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Evaluating the role of GraS and FadXDEBA in promoting Staphylococcus aureus adaptation to host-derived fatty acids encountered at sites of colonization and infection

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Abstract

Staphylococcus aureus is an opportunistic pathogen that asymptomatically colonizes 30% of humans, where it is well adapted to survive on the skin in the presence of innate defense mechanisms such as antimicrobial free fatty acids (FFA). While antimicrobial FFA function to inhibit the growth of *S. aureus*, they also provide a valuable source of lipids for membrane synthesis and energy production. We hypothesized that *S. aureus* possesses a novel antimicrobial FFA resistance pathway that is activated under conditions found on human skin, and that under these conditions, *S. aureus* can metabolize exogenous fatty acids to fuel growth and virulence expression. Working with the endemic strain, USA300, our data show that when grown with cationic antimicrobial peptides or at an acidic pH, conditions encountered on human skin, *S. aureus* becomes extremely resistant to antimicrobial FFA. This resistance is dependent on activation of the sensor kinase GraS, as well as the downstream effector protein MprF. While MprF is known for synthesizing lysyl-phosphatidylglycerol, this antimicrobial FFA resistance is independent of this synthase activity, highlighting a novel function for MprF. Once resistant to high levels of host derived fatty acids, expression of putative ß-oxidation genes, *fadXDEBA*, occurs. Expression is upregulated by exogenous FFA in a concentration dependent manner, and is repressed by glucose. Additionally, expression appears to be regulated by the gene directly upstream of the *fad* locus, *prsW*, which is a membrane protease proposed to modulate the function of a stress response Sigma Factor. Interestingly, growth with exogenous FFA enhances the growth and protease expression of wildtype *S. aureus*, but severely impairs growth and viability in a *fadXDEBA* deletion mutant. Finally, we show that knocking out either *graS* or *fadXEDBA* results in reduced virulence in a murine abscess model, indicating both resistance and metabolism of host derived fatty acids are important during infection. While antimicrobial FFA encountered during colonization and infection of a host normally function to inhibit bacterial growth, *S. aureus* has evolved to thrive in this environmental niche through the use of GraS, MprF, and FadXEDBA.

Keywords

Staphylococcus aureus; MRSA; innate immunity; antimicrobial fatty acids, fatty acid resistance, fatty acid metabolism, GraRS, FadXDEBA

Summary for Lay Audience

Staphylococcus aureus is an opportunistic pathogen that asymptotically colonizes approximately 30% of the population, primarily in our nose or on our skin. Those who are colonized have a much greater risk of then becoming infected by *S. aureus*, and it is frequently the strain that colonizes us that then subsequently infects us. To combat *S. aureus* colonization, our skin produces a variety of antimicrobial compounds that function to inhibit the growth of this bacterium. Of these, are antimicrobial fatty acids, which can compromise the membrane integrity of *S. aureus*. However, these fatty acids are also a valuable energy source for the bacteria on our skin. We found there is a protein in the membrane of *S. aureus*, GraS, that can sense the antimicrobial conditions of human skin, and activate a robust response to resist these compounds. Specifically, activation of the protein GraS leads to high levels of resistance to antimicrobial fatty acids. Furthermore, once *S. aureus* is resistant to these fatty acids, we have identified a pathway in *S. aureus* that can metabolize these fatty acids to provide energy for the bacteria. This metabolism occurs through the proteins FadDEBA, which are predicted to conduct a metabolic process known as β-oxidation. Together, our findings show that although our skin produces antimicrobial fatty acids to inhibit the growth *S. aureus*, the bacteria has evolved to sense the environment of human skin and upregulate a robust resistance to these antimicrobial fatty acids, as well as metabolize these fatty acids as an energy source.

Co-Authorship Statement

All studies presented in this thesis were completed by Robert Kuiack in the laboratory of Dr. Martin McGavin, with assistance from co-authors as listed below. Dr. Martin McGavin contributed to the conceptualization and design of the experiments, and to manuscript preparation.

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Dr. Stephen W. Tuffs and Dr. Karine Dufresne conducted the murine infection experiment, under the supervision of Dr. John K. McCormick.

Dedication

To my dad, who always encouraged me to embrace my curiosity.

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List of Abbreviations

%	Percent
°C	Degrees Celcius
Δ	Delta or Deletion
Δ*fadXDEBA*	Deletion of *fadXDEBA* genes
Δ*fakA*	Deletion of *fakA* gene
Δ*fakA*Δ*farER*	Deletion of *fakA* and *farER* genes
Δ*farER*	Deletion of *farER* genes
Δ*graR*	Deletion of *graR* gene
Δ*graS*	Deletion of *graS* gene
Δ*pro*	Protease deficient mutant
<	Less than
>	Greater than
~	Approximately
A	Alanine
A_{530}	Absorbance at 530 nm
AA	Arachidonic acid (Chapter 2) or Arachidic acid (Chapter 3)
Aas	Acyl-ACP synthase
ACME	Arginine catabolic mobile element
ACP	Acyl-carrier protein
ADI	Arginine deiminase pathway
agr	Accessory gene regulator
AIP	Autoinducing peptide
AMP	Antimicrobial peptide
Amp	Ampicillin
ANOVA	Analysis of variance
aTc	Anhydrotetracycline
Aur	Aureolysin
bp	base pair
C#$_1$:#$_2$	Carbon (#$_1$ indicates carbon chain length, #$_2$ indicates units of unsaturation)
CA-MRSA	Community-associated methicillin-resistant Staphylococcus aureus
$CaCl_2$	Calcium chloride
CAMPs	Cationic antimicrobial peptides
CFU	Colony forming units
CHIP	Chemotaxis inhibitory protein
Cm	Chloramphenicol
CoA	Coenzyme A
CWA	Cell wall anchored
D	Aspartic acid

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Erm	Erythromycin
ETs	Exfoliative toxins
F	Phenylalanine
fab	Fatty acid biosynthesis
fad	Fatty acid degradation
FakA	Fatty acid kinase A
FAME	Fatty acid modifying enzyme
FarE	Fatty acid resistance efflux protein
FarR	Fatty acid resistance regulator protein
FASII	Type II bacterial fatty acid synthesis
Fc	Fragment crystallizable
FFA	Free fatty acid
fur	Ferric uptake regulator
g	grams
G	Glycine
G3P	Glycerol-3-phosphate
Geh	Glycerol ester hydrolase
h	Hour
H^+	Hydrogen ion
HCl	Hydrogen chloride
I	Isoleucine
IgG	Immunoglobulin G
JAK- STAT	Janus kinase-signal transducer and activator of transcription
K	Lysine
kb	Kilobase
kDa	Kilodalton
L	Litres or Leucine
LA	Linoleic acid
LAC	Los Angeles County clone
LB	Luria Bertani
LL-37	Human cathelicidin
lux	Luciferase
Lysyl-PG	Lysyl-phosphatidylglycerol
m	Milli
M	Molar
MES	Morpholinoethanesulfonic acid
mg	Milligram
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration

min	Minutes
mL	Millilitre
mm	Millimetre
mM	Millimolar
MOPS	Morpholinopropanesulfonic acid
MRSA	Methicillin-resistant Staphylococcus aureus
MSA	Mannitol salt agar
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
n	Nano
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NHE-1	Sodium-hydrogen antiporter 1
nm	Nanometre
OA	Oleic acid
OD_{600}	Optical density at a wavelength of 600 nm
P	Proline
PA	Palmitoleic acid (Chapter 2) or Palmitic acid (Chapter 3)
PAMPs	Pathogen-associated molecular pattern molecules
PBP	Penicillin-binding proteins
PBP2a	Penicillin-binding protein 2a
PCR	Polymerase chain reaction
PG	Phosphatidylglycerol
pH	Potential of hydrogen
PmB	Polymyxin B
PMNs	Polymorphonuclear leukocytes
PO_4	Phosphate
PSMs	Phenol-soluble modulins
PtdOH	Phosphatidic acid
$P_{xyl/tetO}$	Tetracycline-inducible promoter
RLU	Relative light units
RNA	Ribonucleic acid
RNAIII	Regulatory RNA transcribed by the P3 promoter of *agr* system
RNAP	RNA polymerase
Rot	Repressor of toxin
rpm	Revolutions per minute
rRNA	Ribosomal RNA
S	Serine
SA	Sapienic acid (Chapter 2) or Steric acid (Chapter 3)
SARS CoV-2	Severe acute respiratory syndrome coronavirus 2
SCC*mec*	Staphylococcal cassette chromosome *mec*
SCIN	Staphylococcal Complement Inhibitor

Scp	Staphopain
SDM	Site directed mutagenesis
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SE	Standard Error
SERAMs	Secretable expanded repertoire adhesive molecules
sFFA	Saturated free fatty acid
spp.	Species
SSTIs	Skin and soft tissue infections
T	Threonine
T7SS	Type VII secretion system
Tc	Tetracycline
TCA cycle	Tricarboxylic acid cycle
TCS	Two-component system
Tet	Tetracycline
Tn	Transposon
TSA	Tryptic soy agar
TSB	Tryptic soy broth
uFFA	Unsaturated free fatty acid
v/v	Volume per volume
x g	Relative centrifugal force measured as multiples of gravity
Y	Tyrosine
α	Alpha
β	Beta
β	Beta
γ	Gamma
δ	Delta
µ	Micro
µg	Microgram
µL	Microlitre
µM	Micromolar
σ	Sigma

Chapter 1

1 Introduction

1.1 *Staphylococcus aureus* overview

Staphylococcus aureus is a Gram-positive bacterium, part of the Firmicute phylum, which asymptomatically colonizes 30% of the population (1). This bacterium primarily colonizes the anterior nares but can also colonize other areas of the body including the throat, perineum, vagina, and gastrointestinal tract (2–5). While part of the normal skin flora, *S. aureus* is an opportunistic pathogen that can cause a broad spectrum of infections, including severe soft tissue infections, endocarditis, osteomyelitis, pneumonia, and sepsis, due to the multitude of virulence factors at its disposal (6–8).

S. aureus was first discovered in 1880 by Dr. Alexander Ogston in a knee joint abscess, who named the cocci bacteria after the Greek word "staphyle" meaning bunch of grapes, based on its clustered appearance under the microscope (9–12). Later in 1884, Dr. Friedrich Julius Rosenbach differentiated *S. aureus* from *Staphylococcus albus* (later renamed *Staphylococcus epidermidis*), using the Latin words "aurum" meaning gold, and "album" meaning white, based on the colour of the colonies (12, 13). Once discovered, the mortality rate for *S. aureus* bacteremia was approximately 80%, as antibiotics had not yet been discovered (14). Although the discovery of penicillin to treat *S. aureus* infections was initially promising, resistance through β-lactamases quickly arose, and by the 1950s, resistant isolates had been identified (15, 16). Methicillin was used next, but acquisition of the *SCCmec* cassette led to methicillin resistant *S. aureus* (MRSA) strains that are resistant to all available β-lactam drugs on the market, making it one of the leading causes of nosocomial infections worldwide (17–19).

1.2 Development of antibiotic resistance in *S. aureus*

The first case of antibiotic resistance in *S. aureus* occurred through acquisition of the *blaZ* β-lactamase gene, encoding an enzyme that inactivates penicillin by hydrolysis of its β-lactam ring (20, 21). In the absence of β-lactam antibiotics, BlaI represses expression of *blaZ* (22). However, upon exposure to a β-lactam, the transmembrane protease BlaR1 is activated, degrading the BlaI repressor, leading to *blaZ* expression (23, 24). To overcome this resistance, modified β-lactam antibiotics, such as methicillin, were used which were resistant to BlaZ inactivation. However, methicillin resistance was also

acquired through horizontal gene transfer of the *mecA* gene, encoding an alternative penicillin-binding protein (PBP), PBP2a. PBPs are transpeptidases that catalyze the cross-linking of peptidoglycan in the cell wall, but are highly susceptible to binding and subsequent inactivation by β-lactam antibiotics; however, PBP2a exhibits low affinity to the β-lactam class of antibiotics, providing resistance to a broad range of β-lactam antibiotics (25, 26). As with the β-lactamase gene *blaZ*, *mecA* is regulated by a transmembrane protease MecR1, which senses β-lactam antibiotics and degrades the MecI repressor, leading to *mecA* expression (27, 28). Additionally, MecR2 can destabilize the binding of MecI to the *mecA* promoter, leading to proteolytic degradation of MecI independent of MecR1 (29). MRSA have continued to evolve as new families of antibiotics are introduced into clinical practice, such that many MRSA strains are now resistant to a wide variety of commonly used antibiotics as outlined in Table 1.1, through mechanisms that include both horizontal transfer of genes as well as mutations to native genes (30).

Table 1.1. Mechanisms of antibiotic resistance in *S. aureus*

Antibiotic:	**Resistance Mechanism:**	**Reference:**
β-lactams	BlaZ β-lactamase, encoding an enzyme that inactivates certain β-lactams through hydrolysis of the β-lactam ring	(20, 21)
	The MecA alternative penicillin-binding protein, PBP2a, exhibits low affinity to β-lactams	(25, 26)
Fluoroquinolones	Mutations in the conserved quinolone resistance-determining regions of topoisomerases which reduce fluoroquinolone affinity	(31–33)
Vancomycin	Acquisition of the *vanA* gene cluster, transferred from vancomycin-resistant enterococcus, alters the peptide target that vancomycin and closely related antibiotics bind to inhibit cell synthesis	(34–36)
Daptomycin	Variety of mutations in genes that provide general resistance to CAMPs and cell envelope damage.	(37–42)
Macrolides, Lincosamides, and Streptogramis	Erythromycin resistance methyltransferase, ErmA, transfers a methyl group to the 23S ribosomal RNA, blocking the antibiotic-binding site.	(43–45)
Tetracycline	TetK and TetL efflux of intracellular tetracycline.	(46, 47)
	TetO and TetM bind to the ribosome and dislodge tetracycline from its binding site.	(46–48)

Aminoglycoside	Cytoplasmic aminoglycoside modifying enzymes that catalyze modifications to the antibiotics, rendering them unable to bind the ribosome.	(47, 49)
Oxazolidinones (Linezolid), Chloramphenicol, and Florfenicol	Cfr methyltransferase modifies the 23S rRNA, blocking the binding site for antibiotics	(50, 51)
	Mutations to ribosomal proteins L3 (RplC), L4 (RplD), and L22 (RplV) are proposed to inhibit antibiotics through structural changes in the ribosome	(52–54)
Chloramphenicol	Chloramphenicol acetyltransferase inactivates chloramphenicol by acetylation (acetyl-CoA + chloramphenicol → chloramphenicol 3-acetate + CoA)	(55, 56)
Fusidic acid	Amino acid substitutions in FusA (elongation factor G), altering the drug target	(57–59)
	FusB and FusC promote the dissociation of stalled ribosome caused by fusidic acid impairment, allowing the ribosomes to resume translation	(60, 61)
Polymyxin B and Colistin	Intrinsic resistance through GraXRS regulated changes to membrane surface charge including D-alanylation of the lipoteichoic acids and production of lysyl-phosphatidylglycerol	(62–66)

1.3 Community-associated methicillin resistance *S. aureus*

Previously, the majority of MRSA infections were confined to healthcare settings (67); however, beginning in the 1990s, there has a been a dramatic rise in the number of community-associated MRSA (CA-MRSA) strains that are rapidly spread among the general population, regardless of exposure to healthcare settings (8, 68). There are five CA-MRSA strains that account for the vast majority of CA-MRSA infections worldwide (69): the Midwest clone (70, 71); the Southwest Pacific/Oceania clone (72–74); the European clone (75, 76); the Pacific clone (77, 78); and the USA300 clone (79, 80). However, amongst the five clones, the USA300 clone dominates and displaces the locally endemic clones once introduced into the population (80–83). In many regions, USA300 accounts for over 50% of CA-MRSA infections, and in North America specifically, USA300 accounts for over 98% of infections presented to emergency departments (84). The success of USA300 can in part be attributed to an enhanced ability to colonize skin due to the acquisition of the arginine catabolic mobile element (ACME), thought to have been transferred horizontally from *S. epidermidis* (85). ACME provides resistance to polyamines through the detoxifying spermidine acetyltransferase, *speG*, and to acidic pH through an arginine deaminase pathway that neutralizes acidic environments (86, 87). An increased ability to colonize individuals poses serious concerns for healthcare systems, as transmission can occur more rapidly when individuals are colonized, and studies have shown that there exists a strong correlation between the *S. aureus* strains isolated from the blood and foci of infection, with the strains colonizing the anterior nares (1, 88). Furthermore, those colonized by *S. aureus* have a significantly greater risk of developing blood stream infections than non-carriers when hospitalized (88–90). Together, the rapid spread of CA-MRSA among the general population poses a serious health concern due to the increased risk of infection of subsequent infections.

1.4 Disease progression of *S. aureus*

While usually an asymptomatic colonizer of humans, *S. aureus* is an opportunistic pathogen that can cause serious infection, ranging from relatively benign skin and soft tissue infections (SSTIs) to life-threatening bacteremia (91). This broad range of infections is facilitated by a vast array of virulence factors to combat the host immune

system, as well as a diverse repertoire of adhesions factors allowing for colonization and infection of various sites throughout the body (6, 92).

1.4.1 Adhesion of *S. aureus* to host tissues

For *S. aureus* to colonize and infect the host, the first step is adhesion, which can occur through both protein and non-protein adhesion factors. One primary family of adhesion factors is the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which allow for adhesion to a variety of different components of the extracellular matrix of the host including fibrinogen, fibronectin, keratin, collagen, elastin, and plasminogen, among others (92, 93). For example, the MSCRAMM fibronectin binding proteins alone can bind fibronectin, corneodesmosin, elastin, and plasminogen (94–98). In addition to MSCRAMMs, *S. aureus* possesses a variety of other cell wall anchored (CWA) adhesion proteins, secreted repertoire of adhesive molecules (SERAMs), polysaccharide intercellular adhesin, and wall teichoic/lipoteichoic acids, that together allow *S. aureus* to adhere to a wide range of niches within the human body, leading to long term colonization or infections of various tissues (92).

1.4.2 Development of SSTIs and abscesses

Once adherent, *S. aureus* can remain as a colonizer of skin, or progress to an infection if there is a breach in the epithelial barrier, with the most common manifestation of infection being SSTIs. *S. aureus* is the leading cause of SSTIs (99), which can manifest as cellulitis, impetigo, furuncle, carbuncle, staphylococcal scalded-skin syndrome, and abscesses; however, abscesses and cellulitis are the most common clinical presentations, accounting for approximately 63% of *S. aureus* SSTIs (99). A breach to the epithelial layers of the skin allows for penetration of *S. aureus* into the underlying tissues. Circulating polymorphonuclear leukocytes (PMNs) are rapidly recruited to the site of infection, in response to both tissue damage and host proinflammatory molecules elicited by the bacteria (100, 101), which phagocytose the invading pathogen. However, as outlined in Section 1.7., *S. aureus* has a variety of virulence factors and immune evasion mechanisms to combat the infiltrating leukocytes and phagocytosis. Typically, PMNs undergo apoptosis and are removed from circulation by macrophages in a process called

efferocytosis (102); however, *S. aureus* can divert PMNs from this apoptotic pathway in favour of lysis through programmed necrosis (103, 104). Additionally, a variety of secreted toxins from *S. aureus*, including Panton-Valentine leukocidin, leukocidin GH, or leukocidin DE, can cause lysis of neutrophils (105). This on-going battle leads to the development of an abscess, which when matures, forms a fibrous capsule at the periphery to contain the infection (106). These abscesses can resolve spontaneously, but often require treatment through antibiotics or surgical intervention to remove the *S. aureus* infection.

1.4.3 Development of *S. aureus* blood stream infections

In contrast to SSTIs, if *S. aureus* is able to penetrate deeper into the body, or accesses the blood stream, a much more severe infection can take place. While SSTIs are the most frequent type of *S. aureus* infections, *S. aureus* is also the second leading causing of bloodstream infections behind *Escherichia coli* (1–3). Upon entering the blood stream, *S. aureus* is initially cleared by Kupffer cells in the liver (4, 5). While effective in sequestering the infection, this is only a temporary solution, as a portion of the phagocytosed *S. aureus* will survive and begin to replicate (5, 6). Replicating *S. aureus* will eventually cause lysis of the Kupffer cells, releasing bacteria into the peritoneum and the blood, where they are phagocytosed again by peritoneal macrophages and bloodstream neutrophils (5, 6). However, once again a portion of *S. aureus* will survive and begin replication, while these phagocytes migrate throughout the body, leading to systemic dissemination and further disease manifestations (5–7).

Although *S. aureus* can cause a wide array of additional infections not outlined in this section, the lifecycle of *S. aureus* in both abscesses and bacteremia demonstrate the incredible ability of *S. aureus* to combat the immune system. This resistance comes from the myriad of virulence factors at *S. aureus* disposal (8, 9), and the ability to regulate expression of these virulence factors or resistance mechanisms in response to external stimuli (10).

1.5 Comorbidities associated with *S. aureus* infections

Although *S. aureus* infections are severe alone, they can also exacerbate co-infections with other bacteria or viruses, which has become much more prevalent during the onset of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS CoV-2) pandemic. SARS CoV-2 infections can be significantly complicated with secondary bacterial infections, which occur in approximately 5% of cases in the general population, but up to 30% of hospitalized cases (115–118). In particular, *S. aureus* is the most frequent cause of secondary bacterial infections (115–117, 119). This may in part be due to the *S. aureus* protein IsdA manipulating host Janus kinase-signal transducer and activator of transcription (JAK- STAT) signaling which enhances SARS CoV-2 replication and production of infectious viral particles (120). Similarly, Influenza A viral infection can also be complicated with secondary bacterial infections, with *S. aureus* again being one of the main co-infections (121). Specifically, the *S. aureus* secreted lipase-1 protein can enhance Influenza A replication through positive modulation of virus budding (122). With regards to other bacterial infections, *S. aureus* and *Pseudomonas aeruginosa* frequently exist as a co-infection in airways of cystic fibrosis patients and in chronic wounds, and can enhance antibiotic resistances by forming mixed-species biofilms (123–125). Additionally, *S. aureus* infections can be made worse by a variety of other comorbidities including cardiovascular diseases, diabetes, malignant diseases, immunosuppression, and human immunodeficiency virus infections (126–129). Together, *S. aureus* is a dangerous opportunistic pathogen that is made even more severe when combined with other diseases.

1.6 Competition between *S. aureus* and skin commensals

Although *S. aureus* has been shown to coexist with *P. aeruginosa* through a mutually beneficial relationship in the airways of cystic fibrosis patients and in chronic wounds (123–125), bacteria frequently have mechanisms to impair the growth of other bacteria in order to optimize growth. For example, *S. aureus* must compete with resident commensal bacteria in order to effectively colonize human skin. Commensal bacteria can compete with *S. aureus* through a variety of mechanisms including competition for adhesion sites on the host epithelium, competition for limited nutrients on the skin,

competition through antibiosis and the production of antimicrobials, and competition through induction of host defense mechanisms (130). While commensal microbes can directly compete with *S. aureus* to bind certain host epithelial ligands, *S. epidermidis* can directly inhibit *S. aureus* adhesion by secreting high levels of extracellular proteases that degrade *S. aureus* adhesion factors (131, 132). Due to the limited nutrients available on the skin, commensal bacteria must also compete for these resources. For example, the variety of iron acquisition systems in Corynebacteria, Enterobacteria, and coagulase-negative Staphylococci are in direct competition with *S. aureus*, and compete for the limited iron that is available (133–135). The list of antimicrobials produced by skin commensals which impede *S. aureus* colonization constantly expands and highlights the relevance of competition through antibiosis (130). For example, coagulase-negative Staphylococci produce a myriad of antimicrobials including lantibiotic-α and -β produced by *Staphylococcus hominis* (136), lugdunin produced by *Staphylococcus lugdunensis* (137), and 6-thioguanine produced by *Staphylococcus chromogenes* (138), which all severely impair the growth of *S. aureus*. More recently, studies have also shown that probiotic strains of *B. subtilis* which produce Fengycin can be used to inhibit quorum-sensing of *S. aureus* and restrict nasal colonization (139, 140). Finally, commensal bacteria can stimulate a variety of immune cells and immune functions to further restrict colonization by pathogenic *S. aureus* (141, 142). Together, commensal bacteria have a variety of mechanisms to both directly and indirectly compete with *S. aureus* on the skin.

With *S. aureus* still able to colonize approximately 30% of the population, it must have a variety of mechanisms to resist these competitive effects of commensal bacteria. For example, the diverse repertoire of adhesion factors produced by *S. aureus* allows for adhesion to a wide range of different host epithelial ligands, circumventing competition for any one specific ligand (92, 93). To counteract nutrition competition with commensal bacteria, *S. aureus* can upregulate a variety of metabolic pathways in order to optimally acquire nutrients on the skin and in the nose, allowing for survival in very nutrient limited environments (130, 143, 144). Furthermore, *S. aureus* has been shown to uptake foreign siderophores produced by other bacteria as a mechanism to directly compete for iron acquisition (145). In response to antibiosis competition, *S. aureus* can produce its own antimicrobials to target skin commensals. For example, phenol-soluble modulins (PSMs)

have been shown to have direct antimicrobial activity against *S. epidermidis*, *E. coli*, and *S. pyogenes* (146). Finally, although skin commensals can induce host defense mechanisms and stimulate immune function, *S. aureus* has a diverse repertoire of immune evasion strategies to resist the host immune response as outlined in Section 1.7. Together, the ongoing competition between *S. aureus* and skin commensals has resulted in an evolutionary arms race, where bacteria continue to evolve in order to best grow and persist on human skin.

1.7 Immune evasion strategies of *S. aureus*

As mentioned previously, the success of *S. aureus* to colonize and infect humans comes from a diverse repertoire of virulence factors and resistance mechanisms used to combat the host immune system (6, 7, 92). Some of these virulence factors include toxins, such as hemolysins (α, β, γ, and phenol-soluble modulin δ toxin), leukocidins (LukAB, LukDE, and Panton-Valentine leukocidin LukSF), and staphylococcal exfoliative toxins (ETs), which have direct activity against a variety of host tissues, as well as red and white blood cells (105). Targeting of host cell membranes can allow for tissue penetration and deeper infection, lysis of cells can provide access to essential nutrients like iron from red blood cells, and lysis of immune cells can combat immune cell phagocytosis and killing (105). In contrast to toxins which directly target host cells, *S. aureus* also possesses a variety of immune evasion mechanisms outlined in Table 1.2, that can dysregulate immune system activation, impair phagocytosis mechanisms, or resist antimicrobial compounds produced by the immune system (147, 148). However, production of these factors imposes a high energetic burden, and therefore the successful elaboration and coordination of these toxins and immune evasion factors relies on *S. aureus* sensing environmental stimuli and regulating gene expression, to optimally combat the host immune system.

Table 1.2. Immune evasion mechanisms in *S. aureus*

Immune Evasion Mechanism:	***S. aureus* Effector System:**	**Reference:**
Bind Fc fragments of IgG to inhibit opsonization	Protein A	(149, 150)
Cleave fibrinogen to form fibrin clots to create bacterial aggregates and inhibit leukocyte infiltration	Coagulase and von Willebrand factor-binding protein	(151–153)
Inhibit neutrophil migration and activation by PAMPs	Staphopain protease A (Scp) and glycerol ester hydrolase (Geh)	(154, 155)
Degradation of immune defense proteins and nutrient acquisition to combat nutritional immunity	Proteases (SspA, SspB, Aur, Scp)	(156–162)
Inhibit the complement system and neutrophil/monocyte chemotaxis	Staphylococcal complement inhibitor (SCIN) and chemotaxis inhibitory protein (CHIP)	(163–166)
Iron acquisition to combat nutritional immunity	Iron regulated surface determinant system (Isd) and Staphyloferrin A and B	(167–170)
D-alanylation of the lipoteichoic acids to repel CAMPs and resist neutrophil killing	DltABCD	(171–173)
Production of lysyl-phosphatidylglycerol to repel CAMPs and resist neutrophil killing	Multiple peptide resistance factor F (MprF)	(64, 172, 174)

Resistance to reactive oxygen species	Staphyloxanthin, Catalase (KatA), alkyl hydroperoxide reductase (AhpC), and superoxide dismutase	(175–179)
Inhibit the complement system and phagocytosis	Capsular polysaccharides	(180, 181)
Inactivate defensins and inhibit opsonization	Staphylokinase	(182, 183)
Non-specific hyperactivation of T-cells to dysregulate proper immune responses	Toxic Shock Syndrome Toxin 1 and related superantigen enterotoxins	(184–187)

1.8 Genetic regulation in *S. aureus*

With *S. aureus* capable of both asymptomatic colonization and infectious states, there must be tight regulation of gene expression to coordinate these processes and optimize gene expression in response to environmental parameters. One of the most notable regulators of gene expression is the accessory gene regulator (*agr*) system, which regulates gene expression through a quorum sensing mechanism. The *agr* system involves four genes, *agrABCD*, and RNAIII (188). The system begins with AgrD, a precursor for the quorum signal of Agr known as the autoinducing peptide (AIP), being processed and effluxed from the cell by AgrB. In the extracellular space, the AIP is furthered processed by the signal peptidases SpsB into its final active form (189–192). The final two genes in the *agr* system encode a two-component sensor system, with the histidine kinase sensor AgrC binding to AIP, leading to phosphorylation and activation of the response regulator AgrA. AgrA upregulates expression of PSMs, as well as RNAIII, a regulatory RNA molecule that promotes expression of a variety of secreted virulence factors (proteases, lipase, hemolysins, etc.) while repressing expression of cell-surface associated proteins (fibronectin binding proteins, Protein A, *etc*.) (193–198).

While *agr* causes a global shift in gene expression to facilitate a transition from colonization to infection, there are variety of other genetic regulatory mechanisms *S. aureus* employs to sense and respond to the environment, including alternative sigma factors (Table 1.3), additional two-component sensing systems (Table 1.4), SarA family DNA binding proteins (*ie*. SarA (197–204) and Rot (205–207), which play a fundamental role in regulating toxins and immune evasion mechanisms outlined in Table 1.2), and metal-dependent DNA binding proteins such as ferric uptake regulator Fur (208–210), which regulates expression of a variety of genes in response to iron availability. Together, this complex system of genetic regulation allows *S. aureus* to optimally colonize, infect, and resist immune system function, in a wide range of different environments.

Table 1.3. Sigma factor transcriptional regulation in *S. aureus*

Sigma Factor:	Function:	Reference:
σA (SAUSA300_1521)	*Primary (housekeeping) sigma factor:* Directs the transcription of the bulk cellular RNA.	(211)
σB (SAUSA300_2022)	*General stress response sigma factor:* Responds to a variety of stressors including non-optimal temperatures, high salt, ethanol, oxidative stress, cell wall-active agents, and acid stress, to regulate virulence and resistance factors involved in cell envelope composition, membrane transport, protein secretion, biofilm formation, metabolism, and drug resistance.	(212–216)
σH (SAUSA300_0519)	*Bacterial competence sigma factor:* Regulates genes important for competency, integration and excision of prophage, and stabilizes lysogeny in host cell.	(217, 218)
σS (SAUSA300_1722)	*Extracytoplasmic function sigma factor:* Regulates genes important for survival during starvation, growth at elevated temperatures, and in response to membrane disrupting agents.	(219–221)

Table 1.4. Two-component sensor system transcriptional regulation in *S. aureus*.
Sensor kinases are listed in red, and response regulators are listed in blue.

Genes:	Accessory Genes:	Signal:	Function:	Reference:
walR (SAUSA300_0020) *walK* (SAUSA300_0021)	*walH* (SAUSA300_0022) *walI* (SAUSA300_0023)	Unknown	Cell wall metabolism, cell wall division, biofilm formation, virulence expression	(222–224)
hptS (SAUSA300_0218) *hptR* (SAUSA300_0217)	*hptA* (SAUSA300_0219)	Glucose-6-phosphate	Glucose-6-phosphate import	(225–227)
lytS (SAUSA300_0254) *lytR* (SAUSA300_0255)	None	Membrane potential	Cell wall autolysis, CAMP resistance	(228–230)
graS (SAUSA300_0646) *graR* (SAUSA300_0645)	*graX* (SAUSA300_0644) *vraF* (SAUSA300_0647) *vraG* (SAUSA300_0648)	CAMPs (nisin, LL-37, colistin, polymyxin B, etc.), acidic pH	Resistance to: CAMPs; acidic pH; growth at high temperatures; oxidative stress; glycopeptide antibiotics	(63, 66, 231–237)

saeS (SAUSA300_0690) *saeR* (SAUSA300_0691)	*saeQ* (SAUSA300_0692) *saeP* (SAUSA300_0693)	Human neutrophil peptides 1–3	Virulence expression	(238, 239)
desK (SAUSA300_1219) *desR* (SAUSA300_1220)	SAUSA300_1217 SAUSA300_1218	Low temperature	Not evaluated in *S. aureus*	(240)
arlS (SAUSA300_1307) *arlR* (SAUSA300_1308)	None	Low manganese, additional unknown stimuli	Expression of cell wall surface proteins, ß-lactam antibiotic resistance, virulence expression, manganese homeostasis	(233, 241–246)
srrB (SAUSA300_1441) *srrA* (SAUSA300_1442)	None	Hypoxia and nitric oxide concentrations	Anaerobic metabolism, nitrosative stress resistance, virulence factor repression, growth at	(233, 247–249)

			lower (28ºC) temperatures	
phoR (SAUSA300_1638) *phoP* (SAUSA300_1639)	None	Low inorganic phosphate levels	Phosphate import and homeostasis	(250)
airS (SAUSA300_1799) *airR* (SAUSA300_1798)	None	Oxidation signals	Oxidative stress resistance, autolysis resistance, vancomycin resistance, survival in blood through expression of *sspABC*	(251–254)
vraS (SAUSA300_1866) *vraR* (SAUSA300_1865)	*vraT* (SAUSA300_1867) *vraU* (SAUSA300_1868)	Cell wall damage	Cell wall biosynthesis, resistance to cell wall synthesis inhibitors	(233, 255–257)
agrC (SAUSA300_1991) agrA (SAUSA300_1992)	*agrB* (SAUSA300_1989) *agrD* (SAUSA300_1990)	Autoinducing peptide quorum sensing	Virulence expression, repression of surface binding proteins	(258, 259)

kdpD (SAUSA300_2035) *kdpE* (SAUSA300_2036)	None	Cyclic-di-AMP	Potassium transport, virulence expression	(260–264)
hssS (SAUSA300_2309) *hssR* (SAUSA300_2308)	None	Hemin toxicity	Heme detoxification	(265, 266)
nreB (SAUSA300_2338) *nreC* (SAUSA300_2337)	*nreA* (SAUSA300_2339) *narI* (SAUSA300_2340) *narJ* (SAUSA300_2341) *narH* (SAUSA300_2342) *narG* (SAUSA300_2343)	Low oxygen, high nitrate	Nitrate and nitrite reduction	(233, 267, 268)
braS (SAUSA300_2558) *braR* (SAUSA300_2559)	*braE* (SAUSA300_2556) *braD* (SAUSA300_2557)	Bacitracin, Nisin	Bacteriocin resistance	(269–271)

1.9 Innate immune conditions of the skin

In addition to the myriad of factors to resist host immune cells outlined in Table 1.2, *S. aureus* must also overcome the innate immune compounds of human skin in order to establish colonization. As mentioned earlier, acquisition of the *speG* encoded spermidine acetyltransferase by USA300 is essential for its success, since the polyamines spermidine and spermine found on human skin are highly toxic to other strains of *S. aureus* (86, 272). In addition to polyamines, there are a variety of other innate conditions that must be overcome to establish colonization including antimicrobial unsaturated free fatty acids (uFFA), acidic pH, and cationic antimicrobial peptides (CAMPs), which normally restrict colonization and infection by pathogens (273). Antimicrobial uFFA, found in the sebum from sebaceous glands, are toxic to bacteria through mechanisms that include inhibiting the electron transport chain and proton motive force (274–276), uncoupling oxidative phosphorylation (277), altering cell membrane permeability and fluidity causing lysis (276, 278–281), release of low molecular weight proteins through the creation of small pores (282), and oxidative stress (283, 284). Similarly, acidic pH can disrupt the plasma membrane, inhibit the activity of enzymes and membrane transport proteins, and lead to significant alterations in the phospholipid composition of the membrane (285, 286). With uFFA able to alter membrane permeability and allow for penetration by H^+ ions, uFFA and acidic pH conditions may work synergistically in combatting microbial colonization (287). Finally, CAMPs are a diverse set of molecules that primarily function by disrupting the phospholipid membrane; however, certain CAMPs can traverse the membrane and act on intracellular targets as well (288, 289). Of interest, all three mechanisms can affect the integrity of the phospholipid membrane, albeit through different mechanisms. Together, these mechanisms of innate immunity inhibit colonization by most pathogens; however, *S. aureus* can circumvent these defense mechanisms using a variety of proteins and virulence factors in order to effectively colonize the skin (287, 288, 290).

1.9.1 Response of *S. aureus* to acidic pH

The pH of human skin ranges from a 4.1–5.8 (291), inhibiting the colonization of a majority of pathogens (292, 293). This acidic environment is maintained through a variety of mechanisms including degradation of filaggrin into trans-urocanic acid (294, 295), metabolism of sebum triglycerides by both host and bacterial lipases to release free fatty acids (296–298), production of acidic electrolytes and lactic acid from sweat glands (299), and the sodium-hydrogen exchanger 1 (NHE-1) expressed in keratinocytes directly contributing to the acidification of the stratum corneum (300–302). To combat the acidity of skin, *S. aureus* utilizes urease activity and the arginine deaminase pathway (Figure 1.1). Low environmental pH highly upregulates expression of urease genes, which metabolize urea to ammonia and carbon dioxide (303–305). The ammonia produced can then be used to neutralize the pH of the bacterial surroundings (306). Additionally, the arginine deaminase pathway can metabolize arginine into L-ornithine, ammonium, and carbon dioxide, again functioning to neutralize acidic environments (87) (Figure 1.1). Interestingly, *S. aureus* USA300 possesses both a native arginine deaminase pathway, as well as an accessory arginine deaminase pathway within ACME, which allows for higher arginine deaminase activity under skin-like acidic environments (87, 307) (Figure 1.1).

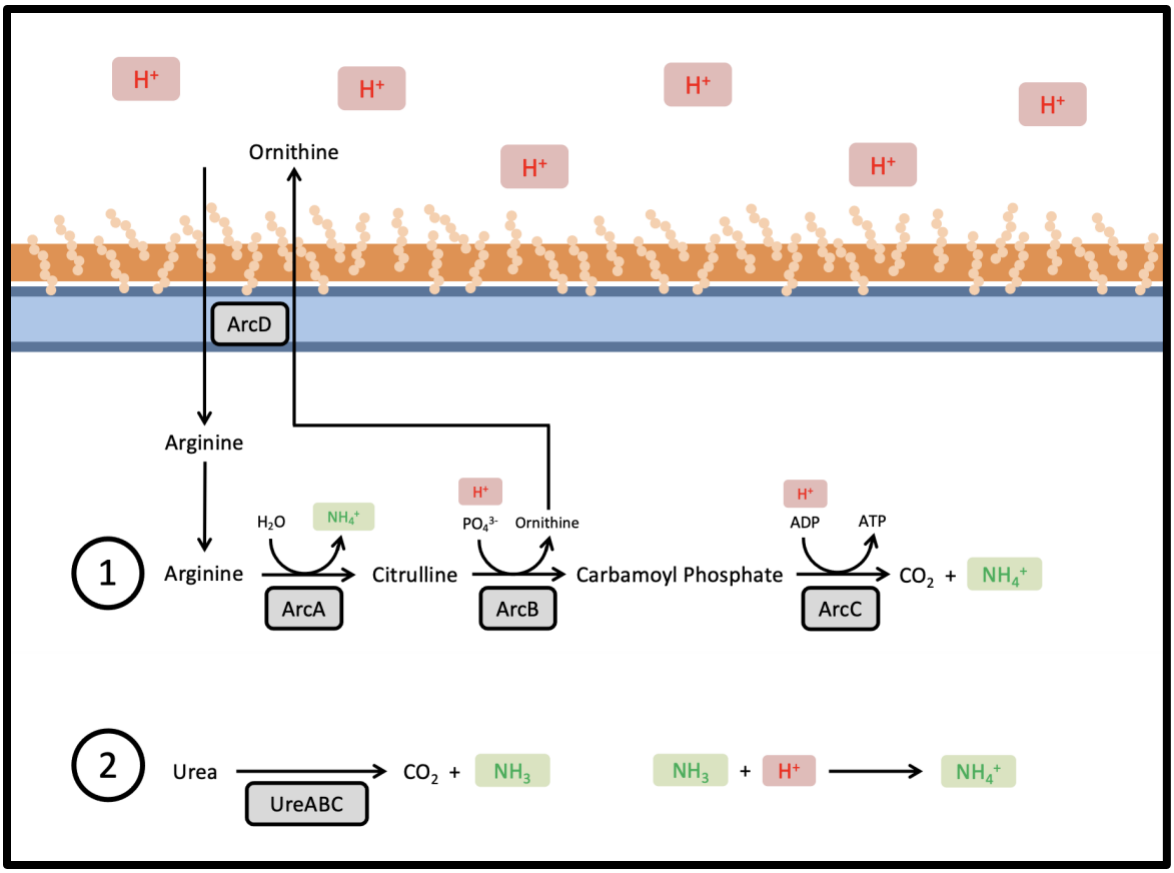


Figure 1.1. Schematic diagram for the neutralization of acidic pH through the arginine deaminase pathway (ADI) and urease. Acidic pH (red) can be neutralized through ADI **(1)** or urease **(2)** by the production of ammonia and ammonium species (green). *S. aureus* USA300 possesses two functional ADI gene clusters, the native ADI system (SAUSA300_2567, SAUSA300_2568, SAUSA300_2569, SAUSA300_2570) and one in ACME (SAUSA300_0061, SAUSA300_0062, SAUSA300_0064, SAUSA300_0065).

1.9.2 Response of *S. aureus* to CAMPs

Resistance to CAMPs can occur through a variety of mechanisms such as thickening the cell wall and altering membrane fluidity, releasing proteases to degrade peptides, and altering the net surface charge of the membrane (289, 308) (Figure 1.2). Noted in both *Enterococcus faecalis* and *S. aureus*, thickening of the cell wall is proposed to act as a sieve, inhibiting CAMPs from accessing the phospholipid membrane (308–310). Additionally, in *S. aureus*, the thickened cell wall contains increased levels of non-amidated peptidoglycan, which have a higher affinity for CAMPs (310). This peptidoglycan can bind and sequester the CAMPs, preventing them from accessing the phospholipid membrane (Figure 1.2). Altering membrane fluidity in response to CAMPs is another mechanism seen in various Gram-positive bacteria (39, 41). In response to CAMPs, *Enterococcus faecium* decreases the amount of unsaturated fatty acids in the membrane to increase membrane rigidity (311). In contrast, *S. aureus* maintains membrane fluidity through the amount of carotenoid and branched chain fatty acids in the cell phospholipid membrane in order to combat CAMP toxicity (39, 312, 313) (Figure 1.2). Production of proteases can directly degrade CAMPs, and is well demonstrated by the *S. aureus* metalloprotease aureolysin, which can directly degrade LL-37 (159) (Figure 1.2). Finally, reducing the negative net surface charge of the membrane in order to repel CAMPs occurs through two mechanisms in *S. aureus*; D-alanylation of the teichoic acids by DltABCD, and the production of lysyl-phosphatidylglycerol by MprF (64, 65, 314). In addition to creating a more positive net charge on the membrane, lysyl-phosphatidylglycerol also stabilizes the membrane, counteracting the effects of many CAMPs (315) (Figure 1.2). In *S. aureus*, both the *dlt* operon and *mprF* are regulated by the two-component regulatory system, GraRS (66).

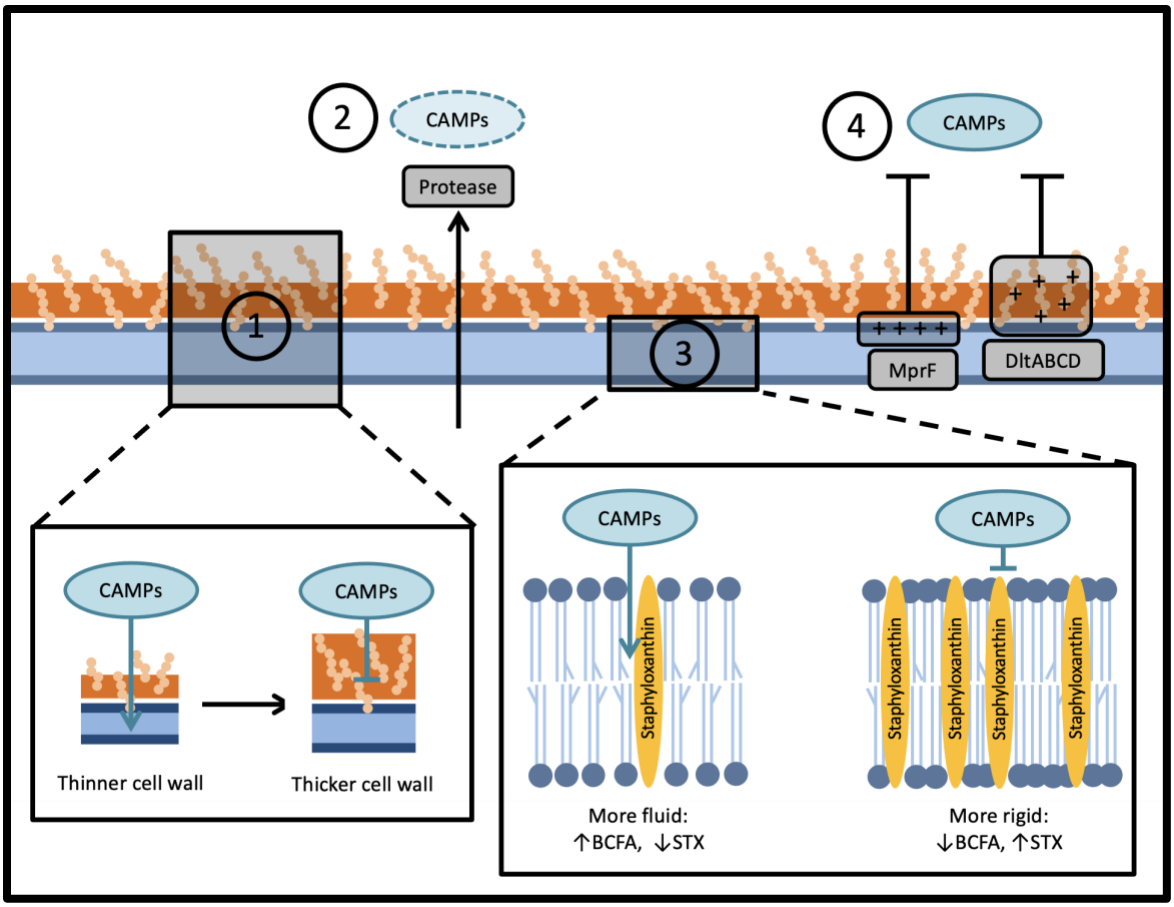


Figure 1.2. Schematic diagram of *S. aureus* CAMPs resistance mechanisms. *S. aureus* possesses a variety of mechanisms to resist CAMPs including: **(1)** Thickening of the cell wall to function as a sieve and prevent penetration of CAMPs; **(2)** Secretion of proteases to degrade CAMPs; **(3)** Increasing membrane rigidity by decreasing the amount of branched chain fatty acids (BCFA) and increasing the amount of staphyloxanthin (STX); and **(4)** increasing the cell surface charge through the production of lysyl-phosphatidylglycerol by MprF and D-alanylation of the teichoic acids by DltABCD. Additionally, lysyl-phosphatidylglycerol produced by MprF has been attributed to stabilizing the membrane to counteract the effects of CAMPs.

1.9.3 The *S. aureus* two-component glycopeptide resistance associated GraRS system

As outlined in Table 1.4, the GraRS system responds to a variety of conditions encountered during colonization and infection of a host, but its primary function in the literature is to respond to CAMPs (63, 66, 231–237) (Figure 1.3). Sensing of CAMPs occurs through the binding of CAMPs to a short negatively charged extracellular loop in the histidine kinase GraS (316), leading to phosphorylation of the response regulator GraR, and transcription of genes that provide resistance to CAMPs, including *mprF* and *dltABCD* mentioned previously (66, 237) (Figure 1.3). In contrast to classic TCS systems, the GraRS system requires additional accessory proteins to function including GraX and VraFG (236). GraX is proposed to act as a scaffold to enhance the interaction of GraR and GraS (232, 317), while VraFG is thought to direct the binding of CAMPs to activate GraS (63, 236, 269). Interestingly, VraFG comprises an ABC-transporter; a large family of proteins that are frequently associated with TCS systems, and function to efflux compounds specific to the TCS system (318). However, VraFG does not appear to function as an efflux pump, but rather functions through binding interactions with CAMPs (236, 269).

