SMALL MOLECULE MANIPULATION OF METHICILLIN-RESISTANT STAPHYLOCOCCUS *AUREUS* METABOLISM

By

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ABSTRACT

Staphylococcus aureus is a Gram positive coccoid shaped bacterium that is a normal inhabitant of the human skin microbiome and nasal passages. *S. aureus* can cause a plethora of diseases ranging from skin and soft tissue infections to osteomyelitis and toxic shock syndrome. Outside of the average difficulty treating *S. aureus* infections, the ability of *S. aureus* to resist antibiotic treatment further increases the clinical challenges, Methicillin-resistant S. *aureus* is extremely difficult to clear. Another of the difficulties of treating *S. aureus* infections lies in its ability to colonize a huge variety of host tissues because of its versatile metabolism. *S. aureus* has a branched respiratory chain, meaning it has more than one way to generate energy. If energy generation is interrupted completely, the bacterium would not be able to complete basic cellular functions resulting in cell death. Taking advantage of these pathways for treatment is an extremely difficult task because *S. aureus* can switch to fermentative metabolism adds an extra energy generating pathway. As it stands, there are two phenotypes of *S. aureus* a drug or drug cocktail must be able to inhibit or kill, fully functional respiring *S. aureus* and fully respiration-arrested (fermenting) *S. aureus*. In the clinic, aminoglycoside antibiotics have been used to treat *S. aureus* infections, notably these antibiotic treatments shift bacterial metabolism into a fermentative state, *S. aureus* mutants that can only generate energy using fermentation can be isolated from patients treated with aminoglycosides. We hypothesize that targeting pathways that support fermentative metabolism can lead to total eradication of *S. aureus* infection. Chapter 1 will assess the clinical burden caused by *S. aureus* giving an in-depth review of the genetic and physiological components that contribute to successful infection.

Chapter 2 describes the results of a small molecule screen using the GSK PKIS kinase inhibitor compound library and a novel terpenoid extract library against a menaquinone-deficient strain of *S. aureus* locked in a fermenting state. This screen identified 4 compounds that uniquely inhibit fermenting *S. aureus* growth and several compounds that promote growth in a Wild Type strain. Another important factor of this screen is that it was performed in the Je2 background, a USA300 strain similar to the current endemic stain. While growth promotion of a dangerous strain is not the ideal outcome of a small molecule inhibiting screen, this phenotype can still help identify crucial pathways beneficial to the growth of *S. aureus* and identifying these pathways can lead to better drug development in the near future.

Chapter 3 discusses a potential 'druggable' target of *S. aureus*, the cellular membrane. While unsaturated fatty acids have been observed to have modest anti-staphylococcal activity on respiring *S. aureus*, saturated fatty acids have been shown to be largely ineffective in inhibiting staphylococcal proliferation. These studies show that a range of saturated fatty acids specifically inhibits respiration-arrested bacterial cells. A thorough investigation of the mechanism by which this inhibition occurs is described in this chapter.

Chapter 4 summarizes the findings and implications of this dissertation. Reflecting on the discoveries herein, discussing the results and the potential use of these data. This chapter provides future directions of this research and the impacts this work has on the field of microbiology, specifically in treating *S. aureus* in the clinic. In all, this dissertation reveals several novel findings that can lead directly to positive clinical outcomes.

This dissertation is dedicated to the list of things I couldn’t do. And to my parents, siblings, aunts and uncles, grandparents, and friends who all made it clear that that list was real short.

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To my parents: You gave me unyielding observation and undying compassion. A combination that enabled me to be painfully aware of more things than I can ever be ready for. I've been trying to help everywhere I can ever since. Everyone else on this list knows the legend of me, because of the legends you both are. You share this victory with me.

Maaaaa: Somehow you look at me and still see an innocent baby boy. I haven't felt worthy of the love you give out since I went away for school, probably even before that. I don't know how you got stuck with me but I'm glad I was lucky enough to get stuck with you. Thank you for all the support and motivation that you've given me already and all that will come in the future. I don't know how I can repay you for it but for now my love will have to do.

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their dreams, and I have never stopped trying to be like you. Thank you for the inspiration.

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To the select few folks: Who get to deal with my unfiltered thoughts at all hours of the day, every day of the week, every week of the month, every month of the year, for the rest of all time. Thank you. Don't get married because an army won't be able to stop me from getting on the microphone and exposing you all in front of our families.

To the gang, that is all my relatives I don't share blood with: It started with some doubt. But you all were among the first to realize the words I said weren't hyperbole.

To the plethora of settings that have become impromptu laboratories (or more accurately for me), a playground: the kitchen turned chemistry lab, where I tried to re-catalyze an experiment… by adding heat… in a plastic bottle. To the classrooms of EHS where I drafted up my first formal science project. To the physics classrooms at

Deerfield where I discovered that electric currents hurt… a lot. To my first real microbiology lab at RIT where I was given free range over amber mutants. Somehow you lead me here.

To the men that called me house mom, you're insufferable. But I want nothing but the best for you all, keep striving to be the best you can be, LEDN.

To the old days that pushed me forward: To the rice pizzas flush with ketchup, corn, and Vienna sausages as makeshift pepperoni slices. To an endless supply of ramen noodles, don't sleep on the blue pack. To avoiding fights and bullets on the walk home from the 6 train. To the Hill, where I realized that 12-year-old black boys weren't safe or innocent in some people's eyes. And to the many futures I've missed completely with words or decisions. I'll make it up to you. Fingers crossed this was the right path.

To you all: I'm sorry school has been the only word associated with me for the past 15 years. I only desire the time to make it up to you.

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To those who couldn’t make it here with me: The pain of your loss never goes away, but neither does the warmth of your hearts. Please, keep watching over me.

And finally, to me: From three fifths to this. Next time you read this, do it with pride and joy. Throw in some happy tears because you deserve to know what that feels like. You’ve got a lot of hugs and smiles to make up for. Get to work.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

SSTI	Skin and soft tissue infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
Fe	Iron
Zn	Zinc
AdsA	Adenosine synthase A
IL-10	Interleukin 10
SPA	Staphylococcal protein A
MK	Menaquinone
O_2	Dimeric oxygen
H_2O	Water
PMF	Proton motive force
PAMPs	Pathogen-associated molecular patterns
NO	Nitric Oxide
iNOs	Inducible nitric oxide synthase
SCV	Small colony variant
OD	Optical density
PKIS	Published kinase inhibitor set
WT	Wild type
NM	nanometers
GSK	Compound GSK1487252A
SB	Compound SB-707548-A
‘893	Compound GW809893

‘841	Compound GW405841X
IC_{50}	Inhibitory concentration of 50% of culture
SULF	Sulfanilamide
PABA	Para-aminobenzoic-acid
TSB	Tryptic soy broth
C 14:0	Myristic Acid
PG	Phosphatidyl-glycerol
LPG	Lysyl-phosphatidyl-glycerol
CL	Cardiolipin
ACP	Acyl carrier protein
FAB	Fatty acid biosynthesis
G3P	Glycerol-3-phosphate
UFA	Unsaturated fatty acid
SFA	Saturated fatty acid
$DIOC_2$	3-3 diethyloxacarbocyanine iodide
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
C 18:0	Stearic Acid
CID	Collision induced dissociation
LD	Lipid droplet

CHAPTER 1: *STAPHYLOCOCCUS AUREUS*: PATHOGENIC POTENTIAL OF A CONSISTENTLY GROWING THREAT TO THE MODERN WORLD.

CLINICAL BURDEN OF *STAPHYLOCOCCUS AUREUS* INFECTION

Staphylococcus aureus was first isolated by Alexander Ogston, a surgeon, in the late 1800s and it has been a persistent problem for the following 150 years. Ogston noticed cocci shaped organisms from a pus-filled abscess in a human leg, immediately identifying two key features of *S. aureus* its small round, coccoid shape, and its ability to form abscesses in the mammalian host (1,2). As a commensal bacterium, *S. aureus* innocuously colonizes around 30% of the population (3). For the most part, this leads to colonization of the skin and a harmless commensal relationship with the host. Severe infections can present in blood stream, systemic infection can lead to numerous symptoms including death (4,5). To further the impact of this threat, *S. aureus* is a leading cause of skin and soft tissue infections (ssti) such as cellulitis and folliculitis (6,7). In both the hospital and the community, *S. aureus* infections cause between 11.6 and 14.2 million infections per year in the United States alone, highlighting the burden on the healthcare system *S. aureus* causes (8).

Infections are increasing in both incidence and difficulty to treat as antibiotic resistant strains present themselves around the world (9). Methicillin-resistant *S. aureus* has become a common adversary in the health care environment (9,10). The ability of *S. aureus* to acquire antibiotic resistance genes through horizontal gene transfer is astounding. This ability to gain resistance genes has caused *S. aureus* infections to reach epidemic levels (11). Currently there are strains that are resistant to vancomycin, gentamicin, penicillin, streptomycin, erythromycin, and tetracycline (11). The genetic versatility of *S. aureus* is one of the most difficult threats to combat in the clinic as even newer antibiotics can be useless against MRSA in just 6 months' time (11). The ability

of the bacterium to acquire resistance has led pharmaceutical developers to abandon the idea of antibiotics at all. The need for mechanisms to keep MRSA in check grows daily.

THERAPEUTIC TREATMENT

Treatment of this opportunistic pathogen has been difficult since the first therapeutic, penicillin, arrived in the early 1940's (9). Penicillin-resistant *S. aureus* was caused by the bacteria's ability to acquire new genes and retain them (12). After penicillin resistant clones took over as the dominant hospital and community acquired strain, methicillin was developed and introduced (9,13). One key difference between these resistance adaptations was that penicillin resistance specifically inhibited only the effects of penicillin. The resistance mechanism to methicillin was broad spectrum, meaning that resistance was applicable to the entire class of beta-lactam antibiotics (9,14). Development of so-called Methicillin-resistant *S. aureus* (MRSA) strains necessitated additional reliance on vancomycin (15,16). Soon after, MRSA became increasingly common in the community, still able to cause immense infection and death (17). During this push to the community, the current endemic strain of MRSA (USA300) first arose (18). Since then, USA300 clones have run rampant across America (61). The strains described in this dissertation are from the USA300 genetic background. Current treatments lag behind the evolution of this troublesome pathogen. Treatments include daptomycin or vancomycin and even fully resistant or intermediately resistant clones of *S. aureus* exist to both of these therapies (19). The alternative to these treatments is ceftaroline (20). While these can usually control infections, the need for novel therapies is clear, as the incidence of these infections is still significant (21).

STAPHYLOCOCCAL HOST ADAPTATION AND IMMUNE EVASION

S. aureus has been evolving with the human host for 100s of thousands of years (1,3). This co-evolution has allowed *S. aureus* to become extremely skilled at several functions key to survival in the host. Generating resistance to antibiotics is a critical aspect of *S. aureus* physiology, but other fundamental traits of pathogens include host immune evasion and nutrient acquisition. All organisms require many nutrients to survive and propagate, *S. aureus* is no exception; some well-studied examples include iron (Fe) and zinc (Zn) (22). As these nutrients are essential for *S. aureus* survival, it is no surprise that there are entire genetic systems that have been developed to acquire these nutrients. Examples of this are the zinc uptake regulator and ferric uptake regulator that respond to the environmental presence of metal ions and regulate uptake and transport (22,23). These well-developed systems illustrate a small fraction of how well evolved *S. aureus* is to the human host.

Another example of *S. aureus* co-evolution with the human host is its ability to disrupt functions of the immune system. The first example of this is the chosen niche of *S. aureus*, the human skin. The skin is the largest organ as well as our primary defense against internal bacterial infection. *S. aureus* colonizes and propagates on our skin (24). Much of the epithelial surface produces mucin or glycoproteins to prevent physical adherence by microbes (24). Wall teichoic acid, a cell surface expressed polymer of the staphylococcal cell envelope, allows for direct epithelial adhesion even under sheer stress that would be experienced when interacting with epithelial cells (25). Along the surface of most epithelial cells reside the defensins, a generalized antimicrobial powerhouse that are also abundant in neutrophils where they help facilitate bacterial

killing (26,27). Defensins are a multi-faceted molecule and offer many ways to prevent pathogenic propagation. They can interfere with cell wall synthesis, but in *S. aureus* they specifically bind to important precursor molecules for cell wall components. To further prove defensin versatility, in other bacteria they can even block export pumps to prevent release of toxic analogs and cause pathogenic bacterial cell death (26). *S. aureus* is able to survive on the surface of epithelial cells despite the presence of these defensins.

Once the immune system elicits an innate response to threats it can identify, *S. aureus* has more abilities to allow for survival after being engulfed by the immune cells. One tool is adenosine synthase A (*adsA*), a gene required for *S. aureus* survival in the host, is a cell surface expressed staphylococcal protein that bolsters the amount of free adenosine monophosphate in the extracellular space (28). This increase in free adenosine reduces successful killing of bacteria that have been phagocytosed by altering host production of inflammatory cytokines (28). In both *S. aureus* and *Bacillus anthracis*, the presence of the adenosine synthase promotes bacterial survival upon phagocytosis and the absence of the gene leads to bacterial cell death (28). Adenosine reduces production of hydrogen peroxide in the presence of inflammatory stimuli (29). The tempered inflammatory response is due to increased production of interleukin 10 (IL-10), an anti-inflammatory cytokine that is produced in response to activation of an adenosine receptor, A_2B. Therefore, bacterial production of adenosine by AdsA increases IL-10 production, decreasing inflammation, and promotes bacterial survival (30,31). Adenosine also causes decreased platelet aggregation, another inflammatory response (32,33). Thus, the ability to increase the amount of adenosine potently

dampens the innate immune response to staphylococcal infection and underscores the intimate co-evolution between host and microbe that directly leads to *S. aureus* pathogenicity.

Prolonged infection eventually leads to the highly specific adaptive immune response, but *S. aureus* has also evolved mechanisms to thwart this precisely orchestrated host attack. A critical aspect of the adaptive immune response is the production of highly specific immunoglobulins, proteins produced by plasma cells in response to specific pathogens, for the intents and purposes of this thesis, bacteria (34). *S. aureus* produces staphylococcal protein A (Spa) which binds to immunoglobulins. This binding caused precipitation in the blood which began the identification of protein A (35). Protein A has four highly homologous regions that are specific to binding immunoglobulin G (36). Normally, upon further processing, immunoglobulin M (IgM) is recognized and trafficked into B-cells for downstream production of specific antibodies (37,38). Protein A can also bind loose IgM on the surface of B-cells, interrupting IgM trafficking (39,40).

Bacterial recognition by the immune system stimulates the complement cascade. The complement cascade is a series of host derived proteins that form pores in the membrane of invading cells and cause the cells to lyse (41). To cope with this, *S. aureus* produces another abundant surface-expressed protein, staphylococcal binder of immunoglobulin (Sbi) (42). As the name suggests, this protein binds immunoglobulins similar to staphylococcal protein A; an added function of this protein is its ability to bind complement and disrupt the function of host complement proteins (43). Sbi, can form a complex with factor H, a major regulator of the complement system, preventing human

complement factor H from completing its function of upregulating the complement cascade (43). Expression of both Spa and Sbi promotes potent immune evasion. These factors highlight the pathogenic potential of *S. aureus* but also underscore why it has been difficult to generate protective immunity via vaccination. Therefore, current therapeutic strategies rely on antibiotics, but this requires consistent development of new molecules and regimens to stay one step ahead of the inevitable evolution of resistance.

S. AUREUS ENERGY GENERATION DURING INFECTION

A potential therapeutic target is inhibiting the metabolic pathways that produce energy needed for proliferation in the host. However, *S. aureus* is endowed with metabolic versatility and has more than one way to generate energy for its survival and propagation. When oxygen is present, bacteria can utilize the electron transport chain to perform oxidative phosphorylation, generating energy. In the absence of oxygen, but in the presence of another electron acceptor, anaerobic respiration is an available energy generating pathway. In the absence of any terminal electron acceptor, some bacteria can ferment, although the return on investment is considerably less than either aerobic or anaerobic respiration (44,45,46). As a facultative anaerobe, *S. aureus* is capable of all three. The fact that *S. aureus* encodes two terminal oxidases that both contribute to aerobic respiration in the host further emphasizes its metabolic versatility (**Figure 1.1A**) (47). These terminal oxidases, QoxABCD and CydAB, are able to function independently or in conjunction with each other. Numerous bacteria concomitantly express multiple terminal oxidases which results in a branched electron transport chain (47). This versatility allows bacteria to rapidly adapt to different environments and

changing oxygen concentration, a trait that is beneficial to a pathogen like *S. aureus* that colonizes multiple distinct tissue sites. The importance of a branched respiratory chain was established experimentally using a systemic mouse model of infection (47). In this model, mice infected with a cydB mutant strains harbored less bacteria in the heart while mice infected with a qoxB mutant exhibited decreased bacterial colonization of the liver. These results demonstrate that each terminal oxidase promotes colonization of distinct host organs (47,48).

