Characterization of fatty acid resistant Staphylococcus aureus mutants containing SNPs in cvfA

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Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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Characterization of fatty acid resistant *Staphylococcus aureus* mutants containing SNPs in *cvfA*

(Thesis format: Monograph)

by

Melissa N. Loyzer

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Upon colonization, Staphylococcus aureus must withstand the actions of many host defense mechanisms, including the unsaturated free fatty acids (uFFAs) secreted in sebum. Linoleic acid, a representative uFFA, is toxic to S. aureus at concentrations ≥50 µM; however, we selected for mutants capable of growing at elevated concentrations of linoleic acid. Five mutants had single nucleotide polymorphisms (SNPs) in the cvfA gene encoding an RNase that is a component of the RNA degradosome, and expression of the SNP-containing cvfA genes from a plasmid engendered S. aureus with high level resistance to linoleic acid. The SNPs were located within a region of the protein with unknown function, between the RNA-binding and catalytic domains. We have confirmed that at least one of the SNPs does not impact CvfA catalytic activity. Further research is required to determine the mechanism by which SNPs in cvfA confer increased resistance to uFFAs.

Keywords

Staphylococcus aureus, USA300, fatty acids, RNase, CvfA, degradosome

Co-Authorship Statement

Experiments leading to the identification of SNP-containing strains were performed by Benjamin Arsic.
Acknowledgments

I have learned more than I thought possible over the past two years and I am greatly appreciative to my supervisor, Dr. David Heinrichs, for giving me this opportunity. It is because of his guidance, support, and advice that I have grown as a researcher and feel prepared to continue to the next phase of my career. I have also benefited tremendously from having supportive and motivating lab-mates and am very thankful for all of their assistance with daily obstacles in the lab and for their friendship outside of the lab. Additionally, I would like to thank Dr. Jimmy Dikeakos and Dr. Martin McGavin for their direction as committee members. I have truly valued their understanding and willingness to help throughout the hurdles of my project.

None of this would have been possible without the support of my family. My parents have made many sacrifices to ensure that my brothers and I could pursue whatever career we chose, and I am eternally grateful for all of their help and support. I know that no matter where I end up in life, I will always be able to lean on them. Finally, I would like to thank my husband, Huck. Whether I have a bad day or a great day, he is always there for me. I am so lucky to have him in my life supporting and encouraging me in whatever endeavour I choose to embark on next.
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<tr>
<td>4-NP</td>
<td>4-nitrophenyl</td>
</tr>
<tr>
<td>$A_{405\text{ nm}}$</td>
<td>Absorbance at 405 nanometers</td>
</tr>
<tr>
<td>ACME</td>
<td>Arginine catabolic mobile element</td>
</tr>
<tr>
<td>agr</td>
<td>Staphylococcal accessory gene regulator</td>
</tr>
<tr>
<td>AIP</td>
<td>Autoinducing peptide</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>bis-4-NPP</td>
<td>Bis-4-nitrophenyl phosphate</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>Community-acquired MRSA</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CvfA</td>
<td>Conserved virulence factor A</td>
</tr>
<tr>
<td>DDM</td>
<td>n-dodecyl β-D-maltoside</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Ess</td>
<td>ESAT-6 secretion system</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid modifying enzyme</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>HA-MRSA</td>
<td>Healthcare-associated MRSA</td>
</tr>
<tr>
<td>hBD-1</td>
<td>Human β-defensin 1</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LRGC</td>
<td>London Regional Genomics Center</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MSCRAMM</td>
<td>Microbial surface components recognizing adhesive matrix molecule</td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin sensitive <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OG</td>
<td>Octyl-β-D-glucopyranoside</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PVL</td>
<td>Panton-Valentine leukocidin</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RPKM</td>
<td>Reads per kilobase of transcript per million mapped reads</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>Sae</td>
<td><em>S. aureus</em> exoprotein expression</td>
</tr>
<tr>
<td>SarA</td>
<td>Staphylococcal accessory regulator A</td>
</tr>
<tr>
<td>SCCmec</td>
<td>Staphylococcal cassette chromosome <em>mec</em></td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
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</table>
TSB  Tryptic soy broth
TSST-1  Toxic shock syndrome toxin-1
uFFA  Unsaturated free fatty acid
VISA  Vancomycin intermediate *Staphylococcus aureus*
VRE  Vancomycin resistant enterococci
VRSA  Vancomycin resistant *Staphylococcus aureus*
WTA  Wall teichoic acid
Chapter 1

1 Literature Review

*Staphylococcus aureus* is a common human pathogen that poses a significant burden to health care systems worldwide. It is one of the ESKAPE superbugs, a group of pathogens encompassing *Enterococcus faecium, S. aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species (1).

These pathogens are responsible for a majority of nosocomial infections and are capable of ‘escaping’ the actions of antibiotics. *S. aureus* employs a multitude of virulence and colonization factors that contribute to its success in causing infections, in part by helping withstand attack by the human immune system. The antibacterial free fatty acids on skin and in nasal secretions are among our first lines of defense against *S. aureus* and are known to dramatically increase in concentration during abscess formation (2). Further characterization of the mechanisms employed by *S. aureus* to overcome this component of innate immunity will increase our understanding of the first stages of infection. This will provide insight into the role of fatty acids in controlling infection, and is the subject of this thesis work.