The requirement of VraFG for proper activation of GraRS is not novel however, as there are a variety of other examples of TCS systems that sense antimicrobial peptides which also require an ABC-transporter for proper signalling activity. Interestingly, these systems are found almost exclusively in *Firmicutes* bacteria (319). The majority of these TCS systems are referred to as BceRS-like TCS, based on the well documented system in *B. subtilis* where the TCS BceRS requires the ABC-transporter BceAB for proper activation in the presence of bacitracin (320–323). BceS is an intramembrane sensor kinase that lacks an extracellular sensory domain (319, 324). Instead, the ABC-transporter BceAB communicates with sensor kinase BceS in the presence of bacitracin to activate signalling, however the exact mechanism for this communication remains unknown (320–322). *B. subtilis* possesses three such BceRS-like TCS systems: BceRS-BceAB which responds to bacitracin (320–322, 325); PsdRS-PsdAB which responds to nisin (325–327); and YxdJK-YxdLM which responds to LL-37 (325, 328, 329). *S. aureus*

only possesses two BceRS-like TCS systems: BraRS-BraDE which responds to both bacitracin and nisin (269, 330); and the aforementioned GraRS-VraFG which responds to a variety of cationic antimicrobial peptides such as LL-37, RP-1, polymyxin B, and indolicidin (66, 232, 237).

In addition to providing CAMP resistance through *mprF* and *dltABCD,* the *gra* system regulates transcription of various other genes involved in envelope modification, oxidoreduction processes, global regulation, virulence factors, as well as additional antimicrobial resistance factors, making this system essential to resist innate immune conditions and for *S, aureus* to survive *in vivo* (Table 1.4).

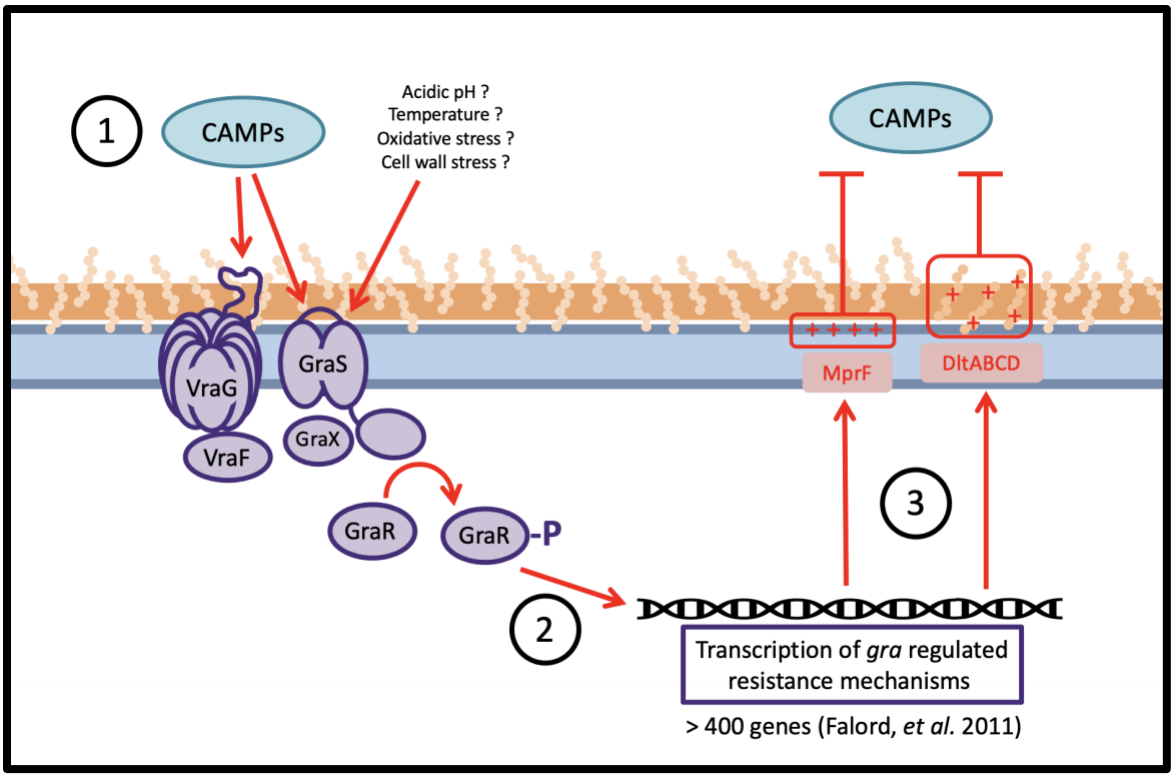


Figure 1.3. Schematic diagram for GraRS activation and resistance to cationic antimicrobial peptides (CAMPs). (1) CAMPs bind the negatively charged extracellular loop of histidine sensor kinase GraS with assistance from the ABC transporter VraFG. Activation of GraS has been shown to play a role in acidic pH, temperature, oxidative, and cell wall stresses, indicating there may be additional stimuli which GraS can sense. **(2)** Upon activation of GraS, the response regulator GraR is phosphorylated and regulates the transcription of a wide range of different genes. The phosphorelay between GraS and GraR required the scaffold protein GraX. **(3)** Two of the genes regulated by GraRS include MprF, which produces lysyl-phosphatidylglycerol, and DltABCD, which produces D-alanylated teichoic acids, that create a positive cell surface charge to repel CAMPs.

1.9.4 Response of *S. aureus* to uFFA

Whether colonizing the anterior nares or skin (276, 331–333), or developing into an abscess infection (334–336), *S. aureus* encounters high concentrations of host-derived antimicrobial uFFA. As such, *S. aureus* has a variety of resistance factors to counteract uFFA ranging from efflux of the uFFA from the cell, changes to the cell wall structure to prevent penetration of uFFA or to stabilize membrane structure to resist the toxic effects of uFFA, and metabolism of the uFFA into non-toxic derivates (Table 1.5). The sheer number of resistance mechanisms to combat uFFA highlights the importance of uFFA resistance in the ability of *S. aureus* to colonize and infect humans. Furthermore, deficiencies in uFFA production correlate with increased colonization rates of *S. aureus*, indicating that uFFA play an important role in controlling *S. aureus* growth (333). However, although these host-derived lipids are toxic to *S. aureus*, they also constitute a potential energy source for the bacteria, and can also be incorporated into membrane phospholipid to conserve energy of *de novo* phospholipid synthesis.

Table 1.5. *S. aureus* resistance mechanisms to combat uFFA toxicity

Genes of Mechanism:	Function:	Reference:
FarER	Efflux of uFFA through the FarE efflux pump, regulated by the TetR-Family regulator FarR	(337–339)
Tet38	An additional efflux pump to decrease intracellular levels of uFFA	(340)
Staphyloxanthin (CrtOPQMN)	Carotenoid production improves membrane stability to resist toxic effects of uFFA	(280, 281)
Wall teichoic acids	Proposed to act as a filter to impair passage of uFFA across the cell wall	(282, 314)
IsdA	Decreases cell wall hydrophobicity to resist uFFA penetration into the cell	(342)
Type VII secretion system (T7SS)	Contributes to membrane integrity and homeostasis in response to uFFA	(343, 344)
Fatty acid modifying enzyme (FAME)	Esterification of uFFA	(345–347)
OhyA	Detoxification of uFFA through hydroxylation	(348, 349)
FakA	Phosphorylation of uFFA and incorporation into phospholipid	(350–354)

1.10 Phospholipid membrane composition and synthesis

In *S. aureus*, the phospholipid membrane consists primarily of phosphatidylglycerol, lysyl-phosphatidylglycerol, and cardiolipin (355). Phospholipid synthesis begins with the acyl groups of acyl-acyl carrier proteins (acyl-ACP) being extended through the type II fatty acid synthesis (FASII) cycle (Figure 1.4). The acyl-ACP is converted into acyl-phosphate (acyl-PO_4) by PlsX, and then used by PlsY to catalyze the acylation of glycerol-3-phosphate (G3P) at the *sn*-1 position, into an acyl-G3P. PlsC then transfers an additional fatty acid from an acyl-ACP to the acyl-G3P molecule at the *sn*-2 position, with an extremely strong preference for C15 acyl chains, to produce the universal bacterial phospholipid precursor, phosphatidic acid (PtdOH) (356, 357). Then, through a series of enzymatic reactions by CdsA, PsgA, and PgpP, PtdOH is converted into phosphatidylglycerol, the major phospholipid in *S. aureus* (356) (Figure 1.4). However, phosphatidylglycerol can be further processed into lysyl-phosphatidylglycerol by MprF, or cardiolipin by Cls1 or Cls2 (64, 174, 358, 359) (Figure 1.4). The fatty acids that make up these phospholipids play an important role in regulating membrane fluidity, and this fluidity is critical to responding to antimicrobial conditions of the skin (360). In many bacteria, such as *B. subtilis*, membrane fluidity is maintained through the combination of saturated and unsaturated fatty acids (361). Unlike *B. subtilis*, *S. aureus* lacks a fatty acid desaturase enzyme, and therefore cannot produce unsaturated fatty acids (361). Instead, *S. aureus* produces branched chain fatty acids in combination with straight chain fatty acids to alter membrane fluidity (360). Branched chain fatty acids make up approximately 55-65% of the total fatty acids found in the membrane of *S. aureus*, and are synthesized using the branched chain amino acids leucine, valine, and isoleucine as precursors (360, 362). The pathways for producing both straight chain and branched chain fatty acids are energetically expensive, and therefore, incorporating host-derived fatty acids into phospholipid synthesis would be favourable for the bacteria.

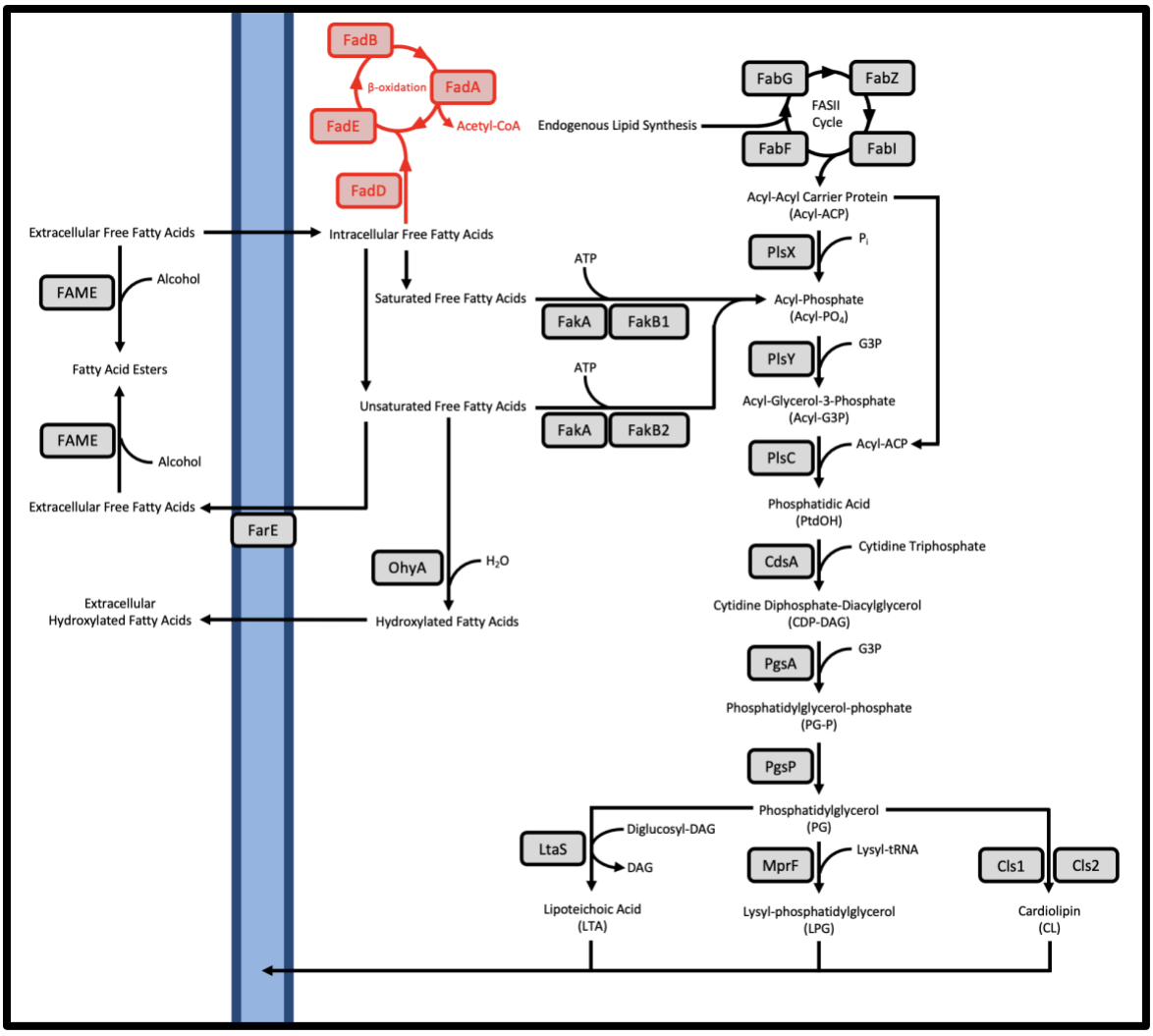


Figure 1.4. Overview of phospholipid synthesis and fatty acids metabolism in *S. aureus*. An overview of phospholipid synthesis and the genes involved in each step of phospholipid synthesis is detailed by Kuhn *et. al.* 2015 (356) and summarized in this figure. Additionally, the known metabolic or efflux pathways for exogenous fatty acids including fatty acid modifying enzyme (FAME), the fatty acids efflux pump (FarE), the oleate hydratase (OhyA), and the fatty acid kinase (Fak), are shown. Although *S. aureus* possesses the genes capable of β-oxidation (red), this pathway has not yet been elucidated. All metabolic steps in grey have been confirmed to occur in *S. aureus*.

1.11 *S. aureus* fatty acid kinase, FakA

Another technique to alter the membrane fluidity is by incorporating exogenous uFFA into phospholipid through the use of FakA, which reduces the energetic burden of producing branched chain phospholipids *de novo* (351) (Figure 1.4). Extracellular fatty acids translocate across the cytoplasmic membrane using the concentration gradient (363). Once on the inner leaflet of the membrane, the fatty acids are bound by either FakB1 or FakB2, and then phosphorylated by FakA (351) (Figure 1.4). FakB2 has specificity for unsaturated fatty acids, while FakB1 has specificity for saturated fatty acids (351). The acyl-PO_4 produced through the Fak system can be converted into an acyl-ACP by PlsX and enter the FASII cycle for extension, or be used by PlsC to acylate the acyl-G3P to produce PtdOH (350). Additionally, the acyl-PO_4 can directly be used by PlsY to produce an acyl-G3P (350) (Figure 1.4). This incorporation of exogenous fatty acids into membrane phospholipid explains why FASII inhibitors are not promising targets for antibiotic development in *S. aureus* (364). Interestingly, while FakA functions to incorporate exogenous fatty acids into the membrane, knockouts of *fakA* result in reduced toxin production, increased protease expression, altered phospholipid membrane composition, decreased susceptibility to dermcidin, and increased virulence in certain animal models (354, 365). Furthermore, expression of the type VII secretion system, which is responsible for exporting various virulence factors, is decreased in a *fakA* deletion mutant (343). Consequently, it appears that numerous phenotypic traits are linked to metabolism of exogenous fatty acids through the FakA pathway.

Although FakA incorporates uFFA into the phospholipid as a detoxification strategy, deletion of *fakA* results in an increased resistance to uFFA; however, this increased resistance is likely due to a higher constitutive expression of the uFFA efflux pump, *farE,* documented in a *fakA* deletion background (366). Deletion of *fakA* also results in an altered profile of cellular metabolites, and an increased pool of cytoplasmic free fatty acids (353, 367). Together, these studies show that alterations to lipid metabolism by deleting *fakA* leads to pleiotropic effects which may explain the variety of phenotypes that occurs in Δ*fakA* strains (343, 354, 365).

1.12 Additional metabolic fates of host-derived fatty acids

FakA is not the only metabolic fate in *S. aureus* for exogenous fatty acids (Figure 1.4). Oleate hydratase OhyA can hydroxylate host-derived uFFA, inactivating the antimicrobial nature of these lipids (Figure 1.4). However, this metabolic process does not provide a nutritional benefit to the bacteria, rather it strictly functions as a detoxification method. Similarly, the fatty acid modifying enzyme (FAME) is an extracellular enzyme responsible for detoxifying antimicrobial fatty acids through an esterification mechanism (Figure 1.4), but again does not provide a nutritional benefit for the bacteria (345–347). Therefore, the literature currently indicates that FakA is the only metabolic pathway used by *S. aureus* where exogenous fatty acids can be used for an energetic benefit to the bacteria (350, 357).

Although FakA is ubiquitous in Gram-positive bacteria and is effective in promoting incorporation of exogenous fatty acids into phospholipid (351, 368–371), β-oxidation is another metabolic pathway bacteria can use to energetically benefit from host-derived fatty acids. β-oxidation is a cyclic process that degrades long chain fatty acids through the release of acetyl-CoA, which can then be fed into the tricarboxylic acid (TCA) cycle to generate large amounts of energy. Although β-oxidation has not previously been noted in *S. aureus* (350, 357), it has been demonstrated in *Staphylococcus carnosus* (372). Furthermore, *S. aureus* possesses a locus of genes, *fadDEBA*, orthologous to those in *E. coli* and *B. subtilis* that allow for β-oxidation. Additionally, these gene are highly upregulated in bacteria internalized by human bronchial epithelial cells, an environment rich in host-derived lipid species (373, 374). Together, we believe β-oxidation is an additional metabolic pathway for host-derived fatty acids, that works in conjunction with the established FakA pathway (Figure 1.4).

1.13 Fatty acid biosynthesis and degradation in *E. coli* and *S. aureus*

In *E. coli*, fatty acid biosynthesis occurs through the Fab proteins, while fatty acid degradation occurs through the Fad proteins. A simplified representation of both pathways can be seen in Figure 1.5. Both pathways are cyclic in nature, and either

increase or decrease the acyl chain length by two carbons per cycle. Since these pathways have opposing functions, they must be tightly regulated for proper metabolic balance in the bacteria. In *E. coli*, this regulation occurs primarily through FadR (375, 376). In the absence of acyl-CoA, FadR induces expression of expression of fatty acid biosynthesis genes (*fab*) and represses expression of fatty acid degradation genes (*fad*) (Figure 1.5). The net result is increased fatty acid biosynthesis to generate more acyl-CoA (57, 375). However, in the presence of acyl-CoA, FadR is inactivated and will dissociate from *fab* and *fad* promoters, stopping induction of *fab* and resulting in derepression of *fad* (375, 376). The net result is increased fatty acid degradation to metabolize excess acyl-CoA. There is an additional regulator of *fab* called FabR; however, FabR is responsible for regulating additional *fab* genes not listed in Figure 1.5, *fabA* and *fabB*, which function in the FASII cycle to produce unsaturated acyl-ACP rather than saturated acyl-ACP (377–380).

In *S. aureus*, fatty acid biosynthesis occurs in a similar manner, where Fab proteins will extend an acyl chain by two carbons every cycle (Figure 1.5). However, even though *S. aureus* possesses genes orthologous to FadDEBA in *E. coli*, there is no orthologue to FadR, meaning regulation of *fab* occurs through a different mechanism. Similar to *B. subtilis*, regulation of the fatty acid biosynthesis genes occurs through FapR (381–383). FapR normally functions to represses expression of fatty acid biosynthesis genes (Figure 1.5); however, elevated malonyl-CoA, a lipid precursor of the FASII cycle, will cause FapR to dissociate from the operator site of fatty acid biosynthesis genes, leading to expression of these genes (381). Interesting, FapR not only regulates expression of the *fab* genes, but also expression of *plsX* and *plsC* which are involved in phospholipid synthesis (381, 383).

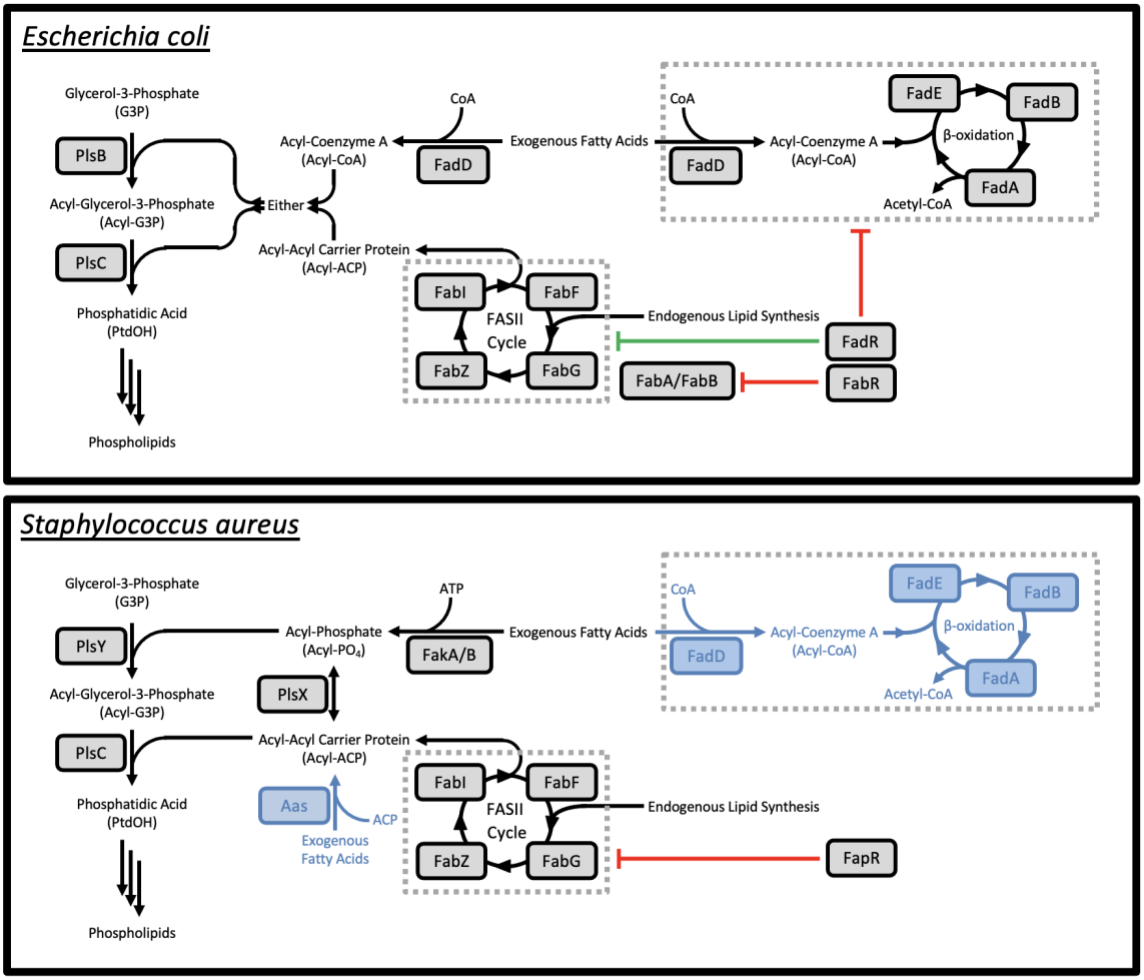


Figure 1.5. Schematic diagram for fatty acid biosynthesis (Fab) and fatty acid degradation (Fad), and the relationship to phospholipid synthesis, in *E. coli* and *S. aureus*. Simplified versions of fatty acid biosynthesis or degradation pathways are outlined with grey dotted lines, and the metabolic connection between these pathways is shown. Red lines indicate repression and green lines indicate induction of genes involved in each pathway. Triple arrows indicate multiple metabolic steps, outlined in Figure 1.4. Pathways in blue indicate proposed pathways mentioned in this thesis, but which have not been confirmed in the literature.

1.13.1 Activation of acyl chains for phospholipid synthesis

In *E. coli*, acylation of the glycerol backbone occurs with both acyl-CoA and acyl-ACP (Figure 1.5). G3P is successively acylated by PlsB at the *sn*-1 site (384, 385), and then PlsC at the *sn*-2 site (386), to create PtdOH. In *S. aureus* acylation of the glycerol backbone occurs with both acyl-PO_4 and acyl-ACP. However, the enzymes responsible for acylation of G3P are substrate specific in *S. aureus*, with PlsY acylating with acyl-PO_4 at the *sn*-1 site, followed by PlsC acylating with acyl-ACP at the *sn*-2 site, to create PtdOH (356, 387, 388). The PO_4 or ACP attached to the acyl chain can however be exchanged through PlsX (356, 387, 388). As seen in Figure 1.5, deletion of *plsX* removes the ability for *S. aureus* to synthesize acyl-PO_4 endogenously, causing these mutants to be auxotrophic for exogenous fatty (350). The use of acyl-PO_4 or acyl-CoA differs between these two systems, and changes the way in which exogenous fatty acids are incorporated into phospholipids.

As mentioned previously, in *S. aureus*, FakA phosphorylates exogenous fatty acids to create acyl-PO_4, which can then be incorporated into phospholipid through PlsY or PlsX/PlsC (351). In *E. coli*, FadD synthesizes acyl-CoA from exogenous fatty acids, which can then be incorporated into phospholipid through PlsB or PlsC (384–386, 389). Additionally, acyl-CoA produced from FadD can funnel into β-oxidation, through FadDEBA (390, 391). Although *S. aureus* possesses orthologous genes to FadDEBA, there have been no studies to date that have identified generation of acyl-CoA by FadD or subsequent β-oxidation function in *S. aureus*.

1.13.2 Role of acyl-ACP synthases in bacterial phospholipid synthesis

An additional family of enzymes capable of generating acyl-ACP for phospholipid synthesis are acyl-ACP synthases (Aas) (Figure 1.6). For example, in *Neisseria gonorrhoeae* and *Chlamydia trachomatis*, exogenous fatty acids are converted to acyl-ACPs through AasN and AasC respectively as a mechanism to activate fatty acids for phospholipid synthesis (392, 393) (Figure 1.6). *E. coli* also possesses an acyl-ACP synthase; however, this enzyme is stringently linked to reacylation of lysophospholipids

generated by lipoprotein synthesis, and has not been shown to function in the general phospholipid synthesis pathway (394–396). Interestingly, acyl-ACP synthase activity has been detected in *S. aureus* lysates, but no gene has yet been identified to possess this function (357). This may be in part due to acyl-ACP synthases being highly similar to long chain fatty acyl-CoA ligases, with the only difference being the addition of a CoA or ACP group to the acyl chain respectively. In some bacteria, such as *Synechocystis* and *Thermus thermophilus*, genes annotated as long chain fatty acyl-CoA ligases can synthesize both acyl-ACP and acyl-CoA (397). Therefore, while the gene responsible for acyl-ACP synthase function in *S. aureus* remains elusive, the two annotated long chain fatty acyl-CoA ligases, *fadD* (SAUSA300_ 0228) and *vraA* (SAUSA300_0559) may be capable of this function. Furthermore, it is not clear if production of acyl-ACP by these proteins can directly enter into phospholipid synthesis, as is the case for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (Figure 1.6), or if these generated acyl-ACP serve a more specialized role, as is the case for *E. coli*.

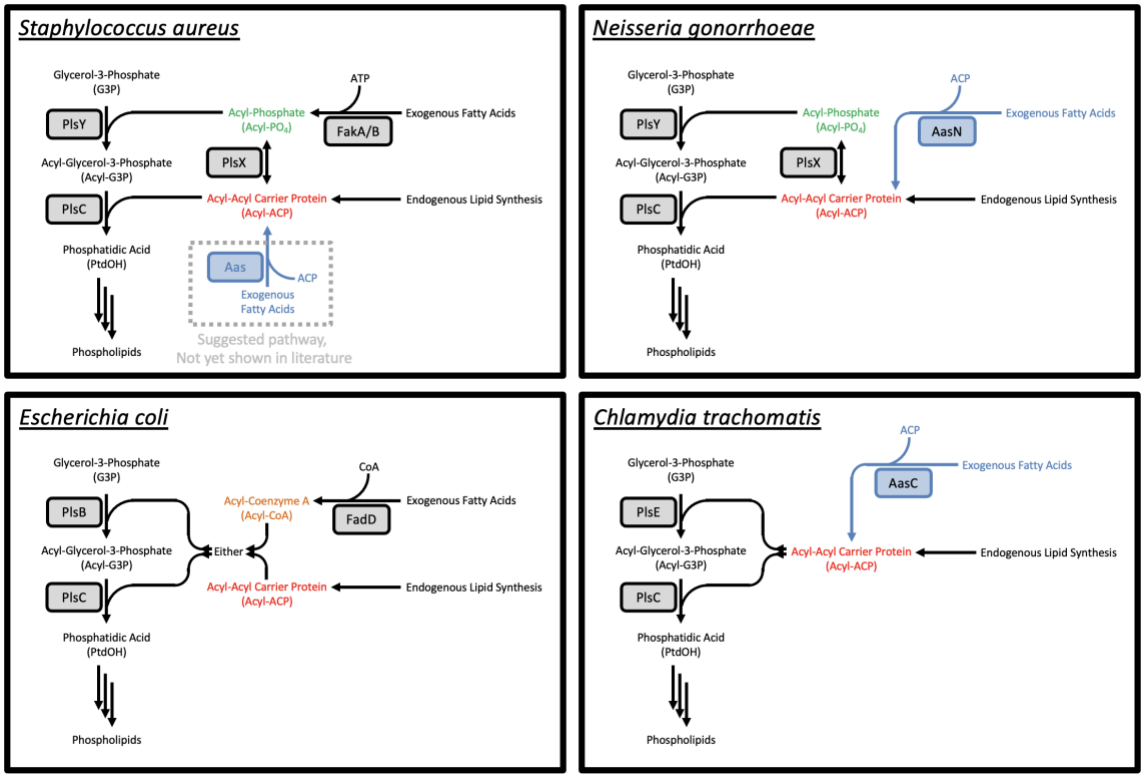


Figure 1.6. Activation of fatty acids for incorporation into phospholipid synthesis in *S. aureus*, *E. coli*, *N. gonorrhoeae*, and *C. trachomatis*. Activation of fatty acids for phospholipid synthesis can come from generation of acyl-phosphate (green), acyl-acyl carrier protein (red), or acyl-Coenzyme A (orange). Activity of acyl-ACP synthases (Aas) are highlighted in blue. The grey dotted box in the *S. aureus* pathway indicates a proposed metabolic step that has not yet been shown in the literature. Triple arrows indicate multiple metabolic steps, outlined in Figure 1.4.

1.14 Rationale and Hypothesis

When colonizing human skin, *S. aureus* encounters a variety of different host-derived fatty acids (276, 398, 399). We hypothesized that *S. aureus* has evolved to thrive in this environmental niche, upregulating novel resistance pathways to resist the toxic effects of these compounds, as well as upregulating fatty acid metabolic pathways to energetically benefit from these fatty acids. A list of the fatty acids used in this dissertation, and their physiological relevance, is outlined in Table 1.6.

My research focuses on how *Staphylococcus aureus* can adapt to the antimicrobial conditions encountered on human skin, in particular acidic pH, cationic antimicrobial peptides, and host-derived free fatty acids, allowing for asymptomatic colonization of 30% of the population. Furthermore, while uFFA function to inhibit the growth of *S. aureus* (290), they also provide a valuable source of lipids for membrane synthesis and energy production, and human skin also provides a rich source of less toxic saturated free fatty acids (sFFA) (399). We hypothesized that *S. aureus* possesses novel uFFA resistance pathways that are activated under conditions found on human skin, and that under these conditions, *S. aureus* can metabolize exogenous FFA to fuel growth and virulence expression.

My first objective is to evaluate how *S. aureus* senses and responds to the antimicrobial uFFA that would be encountered on human skin. While many mechanisms for uFFA resistance have been studied in *S. aureus* (Table 1.5), these resistance mechanisms have been studied independently of other innate immune conditions encountered on human skin, specifically acidic pH and CAMPs. As noted in Tables 1.3 and 1.4, *S. aureus* has a diverse network of regulatory mechanisms that can sense and respond to environmental signals. Therefore, we hypothesized that *S. aureus* can sense the innate immune conditions encountered on human skin, to upregulate robust uFFA resistance pathways, in order to effectively colonize skin. Although VraRS (255, 256) and BraRS (270) both respond to cell wall damage that could be inflicted by acidic pH and CAMPs, we are specifically interested in the GraXRS system as GraS is known to sense and directly respond to CAMPs (66). Furthermore, more recent studies have shown that acidic pH can enhance the GraXRS response to CAMPs, and that GraXRS may play a role in resistance

to acidic pH (233, 400). We hypothesize that in addition to providing resistance to CAMPs and acidic pH, activation of the GraXRS provides resistance to uFFA.

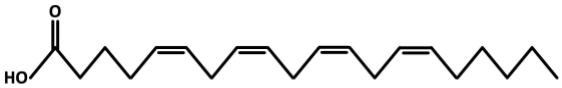
My second objective is to study how *S. aureus* can thrive in the presence of host-derived fatty acids. Once toxicity of host derived fatty acids has been overcome, we hypothesize that the *fadXDEBA* genes in *S. aureus* should have a fundamental role in metabolizing host-derived fatty acids to provide additional energy for growth and virulence expression. We hypothesize this pathway has been underappreciated in *S. aureus* due to TSB, the primary media used to study *S* aureus, containing glucose and therefore repressing expression of β-oxidation machinery (401, 402). Furthermore, studies show that host-derived fatty acids can induce the staphylococcal proteolytic cascade (403), and that the metalloprotease aureolysin induced under these conditions processes the 72-kDa proSAL2 precursor to the 44-kDa mature SAL2 lipase (298). This lipase has been shown to liberate free fatty acids from host-derived triglyceride molecules, indicating that *S. aureus* may benefit from increased concentrations of host-derived free fatty acids (298). Together, we believe these findings indicate *fadXDEBA* plays a fundamental, but currently unelucidated, role in metabolism of host-derived fatty acids, which will provide more insight into the mechanisms used by *S. aureus* to survive in the nutrient limited environment of human skin.

We believe a better understanding of how *S. aureus* responds to host-derived fatty acids to both resist the toxicity of these lipids, as well as benefit from these lipids by metabolizing them to liberate a valuable energy source, is fundamental to understanding how *S. aureus* colonizes human skin. Studies have shown that there exists a strong correlation between the *S. aureus* strains isolated from the blood and foci of infection, with the strains isolated in the anterior nares (1, 88), and that those colonized by *S. aureus* have a significantly greater risk of developing blood stream infections than non-carriers when hospitalized (88–90). Therefore, with an increasing rise in antibiotic resistance, a better understanding of the mechanisms used by *S. aureus* during colonization may lead to novel therapies or adjuvants that prevent persistent colonization and subsequent infections, reducing the serious health burden of MRSA.

Table 1.6. Free fatty acids used in this dissertation.

Fatty Acid	Structure and Physiological Relevance
Saturated Fatty Acids	
Myristic Acid (tetradecanoic acid; 14:0)	 Myristic acid can inhibit the growth of *S. aureus* (282), and can enhance the toxicity of certain unsaturated fatty acids towards *S. aureus* when encountered in combination (404). It is present in human nasal fluid (405, 406) and plasma (407).
Palmitic Acid (hexadecanoic acid; 16:0)	 Palmitic acid does not have major inhibitory activity towards *S. aureus*, and must be encountered at very high concentrations to impair growth (282). It is a major lipid component of both human nasal fluid (405, 406) and plasma (407). Furthermore, it is the most prevalent saturated fatty acid in human sebum (408). It is a poor substrate for FakA-mediated metabolism, and must be extended to a C18 fatty acid before incorporation into the membrane can occur efficiently (282).
Stearic Acid (octadecanoic acid; 18:0)	 Stearic acid does not have major inhibitory activity towards *S. aureus*, and must be encountered at very high concentrations to impair growth (282). It is present in human nasal fluid (405, 406) and plasma (407).
Arachidic Acid (icosanoic acid; 20:0)	 Arachidic acid does not have major inhibitory activity towards *S. aureus*, and must be encountered at very high concentrations to impair growth (282). It is present in human nasal fluid (406) and plasma (407).

Behenic Acid (docosanoic acid; 22:0)	
	Behenic acid has not been shown in the literature to be inhibitory to *S. aureus* growth. It is present in human nasal fluid (406) and plasma (407).
Unsaturated Fatty Acids	
Sapienic Acid (*cis*-6-hexadecenoic acid; 16:1)	
	Sapienic acid is unique to humans and is highly inhibitory to growth of *S. aureus* (282). It is present in human nasal fluid (406), and is the most prevalent unsaturated fatty acid in human sebum (408). It is a poor substrate for FakA-mediated metabolism, and must be extended to a C18 fatty acid before incorporation into the membrane can occur efficiently (282).
Palmitoleic Acid (*cis*-9-hexadecenoic acid; 16:1)	
	Palmitoleic acid is an isomer of sapienic acid and is also highly inhibitory to growth of *S. aureus* (282). It is present in human nasal fluid (405) and plasma (407), and is functionally equivalent to sapienic acid in murine sebaceous secretions (409, 410). It is a poor substrate for FakA-mediated metabolism, and must be extended to a C18 fatty acid before incorporation into the membrane can occur efficiently (282). Expression of the *S. aureus* efflux pump *tet38* provides high levels of resistance to this fatty acid (340).
Oleic Acid (*cis*-9-octadecenoic acid, 18:1)	
	Unlike other uFFA, oleic acid does not exhibit major inhibitory activity towards *S. aureus* (282). It is present in human nasal fluid (405, 406) and is a major lipid component of human plasma (407). Due to the unsaturated nature and low toxicity of this fatty acid, it is frequently used to study *S. aureus* lipid metabolism (350, 351, 357).

Linoleic Acid (*cis, cis*-9,12-octadecadienoic acid; 18:2)	 Linoleic acid is highly inhibitory to the growth of *S. aureus* (282). It is a major lipid component of both human nasal fluid (405, 406) and human plasma (407). It is a strong inducer of the FarE efflux pump, which provides *S. aureus* with resistance to uFFA (338, 339).
Arachidonic Acid (*cis,cis,cis,cis*-5,8,11,14-eicosatetraenoic acid; 20:4)	 Arachidonic acid is highly inhibitory to the growth of *S. aureus* (282), and has been shown in inhibit *S. aureus* growth through lipid peroxidation (283). It is present in human nasal fluid (406) and human plasma (407), and is produced in large amounts by both macrophages and neutrophils during inflammation (411, 412). Furthermore, metabolism of arachidonic acid by the host produces potent inflammatory mediators such as prostaglandins, hydroxytetraenoic acids, and leukotrienes (413–415). It is also a strong inducer of the FarE efflux pump, which provides *S. aureus* with resistance to uFFA (338, 339).

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Chapter 2

2 Novel Functions and Signaling Specificity for the GraS Sensor Kinase of *Staphylococcus aureus* in Response to Acidic pH[1]

[1] This chapter (with the exception of Section 2.1 (published as indicated), 2.4.5, and minor alterations to 2.5) has been previously published. Kuiack RC, Veldhuizen RAW, McGavin MJ. 2020. Novel functions and signaling specificity for the Gras sensor kinase of *Staphylococcus aureus* in response to acidic pH. J Bacteriol 202: e00219-20.

2.1 Chapter Preface

2.1.1 Discovery of an antivirulence compound that reverses β-lactam resistance in MRSA

This collaborative work was published in 2020, in Nature Chemical Biology (1). As mentioned previously, the *graXRS* system was first discovered in *S. aureus* for its role in providing resistance to CAMPs and the glycopeptide vancomycin (2, 3). We built upon this discovery by showing the GraXRS system also plays an important role in β-lactam resistance. Specifically, we showed that an inhibitor of GraR, MAC-545496, reverses β-lactam resistance in the MRSA strain USA300. Furthermore, MAC-545496 was able to synergize with components of the innate immune system such as CAMPs, oxidative stress, and lysozyme, to impair growth of *S. aureus*, inhibit biofilm formation, abrogate intracellular replication in macrophages, and attenuate virulence *in vivo*. Together, this paper indicates that MAC-545496, and inhibition of GraXRS function, is a promising new option to fight drug resistant *S. aureus* infections, and that a better understanding of GraXRS function could be paramount to identifying novel mechanisms to both inhibit *S. aureus* survival and sensitize *S. aureus* to existing innate immune mechanisms. My contribution in this publication was the construction of Δ*graS* and Δ*graR* strains, as well as the construction of the pGY*mprF::lux* reporter vector to measure GraRS activation.

2.1.2 *S. aureus* uses the GraXRS regulatory system to sense and adapt to the acidified phagolysosome in macrophages

This collaborative work was published in 2018, in mBio (4). Even though the phagolysosome of a macrophage is a highly microbicidal environment, *S. aureus* has evolved to both survive and replicate within this niche (5, 6). In addition to playing an essential role in combating cationic antimicrobial peptides (7), the GraXRS system has also been implemented in resistance to acidic pH (8) and oxidative stress (9), both of which are important for phagolysosome function. Therefore, we investigated the role GraXRS plays in the ability for *S. aureus* to combat macrophage eradication. We determined that the GraXRS system, and downstream expression of MprF, were essential for *S. aureus* survival and replication in the phagolysosome of a macrophage, but the SaeRS and AgrAC two-component regulatory systems, as well as the α-phenol soluble

modulins, were dispensable for this survival. More specifically, we determined that acidic pH is a novel stimulus that can directly activate GraS signalling, and that activation of GraS by acidic pH was required for survival in macrophages, as well as resistance to cationic antimicrobial peptides and reactive oxygen species. Interestingly, *S. aureus* was also unable to replicate in cathelicidin-deficient murine macrophages, indicating GraS must be activated by both acidic pH and CAMPs concurrently in order to properly signal, and promote *S. aureus* adaptation to the antimicrobial conditions of the phagolysosome. Finally, we confirmed the importance of GraS signalling and MprF activation *in vivo* by showing both these genes are required for early-stage survival of *S. aureus* within the murine liver. Together, this paper broadened our understanding of the importance for GraXRS in combating the innate immune system, as well as identified a novel stimulus, acidic pH, that could directly activate GraS signalling. My contribution in this publication included constructing the *graS* deletion strain, characterizing the phenotypes of Δ*graS* in response to various antimicrobial conditions *in vitro*, designing the assays and strains to measure GraS activation through *mprF* expression, and identifying the novel signal, acidic pH, which can directly activate GraS signalling.

2.1.3 Rationale for Investigation

Our collaborative papers, in addition to established literature, highlight an essential function for GraXRS and MprF in combating the innate immune system of humans. GraXRS, and induction of *mprF* expression, have been well established to combat cationic antimicrobial peptides (7), but more recent findings indicate that GraXRS also plays an important role in sensing and responding to acidic pH (4, 8). Additionally, studies show that concurrent activation of GraS by both cationic antimicrobial peptides and acidic pH are required for optimal signalling through this system (4, 10), indicating this system has evolved to optimally respond to conditions encountered on human skin during colonization and subsequent infection. In addition to cationic antimicrobial peptides and acidic pH, antimicrobial uFFA are another innate immune condition encountered on human skin that are highly inhibitory to *S. aureus* (11, 12). With GraXRS playing such a fundamental role in combating the innate immune conditions of human skin, we queried if GraXRS also plays a role in combating uFFA. Furthermore, previous

work into uFFA resistance had not considered what occurs when *S. aureus* encounters combined antimicrobial conditions simultaneously, and focused the ability of *S. aureus* to sense and resist uFFA toxicity alone. Therefore, we believed this investigation would provide valuable insight into what occurs when *S. aureus* is colonizing human skin, and could identify novel resistance pathways that have been previously undiscovered or underappreciated.

2.2 Introduction

Staphylococcus aureus is a Gram-positive opportunistic pathogen that asymptomatically colonizes up to 30% of humans (13) but is also a leading cause of infectious morbidity and mortality, such that death attributed to *S. aureus* in the United States has now exceeded that caused by AIDS, tuberculosis and viral hepatitis combined (14). Its preferred site of colonization in asymptomatic carriage is the anterior nares and among those who exhibit nasal carriage, the bacteria are also frequently found on exposed skin, including the hands, perineum, and axillae. This asymptomatic colonization plays a key role in the epidemiology of *S. aureus* disease, since infections are nearly always caused by the endogenous nasal carriage strain (15). Congruently, the ability of *S. aureus* to resist local innate immunity at sites of colonization is critical to its success as a pathogen, and our recent work has focused on how *S. aureus* is able to sense and respond to these signals of innate immunity (4, 16–18).

The anterior nares of the nose are exposed to secretions of the upper respiratory tract, including antimicrobial unsaturated free fatty acids (uFFA) of which linoleic acid (C18:2) is the most abundant in human nasal secretions (19), while the major antimicrobial uFFA on skin is sapienic acid (C16:1); an isomer of palmitoleic acid that is uniquely produced by human sebaceous glands (20, 21). We found that these antimicrobial uFFA induce the expression of secreted proteases and also an RND family efflux pump that contributes to resistance (16, 17, 22). Other environmental signals relevant to innate immunity on skin and the anterior nares include acidic pH (23–25) and antimicrobial peptides (26–29). Extracellular calcium also has an important role in maintaining the dermal barrier function of the skin (30). Nevertheless, although *S. aureus* is concurrently exposed to multiple environmental signals and mediators of innate immunity at sites of colonization,

its ability to sense and respond to these signals is typically studied in a singular manner (7, 31, 32). In this context, our recent work has alluded to the possibility that *S. aureus* could effectively multi-task in response to sensing disparate environmental signals through the GraS sensor kinase (1, 4).

GraS and its cognate response regulator GraR are part of a five-component signaling system comprised of the co-transcribed *graXRS* genes and co-associated *vraFG*, where GraX is a cytoplasmic accessory protein and VraFG comprise a two component ABC transporter (7, 33–35). GraS is known for its role in sensing cationic antimicrobial peptides (CAMPs), attributed to a short extracellular sensor loop, leading to autophosphorylation and phospho-relay to the response regulator GraR, which in turn promotes expression of genes required for resistance, including *mprF* and *dltABCD* (10, 36, 37). MprF promotes synthesis of lysyl-phosphatidylglycerol (lysyl-PG) while the *dlt* genes promote D-alanylation of teichoic acids (36, 38), and these two activities confer a positive charge to the cell envelope which repels CAMPs. The role of the accessory GraX and VraFG proteins is less well understood, although GraX likely functions as a scaffold to promote protein interactions with GraS, GraR, and VraFG to fine tune the signaling mechanism (34, 35). Adding to the complexity, GraS is considered to belong to the intramembrane sensor kinase family of proteins, which signal in response to membrane perturbation and are characterized by having a minimal extra-cytoplasmic sensor segment, and their co-association with two component ABC transporters or other accessory signaling proteins (39–41).

Recent work by ourselves and others has expanded the sensory capabilities of GraS to include acidic pH, including its requirement for growth at the pH extreme of 4.5, activation of GraS-dependent expression of MprF at pH 5.5, and a requirement for growth in acidified macrophage phagosomes (4, 8). In the current model of GraS function, acidic amino acids in its short nonapeptide extracellular sensor loop promote recognition of and signaling in response to CAMPs (37). However, exposure to acidic pH should reduce the charge on these acidic amino acids and also affect the properties of membrane phospholipid, which would include a reduction in repulsive forces between polar lipid head groups and tighter lateral packing (42). As such, signaling through GraS

at acidic pH may not be critically dependent on these acidic amino acids that contribute to recognition of CAMPs.

In view of these considerations, the purpose of our present study was two-fold. First, since concurrent exposure to antimicrobial uFFA, acidic pH, calcium and antimicrobial peptides should be key environmental features of *S. aureus* persistence on skin and the anterior nares, we queried how the interplay of these environmental signals would influence its resistance to antimicrobial uFFA. Second, we investigated the role of the GraS sensor kinase and signaling mechanism in response to these combined environmental signals. Our experiments were conducted with the hyper-virulent and pandemic USA300 strain of community acquired methicillin-resistant *S. aureus* (CA-MRSA), which is known for its efficient community transmission (43). Herein we report that acidic pH, antimicrobial peptides and environmental calcium all promote increased resistance of *S. aureus* USA300 to antimicrobial uFFA, and that during growth at acidic pH, this was dependent on signaling through GraS independently of acidic amino acids in its extracellular sensor loop. We further reveal a role for GraS in promoting the production of *S. aureus* secreted proteases in response to acidic pH. Cumulatively, these findings are consistent with the function of GraS as an intramembrane sensor kinase.