A common requirement of CydAB and QoxABCD are the cofactors heme and menaquinone (MK). CydAB is composed of a cytochrome bd while QoxABCD contains a cytochrome aa3. Cytochromes are heme-containing polypeptides; heme is a porphyrin ring containing iron (49). In addition to the requirement of oxygen-binding via heme, both terminal oxidases also receive electrons from the MK pool (49). The electrons are eventually passed onto oxygen (O_2) generating water (H_2O). In contrast to CydAB, QoxABCD reduction of O_2 to H_2O contributes to the generation of the proton motive force (PMF), which is converted to chemical energy in the form of ATP by the F_1F_0 ATPase (49). CydAB does not directly contribute to the PMF (50).

As a facultative anaerobe, *S. aureus* is capable of generating ATP in the absence of oxygen via fermentation (51). The ability to ferment glucose offers a valuable energy-generating pathway when *S. aureus* colonizes environments that are not conducive for aerobic respiration (52). For example, a distinct feature of *S. aureus* infections are tissue abscesses, which are macroscopic representations of the innate immune response. Neutrophils are recruited to the site of infection where they attempt to clear *S. aureus* utilizing various methods. Neutrophils recognize pathogen associated

molecular patterns (PAMPs) and respond to them by generating reactive oxygen species and cytokine release, both eventually leading to bacterial cell death (53). Recruitment and activation of neutrophils by bacteria causes abscesses to form in response to the invading species (54). Abscesses are limiting for both oxygen and iron, indicating that fermentation is the predominant metabolic pathway active in this unique environment (**Figure 1.1B** and **1.1C**) (55,56,57). Iron is required to produce heme; therefore, the abscess environment also skews the metabolic preference towards fermentation. Additionally, a major component of the innate immune response is the generation of the reactive nitrogen species like nitric oxide (NO), which compete with oxygen for binding to the terminal oxidases. Consequently, in response to NO exposure, *S. aureus* upregulates *ldh1* a lactic acid dehydrogenase that supports lactic acid fermentation (53,54). A murine sepsis model revealed that infection with the ldh1 mutant leads to reduced mortality, decreased abscess formation, and a competitive disadvantage compared to WT *S. aureus*. Notably, attenuation of the *ldh1* mutant was restored to WT-like levels when the mutant infected mice lacking inducible NO synthase (iNOS) (58). These results underscore the importance of fermentation in response to host-derived NO during sepsis and overall, fortify the conclusion that *S. aureus* utilizes two major energy-generating pathways to proliferate in the host, aerobic respiration and fermentation. Therefore, therapeutic strategies that inhibit fermentation should synergize with those that inhibit aerobic respiration.

THE SMALL COLONY VARIANT (SCV) PHENOTYPE

Decreased ATP production that results as a consequence of fermentation causes a unique colony morphology called the small colony variant (SCV). SCVs can be the

etiological agent of persistent infection. In persistent infections, the host can impose pressure for distinct metabolically restricted *S. aureus* cells (57,59). Generating energy through fermentation allows for *S. aureus* survival within the host but these cells can return to their normal phenotype if the environment changes to less restrictive conditions (56,58,59). SCVs have been isolated in culture since 1944 and from the host since 1995 (57,59).

SCVs develop as result of exposure to aminoglycoside antibiotics. Aminoglycosides require the PMF to access the cytoplasm and inhibit translation. In response, *S. aureus* develops inactivating mutations in pathways that support respiration, such as heme and MK, that decreases the PMF. While the mutated cells are resistant to aminoglycoside antibiotics, a fitness cost is imposed as the mutants are restricted to fermentation, resulting in the SCV phenotype (**Figure 1.2**). As the name suggests, SCVs are considerably smaller than their WT counterparts (60,61). SCVs are around one tenth the size of the WT of the same strain (60,61). Another consequence of the SCV phenotype is decreased growth in liquid media, as the time required for SCVs to reach an overall reduced terminal optical density (OD) in vitro is longer (62). Another key aspect in identifying SCVs is their lack of pigmentation.

Sometimes these hallmark phenotypic traits are permanent, and the phenotype cannot switch back to the respiring, WT colony physiology. Up to 50% of cystic fibrosis patients can have SCVs isolated from them (63). In total, the prevalence of SCVs in patients treated with aminoglycoside antibiotics highlights our ability to manipulate *S. aureus* metabolism during infection (64,65). Therefore, identifying pathways that support

SCV proliferation and the subsequent development of inhibitors for those pathways generates a feasible therapeutic strategy for *S. aureus* infections.

DISSERTATION OVERVIEW AND CONCLUSIONS

Though *S. aureus* has been around for hundreds of years, it continues to be a substantial burden to the healthcare system and new targets and treatment options need to be explored to combat this threat. The metabolic diversity to colonize host tissues, the persistent SCV phenotype, and the problematic resistance to antibiotics are too serious a threat to continue to go unchecked.

The transition between respiring, WT cells to persistent, fermenting SCVs is an antibiotic resistance strategy that also poses a unique opportunity to treat *S. aureus* infections with the clinical potential to cure infections completely. Chapter 2 of this dissertation aims to exploit this unique pathway. Treating with an aminoglycoside antibiotic, encouraging development of the SCV phenotype which can then be targeted with a therapeutic that specifically inhibits fermentative metabolism could provide a complete treatment for MRSA infections (**Figure 1.3**).

This dissertation aims to illuminate vulnerabilities in one of the most potent weapons of *S. aureus*, the SCV. The screen described in Chapter 2 highlights four compounds with anti-SCV specific activity and explores their mechanisms of action: highlighting the potential for new therapeutics. Chapter 3 utilizes the same metabolically specific vulnerability, identifying new SCV-specific inhibitors, saturated fatty acids. This growth defect is likely due to inhibition of a pathway distinct from the one targeted by the four compounds identified in Chapter 2 and offers another avenue to pursue a potential

dual therapy. The final chapter of this dissertation summarizes our findings and proposes future experiments to continue this work, moving towards therapeutics.

FIGURES

Figure 1.1. The host imposes metabolic restriction of *S. aureus* respiration. Three examples of the mammalian host imposing restrictions upon *S. aureus* metabolism. **A.** The two terminal oxidases of *S. aureus*, embedded in the cellular membrane, function by transferring electrons from oxygen to water. Host generated nitrous oxide competitively inhibits oxidase function by mimicking oxygen. **B.** Immunohistochemistry of a probe that responds to hypoxia. Over time the environment inside of the abscess becomes hypoxic. **C.** After 4 days of infection, mouse kidneys were removed and imaged. Arrows represent the location of abscesses. Images show mouse kidneys harboring *pXen1* (a luminescent empty vector) or *pisdlXen1*. The luminescent reporter is controlled by expression of *isdl,* which is upregulated in the absence of iron. Iron is another requirement for terminal oxidase activity.

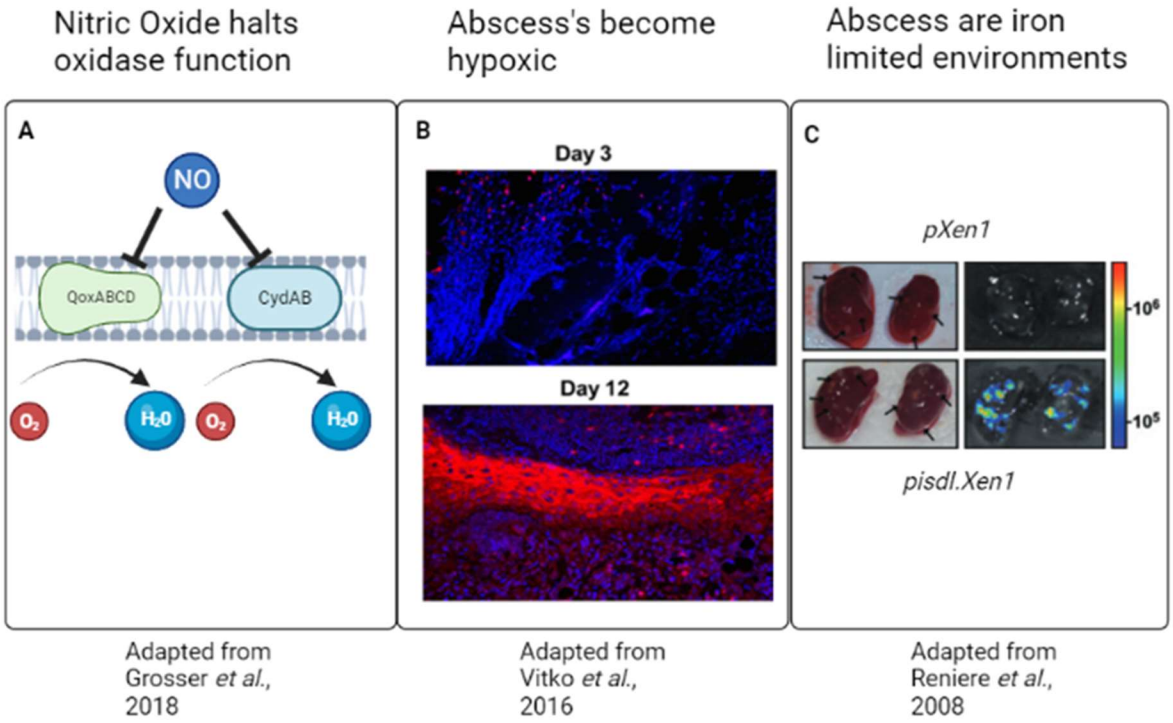


Figure 1.2. Wild type and fermenting SCV colonies. Examples of both the WT and SCV phenotypes on a tryptic soy agar plate, the SCV colonies are smaller and more transparent than WT counterparts.

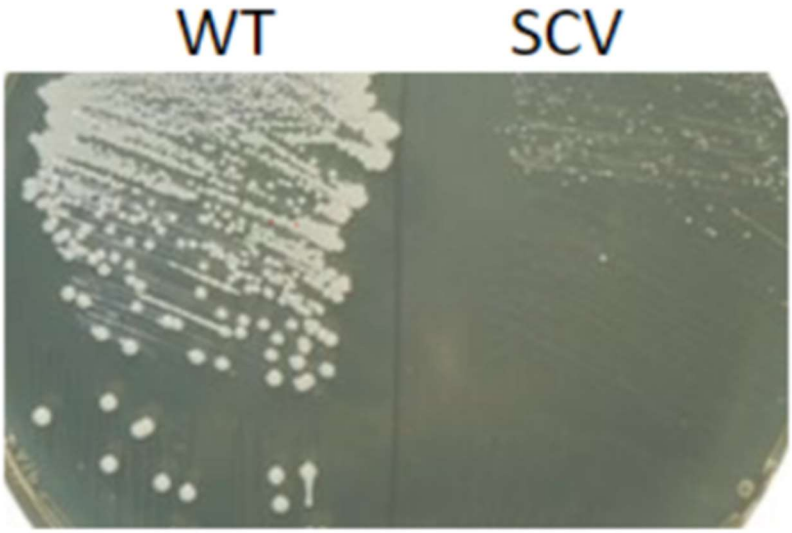
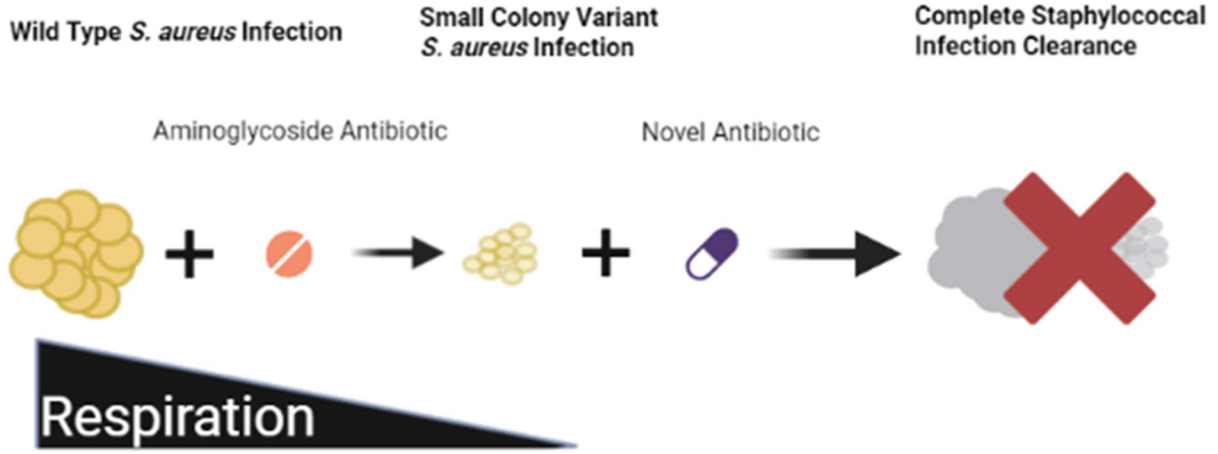


Figure 1.3. Development of a potential dual therapeutic treatment for *S. aureus* infections. A graphical display of a potential treatment regimen for *S. aureus* infections in the clinic. A decrease in respiration, triggered by aminoglycoside antibiotic use, in combination with another new antibiotic that exhibits bacteriostatic or bactericidal activity on fermenting *S. aureus* cells could fully clear infections in the clinic.



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CHAPTER 2: SCREENING OF ANTIMICROBIAL COMPOUNDS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*.

ABSTRACT

Staphylococcus aureus is a Gram-positive cocci bacterium that causes a wide range of diseases. It is the leading cause of endocarditis, toxic shock syndrome, osteomyelitis, and bacterial sepsis in the United States. *S. aureus* is environmentally abundant and asymptomatically colonizes around 30% of the population. Additionally, this pathogen modulates its metabolism to adapt to the host environment. Throughout infection, a consequence of metabolic adaptation is switching to fermentation, a less energetically productive process that leads to the small colony variant (SCV) phenotype. This modulation is especially important in the clinic because SCVs are more resistant to aminoglycoside treatment. We hypothesize that dedicated physiological pathways sustain the SCV phenotype. Therefore, identifying these pathways and developing cognate inhibitors has the potential to restrict *S. aureus* metabolic versatility, growth, and its ability to resist aminoglycosides. To reveal druggable pathways in SCVs, several screens were performed. The GSK Published Kinase Inhibitor Set (PKIS) library as well as two chemical libraries composed of novel plant-inspired compounds were screened for molecules that inhibit growth of a menaquinone-deficient *S. aureus* SCV mutant called *menE*. Notably, this strain was generated in the methicillin resistant, USA300 laboratory derived strain JE2. Our preliminary screen identified nine compounds that significantly inhibit SCV growth. A secondary screen was conducted using a wild type (WT) strain as well as the *menE* SCV mutant strain to determine whether the compounds were selective for SCVs or broadly inhibitory against *S. aureus*. This analysis demonstrated two distinct classes of compounds: those that inhibit both WT and SCV mutant growth and those that selectively inhibit the SCV mutant but appear to

increase growth of WT *S. aureus*. These compounds provide evidence that specific energy generating pathways can be targeted for antibiotic treatment, helping to identify sensitive pathways in the SCV phenotype leading to better treatment outcomes in the future.

INTRODUCTION

The Gram-positive pathogen *Staphylococcus aureus* poses significant clinical challenges. *S. aureus* is abundant within humans as it asymptomatically colonizes approximately 30% of the population (1). The Centers for Disease Control categorizes Methicillin-resistant *S. aureus* (MRSA) as a serious threat to public health, responsible for more than 30,000 infections in the U.S. yearly (1,2). It is the leading cause of skin and soft tissue infections endocarditis, toxic shock syndrome, osteomyelitis, and septicemia in the United States (3,4). Importantly, treatment of staphylococcal infections is complicated by the prevalence of antibiotic resistant strains (4). Additionally, this pathogen can modulate its metabolism to adapt to the host environment. Throughout infection this adaptation is often expressed through fermentation, leading to the SCV phenotype (3,6). Modulating metabolism is especially important in the clinic because SCVs are more resistant to aminoglycoside treatment allowing them to survive exposure to the antibiotic (3). *S. aureus* infection can also lead to abscess formation within major organs such as the heart, kidney, lungs, and liver creating an anoxic environment that benefits fermenting cells (7). This metabolic versatility becomes an even bigger issue when it coincides with antibiotic resistant strains of *S. aureus* such as Methicillin-resistant *S. aureus* (MRSA), which poses a much greater health risk (1,2).

Increasing our understanding of metabolic versatility will allow us to discover clinically relevant treatment strategies.

While Methicillin-resistant *S. aureus* represents a considerable clinical challenge, this pathogen increases resistance further by modulating metabolism (1,2). Reducing metabolic potential by switching to fermentation results in the SCV phenotype which can be the etiological agent of persistent infections (3,8). SCVs are characterized by slower growth, which manifests as a smaller, semi-transparent colony morphology when the bacterial cells are cultured on agar plates. In the clinic, SCVs can be isolated from up to 50% of cystic fibrosis patients (3). Previous work also shows that without surgical removal, prosthetic joint *S. aureus* infections cannot be treated with antibiotics, resulting in a chronic infection (3,8).

