fatty Acids Induce Changes to the Transcriptome of *Enterococcus faecalis*. 2020/2021. William Brewer and Elizabeth Fozo.

Growth of *Enterococcus faecalis* and transcriptome analysis was performed by William Brewer. RNA extraction was done by William Brewer and Elizabeth Fozo. Complimentary DNA library preparation, RNA removal, and MIseq run performed by the Genomics Core at the University of Tennessee Knoxville.

Introduction

Enterococcus faecalis is a Gram-positive facultative anaerobe primarily existing as a commensal in mammalian intestines. Given this, enterococci species are used as indicators of fecal contamination, and in some cases, key organisms for the fermentation of specific food products [1]. In immunocompromised individuals, however, uncontrolled bacterial growth leads to diseases such as septicemia and endocarditis [2]. Surgical wounds also act as a point of entry for *E. faecalis*, causing infections that are difficult to eradicate. *E. faecalis* is also resilient to a variety of environmental stressors, increasing the time it can survive outside of a mammalian host [3]. Along with being resilient to environmental stress, *E. faecalis* is resistant to different classes of antibiotics, complicating treatment strategies.

Multiple antibiotics are currently in use to treat enterococcus infections, including vancomycin, linezolid, and daptomycin. Vancomycin is a glycopeptide often used for intestinal *Clostridium difficile* or systemic Gram-positive infections. It acts by binding to the D-ala D-ala residues that are the binding substrates for peptidoglycan cross-linking by Penicillin binding protein (PBP). PBP cross-links the D-ala D-ala residues of peptides, securing glycan molecules together forming a complete peptidoglycan wall. When PBP is outcompeted by vancomycin, the wall is not formed and cell division results in a spheroplast that is incredibly sensitive to environmental damage. The discovery of vancomycin resistant enterococci in the 1980's led to a search for other drugs effective against Gram-positive bacteria. In 2000, linezolid was approved for clinical use for short durations as long term can lead to nerve damage and permanent vision loss. Linezolid is an oxazolidinone drug that binds to the 50s ribosomal subunit, halting protein synthesis at the initiation step of peptide elongation. Due to the spread of vancomycin resistance, and the limited use and harsh side effects of linezolid, daptomycin is an alternative choice for treating Gram-positive infections. Studies have reported that daptomycin inserts into the cytoplasmic membrane, oligomerizes forming a pore that leads to membrane depolarization. However, other studies have noted additional possible outcomes upon drug treatment, suggesting that the mechanism of killing may have multiple layers. Work with *Bacillus subtilis* noted that daptomycin localizes to areas of higher membrane fluidity, possibly effecting the localization of cell

membrane and cell wall synthesis machinery. Upregulation of the *dlt* operon, responsible for adding positively charged residues to wall teichoic acids, in Staphylococcus aureus has been shown to increase resistance to daptomycin [4]. Interestingly, a thicker cell wall in S. aureus also increases resistance to daptomycin [5]. Disruption of multiple pathways may be the reason the mechanism of killing for daptomycin is so difficult to understand.

Daptomycin resistance has been reported in a variety of clinical species. An invaluable resource for understanding drug activity and resistance specifically in *E. faecalis* has been a daptomycin resistant clinical isolate pair obtained before and after antibiotic treatment. Comparing genomic sequences of the pair revealed mutations in the genes in cardiolipin synthase 1(*cls*), glycerophosphoryl diester phosphodiesterase (*gdpD*) and lipid II interacting antibiotic component (*liaF*) in the resistant isolate, all of which are believed to influence cell membrane phospholipid composition. The major contributor to resistance, however, was determined to be the mutation in the *liaF* gene. LiaF represses signaling via the LiaS sensor to the response regulator LiaR which in turn activates expression of the *lia*HIGFSR operon; thus, mutation in *liaF* results in heighten expression of the *lia*HIGFSR. After transcriptional upregulation, LiaI and LiaH localize to the membrane preventing damage by an unknown mechanism [6-8].

Cardiolipin synthase (*cls1*) is responsible for making the phospholipid cardiolipin, unique in that it is composed of a single glycerophosphate head and four acyl chain tails. This shape likely has a specific function in cell curvature due to its narrow head and wide tail region. Having four acyl tails to a single glycerophosphate head also decreases the overall charge further than that of its precursor, phosphatidylglycerol. Cardiolipin has been noted to localize to cell poles and septa in *E. faecalis*, both being regions of high cell membrane curvature. The essential membrane division protein DivIVA localizes to regions of high membrane curvature likely due the strong negative charge from cardiolipin. The mutation observed in *cls1* is thought to increase the function and subsequently the proportion of cardiolipin in the membrane. GdpD is responsible for the production glycerol-3-phosphate (G3P) by recycling glycerophosphodiesters of varying structure [9]. Mutation in *gdpD* alone does not increase the minimal inhibitory concentration (MIC) of *E. faecalis* to daptomycin, but it is

synergistic with gain of function mutation in *cls1*. These genes represent the known mechanisms of genetic resistance, but *E. faecalis* also shows tolerance to daptomycin in certain environments rich in mammal associated fatty acids.

Work by Saito and colleagues [10, 11] showed that growing *E. faecalis* in host fatty acids drastically increased survival after challenge with daptomycin. Further, only unsaturated fatty acids oleic acid and linoleic acid present in serum or bile induced protection. Work by Harp and colleagues [12] demonstrated that this tolerance induction by oleic or linoleic acids occurs independently of *liaR*, indicative that the protective fatty acids are not turning on this signaling pathway. Further work by Harp and colleagues also showed that the fatty acid induction of tolerance occurred independently of the known cardiolipin synthase genes (both *cls1* and *cls2*), identifying this as a unique mechanism not based on known genetic resistance mechanisms (Harp, Unpublished). We hypothesize that oleic acid induces a unique transcriptional response that leads to daptomycin tolerance. Within, we use RNA-Seq to examine changes in the transcriptome of *E. faecalis* OG1RF in response to exposure to the daptomycin-tolerant inducing fatty acid oleic acid as well as several control fatty acids.

Materials and Methods

Growth conditions. *E. faecalis* OG1RF was grown statically at 37°C in brain heart infusion (BHI) for all experimental conditions. Overnight cultures were diluted to OD_{600nm} 0.01 before experimentation. When cultures reached OD_{600nm} 0.25, cultures were split into 50 ml aliquots, leaving a no supplemented control, and supplemented with a solvent control (ethanol 0.2%) and fatty acids of the following concentrations: cis-vaccenic acid ($C_{18:1\ cis11}$)(20 μg ml^{-1}), oleic acid ($C_{18:1\ cis9}$)(20 μg ml^{-1}, 70.8), stearic acid (C_{18})(10 μg ml^{-1}), and oleyl sulfate ($C_{18:1\ cis9}SO_4$)(20 μgml^{-1}). After 15 minutes of incubation, cells were spun down at 2,739g, washed with 1x PBS twice, pelleted and stored at -80°C for RNA extraction.

RNA extraction. RNA was extracted using the hot phenol: chloroform extraction method described previously [13]. RNA was treated with DNase and the final chloroform extraction step was repeated. 3M sodium acetate and 99% were added to the supernatant, followed by ethanol precipitation. RNA pellet was hydrated with RNase free water.