2.3 Materials and Methods

2.3.1 Bacterial Strains and Growth Conditions

Bacteria and plasmids that were used or constructed in this study are listed in Table 2.1. *S. aureus* cultures were maintained as frozen stocks (-80°C) in 20% glycerol and streaked on TSB agar (TSA) when required. Tryptic soy broth (TSB; Difco) used for this study contained 2.5 g/L glucose (~ 14 mM). Metabolism of glucose can acidify the culture medium as glucose is consumed reaching a minimum of ~ 5.9 in early stationary phase and then increasing again as the acetate byproduct is consumed (44). In consideration of this, we conducted pH measurements during growth in TSB, and confirmed that pH was not affected during early exponential growth when bacteria are highly susceptible to antimicrobial uFFA. TSB or TSA was supplemented, when needed, with 10 µg/mL erythromycin or chloramphenicol, and 2 µg/mL tetracycline for propagation of strains

bearing resistance markers. Where indicated, TSB or TSA were supplemented by addition of 0.1 M MES buffer (Bio Can Scientific) and adjusted to pH 5.5 with HCl prior to autoclaving. To supplement media with fatty acids, a 10 mM stock concentration was first prepared in TSB containing 0.1% dimethyl sulfoxide (DMSO) and then diluted into TSB or warm TSA plus 0.1% DMSO to achieve the desired concentration of fatty acids. Linoleic acid (*cis*, *cis*-9,12-octadecadienoic acid; 18:2) was purchased from Sigma, arachidonic acid (*cis*,*cis*,*cis*,*cis*-5,8,11,14-eicosatetraenoic acid; 20:4) and palmitoleic acid (*cis*-9-hexadecenoic acid; 16:1) were purchased from Cayman Chemicals, and sapienic acid (*cis*-6-Hexadecenoic acid; 16:1) was purchased from Abcam. As required, media were also supplemented with Polymyxin B (Sigma), or CATH-2 (45). *E. coli* strains were grown on LB agar or LB broth supplemented with 100 µg/mL ampicillin when needed. Unless otherwise stated, all cultures were grown at 37°C, and liquid cultures were incubated on an orbital shaking platform at 220 rpm. For all experiments, a minimum of at least two biological replicates were used to confirm findings.

Table 2.1. Strains and plasmids used in Chapter 2

Strain or Plasmid:	Description:	Citation:
S. aureus:		
USA300 LAC	Community associated MRSA; wild type strain cured of resistance plasmids	(16)
RN4220	r_K^- m_K^+; capable of accepting foreign DNA	(46)
USA300Δ*graS*	USA300 with markerless *graS* deletion	(4)
USA300Δ*farER*	USA300 with markerless *farER* deletion	(22)
USA300Δ*fakA*	USA300 with markerless *fakA* deletion	(22)
USA300Δ*pro* (Δ*sspABC-aur::lacZ)*	USA300 deficient in Aureolysin metalloprotease and *sspABC* serine protease operon; *Erm*^{r} *Tc*^{r}	(16)
USA300*mprF*::*Tn*	φNΣ1360 allele from Nebraska transposon library transduced into USA300 LAC*; Erm*^{r}	(4, 47)
USA300vraF::*Tn*	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *vraF*::φNΣ; Erm^r	(4, 47)
USA300*vraG*::*Tn*	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *vraG*::φNΣ; Erm^r	(4, 47)
E. coli:		
DH5α	F$^-$ Φ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^-mK$^+$) *supE44 relA1 deoR* Δ(*lacZYAargF*)*U169 phoA*	Invitrogen
Plasmids:		
pALC2073	Shuttle vector used for expression of genes under control of tetracycline-inducible $P_{xyl/tetO}$ promoter in *S. aureus*; genes are expressed at a basal level in absence of induction	(48)
p*graS*	Promoterless *graS* gene under transcriptional control of $P_{xyl/tetO}$ promoter of pALC2073	(4)
p*graS*$^{3D>G}$	p*graS* after mutagenesis with *graS*-SDM-F and *graS*-SDM-R primers; Aspartic acid to Glycine substitution at D35, D37, and D41	This study
p*mprF*	Promoterless *mprF* gene amplified by PCR with primers *mprF*-pALC-F and *mprF*-pALC-R, and cloned into *BamHI* site of pALC2073	This study

p*mprF*^{K547A}	p*mprF* after mutagenesis with *mprF*-SDM-F and *mprF*-SDM-R primers; Lysine to Alanine substitution at K547 to inactivate lysyl-transferase activity	This study

2.3.2 Strain and Plasmid Construction

Genetic manipulation of *S. aureus* was conducted following established guidelines, and as described in previous work (16, 22, 46). All recombinant plasmids were initially constructed in *E. coli* DH5α. The integrity of plasmids was confirmed through nucleotide sequencing of cloned DNA segments prior to electroporation into USA300 or isogenic derivatives, using *S. aureus* RN4220 as an intermediate host. Primer sequences used for PCR amplification of gene segments for plasmid construction, or site directed mutagenesis of cloned genes, are defined in Tables 2.1 and 2.2 and are based on the reference genome sequence of USA300 FPR3757 (49). Plasmid pALC2073 which provides a basal level of gene expression from the $P_{xyl/tetO}$ promoter and a stronger inducible level of expression with anhydrotetracycline (48, 50) was used for ectopic expression *graS* and *mprF*. Site directed mutagenesis was conducted on p*graS* using primers *graS*-SDM-F and *gra*S-SDM-R with Phusion DNA polymerase, to produce p$graS^{3D>G}$ where codons for Asp35, Asp37 and Asp41 are changed to glycine, using guidelines described in the Stratagene QuickChange manual. Similarly, p*mprF* was used as template with mutagenic primers *mprF*-SDM-F and *mprF*-SDM-R to produce p$mprF^{K547A}$ where the codon at Lys547 is altered to encode alanine.

Table 2.2. Oligonucleotides used in Chapter 2

Oligonucleotide:	**Description:**
graS-SDM-F	CATTAGTCTAATCGGTTATGGTTTTCCAATAGGCAGTTTATTTTATATTGTTTC
graS-SDM-R	GAAACAATATAAAATAAACTGCCTATTGGAAAACCATAACCGATTAGACTAATG
mprF-pALC-F	GATTTATAACAGAAAGGATCCGAGGAGGTGTGAAAAAATGAATCAGGAAG
mprF-pALC-R	TTTGGATCCCGCATCAGGCATAACTGT
mprF-SDM-F	GATATATAGTGGTGACGCGCAGTTTTTCACTAATGA
mprF-SDM-R	GCTGTTTTATTTTCATTAGTGAAAAACTGCGCGTCA

* Underlining indicates restriction cut sites

2.3.3 Growth, Viability, and MIC Assays

For growth analyses, cultures of *S. aureus* were prepared by inoculating 3 mL of TSB in a 13 mL polypropylene tube containing antibiotic as required, and grown overnight for 16 hours. After determining the optical density at 600 nm (OD_{600}), aliquots were sub-cultured into 125 mL capacity flasks containing 25 mL of TSB or TSB modified by addition of buffer, fatty acid, antimicrobial peptide, or cation supplements, to achieve an initial OD_{600} of 0.01. Growth (OD_{600}) was monitored at hourly intervals. Alternately, bacteria were sub-cultured into 200 µL of medium in wells of 96 well flat bottom assay plates (Fisher) to OD_{600} of 0.01, and growth was monitored at 37°C using a Synergy H4 temperature-controlled microplate reader (BioTek Instruments) with measurement of OD_{600} every 20 minutes for 18-24h. For viability assays, *S. aureus* inoculum cultures in polypropylene tubes as described above were grown for 4h, and diluted to OD_{600} of 0.01 in fresh TSB, followed by preparation of serial 10^{-1}, 10^{-2}, and 10^{-3}. Triplicate aliquots of 10 µL from each dilution were then plated on different formulations of TSA, and viable bacteria were enumerated after 24h of growth.

For MIC assays, inoculum cultures were grown to mid-exponential phase in flasks as for growth assays, then subcultured at OD_{600} of 0.01 into triplicate 20- by 150-mm glass culture tubes containing 3 mL of medium supplemented with 0.1% DMSO and indicated concentrations of linoleic or palmitoleic acid. Cultures were incubated at 37°C with vigorous shaking, and OD600 values were determined after 24h.

2.3.4 Cytochrome C Binding Assay

Cell surface charge was measured as a function of cytochrome C binding as previously described (51). Briefly, bacterial cultures were grown to an OD_{600} of 0.5, before being washed twice in MOPS buffer (20 mM, pH 7.0). Cells were resuspended to an OD_{600} of 7.0 in MOPS buffer, and 360 µL aliquots were mixed with 40 µL of bovine cytochrome C (Sigma) to a final concentration of 0.5 mg/ml. Samples were incubated for 15 minutes at 37ºC, followed by centrifugation at 6,000xg for 8 min, and the remaining unbound cytochrome C was quantified by measuring absorbance at 530 nm (A_{530}) relative to a MOPS buffer blank containing 0.5 mg/mL cytochrome C.

2.3.5 SDS-PAGE and Zymography

For SDS-PAGE analysis of secreted protein profiles, *S. aureus* cultures were grown for 20h and proteins in cell free culture supernatant were precipitated by mixing with an equal volume of ice-cold 20% TCA, washed in ice-cold 70% ethanol, and then air dried prior to dissolving in SDS-PAGE reducing buffer as described previously (52). Protein equivalent to 3.0 OD_{600} units of culture supernatant was then loaded for protein separation on a 10% acrylamide gel using the Laemmli buffer system (53), and after electrophoresis, proteins were stained using Coomassie Blue. For detection of protease activity by zymogram assay, the resolving gel was co-polymerized with 1 mg/mL casein and protein equivalent to 0.075 OD_{600} units was applied to each lane. Details on sample processing, electrophoresis and zymogram development are as described previously (54).

2.3.6 Data Analysis

Prism 8 version 8.4.0 was used to create all graphs and perform statistical analyses in this study. In all experiments, triplicate cultures were used and mean ± SE is used to represent the data in graphs. Unpaired one-tailed t tests, one-way ANOVA with multiple comparisons, or two-way ANOVA with multiple comparisons were used to test statistical significance depending on the nature of the experiment. Significance was defined as stated in the figure legends.

2.4 Results

2.4.1 *S. aureus* sensitivity to antimicrobial uFFA is differentially influenced by carbon chain length and acidic pH.

Linoleic acid (C18:2) is the major unsaturated free fatty acid (uFFA) in tissue abscesses and nasal secretions, while sapienic acid (C16:1) is predominant in sebaceous secretions. Its isomer palmitoleic acid is the major antimicrobial uFFA in skin of mice and other mammals, and is also abundant in adipose triglyceride and membrane phospholipid (19, 21, 55). Since the skin and nasal mucosa are maintained at acidic pH (24, 25), we evaluated how this affects *S. aureus* resistance to antimicrobial uFFA by conducting growth assays in unmodified TSB (initial pH 7.3; hereinafter referred to as TSB), or TSB buffered at pH 5.5. Consistent with our previous work (17), *S. aureus* USA300 grew well

in TSB + 25 µM linoleic acid, but exhibited an extended lag phase with 50 µM linoleic acid (Figure 2.1A). At pH 5.5, USA300 grew equally well in 25- or 50 µM linoleic acid (Figure 2.1A), and also grew with 250 µM linoleic or arachidonic acid (C20:4) (Figure 2.1B). Surprisingly, an opposite effect was observed with C16:1, where USA300 grew well in TSB + 25 µM palmitoleic- or sapienic acid, but not with this same concentration at pH 5.5 (Figure 2.1C). However, if the incubation time was extended, acidic pH permitted outgrowth after 48 h at palmitoleic acid concentrations ranging from 200–500 µM, and this was not evident in unbuffered TSB (Figure 2.2). Therefore, we conclude that acidic pH favors increased resistance to C18:2 and C20:4 antimicrobial uFFA but appears to have a bimodal effect with C16:1, initially potentiating the inhibitory activity but also permitting outgrowth on extended incubation.

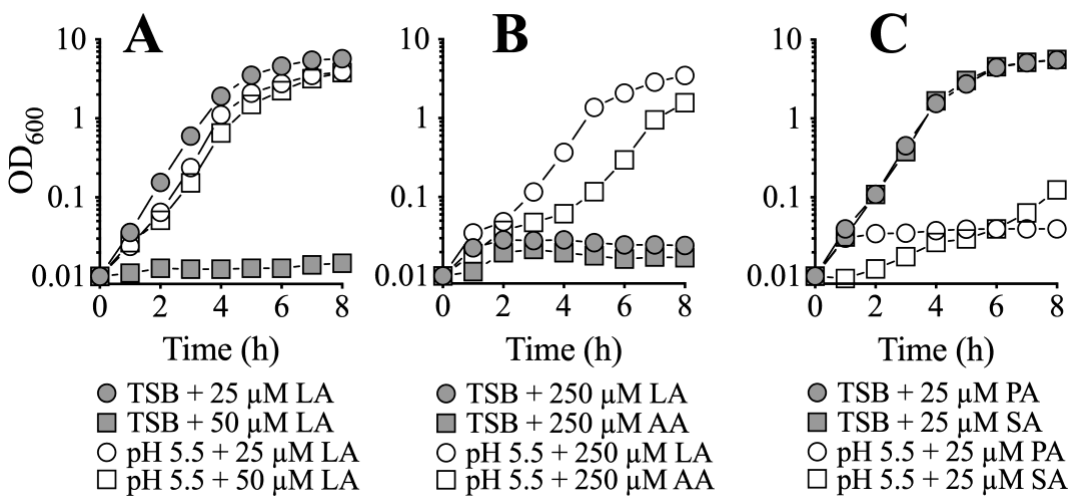

Figure 2.1. Influence of acidic pH on growth of *S. aureus* USA300 in presence of linoleic acid arachidonic acid, palmitoleic acid, or sapienic acid. Triplicate flasks of TSB or TSB pH 5.5 were supplemented with indicated concentrations of linoleic acid (LA; C18:2) **(A)**, linoleic acid (LA; C18:2) or arachidonic acid (AA; C20:4) **(B)**, and palmitoleic acid (PA; C16:1) or sapienic acid (SA; C16:1) **(C)** and inoculated to an initial optical density measured at 600 nm (OD_{600}) of 0.01. Growth (OD_{600}) was assessed at hourly intervals, and each data point represents the mean ± SE of triplicate flasks.

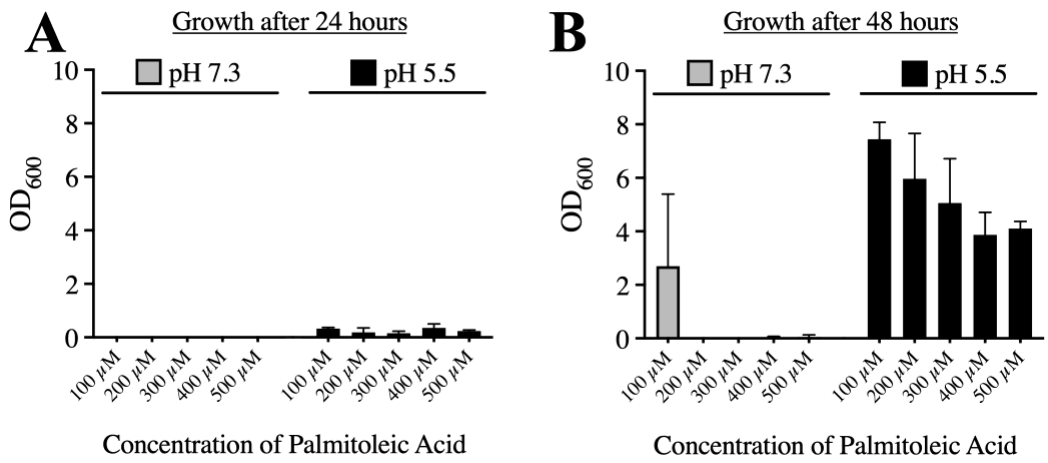

Figure 2.2. Following an extended lag phase, acidic pH makes *S. aureus* more resistant to C16:1 antimicrobial uFFA. Minimum inhibitory concentration assay with palmitoleic acid in TSB and TSB pH 5.5 after 24 hours **(A)** and 48 hours **(B)** of growth. Each data point represents the mean ± SE of triplicate 3 mL cultures.

2.4.2 Cationic antimicrobial peptides (CAMPs) and extracellular calcium also stimulate increased resistance to antimicrobial uFFA.

Although acidic pH promoted increased sensitivity to C16:1 antimicrobial uFFA, we considered that *S. aureus* is concurrently exposed to multiple environmental signals on human skin, including antimicrobial peptides, and extracellular calcium which has a key role in promoting the structural integrity of the dermal barrier, and has been measured in human sweat at 16 µg/mL, equivalent to 0.4 mM (30, 56). We therefore tested whether these additional signals alone or in combination with acidic pH would influence *S. aureus* resistance to antimicrobial uFFA. Using polymyxin B (PmB) as a model cationic antimicrobial peptide, we observed that sub-inhibitory PmB eliminated the lag phase that normally occurs in TSB + 50 µM linoleic acid (Figure 2.3A), and the same effect was achieved with 0.5 mM calcium, but not sodium or magnesium (Figure 2.3B).

Supplemental PmB or calcium also promoted growth at higher concentrations of uFFA, including 200 µM of linoleic, arachidonic or palmitoleic acid and 100 µM sapienic acid (Figure 2.3CD). Moreover, although acidic pH impaired *S. aureus* growth in 25 µM palmitoleic or sapienic acid (Figure 2.1C), this effect was eliminated in TSB pH 5.5 supplemented with either PmB or calcium, which permitted growth in 50 µM palmitoleic acid and 25 µM sapienic acid (Figure 2.3EF). To determine the full extent of resistance, we conducted MIC determinations with different modifications to basal TSB (Table 2.3). In unbuffered TSB, the MICs of palmitoleic and linoleic acid were 100- and 400 µM respectively. At pH 5.5, the MIC of palmitoleic acid decreased to 75 µM, while linoleic acid increased to 1200 µM. At pH 5.5, addition of supplemental calcium or subinhibitory polymyxin B increased the MIC for palmitoleic acid to 300 µM and 800 µM respectively, compared to 1600- and >2500 µM for linoleic acid under the same conditions.

Although subinhibitory PmB promoted enhanced resistance to antimicrobial uFFA, we considered that this could be due to it being a lipopeptide, which might exhibit lipid mediated interactions with free fatty acids to render them less effective. However, this was considered unlikely, since 10 µg/mL PmB is equivalent to 7.7 µM, which promoted *S. aureus* growth in concentrations of linoleic- and palmitoleic acid that were far in

excess of PmB. To provide additional evidence that subinhibitory CAMPs confer increased resistance of *S. aureus* to antimicrobial uFFA, we conducted experiments with CATH-2. Although CATH-2 is of chicken origin, it belongs to the cathelicidin family of CAMPs, has high activity towards *S. aureus*, and compared to LL-37 its antimicrobial activity is less sensitive to variations in acidity and salt (57–59). Growth assays were conducted in microtitre plates, with preliminary experiments establishing a higher threshold of resistance to uFFA compared to flask cultures (Figure 2.4AB), and that CATH-2 was inhibitory above 6.0 µM (Figure 2.4C). Based on these trials, we compared the ability of 10 µg/mL PmB or 1.5 µM CATH-2, to influence growth in TSB containing 500 µM linoleic acid or 300 µM palmitoleic acid. Although we cannot do a direct physiologic comparison for CATH-2, dermcidin is an antimicrobial peptide that has been measured at 2.1 µM in eccrine gland secretions (60), comparable to our use of 1.5 µM CATH-2. As expected, PmB eliminated the lag phase with both uFFA (Figure 2.5), while CATH-2 reduced the lag phase from 20 h to 6 h in 500 µM linoleic acid, and eliminated the lag phase in 300 µM palmitoleic acid (Figure 2.5).

As an additional test of specificity, we queried whether enhanced resistance could be achieved with vancomycin, a cationic antimicrobial glycopeptide that does not stimulate signaling through GraS (61). In unbuffered TSB, vancomycin was inhibitory beyond 0.5 µg/mL (Figure 2.6A), and in contrast to PmB, subinhibitory vancomycin did not confer enhanced resistance to either linoleic or palmitoleic acid (Figure 2.6BC). Subinhibitory vancomycin also did not stimulate growth in 100 µM palmitoleic acid at pH 5.5, whereas PmB and CATH-2 both stimulated growth to a similar extent (Figure 2.6D). Cumulatively, these findings establish that selected CAMPs or supplemental calcium both promote increased resistance of *S. aureus* to antimicrobial uFFA, including amelioration of C16:1 toxicity at acidic pH.

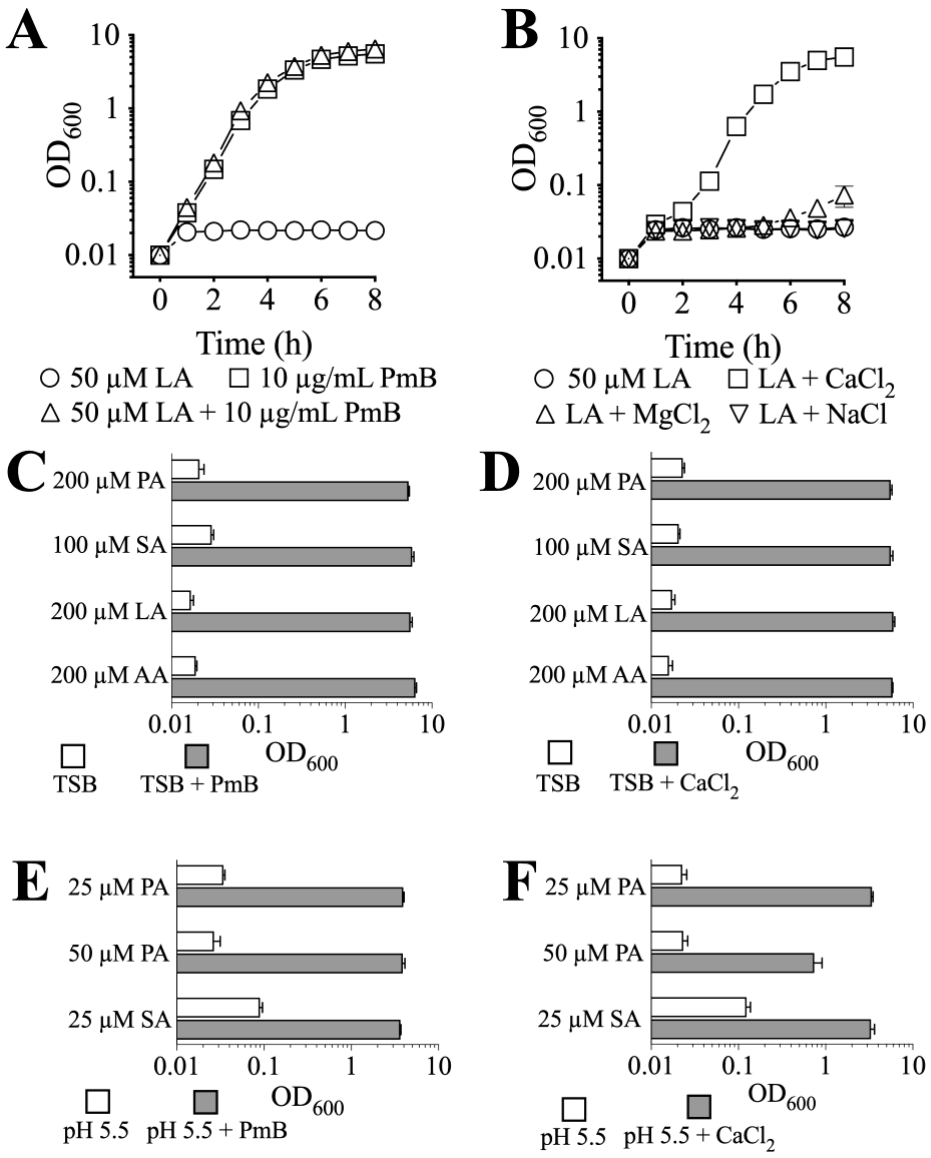

Figure 2.3. Influence of Polymxin B (PmB) and cation supplements on growth of *S. aureus* USA300 in the presence of antimicrobial uFFA. (A) Growth in TSB supplemented with 50 µM LA, 10 µg/mL PmB, or 50 µM LA + 10 µg/mL PmB as indicated; **(B)**, growth in TSB supplemented with 50 µM LA, or 50 µM LA containing 0.5 mM $CaCl_2$, $MgCl_2$ or NaCl; **(C)**, growth in TSB + 10 µg/mL PmB, containing indicated concentrations of PA, SA, LA or AA; **(D)**, growth in TSB + 0.5 mM $CaCl_2$ containing indicated concentrations of PA, SA, LA or AA; **(E)**, growth in TSB pH 5.5 or TSB pH 5.5 + 10 µg/mL PmB, with indicated concentrations of PA or SA; **(F)**, growth in TSB pH 5.5 or TSB pH 5.5 + 0.5 mM $CaCl_2$, with indicated concentrations of PA or SA.

All data points represent the mean ± SE of triplicate cultures. For panels A and B, OD_{600} was monitored at hourly intervals, while for C-F, growth was monitored after 12h. For panel C-F, statistical significance between media alone and media supplemented with PmB or $CaCl_2$ was measured using an unpaired one-tailed t-test. In all cases $p<0.0001$, with the exception of panel F 50 μM PA and 25 μM SA, where $p=0.066$ and $p=0.0003$ respectively.

Table 2.3. Influence of acidic pH, subinhibitory PmB and supplemental calcium on minimum inhibitory concentration (MIC) of linoleic and palmitoleic acid for *S. aureus* USA300

	MIC (μM) of:	
Medium	**Linoleic acid**	**Palmitoleic acid**
TSB	400	100
TSB + 10 μg/mL PmB	1200	300
TSB + 0.5 mM $CaCl_2$	1800	800
TSB (pH 5.5)	1200	75
TSB (pH 5.5) + 20 μg/mL PmB	1600	700
TSB (pH 5.5) + 0.5 mM $CaCl_2$	>2500	300

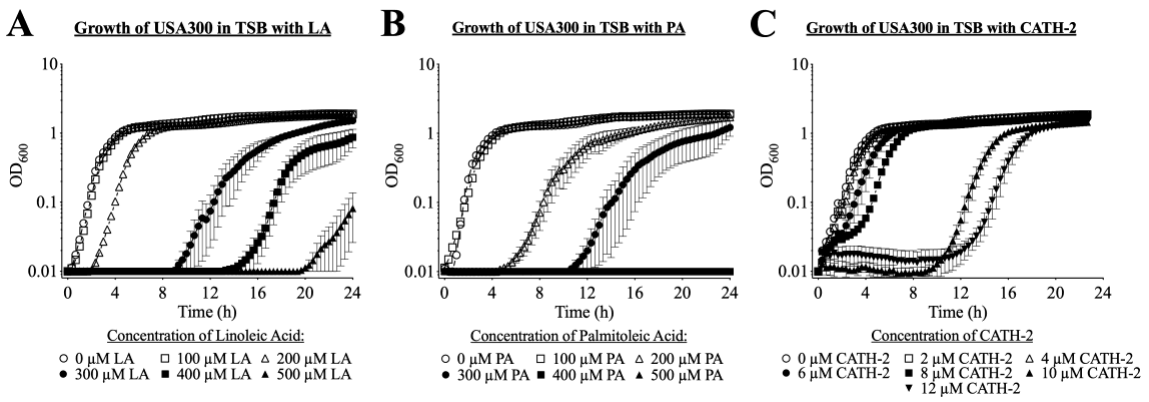

Figure 2.4. Determining the inhibitory concentrations of LA, PA, and CATH-2 for *S. aureus* USA300 in a 96-well plate assay. Growth of USA300 in TSB with varying concentrations of LA **(A)** , PA **(B)**, and CATH-2 **(C)**. Inhibitory concentrations were used for assays in Figure 2.5. Each data point represents mean ± SE of triplicate cultures.

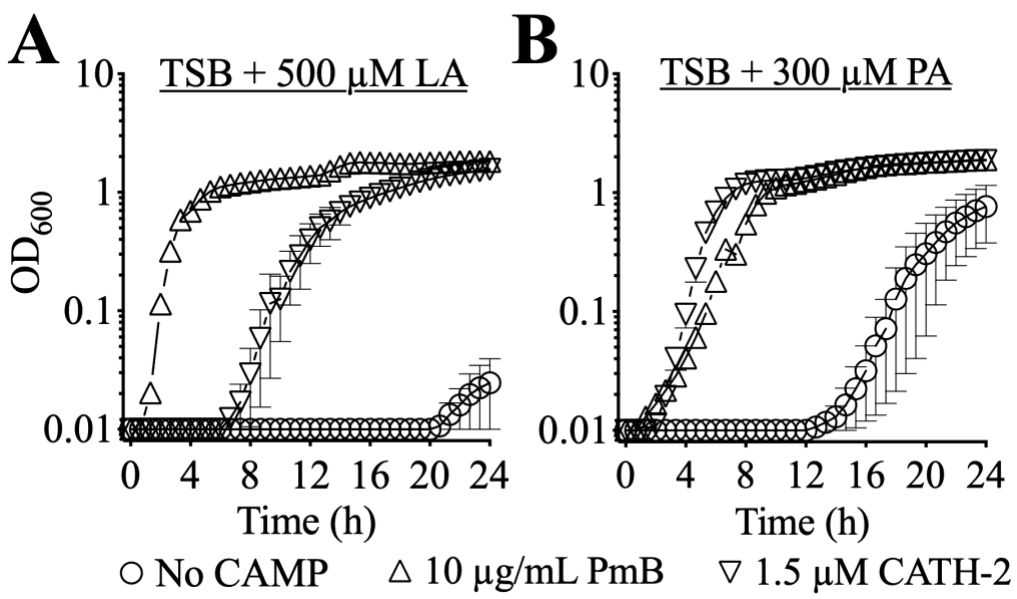

Figure 2.5. Influence of cationic antimicrobial peptides PmB and CATH-2, on growth of *S. aureus* USA300 under inhibitory concentrations of antimicrobial uFFA. Growth of USA300 in TSB with 500 µM LA **(A)** or 300 µM PA **(B)**, supplemented with subinhibitory concentrations of 10 µg/mL PmB or 1.5 µM CATH-2. Each data point represents mean ± SE of triplicate cultures.

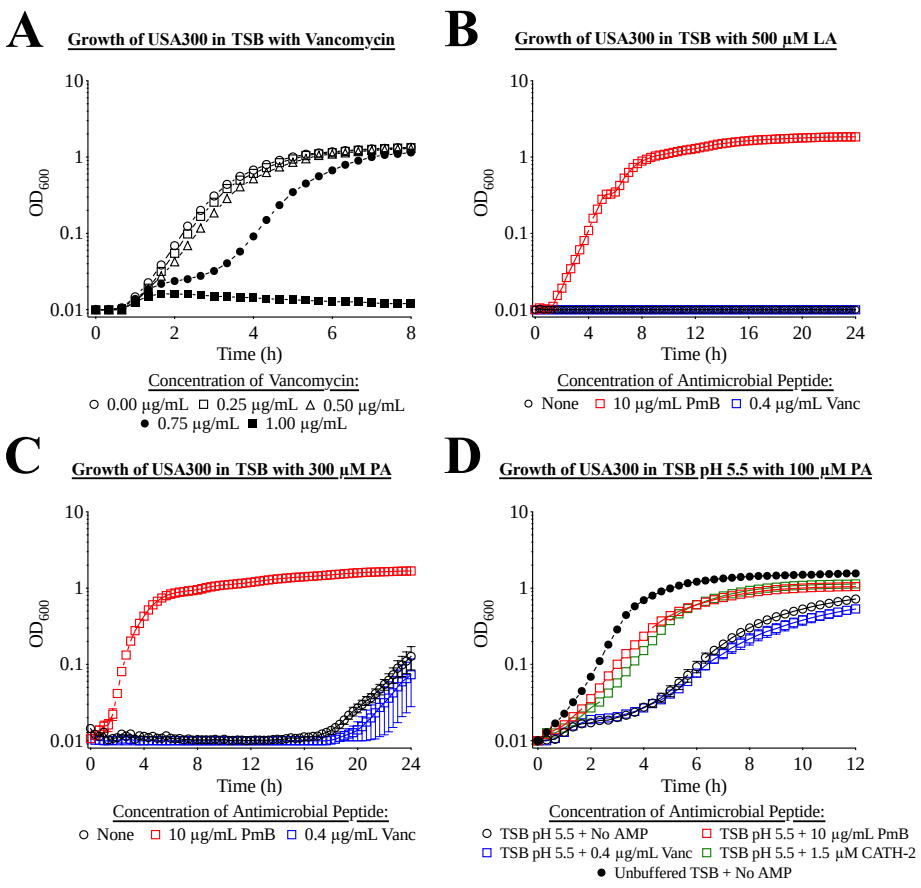

Figure 2.6. Subinhibitory vancomycin does not stimulate resistance to antimicrobial uFFA at neutral or acidic pH conditions. (A) Growth of USA300 in TSB with varying concentrations of vancomycin. **(B)** Growth of USA300 in 500 µM linoleic acid, supplemented with no antimicrobial peptide, or with subinhibitory concentrations of Polymyxin B or vancomycin. **(C)** Growth of USA300 in 300 µM palmitoleic acid, supplemented with no antimicrobial peptide, or with subinhibitory concentrations of Polymyxin B or vancomycin. **(D)** Growth of USA300 in TSB pH 5.5 with 100 µM PA, supplemented with no antimicrobial peptide, or with subinhibitory concentrations of Polymyxin B, vancomycin, or CATH-2. Growth in unbuffered TSB + 100 µM PA with no antimicrobial peptides is also included for comparison. Growth as assessed in 96 well plate format, and each data point represents mean ± SE of quadruplicate cultures.

2.4.3 GraS and the GraS-regulated gene *mprF* are required for resistance to antimicrobial uFFA at acidic pH.

We recently established that efflux pump FarE is induced by and required for resistance to antimicrobial uFFA through a mechanism that is dependent on the fatty acid kinase *fakA* (17, 22), which is also required for metabolic incorporation of uFFA into phospholipid (62), while GraS responds to acidic pH in macrophage phagosomes (4). We therefore queried the role of these genes in enhanced resistance to antimicrobial uFFA that is manifested at acidic pH. We first assessed viability by plating exponential phase cultures of USA300 and isogenic variants on TSA, TSA + 200 µM LA, TSA pH 5.5, or TSA pH 5.5 + 500 µM LA (Figure 2.7). As expected, USA300Δ*farER* exhibited a loss of viability on TSA + 200 µM LA. However, on TSA pH 5.5 + 500 µM LA, there was no loss of viability for either USA300Δ*farER* or USA300Δ*fakA*, whereas USA300Δ*graS* exhibited a severe loss of viability (Figure 2.7). The requirements for *graS* and the GraS-regulated gene *mprF* were then evaluated through growth analyses in TSB pH 5.5 + 250 µM LA, under which condition both mutants showed severely impaired growth, and growth was restored with the respective p*graS* and p*mprF* complementation vectors (Figure 2.8A). Both genes were also required for resistance to 25 µM palmitoleic acid in TSB pH 5.5 + 0.5 mM calcium (Figure 2.8B). Under non-acidic growth conditions, the *farER* genes were once again required for resistance to 250 µM LA in TSB + 0.5 mM calcium, whereas USA300Δ*graS* exhibited unrestricted growth (Figure 2.8C). From these data it is evident that FarE mediated efflux contributes to enhanced resistance that is manifested in response to supplemental calcium under non-acidic growth conditions, whereas GraS and MprF are both essential for enhanced resistance to antimicrobial uFFA at acidic pH.

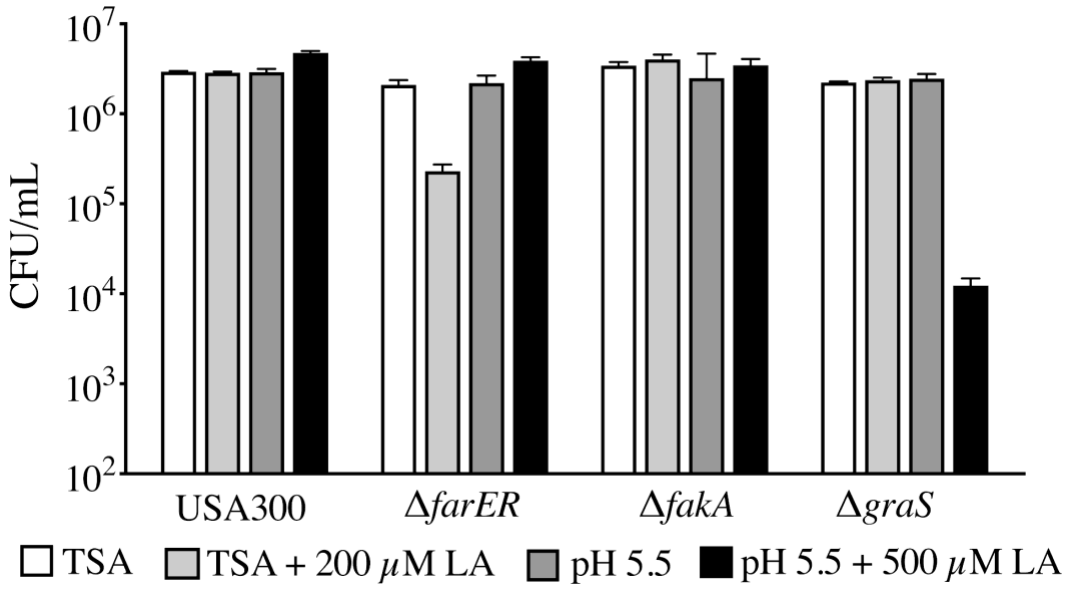

Figure 2.7. Genetic requirement for resistance to antimicrobial uFFA differs depending on the pH of the media. Viability (CFU/mL) of USA300 and isogenic Δ*farER,* Δ*fakA* or Δ*graS* mutants after plating on TSA, TSA + 200 µM LA, TSA pH 5.5, and TSA pH 5.5 + 500 µM LA. Mid-exponential phase triplicate cultures grown in TSB were diluted in fresh TSB to OD_{600} = 0.01. Serial dilutions were then prepared and plated on different TSA formulations as indicated, to determine viability after 24h of incubation. Each set of serial dilutions were done in triplicate, and the average CFU/ml for each culture was calculated. Data graphed as mean ± SE of triplicate cultures.

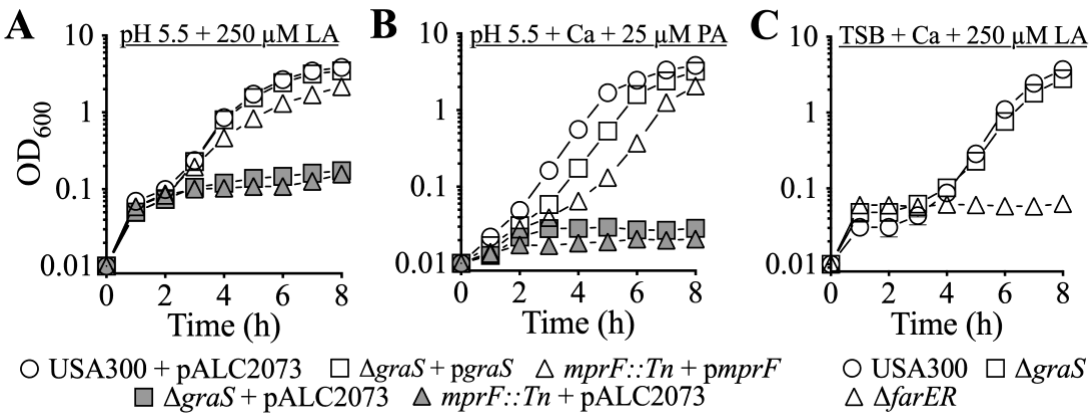

Figure 2.8. Genetic requirements for enhanced resistance of *S. aureus* USA300 to linoleic acid or palmitoleic acid in response to calcium and acidic pH. Growth of USA300, isogenic Δ*graS* or *mprF::Tn* mutants, and respective p*graS* or p*mprF* complemented strains in TSB pH 5.5 + 250 µM LA **(A)**, or TSB pH 5.5 + 0.5 mM $CaCl_2$ and 25 µM PA **(B)**; Growth of USA300 and isogenic Δ*graS* or Δ*farER* mutants in TSB + 0.5 mM $CaCl_2$ and 250 µM **(C)**. Each data point represents the mean ± SE of triplicate cultures.

2.4.4 GraS exhibits unique requirements for signaling at acidic pH.

Since signalling through GraS in response to cationic antimicrobial peptides depends on three aspartate residues in a short nonapeptide extra-cytoplasmic loop (37), we queried whether this requirement is maintained at acidic pH, using polymyxin B (PmB) as a model CAMP. As expected, USA300Δ*graS* failed to grow in TSB + 10 µg/mL PmB, and although there was a significant difference in the stationary phase cell densities of USA300 and USA300Δ*graS* + p*graS*, there was successful complementation of growth. However, no complementation was evident in USA300Δ*graS* + p*graS*$^{3D>G}$ encoding a variant GraS where three aspartate residues in the extra-cytoplasmic sensor loop are substituted with glycine (Figure 2.9A, Figure 2.10A). Although this supports a role for these acidic amino acids in recognition of and signaling in response to CAMPs (37), when growth was assessed in TSB pH 5.5 + 20 µg/mL PmB, both p*graS* and p*graS*$^{3D>G}$ were equally effective in restoring growth of USA300Δ*graS* (Figure 2.9A, Figure 2.10B). Both complementation vectors also restored growth of USA300Δ*graS* in TSB pH 5.5 + 250 µM LA (Figure 2.9B). Similar results were obtained in TSB pH 5.5 supplemented with 20 µg/mL PmB and 25 µM palmitoleic acid, where USA300Δ*graS* failed to grow, but growth was restored with both p*graS* and p*graS*$^{3D>G}$ vectors (Figure 2.9C).

Since signaling through GraS promotes expression of MprF, which confers resistance to CAMPs through lysine modification of membrane phospholipid (63), we conducted assays of cytochrome C binding to monitor MprF-dependent modification of cell surface charge. Consistent with our previous work where acidic pH promoted a GraS-dependent increase in transcription of an *mprF::lux* reporter (4), growth at pH 5.5 also promoted a GraS-dependent increase in cell-surface positive charge, as evident in reduced binding of cytochrome C (Figure 2.11). Once again, p*graS* and p*graS*$^{3D>G}$ were equally effective in restoring cell surface charge to USA300Δ*graS* grown at acidic pH (Figure 2.11). Cumulatively, these data support the established paradigm for signaling through GraS during growth in unbuffered TSB, where acidic amino acids in the extracellular sensor loop contribute to sensing of antimicrobial peptides (37). However, our experiments now reveal a novel specificity at acidic pH, where these same amino acids are dispensable for resistance to PmB and antimicrobial uFFA.

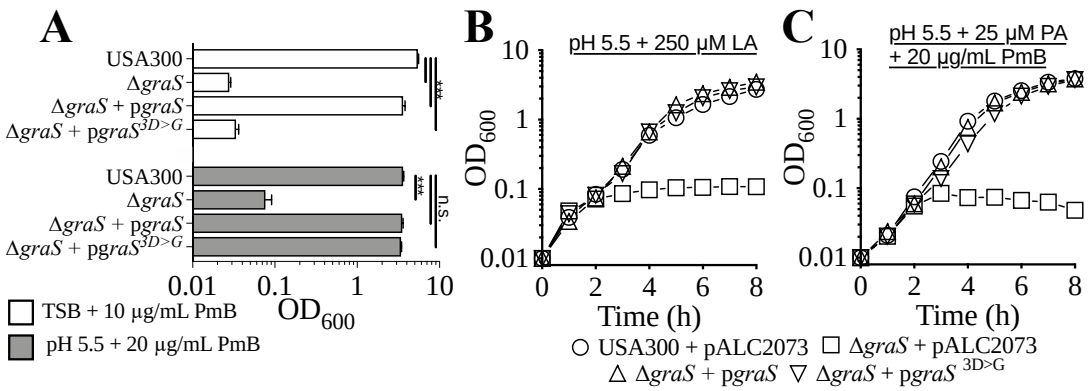

Figure 2.9. Requirement for acidic amino acids in extracellular sensor loop of GraS is dependent on growth conditions. (A) OD_{600} after 12 hours of growth in TSB + 10 µg/mL PmB, or TSB pH 5.5 + 20 µg/mL PmB; **(B)** growth in TSB pH 5.5 containing 250 µM LA; **(C),** growth in TSB pH 5.5 supplemented with 20 µg/mL PmB and 25 µM palmitoleic acid. Each data point represents the mean ± SE of triplicate cultures. Statistical significance for panel A was measured using one way ANOVA; *** $p<0.001$, n.s. not significant.

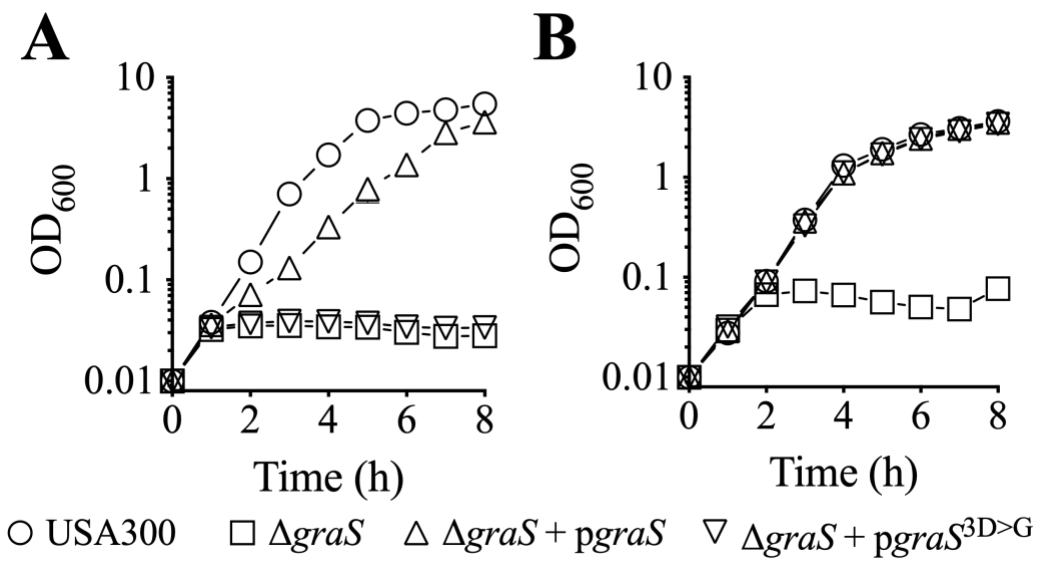

Figure 2.10. Role of GraS and GraS$^{3D>G}$ in resistance to PmB at neutral or acidic pH growth conditions. Growth of USA300 and isogenic Δ*graS* mutant complemented with pALC2073, p*graS*, or p*graS*$^{3D>G}$ in TSB + 10 µg/mL PmB **(A)** or TSB pH 5.5 + 20 µg/mL PmB **(B)**. OD_{600} was measured at hourly intervals for the first 8 hours of the end point growth assay represented in Figure 5A. Each data point represents the mean ± SE of triplicate cultures.

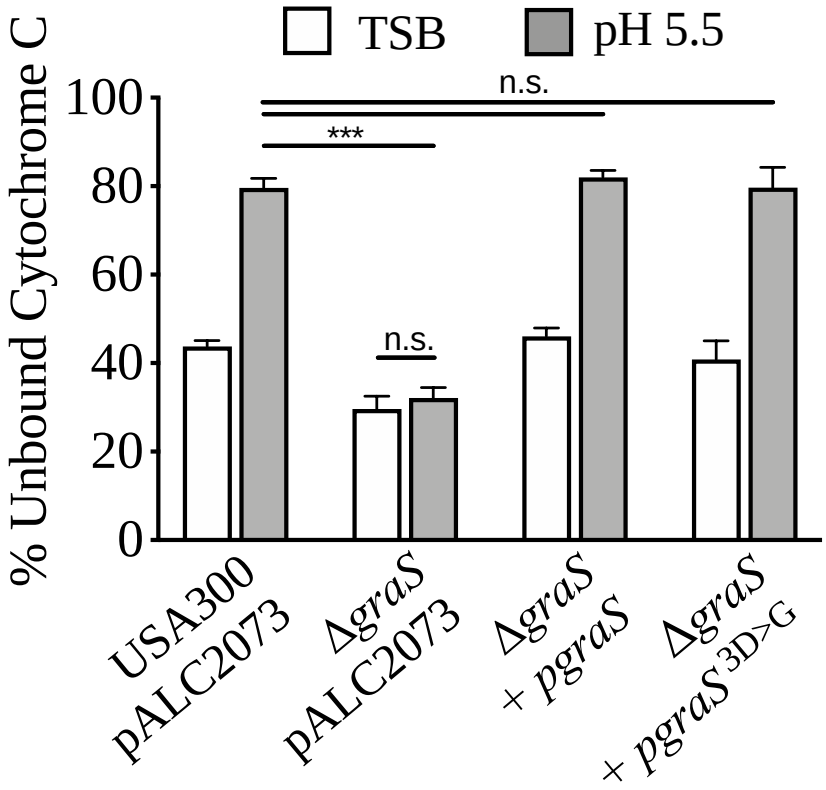

Figure 2.11. Restoration of cell surface positive charge is independent of acidic amino acids in the extracellular sensor loop of GraS during growth at acidic pH. Cultures were grown to an OD_{600} of 0.5 in TSB or TSB pH 5.5, and then processed for assay of cytochrome C binding. Each data point represents the mean ± SE of three replicate determinations from each of three cultures. Statistical significance was measured using two-way ANOVA; *** $p<0.001$, n.s. not significant.