One of the most common types of SCVs isolated from cystic fibrosis patients are those inactivated for menaquinone (MK) or vitamin K2 synthesis (3). MK is an essential electron carrier in the *S. aureus* electron transport chain. Therefore, MK mutants are restricted to fermentation to support growth, resulting in the SCV phenotype. Heme biosynthesis mutants are another type of SCV isolated frequently from patients (3). During electron transport, reduction of heme cofactors within cytochromes of the terminal oxidases provides vital electrons to help complete aerobic respiration. Heme mutants do not synthesize heme and are forced to ferment due to loss of terminal oxidase activity (5). In the clinic, SCVs are described by the compound which when added to media restores WT growth and virulence (3,8). Though the occurrence of heme and MK mutants has been established, SCVs are not understood well enough clinically because it is hypothesized that they appear at different times during infection.

Observing genetically distinct SCVs provides the opportunity to study clinically relevant antibiotic resistant *S. aureus* strains that closely mimic those in a hypoxic-like state in the host environment. Therefore, understanding genetic factors that drive this metabolic state will provide new therapeutic approaches for treating all possible *S. aureus* infections.

Current screening methods often consist of smaller screens of small molecule inhibitors (9-12). This is problematic for two main reasons, the need for MRSA treatments is urgent and screening low numbers of compounds is less efficient in producing new drugs (9-12). Secondly, many molecules in these screens are too flexible and cannot retain functional shape when entering ligand binding sites, two dimensional molecules are easily misshapen. This causes a decrease in efficacy both in vitro and in vivo. A great therapeutic prospect that faces this issue are small interfering RNA molecules (13,14). To fully utilize these molecules, significant time is spent developing delivery methods that further delay the therapeutic from entering the clinic (14). To overcome these major issues that plague screening methods, high throughput screening techniques are being developed to enable a much faster drug development process (15). These screens enable many more drugs to challenge the bacteria and often return more positive results than traditional screens. Since so many compounds are being screened at once, classes of compounds that inhibit proliferation of bacterial species can be identified from these screens (16,17). To attempt to address some traditional screening issues, a different pool of products that are architecturally inspired by plants will be screened. Over 60,000 terpenoids have been isolated from nature and some have antimicrobial action against a diverse set of bacteria (18). Terpenoids are a

natural structure found in plants and are all commonly derived from a five-carbon isoprene (18,19). Even though these structures are abundant in nature, a synthetic approach can be utilized to generate novel terpenoids not found in nature (20-22).

RESULTS

High throughput screening reveals SCV-specific inhibitors of growth.

Developing SCV-specific inhibitors will increase our capacity to effectively treat persistent staphylococcal infection. Additionally, a dual therapeutic composed of an aminoglycoside, which promotes SCVs, with a SCV-specific drug could prove to be a potent addition to anti-*S. aureus* clinical strategies. To determine whether SCV inhibitors could be identified, we screened the 650 compounds of the Glaxo Smith Kline Published Kinase Inhibitor Set as well as two plant-inspired compound libraries developed at Michigan State holding a total of 150 compounds. This was accomplished with help from the Assay Development and Drug Repurposing Core at Michigan State University (**Figure 2.1**) (23). The phenotypic screen monitored growth of both Je2 WT and the *menE* SCV mutant by quantifying absorbance at optical density 600 nanometers (nm) over time. Je2 is a lab-derived USA300 strain which is currently endemic in the United States. Therefore, using this strain provides us with information directly pertinent to the clinic (24).

Compounds GW405841X and GW809893X selectively inhibit SCV proliferation.

Screening 640 compounds revealed five that inhibited *menE* growth (GSK1487252A (GSK), SB-707548-A (SB), GW405841X, GW809893X, and MSU 9052) (**Figure 2.1**). Of these compounds four were not previously known to inhibit *S. aureus* growth. To determine whether the compounds generally inhibit *S. aureus*

proliferation or are specific for *menE*, WT growth in the presence of each compound was quantified. This analysis revealed that two of the compounds, GSK1487252A and SB-707548-A, inhibited WT proliferation (referred to as SB and GSK, respectively). However, the other two compounds, GW405841X and GW809893X (referred to as '841 and '893 throughout), promoted growth of WT *S. aureus* (**Figure 2.2 A-D**). This screen also confirmed findings of a previously discovered inhibitor of *S. aureus* growth, MSU 9052 that was previously observed (25). Notably, MSU 9052 was not tested against a strain in the endemic USA300 background or in a SCV (25). All of these compounds have a 50% Inhibitory Concentration (IC_{50}) below 10 μM except for those that promote growth in the WT (**Table 2.1**).

Structural analysis of WT growth-promoting compound GW809893X.

The WT growth-promoting compound, '893, contains moieties similar to other known antibiotic compounds. Para-aminobenzoic acid (PABA) and sulfanilamide (Sulf) are similar to the individual components that comprise compound '893 (**Table 2.2**). PABA is essential in *S. aureus* because it is an intermediate in folic acid synthesis (26). Folic acid is a constituent important for synthesis of pyrimidine nucleic acids used in DNA synthesis (**Table 2.2**) (26). Sulf is a competitive inhibitor of the enzyme that utilizes PABA in folic acid synthesis (27). Sulf derivatives have appeared in antibiotic screens before with sulfamethoxazole eventually being utilized in the clinic (28). Prior to this study, these compounds have never been shown to promote bacterial growth. Yet, we observed that PABA and SULF promote growth of WT *S. aureus* but have no effect on *menE* proliferation (**Figure 2.3 A, C**). I hypothesize that the differential response to '893 and '841 is due the ability of WT to catabolize the compounds into growth promoting

factors. I predicted that the individual moieties are growth promoting factors. To test this, I cultured WT in the presence of both PABA and Sulf, I found that compared to untreated cells PABA and Sulf promote WT growth but did not affect SCV growth (**Figure 2.3 B, D**).

Mutating the putative PABA transporter, AbgT, does not affect PABA growth promotion.

In other species of bacteria, PABA is imported via the AbgT transporter (29,30). To determine whether a similar mechanism is active in *S. aureus*, the *abgT* mutant was isolated from the Nebraska transposon mutant library (24). The *agbT* mutant exhibited similar growth in media supplemented with PABA and Sulf compared to WT (**Figure 2.4** A, B) (31). These results indicate that PABA and Sulf are entering the cell via one or more other transporters. Identifying these key PABA and Sulf importers is essential to provide additional insight into the factors that facilitate this unique growth promoting phenotype.

Novel plant inspired therapeutics produce anti-staphylococcal drug mixtures.

In addition to the PKIS library screen, around 150 novel terpenoid compound mixtures produced by the Hamberger laboratory at Michigan State University were also screened for anti-staphylococcal activity. Both the WT and the *menE* fermenting mutants were screened for antimicrobial activity by monitoring growth. While none of the extracts conclusively inhibited WT growth in primary screens, 14 of these novel terpenoid mixtures prevented at least 50 percent of average *menE* mutant proliferation (**Figure 2.5**). Since these were complex mixtures of compounds and not specific

terpenoids, further work is required to determine which structures have anti-SCV proliferation activity.

DISCUSSION

Aerobic respiration and fermentation coordinate *S. aureus* energy generation during colonization of host tissues. In response to aminoglycoside antibiotics, *S. aureus* switches to fermentation, proliferating as a SCV. I identified several novel anti-SCV compounds. Compounds ‘841 and ‘893 also promote WT growth. Isolating two distinct compounds ‘841 and ‘893 that are considerably different structurally but cause similar phenotypes raises several questions. Primarily, how do these compounds inhibit SCV growth, and how do they promote WT *S. aureus* growth? Compounds within the GSK-PKIS inhibit kinase activity in mammalian cells. Kinases are a class of enzymes that activate biological reactions through phosphorylation (23). GSK1487252A targets kinase group AGC which are cytoplasmic serine/threonine kinases. SB-707548-A targets receptor interacting protein kinases that are important to innate immunity. GW405841X inhibits a serine/threonine kinase that assists in cell fate determination. GW809893X acts upon VEGFR1, a tyrosine protein kinase that is essential for vascular growth in embryos. While the activities of these kinase inhibitors are known in some human and animal cells, their role in *S. aureus* is not known as these enzymes are not present in *S. aureus* (**Table 2.2**).

We hypothesize that these compounds are being metabolized differently in the respiring WT strains than in the fermenting SCVs. Further investigation could therefore identify new mechanistic differences between WT and SCVs beyond the overt metabolic distinctions. To dive deeper into the mechanisms of action of each compound, similarly

structured compounds were tested to identify any key moieties in compound activity. Even though compound MSU-9052 is chemically similar to ‘841 their activity is not the same. MSU-9052 inhibits the growth of both WT and *menE* while ‘841 only inhibited *menE* proliferation. This result shows that the change of a carbonyl group to an iodine significantly alters activity. Supplementing the growth medium with PABA and Sulf, chemicals similar to ‘893 moieties, did not affect proliferation of *menE*. This finding indicates that the toxic effects of ‘893 do not stem from the part of the molecule that strongly resembles Sulf. When added to WT cultures, Paba and Sulf managed to promote growth which supports the idea that the growth enhancement phenotype is due to ‘893 catabolism. The *agbT* mutant also exhibited growth enhancement in the presence of PABA or Sulf, indicating that there may be another transporter for this class of compounds. This simplified structure activity relationship analysis offered much insight into the mechanisms of action of ‘841 and ‘893 but further work is required to identify specific proteins and genes responsible for the observed phenotypes.

Combating resistant strains can be especially difficult because *S. aureus* has the propensity to become further recalcitrant to therapies by altering its metabolism via induction of the SCV phenotype (3). SCVs are isolated from patients afflicted with persistent infections (3). In the clinic, some persistent *S. aureus* infections are also caused by biofilm formation and not the planktonic SCV phenotype. These are difficult to treat such that they often necessitate surgical removal from patients (8). Much like the SCV phenotype, these biofilms thrive in hypoxic and anoxic environments which have been observed during infection (32). Thus, there is a need to identify compounds that can effectively penetrate and treat biofilms. Due to the similarity in the conditions

that promote both SCV and biofilm growth, I hypothesize that compounds ‘841 and ‘893 will be effective against *S. aureus* WT biofilms. Therefore, increasing our understanding of *S. aureus* metabolism has the potential to mitigate resistance in the clinic. A more complete understanding of the SCV phenotype will lead to more effective treatments for *S. aureus* infections. Throughout the course of infection, anoxic or hypoxic environments in the host promote a fermentative, SCV-like state, providing an opportunity for therapeutic intervention (7). Isolating compounds that inhibit SCV proliferation is the first step but identifying the targets of anti-SCV compounds stimulates the design of second-generation compounds with increased specificity. Terpenoids and other natural products have been screened for anti-staphylococcal activity in prior assays but compounds specifically inhibiting fermenting *S. aureus* have not been a focal point of such screens. The findings in this screen open a clear framework for the isolation of more compounds that target fermenting *S. aureus* cells specifically. Further development of these drugs could prove extremely beneficial in treating infections in the clinic.

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MATERIALS AND METHODS

Bacterial strains

Laboratory derivatives of the Methicillin-resistant *S. aureus* USA300 LAC strain, JE2 was used as wild type (WT) throughout this work (34). The Nebraska transposon mutant library provided the *agbT* transposon mutant (35,36).

High through put antimicrobial compound screens

Assessment of growth will be performed using absorbance values measured in a spectrophotometer as previously described. The screening approach will include both an untreated positive growth control and a previously identified effective antibiotic (chloramphenicol) as a negative growth control for WT and the SCV mutant. SCVs will be cultured in an orbital shaker overnight (24 hours) and absorbance values recorded every hour. To ensure that the results are reproducible, the SCV growth assays will be repeated multiple times in the 384 well plate format. WT growth will also be monitored in the presence of these compounds to ensure that all compounds that proceed are SCV specific. The initial inhibition screen will be performed by the Assay Development and Drug Repurposing Core (DDRC) facility at MSU.

96-well plate growth curves

Strains were grown up overnight at 37° C shaking at 225 rpm. An absorbance at 600 nm was taken using a BioTek Epoch 2 microplate spectrophotometer and bacteria at a final concentration of 0.02 optical density (OD) 600 nm in fresh liquid media was added to a disposable trough. Another trough of fresh tryptic soy broth (TSB) with 2x the final desired concentration of inhibitory compound in μM was made. 100 μL of each trough was added to a 96 well clear round bottom plate and incubated shaking overnight at 37°

C. The plate reader was set to orbital shaking and measured absorbance at OD 600 nm every hour for 20 hours. After the final time point each well was perturbed by pipetting up and down and a final endpoint OD 600 nm was measured.

Inhibitory concentration 50% quantification

IC_{50} values of the bacterial culture's growth was calculated using GraphPad prism software. Terminal absorbance values were taken at 24 hours in TSB to establish 100 percent growth in untreated WT and *menE* bacterial cultures. Bacterial cultures were treated with a range of compound concentrations from 3.125 µM to 100 µM. Absorbance values at 600 nanometers were fit to a curve and the compound concentration at 50% total growth was recorded.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

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Contributions

Conceptualization: RAC, NDH. *Methodology*: RAC, NDH. *Software*: NDH. *Validation*: NDH. *Formal Analysis*: RAC, NDH. *Investigation*: RAC, NDH. *Resources*: NDH. *Data Curation*: RAC, NDH. *Writing*: RAC, NDH. *Visualization*: RAC, NDH. *Supervision*: NDH. *Project Administration*: NDH. *Funding*: NDH.

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Figure 2.1. Screening funnel for SCV specific inhibitors. A high throughput screen of 640 compounds was performed to identify inhibitors for *S. aureus menE* SCV mutant growth.

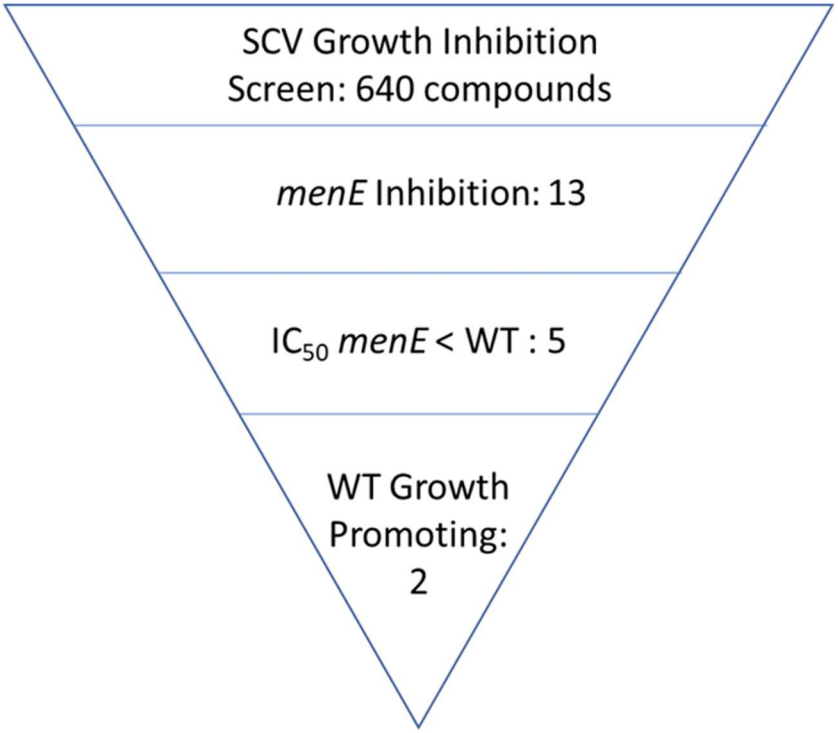


Figure 2.2. WT and fermenting *S. aureus* strains display divergent growth phenotypes in response to compounds ‘893 and ‘841. WT and *menE* growth dynamics over a 20-hour period with absorbance values being measured every hour at 600 nm Cells are grown in Tryptic soy broth with and without 20 μM ‘841 or ‘893 being added to the media. **A**, **B**. Growth in the presence of compound ‘893. C, D. Growth in the presence of compound ‘841. The same untreated, WT growth control is presented in A and C. The same untreated *menE* growth control is used in B and D. The error bars represent ± 1 standard error of the mean.