RNA Sequencing. After RNA quality was confirmed via agarose gel and analysis on a Bioanalyzer (give product name/number), ribosomal RNA was reduced using a Ribominus transcriptome isolation kit for yeast and bacteria (Invitrogen). Complimentary DNA (cDNA) library was prepared using the TruSeq stranded mRNA sample kit (Illumina). cDNA was run on a Miseq V3 150 flow cell lane, producing paired end 75 base pair reads. cDNA library prep, RNA removal, and Miseq was performed by the Genomics Core at the University of Tennessee Knoxville,

Read analysis. Reads were trimmed using Trimmomatic [14] with a PHRED score cutoff of 34 and a length minimum of 55 base pairs. Reads below these limits were dropped from the pool of eligible reads. After trimming, eligible reads were mapped to the OG1RF reference genome (NC_017316.1) using STAR [15] and the reference genome annotation (ASM17257v2.46) from Ensemble genomes. Uniquely mapped reads were counted using HTSeq [16]. Counts were exported to Rstudio (Rstudio, 2015) for differential expression analysis using EdgeR. Differentially expressed genes were determined using a false discovery rate cutoff of 0.05. All figures comparing differentially expressed genes were constructed using Rstudio. Housekeeping gene counts were exported from count files for analysis. The average TPM (Transcripts Per Kilobase Million) and standard deviation across all conditions was calculated for each of the shown housekeeping genes.

Results

RNA isolation from cells treated with protective and non-protective fatty acids. Previously, we noted that when OG1RF is pre-grown in either bile or serum, the cells are protected from high concentrations of daptomycin. This protective response was further elucidated to be due to the presence of either oleic acid ($C_{18:1cis9}$) or linoleic acid ($C_{18:2cis9,12}$). Interestingly, supplementation with fatty acids of similar length of unsaturation did not induce protection: addition of either stearic acid (C_{18}), which is also found in bile or serum and natively in the membrane of OG1RF, or cis-vaccenic acid ($C_{18:1cis11}$) which is one of the major, native fatty acids found in the membrane of OG1RF, did not. Additional work using oleyl sulfate ($C_{18:1\ cis9}\ SO_4$), an analog of oleic acid having a sulfate group instead of a carboxylic group, revealed that it could also induce protection from daptomycin. However, tolerance induction by these fatty acids

occurred independently of the *liaFSR* signaling cascade which is critical for genetic resistance to daptomycin.

As protective fatty acids could be inducing signaling pathways and other gene expression changes in OG1RF, we performed a global transcriptome analysis. Cells were grown to exponential phase in BHI (Brain heart infusion) media, then divided and either received no addition, solvent control, oleic acid (protective; $C_{18:1cis9}$), cis-vaccenic acid (non-protective, $C_{18:1cis11}$), stearic acid (non-protective, $C_{18:0}$), or oleyl sulfate (protective, $C_{18:1SO4cis9}$) for 15 minutes to capture primary transcriptional responses. Sequencing of cDNA resulted in 150 million reads, 39 million of those mapping uniquely to the OG1RF genome. This gave a depth of coverage of 14.2, lower than desired, but unavoidable considering poor efficiency of the ribosomal RNA removal. All runs contained high quality reads, averaging around PHRED score of 35. Trimming removed a small portion (<1%) of reads based on quality and length.

Transcription is consistent across conditions.

To validate the consistency of our treatment groups, we examined whether select housekeeping genes varied among the biological replicates across a treatment group. These genes were selected based on roles in essential processes. Genes selected include DNA gyrase subunit A (OG1RF_RS00040, gyrA), serine hydroxymethyltransferase (OG1RF_RS09890, glyA), penicillin binding protein (OG1RF_RS03750, PBP), Div1B cell division protein (OG1RF_RS03770, div1B), and α/β hydrolase (OG1RF_RS00480). Examining the average TPM (transcripts per kilobase million) (Figure 1) across conditions verifies that differentially expressed genes are targeted and not an overall change to the transcriptome.

Basal transcriptional changes in solvent control cells.

As our fatty acid supplements are reconstituted in ethanol, we included a solvent control to rule out the effects of ethanol on transcriptional changes. We observed a small, but significant group of transcripts altered when cells were given ethanol versus those cells with no addition (Figure 3.1). Interestingly, OG1RF_RS05020, one of four homologs of *fakB* in *Staphylococcus aureus* responsible for binding exogenous fatty acids for later

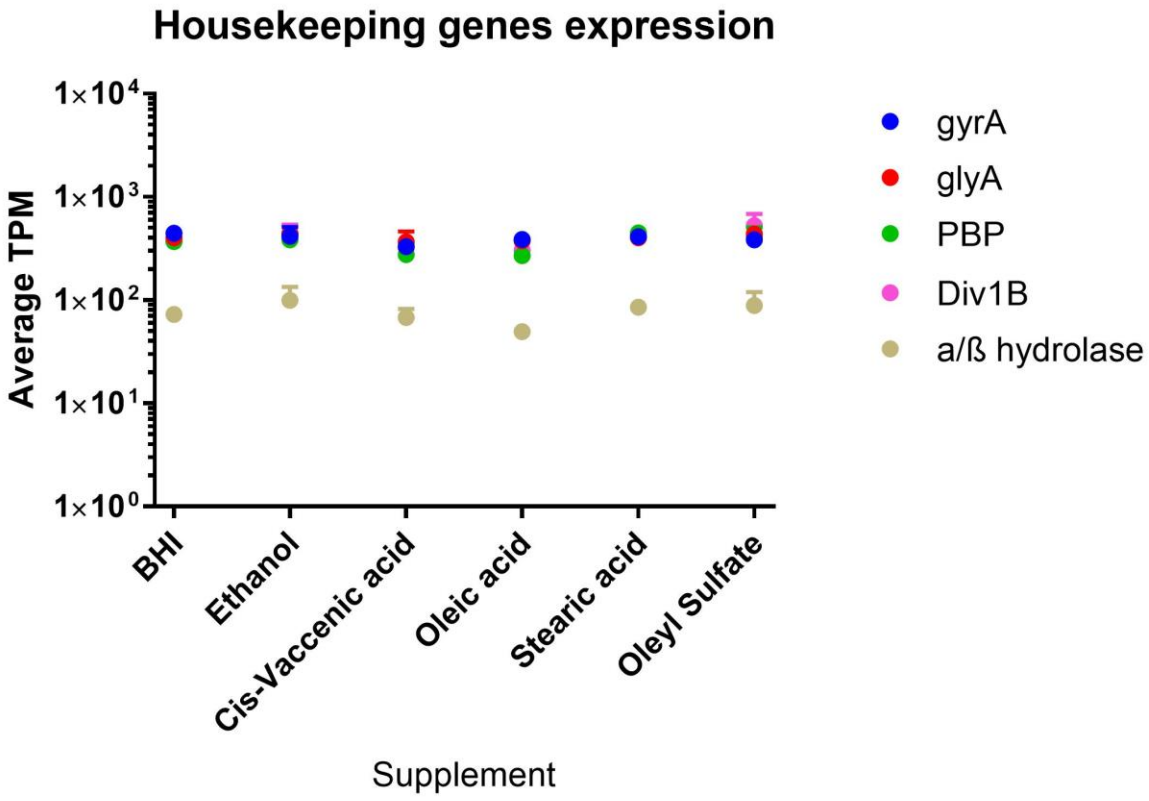

Figure 3.1: Housekeeping gene expression across fatty acid conditions.

Average Log TPM of housekeeping genes after exposure to a fatty acid supplement for 15 minutes. Expression is stable across conditions, indicating the validity differentially expressed genes.

use in lipid synthesis, was reduced in ethanol compared to no supplement addition. The *fakB* homolog was also reduced in all fatty acid conditions except stearic acid.