1.1 Staphylococci and *Staphylococcus aureus*

The staphylococci are gram-positive firmicutes characterized by their spherical morphology and tendency to grow in grape-like clusters. They are facultative anaerobes, highly halotolerant with growth able to occur in >2M NaCl, and are catalase-positive,
allowing them to be distinguished from the catalase-negative streptococci (3). The majority of *S. aureus* strains are coagulase-positive while many other staphylococcal species are coagulase-negative. *S. aureus* is a common cause of infectious diseases and coagulase aids in prevention of host cell attack by acting within the blood to convert fibrinogen to fibrin which then adheres to *S. aureus* cells, shielding them from the host immune system (3). The coagulase-negative staphylococci had previously been regarded as non-pathogenic, or associated with low pathogenicity, however this view has since changed and many coagulase-negative staphylococci are now known to be responsible for causing opportunistic invasive infections (4).

There are over 45 staphylococcal species (4) and while many are able to colonize humans, arguably the most well-known are *S. epidermidis* and *S. aureus*. *S. epidermidis* is non-pigmented and less commonly associated with infections whereas *S. aureus* produces a golden colour and is a leading cause of both hospital- and community-acquired infections. The golden pigment after which *S. aureus* was named is due to the biosynthesis of staphyloxanthin, an orange carotenoid (5). A major role for staphyloxanthin is that of an antioxidant, defending *S. aureus* against reactive oxygen species thereby increasing its fitness and virulence (6, 7). Aside from staphyloxanthin, the staphylococci are known to harbour a diverse arsenal of virulence factors, further discussed in section 1.1.2, that aid in their remarkable success as pathogens.

### 1.1.1 Clinical relevance

*S. aureus*, both a commensal and a dangerous pathogen, presents a significant healthcare burden as it is the most common cause of infective endocarditis in industrialized nations
(8) and lower respiratory tract and surgical site infections (9). It is also the second most common cause of nosocomial bacteremia (10), cardiovascular infections, and pneumonia in the United States (9). However, it is also a significant cause of many other infections, such as skin and soft tissue infections, osteomyelitis, and cellulitis. Interestingly, the most common cause of nosocomial bacteremia was the coagulase-negative staphylococci and therefore, if all staphylococci are considered as a single group, they were found to be responsible for 51% of hospital-acquired bloodstream infections (10).

While the coagulase-negative staphylococci are becoming increasingly more common causes of infections, *S. aureus* reigns supreme in its prevalence and ability to cause infections. High colonization rates of *S. aureus* are in part responsible for its success as a pathogen, as individuals who are colonized serve as reservoirs for *S. aureus* transmission within the community. The most common bodily site colonized by *S. aureus* is the anterior nares, but it also regularly colonizes the skin, perineum, axillae, and pharynx (11). It is considered an opportunistic pathogen as many individuals are asymptomatically colonized – approximately 20% of the population is persistently colonized, 30% are transiently colonized, and 50% are non-carriers (12). Although individuals that are colonized have a higher likelihood of infection, they also have a better prognosis if they do contract an infection (13). Specifically, carriers were found to have a 3-fold greater risk of nosocomial *S. aureus* bacteremia over non-carriers; however, mortality was higher in the non-carriers at 46% compared to the carriers at 18% (13). Additionally, colonized individuals are more likely to become infected with their resident *S. aureus* strain as opposed to a foreign strain (14).
Unfortunately, infections caused by *S. aureus* are not simple to treat as this species has acquired resistance to many antibiotics. It was not long after the introduction of methicillin in the clinic that methicillin resistant *S. aureus* (MRSA) was first identified in the United Kingdom in 1961 (15). This was followed by relatively infrequent outbreaks of MRSA in the UK and the first hospital outbreak in the United States in Boston, MA in 1968 (16). In US hospitals in 2003, the economic burden of *S. aureus* infections was $14.5 billion while the overall mortality rate for inpatient stays was 5.6% (17). However, for *S. aureus* bacteremia cases, the mortality rate was found to be as high as 42% when caused by MRSA and 28% for methicillin-sensitive *S. aureus* (MSSA) (18). These devastating statistics highlight the significant impact of *S. aureus* infections on society and outline the need for additional and improved therapeutics.

While the hospital outbreaks are concerning, there has been another source of serious concern developing over the past couple decades regarding the emergence of community-acquired MRSA (CA-MRSA). CA-MRSA infections are those obtained outside of the healthcare setting in individuals lacking classic risk factors. The first reported case of CA-MRSA was in 1980 in Detroit, Michigan (19) but it wasn’t until the mid-1990s that a significant number of reports on the subject began to surface (20–24). Since then, cases of CA-MRSA have risen dramatically. For example, one study based out of an Atlanta, Georgia hospital and outpatient clinics found CA-MRSA strains to be responsible for 87% of MRSA infections (25). It is important to note, however, that the line between CA-MRSA and healthcare-associated MRSA (HA-MRSA) strains has blurred and both strains can be found in the community and in hospital settings (26, 27).
Traditionally, HA-MRSA strains were considered to be resistant to a greater number of antibiotics than CA-MRSA strains, while the CA-MRSA strains generally have increased virulence potential (28, 29). These differences led researchers to question the initial assumption that CA-MRSA strains evolved from HA-MRSA strains and it was determined that CA-MRSA was more likely to have arisen from MSSA strains that were already circulating in the community (30–32). Although CA-MRSA strains have historically been susceptible to non-β-lactam antibiotics, it is becoming increasingly common for these strains to be resistant to other antibiotics, such as erythromycin and clindamycin (33). Regardless, CA-MRSA strains are very successful pathogens that are capable of causing serious and even fatal infections (20).