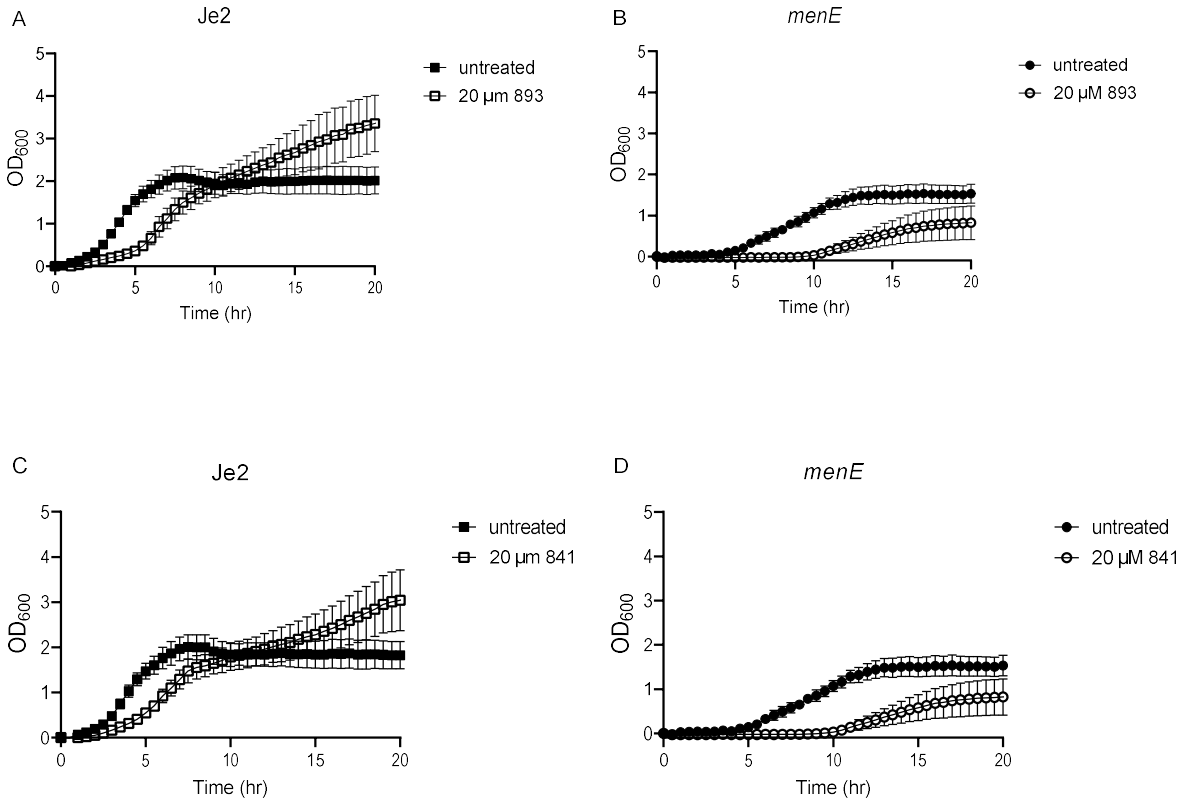


Table 2.1. IC50 values of SCV growth inhibiting compounds.

Compound	WT IC_{50}	*menE* IC_{50}
GSK1487252A	1.94 μM	0.97 μM
SB-707548-A	9.55 μM	8.92 μM
GW405841X	N.D.	7.12 μM
GW809893X	N.D.	4.16 μM
MSU-9052	2.04 μM	0.97 μM

Table 2.2. Structures of pertinent compounds

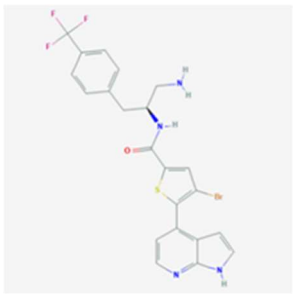
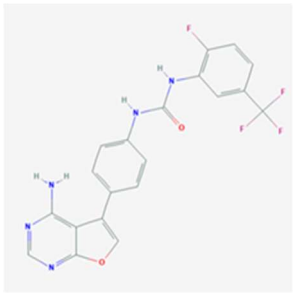
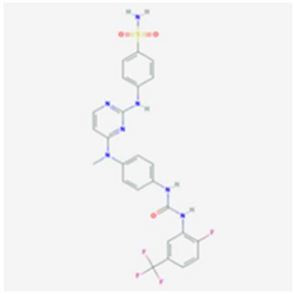
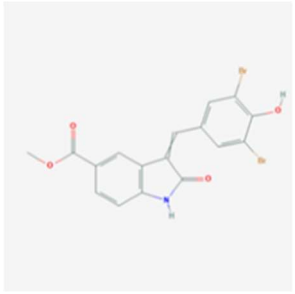
Compound	Structure
GSK1487252A	
SB-707548-A	
GW809893X	
GW405841X	

Table 2.2. (cont'd)

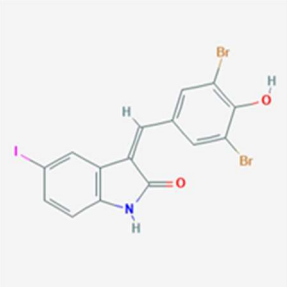
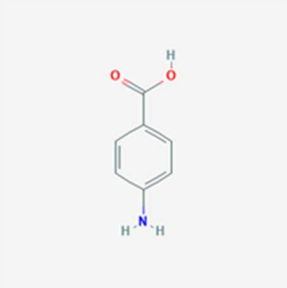
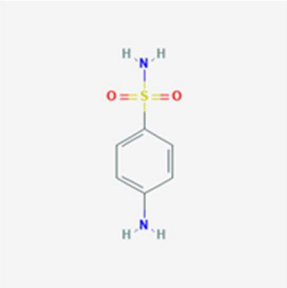
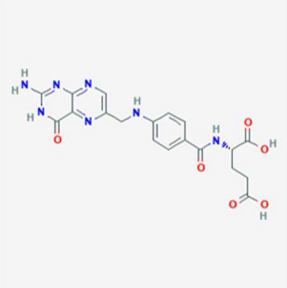
Compound	Structure
MSU-9052	
Para - Aminobenzoic Acid	
Sulfanilamide	
Folic Acid	

Figure 2.3. PABA and Sulf have different effects on WT and *menE* *S. aureus* proliferation. WT and *menE* growth dynamics over an 18-hour period with absorbance values being measured every hour at 600 nm Cells are grown in Tryptic soy broth with and without 100 μM Paba or Sulf being added to the media. **A**, **B**. Growth in the presence of PABA. **C**, **D**. Growth in the presence of compound Sulf. WT and fermenting *S. aureus* strains display divergent growth phenotypes in response to compounds ‘893 and ‘841. The same untreated, WT growth control is presented in A and C. The same untreated *menE* growth control is used in B and D. The error bars represent ± 1 standard error of the mean.

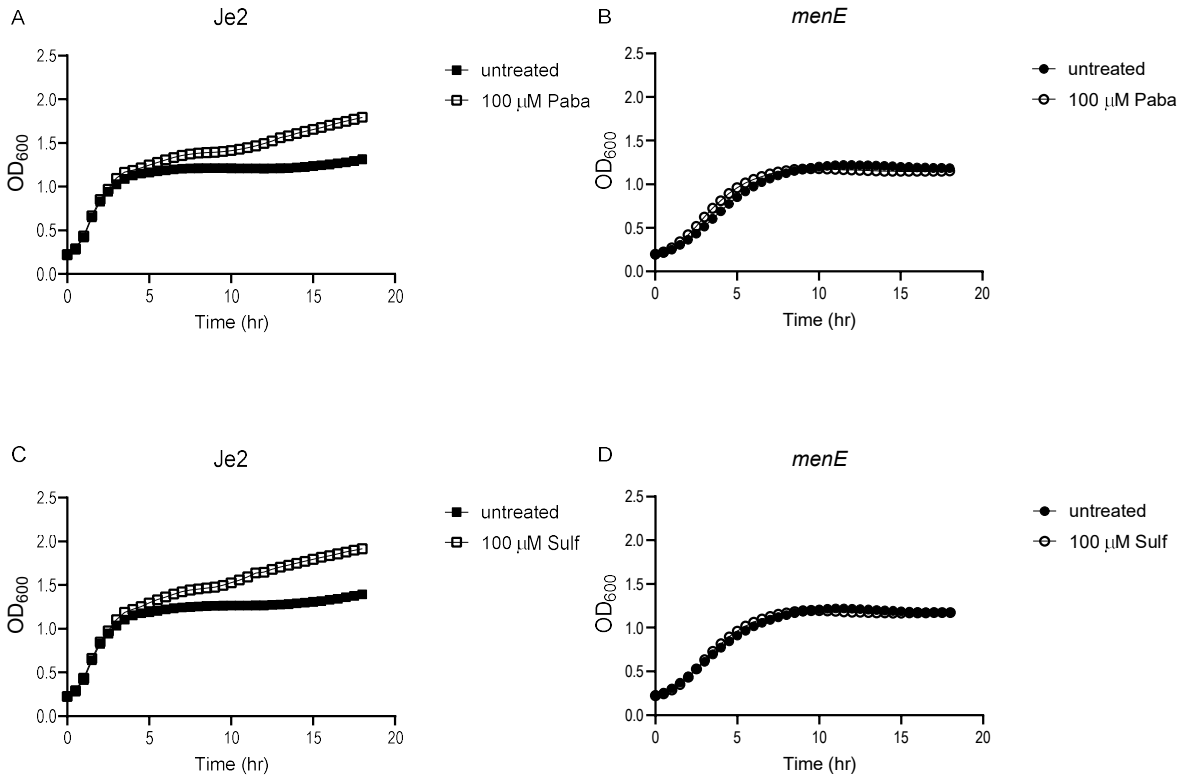


Figure 2.4. Transporter AbgT is not the cause of the growth promotion phenotype. WT and *abgT* growth dynamics over an 18-hour period with absorbance values being measured every hour at 600 nm Cells are grown in Tryptic soy broth with and without 100 µM Paba or Sulf being added to the media. **A**. Growth in the presence of PABA. **B**. Growth in the presence of compound Sulf. The error bars represent ± 1 standard error of the mean.

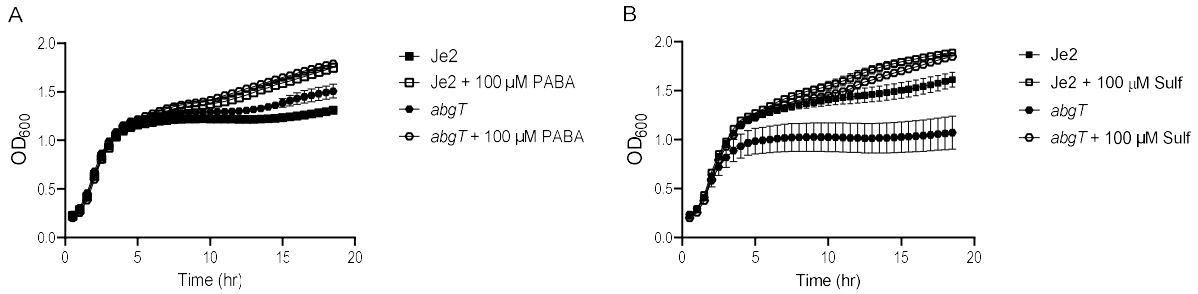
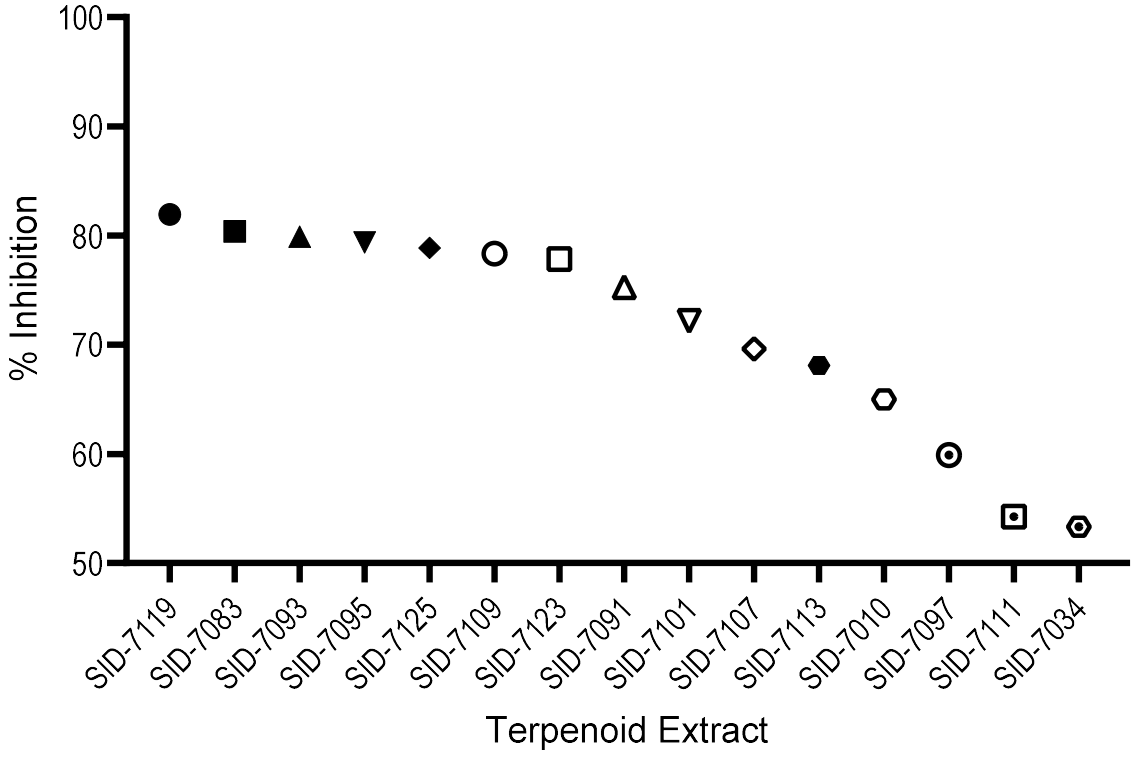


Figure 2.5. Terpenoid extracts inhibit proliferation of at least 50% of fermenting *S. aureus* cells. 14 different compound extracts that each displayed at least 50 percent inhibition against fermenting *menE* cells.



CHAPTER 3: EXOGENOUS FATTY ACIDS IMPEDE PROLIFERATION OF RESPIRATION-ARRESTED *STAPHYLOCOCCUS AUREUS*.

PUBLICATION NOTICE

The following chapter contains work that is currently in preparation for publication. Authors of this work are: Rajab Curtis[1,2], Phillip Delekta[2], Gabrielle Krzyske[2], Janani Ravi[3], Todd Lydic[4], and Neal D. Hammer[2]. [1] Indicates contributors from the Department of Comparative medicine and Integrative Biology, College of Veterinary Medicine, Michigan State University. [2] Indicates contributors from the Department of Microbiology and Molecular Genetics, College of Osteopathic Medicine, Michigan State University [3] Indicates contributors from the Department of Biomedical Informatics, University of Colorado. [4] Indicates contributors from the Department of Physiology, Michigan State University.

ABSTRACT

Staphylococcus aureus poses a major threat to human health due to its ability to cause a multitude of diseases and the prevalence of antibiotic resistant strains. Consistent with this, *S. aureus* is the leading cause of skin and soft tissue infections, septic arthritis, osteomyelitis, and endocarditis. To colonize distinct host tissues, *S. aureus* relies primarily on two energy-generating pathways, aerobic respiration and fermentation. Fermentation also supports development of the small colony variant (SCV) phenotype, a metabolically altered cell-type that is associated in the clinic with aminoglycoside antibiotic treatment and persistent infection. We hypothesize that dedicated physiological pathways sustain the SCV phenotype. Therefore, identifying these pathways and developing cognate inhibitors has the potential to restrict *S. aureus* metabolic versatility, growth, and its ability to resist aminoglycosides. To find new therapeutics we tested the antibiotic potential of saturated fatty acids against metabolically restricted *S. aureus* to decipher if *S. aureus* becomes sensitized to exogenous fatty acids. We screened biologically relevant fatty acids against respiring and fermenting *S. aureus* cells and show that fermenting *S. aureus* is sensitive to saturated fatty acids while respiring cells are not. Exploring SFA sensitivity has revealed that myristic acid (C14:0) is inhibitory towards fermenting *S. aureus*. Additional work demonstrates that critical membrane function is greatly impacted. Compared to untreated controls, SFA-treated cells exhibit decreased membrane potential and total ATP concentration is inversely affected in respiring and fermenting cells. Global lipidomics determined that respiring cells have altered phospholipid composition compared to fermenting cells and that SFA treatment increased the quantity of free fatty

acid in both metabolic types. While only fermenting cells are inhibited in growth, transmission electron microscopy revealed the presence of lipid droplets in respiring wild type treated with myristic acid implicating lipid droplet formation as a mechanism for protection. Overall, these data are consistent with a model that predicts *S. aureus* transition between different metabolic states relies on dedicated physiological pathways that are sensitive to SFAs. Identifying mechanisms by which SFAs elicit toxicity towards fermenting staphylococci will identify new therapeutic targets for this evasive threat.

INTRODUCTION

Staphylococcus aureus has been a burden on the healthcare system for hundreds of years amounting to hundreds of thousands of infections in both health care and community-associated environments (1,2,3). This has led the CDC to categorize Methicillin-resistant *S. aureus* as a serious threat. It is currently the leading cause of skin and soft tissue infections, and it can also cause bacteremia infections both being apt threats (4). In 2017 an endemic strain of *S. aureus* spread in the United States with an almost 10 percent death rate (1,5). This outbreak proves the need for novel methods of treatment for this prevalent microbial threat.