Unsaturated fatty acid supplementation has an impact on transcription.

Of the supplemented fatty acids, both the readily incorporated [10, 11] cis-vaccenic acid and oleic acid caused a varied response to the transcriptome of OG1RF. We noted genes associate with metal transport, *clpB* involved in general stress response, and interestingly glycerophosphoryl diester phosphodiesterase (*gdpD*) were all upregulated in oleic acid supplemented cells (Table 3.1). A gene of interest, *clpB* has been shown to act as part of the stress response across bacterial species, degrading aggregates of non-functional proteins. In *Bacillus* species, *gdpD* is responsible for the generation of glycerol-3-phosphate using a variety of substrates, including wall macromolecules in *Bacillus pumilus* [6]. Deletion of the glycerophosphoryl diester phosphodiesterase *yqiK* in *B. subtilis* renders the cell susceptible to high saline stress, implying a role in osmoprotection [6]. Upregulation of this gene is of particular note due to its role in genetic resistance to daptomycin in the *E. faecalis* clinical strain mentioned above. Unfortunately, our analysis revealed no differentially expressed genes between oleic acid and cis-vaccenic acid. Instead, comparison of genes found in supplements against solvent control (Table 3.1) were used to find the upregulation of OG1RF_RS05275, uniquely regulated in oleic acid supplemented cells. This gene encodes a glycoside hydrolase family 1 protein, necessary for the cleavage of bonds between carbohydrates. Research has primarily been focused on cellulose metabolism, but it has been reported that substrate specificity for protein members of this is low [17].

Table 3.1 Differentially expressed (DE) genes of O1RF in fatty acid supplements compared to solvent control. Left column contains gene names. Middle column contains the Log_2 fold change (LogFC) of the gene relative to solvent control cells. Right column contains false discovery rate P-value (FDR) as determined by the EdgeR differential expression analysis R package.

A. DE genes of cells supplemented with BHI compared to solvent control cells

BHI DE genes		logFC	FDR
OG1RF_RS05020	DegV family protein	4.27	0.0096
OG1RF_RS08730	type I glutamate--ammonia ligase	2.53	0.017
OG1RF_RS05015	DUF1836 domain-containing protein	3.94	0.039

B. DE genes of oleic acid supplemented cells compared to solvent control.

Oleic acid DE genes		logFC	FDR
OG1RF_RS01350	copper-translocating P-type ATPase	4.51	0.00078
OG1RF_RS05915	magnesium-translocating P-type ATPase	2.81	0.0016
OG1RF_RS03080	YhgE/Pip domain-containing protein	3.4	0.0016
OG1RF_RS06160	cadmium-translocating P-type ATPase	5.89	0.0021
OG1RF_RS01345	CopY/TcrY family copper transport repressor	4.16	0.0021
OG1RF_RS02615	MgtC/SapB family protein	6.41	0.0025
OG1RF_RS02700	glycerophosphoryl diester phosphodiesterase	2.58	0.013
OG1RF_RS09175	ATP-dependent chaperone ClpB	2.98	0.013
OG1RF_RS02365	ATP-dependent Clp protease ATP-binding subunit	3.6	0.018
OG1RF_RS02610	heavy metal translocating P-type ATPase	6.2	0.023
OG1RF_RS05275	glycoside hydrolase family 1 protein	1.35	0.026
OG1RF_RS02705	NUDIX hydrolase	2.46	0.026
OG1RF_RS01355	heavy-metal-associated domain-containing protein	3.45	0.035

Table 3.1 continued

C. DE genes of cis-vaccenic acid supplemented cells compared to solvent control.

Cis-vaccenic DE genes		logFC	FDR
OG1RF_RS01350	copper-translocating P-type ATPase	3.88	0.0068
OG1RF_RS06160	cadmium-translocating P-type ATPase	5.75	0.0068
OG1RF_RS02700	glycerophosphoryl diester phosphodiesterase	2.82	0.0068
OG1RF_RS02615	MgtC/SapB family protein	5.96	0.011
OG1RF_RS05915	magnesium-translocating P-type ATPase	2.319	0.026
OG1RF_RS03080	YhgE/Pip domain-containing protein	2.80	0.026
OG1RF_RS02705	NUDIX hydrolase	2.50	0.026
OG1RF_RS02365	ATP-dependent Clp protease ATP-binding subunit	3.60	0.026
OG1RF_RS01345	CopY/TcrY family copper transport repressor	3.41	0.026
OG1RF_RS00125	hypothetical	3.28	0.026
OG1RF_RS04400	universal stress protein	2.19	0.026
OG1RF_RS03430	UvrD-helicase domain-containing protein	2.43	0.030
OG1RF_RS02610	heavy metal translocating P-type ATPase	5.91	0.034
OG1RF_RS09175	ATP-dependent chaperone ClpB	2.67	0.037
OG1RF_RS06755	GNAT family N-acetyltransferase	2.40	0.048

D. DE genes of stearic acid supplemented cells compared to solvent control.

Stearic acid DE genes		logFC	FDR
OG1RF_RS05020	DegV family protein	3.91	0.043

Discussion

To better understand the consequences of fatty acid supplementation upon *E. faecalis* and their possible contributions to daptomycin tolerance*, E. faecalis* was supplemented with different fatty acids, all 18 carbons in length, that differed in their abilities to induce tolerance. To rule out variation across replicates, we employed a similar strategy used by Martin [18] by comparing reads of the same housekeeping genes across conditions to ensure valid detection of differentially expressed genes. We do note that the solvent control did impact gene expression compared to media control; however, we compared gene expression of our fatty acid supplements to the solvent to better determine unique effects from the fatty acids.

When compared to the solvent control, we found that supplementation with unsaturated fatty acids induced upregulation of genes associated with stress response, likely due to the unsaturated fatty acid dominated membrane having altered physiological properties. Also noted was the upregulation of genes for metal transport, similar to OG1RF treated with ampicillin, bacitracin, cephalosporin, or vancomycin. Given the wide range of stresses caused by fatty acid supplementation and the mentioned antibiotics, metal transport maybe part of general stress response in *E. faecalis* in response to cell envelope alterations [19]. Another gene upregulated in both unsaturated fatty acids encodes for a NUDIX hydrolase. Recent work has shown that *Mycobacterium smegmatis* uses specific NUDIX hydrolases to control the amount of acyl-CoA in the cell, diverting the chains towards ADP synthesis. While NUDIX hydrolases have been implicated in the inactivation of many molecules, including mutagenic nucleoside triphosphates, sugar molecules, and signaling molecules, each hydrolase is specific to its substrate. This specificity necessitates many different NUDIX hydrolase genes, for example 30 different NUDIX hydrolase genes are observed in *Bacillus anthracis* [20]. Understanding the role of NUDIX hydrolase in *E. faecalis* will require work specific to the organism and this particular hydrolase.

Given protection is only induced by oleic acid and not the similar fatty acid cis-vaccenic acid, it was interesting to find no differentially expressed genes within this comparison. Oleic acid and cis-vaccenic acid only differ in the placement of the double

bond, carbon 9 in oleic acid and carbon 11 in cis-vaccenic acid, but this difference is sufficient to induce protection. Although direct comparison produced no genes of interest, indirect comparison of differentially expressed genes between oleic acid to solvent control and cis-vaccenic to solvent control produced one gene that encodes for a glycoside hydrolase family 1 protein. Mostly researched for the industrial production of glucose from cellulose, members of this protein family are not very specific and can use different carbohydrates as substrate [17]. However, no work, that we are aware of, in *E. faecalis* indicates its possible contribution to protection from daptomycin.