### 1.1.2 Virulence factor regulation and pathogenesis

The ability of *S. aureus* to cause severe and diverse infections is due in part to its large arsenal of virulence factors. Three important regulators of virulence in this pathogen are the *agr* (staphylococcal accessory gene regulator), *sarA* (staphylococcal accessory regulator), and *sae* (*S. aureus* exoprotein expression) systems. Control of virulence is complex and the regulons of many virulence regulators are interconnected, with overlap in the factors whose expression they control. These global regulators are particularly crucial *in vivo* where their activity allows for rapid adaptation to the changing environments encountered by *S. aureus*, ensuring expression of appropriate factors that allow for colonization and subsequent dissemination.

The *agr* quorum-sensing system is crucial for cell-cell signaling and regulation of virulence factors throughout various growth stages (34). It is responsible for increasing
the expression of exoproteins (e.g. toxins, exoenzymes) while decreasing the expression of cell surface proteins (e.g. extracellular matrix adhesins and IgG binding proteins) during the late-exponential and stationary growth phases (35). The agr system comprises two transcripts, RNAII and RNAIII, that are divergently transcribed from the P2 and P3 promoters, respectively (36, 37). RNAII encodes 4 proteins that are responsible for the quorum sensing functions of this system, AgrB, AgrD, AgrC, and AgrA. AgrB is a membrane protein that serves to process AgrD into the autoinducing peptide (AIP) as well as to translocate it into the extracellular environment during exponential phase. AgrA and AgrC form a two-component sensing system whereby AgrC is the transmembrane receptor for the AIP. Upon binding of the AIP to AgrC, AgrC undergoes a conformational change, resulting in homodimerisation and autophosphorylation. This phosphate is then transferred onto AgrA, a cytoplasmic protein, causing conformational changes within AgrA that then allows it to bind the P2 and P3 promoters, resulting in increased transcription of these operons and completing the positive feedback loop. The RNAIII transcript is the effector of the agr system and also encodes hld, δ-hemolysin (37). As the effector, RNAIII increases the transcription and/or translation (38) of various secreted virulence factors such as toxic shock syndrome toxin-1 (TSST-1), Panton-Valentine leukocidin (PVL) (39), and a variety of hemolysins and proteases (34, 40). It also decreases the expression of select surface-associated proteins, such as Protein A and fibronectin-binding protein (41, 42).

In addition to the positive feedback mechanism mediated by AgrA, SarA activates expression of the agr operon (43), and SarR acts to repress RNAII transcription (44). The SarA protein family consists of DNA binding proteins that alter expression of
virulence genes. The *agr* operon and SarA protein family are intricately entwined as *agr* activation results in repression of *sarS* and *sarT* transcription, which activate protein A and repress α-hemolysin, respectively (45, 46).

Another important virulence regulator is the *saePQRS* system, which is composed of four genes transcribed from two promoters, producing a total of four transcripts (47, 48). The T1 transcript is transcribed from the P1 promoter and includes all four genes. It is processed by endonucleolytic cleavage by CvfA (RNase Y), producing the T2 transcript which no longer contains *saeP* (49). The T3 transcript is transcribed from the P3 promoter and contains *saeR* and *saeS*, and the T4 transcript is monocistronic, containing only *saeP*. SaeS and SaeR form a two-component system consisting of a sensor histidine kinase and response regulator, respectively. The sensor, SaeS, is membrane-bound and upon sensing an environmental signal, it autophosphorylates and subsequently transfers this phosphate to the regulator, SaeR (50). It has been suggested that SaeS monitors disruptions to the membrane, but this has not been conclusively shown (51). Phosphorylation of SaeR results in a conformational change that allows it to bind DNA, influencing the expression of numerous targets including α-hemolysin, β-hemolysin, coagulase, DNase, and extracellular protein A (47, 52). The role of SaeP and SaeQ has only recently been elucidated and they have been shown to act in concert to turn off SaeS by activating its phosphatase activity, resulting in the dephosphorylation of SaeR (53).

The virulence factors governed by these regulators are numerous and fundamental to the pathogenesis of *S. aureus* infections. Virulence factors such as the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) are important in
order for \textit{S. aureus} to establish colonization, and factors such as hemolysins, proteases, and lipases are important for tissue invasion and destruction (54). There are also various toxins responsible for specific diseases, such as toxic shock syndrome caused by TSST-1, food poisoning caused by staphylococcal enterotoxins, and scalded skin syndrome caused by exfoliative toxins. TSST-1 and the enterotoxins are superantigens which are exoproteins that bind to MHC class II molecules regardless of antigen specificity and link them to T-cell receptors. This action results in a substantial increase in the number of activated T cells and causes the release of massive amounts of cytokines, which are responsible for the symptoms associated with the disease. The exfoliative toxins cleave desmoglein-1, a human protein involved in keratinocyte cell-cell adhesion, thereby disrupting the integrity of the skin and causing the blistering that is characteristic of scalded skin syndrome (55). Evidently, \textit{S. aureus} is very well-equipped in terms of virulence factors producing disease; however, another important factor in the pathogenesis of \textit{S. aureus} infections is the ability of this pathogen to evade detection and destruction by the host’s immune system.