Furthering this already troublesome threat, *S. aureus* is metabolically versatile. Two terminal oxidases, CydAB and QoxABCD, allow for widespread host colonization by supporting respiration. These oxidases pass electrons to oxygen, completing respiration in respiring cells. Genetic mutation of the oxidases individually has shown organ specific colonization in a mouse model (6,7). In hypoxic conditions, like abscesses that can form in the host throughout infection, changes in the host environment lead to a completely respiration-arrested cell phenotype that relies fully on fermentation to drive energy generation (8,9). This unique smaller morphology is referred to as the small colony variant phenotype (SCV). Due to a decrease in membrane potential, these SCVs have increased resistance to antibiotics that need to be trafficked into the cell and SCVs are the etiological agent of persistent *S. aureus* infections (10,11). The ability to rely on two separate oxidases allows for further dissemination of *S. aureus* throughout the host and offers increased protection against

the immune system (6,12). In an already powerful infection, the SCV phenotype is a bacterial strategy that can often lead to worsening outcomes for infected individuals.

To treat or prevent infection the cell wall is often viewed as a potential therapeutic target, beta-lactam antibiotics are a popular class of antibiotics that target the cell wall (13,14,15). The glycoplomers wall teichoic acid (WTA) and lipoteichoic acid (LTA) are anchored into the phospholipid bilayer of the Staphylococcal cell membrane (17). The phospholipid bilayer separates important biochemical functions that are vital to the cell from the extracellular environment. As such, the construction of the phospholipid bilayer is an extremely involved process for the bacterial cell. The phospholipid bilayer is comprised of three main biosynthesized phospholipids; phosphatidyl-glycerol (PG), lysyl-phosphatidyl-glycerol (LPG), and cardiolipin (CL) (19,20). While PG and CL are associated with negatively charged polar heads, LPG has a positively charged aminoacyl group that protrudes externally from the phosphate group of the PG molecule (20). This process begins with de novo fatty acid synthesis type 2, also known as the bacterial FASII system.

FASII initiates with acetyl-CoA being carboxylated by the acetyl-CoA carboxylase (AccABCD) operon turning the acetyl-CoA into malonyl-CoA (22,23). Acyl carrier protein (ACP) then takes the malonate group and makes malonyl-ACP. Malonyl-ACP is then condensed with acetyl-CoA to form beta-ketobutyryl-ACP by the fatty acid biosynthesis (FAB) family of proteins. The (PLS) protein family then carries out a series of reduction, condensation, and dehydration reactions on the common precursor molecule glycerol-3-phosphate (G3P) to form the desired length acyl-ACP (22,24). The FASII system has been studied as an antibiotic target with mixed reception (23,25.26). In *S. aureus*, *de*

novo fatty acid synthesis can be overcome with exogenous fatty acid supplementation (27). However, inhibiting FA synthesis to disrupt bacterial proliferation during infection is contentious because Gram positive organisms such as *S. aureus* are adept at incorporating exogenous fatty acids into the phospholipids. Thus, inhibitors of FA synthesis select for FA auxotrophs that are dependent on exogenous FAs present in host tissues for growth (27). Due to this, there is more research required to fully understand how fatty acid metabolism can be utilized to inhibit *S. aureus* growth in host tissues.

In this report, we assess the sensitivity of *S. aureus* to fatty acids present in abscesses that are potentially encountered during infection. We take a closer look at the composition of the PG in the lipid bilayer between respiring and fermenting *S. aureus*. We explore bacterial responses when fatty acids are added to growth media and categorize this response using biochemical assays. Finally, we examine the difference in mechanistic responses to FA in fermenting and respiring *S. aureus* cells.

RESULTS

S. aureus exhibits metabolic-dependent sensitivity towards abscess associated fatty acids.

Previous reports demonstrated that tissue abscesses are hypoxic, iron limiting environments enriched with fatty acids (28). Within an abscess unsaturated fatty acids (UFA) comprise over 50% of the total fatty acids and UFAs exhibit greater anti-staphylococcal activity than SFA (28). However, the minimum inhibitory concentration of UFAs were quantified under conditions that promote bacterial respiration (29,30,31,32). Given the hypoxic nature of the abscess we sought to determine whether respiration-

arrested staphylococci display differential sensitivities towards abscess-associated fatty acids. To test this, WT and a fermentation reliant MK-deficient small colony variant *menE* were cultured in a rich medium (TSB) supplemented with either UFAs or saturated fatty acids (SFAs). In these conditions, the WT mechanism of ATP generation is aerobic respiration, whereas the *menE* mutant ferments (33). Consistent with previous results, WT cells exhibit an increased lag phase upon exposure to unsaturated C18:2 linoleic acid (**Figure 3.1A**). The *menE* mutant failed to grow after 18 h of incubation, indicating the SCV is more sensitive to UFA. WT proliferation in the presence of saturated C14:0 myristic acid was similar to untreated cells (**Figure 3.1B**). This result is also in keeping with previous reports; however, the *menE* mutant displayed reduced proliferation in the presence of C14:0, supporting the conclusion that fermenting staphylococci are more sensitive to exogenous FA. To determine whether the FA toxicity was specific to the lack of menaquinone or broadly applicable to other types of respiration-arrested SCVs, growth of a heme deficient *hemB* mutant in the presence of unsaturated C18:2 and saturated C14:0 was quantified. Similar to the *menE* results, the *hemB* mutant also demonstrated reduced proliferation in the presence of either fatty acid (**Figure 3.1C and 3.1D**). A component of UFA toxicity is lipid peroxidation, which is dependent on the presence of oxygen (34). To discern whether SFA toxicity towards fermenting SCVs involves oxidative stress, WT cells were incubated in the presence of C14:0 under fermenting, anaerobic growth conditions. In the absence of oxygen, WT growth is decreased upon exposure to C14:0 (**Figure 3.1E**). Consistent with this, in fermenting conditions the IC_{50} of C14:0 was between 16 and 32

μM while respiring cells maintained consistent proliferation even in higher concentrations of SFA (**Figure 3.2**).

Lipidomics reveals differentially abundant phospholipids in respiring and fermenting *S. aureus*.

A potential source of the differential SFA toxicity between WT and SCVs is their basal membrane composition. A lipidomic analysis of WT and *menE* mutant cells cultured aerobically was conducted to quantify total phospholipids. Cells were harvested at mid-exponential growth in rich medium (TSB), washed and analyzed. Previous reports demonstrate that the dominant phospholipid species in *S. aureus* is 33:00 (35). Consistent with previous reports, the predominant PG in WT cells are 35:00 and 33:00, each containing FA 15:00 and 18:00 or 20:00 respectively (35) (**Figure 3.3A**). However, the dominant PG species in the *menE* mutant are 33:0 followed by 32:0 and 31:0 (**Figure. 3.3A**). Overall, *menE* cells contain a significantly greater abundance of phospholipids containing shorter fatty acids (30:00, 31:00 and 32:00) compared to WT; whereas WT harbors greater abundance of phospholipids with longer fatty acids (33:00 and 35:00) (**Figure 3.3A**). Additionally, *menE* cells harbor greater abundance of UFAs compared to WT (**Figure 3.3B**). Phospholipids containing short, UFAs increase membrane fluidity (36). Therefore, membrane fluidity was quantified in WT, as well as the *menE* and *hemB* SCVs using a lipid mimicking dye, diphenyl hexatriene. Consistent with its increased abundance of shorter, UFA-containing phospholipids, the *menE* strain demonstrates significantly enhanced membrane fluidity compared to WT (**Figure 3.3C**). A *hemB* SCV mutant also exhibited increased membrane fluidity, implying that phospholipids containing short, UFAs is a conserved trait across distinct SCVs (**Figure**

3.3B). Increased abundance of shorter fatty acids incorporated into phospholipids was also conserved in the second, PBS condition. In this condition cells were cultured to mid-exponential phase, washed, and resuspended in PBS and incubated for an additional 40 min (**Figure 3.3D**). Overall, these findings underscore differences between PG composition and membrane fluidity between WT and the *menE* SCV. These differences may account for the increased SFA sensitivity of SCVs.

SFA C14:0 is not incorporated into WT or SCV phosphatidyl-glycerol.

Lipidomics was also performed on cells resuspended in PBS in the presence or absence of C14:0 to determine whether C14:0 exposure further altered PG composition. In fact, C14:0 treatment did not alter PG composition of WT or *menE* cells (**Figure 3.3E**). While PG composition between treated and untreated WT or *menE* remained similar, the analysis revealed a significant percent decrease in total PG upon exposure to C14:0 (**Figure 3.3F**). The decrease in total percent PG correlated with significantly increased free (i.e., non-esterified) fatty acids (**Figure 3.3G**). These results show that C14:0 was not incorporated into *S. aureus* PG in either respiring, WT cells or fermenting *menE* SCVs. Additionally, free C14:0 was the most abundant fatty acid that increased in abundance upon exposure, indicating it was not further modified by elongation (**Figure 3.3H and 3.3I**). In total, these results demonstrate that C14:0 is not incorporated into *S. aureus* phospholipids and is not otherwise modified, indicating that C14:0 is the most likely cause of toxicity.

Multiple peptide resistance factor promotes fermentation-dependent proliferation in the presence of myristic acid.

Notably, a trend towards increased positively charged lysyl-PG was also observed in C14:0-treated WT and *menE* cells (**Figure 3.4A**). While this difference was not statistically significant, the importance of lysyl-PG in SFA toxicity was directly tested using a mutant strain devoid of lysyl-PG. This strain harbors a transposon within the *mprF* gene, which encodes the enzyme that transfers L-lysine to PG. This mutant is isogenic with the USA300 derivative JE2. Growth of the *mprF* mutant was similar to WT JE2 in aerobic conditions in the presence or absence of C14:0. Neither strain exhibited growth defects (**Figure 3.4B**). However, in anaerobic conditions while both strains showed decreased proliferation over time, the *mprF* mutant displayed an exacerbated growth defect in response to C 14:0 addition, suggesting lysyl-PG and membrane charge play a protective role in response to fermenting SFA toxicity (**Figure 3.4C**).

SFA affects ATP production in WT and SCVs but reduces membrane potential of respiring, WT cells.

After establishing a clear pattern of SFA growth inhibition in respiration-arrested, fermenting *S. aureus* and determining that C14:0 is likely the toxic species, a mechanistic explanation for SFA was explored. Previous work has revealed several UFA toxicity mechanisms ranging from disruption of the membrane potential, membrane pore formation, or lipid peroxidation (29,30,31,32). Based on the anaerobic growth inhibition, oxidative stress was ruled out as a mechanism of toxicity. A TnSeq approach revealed that genetic inactivation of the *S. aureus* F_1F_0 ATPase increases sensitivity to the respiration inhibitor nitric oxide (8,9). During respiration, the F_1F_0 ATPase harnesses

potential energy from the proton motive force (PMF) to synthesize ATP. However, in the presence of the respiratory inhibitor nitric oxide, the F_1F_0 ATPase functions in reverse, consuming ATP and pumping protons out of the cytoplasm to maintain cytoplasmic pH (8,9). Based on this model, we hypothesize that SFAs inhibit the F_1F_0 ATPase. Consequently, we predict that ATP concentrations will increase when SCVs are exposed to SFAs but decrease in respiring cells. Bac-Titer-Glo was used to measure ATP in SFA treated respiring WT and fermenting *menE* and *hemB* SCVs. This analysis revealed that ATP increases in abundance in SFA-treated SCVs compared to untreated cells but decreases in WT cells upon SFA exposure (**Figure 3.5A**) These results suggest that SFAs inhibit F_1F_0 ATPase. A consequence of inhibiting the F_1F_0 ATPase would be a concomitant increase in the membrane potential. Membrane potential was quantified using the cationic cyanine dye 3-3 diethyloxacarbocyanine iodide (DiOC2 (3)). At low concentrations the dye emits in the red wavelength. When imported into cells a higher concentration is reached, the dye forms a complex with itself and emits in the green wavelength. Import of DiOC2 is dependent on the membrane potential and previous findings show membrane potential is decreased in SCVs (37). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) dissipates the membrane potential and is a control for reduced membrane potential. WT cells treated with C14:0 exhibit decreased membrane potential (**Figure 3.5B**). Membrane potential measured in SCVs treated with C14:0 was not statistically different from untreated cells (**Figure 3.5B**), the membrane potential in these conditions appears to be close to the limit of detection. The disparate finding that C14:0 decreases ATP production in respiring, WT cells without a concomitant increase in membrane potential implies that C14:0 targets a process other than or in addition to the

F_1F_0 ATPase. Notably, we surmise that this alternative, ATP-generating pathway is seemingly dispensable in WT cells but consumes ATP in a reaction essential for SCV propagation.

SFA synergizes with the aminoglycoside gentamicin.

To overcome the observed respiring WT resistance to SFA, we considered antibiotics with the potential to synergize with the SFA toxicity. Aminoglycoside antibiotics inhibit translation but require the PMF to gain entry to the cytoplasm (38). In keeping with this, *S. aureus* responds to aminoglycoside treatment by mutating pathways that support respiration, which in turn reduce PMF and decreases antibiotic import (38). Respiration requires the cofactors heme and menaquinone; thus, heme and menaquinone biosynthesis mutants that are fermenting exhibit increased aminoglycoside resistance (38). These facts support the hypothesis that exposure to the aminoglycoside gentamicin will sensitize WT, respiring *S. aureus* to SFAs. To test this, we incubated WT *S. aureus* in the presence and absence of gentamicin. Gentamicin exposure causes a four-log reduction in viable CFUs (**Figure 3.6**). As expected, the SFA C14:0 did not affect *S. aureus* viability as the conditions promote respiration; however, incubation of *S. aureus* with C14:0 together with gentamicin resulted in a six-log reduction in CFUs which is greater than gentamicin alone (**Figure 3.6**). These results demonstrate that SFA-resistant, respiring *S. aureus* can be sensitized to SFAs with gentamicin. The presence of fatty acids within tissue abscess implies that treating with gentamicin with, or potentially without, SFA supplementation could be a means to combat recalcitrant staphylococcal infections.

SFA treatment induces lipid droplet formation in WT respiring cells.

Transmission electron microscopy was performed to investigate and potentially visualize how WT cells respond to SFA. Treated and untreated WT and SCV cells were prepared for observation using an electron microscope. The cytoplasm of C 14:0-treated WT cells contained an increased abundance of electron sparce regions or foci compared to untreated cells (**Figure 3.7A-D**). Further examination revealed that these electron sparce foci were not present in the *menE* SCV mutant (figure not shown). The number of electron sparse foci were quantified across 572 cells and confirmed that at least 20% of WT cells incubated in the presence of C14:0 harbored at least 2 electron sparse foci (**Figure 3.7E**). C 14:0 also increased the numbers of these electron sparce foci per WT cell (**Figure 3.7E**). To characterize the electron sparce areas further, cells were treated with a neutral lipid fluorescent stain LipidTOX Red that targets lipid droplets. Fluorescent LipidTOX Red staining is apparent in WT cells and increased in WT cells exposed to C14:0 (**Figure 3.7 F-I**). These results indicate that the electron sparce foci visualized by TEM are lipid droplets. The fact that electron sparse foci and LipidTOX Red positive staining are both considerably reduced in C 14:0-treated SCVs suggests that fatty acid trafficking and lipid droplet formation is mechanism to minimize toxicity in WT cells (not imaged).

DISCUSSION

Though much research has been performed to understand the fatty acid synthesis mechanism of *S. aureus*, particularly in the context of identifying antimicrobial targets, there is much to be desired in understanding how staphylococcal metabolism effects sensitivity to antimicrobial compounds (39). UFAs have been previously

observed to be toxic to *S. aureus*, but significantly greater quantities of SFA are required to inhibit staphylococcal growth, indicating decreased toxicity compared to the UFAs. Previously work revealed that the mechanisms of UFA toxicity are disruption of the membrane potential, membrane pore formation, or lipid peroxidation (29,30,31,32). Experiments supporting these results were conducted using standard laboratory growth conditions that promote aerobic respiration as the primary mechanism of generating energy. Given that *S. aureus* likely encounters environments, such as tissue abscesses, that promote fermentation as a primary driver of energy, we sought to determine whether fermenting *S. aureus* was more or less sensitive to UFAs and SFAs.

In this work, we show that both UFA and SFA inhibit growth of respiration-arrested, fermenting *S. aureus*. This is shown clearly through IC50 values as well as growth kinetics. The amount of SFA required to inhibit genetic *hemB* and *menE* SCV mutants is 8x lower than the amount necessary to inhibit WT cells. WT cells proliferate normally with the addition of SFA to the media while respiration-arrested cells exhibit decreased yield compared to untreated cells. WT cells cultured anaerobically also display inhibited growth in the presence of SFA. These results are consistent with those showing mutant SCVs cultured aerobically are sensitive to SFA. Also, these findings rule out lipid peroxidation as a mechanism of toxicity as peroxidation requires oxygen (40). *S. aureus* cells grown in the presence of an aminoglycoside antibiotic can resist cell death by decreasing the proton motive force and fermenting (41). However, viability of cells cultured in the presence of both gentamicin and SFA show that the selective pressure applied by the antibiotic sensitizes respiring *S. aureus* to SFA. These results indicate that fermenting *S. aureus* cells are sensitive to SFA.