2.4.5 The ABC-transporter VraFG is required for novel GraS activation mechanism in response to acidic pH

Activation of GraR by GraS has also been shown to require two-component ABC-transporter, VraFG. Unlike typical ABC-transporters associated with two-component systems which have efflux capabilities, VraFG has not been shown to function as a detoxification module (34). Rather, VraFG is proposed to fine tune the signalling mechanism of GraS, as the extracellular loop of VraG has been shown to directly impact CAMP specificity for GraS signalling (64). Therefore, we queried whether VraFG is also required for activation of GraS in response to acidic pH. As expected, knockout of *vraF* or *vraG* caused increased susceptibility to the CAMP polymyxin B when grown in TSB buffered at pH 7.3, similar to a *graS* deletion mutant (Figure 2.12A). Interestingly however, when grown in TSB pH 5.5, *vraF* and *vraG* remained essential for growth in the presence of PmB (Figure 2.12B), conditions that did not require the negatively charged extracellular region of GraS (Figure 2.10). Furthermore, knockout of *vraF* or *vraG* phenocopied a *graS* deletion mutant when grown in TSB pH 5.5 + 250 µM linoleic acid (Figure 2.12C), indicating GraS may not be being activated under these conditions. Together, these findings indicate that activation of GraS at acidic pH does not require the negatively charged amino acids in the extracellular region of GraS, but does require the two-component ABC-transporter VraFG.

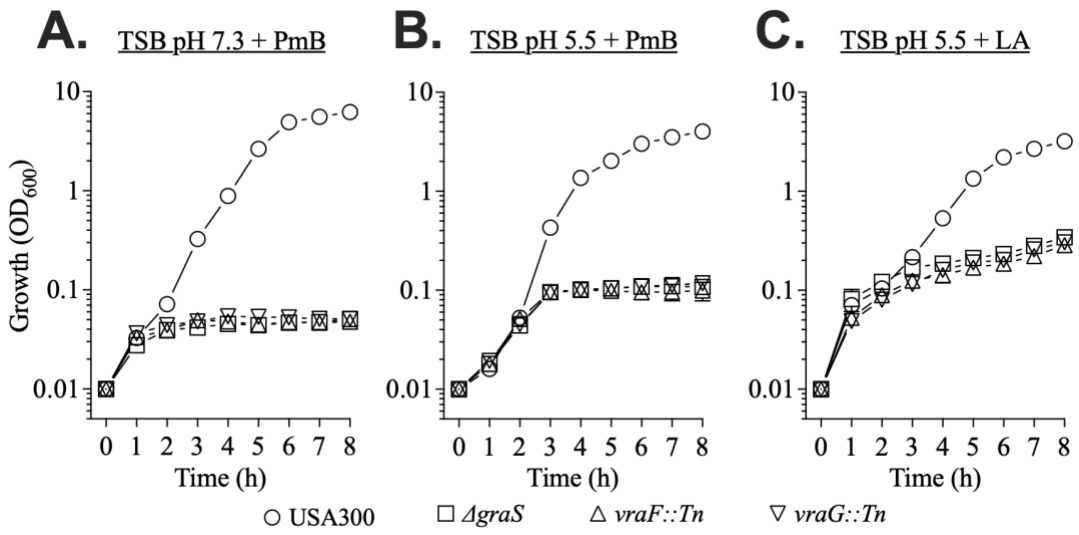

Figure 2.12. The ABC-transporter VraFG is required for GraS signalling in response to both CAMPs and acidic pH. Triplicate flasks of TSB buffered at pH 5.5 or 7.3 were supplemented with 10 µg/mL polymyxin B (PmB) **(A)**, 20 µg/mL PmB **(B)**, or 250 µM linoleic acid (LA) **(C)**. Growth (OD_{600}) was assessed at hourly and each data point represents the mean ± SE from triplicate flasks.

2.4.6 The lysyl-transferase function of MprF is not required for resistance to antimicrobial uFFA during growth at acidic pH.

MprF confers resistance to CAMPs through its ability to promote lysine modification of membrane phospholipid. This occurs through a two-step mechanism whereby the lysyl-phosphatidylglycerol synthase domain transfers lysine to phosphatidylglycerol on the inner surface of the cytoplasmic membrane, after which a flippase domain translocates the nascent lysyl-PG to the outer leaflet of the membrane (38). Since MprF was required for enhanced resistance to antimicrobial uFFA during growth at acidic pH, we queried whether this was dependent on its lysyl-transferase function. We observed that during growth at acidic pH, cell surface positive charge was maintained up to 100 µM linoleic acid, followed by a sharp decrease at 200 µM (Figure 2.13A), consistent with a reduction in cell surface lysyl-PG. From this we surmised that the lysyl-PG-synthase function of MprF should be dispensable for *S. aureus* resistance to 250 µM linoleic acid during growth at pH 5.5, and to assess this hypothesis, we constructed a K547A substitution in MprF, which eliminates lysyl-transferase activity without affecting the stability of the protein (38).

To confirm the MprF^{K547A} phenotype we first evaluated growth of USA300*mprF::Tn* complemented with p*mprF* or p*mprF*^{K547A}, in TSB + 10 µg/mL PmB. As expected, USA300*mprF::Tn* exhibited impaired growth, which was restored with pALC*mprF*, but not pALC*mprF*^{K547A}, consistent with abrogation of lysyl-PG-synthase activity (Figure 2.13B). This was confirmed through cytochrome C binding, where p*mprF* restored cell surface charge to USA300*mprF::Tn*, but p*mprF*^{K547A} did not (Figure 2.13C). These data are consistent with abrogation of lysyl-PG-synthase activity in MprF^{K547A} as previously reported (38). Nevertheless, p*mprF*^{K547A} was able to rescue growth of USA300*mprF::Tn* in TSB pH 5.5 + 250 µM linoleic acid (Figure 2.13D). Therefore, MprF is needed to support *S. aureus* resistance to antimicrobial uFFA during growth at acidic pH, through a mechanism that is independent of its lysyl-transferase function.

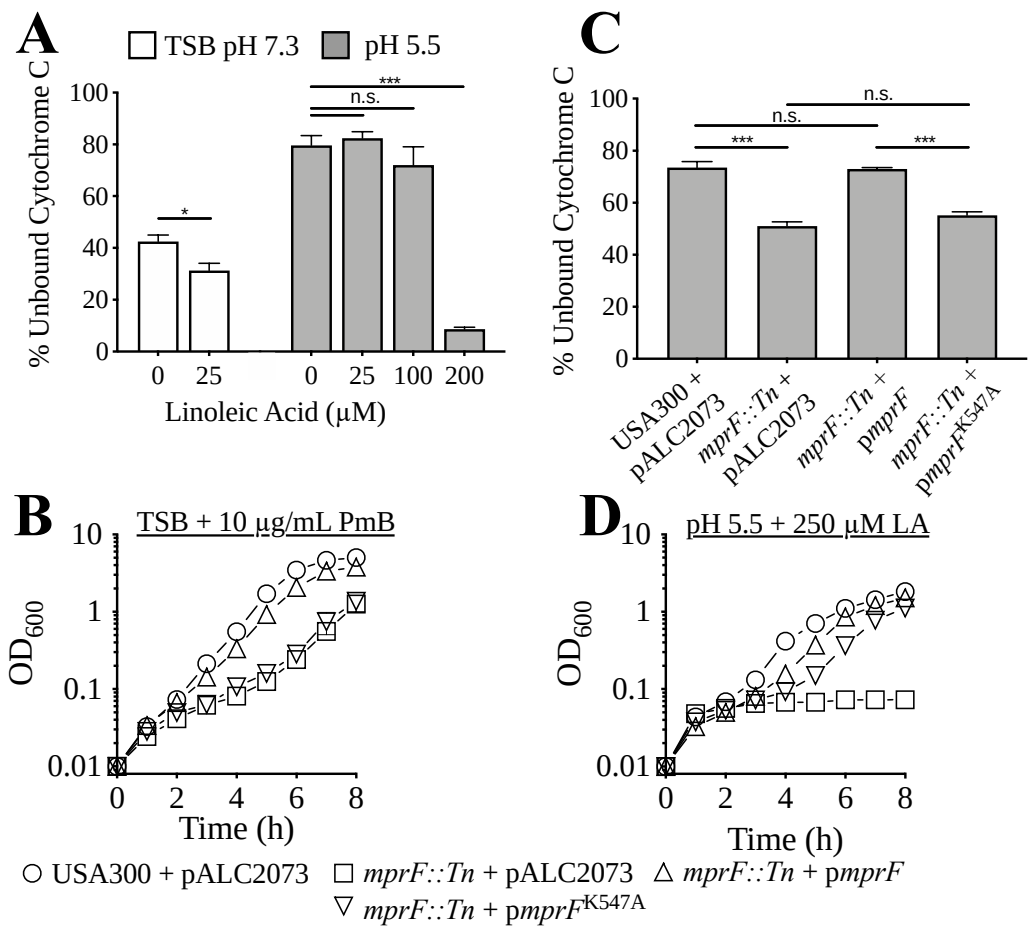

Figure 2.13. The LPG-synthase function of MprF is not required for *S. aureus* USA300 resistance to 250 μM linoleic acid during growth at acidic pH. (A) Cytochrome C binding as a measure of MprF function in cultures of USA300 grown in TSB or TSB pH 5.5 containing the indicated concentration of linoleic acid, LA; **(B)** Growth in TSB + 10 μg/mL PmB, of USA300 and isogenic *mprF::Tn* mutant complemented with pALC2073, p*mprF* or p*mprF*^{K547A}; **(C)**, Cytochrome C binding after growth in TSB pH 5.5, of USA300 and isogenic *mprF::Tn* mutant complemented with pALC2073, p*mprF* or p*mprF*^{K547}. Cultures were supplemented with 120 ng/mL aTc to induce expression from the $P_{xyl/tetO}$ promoter; **(D)** Growth assay as described for panel B, except that cultures were grown in TSB pH 5.5 + 250 μM LA. Statistical significance was measured using two-way ANOVA in panel A, and one-way ANOVA in panel C. * $p<0.05$, *** $p<0.001$, n.s. not significant.

2.4.7 Signaling through GraS also contributes to production of secreted proteases during growth at acidic pH.

Having established that signaling through GraS is required for *S. aureus* resistance to antimicrobial uFFA at acidic pH, we considered whether other phenotypic traits might also exhibit a GraS-dependent phenotype at acidic pH. Foremost, our previous work revealed that antimicrobial uFFA induce expression of secreted proteases (16), producing a characteristic change in the profile of secreted proteins attributed to the Staphylococcal proteolytic cascade. Notably, the 72 kDa precursor of glycerol ester hydrolase proGeh is converted to a mature 40 kDa form by the metalloprotease Aureolysin, which is also required for maturation of the SspA serine protease, and SspA then activates the proSspB cysteine protease precursor producing a 20 kDa mature SspB (16, 18, 52, 65). Knowing that *S. aureus* is concurrently exposed to antimicrobial uFFA and acidic pH on human skin, we queried whether acidic pH could also stimulate protease expression. Accordingly, compared to growth in unbuffered TSB, culture supernatant from USA300 grown in TSB pH 5.5 exhibited the signature protein profile of the Staphylococcal proteolytic cascade, including a reduction in proGeh, and appearance of new proteins consistent with production of SspA and SspB. These changes were not evident in USA300Δ*graS*, or in USA300Δ*pro* where *aur* (Aureolysin) and the *sspABC* serine protease operon are inactivated (Figure 2.14AB), but were restored in USA300Δ*graS* with both p*gra*S and p*graS*$^{3D>G}$ (Figure 2.14B). These observations were mirrored in an accompanying casein hydrolysis zymogram for detection of the SspA serine protease (Figure 2.15AB). We further note that during growth in non-buffered TSB, induction of *graS* expression in USA300Δ*graS* + p*graS* was not sufficient to induce protease production, indicating that acidic pH is a prerequisite for signaling through GraS to induce production of secreted proteases.

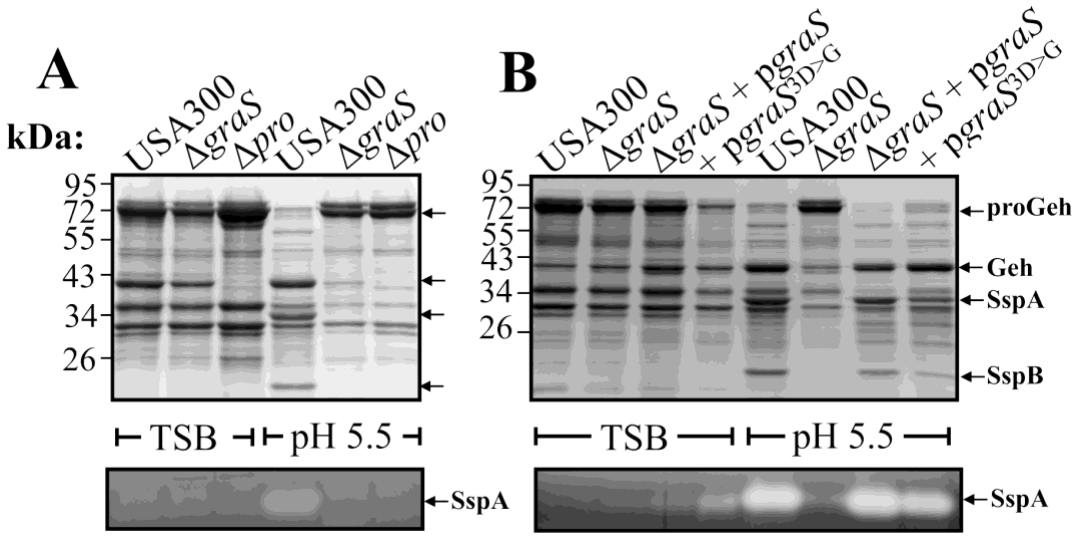

Figure 2.14. Signaling through GraS contributes to *S. aureus* USA300 production of secreted proteases during growth at acidic pH. (A) SDS-PAGE profile of secreted proteins (upper panel) and zymogram for detection of SspA serine protease (lower panel), in culture supernatants of USA300 and isogenic Δ*graS* or protease deficient Δ*pro* mutants after growth for 20 h in TSB or TSB pH 5.5. Arrows on the right margin of each panel indicate the position of proteins that represent the signature protein profile of the Staphylococcal proteolytic cascade, including the precursor of glycerol ester hydrolase proGeh, mature Geh lipase, mature SspA serine protease and SspB cysteine protease. **(B)**, as described for Panel A, with strains USA300 and Δ*graS* complemented with p*graS* or p*graS*3$^{D>G}$. Expression from the $P_{xyl/tetO}$ promoter of pACL2073 was induced using 120 ng/mL aTc. For SDS-PAGE profiles of secreted proteins, TCA precipitated protein equivalent to 3.0 OD_{600} units of culture supernatant were applied to each lane, while for zymogram analyses, a volume of culture supernatant equivalent to 0.075 OD_{600} units was applied to each lane.

2.5 Discussion

In this study, we assessed the genetic requirements for *S. aureus* resistance to antimicrobial uFFA when exposed to signals that would be encountered at sites of colonization and infection, including acidic pH, antimicrobial peptides, and environmental calcium. It is now evident that these commonly encountered environmental signals confer enhanced resistance to antimicrobial uFFA, with different genetic requirements depending on acidic or non-acidic growth conditions, and exposure to calcium or subinhibitory antimicrobial peptides. We previously found that the RND family efflux pump FarE was required for *S. aureus* resistance to antimicrobial uFFA (17, 22), and in our present work this requirement was maintained under non-acidic growth conditions for enhanced resistance to linoleic acid in unbuffered TSB supplemented with calcium. However, at acidic pH, enhanced resistance to both C18:2 and C16:1 uFFA was dependent on GraS and GraS-dependent expression of MprF. Moreover, this occurred independently of acidic amino acids in the extracellular sensor segment of GraS, and the lysyl-transferase function of MprF that are essential for resistance to antimicrobial peptides (37, 38). Growth at acidic pH also promoted GraS-dependent production of secreted proteases, through a mechanism that was again independent of acidic amino acids in the extracellular sensor segment. These novel findings broaden our understanding not only of *S. aureus* mechanisms for resistance to antimicrobial uFFA, but also of mechanisms through which *S. aureus* senses and responds to combined stresses that would be encountered at sites of colonization and infection.

Central to our work is the novel signaling capacity and expanded function of GraS. Previous work on GraS signaling in response to CAMPs focused on acidic amino acids in the extra-cytoplasmic nonapeptide segment DYDFPIDSL, finding that substitution of the three aspartate residues with glycine led to loss of *graS*-dependent expression of *mprF* and *dltA* in response to CAMPs, concomitant with increased sensitivity (37). Our experiments with antimicrobial peptides under non-acidic growth conditions confirmed this requirement, as also reported for the CovS and PhoQ sensor kinases, where acidic amino acids in their large extra-cytoplasmic sensor domain interact with cations on the cytoplasmic membrane to maintain homeostasis, and signaling is initiated when this

interaction is disrupted by CAMPs (66, 67). Nevertheless, these same amino acids were dispensable to GraS function at acidic pH, irrespective of whether the phenotypic readout was cell-surface positive charge, resistance to antimicrobial peptide, resistance to antimicrobial uFFA, or production of secreted proteases. However, a key difference in comparison to CovS and PhoQ is that GraS has a minimal sensor domain, as does the SaeS global regulator of virulence in *S. aureus* (40, 68). Importantly, GraS and SaeS both have a minimal N-terminal sensor domain comprised of two transmembrane helices, separated by an eight or nine amino acid extracellular linker segment, and this organization conforms to a family of intramembrane sensor kinases that sense membrane perturbation (39).

For GraS, this may be especially relevant to signal transduction at acidic pH, which reduces the charge on polar lipid head groups, leading to reduced repulsive forces and tighter lateral packing of phospholipid (42). Acidic pH should also reduce the charge on acidic amino acids in the extra-cytoplasmic linker segment of GraS, which would render them less effective in sensing cationic antimicrobial peptides. It is therefore logical that GraS should have the capacity to signal in response to altered membrane properties at acidic pH as opposed to a strict dependence on acidic amino acids in the extracellular linker segment. In this respect, it is salient to note that antimicrobial peptides also cause changes in membrane structure (69), which could potentially allow GraS to respond to membrane damage caused by these peptides. For SaeS it was proposed that the entire N-terminal sensor domain functions as a trip wire, such that any stimulus that elicits conformational changes in the N-terminal domain would trigger kinase activity, while amino acids in the extracellular linker segment serve to fine tune the response to different stimulants (40, 68). Indeed, deletion of this linker segment in GraS rendered *S. aureus* more sensitive to antimicrobial peptides (10, 37). Therefore, while our present data indicate that acidic amino acids in the extracellular linker segment are dispensable to signaling at acidic pH, it is feasible that the entire N-terminal segment of GraS functions as a molecular tripwire to sense membrane perturbation as described for SaeS (40, 68). As such, changes in phospholipid composition and physical properties in response to acidic pH or antimicrobial peptides would be sufficient to trigger GraS kinase activity independently of the need to recognize a physical antimicrobial peptide ligand. The DesK

sensor kinase of *Bacillus subtilis* operates on such a principle, whereby assembly of a thicker cytoplasmic membrane at low temperature stimulates signaling through DesK independently of the need for an extracellular sensor segment (70).

Although the acidic amino acids in the extracellular segment of GraS were not required for activation in response to acidic pH, the two-component ABC-transporter VraFG remained essential. One plausible explanation for this VraFG requirement is due to the proper assembly of the five-component system requiring all proteins. Multiple studies have shown a high degree of protein-protein interactions in the GraXRS/VraFG system (Figure 2.13), and GraX is proposed to simply act as a scaffold to improve the binding interactions between GraR and GraS (34, 35). Therefore, it is possible protein-protein interactions with VraF or VraG are also required for proper assembly of the sensor system, and to improve the binding and signalling from GraS to GraR. However, this theory remains to be tested in our future studies.

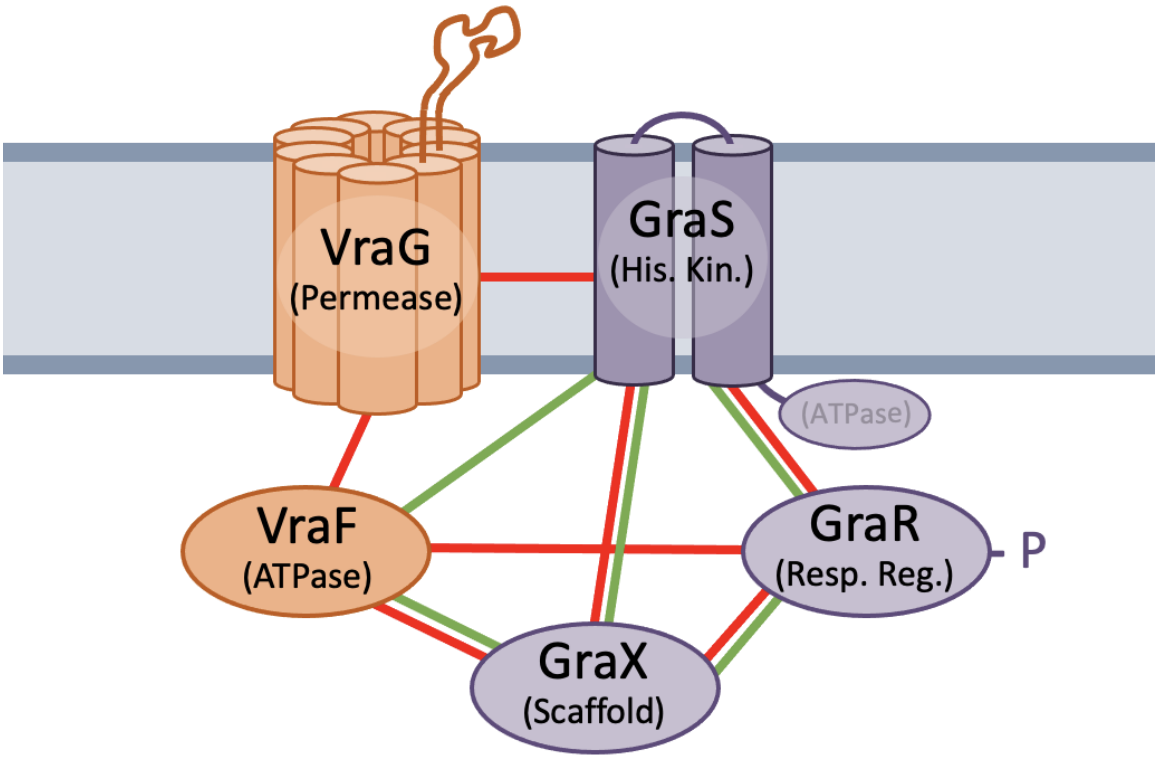

Figure 2.15. Protein-protein interaction in the GraXRS/VraFG five component sensor system. The GraS histidine kinase (His. Kin.) phosphorylates the GraR response regulator (Resp. Reg.) through assistance from the GraX scaffold protein. Proper sensing for this system relies on interactions with the VraG ABC-transporter permease and VraF ABC-transporter ATPase. Two different studies have assessed the binding interactions for each of these proteins, which are shown in red (34) or green (35). Muzamal *et al.* indicated that although GraS binds GraR, this interaction is very weak and requires GraX for proper signal transduction (35).

While previous and present data confirm that signalling through GraS is critical for expression of MprF, which confers resistance to CAMPs through lysine modification of phospholipid (4, 63), we were surprised to find that during growth at acidic pH, MprF was also required for enhanced resistance to both C16:1 and C18:2 antimicrobial uFFA through a mechanism that was independent of its lysyl-transferase function. However, similar observations were noted on the role of MprF in promoting reduced susceptibility to the cationic lipopeptide antibiotic Daptomycin (71, 72). It is not completely understood how daptomycin kills *S. aureus* but current evidence supports a model whereby it targets fluid microdomains in the membrane, followed by oligomerization and translocation to the inner leaflet where it then blocks the interaction between essential membrane proteins and fluid membrane microdomains (73). Although clinical isolates with reduced susceptibility to daptomycin often have non-synonymous polymorphisms in MprF, there is no general consensus that this is due to increased production of lysyl-PG, and one of the most commonly occurring polymorphisms causes a T345A/I/K substitution at the junction of the flippase and lysyl-PG-synthase domains (71).

Notably, a T345A substitution at this juncture is alone sufficient to promote reduced susceptibility to daptomycin, and it was proposed that this may allow the flippase domain to accommodate daptomycin and translocate the antibiotic out of the fluid inner membrane microdomains (71, 72). Since our data indicate that abrogation of the lysyl-PG synthase activity of MprF does not interfere with its ability to complement growth of *S. aureus mprF::Tn* at pH 5.5 in the presence of antimicrobial uFFA, this would be consistent with a mechanism whereby under these conditions, the flippase domain promotes translocation of phosphatidylglycerol containing an unsaturated fatty acid, instead of its physiologic lysyl-PG substrate. In support of this analysis, we note that growth at pH 5.5 promotes a GraS-dependent increase in cell-surface positive charge (Figure 2.11), which is maintained at 25 µM and 100 µM linoleic acid, but then drops sharply at 200 µM linoleic acid (Figure 2.13A), under which condition growth remained dependent on MprF (Figure 2.13D). This could be accounted for if an excess of linoleoyl-PG competed with lysyl-PG for translocation by MprF under these conditions, or alternately if membrane properties under these conditions were not conducive to synthesis of lysyl-PG.

Another novel feature of signaling through GraS and downstream phenotypic traits revealed through our work is its requirement for production of secreted proteases during growth at acidic pH. Although the focus of research on GraS has been its role in signalling through GraR to promote expression of genes that modify cell surface properties as required for resistance to antimicrobial peptides (3, 10, 37), one study alluded to a broader role including promotion of growth at high temperatures and resistance to oxidative stress (9). In this latter respect, it is noteworthy that polyunsaturated arachidonic acid is reported to have bactericidal activity towards *S. aureus* through a lipid peroxidation mechanism (74). Expression profiling also revealed that several major virulence factors exhibited increased GraS-dependent expression in response to antimicrobial peptide, including the accessory gene regulator *agr* as well as secreted hemolysins and cell surface proteins (9). Nevertheless, the gene encoding SspA serine protease was not among those that were reported as being regulated through GraS (9). Another recent study aimed at mapping the global network of extracellular protease regulation in *S. aureus* identified seven major regulators and seven secondary regulators, but GraS and GraR were not among these (75). Consequently, it is likely that the role of GraS in promoting production of secreted protease is limited to signaling at acidic pH, which is encountered by *S. aureus* on human skin and macrophage phagosomes, but also on nasal mucosa (25) and in chronic abscesses (76).

As with acidic pH and CAMPs, our findings revealed that extracellular calcium also promoted increased resistance of *S. aureus* to antimicrobial uFFA, representing a convergence of signals that could promote persistence of *S. aureus* on human skin. Although acidic pH initially promoted increased sensitivity of *S. aureus* to palmitoleic and sapienic acid, this effect was ameliorated with 0.5 mM calcium. While the mechanistic basis of this finding has yet to be elucidated, recent studies have highlighted the role of environmental calcium in promoting microbial persistence strategies, including enhanced biofilm formation in *Vibrio fischeri* through a mechanism that was dependent on the SypS sensor kinase, and calcium dependent activation of the LadS histidine kinase in *Pseudomonas aeruginosa* to induce an acute-to-chronic transition in virulence (77, 78). Therefore, future work will focus on understanding how environmental calcium may influence *S. aureus* persistence strategies, and on elucidating

how changes in membrane properties and composition during growth at acidic pH trigger signaling through GraS.

2.6 References

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Chapter 3

3 The *fadXDEBA* Locus of *Staphylococcus aureus* Contributes to Detoxification of Exogenous Palmitic Acid and *In Vivo* Growth.[2]

[2] This chapter (with the exception of Section 3.1, 3.4.8–10, and minor alterations to 3.5) has been submitted for publication in Molecular Microbiology, and is currently under revision. Kuiack RC, Tuffs SW, Dufresne K, McCormick JK, and McGavin MJ. The *fadXDEBA* locus of *Staphylococcus aureus* is required for metabolism of exogenous palmitic acid and *in vivo* growth. Mol Micro. Under revision.

3.1 Chapter Preface

3.1.1 Rationale for Investigation

In Chapter 2, we outlined a novel resistance mechanism for uFFA resistance activated by GraS signalling and MprF expression. Resistance through this novel mechanism was independent of the established uFFA resistance mechanisms, the fatty acid efflux pump FarE, and the fatty acid kinase FakA (Figure 2.7). One nuance to this novel uFFA resistance mechanism is that even though acidic pH activates GraS and induces MprF expression, acidic pH makes *S. aureus* more susceptible to C16:1 palmitoleic or sapienic acid (Figure 2.1). In contrast, acidic pH makes *S. aureus* significantly more resistant to C18:2 linoleic acid C20:4 arachidonic acid (Figure 2.1). Therefore, there is something specific to C16 uFFA that defies GraS/MprF mediated resistance at acidic pH. We hypothesized this is due to C16 fatty acids being poor substrates for FakA-mediated metabolism of fatty acids (1), and that the membrane disruption caused by a buildup of these fatty acids may make *S. aureus* more susceptible to acidic pH intoxication, as outlined in Chapter 4. Interestingly, we see that deletion of *fakA* rescues growth of *S. aureus* under combined acidic pH and palmitoleic acid conditions (Figure 3.1A). At high enough concentrations, we see acidic pH actually increases resistance to palmitoleic acid in Δ*fakA* (Figure 3.1B). Furthermore, even though deletion of *fakA* causes increased basal expression of the efflux pump FarE (2), this is not responsible for increased palmitoleic acid resistance at acidic pH, as a combined *fakA-farER* deletion was still able to grow under these conditions (Figure 3.1C). Therefore, there must be an additional mechanism in the *fakA* deletion mutant responsible for this resistance.

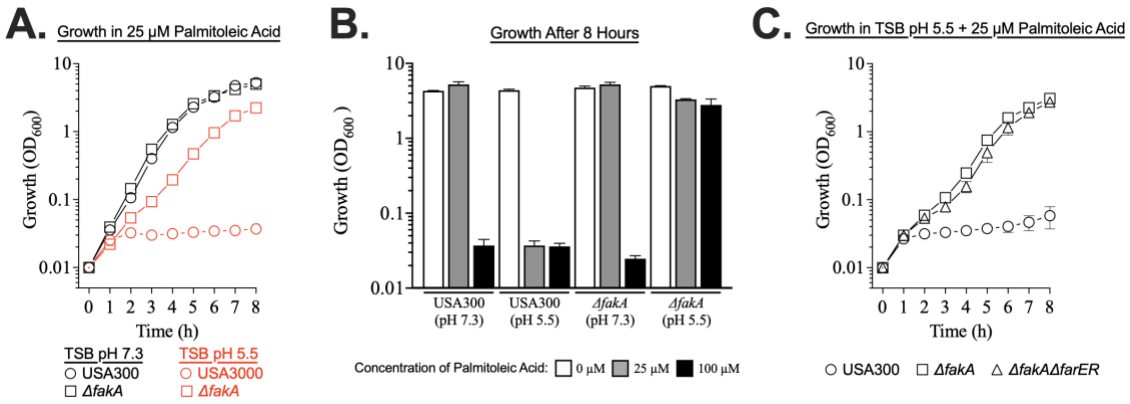


Figure 3.1. US300Δ*fakA* resists toxicity imposed by combined palmitoleic acid and acidic pH conditions. Triplicate flasks of TSB were supplemented with indicated concentrations of palmitoleic acid, and inoculated to an initial optical density measured at 600 nm (OD_{600}) of 0.01. Growth (OD_{600}) was assessed at hourly intervals **(A and C)**, or after 8 hours of growth **(B)**, and each data point represents the mean ± SE from triplicate flasks.

With FakA responsible for metabolizing exogenous fatty acids, we hypothesized that deletion of this metabolic pathway would upregulate a different fatty acid metabolic pathway to compensate; however, metabolism through FakA was thought to be the only metabolic fate for exogenous fatty acids in *S. aureus* (3, 4). In contrast to *S. aureus*, many bacteria such as *E. coli* and *B. subtilis* can use β-oxidation to metabolize exogenous fatty acids for energy (5, 6). Furthermore, β-oxidation has also been seen in other Staphylococci species such as *Staphylococcus carnosus* (7). Therefore, we began to investigate if β-oxidation also occurs in *S. aureus*, and if this may explain the increased resistance we see in Δ*fakA* (Figure 3.1).

Through genomic analysis, we determined that *S. aureus* does possess a *fadDEBA* locus comprising the minimal complement of genes that are necessary and sufficient for degradation of fatty acids through the canonical β-oxidation pathway in other bacteria, such as *E. coli* (6). Furthermore, studies have shown these genes are highly expressed when *S. aureus* is internalized by human bronchial epithelial cells, an environment rich is host-derived lipid species (8, 9). A recent study also demonstrated that *fadX*, an additional gene preceding *fadDEBA* in *S. aureus*, was required for metabolism of short chain (C3) fatty acids (10). Together, these findings led us to believe that *S. aureus* does possesses β-oxidation capabilities, and that this metabolic pathway plays an important role in metabolizing exogenous fatty acids in conjunction with the established FakA metabolic pathway.

3.1.2 Expression data supports a β-oxidation function for FadXDEBA in *S. aureus*

Although the function of the *fadXDEBA* locus has not been directly investigated in *S. aureus*, previous studies have noticed upregulation of the *fad* genes or Fad proteins in a variety of different environments and strains. For example, when internalized in bronchial epithelial cells (8, 9) or liver cells (11), during stationary growth phase (8), or when deleting *ccpA* which regulates gene expression in response to glucose availability (12), *fad* genes/proteins were shown to be expressed at significantly higher levels. With this expression data available, we decided to investigate if there were any common

genes/proteins across these studies expressed in conjunction with *fad*, to elucidate possible interactions of the *fad* system with other pathways in *S. aureus*.

Overall, we see relatively few genes/proteins that are consistently up or downregulated across the multiple studies (Figure 3.2). In agreement with the genomic arrangement of the *fad* genes, upregulation of the *fad* genes occurred in unison, apart from *fadX* in one study (Table 3.1). Interestingly, there was not a similar trend for upregulation of other known fatty acid metabolism genes/proteins (Table 3.1). While no clear trends emerged for genes/proteins consistently downregulated across these studies, there was one group of genes/proteins that were consistently upregulated in conjunction with the *fad* genes, those involved in the tricarboxylic acid (TCA) cycle (Table 3.2). This was specific to the TCA cycle, and not due simply to increased metabolism, as there was not a similar pattern for genes/proteins involved in glycolysis (Table 3.2). Overall, these findings support a system where β-oxidation of fatty acids through FadDEBA produces acetyl-CoA that fuels the TCA cycle. Therefore, we believed the *S. aureus fad* genes warranted further investigation.

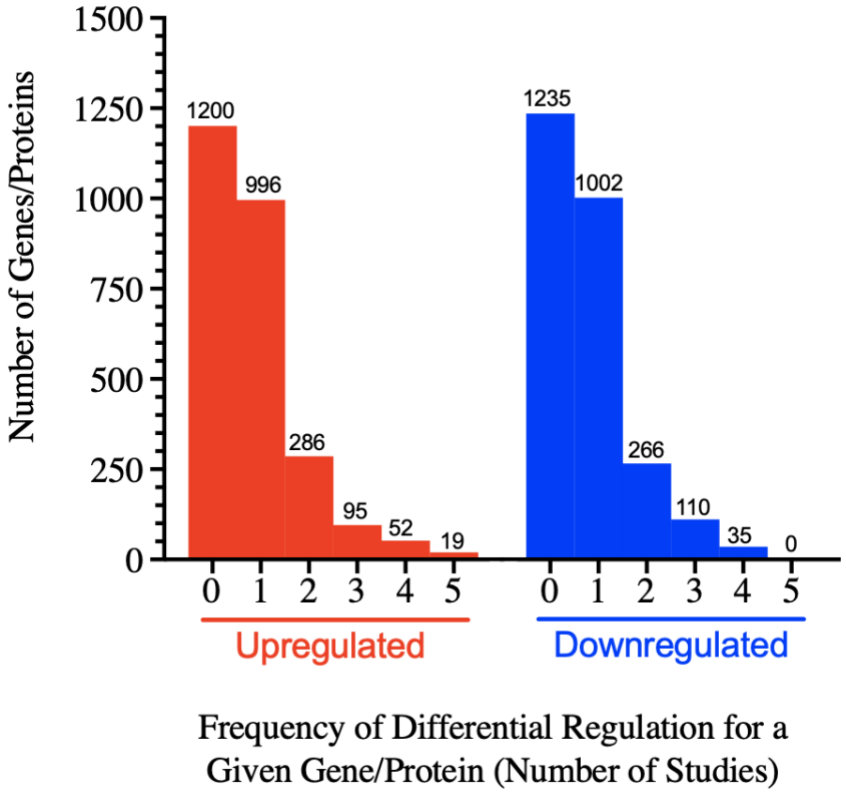


Figure 3.2. Frequency of genes/proteins differentially regulated in studies where increased *fad* expression was observed. Expression data from five different experiments where *fad* genes or Fad proteins were significantly upregulated was amalgamated to investigate genes that were commonly upregulated or downregulated in conjunction with *fad* (8, 9, 11, 12). Genes from different *S. aureus* backgrounds were first aligned to the USA300 genome, and the frequency in which a given gene/protein was significantly upregulated or downregulated across the different studies was recorded. A more detailed breakdown of some of these genes represented in this figure can be found in Tables 3.1 and 3.2.

Table 3.1. Relative expression of fatty acid metabolism genes (mRNA) or proteins (Protein) in *S. aureus* from previous literature where *fad* expression was upregulated. Red indicates upregulation, blue indicates downregulation, and black indicates genes/proteins were not significantly different than controls.

Gene Name	Locus Tag[1]	Bronchial #1 (Protein)[2]	Stationary (Protein)[3]	Bronchial #2 (Protein)[4]	Liver (mRNA)[5]	Δ*ccpA* (mRNA)[6]
β-oxidation Metabolic Pathway						
fadA	SAUSA300_0225	32.67	49.41	3.05	6.20	7.45
fadB	SAUSA300_0226	47.18	27.52	3.32	5.10	7.29
fadE	SAUSA300_0227	21.41	26.05	3.20	5.18	7.93
fadD	SAUSA300_0228	8.40	20.18	2.85	3.25	5.07
fadX	SAUSA300_0229	121.94		2.89	2.22	4.06
Other Fatty Acid Metabolism Pathways						
fakA	SAUSA300_1119	-1.15	-1.64		-2.66	
fakB1	SAUSA300_0733				-2.41	
fakB2	SAUSA300_1318	1.13	1.99		-2.47	
ohyA	SAUSA300_0108				1.28	

[1] Data was collected from a variety of *S. aureus* backgrounds, but aligned to the USA300 genome in order to summarize the data effectively

[2] *S. aureus* proteins significantly regulated when internalized by human bronchial epithelial cells compared to bacteria pre-infection (9)

[3] *S. aureus* proteins significantly regulated in stationary growth phase compared to exponential growth phase (8)

[4] *S. aureus* proteins significantly regulated when internalized by human bronchial epithelial cells compared to non-adherent control (8)

[5] *S. aureus* genes significantly regulated in a murine liver infection following tail vein infection compared to TSB grown controls (11)

[6] *S. aureus* genes significantly regulated in a *ccpA* deletion mutant compared to wildtype control (12)

Table 3.2. Relative expression of metabolism genes (mRNA) or proteins (Protein) in *S. aureus* from previous literature where *fad* expression was upregulated. Red indicates upregulation, blue indicates downregulation, and black indicates genes/proteins were not significantly different than controls.

Gene Name	Locus Tag[1]	Bronchial #1 (Protein)[2]	Stationary (Protein)[3]	Bronchial #2 (Protein)[4]	Liver (mRNA)[5]	Δ*ccpA* (mRNA)[6]
Tricarboxylic Acid Cycle						
pyc	SAUSA300_1014	1.83	2.60		3.30	
sdhC	SAUSA300_1046				2.60	
sdhA	SAUSA300_1047	4.41	9.43	1.96	7.29	
sdhB	SAUSA300_1048	3.58	7.26	1.65	12.00	
sucC	SAUSA300_1138	2.41	7.07	1.79	3.08	2.51
sucD	SAUSA300_1139	2.69	6.80	1.69	4.63	3.51
acnA	SAUSA300_1246	1.23	5.18	1.56	3.46	
sucB	SAUSA300_1305	2.17	10.70	1.92	14.46	3.11
sucA	SAUSA300_1306	4.76	8.24	1.77	11.97	2.59
icd	SAUSA300_1640	3.81	8.03	2.41	28.94	3.42
gltA	SAUSA300_1641	112.99	5.24		21.58	3.00
fumC	SAUSA300_1801	2.89	6.21	1.54	3.00	
mqo	SAUSA300_2312	1.64	9.56	1.85	4.30	
Glycolysis						
NA	SAUSA300_0375				-2.20	
gapA	SAUSA300_0756	-1.33	-1.75		1.29	
pgk	SAUSA300_0757	-1.34			1.17	
gpmI	SAUSA300_0759	-2.17			1.06	
eno	SAUSA300_0760	1.12			1.51	
pgi	SAUSA300_0865	1.30		-1.72	-1.22	
gapB	SAUSA300_1633	31.12	5.69	-1.30	7.38	
pyk	SAUSA300_1644	1.01			-1.03	

pfkA	SAUSA300_1645	-1.23		-1.59	-1.90	
fba	SAUSA300_2079	1.18			-1.52	
pgcA	SAUSA300_2433	2.06	2.60	-1.62	-1.11	

[1] Data was collected from a variety of *S. aureus* backgrounds, but aligned to the USA300 genome in order to summarize the data effectively

[2] *S. aureus* proteins significantly regulated when internalized by human bronchial epithelial cells compared to bacteria pre-infection (9)

[3] *S. aureus* proteins significantly regulated in stationary growth phase compared to exponential growth phase (8)

[4] *S. aureus* proteins significantly regulated when internalized by human bronchial epithelial cells compared to non-adherent control (8)

[5] *S. aureus* genes significantly regulated in a murine liver infection following tail vein infection compared to TSB grown controls (11)

[6] *S. aureus* genes significantly regulated in a *ccpA* deletion mutant compared to wildtype control (12)

3.1.3 Bioinformatic analysis of the *fadXDEBA* genes in *S. aureus*

As previously mentioned, *S. aureus* possesses putative β-oxidation genes orthologous to those found in *E. coli* (Table 3.3, Figure 3.3). While more complex, with a variety of redundant proteins, orthologs for β-oxidation genes in the Gram-positive *B. subtilis* can also be determined (Table 3.4, Figure 3.3). However, although *S. aureus* possesses this putative β-oxidation system, there are a variety of unique differences from the systems in *E. coli* and *B. subtilis*. For example, rather than the β-oxidation genes being distributed throughout the genome, the genes are clustered into a single locus, *fadXDEBA* (Figure 3.3). Additionally, in both *E. coli* and *B. subtilis*, regulation of genes distributed across the genome is coordinated by a transcriptional regulator, FadR; however, in *S. aureus*, there is no ortholog to FadR (Table 3.3 and 3.4). Together, the unique clustering of *fad* genes into a single locus and lack of a conventional FadR regulator indicates a unique regulatory method for expression of the *fad* genes in *S. aureus*, that will be elaborated on in Section 3.4.9.

Additionally, there is a mistake in the annotation of the *fad* genes in *S. aureus*. In *E. coli*, and most β-oxidation pathways in other bacteria, FadD refers to the long chain fatty acyl-CoA ligase whereas FadE refers to the acyl-CoA dehydrogenase. In *S. aureus*, the annotation for these two genes has been switched. For consistency with the established literature, I will be using the nomenclature of the *E. coli* genes to describe the system in *S. aureus* for the remainder of this thesis.

Another unique aspect of the *fad* genes in *S. aureus* is the presence of the short chain acyl-CoA transferase, FadX, being clustered with the canonical β-oxidation pathway, FadDEBA. Like its ortholog YdiF in *E.* coli, FadX plays a role in metabolizing short chain (C2–C4) fatty acids (10, 13). This contrasts β-oxidation pathways which specifically respond to longer chain (greater than C8) fatty acids (5, 14). The unique clustering of these two systems in *S. aureus* further establishes the regulation and function of these *fad* genes may vary from the established system in *E. coli*. This is further supported by no short chain acyl-CoA transferase orthologs being found in *B. subtilis* (Table 3.4), indicating FadX is likely an accessory protein in *S. aureus*, and not

required for standard β-oxidation function. This theory is further supported and elaborated on in Section 3.4.6.

Table 3.3. Genetic homology and function of fatty acid degradation (*fad)* genes in *Staphylococcus aureus* and *Escherichia coli* responsible for β-oxidation.[1]

S. aureus [2]	*E. coli* [2]	Function	Query [3] Coverage	Percent Identity
FadX (525)	YdiF (531)	Short chain acyl-CoA transferase	89%	35.06%
FadD (501)	FadD (561)	Long chain fatty acyl-CoA ligase	92%	27.81%
FadE (403)	FadE (814)	Acyl-CoA dehydrogenase	37%	26.93%
FadB (753)	FadB (729)	3-hydroxyacyl-CoA dehydrogenase, Enoyl-CoA hydratase	58%	32.34%
FadA (394)	FadA (387)	Acetyl-CoA acyltransferase	100%	42.32%
No Ortholog	FadR (239)	GntR-family transcriptional regulator of fatty acid metabolism	-	-

[1] The identification of *fad* genes in *S. aureus* was based on similarities in the amino acid sequence and domain composition with the β-oxidation proteins in *E. coli*; however, identification in this manner does not necessarily imply an evolutionary relationship

[2] Numbers refer to amino acid size for each protein

[3] Query coverage refers to the percentage of the query sequence (*E. coli* protein) overlapped by the subject sequence (*S. aureus* protein)

Table 3.4. Genetic homology and function of fatty acid degradation (*fad)* genes in *Staphylococcus aureus* and *Bacillus subtilis* responsible for β-oxidation.[1]

S. aureus [2]	*B. subtilis* [2]	Function	Query [3] Coverage	Percent Identity
FadX (525)	No Ortholog	Short chain acyl-CoA transferase	-	-
FadD (501)	LcfA (560)	Long chain fatty acid-CoA ligase	90%	29.41%
	LcfB (530)	Long chain fatty acid-CoA ligase	95%	31.36%
FadE (403)	FadE (594)	Acyl-CoA dehydrogenase	52%	29.58%
FadB (753)	FadN (789)	3-hydroxyacyl-CoA dehydrogenase, Enoyl-CoA hydratase	99%	33.58%
	FadB (258)	Enoyl-CoA hydratase	46%	30.95%
FadA (394)	FadA (391)	Acetyl-CoA acyltransferase	100%	52.02%
No Ortholog	FadR (194)	TetR-family transcriptional regulator of fatty acid metabolism	-	-

[1] The identification of *fad* genes in *S. aureus* was based on similarities in the amino acid sequence and domain composition with the β-oxidation proteins in *B. subtilis*; however, identification in this manner does not necessarily imply an evolutionary relationship

[2] Numbers refer to amino acid size for each protein

[3] Query coverage refers to the percentage of the query sequence (*B. subtilis* protein) overlapped by the subject sequence (*S. aureus* protein)

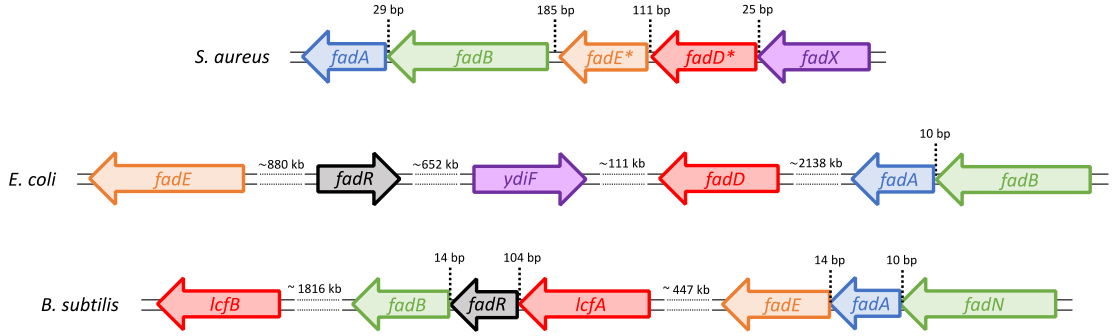


Figure 3.3. Genomic arrangement of β-oxidation genes in *S. aureus*, *E. coli*, and *B. subtilis*. Base pairs (bp) or kilobases (kb) between genes are annotated. Colouring indicates orthologous proteins, with purple representing short chain acyl-CoA transferases, red representing long chain fatty acid-CoA ligases, orange representing acyl-CoA dehydrogenases, green representing 3-hydroxyacyl-CoA dehydrogenases/enoyl-CoA hydratases, blue representing acetyl-CoA acyltransferases, and grey representing transcriptional regulators of β-oxidation metabolism. Asterisks denote misannotation in the *S. aureus* genome, where *fadD* and *fadE* are swapped. This figure, along with the rest of this thesis, use the gene names based on homology to *E. coli* genes, as shown in Table 3.3.