Apart from known functions in producing G3P, a homolog of GdpD in *Bacillus subtilis* (YqiK) was demonstrated to protect from high salt concentrations by regulating wall structure to avoid excess turgor pressure. Another homolog in *Bacillus pumilus* was demonstrated to use wall components such as teichoic acids as a substrate, presumably changing the structure of the wall. Further, work with *Staphylococcus aureus* demonstrated a homolog of GdpdD is required for resistance to β-lactam antibiotics [9]. Given that mutation in *gdpD* alone is not sufficient to increase MIC of daptomycin in *E. faecalis* [21], it is likely that fatty acid induced protection also requires more than just increased transcription of *gdpD*. Previous work in other organisms has shown that glycoside hydrolase family 1 proteins break down cellulose into glucose monomers, mainly useful for metabolism of carbohydrates. As there are published findings supportive that daptomycin maybe targeting features of the cell wall, and not just the cell membrane, it is possible that wall modification by glycoside hydrolase family 1 protein, in conjunction with wall modification by GdpD, leads to the observed daptomycin tolerant phenotype.

Herein we show that exogenous supplementation of *E. faecalis* with fatty acids induces changes to transcriptional regulation. While most changes are not unique to the protective supplement oleic acid, upregulation in *gdpD* and a gene encoding for glycoside hydrolase family 1 protein are a promising future avenue of research into the mechanism of fatty acid induced tolerance to daptomycin.

A.

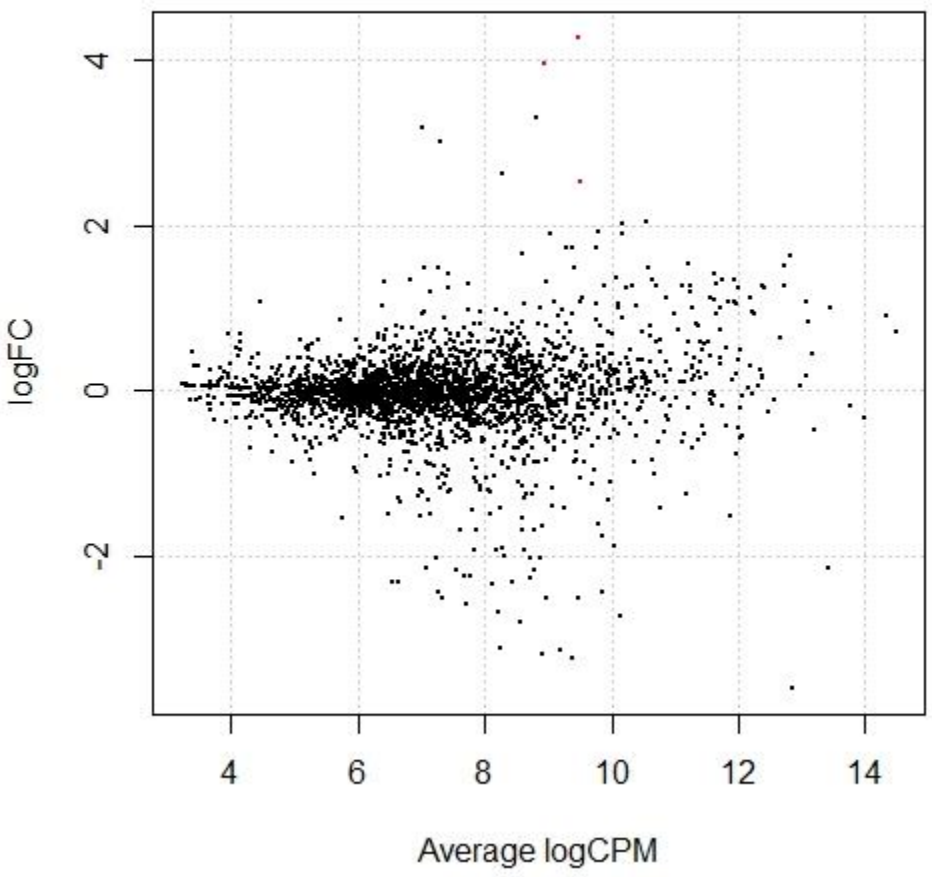

Figure 3.2: Log$_2$ fold change of genes between fatty acid supplements and solvent control. MA plot of differentially expressed genes between supplemented conditions; (A) Ethanol vs BHI (B) Ethanol vs Oleic acid (C) Ethanol vs Cis-vaccenic acid (D) Ethanol vs Stearic acid (E) Ethanol vs Oleyl sulfate. Cutoff for significance is a log fold change of 2 and an FDR of 0.05. Significant genes are shown as red dots.

B.

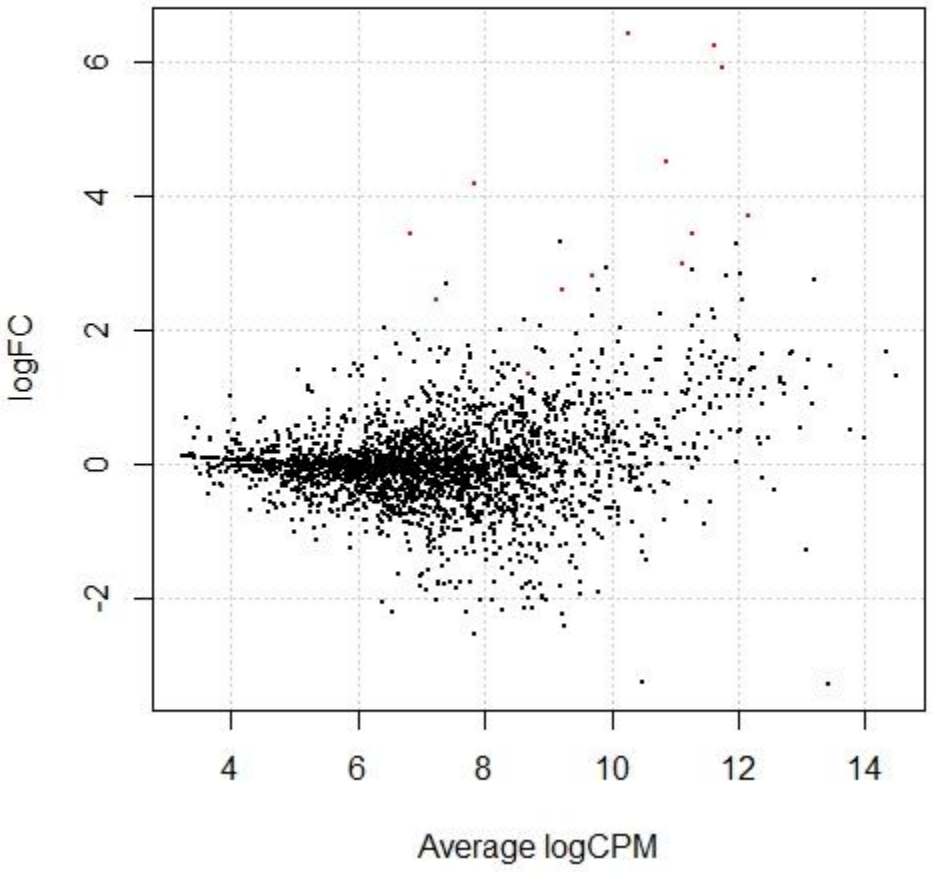

Figure 3.2 continued

C.