To identify the mechanism by which SFA are toxic to fermenting *S. aureus* we first quantified differences in fatty acid and phospholipid content between WT and the *menE* SCV mutant. The lipid composition of the cellular membrane is both important and dynamic in WT *S. aureus* cells (42). Global lipidomics were performed to highlight any key fatty acid and phospholipid changes. In a rich medium like TSB, fermenting *S. aureus* cells favor a shorter phospholipid than respiring cells. The length of the phospholipids could play a role in membrane rigidity and fortify WT cells in the presence of SFA, but further experimentation is required to determine whether or not this is true. The same trend was observed when the cells were placed in PBS in preparation for SFA treatments, but SFA treatments balanced out the abundance of the membrane PG species present in respiring and non-respiring cells. It has already been shown that PG chain length has a direct relationship with membrane stability (36). Membrane fluidity is measured by how easily a lipid mimicking dye can intercalate into the cell's membrane, diphenyl hexatriene is a dye that can intercalate into the membrane (42). The significant increase in fluorescence of the fermenting strains of *S. aureus* indicates a less stable membrane. Unsurprisingly, SFA treatments increased the amounts of free fatty acids in the cells. Surprisingly myristic acid increases the presence of another SFA as well, stearic acid (C 18:0). This increase was observed in both WT and SCVs, so it is unlikely that the selective inhibition is due to the increase in free cellular fatty acid concentrations.

In addition to the stark decreases in phosphatidyl-glycerol quantities in the SFA treated cells, there was a corresponding increase of lysyl-phosphatidyl-glycerol in both the WT and *menE* treated with SFA. To investigate this phenotype further, a multiple

peptide resistance factor, *mprF*, transposon mutant was challenged with SFA in fermenting, anaerobic conditions. MprF is responsible for adding lysine to PG, generating LPG (20,43). Addition of lysine changes the change of PG from negative to positive charge (20,43). We show that a *mprF* mutant exacerbates growth defects fermenting *S. aureus* cultured in presence of SFA more severely than a respiring *S. aureus*. While this increase in LPG was universal, the role of multiple peptide resistance factor should be quantified more directly. Previous work showed respiration-arrested *S. aureus* requires a functional F_1F_0 ATPase, as lactate dehydrogenase and other enzymes required for fermentation cannot function in an acidified cytosol (44). Inhibition of the F_1F_0 ATPase results in an inability to grow in fermenting conditions (41). To identify whether function of the F_1F_0 ATPase is impaired as a consequence of SFA exposure, we measured ATP levels in WT and *menE* cells grown in the presence and absence of SFA. We found that ATP levels decreased in SFA treated WT cells but increased in the *menE* mutant. These data indicated that the F_1F_0 ATPase might be inhibited by SFA. However, inactivation of F_1F_0 ATPase should also increase membrane potential in respiring cells but in our experiment, SFA treatment resulted in a decrease of membrane potential. Measuring the membrane potential was inconclusive as the *menE* and *hemB* cells exhibit decreased membrane potential compared to WT in the absence of SFA. Thus, the limit of detection for the assay restricts the capacity to observe a further depleted membrane potential. These data support a model whereby SFA inhibit the F_1F_0 ATPase in addition to another physiological pathway required for fermenting staphylococci or SFAs inhibit another ATP-generating pathway in respiring *S. aureus* that is consumes ATP during fermentation. Towards the former, efforts to

isolate SFA resistant cells were unsuccessful, indicating that SFAs may target more than one process (44).

Finally, respiring WT *S. aureus* and fermenting *menE* were imaged to observe potential differences in the cell envelope of SFA-treated cells. Transmission electron microscopy was utilized to get a closer look at the cells (45). In a very striking discovery, the cell envelopes appeared to remain similar to their untreated counterparts, but the presence of small, circular, electron sparse areas were abundant within SFA treated WT cells. Upon quantification, it was clear that an increased number of WT cells contained more of these electron sparce foci and the quantity of foci per cell increased significantly upon SFA treatment. To characterize these electron sparce areas, staining SFA treated and untreated WT and *menE* cells with LipidTOX red on cells revealed that these electron sparce areas were likely lipid droplets (46,47). Other clinically relevant bacteria like Mycobacterium tuberculosis utilize the lipid droplet in environmentally harsh conditions for survival (48,49,50). Adjusting to a toxic environment could be a key to the innate ability of *S. aureus* to survive within the host. Taking advantage of the susceptibility of the respiration-arrested cells to SFA offers a tremendous opportunity to combat this pathogen.

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MATERIALS AND METHODS

Bacterial strains

Laboratory derivatives of the Methicillin-resistant *Staphylococcus aureus* USA300 LAC strain, AH1263 provided by the Horswill lab was used as wild type (WT) throughout this work (**Table 1**). The JE2 derivative of USA300 LAC was used as an isogenic WT strain for the *mprF*::Tn mutant growth assessment (**Table 1**).

Growth curves

Strains were cultured overnight at 37° C shaking at 225 rpm. An absorbance at 600 nm was taken using a BioTek Epoch 2 microplate spectrophotometer and bacteria at a final concentration of 0.02 optical density (OD) 600 nm in fresh liquid media was added to a disposable trough. Another trough of fresh tryptic soy broth (TSB) with 2x the final desired concentration of FA in μM was made. 100 μL of each trough was added to a 96 well clear round bottom plate and incubated shaking overnight at 37° C. The plate reader was set to orbital shaking and measured absorbance at OD 600 nm every hour for 18 hours. After the final time point each well was perturbed by pipetting up and down and a final endpoint OD 600 nm was measured. Anaerobic cultures were prepared the same as their aerobic counterparts until incubation. Upon initiation of bacterial growth post cultural inoculation, plates were placed in the BioTek Epoch 2 microplate spectrophotometer in a Coy anaerobic chamber with O_2 parts per million below 3.

Minimum inhibitory concentration assays

Overnight cultures of strains were diluted to an optical density of 0.02 in a 50 mL reservoir. In a separate reservoir a 1mM stock of myristic acid was made in fresh TSB. The leftmost (A1) well was filled with 200 μL of the1 mM concentration myristic acid

stock. All of the other wells (A2-10) were filled with fresh media. 100 µLs of well A1 was taken and transferred to well A2 resulting in a ½ dilution of the initial myristic acid stock. This process was repeated until the last well A10 that was left completely free of myristic acid. All wells were inoculated with 100 µLs of the diluted strains. This resulted in 200 µLs per well of the desired myristic acid concentration and a starting bacterial absorbance of 0.01.

Lipidomics analysis

Samples on dry ice were spiked with 10 microliters of an internal standard and calibration mixture delivering 1 nanomole of di-myristoyl phosphatidyl glycerol (PG). To each sample, 300 microliters of -20C chilled 75% methanol containing 1 mM BHT (an antioxidant) was added along with 0.5 mm zirconium oxide beads. Samples were homogenized briefly in a Bullet Blender tissue homogenizer and placed on ice. 60 microliters of methanol and one mL of MTBE were added to each sample, and samples were then vortexed for 60 minutes at room temperature. 170 microliters of water were added, and the samples were vortexed for an additional 15 minutes and then centrifuged for 15 minutes. The supernatants were collected to new test tubes and precipitated proteins were re-extracted as above. Pooled extracts were dried overnight in a speedvac and resuspended in 200 microliters of isopropanol. Immediately prior to analysis, aliquots of each lipid extract were diluted in isopropanol:methanol (2:1, v:v) containing 20 mM ammonium formate. Full scan MS spectra at 100,000 resolution (defined at m/z 400) were collected on a Thermo Scientific LTQ-Orbitrap Velos mass spectrometer in both positive and negative ionization modes. Scans were collected from m/z 200 to m/z 1200. For each analysis, 10 µL of sample was directly introduced by flow

injection (no LC column) at 10 µL/min using an electrospray ionization source. A Shimadzu Prominence HPLC served as the sample delivery unit. The sample and injection solvent were 2:1 (v: v) isopropanol: methanol containing 20 mM ammonium formate. The spray voltage was 4.5 kV, ion transfer tube temperature was 275 °C, and the ion trap fill time was 100 ms. The autosampler was set to 4 °C. After two minutes of MS signal averaging, the LC tubing, autosampler, and ESI source were flushed with 1 mL of isopropanol, prior to injection of the next sample. Samples were analyzed in random order, interspersed by solvent blank injections, extraction blank injections, and pooled QC samples derived from all study samples. Following MS data acquisition, offline mass recalibration was performed with the "Recalibrate Offline" tool in Thermo Xcalibur software according to the vendor's instructions, using the theoretical computed masses for the internal calibration standards and several endogenous bacterial lipid species. MS/MS confirmation and structural analysis of lipid species identified by database searching were performed using collision induced dissociation (CID) MS/MS at 60,000 resolution and a normalized collision energy of 60 in negative ion mode. MS/MS scans were triggered by inclusion lists generated from putative lipid identifications from initial full scan MS data.

Quantification of membrane potential

Using 3-3 diethyloxacarbocyanine iodide ($DiOC_2$ (3)), wild type and the qox mutant were interrogated for any changes in membrane potential. After a 40-minute incubation with C 14:0, 10 uL of $DiOC_2$ (3) was added to 1 mL aliquots of mid log cells resuspended in PBS. Using carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as a positive control for complete membrane potential ablation, C 14:0 effect on membrane potential was

assessed. After a 15-minute incubation with the $DiOC_2$ dye, fluorescence at both 530 and 610 nanometers was measured using a spectrophotometer. (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp34150.pdf) At a low concentration (low membrane potential) $DiOC_2$ emits at 530 a green wavelength, but when imported, the dye associates with itself and emits in the red 610 wavelength denoting at a high delta PSI and active membrane transportation. By analyzing the green to red ratio, a measure of membrane potential can be monitored. A respiring *S. aureus* cell will have a whole number ratio, implicating more of the dye being imported into the cell. While in a cell treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a compound that ablates membrane potential, the ratio is a fraction, implicating a larger denominator in the ratio and less importing of the dye. Myristic acid was added to mid exponential phase cells and membrane potential was measured.

ATP quantification

Overnight cultures of AH1263 (WT) and respiration-arrested mutant cells (*menE*, *hemB*) were diluted to 1:1000 or 1:10 fresh TSB subcultures. The new cultures were incubated for 4 hours (to mid exponential) and spun down at 4000 rpm for 10 minutes. The supernatant was discarded, and the cultures were resuspended in 1x pbs two times. 1 mL aliquots of the washed subcultures were placed into 1.5 mL eppendorf tubes for FA treatments. 5 µL of 100% ethanol (vehicle) and a 10mM stock of each FA was added to their respective eppendorf tubes. Each sealed eppendorf tube was then placed into one beaker and left shaking at 37 °C for 40 minutes. After this incubation, the tubes were spun at 4000 rpm for 10 minutes to separate the cells and the supernatants. Once separated, the cell portion was resuspended in 800 µL of fresh pbs. 100 µLs of the

original supernatant, and cellular portion from the Eppendorf tubes were plated in triplicate in a black 96 well clear flat bottom plate. Following the instructions of the bac-titer-glo promega kit, 100 µLs of rehydrated enzyme was added to each well, shaken for 5 minutes in the dark and then read for luminescence.

Transmission electron microscopy sample preparation

Following the ATP quantification culture preparation, 30 mL WT and *menE* cultures were grown to mid exponential phase. After a spin at 4000 rpm and resuspending in 15 mLs of pbs, cells were separated into two individual 15 mL cultures representing a FA treatment and a vehicle control group. Each treatment group had 50 µL of 10 mM C 14:0 added to it while each vehicle control group had 50 µL of 100% ethanol added to it. Cells were incubated at 37 °C and shaken at 225 rpm for 40 minutes. After one final spin at 4000 rpm for 10 minutes the cells were resuspended in a 2.5% glutaraldehyde, sodium cacodylate fixing buffer solution and pelleted. These samples were taken to the MSU imaging core and prepared and imaged by Alicia Withrow.

Fluorescent microscopy preparation

Consistent with the TEM preparations, WT and *menE* were grown to mid exponential phase and treated with 50 µM C 14:0 for 40 minutes. After a 10-minute stain with HCS LipidTOX Red, cells were plated onto glass slides atop a 1% gel agarose pad. Slides were fitted with a glass cover slip after addition of cells and images were captured using a Lecia DMI 600 microscope. Images were analyzed using FIJI software.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

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Contributions

Conceptualization: RAC, PCD, NDH. *Methodology*: RAC, PCD, NDH. *Software*: NDH. *Validation*: NDH. *Formal Analysis*: RAC, GRK, PCD, NDH. *Investigation*: RAC, PCD, NDH. *Resources*: NDH. *Data Curation*: RAC, GRK, NDH. *Writing*: RAC, NDH. *Visualization*: RAC, NDH. *Supervision*: NDH. *Project Administration*: NDH. *Funding*: JR, NDH.

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TABLES AND FIGURES

Table 3.1. Strains used in this study.

Strain	Alteration	Source
AH1263	WT	51
menE	*menE*::Tn	52
hemB	*hemB*::Tn	52
Je2	WT	53
mprF	*mprF::*Tn	54

Figure 3.1. FA inhibit growth of SCVs. A,C. WT, *menE*, and *hemB* mutants' growth dynamics over an 18 hour period with absorbance values being measured every hour at 600 nm Cells are grown in Tryptic soy broth with and without 50 μM C 14:0 being added to the media. **B,D**. WT, *menE*, and *hemB* mutants' growth dynamics over an 18 hour period with absorbance values were measured every hour at absorbance 600 nanometers. Cells are grown in Tryptic soy broth with and without 50 μM C 18:2 being added to the media. **E**. Growth dynamics of WT *S. aureus* cells grown in TSB in an anaerobic chamber with or without 50 μM C 14:0 added to the media. The same untreated, WT growth control is presented in A, B, C, and D. The same C 18:2-treated WT control is presented in A and C. The same C 14:0-treated WT control is used in B and D. The error bars represent ± 1 standard error of the mean.

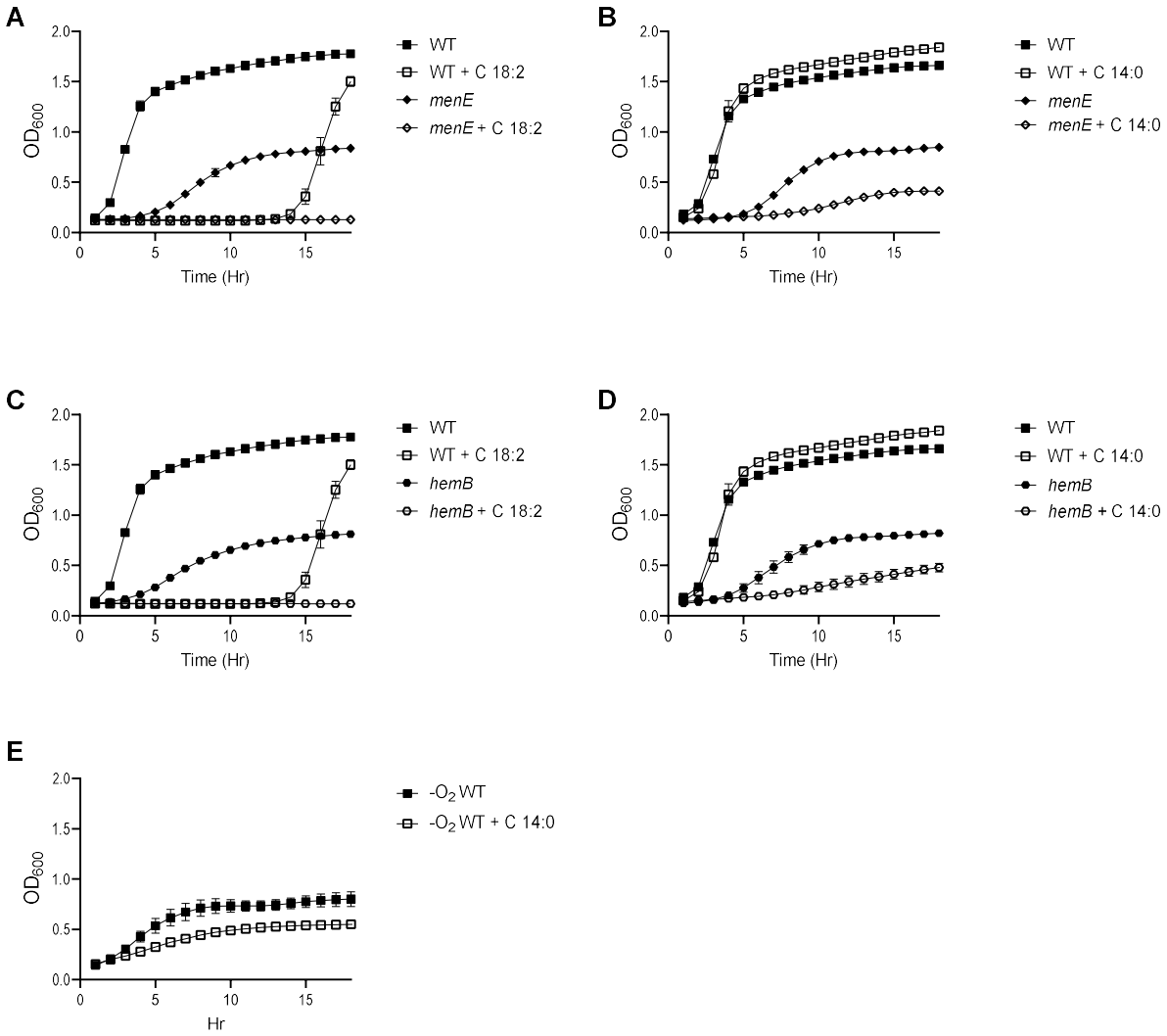


Figure 3.2. Fermenting staphylococci display increased sensitivity to fatty acids. End point absorbance values at 600 nm over a range of C 14:0 concentrations for the WT, *menE*, and *hemB* mutants cultured in tryptic soy broth for 24 hours. The error bars represent ± 1 standard error of the mean.