While orthologs can be established between *S. aureus* and *E. coli* or based on sequence similarity, there is still a large amount of sequence variation between these proteins (Table 3.3). Therefore, we wanted to confirm the proteins in *S. aureus* structurally resembled their counterparts by aligning the AlphaFold predicted protein structures of FadX (YdiF in *E. coli*), FadD, FadE, FadB, and FadA between *S. aureus* and *E. coli*, and saw an incredibly high level of homology (Appendix A–F). While the alignment for FadX, FadD, and FadA are indisputable between *S. aureus* and *E. coli*, FadE and FadB show some variation (Appendix A–F).

The acyl-CoA dehydrogenase FadE in *E. coli* is approximately twice the size of its counterpart in *S. aureus*, and possesses two additional and distinct domains (Figure 3.4). FadE in *E. coli* is predicted to be membrane bound, and as such, the N-terminus of the protein contains a signal peptide and two transmembrane domains, which is not the case for FadE in *S. aureus* (Figure 3.4). Furthermore, FadE in *E. coli* contains an additional C-terminal domain which is functionally uncharacterized, but is found in various other prokaryotic acyl-CoA dehydrogenases (Figure 3.4). However, the central core of both FadE proteins are conserved both functionally and structurally, implying both maintain a similar function overall (Figure 3.4, Appendix C).

The 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase FadB also differs slightly between *E.coli* and *S. aureus*. These enzymes possess dual functions, converting a trans-enoyl acyl-CoA to a hydroxy acyl-CoA through the 3-hydroxyacyl-CoA dehydrogenase domain of the protein, and then converting the hydroxy acyl-CoA to a β-keto acyl-CoA through the enoyl-CoA hydratase domain of the protein. While both domains and subsequent functions are conserved in FadB of *E. coli* and *S. aureus*, the arrangement has been flipped (Figure 3.5), causing a reduced homology result and sequence coverage (Table 3.3), as well as an inability to structurally align both protein domains at the same time (Appendix D and E). However, when aligned separately, both domains have a high level of structural similarity (Appendix D and E).

Together, these findings show that the *fadDEBA* genes in *S. aureus* should be sufficient to allow for β-oxidation of exogenous fatty acids. Therefore, we decided to further

investigate the function of *fadXDEBA* in *S. aureus*, and the role these genes play in metabolism of exogenous fatty acids.

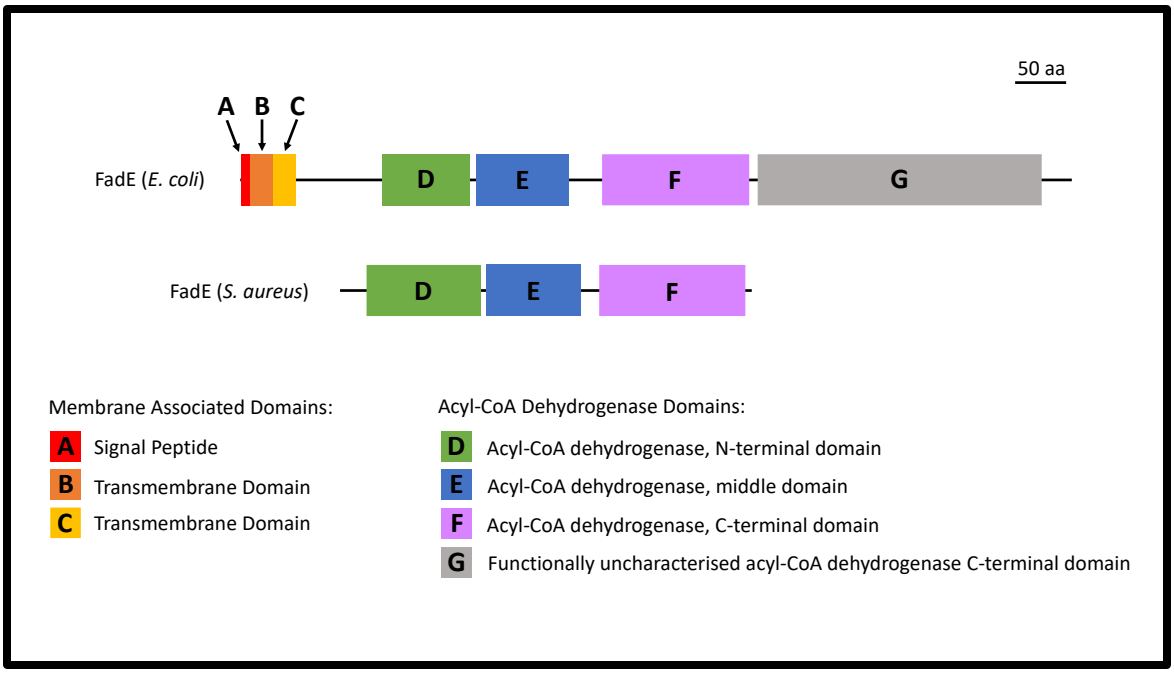


Figure 3.4. Predicted protein domains of the acyl-CoA dehydrogenase FadE in *E. coli* and *S. aureus*. Protein domains were determined using InterProScan, and the relevant domains were reported. The functionally uncharacterized acyl-CoA dehydrogenase C-terminal domain (G) is identified as Pfam PF09317, and is found in a variety of other acyl-CoA dehydrogenase proteins. However, the both domain architectures of FadE in *E. coli* and *S. aureus* are well conserved in acyl-CoA dehydrogenases of other bacteria.

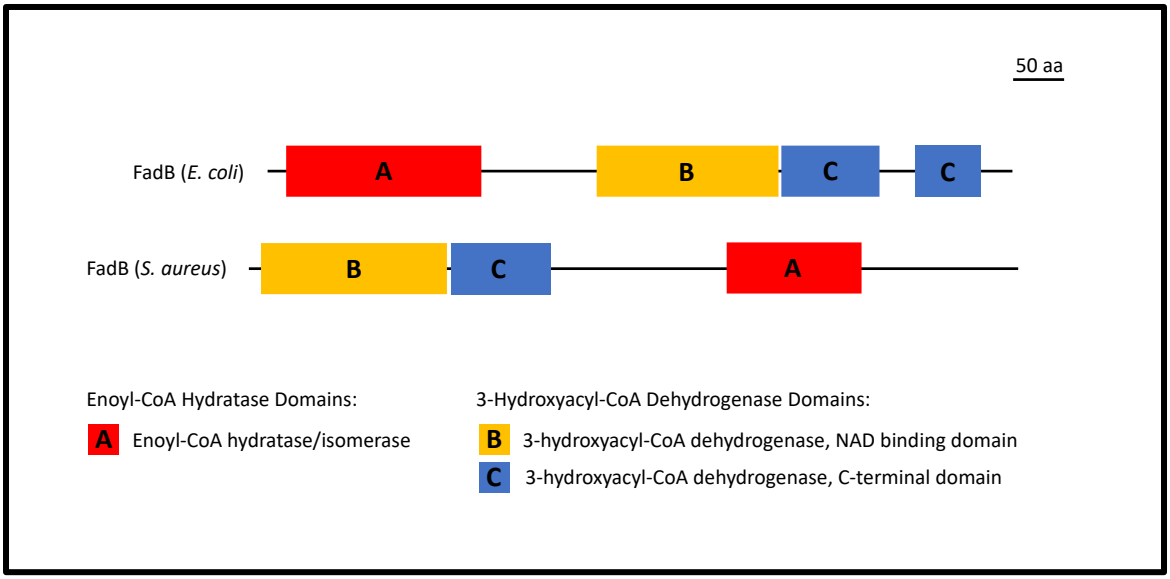


Figure 3.5. Predicted protein domains of the FadB 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase in *E. coli* and *S. aureus*. Protein domains were determined using InterProScan, and the relevant domains were reported. The location of 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase domains are flipped between *E. coli* and *S. aureus*. Furthermore, FadB in *E. coli* has an additional 3-hydroxyacyl-CoA dehydrogenase C-terminal domain. Both the single C-terminal domain and the double C-terminal domains are common domain architectures for 3-hydroxyacyl-CoA dehydrogenases in other bacteria.

3.2 Introduction

Staphylococcus aureus is a Gram-positive opportunistic pathogen that asymptomatically colonizes the anterior nares in approximately 30% of the population (15), and among individuals who exhibit nasal carriage, it is also found on the skin where it is adapted to survive in the presence of a wide range of innate defense mechanisms, including acidic pH, cationic antimicrobial peptides (CAMPs), and antimicrobial unsaturated free fatty acids (FFA) (16–20). Although part of the normal skin microbiota, *S. aureus* also causes a broad spectrum of infections, including invasive soft tissue infections, endocarditis, osteomyelitis, and sepsis, due to the multitude of virulence factors at its disposal (21, 22). It is also well established that *S. aureus* isolates recovered from sites of infection are usually a genetic match to strains recovered from the same patient's anterior nares (15, 23), and individuals who are colonized by *S. aureus* have a significantly greater risk of developing blood stream infections than non-carriers when hospitalized (23–25). Therefore, understanding the mechanisms by which *S. aureus* circumvents the innate immune mechanisms of the skin and anterior nares is of paramount importance.

The response of *S. aureus* to host-derived FFA are of particular interest, as these molecules constitute both a threat to, and opportunity for, its growth. Unsaturated FFA (uFFA) are prevalent on human skin (26, 27) and the anterior nares (28), the primary sites of *S. aureus* colonization, as well as in abscesses (28–30), a common form of infection caused by *S. aureus*. These antimicrobial uFFA can disrupt the phospholipid membrane of *S. aureus*, causing leakage of cellular components (1, 31), or produce reactive oxygen species through lipid peroxidation to intoxicate the bacteria (32). However, exogenous uFFA can also represent a potential nutrient if *S. aureus* is able to overcome their toxicity, and human skin also provides a rich source of less toxic saturated FFA (33) that could also provide a metabolic benefit.

S. aureus has a range of mechanisms that confer resistance to antimicrobial uFFA. Cell wall teichoic acid can function as a barrier to entry of uFFA and can also limit the leakage of cellular components consequent to membrane damage induced by uFFA (1, 34). Iron surface determinant A (IsdA) also functions as a hydrophilic shield to restrict entry of uFFA (35), while fatty acids that penetrate the membrane can be eliminated by

the FarE efflux pump (2, 36), or detoxified through OhyA-dependent hydroxylation (37). Growth at acidic pH also promotes enhanced resistance of *S. aureus* to antimicrobial uFFA through a mechanism that is dependent on the GraS sensor kinase, and GraS-dependent gene MprF (18). Exogenous uFFA can also be detoxified by incorporation into phospholipid, through the fatty acid kinase FakA dependent pathway, which may reduce the energetic cost of *de novo* phospholipid synthesis during growth *in vivo* (3, 38).

Currently, incorporation of uFFA and saturated FFA into phospholipid is thought to be the only option for *S. aureus* to metabolize host-derived fatty acids, since previous studies revealed it to be incapable of metabolizing fatty acids through β-oxidation (3, 4). However, genome sequence data has revealed that *S. aureus* contains the *fadDEBA* locus comprising the minimal complement of genes that are necessary and sufficient for degradation of fatty acids through the canonical β-oxidation pathway in other bacteria (6). Notably, *fad* genes were highly expressed when *S. aureus* was internalized by cultured human bronchial epithelial cells that provide lipid needed for lung surfactant (8, 9), and low human serum antibody titre to FadB was identified as one of a series of biomarkers that was discriminatory in patients with early *S. aureus* bloodstream infection (39). Therefore, we hypothesized that the propensity of *S. aureus* to colonize sites that are rich in host-derived fatty acids reflect an ability to thrive in this environmental niche not only through the established detoxification mechanisms, but also through expression of *fad* genes. To gain a better understanding of the role *fad* plays in *S. aureus* growth, we conducted experiments using the endemic strain of community acquired methicillin-resistant *S. aureus* (CA-MRSA), USA300, known for its high virulence and rapid community transmission (40, 41). We aimed to elucidate the conditions which activate expression of these genes and assess their impact on growth and virulence of *S. aureus.*

3.3 Material and Methods

3.3.1 Bacterial Strains and Growth Conditions

Bacteria and plasmids used or constructed in this study are listed in Table 3.5. Bacterial strains were maintained as frozen stocks (-80 °C) in 20% glycerol and were streaked on Tryptic soy agar (TSA) when required. Tryptic soy broth (TSB) containing 2.5 g/L

glucose (~13.9 mM) or TSB without glucose were supplied by Bacto. TSB or TSA was supplemented, when needed, with 10 μg/mL erythromycin or chloramphenicol for propagation of strains bearing resistance markers. Where indicated, TSB or TSA was supplemented by addition of 0.1 M morpholineethanesulfonic acid (MES) buffer (Bio Can Scientific) and adjusted to pH 5.5 with HCl, or 0.1 M Bis-tris (Sigma) and adjusted to pH 7.4 with NaOH, prior to autoclaving. To supplement media with saturated fatty acids, a 100 mM stock concentration of lauric, myristic, palmitic and stearic acid, or a 75 mM stock concentration of arachidic and behenic acid, was first prepared in 70% ethanol and then diluted into TSB 0.1% dimethyl sulfoxide (DMSO) to achieve the desired concentration of fatty acids. To supplement media with unsaturated linoleic acid, a 10 mM stock concentration was first prepared in TSB containing 0.1% DMSO and then diluted into TSB to achieve the desired concentration of fatty acids. Myristic acid (tetradecanoic acid; 14:0) was purchased from Sigma, palmitic acid (hexadecanoic acid; 16:0) was purchased from Cayman Chemicals, stearic acid (octadecanoic acid; 18:0) was purchased from Sigma, arachidic acid (icosanoic acid; 20:0) was purchased from Acros Organics, behenic acid (docosanoic acid; 22:0) was purchased from TCI Chemicals, linoleic acid (*cis, cis*-9,12-octadecadienoic acid; 18:2) was purchased from Sigma, and methyl-palmitate was purchased from Sigma. *E. coli* strains were grown on LB agar or LB broth supplemented with 100 μg/mL ampicillin when needed. Unless otherwise stated, all cultures were grown at 37°C, and liquid cultures were incubated on an orbital shaking platform at 220 rpm. For all experiments, a minimum of at least two biological replicates were used to confirm findings.

Table 3.5. Strains and plasmids used in Chapter 3.

Strain or Plasmid:	Description:	Citation:
S. aureus:		
USA300 LAC	Community associated MRSA; wild type strain cured of resistance plasmids	(42)
RN4220	r_K^- m_K^+; capable of accepting foreign DNA	(43)
USA300Δ*fakA*	USA300 with markerless *fakA* deletion	(2)
USA300Δ*fad*	USA300 with markerless *fadXEDBA* deletion	This Study
USA300Δ*graS*	USA300 with markerless *graS* deletion	(44)
USA300 *fadX*::Tn	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *fadX*::φNΣ; Erm^r	(45, 46)
USA300 *fadD*::Tn	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *fadD*::φNΣ; Erm^r	(45, 46)
USA300 *fadE*::Tn	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *fadE*::φNΣ; Erm^r	(45, 46)
USA300 *fadB*::Tn	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *fadB*::φNΣ; Erm^r	(45, 46)
USA300 *fadA*::Tn	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *fadA*::φNΣ; Erm^r	(45, 46)
USA300 *prsW*::Tn	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *prsW*::φNΣ; Erm^r	(45, 46)
USA300 *sigS*::Tn	Derivative of *S. aureus* USA300 LAC from the Nebraska transposon library carrying *sigS*::φNΣ; Erm^r	(45, 46)

E. coli:

DH5α	F^- Φ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^-mK^+$) *supE44 relA1 deoR* Δ(*lacZYAargF*)*U169 phoA*	Invitrogen

Plasmids:

pGYLux	*E. coli-S. aureus* shuttle vector harboring promoterless *luxABCDE* operon; Amp^r Cm^r	(47)
pGY*fadX::lux*	pGYLux with the promoter of *fadX* cloned upstream of *luxABCDE*	This Study
pKOR-1	*E. coli/S. aureus* shuttle vector for creation of unmarked gene deletions in *staphylococcal spp*.; Amp^r Cmr	(48)
pKORΔ*fad*	pKOR with regions of homology to delete Promoterless bioluminescent reporter plasmid encoding *luxABCDE;* Amp^r Cm^r	This Study
pLI50	*E. coli-S. aureus* shuttle vector; Amp^r Cm^r	(49)
pLI*fadXEDBA*	pLI50 with *fadXEDBA* expressed from native promoters	This Study

3.3.2 Strain and Plasmid Construction

Genetic manipulation of *S. aureus* was conducted according to established guidelines and as described in previous work (2, 18, 42, 43). All recombinant plasmids were initially constructed in *E. coli* DH5α. The integrity of plasmids was confirmed through nucleotide sequencing prior to electroporation into USA300 or isogenic derivatives, using *S. aureus* RN4220 as an intermediate host. Primer sequences used for PCR amplification of gene segments for plasmid construction or site-directed mutagenesis of cloned genes are defined in Table 3.6 and are based on the reference genome sequence of USA300 FPR3757 (50). Integrity of all the vector constructs were confirmed through DNA sequencing.

USA300Δ*fad* containing a markerless deletion of *fadXDEBA* (SAUSA300_0229, _0228, _0227, _0226, _0225) was generated using pKOR-1 as previously described (48). Briefly, ~ 1kb amplicons that flank the *fadXDEBA* locus were constructed through PCR, using fad-del-UP-*attB1* and fad-del-UP-SacII for the upstream segment, and fad-del-DW-*SacII* and fad-del-DW-*attB2* for the downstream segment. The PCR products were digested with *SacII*, ligated together with T4 DNA ligase, and incorporated in pKOR-1 through use of BP Clonase II (Invitrogen). The resulting pKORΔ*fad* was transformed into USA300 and then subjected to a two-step temperature shift and antisense counterselection, as previously described (48), generating USA300Δ*fad.*

The plasmid pGY*lux* was used to measure expression of the *fadX* gene (47), and pLI50 was used for complementation of genes under control of their natural promoters (49). Primers *fadX*-pro-UP and *fadX*-pro-DW were used to amplify the prompter region of *fadX*, amplified from genomic USA300 LAC DNA. The resulting amplicon, and the pGYlux plasmid, were digested with *BamHI*-HF and *SalI*-HF, and ligated together with T4 DNA ligase, to construct pGY*fadX::lux*. pLI*fadXDEBA* was constructed using *fadXDEBA*-comp-UP and *fadXDEBA*-comp-DW primers. The resulting amplicon and pLI50 were digested with *SacII*-HF and ligated with T4 DNA ligase.

Table 3.6. Oligonucleotides used in Chapter 3.

Oligonucleotide:	**Description:**
fad-del-UP-*attB1*	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGTTAAA CCTCAAGCAACTG
fad-del-UP-*SacII*	GGACCTCCGCGGTATGTGCGTTAGAATGGTTG
fad-del-DW-*SacII*	GGACCTCCGCGGAGTCACCTCCATCACATTT
fad-del-DW-*attB2*	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCCAAT AAAGAACATGATA
fad-del-UP	AGAGGTTAAAACTACGGCTG
fad-del-DW	AATGCCATGTAAAACAACGG
fadX-pro-UP	TTTGGATCCCTGCTTCGTTTTTAGTATGCGG
fadX-pro-DW	TTTGTCGACGCGGGTAAGTTGGCTACAGC
fadXEDBA-comp-UP	TTTGAGCTCAACGGAAATAACCACCATCC
fadXEDBA-comp-DW	TTTGAGCTCGTTTCATGCGCTTTAGCTTC

* Underlining indicates restriction cut sites

3.3.3 Protein Orthology Analysis

A BLASTp analysis was conducted using the protein sequences for FadD, FadE, FadB, and FadA from *Staphylococcus aureus* subsp. *aureus* USA300_FPR3757 and *Escherichia coli* str. K-12 substr. MG1655. For proteins with no obvious ortholog, FadX of *S. aureus* and FadR in *E. coli*, DELTA-BLAST was first conducted to identify potential orthologs.

3.3.4 FadXDEBA Protein Homology in *Staphylococci spp.*

Homology of the different Fad proteins amongst *Staphylococcus spp.* was determined using NCBI tBLASTn. The protein sequences of FadX, FadD, FadE, FadB, FadA of *S. aureus* (NC_007795.1) were aligned to various other *Staphylococcus spp.* with the respective accession numbers outlined. The percent identity of the highest hit was recorded. The threshold for clustering of *fad* genes was set at having at least 3 of the genes occurring in tandem. The phylogenetic tree was derived from Madhaiyan *et. al.* 2020 (51).

3.3.5 Bioluminescence Assay Conditions

Reporter assays for monitoring *fadX::lux* activity were conducted as previously described (44). Briefly, overnight cultures *S. aureus* USA300 or isogenic deletion mutants harbouring the pGY*fadX::lux* plasmid, or the empty pGYlux vector with no promoter inserted, by inoculating 3 ml of TSB in a 13-ml polypropylene tube containing TSB with chloramphenicol for 16 hours. After determining the optical density at 600 nm (OD_{600}), aliquots were sub-cultures into 125-ml-capacity flasks containing 25 ml of TSB with or without glucose, to achieve an initial OD_{600} of 0.01. At hourly intervals, OD_{600} was measured using a spectrophotometer, and quadruplicate luminescence readings (relative light units, RLU) from each flask were taken on a Synergy H4 temperature-controlled microplate reader (BioTek Instruments). RLU readings were done with 200 µL of bacterial culture spiked with 20 µL of decanal to maximize luminescence. The corrected RLU was calculated as the mean of these measurements minus the RLU for each strain carrying empty pGYlux cultured under identical conditions. Where specified, bacteria were alternately sub-cultured into 200 µL of medium in 96-well flat-bottom assay plates

(Fisher) to an OD_{600} of 0.01, and both growth and luminescence were monitored at 37°C using a Synergy H4 temperature-controlled microplate reader (BioTek Instruments) with measurement of OD_{600} every 20 min for 18 to 24 h. Under this assay format, decanal was not used to maximize luminescence readings.

3.3.6 Growth and Viability Assays

For growth analyses, cultures of *S. aureus* were prepared by inoculating 3 mL of TSB in a 13-mL polypropylene tube containing antibiotic as required and grown overnight for 16 h. After determining the OD_{600}, aliquots were sub-cultured into 125-mL-capacity flasks containing 25 mL of TSB, or TSB modified by addition fatty acids, to achieve an initial OD_{600} of 0.01. Growth (OD_{600}) was monitored at hourly intervals, or after 24 hours. For viability assays, *S. aureus* cultures were grown as described above, but aliquots were taken at the time points OD_{600} was measured, and serially diluted from 10^{-2} to 10^{-8}. After serial dilution, quadruplicate 10 µL aliquots were plated on TSA, and bacteria were enumerated after growth for ~16 h at 37°C. All cultures were grown in triplicate unless otherwise stated.

3.3.7 SDS-PAGE and Zymography

For SDS-PAGE analysis of secreted protein profiles, *S. aureus* cultures were grown as outlined for growth and viability assays in TSB without glucose buffered at a pH of 7.3 (0.1M Bis-tris adjusted to pH 7.3 with NaOH) or 5.5 (0.1 M MES adjusted to pH 5.5 with HCl), with and without exogenous palmitic acid, for 24 h. Proteins in cell-free culture supernatant were precipitated by mixing with equal volumes of ice-cold 20% trichloroacetic acid (TCA), washed in ice-cold 70% ethanol, and then air dried prior to dissolving in SDS-PAGE reducing buffer as described previously (18, 52). Protein equivalent to 2.5 OD_{600} units of culture supernatant was then loaded for protein separation on a 10% acrylamide gel using the Laemmli buffer system (53), and after electrophoresis, proteins were stained using Coomassie blue. For detection of protease activity through zymography, the resolving gel was copolymerized with 1 mg/mL casein and protein equivalent to 0.075 OD_{600} units was applied to each lane. Details on sample

processing, electrophoresis, and zymogram development are as described previously (18, 54).

3.3.8 Analysis of Phospholipid Composition

Bacterial lipids were extracted following the Bligh and Dyer method as previously described (55, 56). Briefly, triplicate cultures of bacteria were grown for 4 h, with a bolus of palmitic acid added at 2 h to achieve a concentration of 500 μM. A control with no palmitic acid added was also prepared. Approximately 1.5×10^8 CFUs of culture was pelleted, and washed with 10 mL of PBS three times. The washed samples were resuspended in 1.0 ml of ddH_2O and homogenized with three cycles of bead beating with a FastPrep Speed of 6.0 for 30 seconds, allowing samples to sit on ice for 15 minutes in between cycles. Four ml of chilled 1:2 chloroform/methanol (v/v) was added to samples, and intermittently vortexed for 5 minutes. An additional 1.0 mL of water and 1.0 mL of chloroform were added to induce phase separation, and samples were vortexed for 20 seconds. The samples were then centrifuged at 2000 rpm for 10 minutes at 4°C, and the lower layer containing the lipids was collected and dried using nitrogen gas at 30°C. Lipid samples were analyzed at the Wayne State Lipidomics Core facility using Liquid chromatography–mass spectrometry. PG 15:0/18:1-d5 (Avanti:791640; 15:0-18:1-d7-PG, 1-pentadecanoyl-2-oleoyl(d7)-sn-glycero-3-[phospho-rac-(1'-glycerol)]) was used as the internal standard during analysis.

3.3.9 Murine Abscess Infection Model

Inbred female and male C57BL/6J mice aged between 8–12 weeks were purchased from the Jackson Laboratory (USA). Overnight cultures of *S. aureus* were inoculated 1:50 into fresh TSB broth and cultured for 4 h (to OD_{600} ~ 4) with shaking at 37°C. Staphylococci were harvested by centrifugation, washed, and suspended in sterile HBSS to obtain desired inoculum. Prior to inoculation the animals were anesthetized with isoflurane and 50 μL of dose 5×10^7 (high dose) or 1×10^7 CFU (low dose) was injected intradermally into each lower flank, in a shaved area of skin on the back of each mouse. Infected animals were monitored for health status, weight, and lesion development over a period of 3 days. The area of each skin lesion was measured each day. Animals were sacrificed

at day 3 and bacterial tissue load was determined from each separate excised skin lesion by homogenizing the tissue and enumerating the bacteria by serial dilution and plating onto mannitol salt agar (MSA).

3.3.10 Ethics Statement

All mouse experiments were conducted in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. The animal use protocol number 2020–061 was approved by the Animal Use Subcommittee at the University of Western Ontario (London, ON, Canada).

3.3.11 Data Analysis

GraphPad Prism (version 9.4.1) was used to create all graphs and perform statistical analyses in this study. In all experiments, unless otherwise stated, triplicate cultures were used and data was reported on graphs as means ± standard errors (SEs). Unpaired one-tailed t tests, one-way analysis of variance (ANOVA) with multiple comparisons, or two-way ANOVA with multiple comparisons was used to test statistical significance depending on the nature of the experiment. Significance was defined as stated in the figure legends.

3.4 Results

3.4.1 Genes required for degradative β-oxidation of fatty acids in *E. coli* are conserved in *S. aureus*

The canonical pathway for metabolism of long chain fatty acids through β-oxidation, as first elaborated in *E. coli*, is conserved in a variety of both Gram-positive and Gram-negative bacteria. In most bacteria, including the well-established *E. coli* system (6), *fad* genes are dispersed throughout the genome in a variety of transcriptional units that are coordinately regulated by a GntR family regulator, FadR (Figure 3.6A). Degradation of fatty acids is initiated by FadD, forming a long chain acyl-CoA, that is then converted to trans-enoyl acyl-CoA, hydroxy acyl-CoA, and β-keto acyl-CoA by the consecutive actions of FadE and FadB (Figure 3.6B). In the final step catalyzed by FadA, acetyl-CoA

is released, and the remaining carbon chain is combined with CoA-SH to regenerate a shortened fatty acid that is subjected to additional cycles of β-oxidation (6).

Orthologous genes in *S. aureus* occur in a single locus *fadXDEBA* (Fig. 3.6A). These gene products are matched to orthologous proteins in the β-oxidation pathway of *E. coli* in Figure 3.6B and Table 3.3. The first gene *fadX* is annotated as a putative short chain acyl-CoA transferase, orthologous to *ydiF* in *E. coli* (Figure 3.6A, Table 3.3). While not part of the canonical β-oxidation pathway which shows preferential activity towards long-chain fatty acids, YdiF is proposed to play a role in metabolism of short-chain (< 4 carbon) fatty acids (13). Together, these findings suggest *S. aureus* has the capacity to metabolize long-chain fatty acids through the canonical FadDEBA pathway. This synteny is generally conserved across the *Staphylococcus* genus, although the genes are absent in some well-studied staphylococci such as *S. lugdenensis* and *S. epidermidis* (Figure 3.7).

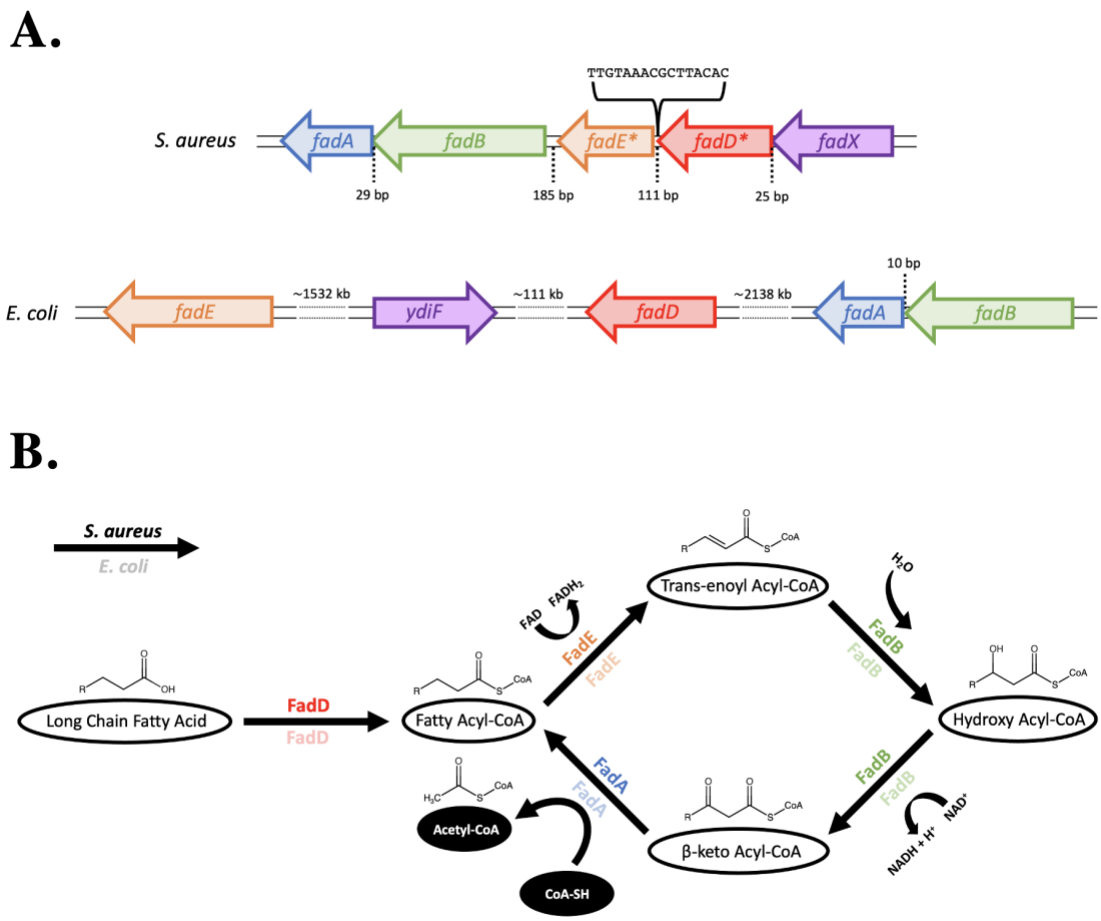
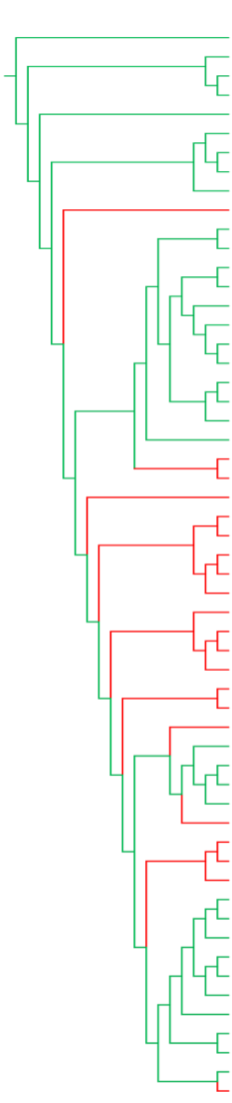


Figure 3.6. Genetic layout and putative function of β-oxidation genes, *fadXDEBA*, in *S. aureus* USA300. Colouring represents homology between genes as determined in Table S1. **(A)** Genetic layout for the β-oxidation genes in *S. aureus* and *E. coli.* Nucleotide sequences indicate a putative *cre*-box *in S. aureus* for glucose repression, and base pair (bp) markers refer to the distance between adjacent genes. * indicates mis-annotation in the genome of *S. aureus*, where *fadE* and *fadD* have been switched. Gene names used in this diagram and paper refer to homology to *E. coli*, rather than the annotation in the *S. aureus* genome. **(B)** Putative β-oxidation pathway in *S. aureus* based on homology to *E. coli*. Bioinformatic analysis indicates *fadX* is orthologous to the short chain acyl-CoA transferase *ydiF* in *E. coli*, which is not part of the canonical β-oxidation pathway.



Species	Accession Number	FadX	FadD	FadE	FadB	FadA	Contains Fad Locus
S. stepanovicii	NZ_LT906462.1	57.09	61.92	79.70	68.26	76.40	Yes
S. lentus	NZ_CP059679.1	58.69	61.72	80.45	70.03	77.47	Yes
S. vitulinus	NZ_CP068061.1	58.69	60.92	79.65	69.76	78.23	Yes
S. fleurettii	GCF_017583105.1	59.92	61.52	78.20	70.16	77.72	Yes
S. sciuri	NZ_CP022046.2	59.07	61.12	79.16	69.50	78.73	Yes
S. aureus	NC_007795.1	100	100	100	100	100	Yes
S. schweitzeri	NZ_LR134304.1	92.00	96.21	98.01	89.77	87.06	Yes
S. argenteus	NC_016941.1	87.05	93.41	97.77	89.11	86.29	Yes
S. simiae	NZ_LT906460.1	72.50	84.57	92.06	80.61	83.76	Yes
S. devriesei	GCF_900458355.1	-	26.61	-	-	33.92	No
S. cohnii	GCF_020144955.1	58.57	66.13	77.69	67.99	77.16	Yes
S. nepalensis	NZ_CP017466.1	57.88	64.13	77.69	68.30	75.38	Yes
S. gallinarum	NZ_CP086207.1	57.09	62.50	76.94	68.83	77.66	Yes
S. arlettae	NZ_AP019698.1	55.41	63.93	77.92	70.42	78.43	Yes
S. xylosus	NZ_CP008724.1	59.04	64.73	76.44	68.57	76.73	Yes
S. pseudoxylosus	NZ_CP075500.1	58.46	64.53	76.44	68.04	76.40	Yes
S. saprophyticus	NZ_CP031196.1	59.62	63.13	77.69	68.04	76.40	Yes
S. edaphicus	GCF_002614725.1	59.04	64.11	76.94	68.83	76.14	Yes
S. succinus	NZ_CP018199.1	59.87	65.32	78.45	68.17	75.63	Yes
S. casei	GCF_002902445.1	58.00	64.92	78.45	67.37	76.14	Yes
S. equorum	NZ_CP066013.1	58.54	62.53	78.20	67.90	76.40	Yes
S. kloosii	NZ_CP027846.1	54.53	60.72	77.44	68.39	79.19	Yes
S. pasteuri	GCF_018407725.1	-	27.99	31.58	-	40.73	No
S. warneri	NZ_CP032159.1	-	27.24	-	23.08	40.21	No
S. lugdunensis	NZ_CP014022.1	-	26.77	23.08	-	33.92	No
S. hominis	NZ_CP033732.1	-	27.53	-	-	35.43	No
S. haemolyticus	NZ_CP013911.1	-	27.42	-	-	35.93	No
S. croceilyticus	GCF_004684875.1	-	26.75	-	24.37	40.84	No
S. pragensis	GCF_004785665.1	-	26.54	-	24.37	40.84	No
S. petrasii	GCF_900458665.1	-	27.94	-	29.65	42.82	No
S. epidermidis	NZ_CP035288.1	-	26.75	-	23.50	41.67	No
S. caprae	NZ_AP018587.1	-	26.73	-	-	39.11	No
S. capitis	NZ_CP086659.1	23.37	26.88	-	24.65	40.56	No
S. saccharolyticus	NZ_CP068029.1	-	26.60	-	26.73	40.36	No
S. argensis	GCF_007682245.1	22.35	27.89	-	23.86	40.05	No
S. pettenkoferi	NZ_CP022096.2	-	25.60	-	-	36.11	No
S. auricularis	NZ_CP065712.1	-	24.69	-	24.75	34.69	No
S. simulans	GCF_003579335.1	-	68.89	80.45	73.71	78.43	Yes
S. carnosus	NZ_CP069582.1	65.71	70.86	80.45	75.43	78.68	Yes
S. condimenti	NZ_CP018776.1	66.20	71.46	80.70	76.10	79.95	Yes
S. piscifermentans	NZ_LT906447.1	65.25	72.65	78.45	75.17	79.95	Yes
S. massiliensis	GCF_000298075.1	-	25.20	-	-	36.80	No
S. rostri	PPRF00000000	-	-	26.47	25.17	-	No
S. microti	GCF_900458705.1	-	25.67	25.79	-	37.41	No
S. muscae	NZ_LT906464.1	-	25.40	30.69	-	36.50	No
S. intermedius	GCF_002374195.1	63.20	79.23	83.13	74.10	80.71	Yes
S. pseudintermedius	NZ_CP065921.1	63.67	80.76	83.13	70.78	79.90	Yes
S. delphini	GCF_002369695.1	62.62	80.04	82.38	70.12	78.88	Yes
S. hyicus	NZ_CP008747.1	64.09	80.44	79.95	73.57	78.68	Yes
S. agnetis	NZ_CP045927.1	65.44	80.24	80.95	73.71	80.15	Yes
S. lutrae	NZ_CP020773.1	63.67	80.56	81.64	71.71	78.17	Yes
S. schleiferi subsp. schleiferi	GCF_011137135.1	63.15	77.96	80.65	69.99	77.16	Yes
S. schleiferi subsp. coagulans	GCF_018616895.1	63.34	78.16	80.89	69.85	77.41	Yes
S. cornubiensis	GCF_900183575.1	63.39	80.04	83.62	73.71	80.96	Yes
S. chromogenes	NZ_CP031274.1	64.88	79.64	81.70	72.24	78.63	Yes
S. felis	NZ_CP027770.1	22.78	26.87	26.53	-	38.50	No

Figure 3.7. Conservation of FadXDEBA proteins across Staphylococcal species. A tBLASTn analysis using the protein sequences from *S. aureus* was conducted, and the percent identity to other *Staphylococcal* species was reported. Phylogenetic relationship was adapted from Madhaiyan *et. al.* 2020 (51), based on 16S rRNA. The threshold to determine if each specie contains the *fad* locus required all base genes (*fadD, fadE, fadB,* and *fadA*) to be present, and these genes to be clustered together in the genome.

3.4.2 Expression of *fadX* is repressed by glucose and induced in response to palmitic acid and cellular metabolic status

To initiate our studies, we constructed a transcriptional *fadX::lux* reporter, and assessed expression in USA300. When grown in flasks with standard TSB containing 2.5 g/L glucose (13.9 mM), *fadX::lux* activity was minimal over a 24h period (Figure 3.8A), while in the absence of glucose, expression peaked in the transition between exponential and stationary phase, followed by a more intense peak in stationary phase. Hereinafter, the designation of TSB refers to glucose free medium, and assays were conducted in microtitre plates to expedite expression assays unless otherwise specified. Activity from the *fadX::lux* reporter exhibited a dose dependent increase in response to exogenous palmitic acid (Figure 3.8B), whereas 250 µM of longer chain length fatty acids, stearic (C18), arachidic (C20) and behenic acid (C22), all failed to induce *fadX* (Figure 3.8C).

To test for the influence of cellular metabolic status, we assessed *fadX::lux* activity in USA300Δ*fakA*. Our rationale was two-fold. First, FakA phosphorylates exogenous fatty acids as the first step in their incorporation into phospholipid, and we reasoned that elimination of FakA would shift metabolism towards the *fad* pathway. Inactivation of *fakA* also promotes accumulation of cytoplasmic FFA (57), which could stimulate *fadX* expression. Indeed, *fadX::lux* activity in USA300Δ*fakA* was enhanced > 4-fold relative to USA300 during growth in TSB, and expression in response to 250 µM palmitic acid was also more than 2-fold enhanced relative to USA300 (Figure 3.8C). The selectivity of induction was also altered, such that expression became responsive to stearic acid, and to a lesser extent arachidic acid, while behenic acid again had no effect. Cumulatively, these data reveal that *fadX* expression is repressed by glucose and selectively induced in response to palmitic acid, and that *fadX* is expressed at a higher level when *S. aureus* is not able to metabolize exogenous fatty acids through incorporation into phospholipid.

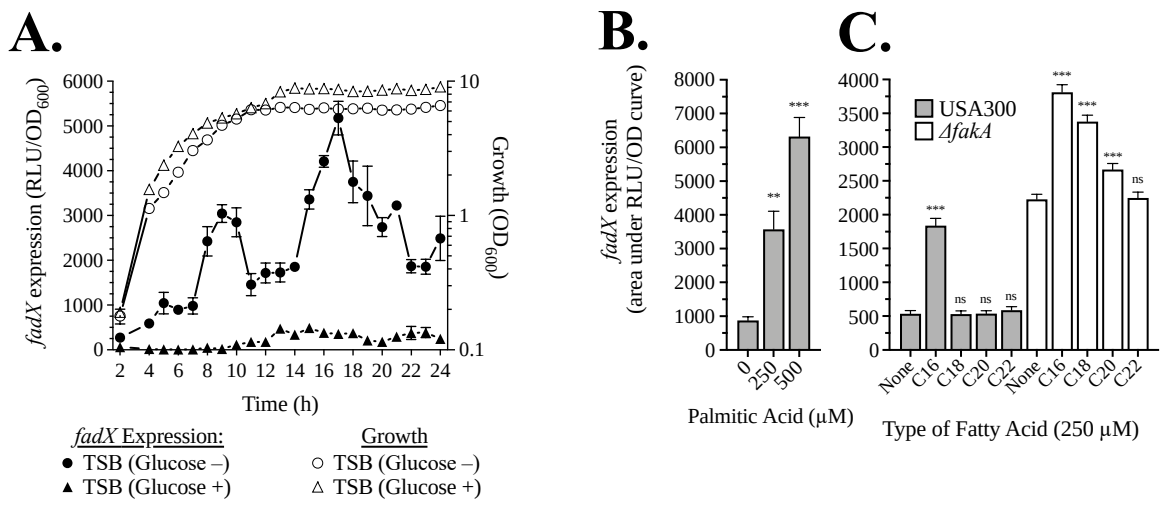


Figure 3.8. Influence of glucose, exogenous fatty acid, and genetic background on expression of *fadX*. Expression of *fadX* was measured as relative luminesce units (RLU) from a pGY*fadX::lux* construct in either USA300 **(A and B)** or USA300 and USA300*ΔfakA* **(C)**. In panel A, cultures were grown in 25 mL of TSB or TSB + glucose in a 125 mL flask, while in B and C, cultures were grown in 200 µl of TSB in 96 well microtitre plates. **(A)** Influence of glucose on temporal expression of *fadX* in TSB. Cultures were inoculated to an initial optical density measured at 600 nm (OD_{600}) of 0.01. Growth (OD_{600}) and *fadX* expression (RLU/OD_{600}) were assessed at hourly intervals, and each data point represents the mean ± SE of triplicate flasks. **(B)** Influence of exogenous palmitic acid on expression of *fadX*. Cultures were inoculated at an OD_{600} of 0.01 and grown for 24 hours in 200 µL of media, n=7. Growth (OD_{600}) and *fadX* expression (RLU) were assessed every 20 minutes, and data was reported as the area under the RLU/OD_{600} curve. Data are reported as mean ± SEM. **(C)** Influence of fatty acid chain length on expression of *fadX* in USA300 and USA300*ΔfakA*. Cultures were grown as in B. Statistical significance was measured using ordinary one-way **(B)** or two-way **(C)** ANOVA with Dunnett's multiple comparisons test, comparing all samples to the 0 µM palmitic acid or no fatty acid conditions ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

3.4.3 Exogenous palmitic acid promotes increased cell density (OD_{600}) and protease expression in *S. aureus*

Having shown that *fadX* is induced in response to exogenous palmitic acid, we next assessed whether this could benefit growth and production of secreted virulence factors. When grown in flasks, concentrations of palmitic acid > 50 µM all promoted increased cell density (OD_{600}) of *S. aureus* USA300 (Figure 3.9A). Previously, we showed that growth in standard TSB containing glucose buffered at pH 5.5, a pH physiologically relevant to human skin, promoted production of secreted proteases (18). We therefore evaluated the profile of secreted proteins produced during growth in glucose free TSB, buffered at pH 7.4 or pH 5.5, to determine if exogenous palmitic acid could stimulate production of secreted protease. Although supplementation with 500 µM palmitic acid did not significantly alter the profile of secreted proteins in TSB pH 7.4, proteolytic activity corresponding to the SspA serine protease was uniquely detected in medium supplemented with palmitic acid (Figure 3.9B). This effect was more pronounced in TSB pH 5.5, where medium supplemented with palmitic acid exhibited more robust production of SspA compared to TSB alone (Figure 3.9B). To determine if the benefit to growth and protease production was *fad* dependent, we constructed a markerless Δ*fadXDEBA* deletion, USA300Δ*fad*. However, as detailed in the next section, exogenous palmitic acid was toxic to USA300Δ*fad,* which did not allow for a meaningful comparison.

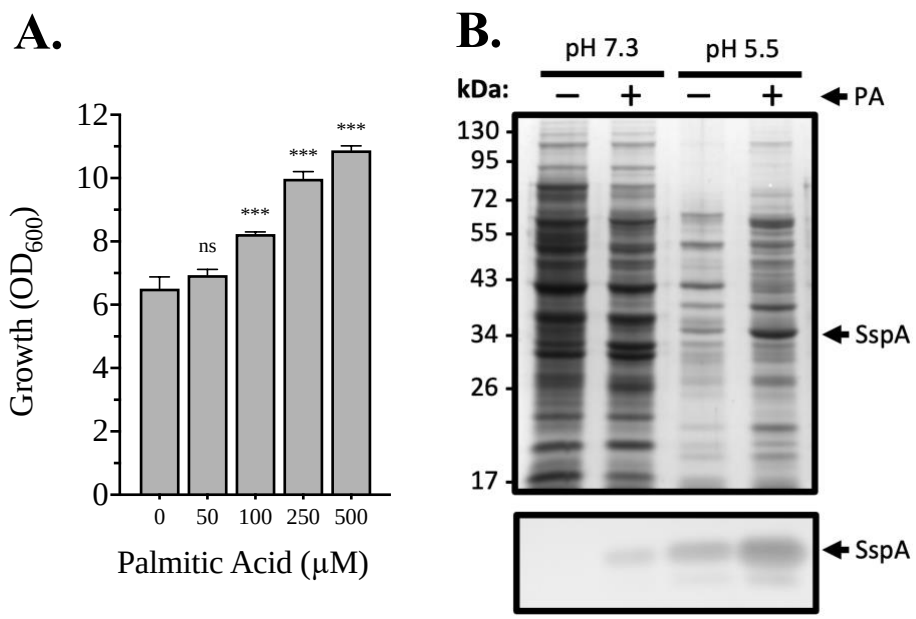


Figure 3.9. Palmitic acid improves growth and protease expression of USA300. (A) Triplicate flasks of 25mL TSB without glucose were supplemented with palmitic acid at the indicated concentration. Cultures were inoculated with bacteria, and growth (OD_{600}) was measured after 24 hours. Data is reported as mean ± SEM. Statistical significance was measured using ordinary one-way ANOVA with Dunnett's multiple comparisons test, comparing all samples to the 0 μM palmitic acid condition. *** $p < 0.001$, ns = not significant. **(B)** SDS-PAGE profile of secreted proteins (top panel) and zymogram for detection of SspA serine protease (bottom panel) in cultures supernatants of USA300 after growth for 24 hours in TSB without glucose, buffered at pH 7.3 or pH 5.5, with or without 500 μM of palmitic acid (PA). Each sample was normalized using OD_{600}, with each lane containing 2.5 or 0.025 OD_{600} units of supernatant for the TCA precipitation and zymography respectively.