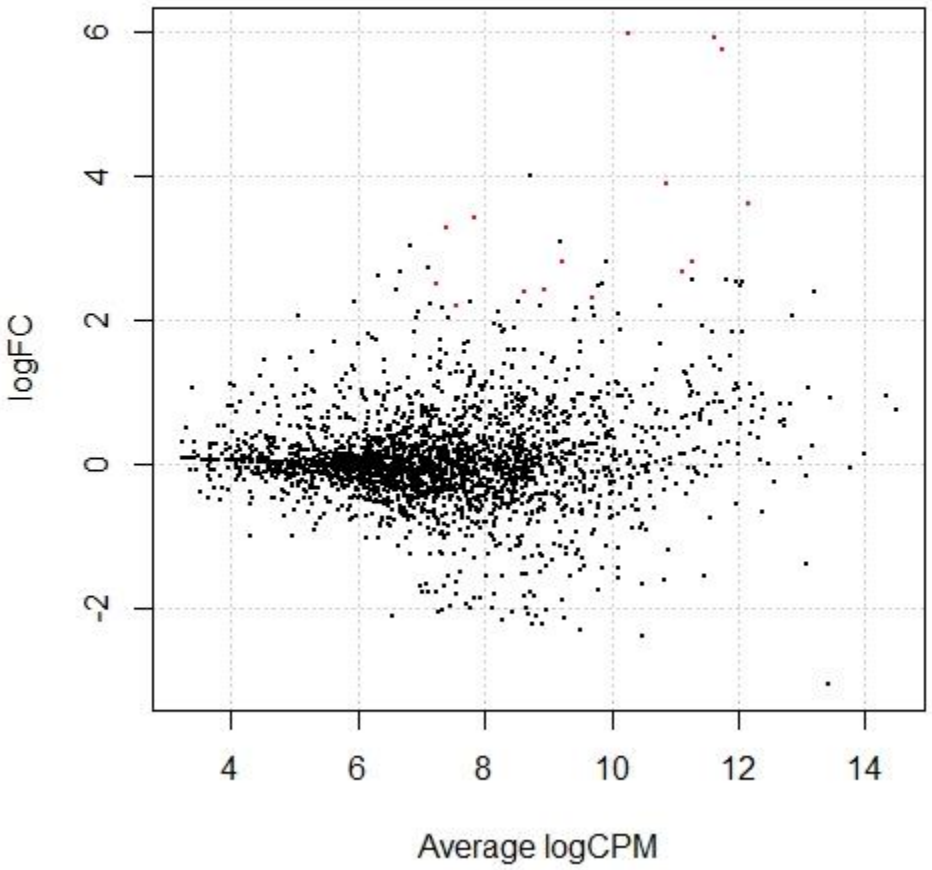

Figure 3.2 continued

D.

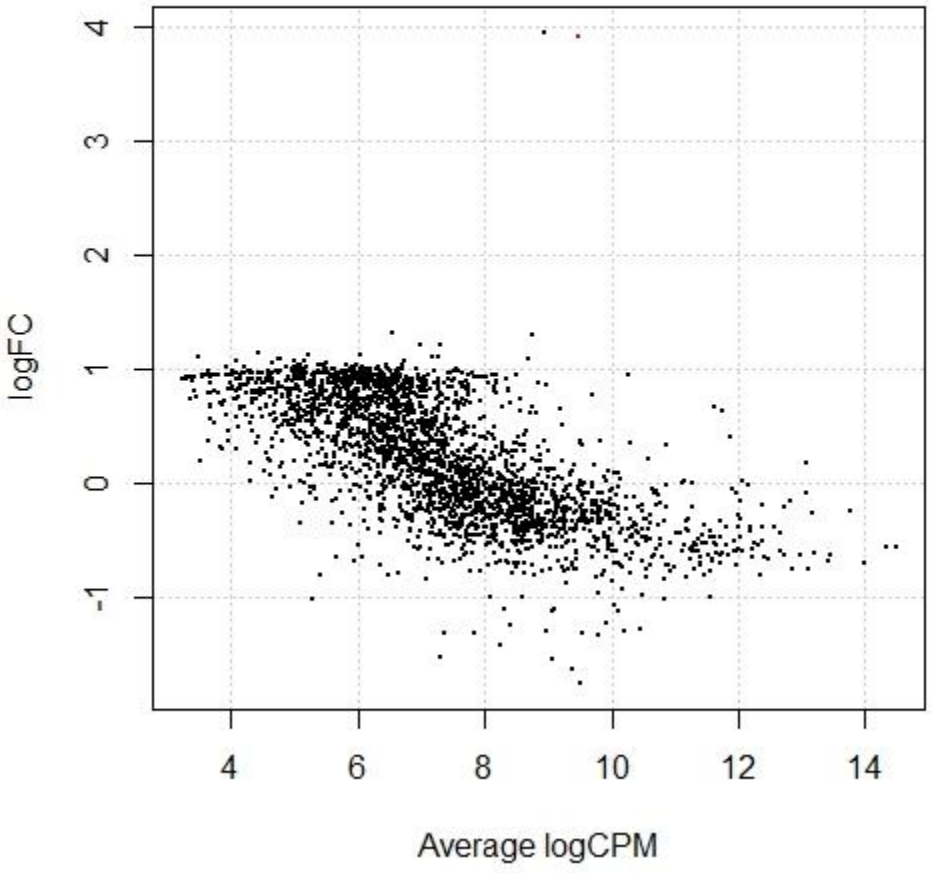

Figure 3.2 continued

E.