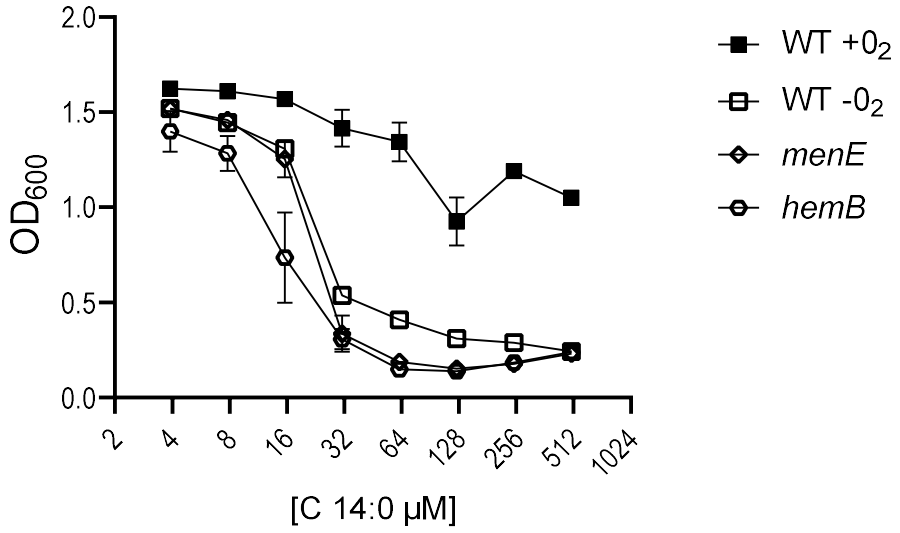


Figure 3.3. Lipidomic analysis shows WT and *menE* response to SFA added to media. Individual phospholipid species in WT *S. aureus* and a fermenting mutant in three different environments. **A**. Saturated lipid content in cells grown to mid exponential phase in TSB. **B.** Unsaturated lipid content in cells grown to mid exponential phase in TSB. **C**. Diphenyl hexatriene fluorescence after 15-minute incubation with mid exponential cells grown in tryptic soy broth. **D-E**. Cellular lipid content in cells grown to mid exponential phase in TSB, then treated in PBS with or without 50 μM C 14:0. Free fatty acid species in WT *S. aureus* and a fermenting mutant in 3 different environments. Global lipidomics reveal FA composition changes after addition of SFA to media. Lipid profile analysis for whole cell suspensions of WT and *menE* mutant cells treated with myristic acid. **F**. Phosphatidyl-glycerol content of the cells expressed as a percentage of total lipid in the cell. **G**. Non-esterified fatty acid concentration in the cells expressed as a percentage of total lipids. **H**. Free fatty acid content in cells grown up to mid exponential phase in TSB. **I**. Free fatty acid content in cells grown to mid exponential phase in TSB, then treated in PBS with or without 50 μM C 14:0. The error bars represent ± 1 standard error of the mean. * Indicates $P<0.0471$ determined from a two-way ANOVA. ** Indicates $P<0.0019$ determined from a two-way ANOVA. *** Indicates $P<0.0004$ determined from a two-way ANOVA. **** Indicates $P<0.0001$ determined from a two-way ANOVA.

Figure 3.3. (cont'd)

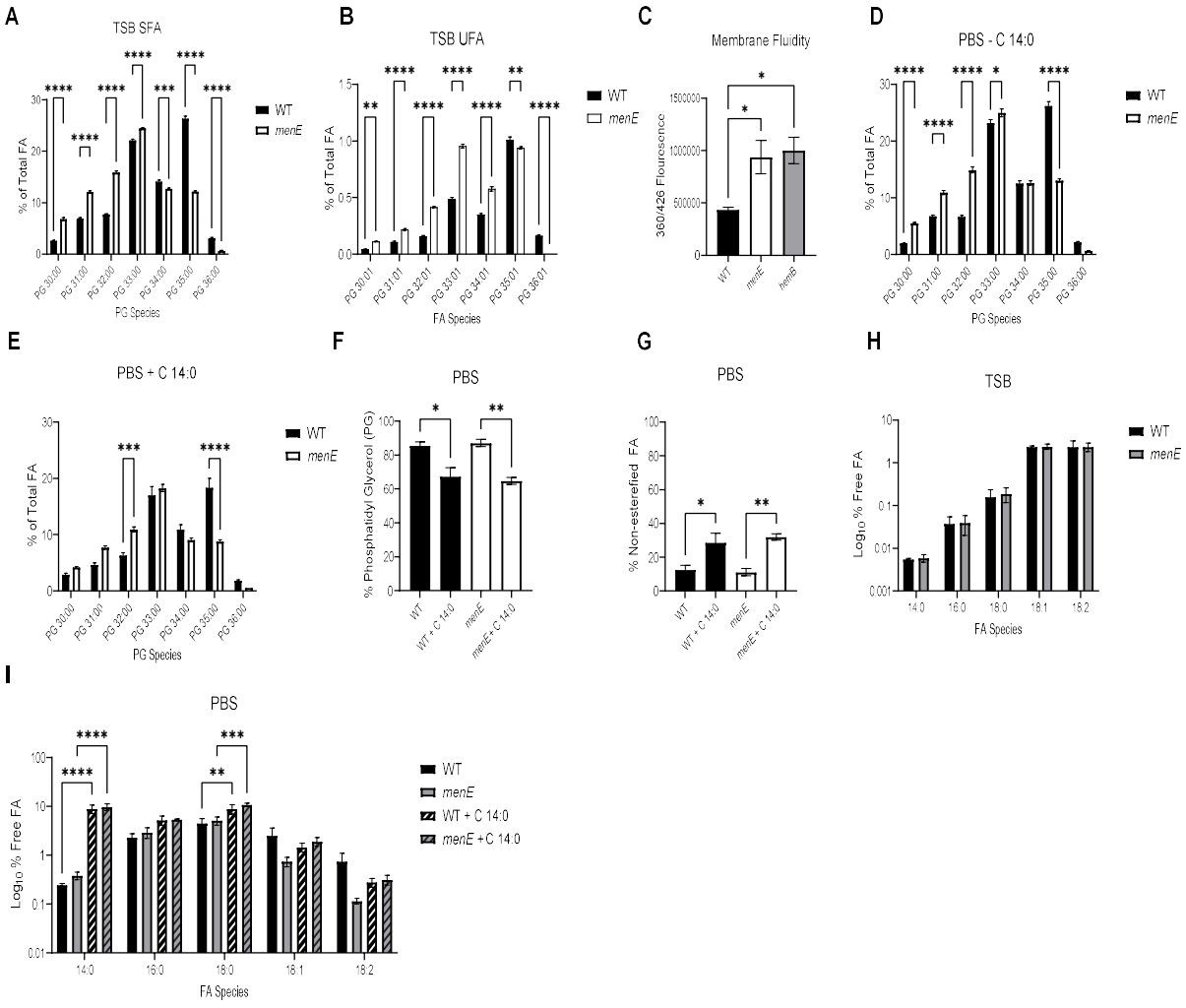


Figure 3.4. Multiple peptide resistance factor implicated in response to SFA. A. Positively charged lysyl-Phosphatidyl-glycerol amounts in each cell expressed as a percentage of total lipid in the cell. Growth phenotypes of the *mprF* mutant in anaerobic and aerobic conditions. **B**. WT and *mprF* mutant grown in TSB over an 18-hours with absorbance values being measured every hour at 600 nm. **C**. Anaerobic *S. aureus* cells incubated in TSB with or without 50 μM myristic acid added to the media. The error bars represent ± 1 standard error of the mean.

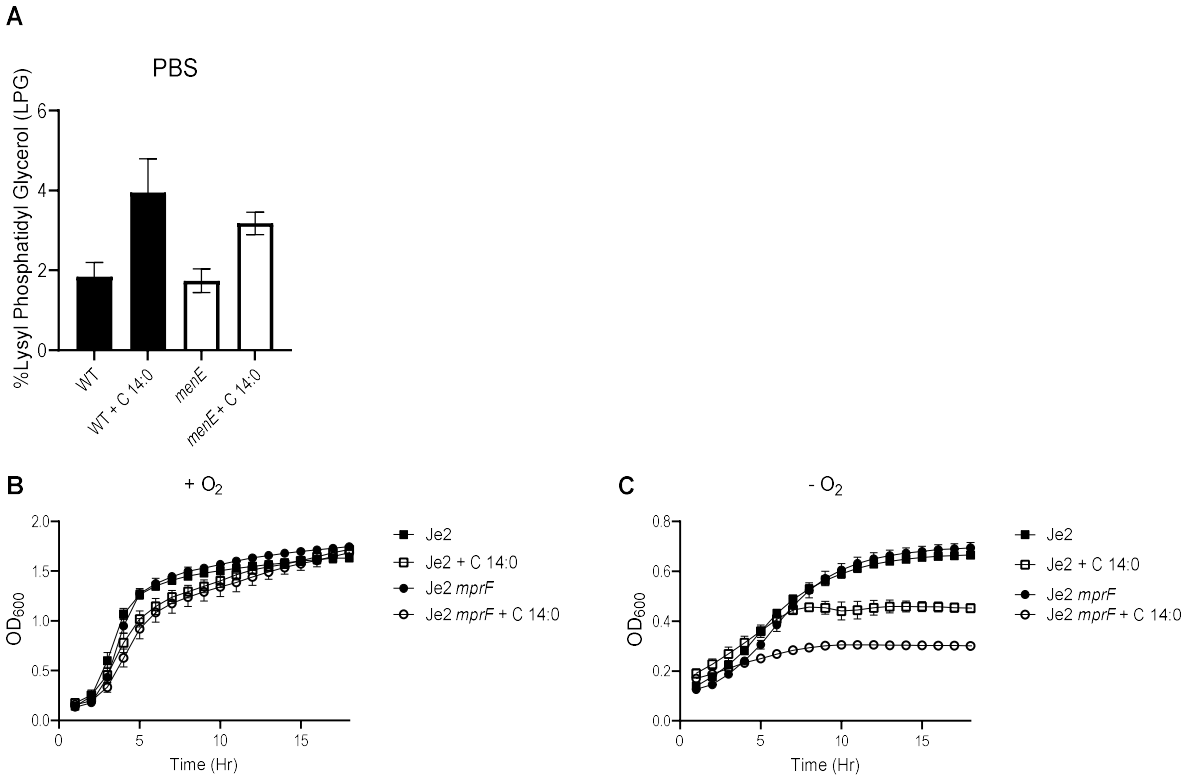


Figure 3.5. Membrane function altered in the presence of SFA. A. Bac-titer-glo ATP quantification assay results of 50 μM C 14:0 treated and untreated cells after 40 min incubation in phosphate buffered saline. **B**. The membrane potential of WT *S. aureus* cells and fermenting mutants in mid-exponential phase measured as the mean ration of red/green fluorescence of 3,3'-Diethyloxacarbocyanine Iodide ($DiOC_2(3)$) after a 40-minute incubation with 50 μM C 14:0 quantified by measuring red or green fluorescence at both 610 nm and 530 nm. The error bars represent ± 1 standard error of the mean. ** Indicates P<0.0079 determined from a two-way ANOVA. *** Indicates P<0.0001 determined from a two-way ANOVA.

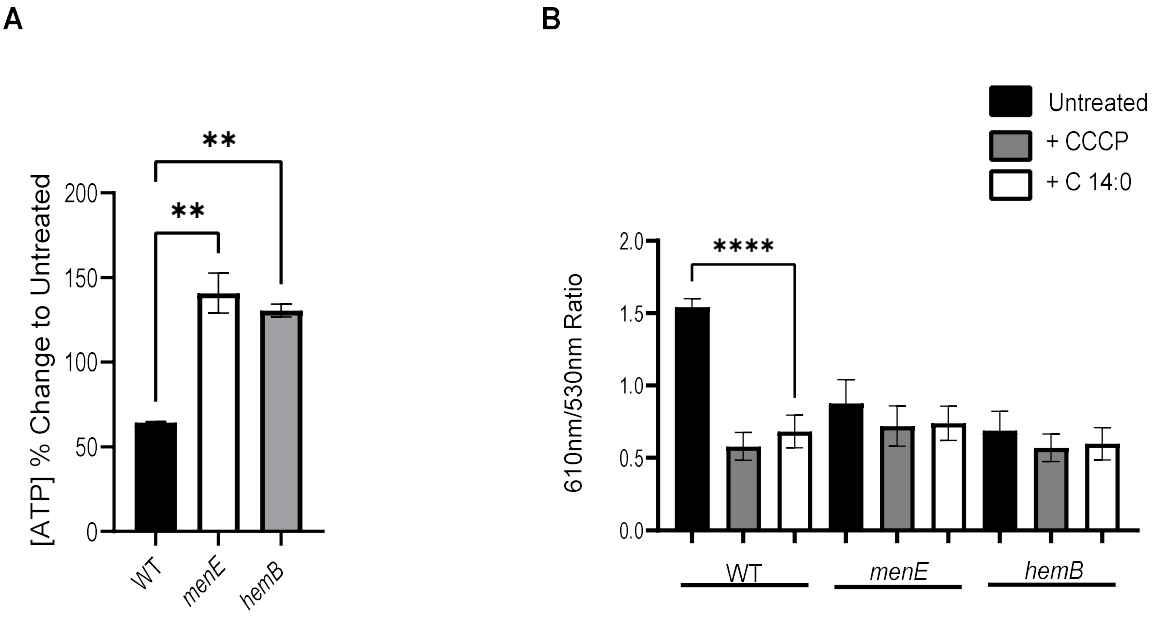


Figure 3.6. Myristic acid potentiates gentamicin activity. Log_{10} colony forming units of *S. aureus* cells plated on 1% tryptone broth with or without 3 µg/mL gentamicin added to the plates. The error bars represent ± 1 standard error of the mean. *** Indicates P<0.0002 determined from a one-way ANOVA.

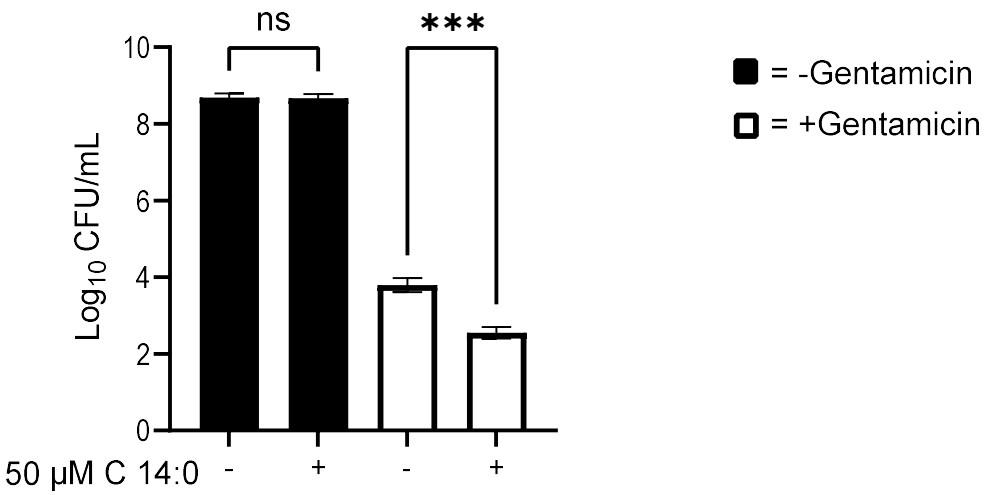


Figure 3.7. Microscopy reveals potential protection mechanism from SFA growth inhibition. A-B. TEM of WT *S. aureus* cells fixed in 2.5% glutaraldehyde, sodium cacodylate fixing buffer after a 40-minute incubation with 50 μM myristic acid. **C-D**. A zoomed in look of images A and B of this figure. **E**. Lipid droplet (LD) quantification of myristic acid treated WT and SCV *S. aureus* cells. **F-G**. Fluorescence microscopy images of WT *S. aureus* cells after a 40-minute incubation with 50 μM myristic acid cells treated with a 1/1000 solution of HCS LipidTox™ red stain for 10 minutes. **H-I**. A zoomed in look of images F and G of this figure. The error bars represent ± 1 standard error of the mean. **** Indicates P<0.0001 determined from a two-way ANOVA.