3.4.4 Exogenous palmitic acid is toxic to USA300Δ*fad*

When growth of USA300 and USA300Δ*fad* was compared in TSB with 100-, 250- or 500 µM palmitic acid, USA300Δ*fad* exhibited a progressive impairment of growth, whereas USA300 was not affected (Figure 3.10A). When cell viability was monitored, USA300 and USA300Δ*fad* both exhibited a similar and slower increase in viability over the first 2 h in TSB + 500 µM PA compared to TSB alone, after which USA300 exhibited an exponential increase in viability equivalent to growth in TSB alone, whereas USA300Δ*fad* exhibited a > 10-fold loss of viability over the same time frame (Figure 3.10B). Consequently, exogenous palmitic acid is toxic to USA300Δ*fad*. Since our expression data indicated that induction of *fadX* was specific to palmitic acid, we next assessed the ability of longer and shorter chain fatty acids to cause loss of viability during growth of USA300Δ*fad*. Reflecting our expression data, palmitic acid caused the greatest impairment at approximately 10-fold (Figure 3.10C), while C18 stearic acid caused an approximate 2-fold impairment, and C20 arachidic acid caused no impairment. Myristic acid (C14) also caused a significant loss of viability (Figure 3.10C). Cumulatively, these data establish a critical role for *fad* genes in metabolizing palmitic acid.

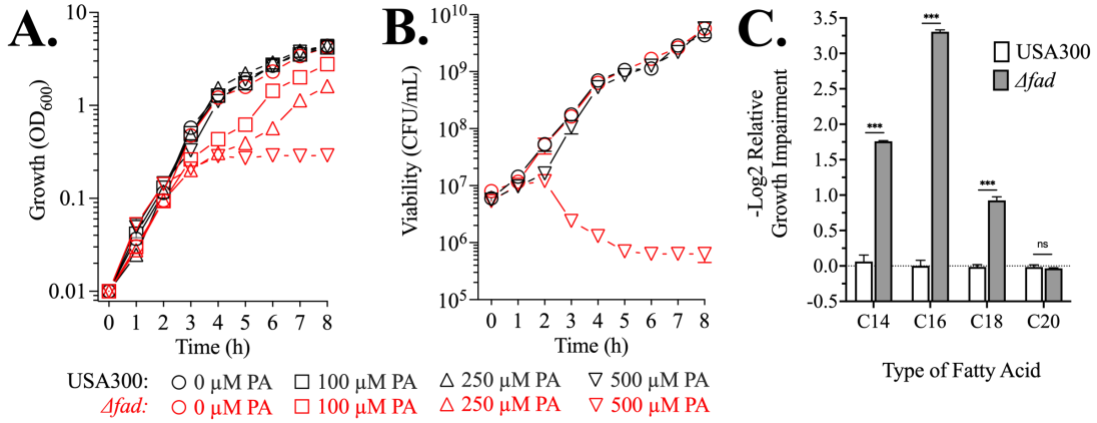


Figure 3.10. Exogenous fatty acids with different chain lengths exhibit differential toxicity towards USA300Δ*fad*. Triplicate flasks of TSB without glucose were supplemented with palmitic acid at the indicated concentration **(A and B)**, myristic acid (C14) at a concentration of 250 µM, or palmitic (C16), stearic (C18), or arachidic acid (C20) at a concentration of 500 µM **(C)**. **(A and B)** Growth (OD_{600}) or viability (CFU/mL) were assessed at hourly intervals, and each data point represents the mean ± SE from triplicate flasks. Viability was measured by plating serial dilutions in triplicate technical replicates. **(C)** Growth (OD_{600}) was measured at hourly intervals for 8 hours, and area under the OD_{600} curve (AUC-OD) was determined. AUC-OD of cultures grown with exogenous fatty acids was compared to the AUC-OD of cultures grown in TSB alone, and data was graphed as the -Log2 ratio of (AUC-OD fatty acid cultures)/(AUC-OD TSB alone cultures). Each data point represents the mean ± SE from triplicate flasks. Statistical significance was measured using an unpaired one-tailed t-test, *** $p < 0.001$, ns = not significant.

3.4.5 USA300Δ*fad* exhibits an altered ability to metabolize palmitic acid through incorporation into phospholipid

Since growth of USA300Δ*fad* was abruptly halted upon challenge with exogenous palmitic acid, we conducted experiments to determine if this could be correlated with altered membrane phospholipid composition. For this purpose, USA300 and USA300Δ*fad* were grown in TSB for 2 hours, and then a bolus of 500 µM palmitic acid was added, followed by monitoring of growth and viability, and collection of cells after 2h for analysis of phospholipid composition. As with our previous findings, addition of palmitic acid halted USA300Δ*fad* growth, accompanied by loss of viability (Figure 3.11).

Phosphatidylglycerol (PG) in *S. aureus* has only saturated fatty acids, where the *sn*-2 position is exclusively C15, while the fatty acid at *sn*-1 ranges between C15 and C20 (3, 4). In TSB alone, USA300 and USA300Δ*fad* exhibited similar PG composition, and the major component was PG32:0 as previously reported (3, 58), which is comprised of C15 at *sn*-2 and C17 at *sn*-1. This together with PG34:0, PG35:0 and PG33:0 which have C19, C20 and C18 fatty acids at *sn*-1 respectively, accounted for > 80% of the total PG (Figure 3.12). When USA300 was grown in TSB + palmitic acid, the PG composition was significantly altered, as evident from the large reduction in PG32:0 and PG34:0, accompanied by a dramatic increase in PG33:0 and PG35:0 (Figure 3.12). This was consistent with previous work documenting a peculiarity in *S. aureus* metabolism of exogenous palmitic acid, whereby the PlsY acyltransferase responsible for incorporation of acyl-phosphate fatty acids into PG does not function effectively with C16, which necessitates its transfer to an acyl carrier protein (ACP). The ACP-C16 then enters the fatty acid synthase (FASII) cycle where it is extended to C18 and C20, which are then incorporated into PG to account for the increase in PG33:0 and PG35:0 (1).

Although the differences were not dramatic, PG recovered from USA300Δ*fad* after exposure to palmitic acid had significantly less PG35:0, a trend for reduced PG33:0, and a significant increase in PG31:0 relative to USA300 (Figure 3.12). This latter product was the least abundant PG species during growth in TSB alone, and its increase upon exposure to palmitic acid reflects direct incorporation of C16 into PG. Consequently, in

the absence of *fad* gene function, the ability of *S. aureus* to metabolize exogenous palmitic acid through incorporation into PG is altered.

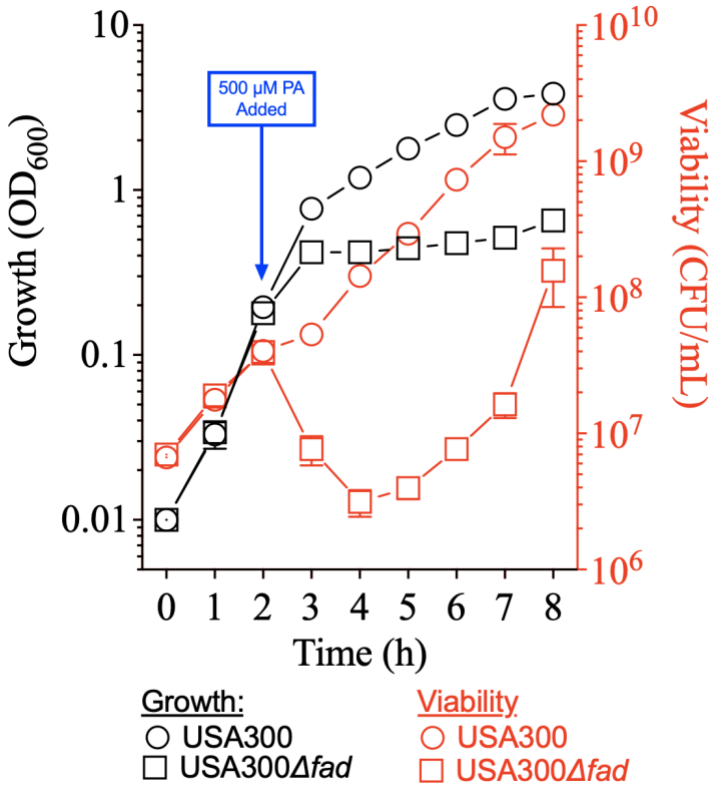


Figure 3.11. Growth and viability of USA300 and USA300Δ*fad* in cultures prepared for membrane lipid analysis. Triplicate flasks of 25 mL TSB without glucose were inoculated to an initial optical density measured at 600 nm (OD_{600}) of 0.01. After 2 hours of growth, a bolus of 500 µM palmitic acid was added to cultures. **(A)** Growth (OD_{600}) and **(B)** viability (CFU/mL) were assessed at hourly intervals, and each data point represents the mean ± SE. Viability was measured by plating serial dilutions in triplicate technical replicates.

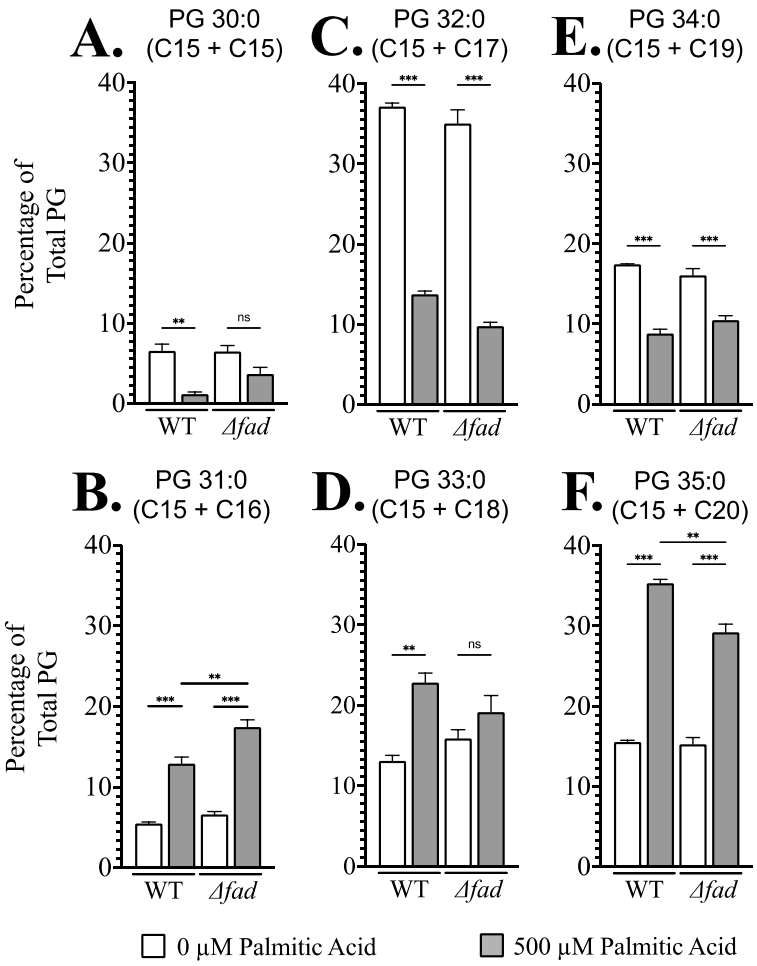


Figure 3.12. Exogenous palmitic acid is differentially incorporated into the phospholipid membrane of USA300 and USA300Δ*fad*. USA300 and USA300Δ*fad* were grown in triplicate cultures to an OD_{600} of 0.15 and a bolus of 500 µM palmitic acid was added as outlined in Figure 3.11. Bacteria were then grown for another 2 hours before samples were collected. Controls with no palmitic acid bolus were also conducted for both strains. Samples were homogenized, and lipids were extracted and analyzed through Liquid chromatography–mass spectrometry at the Wayne State Lipidomics Core Facility. Results are presented as mean ± SEM of the percentage of total PG each lipid species represents. Statistical significance was measured using Two-Way ANOVA with Tukey's multiple comparisons test, ** $p<0.01$, *** $p<0.001$, *** $p<0.001$.

3.4.6 Confirming the role of *fad* in ablating the toxicity of palmitic acid

To validate the role of *fad* genes in metabolism of palmitic acid, the entire *fadXDEBA* locus with endogenous promoters was cloned in pLI50 to complement USA300Δ*fad*. However, at 500 µM palmitic acid, growth wildtype USA300 containing pLI50 alone was severely impaired (Figure 3.13A). However, we were able to partially circumvent this using a less toxic derivative of palmitic acid, methyl-palmitate, which could be supplemented at up to 2500 µM with minimal growth impairment to *S. aureus* containing plasmids (Figure 3.13B). Methyl-palmitate also induced expression of *fadX::lux*, but required 10-fold higher concentrations relative to palmitic acid to obtain similar levels of induction (Figure 3.14A). Since methyl-palmitate is less toxic than palmitic acid, there was less impairment to USA300Δ*fad* growth; nevertheless, at 2500 µM methyl-palmitate, growth of USA300Δ*fad* was impaired relative to USA300, and this was ameliorated with pLI*fadXDEBA* (Figure 3.14B).

To assess the role of individual *fad* genes relative to the entire *fad* locus, we tested individual mutants from the Nebraska Transposon Library (45, 46). Importantly, inactivating any of the core *fadDEBA* genes also led to severe growth impairment establishing that each gene is essential for metabolism of palmitic acid (Figure 3.6, Figure 3.15). In contrast to the core *fadDEBA* genes, the transposon insertion in *fadX* had a less severe impact, although still causing significant impairment (Figure 3.15). From these data, we conclude that the core genes *fadD*, *fadE*, *fadB* and *fadA* are critical to *S. aureus* ability to metabolize exogenous palmitic acid, with a lesser but still significant contribution from *fadX*.

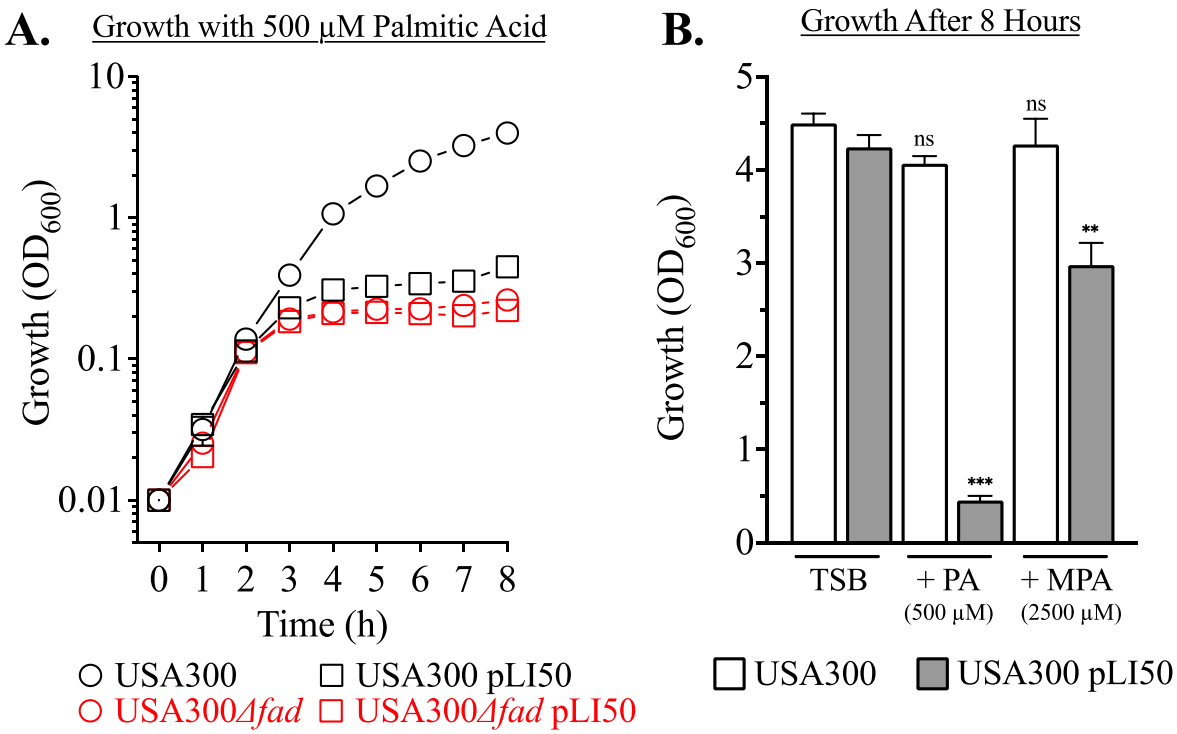


Figure 3.13. Palmitic acid severely impairs growth of *S. aureus* harbouring plasmids. (A) Triplicate flasks of 25 mL TSB without glucose supplemented with 500 µM of palmitic acid were inoculated, and growth (OD_{600}) was assessed at hourly intervals. **(B)** Triplicate flasks of 25 mL TSB without glucose alone, supplemented with 500 µM of palmitic acid (PA), or supplemented with 2500 µM methyl palmitate (MPA), were inoculated and growth (OD_{600}) was assessed after 8 hours. Each data point represents the mean ± SE. Statistical significance was measured using ordinary one-way ANOVA with Dunnett's multiple comparisons test, comparing each strain to the TSB alone condition. ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

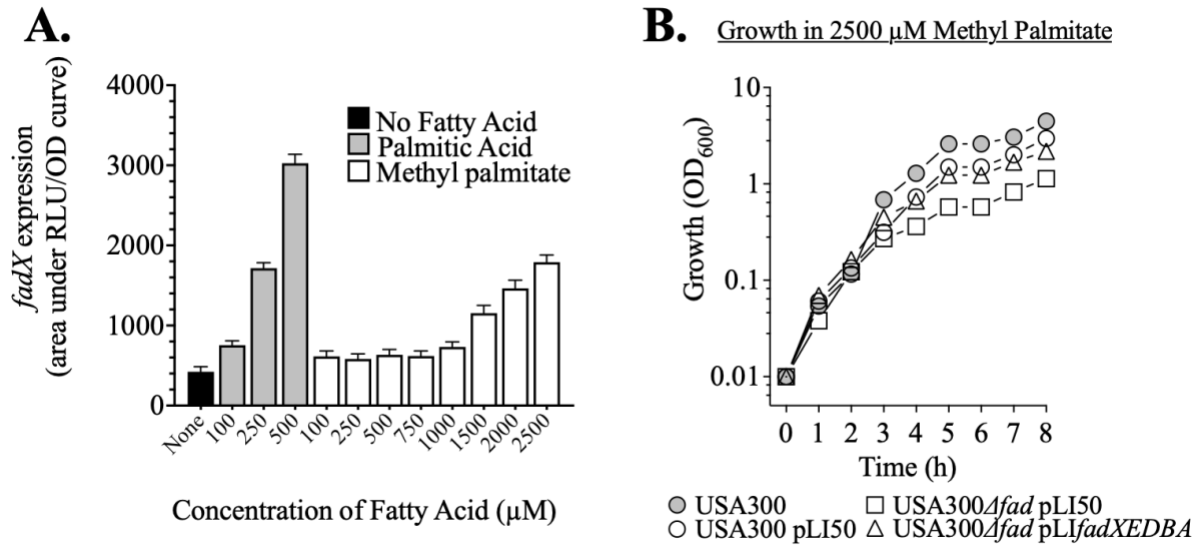


Figure 3.14. Methyl palmitate induces *fadX* expression in a concentration dependent manner and requires *fadXDEBA* for proper growth. (A) Expression of *fadX* was measured as relative luminesce units (RLU) from a pGY*fadX::lux* construct. Cultures were grown for 24 hours in 200 µL of media, n=5, and growth (OD_{600}) and *fadX* expression (RLU) were assessed every 20 minutes. Data was reported as mean ± SEM of the area under the RLU/OD_{600} curve. **(B)** Triplicate flasks of 25mL TSB without glucose were supplemented with 2500 µM methyl palmitate and and growth (OD_{600}) was measured hourly for 8 hours. Data is reported as mean ± SEM.

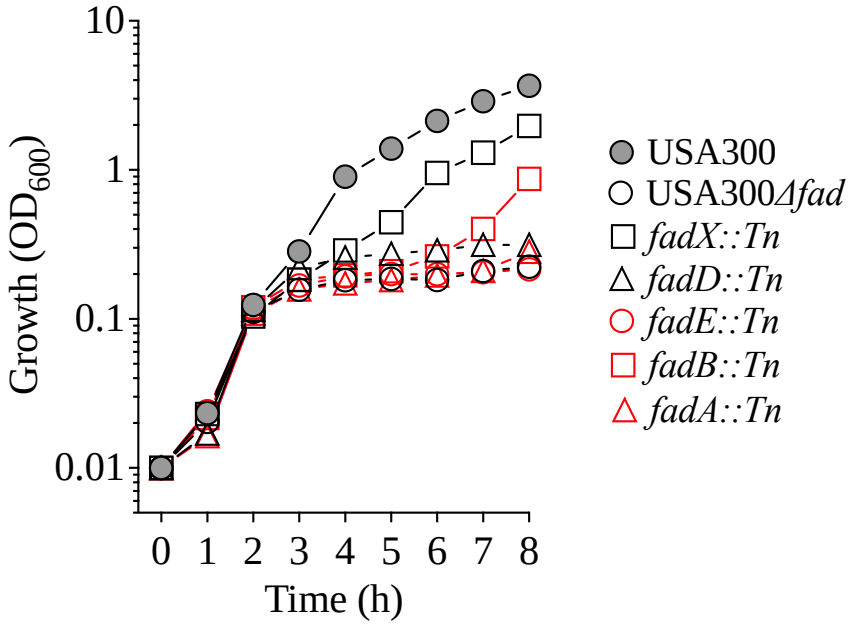


Figure 3.15. Individual knockouts of the *fadXDEBA* genes phenocopy growth impairment in media supplemented with palmitic acid. Triplicate flasks of TSB without glucose were supplemented with 500 µM palmitic acid and inoculated with the stated bacterial strains. Growth (OD_{600}) was assessed at hourly intervals for 8 hours, and each data point represents the mean ± SE from triplicate flasks.

3.4.7 *fad* contributes to *S. aureus* survival *in vivo*

Next, we assessed the *in vivo* requirement for *fad* in a murine subcutaneous abscess model, an environment rich in host-derived lipids. C57BL/6 mice challenged with 5×10^7 CFU of either USA300Δ*fakA* or USA300Δ*fad* exhibited a significantly lower bacterial burden in excised abscess tissue at 72h post-challenge compared to wild-type USA300 (Figure 3.16A). For a comparative measure we also assessed a *graS* deletion mutant which exhibits reduced virulence *in vivo* due to impaired resistance to uFFA (18), cationic antimicrobial peptides (19), and macrophage killing (44). Strikingly, deletion of either *fakA* or *fadXDEBA* caused a reduction in bacterial burden that was comparable to loss of *graS* function (Figure 3.16A). Although there was a reduction in bacterial burden, there were no major differences in mouse weight or abscess size throughout the course of the infection (Figure 3.17AB). When this experiment was repeated with a smaller inoculum of 1×10^7 CFU, we again observed a significant reduction in bacterial burden among each of the mutant strains, but no major difference in mouse weight or abscess size (Figure 3.16B, Figure 3.17CD). These findings confirm the relevance of our *in vitro* characterization and highlight the importance of *fad* genes during infection.

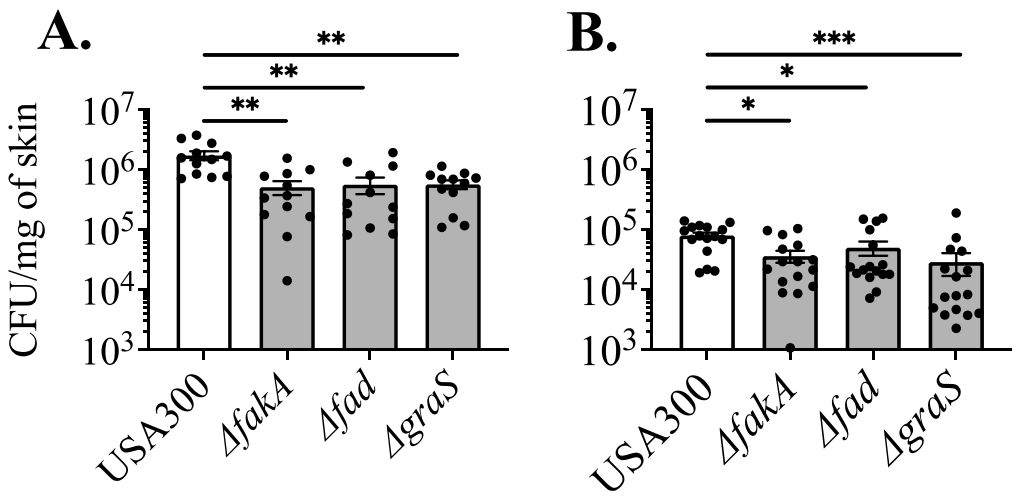


Figure 3.16. Deletion of genes involved in host derived fatty acids resistance and metabolism reduce virulence in a murine infection model. C57BL/6 mice had both hind thighs inoculated with a high dose of $5x10^7$ CFU **(A)** or a low dose of $1x10^7$ CFU **(B)** of the respective bacterial strains in 50 µl Hanks' Balanced Salt Solution. After 72 hours, mice were sacrificed and CFU/lesion was determined, and normalized as CFU/mg of skin. Data is reported as mean ± SEM, n=6 with CFU data derived from abscesses on both the left and right flank of each animal. Statistical significance was determined using Kruskal-Wallis ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

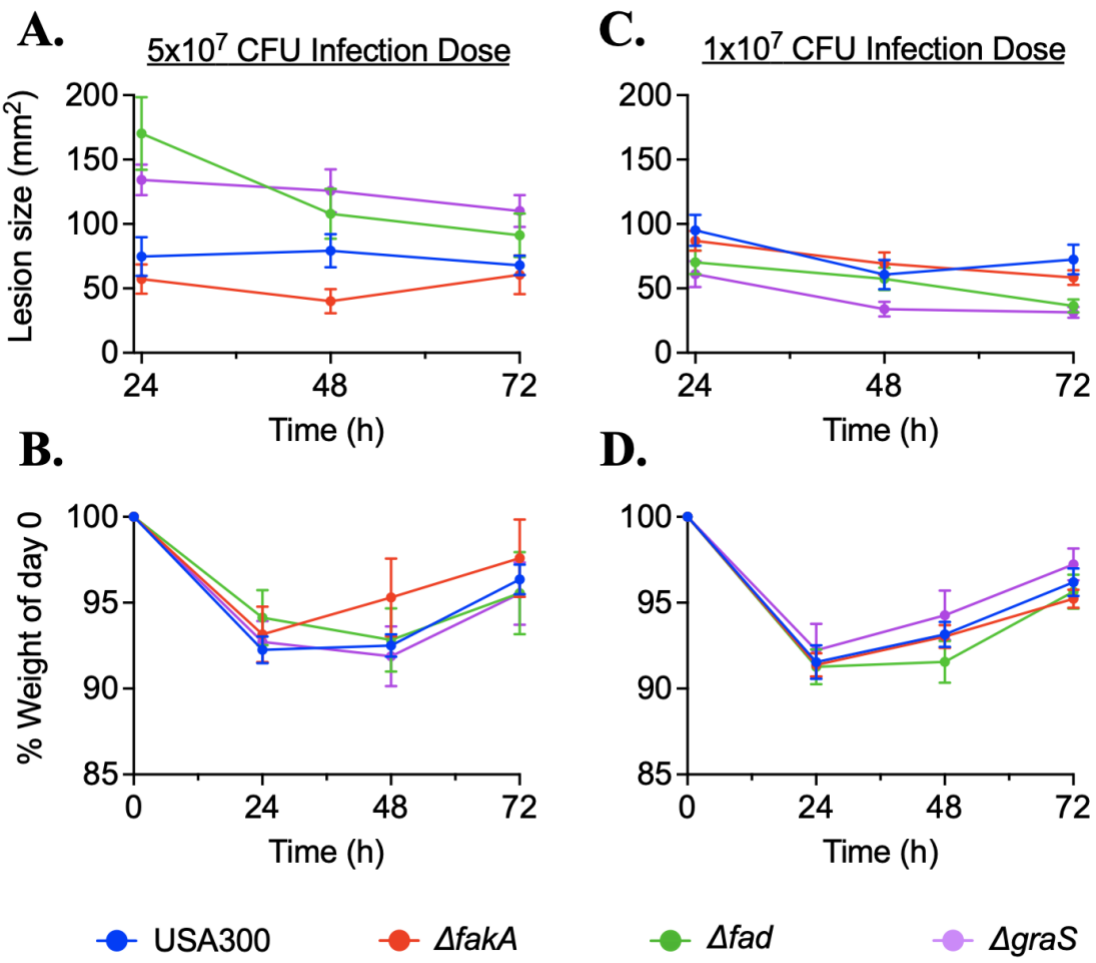


Figure 3.17. Deletion of *fakA*, *fadXDEBA*, or *graS* did not impact lesion size of weight loss in a murine infection model. C57BL/6 mice had both hind thighs inoculated with a high dose of $5x10^7$ CFU **(A and B)** or a low dose of $1x10^7$ CFU **(C and D)** of the respective bacterial strains in 50 µl Hanks' Balanced Salt Solution. Every 24 hours, lesion size (mm^2) and weight loss (% of original weight) were measured. Data is reported as mean ± SEM, n=12 **(A)** or n=6 **(B).**

3.4.8 Expression of *fad* is regulated through an interplay of glucose repression, palmitic acid induction, and acidic pH induction

After determining an *in vivo* role for *fadXDEBA*, we next wanted to better understand its regulation patterns under conditions that would be encountered during an infection, or during colonization of the skin. In particular, we became interested in how *fad* is expressed when *S. aureus* encounters both palmitic acid and glucose simultaneously, as well as when *S. aureus* encounters palmitic acid at an acidic pH.

3.4.8.1 Glucose repression dominates over palmitic acid induction of *fad*

Although we see *fadX* expression is induced by palmitic acid and repressed by glucose (Figure 3.8), we queried how expression patterns would change when encountering both conditions simultaneously. Fasted blood glucose levels range from 4.0–6.0 mM for healthy adults, and this can be elevated after a meal, or in individuals who suffer from diabetes. Therefore, although host cells sequester glucose availability as a fundamental aspect of nutritional immunity to reduce growth and virulence of *S. aureus* (59), *S. aureus* will encounter glucose and palmitic acid simultaneously during an infection.

We hypothesized that glucose repression dominates over fatty acid induction of the *fad* genes, contributing to why this metabolic pathway has not been identified in previous studies (3, 4). In TSB with no glucose, there was no significant difference in growth of USA300 supplemented with 500 µM palmitic acid compared to the no fatty acid control (Figure 3.18). However, in TSB + glucose there was an abrupt exit from exponential growth (Figure 3.18), mirroring the phenotype of USA300Δ*fad* grown in glucose free TSB with palmitic acid (Figure 3.10). This suggests that glucose represses *fad* expression, rendering USA300 susceptible to palmitic acid intoxication. To further support these findings, we assessed *fadX* expression in media containing palmitic acid and varying concentrations of glucose (Figure 3.19).

For this purpose, growth was assessed over 36h in microtitre plates. In TSB with no glucose, USA300 exhibited unimpeded growth in the presence of 500 µM palmitic acid

similar to that observed in flask culture; one notable difference was a dip in optical density at approximately 5-6h followed by resumption of exponential growth (Figure 3.19). Visual inspection of the plates revealed that this correlates with some transient aggregation at this time point. As in flask culture, *fadX* expression peaked in transition between exponential and stationary growth phases, at approximately 8h (Figure 3.19). When cultured in TSB with 10 mM glucose, or 13.9 mM as formulated in conventional TSB with glucose, there was a 6-8h lag phase prior to initiation of unimpeded exponential growth. This was followed by a transient cessation of growth at 10-12h, followed by a resumption in growth that coincided with initiation of *fadX* expression (Figure 3.19). There was also a longer cessation of growth and delay in *fad* expression in TSB + 13.9 mM glucose compared to 10 mM glucose, indicating that *fadX* expression is highly sensitive to the availability of glucose (Figure 3.19). This supports a model where *fad* expression is induced in response to palmitic acid, but requires glucose to be depleted from the environment before this expression can occur.

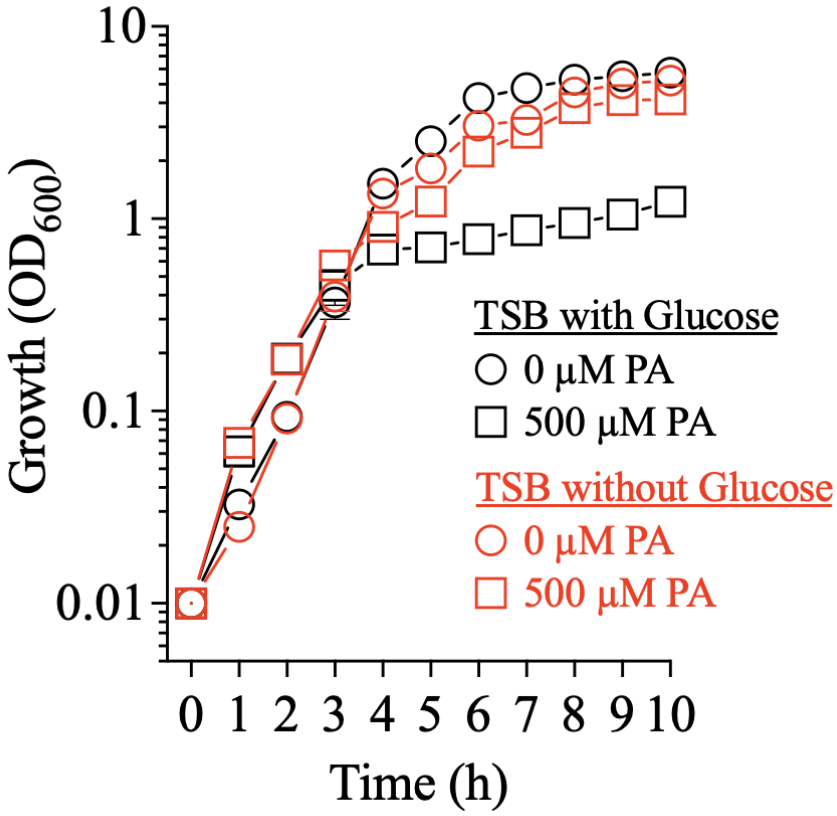


Figure 3.18. USA300 is more susceptible to palmitic acid in media with glucose. Triplicate flasks of TSB with (13.9 mM) or without glucose were supplemented with indicated concentrations of palmitic acid. Cultures we inoculated with bacteria and growth (OD_{600}) was assessed at hourly intervals. Each data point represents the mean ± SE from triplicate flasks.

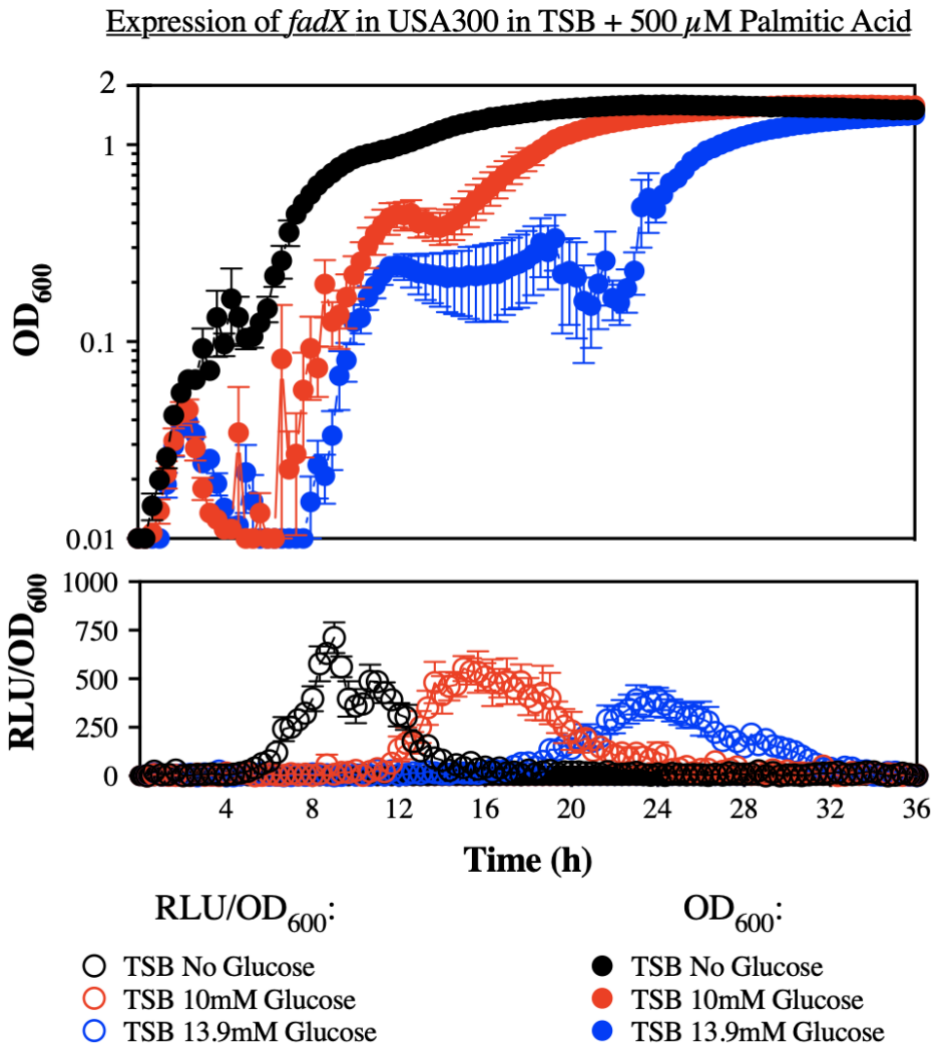


Figure 3.19. Glucose repression of *fad* expression appears to dominate over palmitic acid induction. Microtitre plate wells with 200 µL of TSB with 500 µM of palmitic acid, and varying concentration of glucose, were inoculated with bacteria and growth (OD_{600}) and *fadX* expression (RLU/OD_{600}) from a pGY*fadX::lux* construct were assessed every 15 minutes. Each data point represents the mean ± SE, n = 7.

3.4.8.2 Acidic pH induces expression of *fad*

When colonizing skin, *S. aureus* encounters both host-derived fatty acids and acidic pH concurrently (60), and we have shown acidic pH is a stimulus that enhances resistance to antimicrobial uFFA through GraS signalling (Chapter 2). Therefore, we queried whether acidic pH could also act as an environmental cue to enhance *fadX* expression in *S. aureus*. Compared to growth in unbuffered TSB, acidic pH promoted a significant increase in *fadX* expression, both in the absence and presence of palmitic acid (Figure 3.20A). We next investigated whether GraS plays a role in regulating this response, and found that deletion of *graS* dampens the ability to induce *fadX* expression in response to palmitic acid (Figure 3.20BC). However, regulation of *fadX* expression by GraS was not pH-dependent, as induction of *fadX* expression in response to palmitic acid was attenuated at both neutral and acidic pH in Δ*graS* (Figure 3.20BC). Furthermore, there was no difference in *fadX* expression between USA300 and Δ*graS* in acidic pH alone (Figure 3.20BC). Therefore, while acidic pH induces *fad* expression, this induction does not occur through GraS. Furthermore, these findings indicate GraS does play a role in regulating *fad* expression, and is not the major regulator for this putative β-oxidation system.

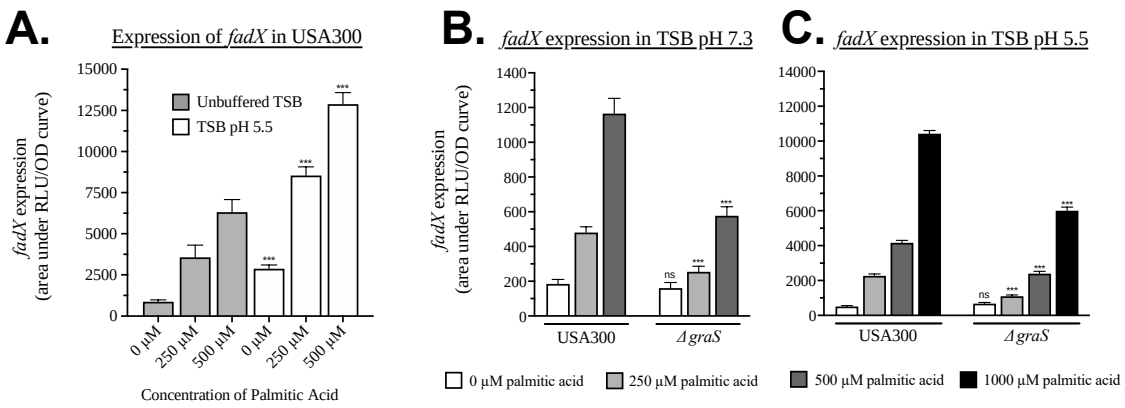


Figure 3.20. Expression of *fadX* is increased at acidic pH, and decreased in a Δ*graS* background. Wildtype or a *graS* deletion mutant were grown in 200 µL of TSB without glucose in a microtitre plate, n=7. Media remained unbuffered, was buffered at pH 7.3 using 0.1 M Bis-tris, or was buffered at pH 5.5 using 0.1 M morpholineethanesulfonic acid. Media was supplemented with the indicated concentration of palmitic acid and growth (OD_{600}) and *fadX* expression (RLU/OD_{600}) from a pGY*fadX::lux* construct were assessed every 15 minutes for 24 hours. Data is reported as mean ± SEM of the area under the RLU/OD_{600} curve. Each panel represents an independent experiment. Statistical significance was measured using an unpaired two-tailed t-test, comparing *fadX* expression in TSB pH 5.5 to unbuffered TSB **(A)** or comparing *fadX* expression in Δ*graS* to USA300 **(B and C)**, *** $p < 0.001$ *** $p < 0.001$, ns = not significant.

3.4.9 The intramembrane protease PrsW is required to induce expression of *fad*

As detailed in Section 3.1.3, the *fad* system in *S. aureus* lacks a conventional FadR transcriptional regulator, which is the key regulator of *fad* expression in *E. coli* and *B. subtilis* (14, 61, 62). Furthermore, the genomic arrangement of the *fad* genes is unusual, as they are clustered into a single locus rather than dispersed throughout the genome (Figure 3.3). Directly upstream of the *fad* locus is a divergently transcribed intramembrane protease, *prsW* (Figure 3.21). While work is still ongoing to determine the exact details of this system, PrsW is proposed to modulate the activity of a stress response Sigma Factor, σ^S, through proteolytic degradation of an anti-sigma factor (Figure 3.22) (63, 64). This proposed interaction is supported by a *prsW* knockout mutant phenocopying a *sigS* knockout mutant in sensitivity to DNA-damaging agents and cell wall targeting antibiotics (63). Due to the proximity of *prsW* to the *fad* genes, we queried whether PrsW plays a role in regulating *fadX* expression. Using a *prsW* knockout mutant from the Nebraska transposon library (45), we found that knocking out *prsW* resulted in an inability to induce *fadX* expression in response to palmitic acid (Figure 3.23). Furthermore, transposon knockouts of both *prsW* and *sigS* behave similarly to a *fad* deletion mutant, showing impaired growth in both 250 µM and 500 µM palmitic acid (Figure 3.24). Interestingly however, it appears that *prsW* and *sigS* mutants are slightly more sensitive to palmitic acid than a *fad* deletion mutant (Figure 3.24BC), likely due to σ^S having a global effect on gene expression, regulating a variety of genes in response to bacterial stress (64, 65). These data are consistent with PrsW being able to respond to membrane stress imposed from exposure to palmitic acid, leading to degradation of an as yet unidentified anti-sigma factor, thereby freeing σ^S to promote expression of *fad* genes (Figure 3.22).

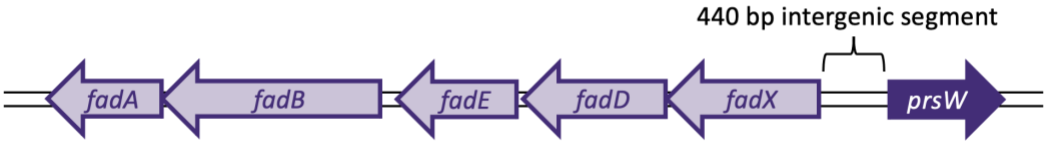


Figure 3.21. Genomic layout of *fadXDEBA* and the divergently transcribed *prsW*. The intramembrane protease *prsW* (SAUS300_0230) is located directly upstream of the *fadXDEBA* (SAUSA300_0225–SAUSA300_0229) locus. PrsW is proposed to modulate the function of a stress response Sigma Factor, σ^S, through proteolytic degradation of an anti-sigma factor (63).

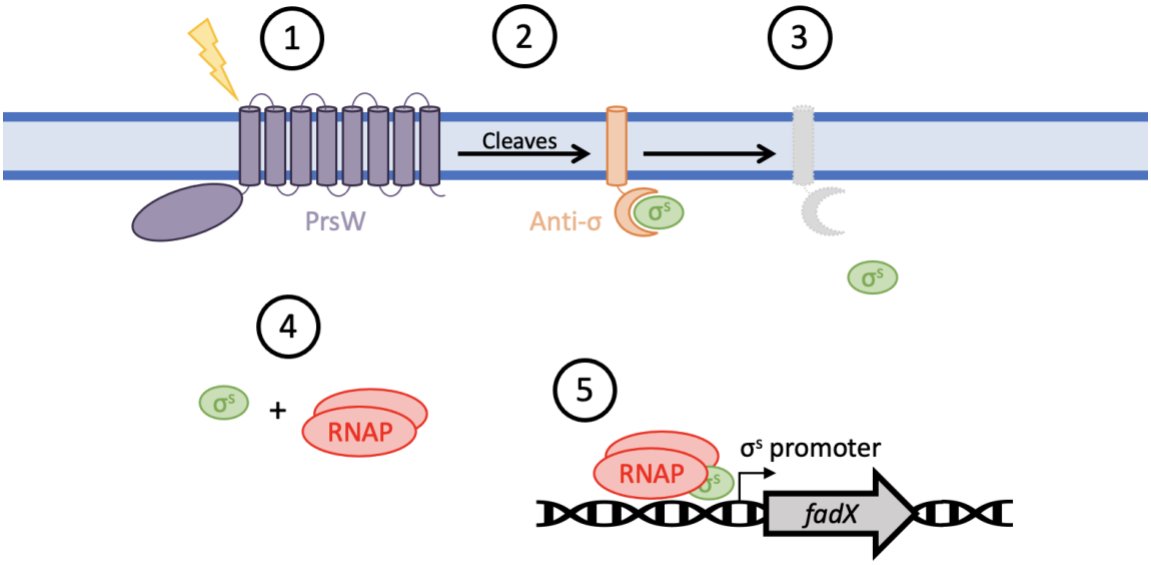


Figure 3.22. Schematic diagram for proposed PrsW and SigS regulation of gene expression. PrsW is activated by through an external stimulus, or by sensing membrane stress **(1)**. Activation of PrsW leads to proteolytic degradation of an anti-sigma factor **(2)**, freeing the sigma factor, σ^s, from anti-sigma factor inhibition **(3)**. σ^s is then able to interact with RNA Polymerase (RNAP) **(4)**, which enables binding of RNA Polymerase to the promoters of specific genes **(5)**. Furthermore, we believe the *fad* genes are a target for σ^s mediated expression **(5)**. This schematic likely represents a simplified version of the intramembrane proteolysis required for inactivation of the anti-sigma factor. For example, in *B. subtilis*, PrsW is the first in a four-part proteolytic cascade required for complete degradation of the anti-sigma factor, RsiW, and subsequent freeing of the sigma factor, σ^w (66).