\subsection*{1.1.3 Immune evasion}

Throughout the course of establishing an infection, a bacterium must survive or avoid assault by the host’s immune system and this is a skill that \textit{S. aureus} has mastered. \textit{S. aureus} evades detection by utilizing proteins such as protein A and clumping factor A. Protein A is a well characterized wall-anchored protein that binds to the Fc portion of IgG. With protein A scattered along the surface of the bacterium, this results in \textit{S. aureus} being covered with IgG antibodies that are oriented in the opposite way such that they
cannot be recognized by Fcγ receptors on phagocytes. The importance of this defense mechanism has been illustrated in both an arthritis and a subcutaneous murine infection model in that the *S. aureus* strain lacking protein A showed attenuated virulence (56, 57).

Clumping factor A (ClfA) is another cell surface protein involved in masking *S. aureus* from the host’s immune system by binding fibrinogen which is a protein found in blood that is converted into fibrin in order for blood clots to form (58). While ClfA is the primary protein involved in this process, other proteins, such as ClfB and the fibronectin-binding proteins also contribute to fibrinogen binding (59, 60). The end result is the bacterium being coated with fibrinogen molecules, protecting *S. aureus* from phagocytosis (61). Conversely, fibronectin has also been shown to form a bridge between the fibronectin binding proteins and β1 integrins on nonprofessional phagocytes, mediating the uptake of bacteria (62). *S. aureus* is able to survive intracellularly and this action allows *S. aureus* to avoid complement and antibody-mediated host defense mechanisms.

There are many other ways that *S. aureus* evades direct attacks by the immune system. For example, when a neutrophil succeeds in phagocytosing *S. aureus*, a large proportion of the bacteria are able to survive the neutrophil’s attack and emerge from the phagocyte (63, 64). One of the ways it is able to do this is by producing staphyloxanthin, which acts as an antioxidant to neutralize reactive oxygen species that are created by neutrophils (6, 7). *S. aureus* also produces a capsule that has been shown to protect itself from phagocytosis by neutrophils *in vitro* (65). Although *S. aureus* is usually considered an extracellular pathogen, phagocytes are not the only cells to which *S. aureus* is able gain access. Previous studies have shown that intracellular survival of *S. aureus* within nasal
epithelial cells, keratinocytes, and endothelial cells, among others, are responsible for persistent infections (66–68). This is at least partly due to the formation of slow-growing, small, hypo-pigmented cells, termed small colony variants, within these host cells (69).

When *S. aureus* is not camouflaging itself within the interior of host cells, it must withstand attack by various bactericidal compounds, such as cationic antimicrobial peptides (AMPs). One way in which it does this is by modifying surface molecules such as wall teichoic acids and membrane phospholipids in order to minimize the negative charge of these molecules thereby lessening the attractive force for these AMPs (70, 71). Additionally, *S. aureus* secretes staphylokinase and aureolysin, two proteins that inactivate AMPs. Staphylokinase binds and neutralizes alpha-defensins which are AMPs that are important in the innate immune system, and this activity has been shown to increase bacterial protection *in vivo* (72). Aureolysin cleaves and incapacitates human cathelicidin LL-37, another AMP (73). All of these factors, along with many others, work together to protect *S. aureus* from attack by the immune system.

1.1.4 Antibiotic resistance

While MRSA was initially associated with hospital-acquired infections, this has changed and now MRSA infections rapidly spread among otherwise healthy individuals. Methicillin resistance first arose in *S. aureus* when it acquired the staphylococcal cassette chromosome *mec* (SCCmec) mobile genetic element (74, 75). SCCmec is a large DNA fragment that inserted near the origin of replication and was acquired relatively recently, compared to other genes, as evidenced by atypical codon usage (76). The SCCmec
element contains many genes including two recombinases, ccrA and ccrB, which are conserved across all types of SCCmec elements identified and are required for its movement (75). Importantly, the element contains the mecA gene as well as others that are involved in the regulation of mecA. The mecA gene codes for penicillin-binding protein 2a (PBP2a) which confers resistance to β-lactam antibiotics due to its binding of β-lactams with a much lower affinity compared to other PBPs; therefore the antibiotics cannot inhibit cell wall crosslinking to the degree that would be necessary for their bactericidal action (77, 78).

Vancomycin is a glycopeptide antibiotic that acts by inhibiting cross-linking in the cell wall by binding to the two terminal D-alanine residues in the peptide chains, and was first made available in 1958 (79). It was considered the drug of last resort for MRSA infections; however, S. aureus strains with varying levels of resistance to vancomycin have been reported. Vancomycin intermediate S. aureus (VISA) was first identified in 1997 and vancomycin resistant S. aureus (VRSA) was reported in 2002 (80, 81). Strains are currently defined as vancomycin susceptible at a broth MIC of ≤2 µg/mL, intermediate at 4-8 µg/mL, and resistant at ≥16 µg/mL (82). VISA strains do not have a resistance mechanism to vancomycin that was acquired by foreign transfer of DNA but rather have an accumulation of mutations that leads them to better resist this antibiotic. Some of these mutations in VISA strains that increase their resistance to vancomycin lead to cell wall thickening, downregulation of protein A (spa), decreased acetate catabolism, and increased capsule production (reviewed in reference 75).

VRSA strains are believed to have arisen from the transfer of the vanA gene from vancomycin resistant enterococci (VRE) to S. aureus. The vanA gene has been found in
VRSA isolates on a plasmid in an 11-kb transposon, Tn1546 (83). VanA is responsible for the formation of D-Ala-D-Lac terminal peptides in place of the D-Ala-D-Ala amino acids normally in peptide chains within the peptidoglycan. This changes the binding site for vancomycin thus drastically decreasing its effectivity.