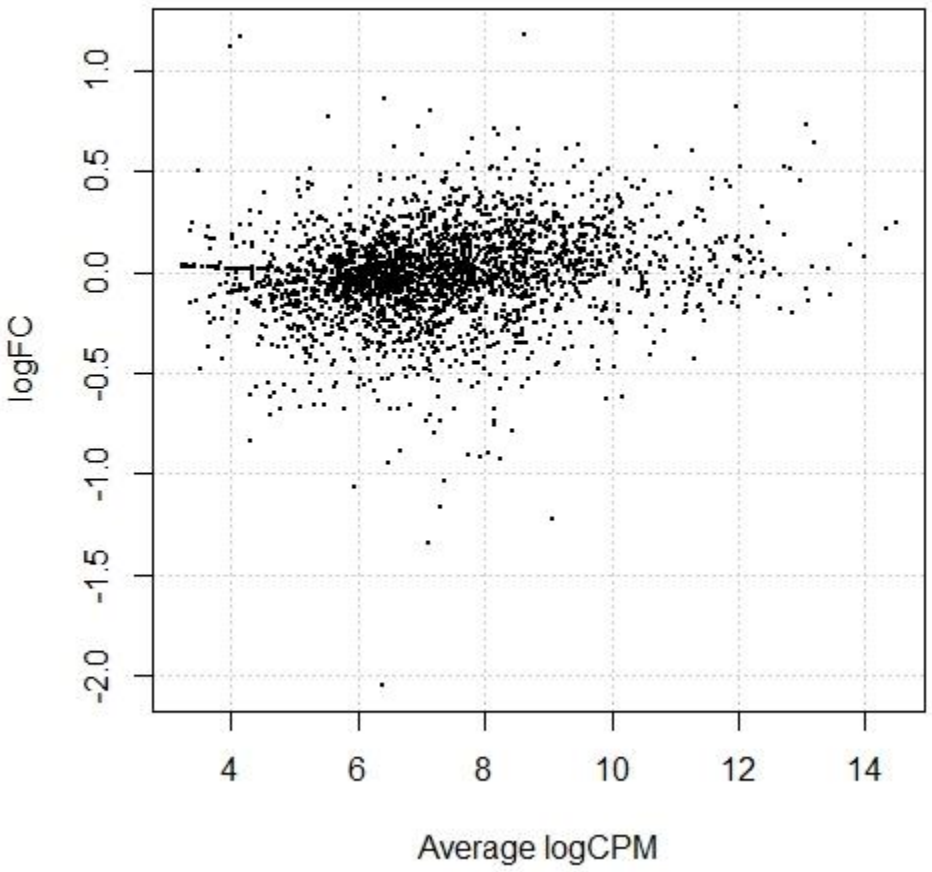

Figure 3.2 continued

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Chapter 4: Conclusions

Past work by members of the lab revealed the *Enterococcus faecalis* can take fatty acids from host fluids to gain tolerance to daptomycin. Further studies noted that each fatty acid present in host fluids causes a different effect to the cell, with only the unsaturated fatty acids oleic acid ($C_{18:1cis9}$) and linoleic acid ($C_{18:2cis9,12}$) inducing tolerance [1, 2]. Further exploration demonstrated that the daptomycin tolerant phenotype is not associated with the major genetic resistance mechanism [3]. The work presented in this thesis aims at understanding the impact of fatty acid supplementation on physiology and transcriptional regulation.

Combination fatty acid supplementation favors protection

Host fluid supplementation induces daptomycin tolerance, yet it is composed of both protective and non-protective fatty acids. The protective fatty acids oleic acid and linoleic acid are both 18 carbons in length and unsaturated. Conversely, the non-protective fatty acids stearic acid (C_{18}) and palmitic acid (C_{16}) are both saturated but differ in length. While I use protective and non-protective to describe the fatty acids, there are negative effects on cell physiology associated with the non-protective fatty acids as well. Linoleic acid causes an increase in generation time when supplemented at 20 $\mu g ml^{-1}$ [1, 2]. Both stearic acid and palmitic acid delay growth and induce heavily disfigured cells, making it all the more curious that host fluids protect while conferring neither of these negative effects.

In order to understand the interplay between protective and non-protective fatty acids, I performed a series of physiological assays after supplementation of *E. faecalis* with combinations (protective and non-protective) of fatty acids. Cell growth, morphology, membrane fluidity, cerulenin rescue, and daptomycin protection induced by fatty acid combinations all followed trends for the protective fatty acid in the mixture. Our combining all four fatty acids to create a “synthetic serum” matched human serum in daptomycin protection, further supporting that fatty acids contained in host fluids are solely responsible for tolerance. While the mechanism is still unclear, we have shown that the presence of a protective fatty acid will protect from daptomycin and the negative physiological effects caused by non-protective fatty acids.

Membrane fluidity as a target for daptomycin

Research with *Bacillus subtilis* has looked into membrane fluidity, noticing daptomycin preferentially binding to fluid microdomains, which leads to decreased fluidity and delocalization of membrane bound cell wall machinery [4]. Our numerous accounts of only specific unsaturated fatty acids inducing daptomycin tolerance indicated that protection is not as simple as increasing membrane fluidity. Previous GC-FAME (gas chromatography fatty acid methyl ester) data showed that cis-vaccenic and oleic acid makes up as much as 70% of the membrane fatty acid tails when supplemented as the sole fatty acid from inoculation [1]. Similarly, anisotropy shows that cis-vaccenic makes the membrane just as fluid as oleic acid when supplemented for 30 minutes during mid-log growth (Fig 4.1), and yet, only oleic acid can induce protection under these conditions [2]. A pilot experiment indicates that daptomycin in fact increases membrane fluidity of *E. faecalis* (Fig 4.2). Note that this measurement is done as a bulk measurement of cells, and that the concentration of daptomycin used was a minimum of 15 $\mu g ml^{-1}$ (MIC 2-4 ug/ml normally for this strain). This is unlike the above-mentioned *B. subtilis* study which relied on lower concentration of daptomycin and fluorescent microscopy using dyes to examine localized regions. Further, the membrane phospholipid and fatty acid composition of B. sub and E. faecalis are different, which could give contradictory results. Regardless, our combined data comparing cis-vaccenic and oleic acid across multiple physiological assays does not seem to support a role for membrane fluidity as controlling/inducing tolerance to daptomycin.

Cell wall as a target for daptomycin

While performing membrane anisotropy, we were concerned about the cell wall possibly interfering with access to the membrane by DPH. Consequently, we generated protoplasts. We also noted that several studies have implied cell wall alterations as a driver for daptomycin protection/resistance. Alterations to cell wall include adding positive charge via the *dlt* operon and thickening of the cell wall in Bacillus subtilis, as well as cell wall homeostasis proteins in Staphylococcus aureus and Enterococcus faecium [5]. We decided then to test the susceptibility of protoplasts to daptomycin with and without pre-growth protective fatty acid supplementation. Surprisingly, we observed dramatic protection from daptomycin from the protoplasts as opposed to whole cells

(Fig 4.3). Cell wall removal increased survival just as much as oleic acid supplementation did for the whole cells. However, in comparing whole cells versus protoplasts formed from cultures pre-grown in oleic acid, there was little to no change in survival of protoplasts.

Fatty acid supplementation alters transcription

After ruling out connections to all known daptomycin genetic resistance mechanisms, we designed an experiment to discover genes associated with tolerance. We used an assortment of fatty acid supplements meant to be similar in form, but different in the effect on cellular physiology, as well as appropriate media and solvent controls. Oleic acid was used as the target for genes of interests. Cis-vaccenic is the same length as oleic acid but differs in the location of the double bond and does not induce daptomycin protection. Oleyl sulfate has the same acyl tail as oleic acid but has a sulfate head group instead of a carboxylic head group, theoretically unable to be incorporated onto a phospholipid head group. Interestingly, even though it cannot be incorporated onto a head group, it is still able to protect from daptomycin: transcriptional changes that overlap with oleyl sulfate and oleic acid, but not with others, could be strong candidates for protective responses. Stearic acid is the same length as oleic acid but is saturated and does not induce protection.

Typically, hypotheses attempting to explain daptomycin mechanism of killing involves the cell membrane, either by creating discrete pores or ion leakage [6]. These proposed mechanisms also relate cell charge and increased proportions of lysyl-phosphatidylglycerol (lysyl-PG) to protection, assuming that daptomycin operates via charge affinity with negatively charge regions of the membrane, but our data suggests no correlation of charge or lysyl-PG levels with daptomycin susceptibility [6] (Harp, in preparation) (chapter 2).