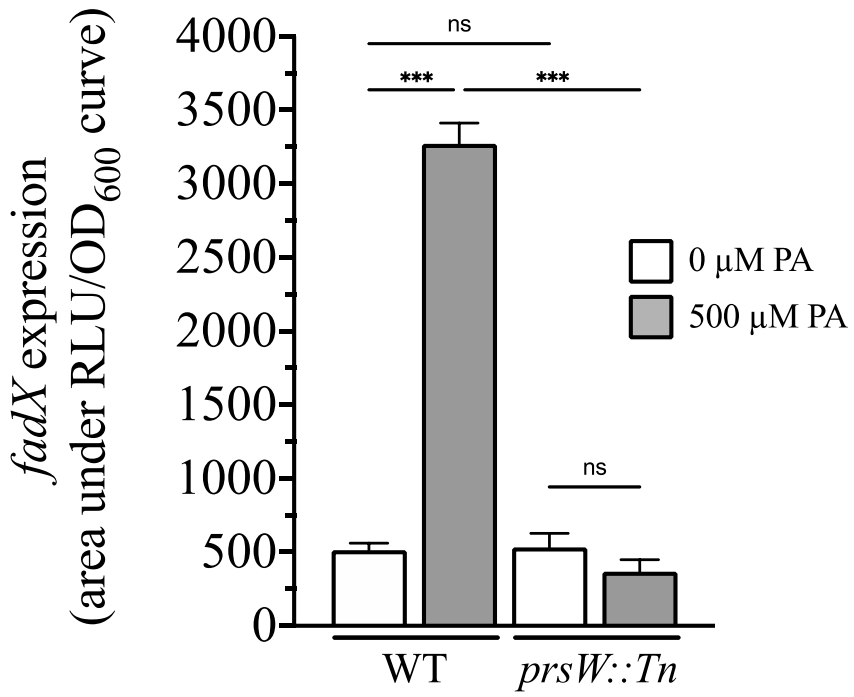


Figure 3.23. Knockout of *prsW* results in an inability to induce *fadX* expression in response to palmitic acid. Wildtype USA300 (WT) and *prsW::Tn*, a mutant from the Nebraska Transposon Library (67) with a transposon insertion in the *prsW* gene, were grown in 200 µL of TSB without glucose in a microtitre plate, n=7. Media was supplemented with 500 µM palmitic acid, or remained as TSB alone as a control. Cultures were with bacteria and growth (OD_{600}) and *fadX* expression (RLU/OD_{600}) from a pGY*fadX::lux* construct were assessed every 15 minutes for 24 hours. Data is reported as mean ± SEM of the area under the RLU/OD_{600} curve. Statistical significance was measured using Two-Way ANOVA with Tukey's multiple comparisons Test, *** $p<0.001$, ns = not significant.

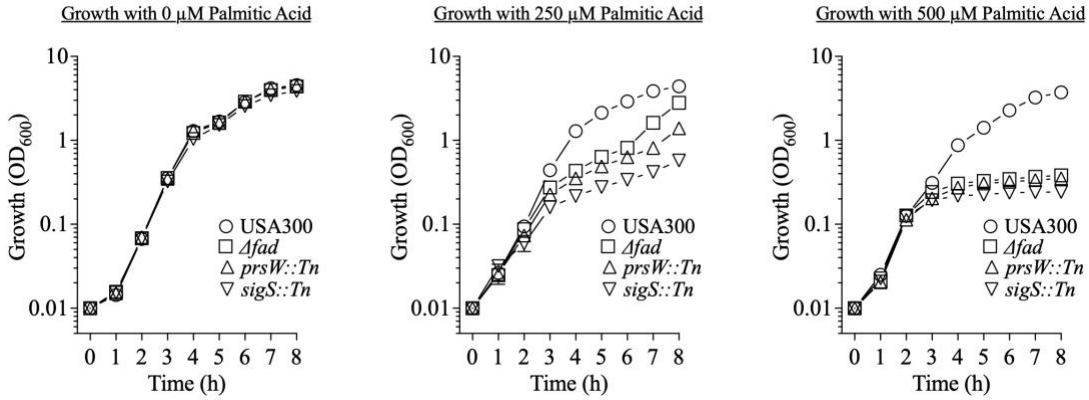


Figure 3.24. Knockout of *prsW* or *sigS* phenocopies Δ*fadXDEBA* in susceptibility to palmitic acid. Wildtype USA300 (WT), *prsW::Tn*, and *sigS::Tn*, mutants from the Nebraska Transposon Library (67) were grown in triplicate flasks of TSB without glucose, supplemented with 0 µM **(A)**, 250 µM **(B)**, or 500 µM **(C)** palmitic acid. Cultures were inoculated with bacteria and growth (OD_{600}) was assessed hourly. Each data point represents the mean ± SE.

3.4.10 *fad* demonstrates an important function in responding to conditions that impose stress on the phospholipid membrane

After demonstrating PrsW is important for inducing *fad* expression, we became interested in the relationship between membrane stress and Fad function. In particular, we investigated Fad function in response at lower temperatures, and in response to antimicrobial peptides. Although the average body temperature is around 37ºC, various regions on the skin range in temperature from around 30ºC to 35ºC (68). The temperature of the nose in particular, the most frequent location of *S. aureus* colonization (69–71), is around 33.5ºC (68). Furthermore, the skin produces a variety of antimicrobial peptides to restrict the growth of various pathogens, that *S. aureus* must combat. Therefore, both growth at lower temperatures and in the presence of antimicrobial peptides represent conditions *S. aureus* would encounter when colonizing human skin, that impose stress on the membrane.

3.4.10.1 Function of *fad* at decreased temperatures

When grown at a lower temperature, the bacterial membrane becomes more rigid. To compensate, the bacteria is forced to increase membrane fluidity by incorporating shorter length, branched chain, or unsaturated fatty acids into phospholipid (72–75). However, *S. aureus* does not possess a membrane phospholipid desaturase, and therefore relies solely on the length or branching nature of phospholipids to regulate membrane fluidity.

Previously, we noted exogenous palmitic acid dramatically altered the phospholipid membrane composition of *S. aureus*, as palmitic acid or extended derivatives of palmitic acid readily replaced natively produced lipid species in the membrane (Figure 3.12). Therefore, we queried whether the combined stress of properly metabolizing exogenous palmitic acid to incorporate into the membrane, as well as altering membrane fluidity to cope with lower growth temperatures, would create a more essential requirement for Fad. Although USA300 grew slower at lower temperatures, the bacteria remained resistant to palmitic acid at all temperatures tested (Figure 3.25). In contrast, USA300Δ*fad* became more susceptible to palmitic acid at lower temperatures, with 100 µM palmitic acid causing complete growth arrest at 30ºC (Figure 3.25), which also occurred in a *prsW*

knockout mutant (Figure 3.25B). Importantly, in the absence of exogenous palmitic acid, USA300, Δ*fad*, and *prsW::Tn* grew equally well at 30ºC, indicating these genes are not required for growth at lower temperatures alone, but rather are required for resistance to palmitic acid toxicity which is further exacerbated at lower temperatures (Figure 3.25B).

Additionally, we investigated the phospholipid membrane composition of USA300 and Δ*fad* grown with exogenous palmitic acid at both 30ºC and 37ºC, as we previously noted incorporation of exogenous palmitic acid, or its extension products from the FASII pathway, dramatically altered the phospholipid membrane composition of *S. aureus* (Figure 3.12). With phospholipid length being important to combat the rigidity associated with growth at lower temperatures, we queried whether palmitic acid would be differentially incorporated into the membrane at 30ºC or 37ºC. As expected, the overall fatty acid chain length of PG was shortened in both USA300 and Δ*fad* grown at 30ºC, which helps maintain membrane fluidity (Figure 3.26A). Supporting this finding, USA300 exhibited a preference for direct incorporation of C16 palmitic acid into the membrane at 30ºC (PG31:0; C15+C16), whereas at 37ºC there was preferential incorporation of the FASII extension product C20 (PG35:0; C15+C20) (Figure 3.26B–D). While USA300Δ*fad* followed a similar trend, it exhibited a reduced capacity to directly incorporate C16 palmitic acid into the membrane at 30ºC, and also less incorporation of the C20 extension product at 37ºC (Figure 3.26B–D). Taken together, these findings indicate USA300 can effectively incorporate palmitic acid into the membrane in a way that optimizes membrane fluidity based on temperature, whereas USA300Δ*fad* has a reduced capacity to perform this function. As the Fad proteins have not been shown to directly incorporate exogenous fatty acids into the membrane, this altered phospholipid profile may be indicative of Fad recycling membrane lipids in order to contribute to membrane homeostasis; a function Fad has been associated with in other bacteria (76).

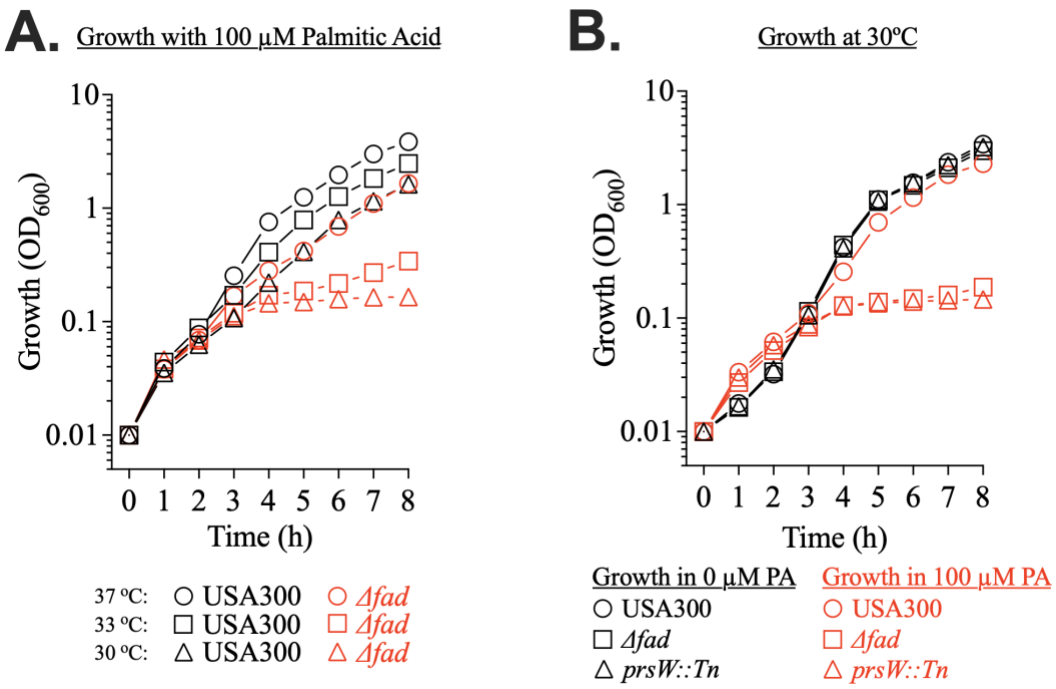


Figure 3.25. Palmitic acid is more toxic to Δ*fadXDEBA* at lower temperatures. Triplicate flasks of TSB without glucose were supplemented with indicated concentrations of palmitic acid, and grown at either 30ºC, 33ºC, or 37ºC as indicated. Growth (OD_{600}) was assessed at hourly intervals, and each data point represents the mean ± SE from triplicate flasks.

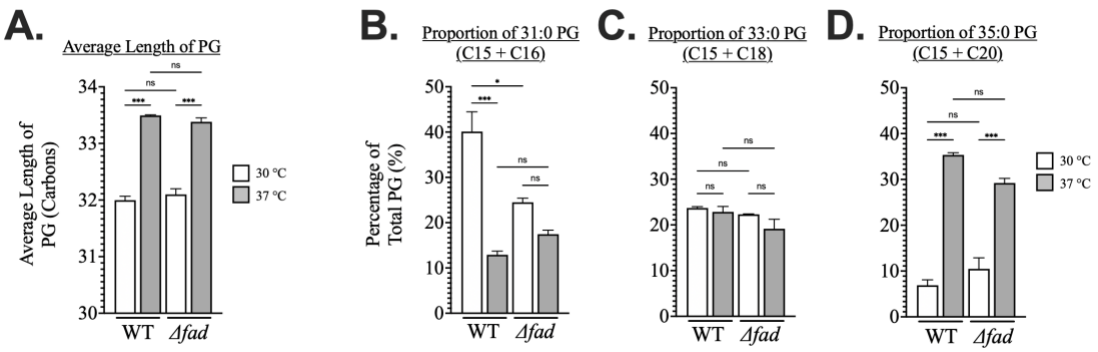


Figure 3.26. Temperature impacts the length of phosphatidylglycerol (PG) in the membrane, and the manner in which palmitic acid is incorporated into the membrane. Wildtype USA300 (WT) and USA300Δ*fad* were grown in triplicate cultures to an OD_{600} of 0.15 and a bolus of 500 µM palmitic acid was added. Bacteria were then grown for another 2 hours before samples were collected. Samples were homogenized, and lipids were extracted and analyzed through Liquid chromatography–mass spectrometry at the Wayne State Lipidomics Core Facility. Results are presented as mean ± SEM of the percentage of total PG each lipid species represents. Statistical significance was measured using Two-Way ANOVA with Tukey's multiple comparisons Test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, *** $p<0.001$, ns = not significant.

3.4.10.2 Function of *fad* in response to antimicrobial peptides

Another common cause of membrane stress when colonizing human skin are antimicrobial peptides. Furthermore, expression of the *fad* genes has been shown to be upregulated in response to a variety of membrane targeting antimicrobial agents (77, 78). Therefore, we wanted to determine if deletion of *fad* would make *S. aureus* more susceptible to antimicrobial peptides. As seen, 16 µg/mL of the lipopeptide polymyxin B slightly impaired Δ*fad* growth relative to USA300; however, this impairment was much more severe at 32 µg/mL, indicating *fad* does play a role in resistance to CAMPs (Figure 3.27A). Although polymyxin B does not represent a CAMP encountered on human skin, this was only a preliminary finding, and we plan to investigate this further to determine if this impairment generalizes to a broad range of CAMPs.

Additionally, we wanted to investigate if USA300Δ*fad* was more susceptible to daptomycin or vancomycin, as a previous study showed increased daptomycin and vancomycin resistance was associated with increased *fad* expression (77). However, our findings demonstrate that USA300Δ*fad* is only marginally more susceptible to daptomycin and vancomycin than USA300 (Figure 3.27BC). Therefore, while increased expression of *fad* may provide increased resistance to these antimicrobial agents (77), the absence of *fad* does not make *S. aureus* markedly more susceptible.

Together, our findings indicate that Fad does play a role in resistance to antimicrobial peptides; however, the specificity of this resistance needs to be further elucidated. Furthermore, based on the predicted function of the Fad proteins (Section 3.1.3), this resistance is likely not due to direct interaction with antimicrobial agents, but rather due to metabolism of fatty acids and recycling or remodelling of the phospholipid membrane to maintain membrane integrity. Future investigations will be focused on better understanding the impact Fad has on membrane composition, and understanding how these impacts can promote resistance to other membrane damaging agents such as CAMPs or lipopeptides.

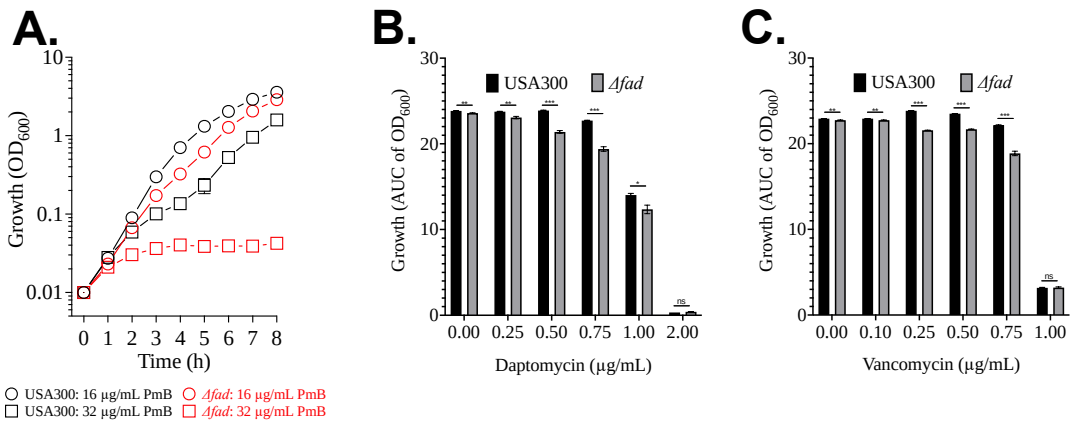


Figure 3.27. FadXDEBA plays a role in Polymyxin B (PmB) resistance and may play a minor role in daptomycin and vancomycin resistance. (A) Triplicate flasks of TSB without glucose were supplemented with indicated concentrations of PmB. Growth (OD_{600}) was assessed at hourly intervals, and each data point represents the mean ± SE from triplicate flasks. **(B and C)** Microtitre plate wells with 200 µL of TSB, and varying concentration of daptomycin or vancomycin, were inoculated with bacteria and growth (OD_{600}) was assessed every 15 minutes for 24 hours. Each data point represents the mean ± SE of the area under the OD_{600} curve, n = 7. Media was supplemented with 0.5 mM $CaCl_2$ for the daptomycin assay **(B)**. Statistical significance was measured using an unpaired two-tailed t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant **(B and C).**

3.5 Discussion

This study has expanded our knowledge of lipid metabolism in *S. aureus*. While the metabolic fate of unsaturated FFA has been well documented in *S. aureus* through proteins such as FakA, OhyA, and FarE (36–38), the fate of saturated FFA has been much less explored, with FakA being the only known route for their metabolism through incorporation into phospholipid (38) (Figure 3.28). Saturated palmitic acid in particular is encountered at high concentrations on human skin (79) as well as in plasma (80), and is also the most abundant saturated fatty acid in adipose triglyceride (81). In consideration of the *in vivo* abundance of palmitic acid, it is surprising that it is a poor substrate for the PlsY acyl-transferase that would otherwise mediate its direct incorporation into phospholipid, and our data point towards a role for the *fad* locus in providing an alternate route for metabolism of exogenous palmitic acid (Figure 3.28).

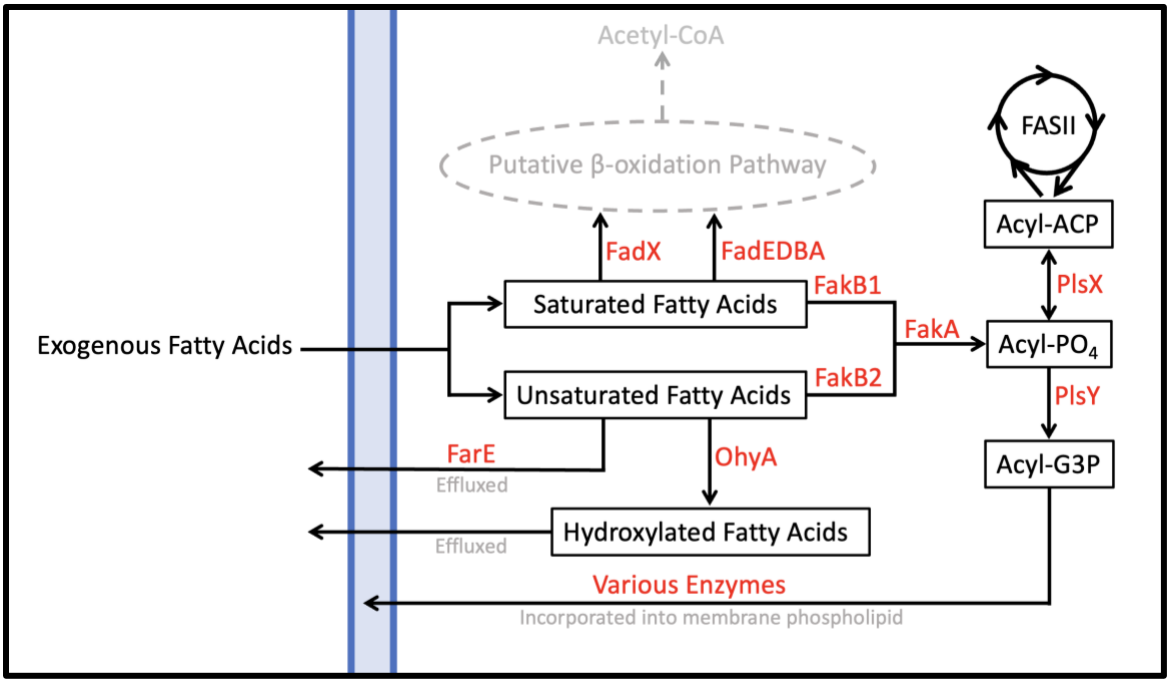


Figure 3.28. Schematic diagram for fatty acid metabolism in *S. aureus*. After entering the bacterial cell, unsaturated fatty acids can be directly effluxed by FarE, detoxified through OhyA-dependent hydroxylation and then effluxed, or bound by FakB2 and phosphorylated by FakA as required for incorporation into membrane phospholipid. Saturated fatty acids instead are bound by FakB1, but follow a similar metabolic fate through FakA and incorporation into phospholipid. Our study documents an additional metabolic fate for saturated fatty acids through the putative β-oxidation proteins, FadDEBA, that may function to reduce the metabolic load on FakA.

The *fad* genes are well documented for their role in β-oxidation of exogenous fatty acids in both Gram-negative and Gram-positive bacteria (5, 6), and biochemical assays have demonstrated metabolic competency for β-oxidation in *S. carnosus* (7), which has the same complement and organization of *fad* genes as in *S. aureus* (Figure 3.7). Antibody to FadB of *S. aureus* was also demonstrated in human serum (39) and *fad* genes are expressed at a high level when *S. aureus* is internalized by cultured human respiratory epithelial cells which are responsible for synthesizing human lung surfactant (8, 9). Nevertheless, previous studies aimed at demonstrating a functional β-oxidation pathway in *S. aureus* were not successful (3, 4). Therefore, it is likely that *fad* function in *S. aureus* has been underappreciated due to growth conditions not being optimized to investigate this system. As such, our data have revealed that expression of *fadX* is strongly repressed by glucose (Figure 3.8), and *fadE* is preceded by a putative *cre*-box (Figure 3.6A), which in other systems functions to repress *fad* expression through carbon catabolite repression (82). Repression by glucose is further supported by recent work where inactivation of the glucose-dependent virulence regulator CcpA resulted in de-repression of all *fad* genes in *S. aureus* MN8 (12). Therefore, with TSB used to study *S. aureus* often containing glucose, previous investigations into additional metabolic pathways for exogenous fatty acids may have inadvertently suppressed expression of the *fad* genes. Furthermore, with *fad* expression not being maximal until later growth phases (Figure 3.8A) and exhibiting a strong preference for C16 palmitic acid (Figure 3.8C), we believe conditions used to optimally study FakA have obfuscated Fad function in past research (3, 4).

Our work has now documented that the *fad* locus, in addition to FakA mediated incorporation into phospholipid, presents a second option for metabolism of exogenous palmitic acid (Figure 3.28). Indeed, *fadX* expression was significantly enhanced in the absence of *fakA*, suggesting that incorporation of exogenous fatty acids into phospholipid is a preferred metabolic pathway. Although previous studies found that C16 acyl-phosphate is a poor substrate for the PlsY acyl-transferase that promotes incorporation of exogenous fatty acids into PG (1), we did nevertheless observe a small amount of PG31:0 in USA300 grown in TSB alone, comprised of PG containing C15 and C16 fatty acids, and PG31:0 was significantly elevated in TSB + palmitic acid (Figure 3.12). This is

consistent with an observation that PG in *S. aureus* recovered from sites of infection also contains a significant amount of PG31:0, indicating that during *in vivo* growth, *S. aureus* does incorporate palmitic acid into phospholipid (58). Of interest, our data revealed that USA300Δ*fad* exhibited significantly more PG31:0 relative to USA300 in TSB + palmitic acid (Figure 3.12B), such that one function of the *fad* locus may be to draw palmitic acid away from incorporation into phospholipid through its conversion to acyl-CoA, which is not used by *S. aureus* as a substrate for phospholipid synthesis. This would help to alleviate a metabolic bottleneck attributed to C16 acyl-phosphate being a poor substrate for incorporation into phospholipid (Figure 3.28).

A second clue to the physiologic function of the *fad* locus is evident in the temporal pattern of *fadX* expression, which when grown in glucose free TSB exhibited one peak of expression in transition between exponential and stationary phase (Figure 3.8A). As such, it may be that peak *fad* expression cannot occur until phospholipid synthesis begins to decrease in this transition phase. Moreover, when grown in flask culture with optimal aeration, there was a second and more intense peak of *fad* expression at approximately 16h, after an extended period in stationary phase (Figure 3.8A). While this will be the subject of future research, it is consistent with studies where *fad* genes have been associated with recycling membrane phospholipids to maintain membrane homeostasis (76). Consistent with such a role, we note that *fad* expression is upregulated when *S. aureus* encounters cell membrane targeting antimicrobial agents such as daptomycin (77) and lignin (78), and was required for proper resistance to polymyxin B (Figure 3.27A).

We have established an important *in vitro* role for *fad* genes in alleviating toxicity attributed to exogenous palmitic acid, which is the most abundant saturated fatty acid in human plasma at a concentration of ~ 100 μM (83, 84), and this physiologic concentration was sufficient to impair growth of USA300*Δfad* (Figure 3.10A). We have also established an *in vivo* requirement for *fad* genes in a C57BL/6J murine subcutaneous abscess model, where attenuated virulence was comparable in impact to deletion of the *graS* sensor kinase, and deletion of *fakA* which is required for incorporation of exogenous fatty acids into phospholipids (Figure 3.16). Although subcutaneous abscess infection models using SKH1 mice have indicated a *fakA* mutant shows increased virulence (85,

86), others observed attenuated virulence in a bacteremia model using C57BL/6 mice (87), congruent with our present findings. Having now demonstrated that deletion of *fakA* or *fad* individually cause a comparable and significant attenuation of virulence, and with our finding that *fadX* expression is significantly elevated in the absence of *fakA*, one focus of our future work will be to assess the impact on *in vivo* survival of *S. aureus* when both of these key pathways for metabolism of host derived fatty acids are eliminated.

One caveat of our analysis is that we used a *fadX*::lux reporter as an indicator of *fad* gene expression, but have not evaluated the transcriptional organization of the *fadXDEBA* locus which as noted in Figure 3.6A, is suggestive of three transcriptional units comprised of *fadXD, fadE*, and *fadBA*. Nevertheless, *fadX* expression was repressed by glucose as expected for *fad* expression in other bacteria, and the central *fadE* is also preceded by a *cre*-box that is likely to mediate glucose mediated repression (Figure 3.6A). Moreover, individual transposon mutants confirmed that each of the core *fadDEBA* genes with orthologs in *E. coli* are needed to alleviate *in vitro* toxicity of exogenous palmitic acid, whereas the requirement for *fadX* was less stringent (Figure 3.15). In other work, *fadX* was required for metabolism of C3 propionic acid (10), consistent with its annotation as a short chain acyl-CoA transferase and homology to YdiF which shows activity to short chain (< 4 carbon) fatty acids (13). Therefore, we cannot exclude the possibility that *fadX* could be subject to other regulatory signals related to accumulation of short chain fatty acid metabolites. However, this could also include short chain fatty acids generated in the final rounds of β-oxidation of palmitic acid through *fadDEBA.*

An additional nuance to the *fad* system in *S. aureus* is that there is no ortholog to the canonical regulator of β-oxidation gene expression, FadR (Table 3.3 and 3.4). Instead, we believe the *fad* genes in *S. aureus* are regulated by the intramembrane protease, PrsW, which is located directly upstream of the *fad* genes (Figure 3.21). While the exact mechanism behind PrsW function is not completely understood, it has been proposed that PrsW functions by degrading the anti-sigma factor for σ^S (Figure 3.22) (63, 64). While we have not directly assessed the proteolytic function of PrsW in this study, we have

confirmed that a knockout of *prsW* is unable to induce *fad* expression in response to exogenous palmitic acid (Figure 3.23), and a knockout of *prsW* or *sigS* phenocopies USA300Δ*fad* in susceptibility to palmitic acid (Figure 3.24). Together these findings provide strong evidence that PrsW, and by extension σ^S, play an important role in regulating *fad* function. Interestingly, PrsW and σ^S are proposed to play a role in resistance to a variety of cell wall stressors including changes in temperature, surfactants, and cell wall targeting antibiotics (63–65), and the *fad* genes have been associated with membrane phospholipid recycling and maintenance of proper membrane composition in other bacteria (76), showing a logical link between these two systems.

This relationship between *fad* and membrane stress is supported by our findings, as growth at lower temperatures increased palmitic acid sensitivity in USA300Δ*fad* (Figure 3.25). Furthermore, deletion of *fad* genes increased the susceptibility of *S. aureus* to membrane the disrupting agent, polymyxin B (Figure 3.27). We also saw exogenous palmitic acid being differentially incorporated into the phospholipid membrane in wildtype USA300 and USA00Δ*fad*, indicating Fad plays a role in regulating the membrane composition of *S. aureus* (Figure 3.12 and 3.26). All together, these findings demonstrate that *fad* likely does play a role in membrane homeostasis in *S. aureus*, however the exact mechanism still needs to be elucidated. Furthermore, this supports a model where an intramembrane protease like PrsW, capable of sensing changes to membrane composition, can regulate expression of *fad.*

Supporting a role in maintaining membrane homeostasis, we see exogenous palmitic acid not only inhibits USA300Δ*fad* from growing, but significantly reduces the viability of the bacteria. We do not have an explanation for the rapid loss of viability on exposure of USA300Δ*fad* to exogenous palmitic acid, upon which it appears that the bacteria may complete one round of replication, concomitant with declining viability (Figure 3.11). We speculate that metabolic signals arising from a disfunction in metabolism of exogenous palmitic acid are responsible for triggering this loss of viability, which is the focus of on-going research. One potential hint is that the PG of USA300Δ*fad* exhibited a significantly higher content of PG31:0 attributed to direct incorporation of palmitic acid into phospholipid at 37 ºC (Figure 3.12). Since the PlsY acyltransferase that would produce

this product does not work efficiently with C16-acylphosphate substrate (1), it is feasible that loss of *fad* function contributes to a bottleneck in phospholipid synthesis that triggers loss of viability. A better understanding of this intoxication by exogenous palmitic acid in the absence of *fad* function would support current efforts to use fatty acids therapeutically as antimicrobial agents (88), or to enhance activity of antibiotic therapies (89, 90) and innate immune function of human skin (91, 92).

Together, our findings have brought to light the importance of *fad* function in *S. aureus*. The *fad* genes play an important role in metabolism of C16 palmitic acid, and to a lesser degree C14 and C18 fatty acids. This metabolic pathway works in conjunction with the established FakA pathway, and knocking out one pathway imposes increased metabolic stress onto the remaining pathway. Our findings also indicate Fad plays an important role in membrane homeostasis and phospholipid recycling, and is regulated by the intramembrane protease PrsW; however, we have not yet fully elucidated the details of these findings. Finally, Fad has been directly associated with virulence regulation in a variety of other bacteria. For example, the virulence factor regulators TcpP in *Vibro cholera* (89–91), TfmR in *Xanthomonas citri* (92), and HilA in *Salmonella* (93), are all regulated by exogenous fatty acids, and require *fad* to metabolize these fatty acids in order to properly express their respective virulence factors. Although we have established an *in vivo* requirement for the *fad* genes in a murine subcutaneous abscess model (Figure 3.11), we have not yet investigated if Fad directly plays a role in regulating virulence factors of *S. aureus.*

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Chapter 4

4 General Discussion and Conclusion

4.1 General Summary

S. aureus USA300 is the current endemic strain of CA-MRSA (1), which has enhanced virulence and ability to colonize human skin, allowing for rapid spread amongst communities (2–4). In order to effectively colonize human skin, *S. aureus* must combat a variety of innate immune conditions including acidic pH, CAMPs, and host-derived uFFA. While uFFAs function to inhibit the growth of *S. aureus*, they also provide a valuable source of lipids for membrane synthesis and energy production, and human skin also provides a rich source of less toxic sFFA. Therefore, the purpose of this thesis was to broaden our understanding of both the resistance and metabolic pathways in *S. aureus* that allow the bacteria to both survive and thrive in the presence of host-derived fatty acids.

Bacteria on human skin are concurrently exposed to acidic pH and antimicrobial fatty acids, and it was a reasonable assumption that these combined conditions would exhibit enhanced antimicrobial activity toward *S. aureus*. Remarkably, our work demonstrated that acidic pH promoted enhanced resistance to antimicrobial uFFA through a GraS dependent mechanism, which occurred independently of the established resistance mechanisms represented by FarE mediated efflux (5, 6), and FakA mediated incorporation into phospholipid (7). Rather, GraS mediated resistance to antimicrobial uFFA during growth at acidic pH was dependent on the flippase function of MprF which was previously found to be essential for translocation of lysyl-phosphatidylglycerol from the inner leaflet of the cytoplasmic membrane to the external surface (8–11). Consequently, our work has revealed a new function for MprF that is distinct from its previously ascribed function in resistance to antimicrobial peptides.

In evaluating the mechanistic basis of signalling through GraS at acidic pH, we established that this occurred independently of acidic amino acids in the short extracytoplasmic segment that had previously been implicated in recognition of antimicrobial peptides (12). Activation of GraS by acidic pH also resulted in robust expression of secreted proteases. Furthermore, our collaborative studies demonstrated that activation of GraS by both CAMPs and acidic pH, allowed *S. aureus* to survive and replicate in macrophages (13). Additionally, inhibiting GraR could reverse β-lactam

resistance in *S. aureus* USA300, and made the bacteria more susceptible to innate immune agents such as CAMPs, oxidative stress, and lysozyme, as well as inhibiting biofilm formation, intracellular replication in macrophages, and *in vivo* virulence (14). Together, these findings indicate that through GraXRS, *S. aureus* has become exceptionally resistant to innate immunity encountered during both colonization and infection of humans.

Once resistant to the toxic effects for host-derived fatty acids through GraS activation, we demonstrated that *S. aureus* can metabolically benefit from exogenous fatty acids through FadXDEBA. Bioinformatic analysis indicates FadDEBA in *S. aureus* is orthologous to the β-oxidation machinery in *E. coli* and *B. subtilis*. These genes are repressed by glucose and induced by palmitic acid and acidic pH. Furthermore, deletion of the established FakA fatty acid metabolic pathway results in heightened expression of *fad*, and a broader range of exogenous fatty acids that can induce *fad* expression. Expression of *fad* appears to be primarily regulated by the intramembrane protease PrsW and the alternative sigma factor σ^S, but GraS also appears to play a minor role in regulating *fad* expression in response to exogenous fatty acids. While exogenous palmitic acid promoted increased growth of USA300, it impaired growth of a Δ*fadXDEBA* mutant, and remarkably palmitic acid was toxic to this mutant.

Interestingly, this Δ*fadXDEBA* growth impairment is exacerbated at lower temperatures. Additionally, Δ*fadXEDBA* appears to be more susceptible to the CAMP polymyxin B. Although palmitic acid is rapidly incorporated into the phospholipid membrane, presumably through the FakA pathway (7), wildtype USA300 and Δ*fadXDEBA* have differing capacities to properly modulate the manner in which palmitic acid is incorporated into the membrane. Together, our findings have demonstrated that the previously unstudied *fadDEBA* genes have an important role in palmitic acid metabolism, a fatty acid physiologically relevant to *S. aureus* colonization of human skin (15, 16), as well as a preliminary role in CAMPs resistance. Our next steps will focus on confirming β-oxidation occurs through FadDEBA, as well as further elucidating the regulatory network responsible for *fad* expression.

Together, through the resistance gained by GraS and MprF, and the metabolic benefit gained through FadXDEBA, we have demonstrated *S. aureus* can both survive and thrive in the presence of host-derived fatty acids. Activation of both these pathways is enhanced under conditions found on human skin, including acidic pH and CAMPs, indicating *S. aureus* has evolved to efficiently sense and respond to environmental conditions that would be encountered on human skin, promoting enhanced resistance to and efficient metabolism of host-derived fatty acids.

4.2 General Discussion, Limitations, and Future Studies

While our study has greatly expanded our understanding of host-derived fatty acid resistance and utilization in *S. aureus* during colonization of human skin, there are some limitations that require further investigation and explanation. Specifically, while we have identified novel host-derived fatty acid resistance and metabolic pathways, work is still required to elucidate the detailed mechanisms behind these pathways.

4.2.1 GraS-mediated resistance to uFFA general discussion

Our findings show that activation of GraS by acidic pH or CAMPs results in robust resistance to uFFA (17); however, our next steps will focus on how this activation occurs, and how resistance to uFFA is mediated.

Activation of the sensor kinase GraS provides resistance to a variety of conditions including CAMPs, acidic pH, growth at high temperatures, oxidative stress, glycopeptide antibiotics, and uFFA (13, 17–25); however, the only established sensing mechanism for GraS occurs through a short negatively charged extracellular segment (12). While this sensing mechanism is compatible with sensing CAMPs, it does not explain how the variety of other stressors associated with GraRS function would activate GraS signalling. GraS belongs to a sub-family of histidine kinases known as intramembrane-sensing histidine kinases which sense disruptions to membrane composition rather than directly binding molecules (26, 27). This classification is supported by our finding that acidic pH activates GraS independently of the negatively charged extracellular segment (17). Furthermore, a variety of CAMPs are known to target and disrupt phospholipid membrane composition (28, 29). Therefore, we believe that in addition to sensing

CAMPs through a short extracellular segment, GraS is capable of sensing membrane perturbations in a currently unelucidated sensing mechanism, explaining its diverse role in resistance to antimicrobial conditions. In particular, we plan to investigate what additional conditions can directly activate GraS signalling, and if a low complexity region directly following the final transmembrane domain in GraS contributes to this sensing mechanism.

Our hypothesis that activation of GraS occurs through a low complexity region is based on research of the sensor histidine kinase DesK in *B. subtilis*. DesK can act as both a kinase and a phosphatase, depending on temperature induced changes to the membrane composition (30, 31). The difference between these two states occurs due to a short linker region, ^{154}RKERERLEEKLE^{165}, which connects the final transmembrane domain to the cytoplasmic domain, and can exist in a random coiled state promoting the phosphatase state, or as a continuous helix promoting the kinase state (30). Using the online Simple Modular Architecture Research Tool, there exists a low complexity region directly following the final transmembrane domain in GraS as well, ^{70}KHFDKDKEIEEIKHKD^{85} (Figure 4.1). Like DesK, we believe this low complexity region can take on different conformations in GraS depending on membrane composition, regulating GraS activation. Acidic pH has been proposed to impact phospholipid membrane composition in various ways including diminishing the repulsive forces between phosphate head groups allowing for tighter packing, reducing membrane curvature, and increasing the thickness of the membrane (32–34), and these changes may be the stimulus used by GraS to respond to acidic pH. Like studies elucidating the mechanism for DesK activation, we plan to introduce a variety of mutations into this low complexity region of GraS to probe the functionality of this region. Once we better understand this novel sensing mechanism, we plan to determine other conditions that activate GraS through this mechanism. As mentioned, GraS has been associated with resistance to high temperatures and oxidative stress (18), both of which would impact membrane composition. By creating mutations in both the extracellular loop and the low complexity region, we will investigate if temperature and oxidative stress are able to directly activate GraS signalling, and the mechanism by which this activation occurs.

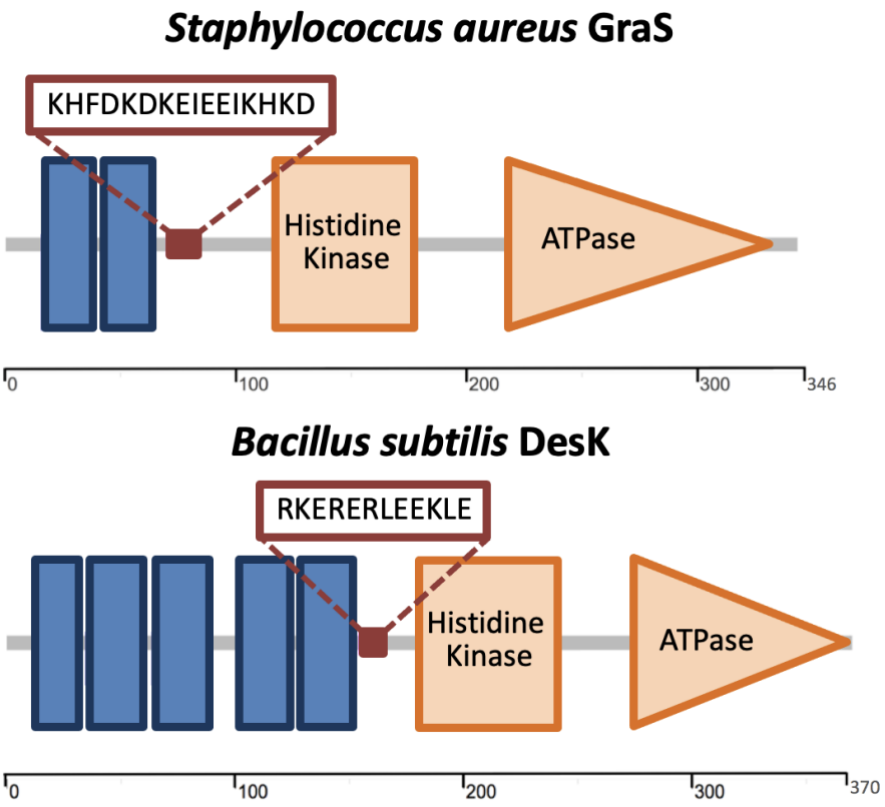

Figure 4.1. Domain architecture of GraS and DesK according to the online Simple Modular Architecture Research Tool. Transmembrane segments are coloured in blue, low complexity region is coloured in maroon, and histidine kinase and ATPase segments are coloured in orange. The amino acid scale is indicated beneath each protein.

Additionally, we want to determine the role the ABC-transporter VraFG plays in this novel GraS sensing mechanism. Preliminary findings indicate that both VraF and VraG are required for GraS to signal in response to acidic pH. However, these findings were based on growth phenotypes that require GraS activation. Therefore, we plan to directly measure *mprF* expression in response to acidic pH, in both *vraF* and *vraG* knockout strains, to confirm VraFG is required for activation of GraS by acidic pH. However, how VraFG is involved in this novel GraS sensing mechanism remains a mystery. Our current hypothesis is that VraFG is required for proper assembly of the sensor system, and signal transduction from GraS to GraR, as is the case for GraX (23, 35). However, the extracellular loop of VraG has been shown to impact CAMP specificity for GraS signalling (36), indicating VraG directly interacts with external stimuli used for GraS signalling. Although acidic pH isn't a physical ligand that can bind to the extracellular loops of GraS or VraG, acidic pH has been shown to still interact with extracellular loops to induce signalling in two-component sensors. For example, the extracellular loop of PhoQ of Salmonella binds to CAMPs to activate the transcriptional regulator PhoP (37–39). However, independent of binding CAMPs, the extracellular loop of PhoQ can undergo conformational changes in response acidic pH that also leads to signalling (40). We have demonstrated mutations in the extracellular loop of GraS does not impact signalling in response to acidic pH, but VraG possesses a large extracellular loop similar to PhoQ, that is better suited for responding to external stimuli. Furthermore, the specific interactions between the extracellular regions of VraG and GraS have not been elucidated. Therefore, we plan to introduce mutations into this extracellular loop of VraG to determine if this extracellular loop is also required for GraS signalling in response to acidic pH.

While activation of GraS and expression of *mprF* are required for the novel uFFA resistance pathway we have discovered, we have not yet elucidated the exact mechanism for this resistance (17). We initially hypothesized the production of lysyl-phosphatidylglycerol by MprF would allow for tighter packing of phospholipids, stabilizing the membrane and counteracting the toxicity associated with uFFA membrane disruption, as has been shown for CAMP resistance (41); however, we demonstrated that the synthesis of lysyl-phosphatidylglycerol was not required for uFFA resistance, rather it

was the flippase domain of MprF that provided the high levels of uFFA resistance (17). In more recent studies, a gain-of-function mutation in the flippase domain of MprF was shown to confer increased resistance to the lipopeptide antibiotic daptomycin (9). The proposed model for this resistance is that the mutation in MprF altered the substrate range of the MprF flippase, leading to translocation of daptomycin from the inner leaflet of the membrane (9). A degree of promiscuity in the substrate specificity of MprF flippase had been established previously, with both alanyl- and lysyl-phosphatidylglycerol able to be translocated by this domain (11). Therefore, our current hypothesis is that this promiscuity of MprF flippase allows this domain to also interact with uFFA incorporated into phospholipid, to improve the distribution of these uFFA in the cell membrane, and better maintain proper membrane integrity.

The final limitation to our GraS-mediated uFFA resistance findings is how generalizable our findings are to other uFFA. We chose a variety of uFFA to study that are physiologically relevant to human skin and nasal colonization, namely linoleic and sapienic/palmitoleic acid (42, 43), as well as uFFA that have demonstrated unique mechanisms to impair *S. aureus*, namely arachidonic acid which causes oxidative stress for the bacteria (44). While these four fatty acids might not generalize to all uFFA *S. aureus* encounters in the environment, we believe they do provide sufficient evidence that this uFFA resistance mechanism is effective for a broad range of physiologically relevant uFFA. One caveat to these findings is that, unlike CAMP-mediated resistance which provides high levels of resistance to all uFFA tested, acidic pH made *S. aureus* more resistance to linoleic and arachidonic acid, but susceptible to sapienic and palmitoleic acid (17). Therefore, we believe it is important to understand why acidic pH makes *S. aureus* more susceptible to sapienic and palmitoleic acid before we can confidently generalize these findings to other uFFA. Our current hypothesis is that the toxicity occurring under combined acidic pH and 16:1 uFFA conditions is due to the acidic pH, rather than the uFFA. Studies have shown that palmitoleic acid causes rapid membrane depolarization and the release low-molecular weight proteins in *S. aureus*, through the production of small pores in the membrane (45). Under these conditions, we hypothesize H^+ can migrate into the cytoplasm, leading to the toxicity seen in our experiments. We plan to directly investigate membrane fluidity and integrity, as well as

intracellular and extracellular pH, under these conditions to determine if intracellular acidification is responsible for growth impairment. Additionally, we plan to determine if increasing membrane rigidity, or expressing high levels of ADI or urease genes, can combat this growth impairment by preventing acidification of the cytoplasm.

Together, our findings have broadened our understanding of GraXRS function in combating the innate immune conditions of human skin. While proud of our findings thus far, we plan to continue these investigations to better understand the nuances of this system, and possibly identify novel targets that will impair *S. aureus* resistance to the innate immune systems of the host.

4.2.2 FadXDEBA metabolism of host-derived fatty acids general discussion

Our study is first to directly assess the capacity of the *fadDEBA* genes in *S. aureus* to metabolize exogenous fatty acids; however, there are still unanswered questions we plan to address, in order to better understand the complex nature of lipid metabolism is *S. aureus*.

The first limitation to our study is we have not directly confirmed that the FadDEBA proteins allow for β-oxidation. While we have demonstrated the Fad proteins show homology to β-oxidation capable genes in *E. coli* and *B. subtilis*, are induced in response to exogenous fatty acids, are required for resistance to high concentrations of exogenous fatty acids, and show importance in an *in vivo* murine abscess infection model, we have not directly assessed if β-oxidation occurs through FadDEBA in *S. aureus*. However, this is a project we are currently working on, by investigating if exogenous palmitic acid is converted to palmitoyl-CoA in a Fad-dependent manner.

Although FadD, the first step in the putative β-oxidation pathway of *S. aureus*, is annotated as long chain fatty acyl-CoA ligase, it may also function as an acyl-ACP synthase, allowing for incorporation of exogenous fatty acids into the phospholipid synthesis pathway. In *Synechocystis* and *Thermus thermophilus*, genes annotated as long chain fatty acyl-CoA ligases can transfer fatty acids to ACP or CoA substrates (46). A dual function for FadD is supported by the fact acyl-ACP synthase activity has been

detected in *S. aureus* lysates, but the gene responsible for this function remains elusive (47), and we see USA300 and Δ*fadXDEBA* have differing capacities to incorporate exogenous palmitic acid into the phospholipid membrane in our own studies. Interestingly, *S. aureus* USA300 has an additional annotated long chain fatty acyl-CoA ligase, *vraA* (SAUSA300_0559), separate from the *fad* locus. VraA likely does not function as a redundant protein to FadD in the β-oxidation pathway of *S. aureus*, as we see a knockout of *fadD* alone phenocopies a complete deletion of the entire *fad* locus. Nevertheless, it is feasible that VraA could still function as an acyl-ACP synthase. Due to long chain acyl-CoA not being a suitable substrate for phospholipid synthesis in *S. aureus* (48, 49), generation of acyl-ACP through acyl-ACP synthase activity may represent an additional mechanism in *S. aureus* to incorporate exogenous fatty acids into phospholipid.

Although palmitic acid is the strongest inducer of *fad* expression, and caused the strongest growth impairment in a Δ*fadXDEBA* mutant, C14 and C18 fatty acids were also shown to impact Fad function. However, the preference of the Fad system for C16 fatty acids is interesting, as C16 fatty acids are poor substrates for FakA-mediated fatty acid metabolism (45), indicating these two systems may have coevolved to optimally metabolize the free fatty acids most frequently encountered on human skin, in human serum, and in the cytoplasm of *S. aureus* (15, 16). To gain insight into the relationship between these pathways, we plan on deleting *fakA* in conjunction with *fadXDEBA* to determine what occurs when both metabolic pathways are deleted. Thus far, we have been unsuccessful in creating this mutant strain, indicating the combined deletion of *fakA* and *fadXDEBA* may be lethal to the bacteria. Therefore, we plan to clone these genes under inducible promoters that allow control of expression, providing us the opportunity to turn off expression and phenotypically create our combined deletion mutants. Additionally, by tracking the presence of acyl-CoA molecules in *S. aureus* grown with a variety of different exogenous fatty acids, we plan to better understand the specificity of Fad to fatty acids other than C16 palmitic acid, and the relationship between fatty acid metabolism through FakA or Fad.