In addition to vancomycin and methicillin resistance, *S. aureus* can obtain resistance to a number of other antibiotics, often mediated by plasmids, but also by transposon insertion into the chromosome or mutations in certain pre-existing genes. For example, chloramphenicol, tetracycline, and streptomycin resistance genes have been found on a number of plasmids isolated from *S. aureus* (84). Resistance to the macrolides, lincosamides, and streptogramins can be mediated by a chromosomal mutation in the 23S rRNA gene, through numerous plasmids, or through a transposon insertion (85–88). Due to the very mobile nature of these elements, the antibiotic resistance profiles of each strain can be quite different, making antibiotic susceptibility testing in healthcare settings very important.

### 1.1.5 USA300

Currently, there is one strain of CA-MRSA that is spreading across the world and is the most common cause of MRSA infections in both the United States and Canada, among other countries (89–92). This strain, USA300, was first identified in 2002 in Los Angeles, California and was resistant to β-lactam antibiotics and commonly to erythromycin; however, additional antibiotic resistance in this strain has been documented (25, 93, 94). Causing even more concern is the emergence of USA300 isolates with increased vancomycin resistance, putting them in the VISA category (95,
In terms of methicillin resistance, USA300 carries SCC\textit{mec} type IV which is smaller than the SCC\textit{mec} types commonly carried by classic HA-MRSA strains (93). Due to its small size, it has been postulated that SCC\textit{mec} type IV is more mobile than other types and has been transferred to MSSA strains over 20 times, presumably from coagulase-negative staphylococci (97).

USA300 is most commonly associated with skin and soft tissue infections but has also been responsible for invasive infections including bloodstream infections, necrotizing fasciitis, and pneumonia (25, 98–100). One reason why USA300 may be such a successful pathogen is its increased production of a number of global virulence regulators and virulence factors, including \textit{agr}, \textit{saeRS}, \textit{sarA}, \textit{hla}, and \textit{pvl} (29). The PVL toxin is encoded by two genes, \textit{lukS} and \textit{lukF} that are secreted and combine to form a pore in polymorphonuclear neutrophils and was first identified in 1932 (101). There has been considerable debate regarding the role of PVL in USA300 and whether it is responsible for the observed increase in virulence and fitness associated with this strain and other PVL\textsuperscript{+} strains (102–106).

Finally, another characteristic feature of the genome in USA300 is the presence of the arginine catabolic mobile element (ACME) which is present in almost all USA300 isolates and is relatively infrequently found in other strains (107). The ACME encodes at least 33 genes including those in the \textit{arc} operon involved in arginine catabolism and the \textit{opp} operon, thought to be involved in oligopeptide uptake (108). It has been shown to improve \textit{S. aureus} survival in acidic conditions intended to mimic human sweat and also to improve general fitness (107, 109). Importantly, it encodes the \textit{speG} gene for spermidine acetyltransferase (110). Spermidine is a polyamine involved in host wound
healing that has a bactericidal effect on *S. aureus*; however, with the *speG* gene product, USA300 is able to overcome sensitivity to spermidine and therefore *speG* may be an important factor in the success of this strain.

### 1.2 Staphylococcal colonization

Various staphylococci are ubiquitously found on the skin of humans as part of the normal flora, of which *S. epidermidis* is the most commonly found species. Other staphylococci that are frequent or occasional colonizers include *S. haemolyticus*, *S. cohnii*, and *S. lugdunensis*, among many others (111, 112). While many individuals colonized with these bacteria will not experience infection caused by them, they are opportunistic pathogens capable of causing a range of infections.

Together with these other staphylococci, *S. aureus* must compete for resources and residency in its preferred habitat at the anterior nares. One study identified a negative correlation between carriage of *Streptococcus pneumoniae* and *S. aureus* (113). In another study, *S. aureus* carriage was found to be associated with a reduction in co-colonizing *S. epidermidis* and *Propionibacterium acnes*, indicating competition between these microbes and suggesting opportunity to exploit this interaction for therapeutic gains (114).

In 2010, a specific protein was identified that could be useful for inhibiting *S. aureus* colonization. The researchers showed that a serine protease secreted by *S. epidermidis*, Esp, is able to inhibit *S. aureus* nasal colonization (115). This was demonstrated in human volunteers who were *S. aureus* carriers. When *S. epidermidis* producing Esp was
added into the nasal cavity of these volunteers, *S. aureus* was eliminated; however, when Esp non-producers were added, there was no impact on *S. aureus* carriage.

This is not the first time that eradication of colonization has been considered as a preventative measure for *S. aureus* infections. Mupirocin has been utilized as a topical antimicrobial for decolonisation of MRSA and acts by inhibiting bacterial protein synthesis. Unfortunately, resistance arose in the form of a point mutation in the *ileS* gene coding for the isoleucyl tRNA synthetase, which is the specific target of mupirocin, and also through the acquisition of the *mupA* gene that codes for an alternate isoleucyl tRNA synthetase to which mupirocin cannot bind (116). Regardless, the effectiveness of mupirocin as a decolonizer or as a postoperative preventative measure has been questioned and is moderate at best (117, 118).