Using solvent control as a baseline for comparison, transcriptomic analysis revealed the upregulation of a gene encoding glycoside hydrolase family 1 protein only when supplemented with oleic acid. Glycoside hydrolase breaks glycosylic bonds between sugar monomers in a carbohydrate, usually studied in respect to glucose

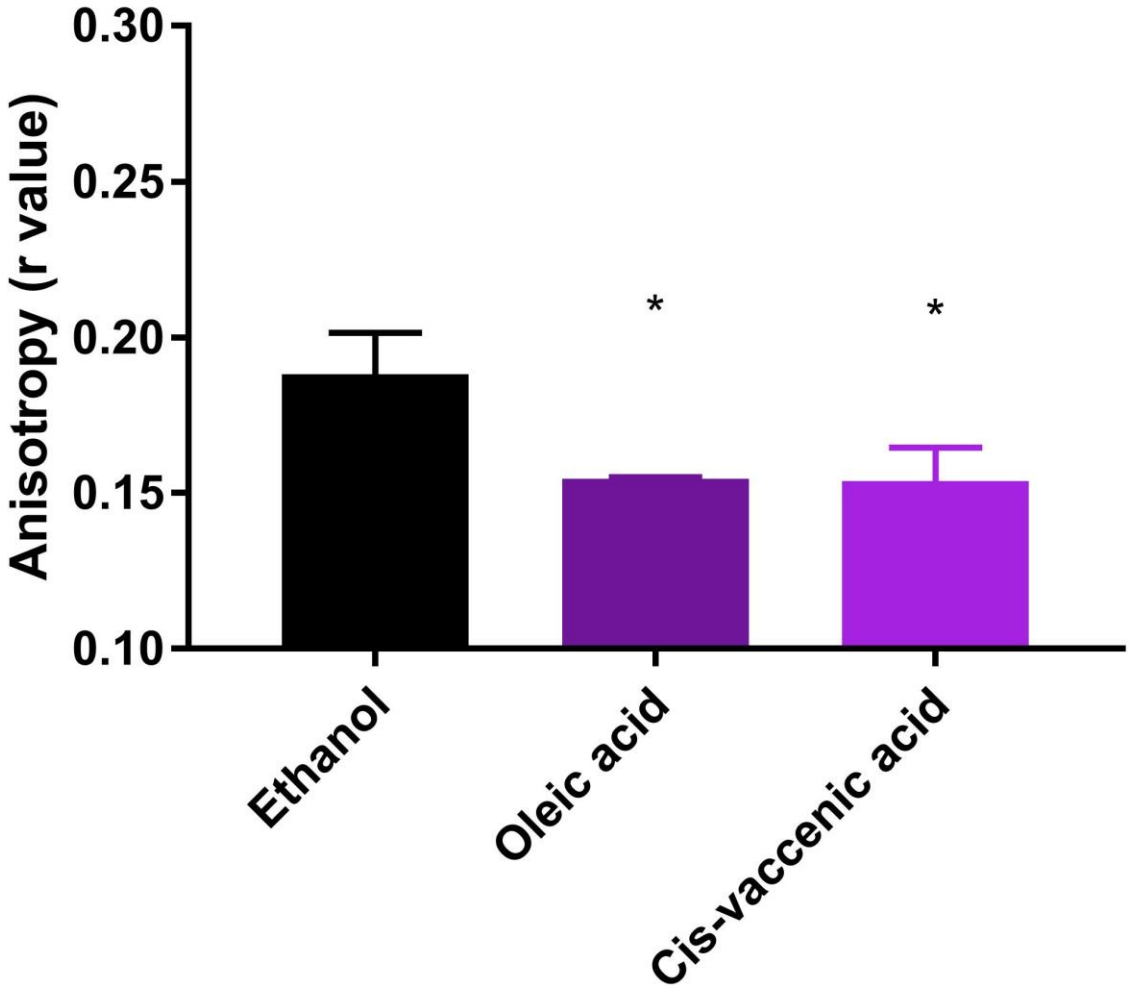

Figure 4.1: Membrane fluidity of OG1RF supplemented with unsaturated C_{18} fatty acids. DPH fluorescence anisotropy of OG1RF after long term supplementation with 0.2% ethanol, 20 $\mu g ml^{-1}$ oleic acid, or 20 $\mu g ml^{-1}$ cis-vaccenic acid. Higher r value indicates a more ordered and less fluid cell membrane. Asterisk indicates difference between indicated condition and solvent control (ethanol) with a P value below 0.05. n=3

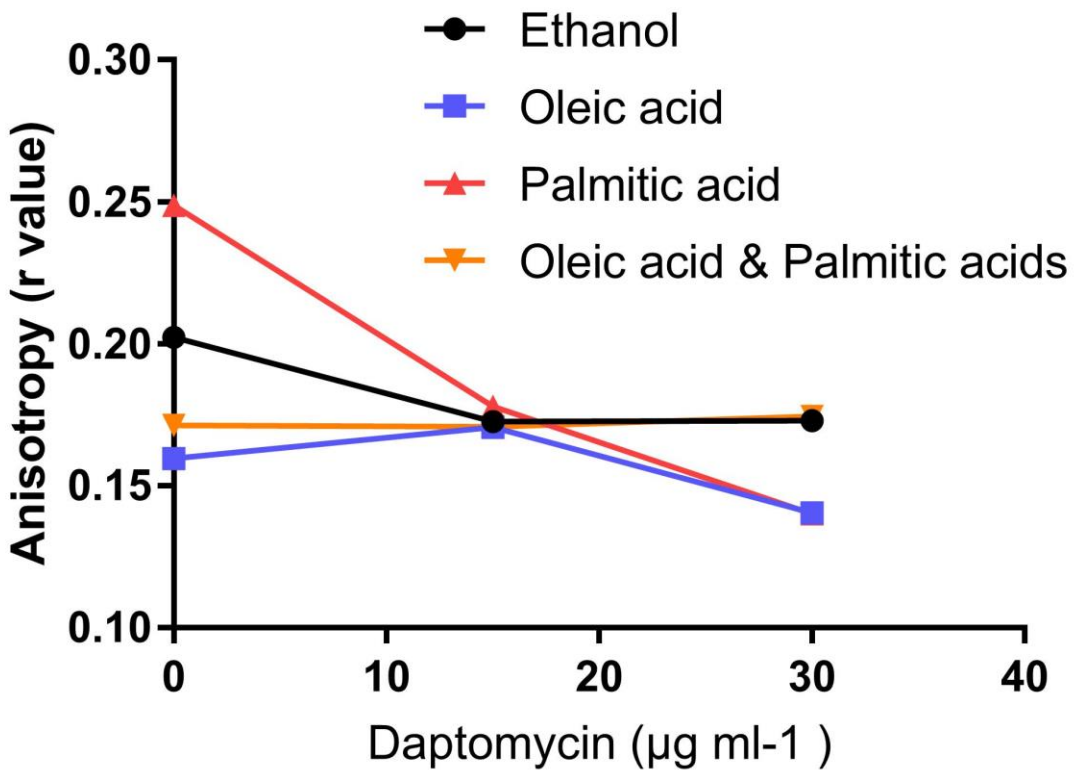

Figure 4.2: Membrane fluidity of OG1RF after daptomycin challenge. DPH fluorescent anisotropy of OG1RF supplemented with 0.2% ethanol or 5 µgml^{-1} of indicated fatty acid(s) for 30 minutes before challenge with indicated concentration of daptomycin. Higher r value indicates a more ordered and less fluid cell membrane. n=1.