The relationship between FakA and Fad metabolism is further evident when looking at sFFA toxicity. Although less toxic than uFFA, we see deletion of *fad* makes *S. aureus* highly susceptible to C16 sFFA intoxication, a fatty acid that is a poor substrate for FakA metabolism (45), with C18 and C20 sFFA, fatty acids that are ideal substrates for FakA metabolism, causing markedly less toxicity. We believe these findings allude to FakA compensating for loss of Fad function and metabolizing C18 and C20 sFFA, but not being unable to compensate for metabolism of C16 sFFA. This compensation between systems is mirrored by C16 palmitic acid being the only sFFA we tested capable of inducing *fad* expression in wildtype USA300, but C18 and C20 sFFA also inducing *fad* expression when *fakA* was deleted. Although we have not yet identified the basis for palmitic acid toxicity in a Δ*fadXDEBA* mutant, we believe this data supports a model where fatty acid metabolism is balanced between FakA and Fad function, and deletion of Fad severely disrupts this balance. As a result, exogenous palmitic acid not only impairs growth, but causes a decrease is bacterial viability.

We have two main hypotheses to explain this palmitic acid toxicity, which vary depending on the role FakA plays in metabolizing C16 fatty acids. Because C16 fatty acids are a poor substrate for the FakA metabolic pathway (7, 45), we hypothesize a bottleneck in lipid metabolism is occurring at two potential points, leading to palmitic acid toxicity. First, if FakA does not efficiently phosphorylate the exogenous fatty acids, a buildup of cytoplasmic palmitic acid may occur. Although saturated fatty acids do not have the same structure of unsaturated fatty acids and therefore to not compromise membrane integrity as easily, intoxication can still occur at high enough concentrations (50). Therefore, we plan to use a variety of membrane integrity assays with our different mutants, to assess if a compromised membrane is responsible for this toxicity. Second, if FakA efficiently phosphorylates the exogenous fatty acids, there may be a rapid depletion in ACP molecules in the bacteria. Because phosphorylated C16 fatty acids are a poor substrate for PlsY, the acyl-PO_4 molecules are first converted to acyl-ACP through PlsX to be extended through the FASII cycle (7, 45), which could deplete the amount of ACPs available in the cell. ACPs are required for endogenous phospholipid synthesis, and the depletion of these proteins due to a bottleneck in C16 fatty acid metabolism may cause toxicity to the bacteria. To assesses this hypothesis, we plan to construct an inducible

ACP vector in order to control ACP levels in the bacteria, and determine if this can circumvent palmitic acid toxicity in Δ*fadXDEBA*. Both of these investigations will provide valuable insight into the metabolic fate of C16 fatty acid in *S. aureus*, and the associated toxicity with these lipid species.

Another question raised by our work is why β-oxidation has not been previously identified in *S. aureus* if it possesses the genes capable of this metabolic pathway. We have three theories for why this pathway was previously missed which include the role of glucose repression, the interaction with FakA, and the temporal regulation of *fad* expression. First, glucose is a known repressor of β-oxidation machinery in other bacteria (51), and this also appears to be the case for *S. aureus* (52). However, the primary media used to study *S. aureus* is Tryptic Soy Broth, which contains 13.9 mM glucose. Second, previous studies into lipid metabolism have inadvertently optimized conditions for FakA function, not FadXDEBA function (47, 53). Namely, studies of FakA function have typically employed oleic acid, which unlike palmitic acid does not induce *fad* expression (Figure 4.2), and have also usually sampled cells in mid-exponential growth, whereas peak *fad* expression occurs in transition between exponential and stationary growth phase. It may be that exogenous fatty acids are preferentially metabolized through incorporation into phospholipid, while *fad* expression occurs later in the growth phase in a role that could be associated with membrane homeostasis. Alternately, it is feasible that the cytoplasmic pool of fatty acids available to induce *fad* expression is kept low during exponential growth due to rapid phosphorylation by FakA, such that *fad* expression only occurs once growth begins to slow. Indeed, both of these explanations may be relevant.

In favour of a role for Fad in maintaining membrane homeostasis, we see a second stronger spike in *fad* expression in late stationary phase, which has been confirmed in other studies (54). This later spike in expression may represent an additional function for Fad in recycling membrane phospholipids to maintain membrane homeostasis, a function attributed to Fad in both *Sinorhizobium meliloti* and *E. coli* (55). We plan to further investigate the role Fad has on membrane phospholipid recycling and composition, especially during stationary growth phases, through a variety of metabolomic and phospholipid analyses.

A role for Fad in maintaining membrane homeostasis is further supported by PrsW, and by extension the alternative Sigma Factor σ^S, playing a significant role in regulating *fad* expression. PrsW is proposed to respond to membrane stress and modulate σ^S activity (56, 57), providing a direct relationship between membrane stress and *fad* expression. In lieu of an ortholog to the FadR regulator, seen for both *E. coli* and *B. subtilis* (51, 58–60), PrsW may function as the primary regulator of *fad* expression in *S. aureus*.

Although we have strong evidence to support PrsW as a regulatory mechanism for *fad* expression, there is still a lot unknown about how this regulation occurs. First, although PrsW is predicted to regulate the function of an alternative Sigma Factor, σ^S, through degrading an anti-sigma factor, no such substrate has been identified for PrsW in *S. aureus*. Furthermore, it is unclear if degradation of this anti-sigma factor occurs through a proteolytic cascade, as is the case for the PrsW associated anti-sigma factor RsiW in *B. subtilis* (61). Therefore, we plan to use copurification techniques to first isolate any potential substrates for proteolytic degradation through PrsW, and if the anti-sigma factor is discovered, we plan to further investigate the proteolytic steps required to free σ^S from this inhibition. Additionally, while we hypothesize degradation of the anti-sigma factor allows σ^S to regulate expression of *fad*, we have not yet directly shown this interaction. Therefore, we plan to use a biotinylated probe of the promoter region for *fadX* to pull down proteins which bind to this region, in both *fad* inducing and repressing conditions. Together, we believe a better understanding of the regulatory network that modulates *fad* expression will provide valuable insights into the nuances of fatty acid metabolism in *S. aureus*, and the relationship between the Fak and Fad pathways.

The ability to properly study the details of sFFA metabolism in *S. aureus* is complicated by high concentrations of fatty acids severely impairing growth of strains containing plasmids. Although we were able to partially overcome this limitation by using less toxic methyl-palmitate, future work will focus on complementation by chromosomal allelic exchange to provide a truer measure of restorative function. At this time, we are not sure why high concentrations of exogenous fatty acids impair the growth of our bacteria harbouring plasmids. However, even in the absence of plasmids, and irrespective of Fad function, we see high concentrations of palmitic acid stimulates increased pigmentation

of *S. aureus*, likely due to increased staphyloxanthin production during late exponential and stationary growth phases (62), and biofilm formation in *S. aureus* cultures grown to late stationary phases (Figure 4.3 and 4.4). Together, these findings show high concentrations of palmitic acid have pleiotropic effects on *S. aureus* growth, one of which being impaired growth when harbouring plasmids.

The final limitation in our study involves the use of the *fadX* promoter, the only gene not involved in the canonical β-oxidation pathway, to represent overall *fad* expression. Our reason for choosing this promoter is that the genetic layout of the *fad* locus favours *fadX* and *fadD* being co-transcribed. With FadD being the first step of β-oxidation, we wanted to assess expression of this gene as a measure of the overall expression of the β-oxidation machinery. Furthermore, previous studies, and our own unpublished RNA-seq data, indicate the *fad* genes are relatively co-expressed under inducing conditions (Table 3.1). However, additional experiments to confirm the inducibility and temporal regulation noted for *fadX* expression occurs for all *fad* genes may be beneficial. Additionally, the unique clustering of a short chain acyl-CoA transferase (FadX) with the canonical β-oxidation pathway in *S. aureus* is interesting. While FadX has been attributed to metabolizing and resisting the toxic effects of short chain (C3) fatty acids (63), it was dispensable in resisting palmitic acid toxicity in our study. Therefore, while FadX and FadDEBA appear to have unique functions, the clustering of genes into a single genetic locus warrants further investigation into the relationship between these pathways. We hypothesize this relationship may be due to FadX metabolizing the final carbon chain remaining following subsequent rounds of β-oxidation through FadDEBA, explaining why these two systems are grouped together in the *S. aureus* genome.

Together, our findings have demonstrated that the previously unstudied *fadDEBA* genes have an important role in palmitic acid resistance, a fatty acid physiologically relevant to *S. aureus* colonization of human skin (15, 16). Although a combination of bioinformatic analysis and our own experiments heavily indicate the FadDEBA proteins are capable of β-oxidation, we are currently working to confirm this theory. These findings provide a new understanding of lipid metabolism in *S. aureus*, and provide a foundation to build upon in future experiments.

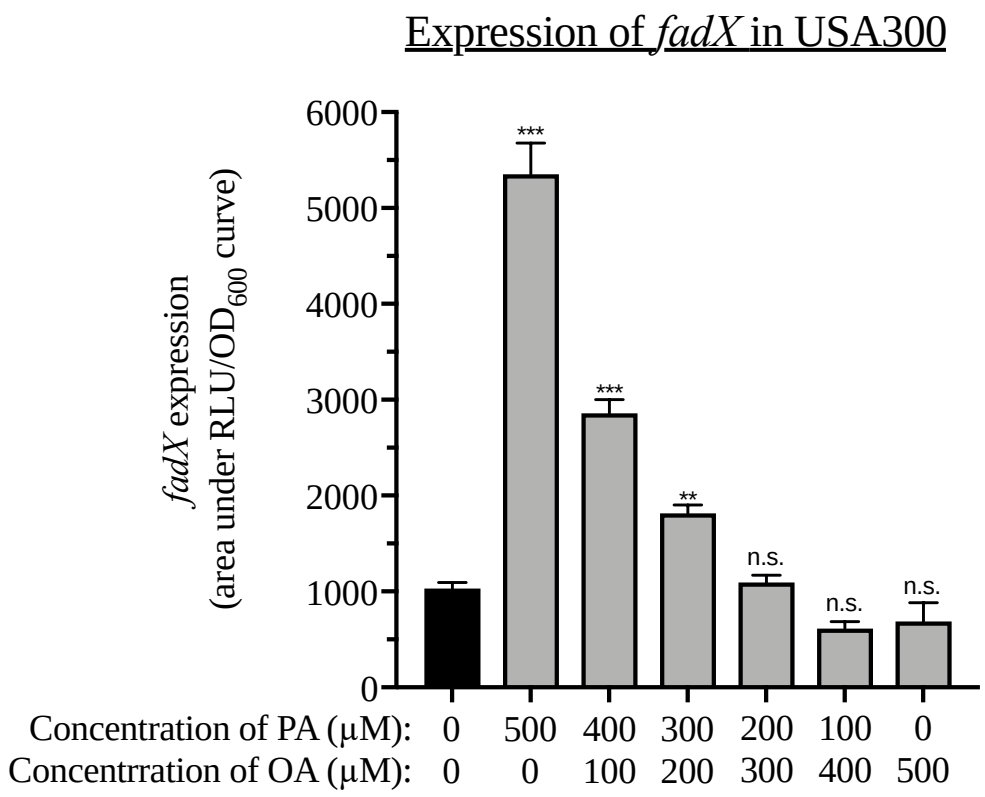

Figure 4.2. Palmitic acid (PA), but not oleic acid (OA), induces *fadX* expression. Expression of *fadX* was measured as relative luminesce units (RLU) from a pGY*fadX::lux* construct. Cultures were grown for 24 hours in 200 µL of TSB without glucose supplemented with the indicated concentration of PA or OA, n=12. Growth (OD_{600}) and *fadX* expression (RLU) were assessed every 20 minutes. Data was reported as mean ± SEM of the area under the RLU/OD_{600} curve. Data is reported as mean ± SEM. Statistical significance was measured using ordinary one-way ANOVA with Dunnett's multiple comparisons test, comparing all samples to the no fatty acid (0 µM PA, 0 µM OA) condition. *** $p < 0.001$, ** $p < 0.01$, n.s. = not significant.

Figure 4.3. Palmitic acid (PA) causes increased pigmentation in *S. aureus* cultures. Cultures of both wildtype USA300 and Δ*fadXDEBA* were inoculated to an initial optical density at a 600 nm wavelength (OD_{600}) of 0.01 in 25 mL of TSB without glucose. Cultures were supplemented with the indicated concentration of palmitic acid. Cultures were grown at 37ºC on an orbital shaker, shaking at 220 rpm, for 24 hours. Optical densities of all cultures were similar, at an OD_{600} of approximately 8.0. Cultures were grown in triplicate flasks, and the image taken is representative of flasks for each condition.

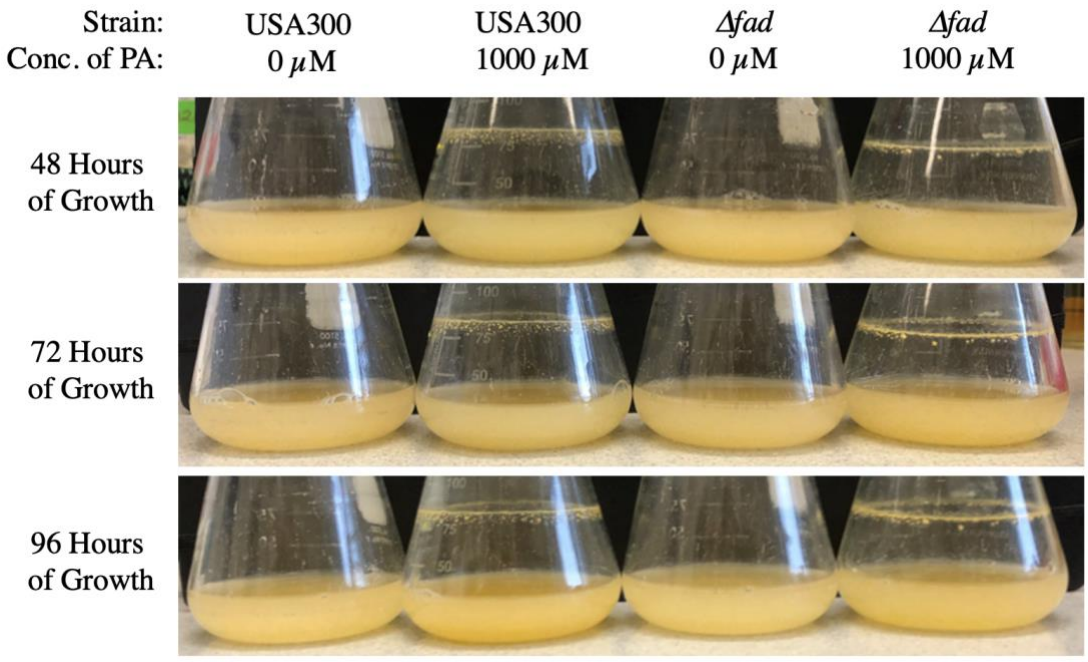

Figure 4.4. Palmitic acid (PA) induces production of biofilms at the air-liquid interface during shaking growth of *S. aureus* cultures. Cultures of both wildtype USA300 and Δ*fadXDEBA* were inoculated to an initial optical density at a 600 nm wavelength (OD_{600}) of 0.01 in 25 mL of TSB without glucose. Cultures were supplemented with the indicated concentration of palmitic acid. Cultures were grown at 37ºC on an orbital shaker, shaking at 220 rpm, and images were taken at 48, 72, and 96 hours. At 24 hours, no visible biofilms had formed in any flasks. Cultures were grown in triplicate flasks, and images were taken of a representative flask for each condition.

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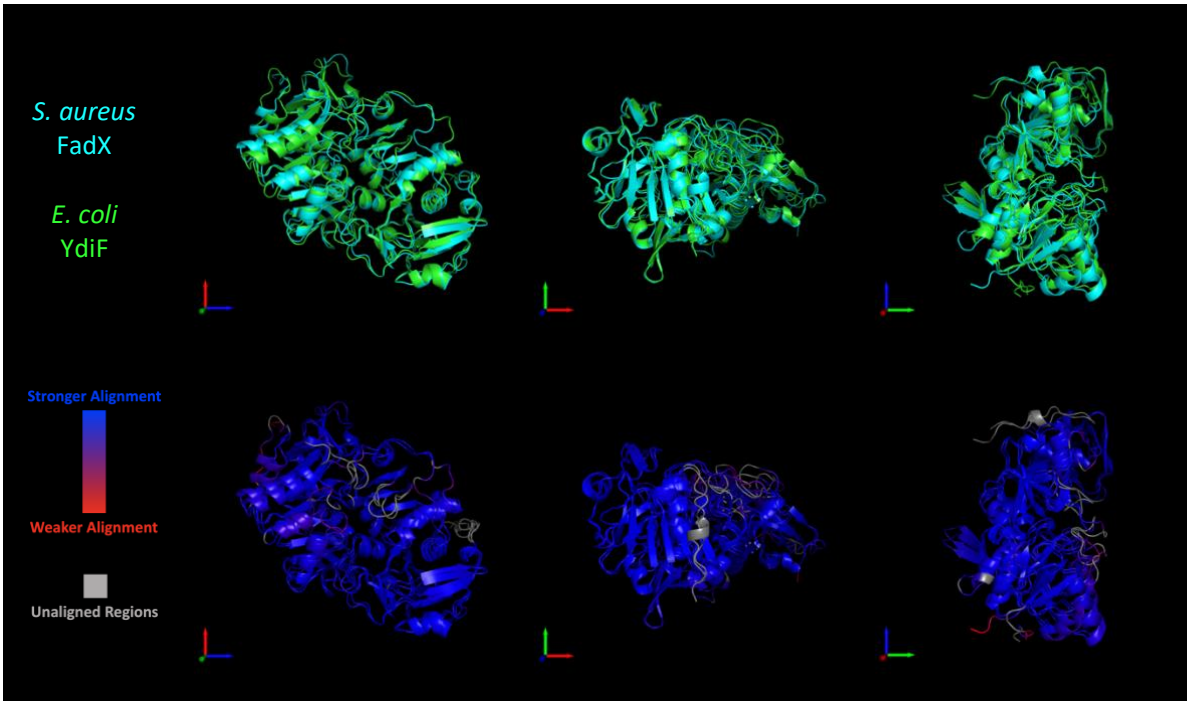
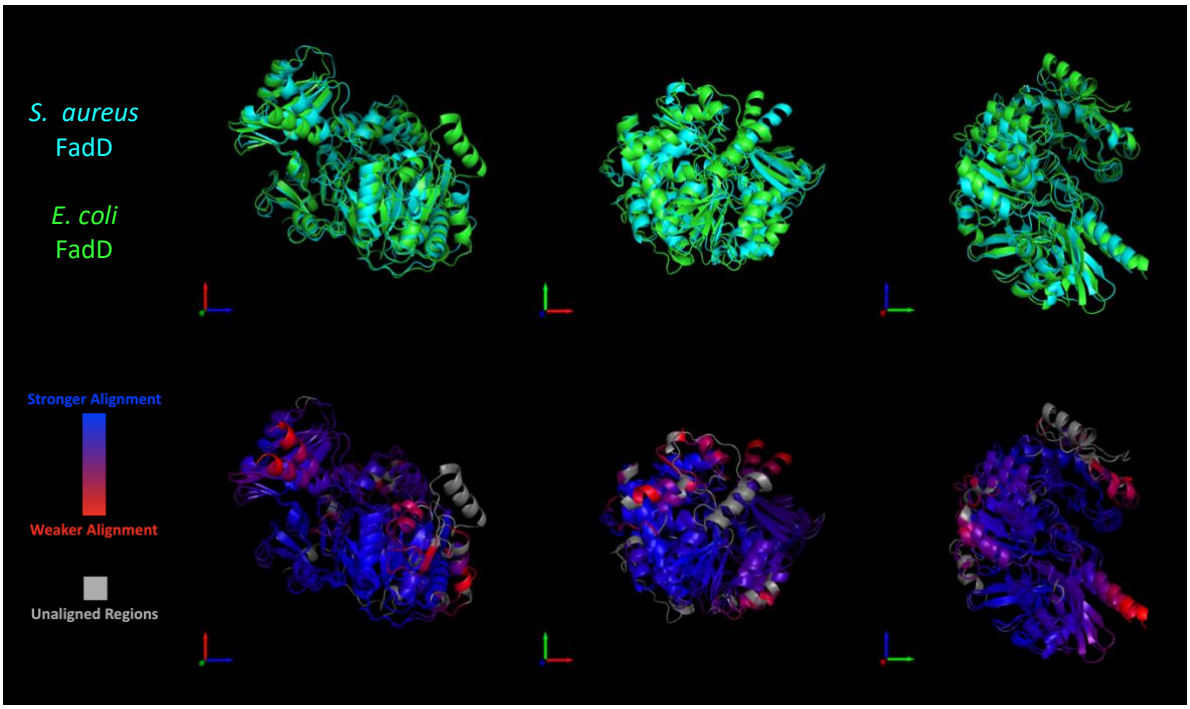
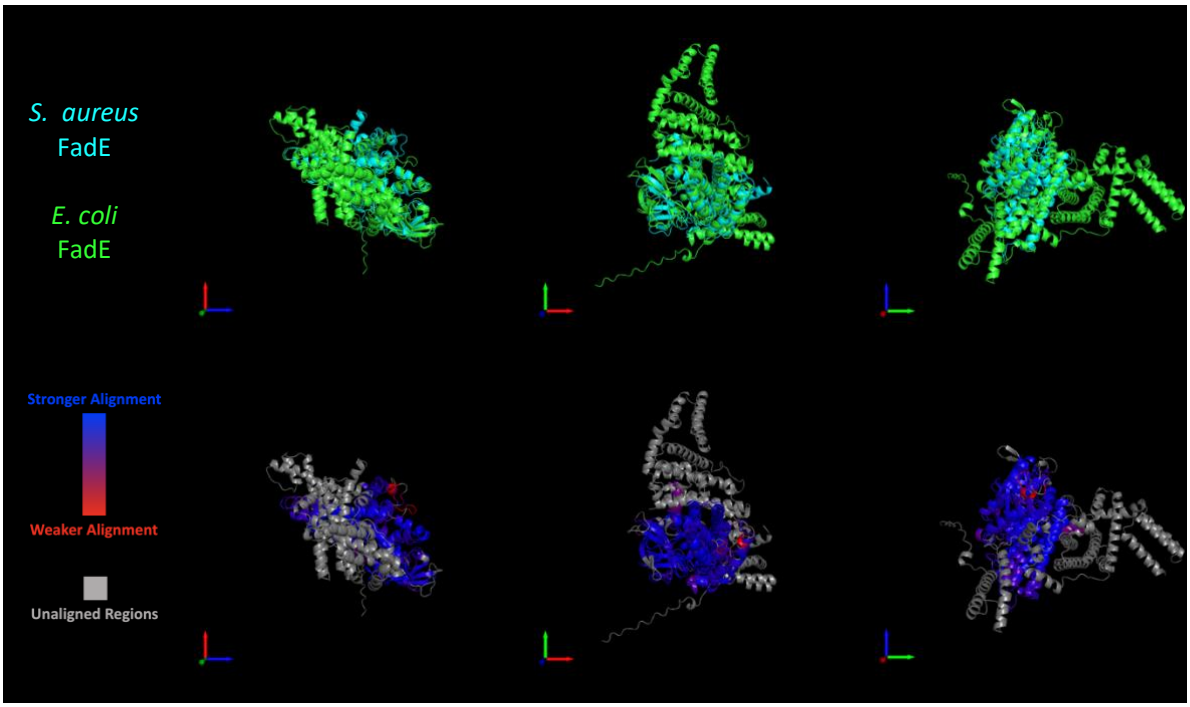
Appendices



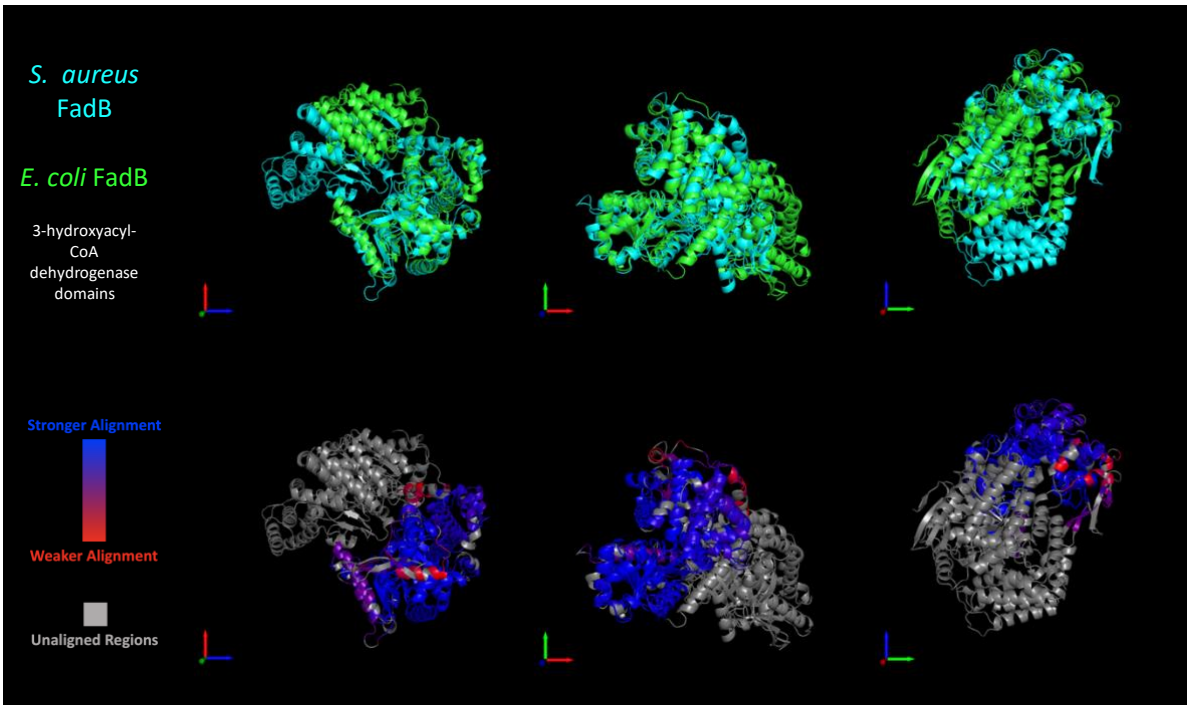
Appendix A. Structural alignment of the short chain acyl-CoA transferase in *S. aureus* (FadX) and *E. coli* (Ydif). Protein structures were predicted through AlphaFold and aligned through PyMol. Top panels show aligned structures of both proteins, with *S. aureus* coloured in cyan and *E. coli* coloured in light green. Bottom panels represent the same images as the top panels, however ColorByRMSD has been run on the protein alignment, applying a gradient of color to the protein residues with blue representing the stronger regions of alignment and red representing the weaker regions of alignment. Grey refers to residues that were not aligned between the proteins.



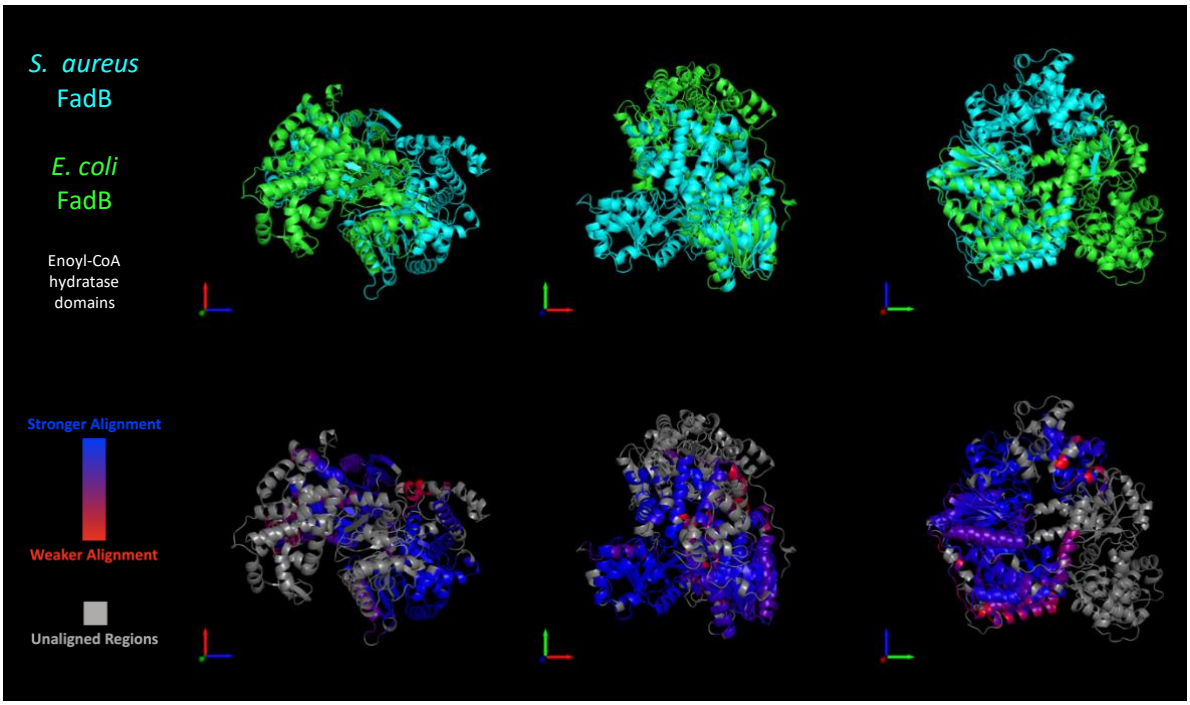
Appendix B. Structural alignment of the long-chain fatty acyl-CoA ligase FadD in *S. aureus* and *E. coli*. Protein structures were predicted through AlphaFold and aligned through PyMol. Top panels show aligned structures of both proteins, with *S. aureus* coloured in cyan and *E. coli* coloured in light green. Bottom panels represent the same images as the top panels, however ColorByRMSD has been run on the protein alignment, applying a gradient of color to the protein residues with blue representing the stronger regions of alignment and red representing the weaker regions of alignment. Grey refers to residues that were not aligned between the proteins.



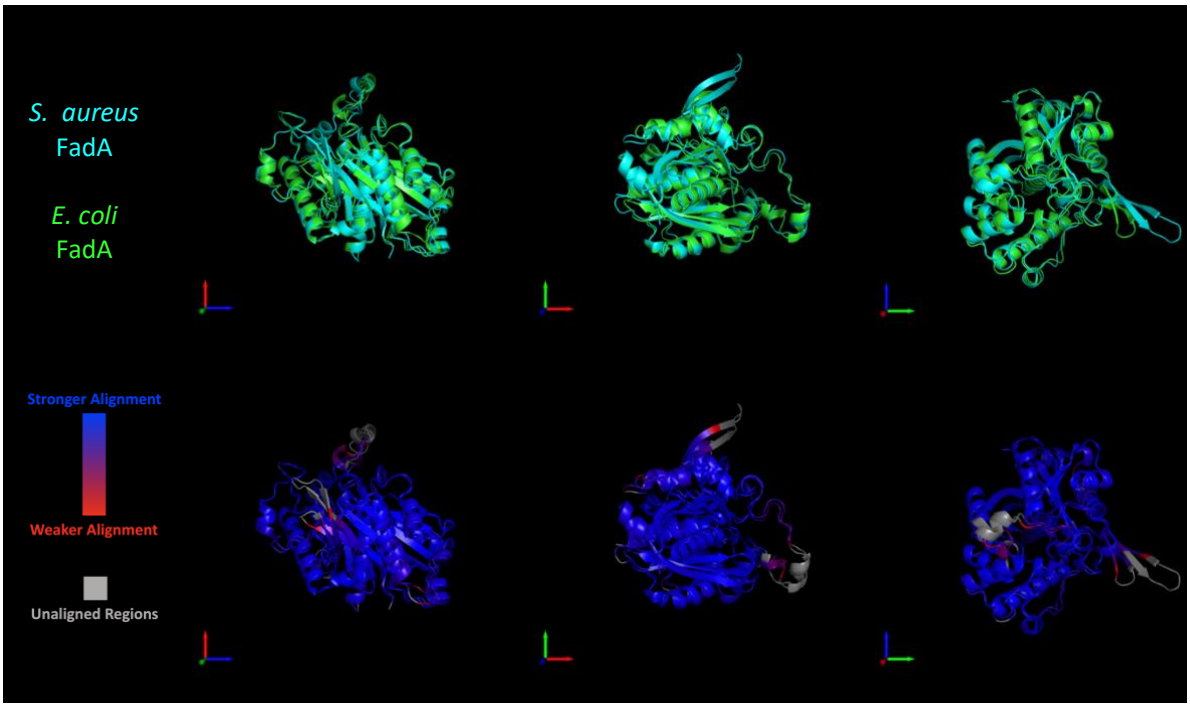
Appendix C. Structural alignment of the acyl-CoA dehydrogenase FadE in *S. aureus* and *E. coli.* Protein structures were predicted through AlphaFold and aligned through PyMol. Top panels show aligned structures of both proteins, with *S. aureus* coloured in cyan and *E. coli* coloured in light green. Bottom panels represent the same images as the top panels, however ColorByRMSD has been run on the protein alignment, applying a gradient of color to the protein residues with blue representing the stronger regions of alignment and red representing the weaker regions of alignment. Grey refers to residues that were not aligned between the proteins.



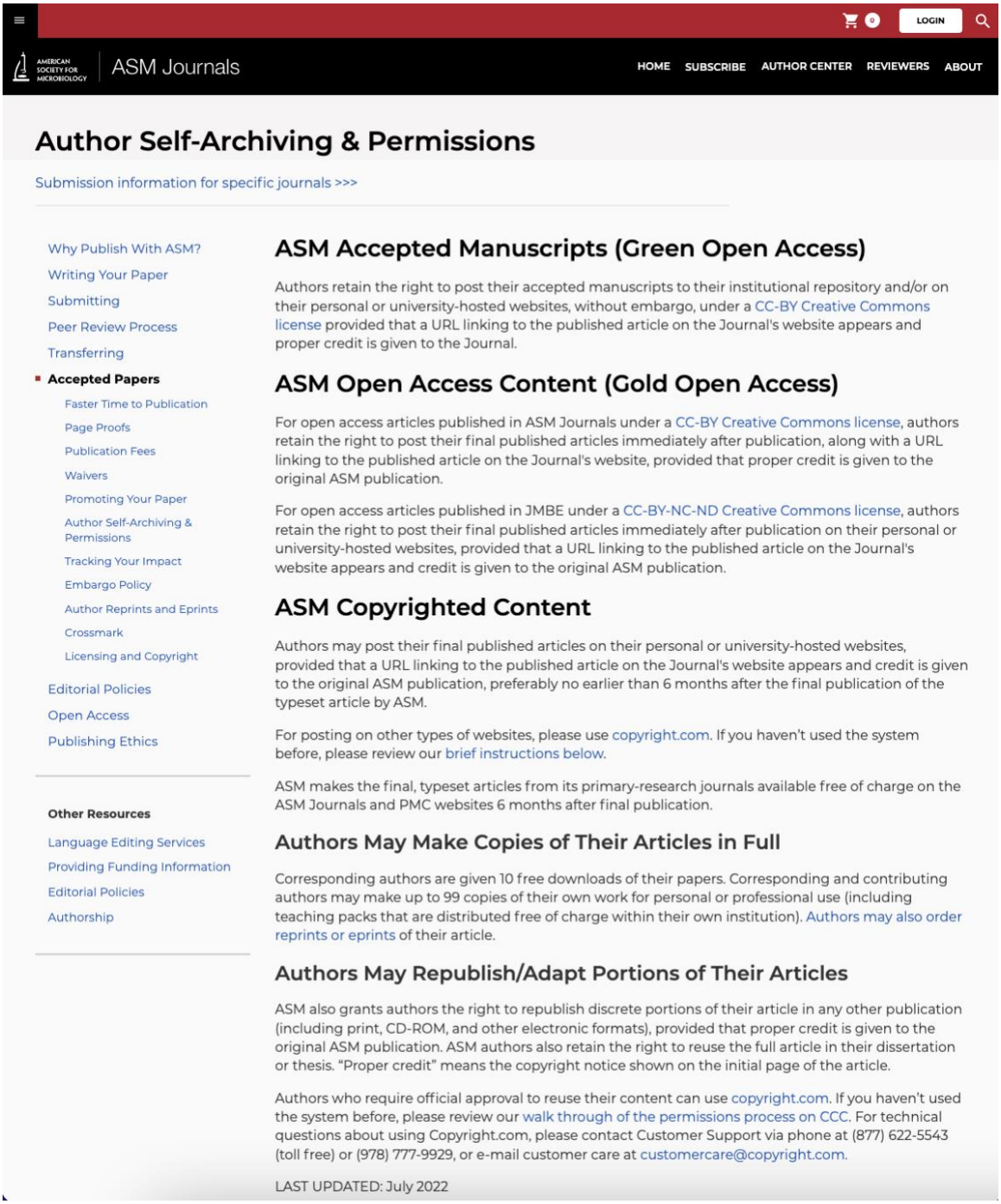
Appendix D. Structural alignment of the 3-hydroxyacyl-CoA dehydrogenase domain of FadB in *S. aureus* and *E. coli.* Protein structures were predicted through AlphaFold and aligned through PyMol. Top panels show aligned structures of both proteins, with *S. aureus* coloured in cyan and *E. coli* coloured in light green. Bottom panels represent the same images as the top panels, however ColorByRMSD has been run on the protein alignment, applying a gradient of color to the protein residues with blue representing the stronger regions of alignment and red representing the weaker regions of alignment. Grey refers to residues that were not aligned between the proteins.



Appendix E. Structural alignment of the enoyl-CoA hydratase domain of FadB in *S. aureus* and *E. coli.* Protein structures were predicted through AlphaFold and aligned through PyMol. Top panels show aligned structures of both proteins, with *S. aureus* coloured in cyan and *E. coli* coloured in light green. Bottom panels represent the same images as the top panels, however ColorByRMSD has been run on the protein alignment, applying a gradient of color to the protein residues with blue representing the stronger regions of alignment and red representing the weaker regions of alignment. Grey refers to residues that were not aligned between the proteins.



Appendix F. Structural alignment of the acetyl-CoA acyltransferase FadA in *S. aureus* and *E. coli*. Protein structures were predicted through AlphaFold and aligned through PyMol. Top panels show aligned structures of both proteins, with *S. aureus* coloured in cyan and *E. coli* coloured in light green. Bottom panels represent the same images as the top panels, however ColorByRMSD has been run on the protein alignment, applying a gradient of color to the protein residues with blue representing the stronger regions of alignment and red representing the weaker regions of alignment. Grey refers to residues that were not aligned between the proteins.



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- Language Editing Services
- Providing Funding Information
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Appendix G. ASM Journals author self-archiving and permissions.

Curriculum Vitae

Education

PhD candidate in Microbiology and Immunology — September 2017–Present
University of Western Ontario, London ON
- Transferred from Master's program in March 2019

Bachelor of Medical Sciences — September 2013–April 2017
University of Western Ontario, London ON
- Honours Specialization in Microbiology and Immunology with Scholars
- Western University Gold Medalist

Publications

Kuiack R, Tuffs S, Dufresne K, McCormick J, and McGavin M. 2023. **The *fadXDEBA* locus of *Staphylococcus aureus* is required for metabolism of exogenous palmitic acid and *in vivo* growth.** Mol Micro (under revisions).

Kuiack R, Veldhuizen R, and McGavin M. 2020. **Novel functions and signaling requirements for the GraS sensor kinase of *Staphylococcus aureus* in response to acidic pH.** J Bacteriol 202: e00219-20.

El-Halfawy O, Czarny T, Flannagan R, Day J, Bozelli Jr. J, Kuiack R, Salim A, Eckert P, Epand R, McGavin M, Organ M, Heinrichs D, and Brown E. 2019. **Discovery of an anti-virulence compound that reverses β-lactam resistance in MRSA.** Nat Chem Biol 16:143–149.

Alnaseri H, Kuiack R, Ferguson K, Schneider J, Heinrichs D, and McGavin M. 2019. **DNA binding and sensor specificity of FarR, a novel TetR family regulator required for induction of the fatty acid efflux pump FarE in *Staphylococcus aureus.*** J Bacteriol 201:e00602-18.

Flannagan R, Kuiack R, McGavin M, and Heinrichs D. 2018. ***Staphylococcus aureus* uses the GraXRS regulatory system to sense and adapt to the acidified phagolysosome in macrophages.** mBio 9:e01143-18.

Awards and Scholarships

Dr. FW Luney Graduate Travel Award in Microbiology and Immunology — Fall 2022
University of Western Ontario, Graduate Scholarship

2022 Canadian Student Health Research Forum Silver Medalist Summer 2022
University of Manitoba, Conference Award

NSERC Canadian Graduate Scholarship – Doctoral (NSERC CGS-D) Spring 2022
University of Western Ontario, Graduate Scholarship
* Converted from NSERC PGS-D awarded in September 2020

Dr. RGE Murray Microbiology and Immunology Graduate Student Seminar Award Fall 2021
University of Western Ontario, Graduate Scholarship

Infection and Immunity Research Forum 2020 – First Place Oral Presentation Fall 2020
University of Western Ontario, Conference Award

NSERC Postgraduate Scholarship – Doctoral (NSERC PGS-D) Fall 2020
University of Western Ontario, Graduate Scholarship
* Converted to NSERC CGS-D in March 2022

Queen Elizabeth II Ontario Graduate Scholarship in Science and Technology Fall 2020 (Declined)
University of Western Ontario, Graduate Scholarship, Declined

Queen Elizabeth II Ontario Graduate Scholarship in Science and Technology Fall 2019
University of Western Ontario, Graduate Scholarship

The Dr. John Robinson Graduate Scholarship Fall 2019
University of Western Ontario, Graduate Scholarship

Dr. RGE Murray Microbiology and Immunology Graduate Student Seminar Award Fall 2019
University of Western Ontario, Graduate Scholarship

Dr. FW Luney Graduate Travel Award in Microbiology and Immunology Summer 2019
University of Western Ontario, Graduate Scholarship

Dr. FW Luney Graduate Travel Award in Microbiology and Immunology Fall 2018
University of Western Ontario, Graduate Scholarship

International Conference on Gram-Positive Pathogens Travel Award Fall 2018
University of Nebraska Medical Center, Conference Award

Alexander Graham Bell Canada Graduate Scholarship - Master's (NSERC CGS-M) University of Western Ontario, Graduate Scholarship	Fall 2018
Queen Elizabeth II Ontario Graduate Scholarship in Science and Technology University of Western Ontario, Graduate Scholarship, Declined	Fall 2018 (Declined)
Dr. RGE Murray Graduate Scholarship in Microbiology and Immunology University of Western Ontario, Graduate Scholarship	Fall 2017
Dr. FW Luney Graduate Entrance Scholarship in Microbiology and Immunology University of Western Ontario, Graduate Scholarship	Fall 2017
Western University Microbiology and Immunology Gold Medal University of Western Ontario, Academic Prize	Spring 2017
Dean's Honour List University of Western Ontario, Academic Prize	Fall 2013– Spring 2017
Western Four Year Continuing Admission Scholarship University of Western Ontario, Undergraduate Scholarship	Fall 2013– Spring 2017
Summer Undergraduate Research Fellowship Award University of Western Ontario, Research Grant	Summer 2015 and 2016

Conference Presentations (Oral)

Kuiack R and McGavin M. From survive to thrive: Evaluating the role of GraS and novel fatty acid metabolic pathways in promoting *Staphylococcus aureus* adaptation to antimicrobial free fatty acids of human skin. 2021 CSM Annual Conference, Virtual Conference.	June 2021
Kuiack R and McGavin M. Evaluating the role of GraS and novel fatty acid metabolic pathways in promoting *Staphylococcus aureus* adaptation to antimicrobial free fatty acids of human skin. Infection and Immunity Research Forum, Virtual Conference.	October 2020
Kuiack R and McGavin M. Evaluating the role of GraS in promoting *Staphylococcus aureus* adaptation to combined antimicrobial conditions of human skin. London Health Research Data, London, Ontario.	March 2019

Kuiack R and McGavin M. Evaluating the role of GraS in promoting *Staphylococcus aureus* adaptation to combined antimicrobial conditions of human skin. International Conference on Gram-Positive Pathogens, Omaha, Nebraska. October 2018

Conference Presentations (Poster)

Kuiack R, Tuffs S, Dufresne K, McCormick J, and McGavin M. From survive to thrive: Evaluating the role of GraS and novel fatty acid metabolic pathways in promoting *Staphylococcus aureus* adaptation to antimicrobial free fatty acids of human skin. Infection and Immunity Research Forum, London, Ontario. November 2022

Kuiack R, Tuffs S, Dufresne K, McCormick J, and McGavin M. From survive to thrive: Evaluating the role of GraS and novel fatty acid metabolic pathways in promoting *Staphylococcus aureus* adaptation to antimicrobial free fatty acids of human skin. 8th International Conference on Gram Positive Pathogens, Omaha, Nebraska. October 2022

Kuiack R, Tuffs S, Dufresne K, McCormick J, and McGavin M. From survive to thrive: Evaluating the role of GraS and novel fatty acid metabolic pathways in promoting *Staphylococcus aureus* adaptation to antimicrobial free fatty acids of human skin. 2022 CSM Annual Conference, Guelph, Ontario. June 2022

Kuiack R, Tuffs S, Dufresne K, McCormick J, and McGavin M. From survive to thrive: Evaluating the role of GraS and novel fatty acid metabolic pathways in promoting *Staphylococcus aureus* adaptation to antimicrobial free fatty acids of human skin. Canadian Student Health Research Forum, Winnipeg, Manitoba. June 2022

Kuiack R, Tuffs S, Dufresne K, McCormick J, and McGavin M. From survive to thrive: Evaluating the role of GraS and novel fatty acid metabolic pathways in promoting *Staphylococcus aureus* adaptation to antimicrobial free fatty acids of human skin. London Health Research Day, London, Ontario. May 2022

Kuiack R, and McGavin M. From survive to thrive: Evaluating the role of GraS and novel fatty acid metabolic pathways in promoting *Staphylococcus aureus* adaptation to antimicrobial free fatty acids of human skin. London Health Research Day, Virtual Conference. May 2021

Kuiack R, and McGavin M. Evaluating the role of GraS in promoting *Staphylococcus aureus* adaptation to combined antimicrobial conditions of human skin. Infection and Immunity Research Forum, London, Ontario. November 2019

Kuiack R, and McGavin M. Evaluating the role of GraS in promoting *Staphylococcus aureus* adaptation to combined antimicrobial conditions of human skin. 2019 CSM Annual Conference, Sherbrooke, Quebec.	June 2019
Kuiack R, and McGavin M. Evaluating the role of GraS in promoting *Staphylococcus aureus* adaptation to combined antimicrobial conditions of human skin. Infection and Immunity Research Forum, London, Ontario.	October 2018
Kuiack R, and McGavin M. Evaluating the role of ApsS in promoting *Staphylococcus aureus* adaptation to combined antimicrobial conditions of human skin. London Health Research Data, London, Ontario.	May 2018
Kuiack R, and McGavin M. Evaluating the role of sensor and metabolic kinases in promoting *Staphylococcus aureus* adaptation to acidic pH and antimicrobial unsaturated free fatty acids. Infection and Immunity Research Forum, London, Ontario.	October 2017
Kuiack R, and McGavin M. Regulation of *Staphylococcus aureus* virulence in response to membrane phospholipid composition. London Health Research Data, London, Ontario.	March 2017
Kuiack R, and McGavin M. Regulation of *Staphylococcus aureus* virulence in response to membrane phospholipid composition. Infection and Immunity Research Forum, London, Ontario.	September 2016

Relevant Work Experience

MICROIMM 3610F Graduate Teaching Assistant University of Western Ontario	Fall 2018, 2019, 2020, and 2021
Research Assistant in the McGavin Lab University of Western Ontario	Summer 2015 and 2016

Relevant Extracurricular and Leadership Activities

Microbiology and Immunology Graduate Studies Committee Representative University of Western Ontario	September 2020–June 2022
Microbiology and Immunology Graduate Student Representative University of Western Ontario	September 2019–June 2022

Society of Graduate Students (SOGS) Representative University of Western Ontario	September 2019– September 2020
Infection and Immunity Research Forum Organizer University of Western Ontario	September 2017– March 2023
Mentor/Supervisor for 4th-year Undergraduate Thesis Students University of Western Ontario	September 2017– Present
Health Occupations Students of America Head Biomedical Laboratory Trainer University of Western Ontario	April 2016– September 2017
Health Occupations Students of America VP Communications University of Western Ontario	January 2016– April 2016
Western Orientation Volunteer (Soph) University of Western Ontario	September 2014– September 2015