There are also host factors that aid or prevent *S. aureus* colonization. One study found that the presence of hemoglobin in human nasal secretions promoted *S. aureus* colonization (119). Additionally, there have been several studies examining the impact of single nucleotide polymorphisms (SNPs) in the host genome and how these relate to *S. aureus* colonization. Associations have been made between persistent carriage of *S. aureus* and SNPs in *IL-4, CRP, CFH* and *NR3C1* (120, 121). Some disease states are also correlated with an increased risk of *S. aureus* infection, such as cystic fibrosis and diabetes (68, 122). There are many other host factors that are known to provide protection against the invasion of pathogens, such as antimicrobial polypeptides (AMPs) and antibacterial free fatty acids (FFAs).
1.2.1 Human skin innate immunity - AMPs

The skin acts as the primary barrier to infection and thus represents our first line of defense against invading microorganisms. In addition to simply being a mechanical barrier, many compounds are secreted from various cell types to help protect us from infection, such as antimicrobial peptides (AMPs). The biggest producers of AMPs in human skin are keratinocytes, however once inflammation occurs and immune cells arrive, leukocytes secrete a majority of the AMPs (123, 124). Two of the most studied AMPs are human β-defensin 1 (hBD-1) and cathelicidin LL-37. HBD-1 is constitutively expressed in keratinocytes but wasn’t thought to have significant antimicrobial activity until it was identified that the reduction of disulphide bonds in this protein was required for its full antibacterial effect (125). The exact mechanism of the antibacterial effect of hBD-1 is not known, although it is generally attributed to its ability to disrupt membranes, possibly due to their positive charge interacting with negatively charged molecules on the surface of bacteria (126). Additionally, hBD-1 is involved in the recruitment of various immune cells to the site of infection, thus it also exerts an indirect antibacterial effect (127).

Conversely, cathelicidin LL-37 is normally expressed at very low levels in keratinocytes but is upregulated in response to infection (128). It is also expressed constitutively in epithelial cells and stored in neutrophil granules (129, 130). Cathelicidin LL-37 is chemotactic for a variety of immune cells, thus recruiting these immune cells to the site of infection (131). Additionally, cathelicidin LL-37 was shown to disrupt bacterial membranes with increased effectiveness against gram-positive bacteria compared to
gram-negatives (132). Cathelicidin LL-37 and hBD-1, along with other AMPs, are important components of the innate immunity of our skin and they act alongside a variety of free fatty acids, also with antimicrobial properties.

1.2.2 Antibacterial free fatty acids

The sebaceous glands are responsible for secreting sebum which has a high concentration of triglycerides but also secretes AMPs (133). Sebaceous glands are found throughout our body with the exception of our palms and soles. The majority of these are associated with hair follicles and as such, are termed pilosebaceous glands while those that are not are termed free sebaceous glands (134). Sebaceous glands secrete sebum as the mature, lipid-filled cells die and lyse (135). The sebum is composed primarily of triglycerides, wax esters, squalene, cholesterol, and free fatty acids (FFAs) and the FFAs are largely produced by hydrolysis of the triglycerides (136, 137). Interestingly, the triglycerides are known to be cleaved into FFAs by bacterial lipases and this further contributes to the antimicrobial effect, as FFAs are the most active antibacterial component of sebum (138, 139).

One of the major FFA constituents of human sebum is sapienic acid (C16:1Δ6) and it has been shown to have potent antimicrobial activity (138). Illustrating the importance of sapienic acid in innate immunity, it was found that individuals with atopic dermatitis often have a deficiency in sapienic acid and this was correlated to an increase in *S. aureus* colonization (140). Other fatty acids, such as linoleic (C18:2Δ9,12), oleic (C18:1Δ9), and palmitic (C16:0) acids were found to accumulate to high levels in staphylococcal abscesses (2). However, in a separate study, oleic and palmitic acids were not found to
be inhibitory toward *S. aureus in vitro* while linoleic acid was inhibitory, suggesting that linoleic acid may be an important defense component in abscess formation (141). The antibacterial effect of many FFAs has been well-documented and impacts a number of factors, both in the host and the bacterium (142).

The characteristics of each fatty acid play a strong role on the strength of its antimicrobial activity. For example, fatty acids of the same chain length are generally more potent when unsaturated compared to saturated and potency is also increased with increasing degrees of unsaturation (143, 144). Additionally, FFAs with double bonds in the *trans* orientation have a larger effect compared to the *cis* orientation, likely due to the shape this gives the molecule (144). In terms of the effect of FFAs on the skin, in addition to a direct antibacterial action, they also create a hostile environment by reducing skin pH and negatively impacting the production of bacterial virulence factors (140, 145, 146).

Although there has been significant evidence of an antimicrobial effect with these FFAs, the mechanism of action has not been definitively elucidated. The majority of the proposed mechanisms involve disruption of the bacterial membrane resulting in trauma to the membrane and/or to the various processes that take place there (reviewed in reference 135). The FFAs likely insert into the bacterial cell membrane and disrupt the electron transport chain and oxidative phosphorylation, impacting energy generation (146–148). This could be achieved by displacing the protein components from the membrane entirely, causing too much distance between them, or binding and inactivating them. Additionally, FFAs may cause cell lysis, inhibit endogenous fatty acid synthesis, or reduce nutrient uptake (142). While the exact direct effect of these FFAs has not been conclusively shown, it is evident that these compounds inhibit bacterial growth likely
through a disrupting effect to the membrane. This begs the question, if these FFAs are so harmful to bacteria, how do they survive in FFA rich environments, such as the skin?