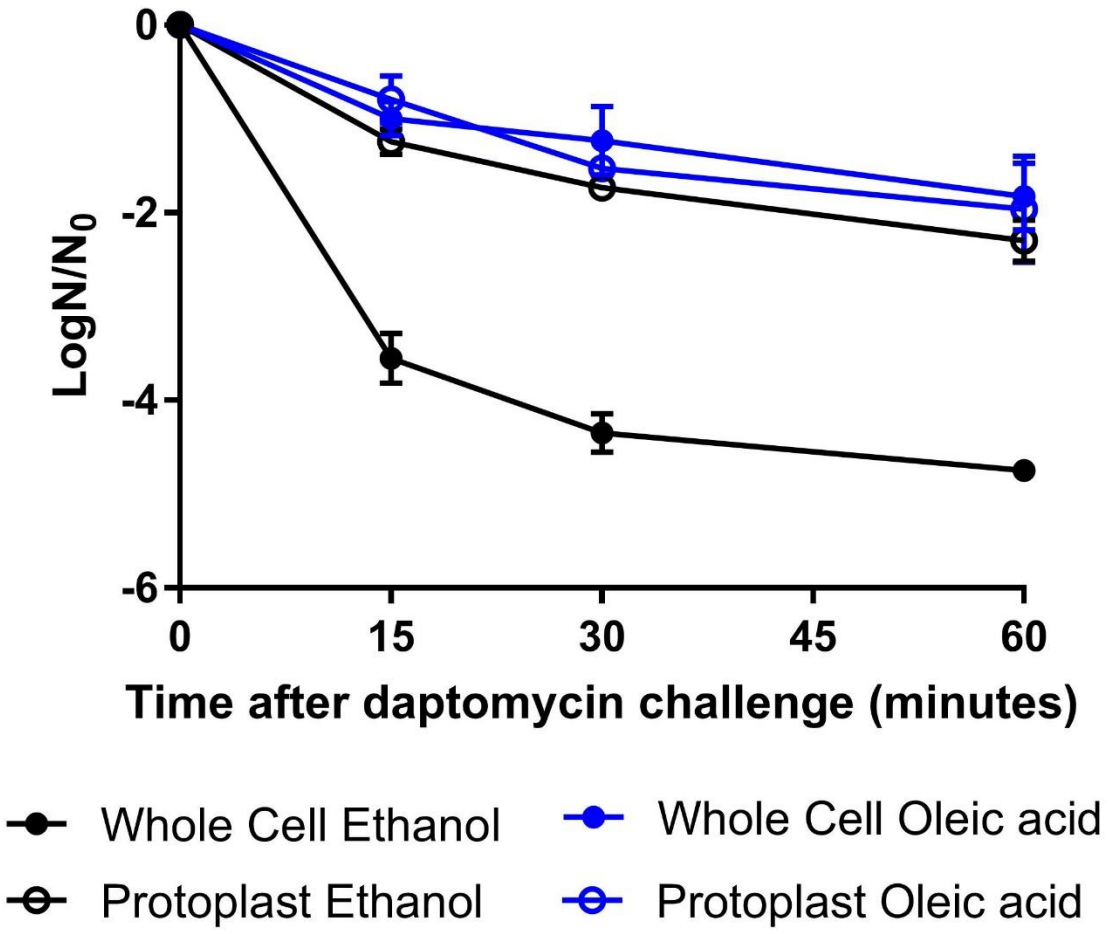

Figure 4.3: Daptomycin challenge of protoplast and whole cell OG1RF. 30 µgml^{-1} Daptomycin challenge of whole cells and protoplasts supplemented with 0.2% ethanol and 20 µg ml^{-1} oleic acid.

production from cellulose [7]. This protein becomes more interesting if the true target (or one of the targets) of daptomycin is the cell envelope. Another gene of note upregulated in oleic acid supplemented cells was glycerophosphoryl diester phosphodiesterase (*gdpD*), upregulated in both cis-vaccenic acid and oleic acid relative to solvent control. GdpD is a known daptomycin resistance gene and responsible for glycerol-3-phosphate production, but having off target, wall modification effects in *Bacillus pumilus [8, 9]*. Although found mutated in a daptomycin resistant enterococcal strain, when the mutation was generated alone, there was no effect on MIC to daptomycin but instead, appears to have a synergistic effect with other daptomycin resistant mutations [8]. If we expand the possibilities of daptomycin targeting to the cell wall, then upregulation in both *gdpD* and glycoside hydrolase could lead to wall modification, making both genes relevant to daptomycin tolerance. Upregulation of *gdpD* seen in cis-vaccenic supplemented cells may not be sufficient for protection, but the combination of upregulation in both *gdpD* and glycoside hydrolase in oleic acid supplemented cells may mirror the reliance of *gdpD* on another gene to protect from daptomycin in the daptomycin resistant clinical isolate. Further work is required to validate this hypothesis.

Future directions

We have shown that protective fatty acids are able to negate any deleterious effects from non-protective fatty acids while protecting from daptomycin but have only been able to rule out mechanisms. Transcriptomic analysis reveals an interesting avenue of research, possibly connecting the genetic resistance gene *gdpD* with the oleic acid induced upregulation of glycoside hydrolase. Creating a mutant lacking *gdpD* and glycoside hydrolase would confirm our suspicions that these genes are vital to the protection seen by oleic acid supplemented cells. Because we know gdpD upregulation alone is not sufficient to induce protection from daptomycin, we should start with a glycoside hydrolase mutant. Then in the same construct lacking a functional glycoside hydrolase, we also inactivate *gdpD*. The purpose is to elucidate the interplay between glycoside hydrolase and *gdpD*. If the glycoside hydrolase mutant alone loses protection from daptomycin then we know *gdpD* is not necessary, however the upregulation of both genes in oleic acid supplemented cells implies both are necessary for daptomycin protection.

In conjunction with molecular verification of cell wall remodeling, the survival of protoplasts after daptomycin challenge could also provide evidence of cell wall modification being responsible for daptomycin tolerance. It is likely that gently removing the cell wall while generating protoplasts also removes an essential target of daptomycin. We know by Gram staining that the cell wall is gone in protoplasts, so the activity of GdpD and glycoside hydrolase may selectively remove a target of daptomycin in a way that keeps the cell wall intact and viable. Daptomycin challenge assays will continue to be a hallmark of this project as the focus narrows to the effector rather than the causative agent, as seen in my work.

If the previously mentioned future experiments fail to identify the cause of daptomycin tolerance, we could investigate the cell wall of *E. faecalis*. Cell wall is clearly important to the mechanism of killing by daptomycin. Given this recent revelation, we should characterize the carbohydrate composition of the cell wall when grown in different fatty acid supplements. Assuming that the cell wall is being remodeled in the presence of protective fatty acids, it stands to reason there will be an observable change in the carbohydrate composition.

Lastly, we have recently sent out samples to a collaborator in order to quantify the lipid species composition of cell membranes after supplementation with fatty acid combinations using mass spectroscopy. Fatty acid composition analysis by GC-FAME of cell membranes supplemented with multiple fatty acids show that in most instances, both protective and non-protective fatty acids are in the membrane. The reason we have used mass spectroscopy in the past and continue to use it is because we can tell if the fatty acid we are detecting is incorporated onto the membrane as a phospholipid or floating free as an acyl chain. Comparing the GC-FAME lipid composition with mass spectroscopy analysis should provide evidence supporting or refuting the hypothesis that it matters for daptomycin protection that the supplemented protective fatty acid is present as a free fatty acid or incorporated onto a phospholipid.

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Vita

William Thomas Brewer V was born in Memphis, Tennessee and escaped to the opposite side of the state at age 18. There he earned a Bachelor of Science degree at the University of Tennessee and moved across the hallway to earn a Master of Science in microbiology. Against the advice of every sane person he talked to, he and his wife decided to make a family. After earning his degree, he is one step closer to his ultimate goal of being a stay at home dad.