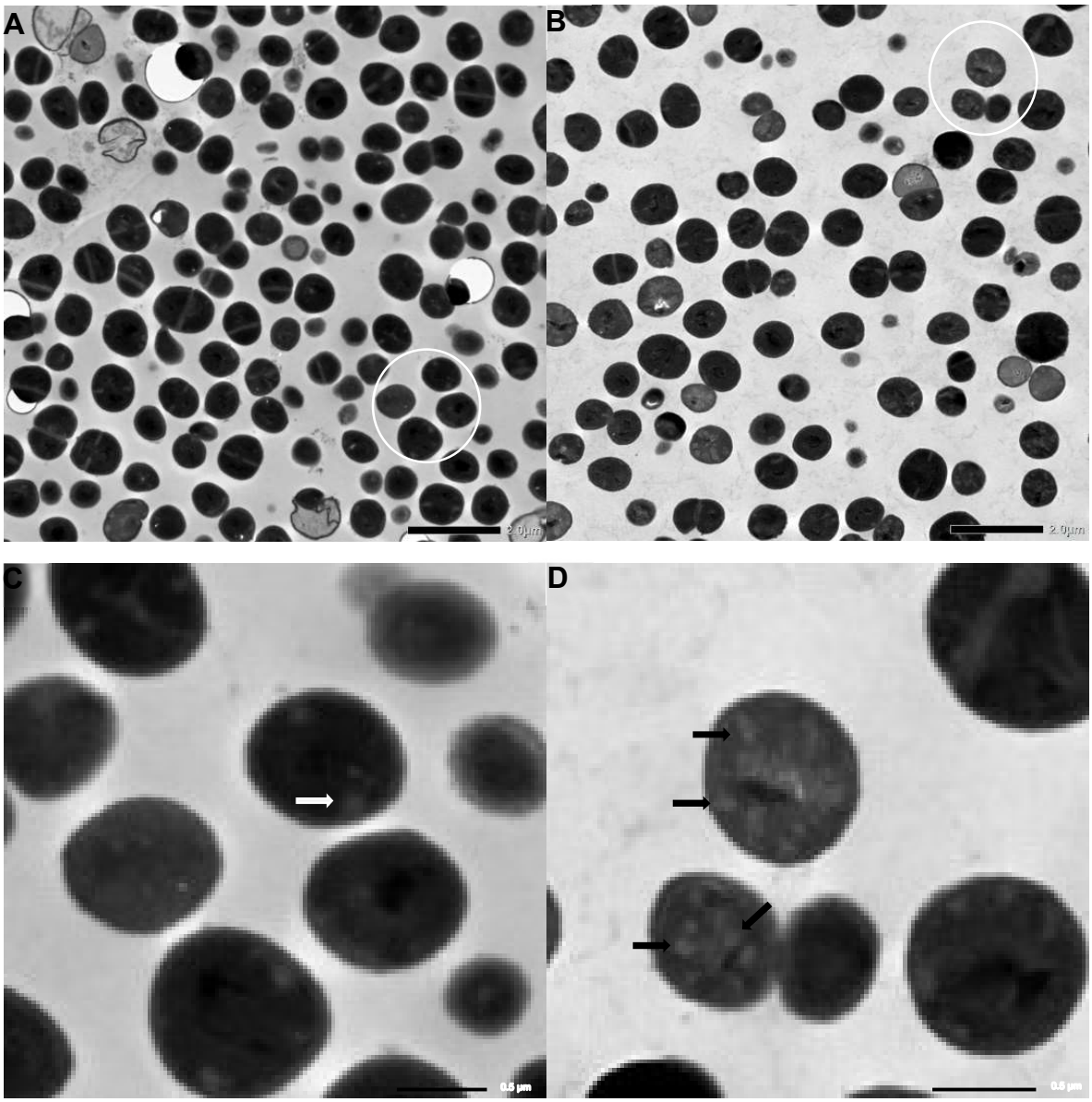


Figure 3.7. (cont'd)

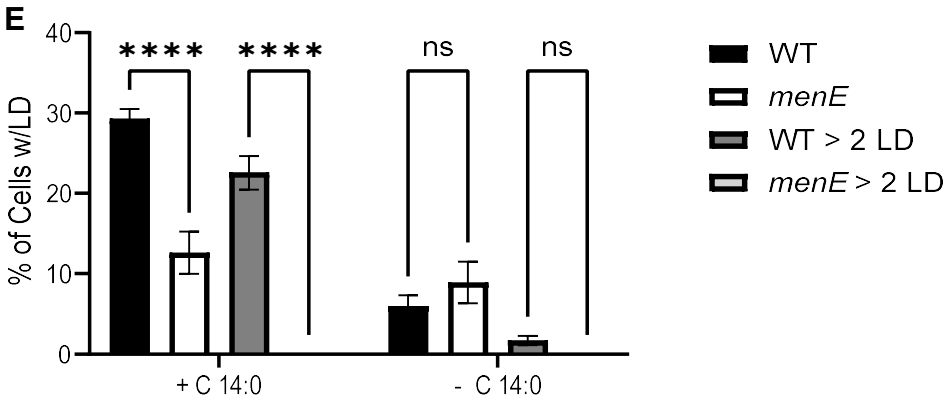
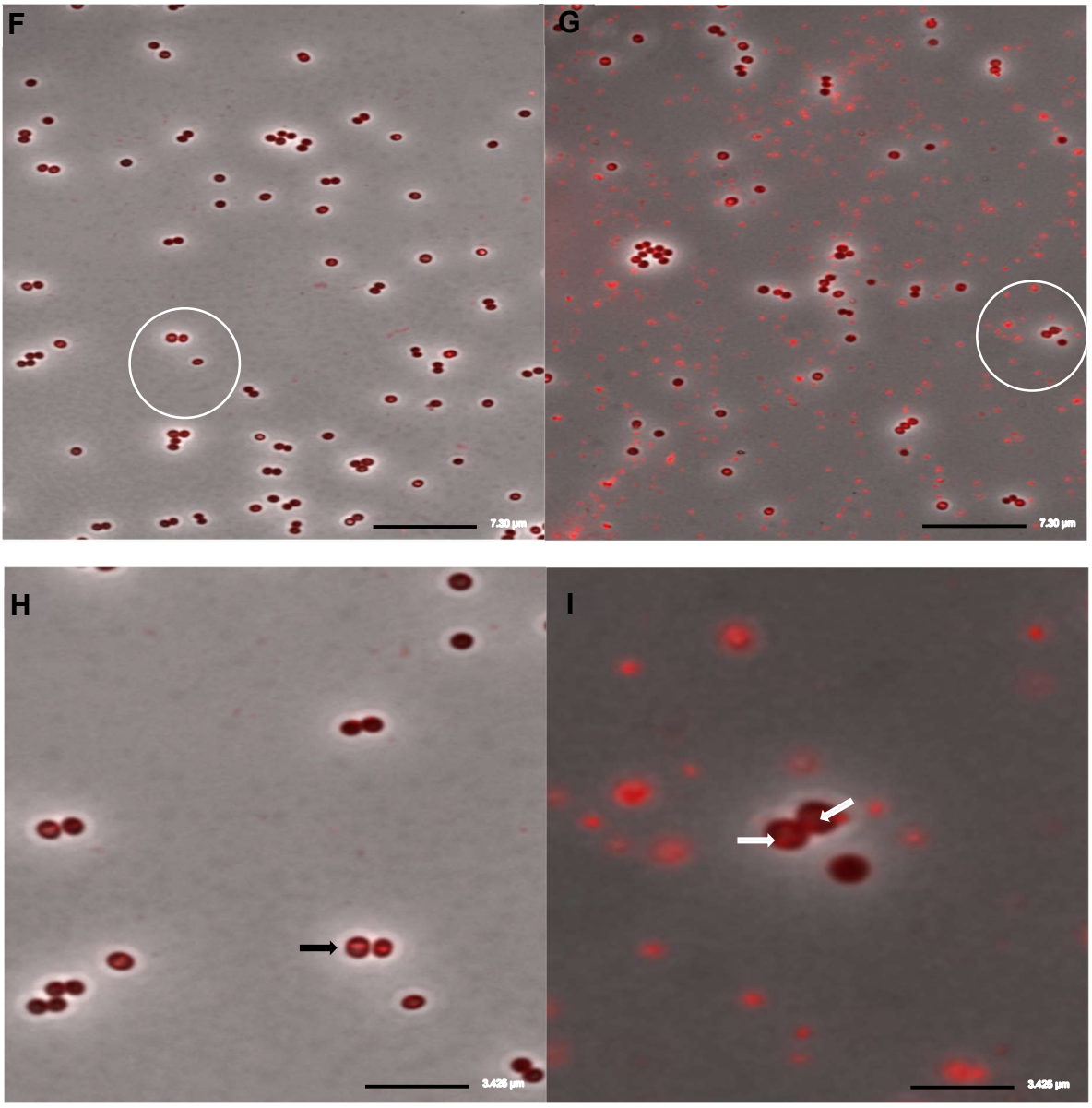


Figure 3.7. (cont'd)



CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS

From before it was discovered to the present day, *Staphylococcus aureus* has been a global threat. As a commensal bacterium, symptomatic colonization of almost a third of the population allows for widespread dissemination of this pathogen (1,2). *S. aureus* can cause a plethora of infections being the leading cause of skin and soft tissue infections but also can cause bacteremia and endocarditis potentially leading to death (2). These infections can be contracted in both community as well as hospital-associated environments (3). For over 150 years, research centered on successful treatment of *S. aureus* has led to limited success, due to its ability to acquire antibiotic resistant genes as well as being able to modulate its metabolism during infection (4). Metabolic versatility allows *S. aureus* to colonize several distinct organs during infection even when respiration is limited (5). Complete respiration arrest leads to the small colony variant phenotype which can be isolated from up to 50% of cystic fibrosis patients (6). It is necessary to find new treatments for this pathogen. In this dissertation I assess the feasibility of a treatment regimen that exploits the metabolic versatility of *S. aureus* to clear clinical infections completely.

In Chapter 2, we establish a new pipeline for the development of anti-staphylococcal drug development. Identifying SCV specific inhibitors is a key step to rectifying the *S. aureus* clinical issues. We performed two library screens for antimicrobial compounds that specifically inhibit the treatment recalcitrant SCV phenotype. These screens identified a total of 19 compounds that do not inhibit WT *S. aureus* growth, or that inhibit growth of the SCV phenotype more potently than the WT. This data supports the theory that distinct metabolic pathways support proliferation of SCVs and that these pathways offer a target for therapeutic interventions. The fact that the F_1F_0 ATPase is

essential for SCV propagation but dispensable for respiring, WT growth under laboratory conditions also supports this theory. Further support is offered in the discovery that two compounds that inhibit SCV growth support WT proliferation and even enhanced WT growth over the untreated control. However, results from the lipidomics experiment presented in Chapter 3 demonstrate a significant, global alteration of the membrane composition between WT and the *menE* SCV mutant. Indicating that numerous metabolic pathways are distinctly active in WT and SCVs. Thus, the theory should be modified to holistically account for differences between respiring and fermenting *S. aureus*. Nonetheless, establishment of a pipeline for drugs that are specific to SCVs provides considerable potential for positive clinical outcomes.

Further experimentation should be performed to provide mechanistic information about these compounds and any others isolated in the same manner. Primarily describing which pathways are being impacted by the compounds via mutagenesis. To identify the targets of ‘841 and ‘893 experimental efforts should be focused on passaging both the *menE* and *hemB* mutants in the presence of increasing concentrations of growth inhibiting compounds. Previous work showed that consistent exposure to sub-inhibitory concentrations of antibiotics enriches for strains with enhanced survival (7). In two settings: severe (highest concentration initially), and gradual (lowest concentration initially), the gradual test bacteria group will slowly accumulate mutations needed for survival in the presence of the antibiotic (7). The severe treatment group also accumulated mutations, but the lack of consistent challenge halted the progression of mutations (7). I hypothesize that constant, selective pressure will result in the isolation of resistant strains harboring mutations in the genes involved in SCV compound sensitivity.

Revealing these genes can lead to the development of more specific and efficacious therapeutics targeting these genetic pathways.

Antimicrobial compound synergy with aminoglycoside antibiotics is another essential study. Compound synergy is a long-studied aspect of drug design, the checkerboard assay is a very effective way to test this (8,9). The checkerboard assay consists of utilizing two compounds mixed at increasing concentrations in culture and quantifying growth by measuring light absorbance with a spectrophotometer (10). Compounds GSK, SB, ‘841, ‘893, and MSU 9052 will be checked against gentamicin in WT as well as the *menE*, *hemB*, and anaerobic WT backgrounds. Gentamicin is known to transition the WT into an SCV physiological state, this chemical condition should produce fermenting SCVs. A mixture of differing concentrations of two compounds, one being an aminoglycoside antibiotic and the other being a novel SCV inhibiting compound, should be added to growth media used to incubate cells. This systematic combination of compounds should allow for calculation of the Fractional Inhibitory Concentration (FIC) (11). The FIC is a formula that is used to quantify synergistic effects between compounds. We hypothesize that a value of less than 0.5 will be calculated indicating a synergistic interaction indicating a synergistic relationship between the compounds and gentamicin.

In Chapter 3 I examined the fatty acid composition of both WT and fermenting *S. aureus* cells using mass spectrometry. Immediately, a difference in fatty acid chain length was observed with fermenting cells favoring a shorter chain length. Longer chain length is correlated with increased rigidity of the membrane (12,13). This data was supported by fermenting *S. aureus* cells exhibiting increased sensitivity to fatty acid treatments, despite both WT and fermenting cells modeling the FA composition of their membranes similarly

in response to SFA addition to growth media. I also observed a difference in membrane fluidity in fermenting *S. aureus* cells when compared to respiring WT cells, increases in shorter fatty acids results in a less stable membrane (14,15). These data further implicate the instability of the membrane as a SCV specific drug target.

Chapter 3 goes on to identify a mechanism of action for SFA activity in fermenting *S. aureus* cells. In respiring cells, inhibition of the F_1F_0 ATPase can be overcome, but fermenting cells require a functional ATPase to survive (16). Enzymes required for fermentation are inactive in highly acidic environments, thus the ATPase removes protons from the cytosol in fermenting *S. aureus* cells. When this process is inhibited, fermenting cells struggle to proliferate (17). I discovered that fermenting cells respond to SFA in a manner consistent with their ATPase function being inhibited.

Finally, chapter 3 reveals how WT cells may be responding to SFA in order to survive exposure. Mass spectrometry also revealed a decrease in negatively charged phospholipids but a corresponding rise in lysylated positively charged phospholipid species was not significant. However, directly assessing the role of lysyl phospholipid was achieved using a lysyl-phosphatidyl-glycerol null mutant called *mprF*. Mutating *mprF* exacerbated the fermentation dependent susceptibility to SFA treatment. This result suggests that cell surface charge could have a critical a role in increased sensitivity to SFA. Another key finding was the production of lipid droplets in respiring cells treated with SFA using electron and fluorescent microscopy. In other clinically relevant bacterium like *Mycobacterium tuberculosis*, the lipid droplet is an essential feature for survival in hostile environments (18,19,20). These lipid droplets were present in both WT and fermenting cells but there was a significant increase in the amount of lipid droplets in WT cells which

grew even larger in response to SFA. Given these disparities, future work should identify differences between WT and fermenting *S. aureus* cellular envelopes. To examine the envelopes closer, cells could be fractionated prior to lipidomic analysis to identify the precise locations of the altered fatty acids. It is possible that the free fatty acid increases we observed in Chapter 3 are being deposited into the membrane. This would be consistent with the interruption of the ATPase model, as adding fatty acids to the membrane would displace the ATPase.

The precise mechanisms by which respiring *S. aureus* resist SFA is still unknown, and identifying this mechanism could be a key in new therapies. The lipid droplet response could be what keeps WT cells proliferating in the presence of SFA. Inhibition of this response could lead to WT cell vulnerability in the presence of SFA. Chemical reagents are available to inhibit lipid droplet production such as triacsin C which has been shown to reduce *Chlamydia trachomatis* growth in vitro (21,22). Reducing the presence of these lipid droplets could promote a similar fermentation SFA toxicity in respiring WT cells. These experiments will elucidate whether lipid droplets provide protection to respiring WT cells.

In this dissertation, I have addressed several important gaps in the field of bacterial pathogenesis and antimicrobial development. In Chapter 2 we established a pipeline for finding suitable drugs to treat fermenting *S. aureus* SCVs that can be isolated from the host throughout infection. We discovered key pathways in fermenting cells that are vulnerable drug targets for future development. Chapter 3 identifies another series of compounds that selectively inhibit fermenting cells, saturated fatty acids. We uncovered the mechanism of action in respiration-arrested cells and focused on the ATPase as a

drug target. In this chapter we also identify key differences in the WT and fermenting cellular envelope, a study that has not been performed before. Finally, we identified a WT cellular response to the presence of SFA using microscopy. Overall, this dissertation provides hope for future therapeutic development for the human pathogen *S. aureus*.

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