1.2.3 Resistance to fatty acids

The fatty acid composition of nasal secretions varies largely by individual but they are found in micromolar concentrations in nasal secretions and accumulate in staphylococcal abscesses (2, 149, 150). Nasal secretions contain FFAs at sub-inhibitory concentrations for *S. aureus* and therefore *S. aureus* can survive under these conditions. Interestingly, other bacterial species are able to utilize host-derived fatty acids as an energy source; however this requires enzymes capable of beta-oxidation to break down the fatty acids into two-carbon acetyl-coA molecules that can enter the TCA cycle. No enzyme with this functionality has been identified in *S. aureus* and therefore, *S. aureus* is not thought to be capable of using exogenous fatty acids for energy derivation (151, 152). As such, inhibitors of fatty acid biosynthesis are potential antimicrobial candidates for *S. aureus*.

One natural resistance mechanism thought to exist in *S. aureus* is the fatty acid modifying enzyme (FAME). While this enzyme has not been purified, nor has a gene been identified, its activity has been documented in approximately 80% of *S. aureus* culture supernatants (153, 154). The FAME functions to esterify the fatty acid to an alcohol resulting in detoxification of the compound (154). Triglycerides containing unsaturated free fatty acids are inhibitors of FAME activity (153) suggesting a potential complementary role between FAME and bacterial lipases whereby lipases degrade triglycerides and FAME detoxifies the resulting FFAs. Another study found that many
coagulase-negative staphylococci also produce FAME and that there was a strong correlation between the production of FAME and lipase (155).

Another way that *S. aureus* is thought to protect itself from FFAs is through the modification of its cell wall or the proteins therein. The cell wall was shown to be important as when bacterial cells lacked their cell wall, they were readily lysed by various FFAs (156). In a similar vein, *S. aureus* mutants lacking wall teichoic acids (WTAs) are more susceptible to FFA killing (157). WTAs are negatively charged and may help to repel fatty acids by decreasing the hydrophobicity of the outer surface of the bacterium. Another mechanism the cell employs to decrease hydrophobicity at its surface is through the upregulation of IsdA, which is a surface-associated protein involved in iron acquisition from hemoglobin (158). Researchers used human volunteers to show that a Δ*isdA* mutant *S. aureus* strain was recovered in significantly lower numbers compared to wild-type when allowed to colonize the forearms of these volunteers (158). Recently, the identification and characterization of a novel FFA resistance mechanism has been described in which *farE*, coding for an efflux pump, is induced by a transcriptional regulator, FarR (159). Deletion of the *farE* gene resulted in increased susceptibility to both linoleic and arachidonic acids, but not palmitoleic acid.

### 1.3 Research Hypothesis

We have previously shown that when grown in 50 µM linoleic acid, *Staphylococcus aureus* experiences a 10-12 hour lag phase, followed by unimpeded growth (141). We hypothesize that the growth observed in the presence of 50 µM linoleic acid is due to the presence of, and selection for, mutants with a genetically-encoded resistance mechanism.
1.3.1 Objectives

**Objective 1:** Obtain linoleic acid-resistant *S. aureus* mutants by performing growth in 50 µM linoleic acid and select single colonies for further analysis.

**Objective 2:** Identify genetic mutations associated with increased resistance by whole genome sequencing.

**Objective 3:** Characterize the mechanism of linoleic acid resistance afforded to these genetically altered *S. aureus* mutants.
Chapter 2

2 Materials and Methods

2.1 Bacterial strains, storage, and growth conditions

Bacterial strains used in this study are listed in Table 1. All *S. aureus* strains were cultured in TSB while *E. coli* strains were cultured in LB and stored at -80°C in 15% glycerol. Strains were streaked on TSB (*S. aureus*) or LB (*E. coli*) agar and supplemented with antibiotics when required: chloramphenicol at 10 µg/mL, kanamycin at 40 µg/mL, or ampicillin at 100 µg/mL. USA300 LAC was a gift from Dr. B. Kreiswirth and was cured of plasmid-mediated resistance by Jessica Sheldon. The resulting strain, USA300, was used as the wild-type strain for all experiments.

For growth in fatty acids, a 5mM stock solution of fatty acid was made by adding an equal volume of fatty acid and DMSO to TSB. To make a 5 ml solution of 5 mM linoleic acid, 5 ml TSB was mixed with 7.78 µL DMSO and 7.78 µL linoleic acid (L1012, Sigma-Aldrich, Oakville, Ontario). The required amount of this stock was then diluted into the assay flasks or tubes containing TSB to reach the desired concentration. Aerobic growth occurred at 37°C with shaking at 220 rpm, unless otherwise noted.
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA300 LAC</td>
<td>Community-acquired MRSA isolate</td>
<td>(99) Dr. Kreiswirth</td>
</tr>
<tr>
<td>USA300</td>
<td>USA300 LAC cured of antibiotic resistance plasmid, wild-type strain</td>
<td>(141)</td>
</tr>
<tr>
<td>RN4220</td>
<td>rK mK⁺; lab strain capable of accepting foreign DNA</td>
<td>(160)</td>
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<tr>
<td>CS1</td>
<td>USA300 CvfA&lt;sub&gt;L269F&lt;/sub&gt;</td>
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</tr>
<tr>
<td>CS2</td>
<td>USA300 CvfA&lt;sub&gt;Q333K&lt;/sub&gt;</td>
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<td>USA300 CvfA&lt;sub&gt;V287D&lt;/sub&gt;</td>
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<td>CS4</td>
<td>USA300 CvfA&lt;sub&gt;S330N&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CS5</td>
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<td>DH5α</td>
<td>F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rl−,m&lt;i&gt;k&lt;/i&gt;⁺) phoAΔsupE44 thi-1 gyrA96 relA1 λ−</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamH1o ΔEcoRI-B int::(lacI::PlacUV5::T7 gene 1) i21Δnin5</td>
<td>(161)</td>
</tr>
<tr>
<td>BL21 + pET</td>
<td>BL21 containing empty pET28a(+) vector; Km&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
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<td>BL21 + pML4</td>
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<td>This study</td>
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</table>
2.2 DNA methodology

2.2.1 Polymerase chain reaction (PCR)

For cloning, PCR was performed using phusion polymerase (New England Biolabs) following the manufacturer’s protocol in 50 μL reactions. For checking transformants, PCR was performed using Taq polymerase (New England Biolabs) and 20 μL reactions. PCRs were performed in the DNA engine Gradient Cycler (Bio-rad) or the MJ Mini Personal Thermal Cycler (Bio-rad). Annealing temperatures were determined using the New England Biolabs online Tm calculator based on primers and polymerase. Primers used in this study are listed in Table 2. Following amplification, PCR products were visualized on a 0.8% (w/v) agarose gel stained with SYBR® safe (Invitrogen) and cleaned using a QIAquick PCR purification kit (Qiagen).
Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
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<td>CvfA_clonR</td>
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<td>noTM_R</td>
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<td>FullCvfA_F</td>
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<td>FullCvfA_R</td>
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<tr>
<td>AddHis_F</td>
<td>TTTAACCGCGGCAAGACCCCGGAAACCAGTTTTGCTATATTCTA CTGCTCTA</td>
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<tr>
<td>AddHis_R</td>
<td>TTTGCCCGGGGCTCACCCTCATCATCATCATCAACCACCACCACCAA CCACTAAGGATCCAAA</td>
</tr>
</tbody>
</table>

aRestriction digestion sites are underlined; KpnI (GGTACC); SacI (GAGCTC); NdeI (CATATG); EcoRI (GAATTC); NcoI (CCATGG); BamHI (GGATCC); SacII (CCGCGG)
2.2.2  Restriction enzyme digestion and DNA ligation

Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer’s directions. Double digests were performed in 50 µL volumes with approximately 1 µg of DNA with the appropriate reaction buffer. The reaction mixture was incubated for 2-4 hours at 37°C and cleaned using a QIAquick PCR purification kit, following manufacturer’s protocols (Qiagen). Digested plasmids were isolated from agarose gels using the QIAquick Gel Extraction Kit (Qiagen). DNA fragments and plasmids were ligated using the T4 DNA ligase Rapid Ligation Kit (Roche) in a 20 µL reaction volume. A 10:1 molar ratio of insert to vector was used for plasmid construction.

2.2.3  DNA isolation from E. coli and S. aureus

Plasmids were isolated using the E.Z.N.A. Plasmid Miniprep Kit (Omega Biotek) following the manufacturer’s protocol and are listed in Table 3. For plasmid preparation from S. aureus, cell pellets were resuspended in 250 µL Solution I/RNase containing 10 µg lysostaphin (Sigma). This mixture was incubated for approximately 1 hour at 37°C before the addition of Solution II.

Genomic DNA from S. aureus was isolated for sequencing using the Invitrogen genomic DNA isolation kit following the manufacturer’s protocol. For checking transformed colonies, DNA was isolated from S. aureus by resuspending a colony in 100 µL lysis buffer (25 mM tris-HCl, 50 mM glucose, 150 mM NaCl, 10 mM EDTA, pH 8.0) containing 4 µg lysostaphin and incubating for approximately 1 hour at 37°C to obtain
complete cell lysis. Two microliters of 10% (w/v) SDS was added before heating to 95°C for 10 minutes and cooling to room temperature. An equal volume (102 µL) of phenol:chloroform was added and the solution was mixed by inversion. Following a 30 minute incubation at room temperature, the sample was centrifuged for 10 minutes at 13000 × g and the aqueous phase was transferred to a fresh tube and diluted 10-fold. This sample contained genomic DNA and was used as the template for subsequent reactions.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pALC2073</td>
<td>(E. coli/S. aureus) shuttle vector with anhydrotetracycline-inducible expression; (\text{Amp}^\text{r}) in (E. coli), (\text{Cm}^\text{r}) in (S. aureus)</td>
<td>(162)</td>
</tr>
<tr>
<td>pΔTetR</td>
<td>pALC2073 with the TetR repressor gene deleted</td>
<td>Jessica Sheldon</td>
</tr>
<tr>
<td>pWT</td>
<td>pΔTetR expressing wild-type CvfA</td>
<td>This study</td>
</tr>
<tr>
<td>pCS1</td>
<td>pΔTetR expressing SNP-containing CvfA from strain CS1 (CvfA(_{L269F}))</td>
<td>This study</td>
</tr>
<tr>
<td>pCS2</td>
<td>pΔTetR expressing SNP-containing CvfA from strain CS2 (CvfA(_{Q333K}))</td>
<td>This study</td>
</tr>
<tr>
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<td>pΔTetR expressing SNP-containing CvfA from strain CS3 (CvfA(_{V287D}))</td>
<td>This study</td>
</tr>
<tr>
<td>pCS4</td>
<td>pΔTetR expressing SNP-containing CvfA from strain CS4 (CvfA(_{S330N}))</td>
<td>This study</td>
</tr>
<tr>
<td>pCS5</td>
<td>pΔTetR expressing SNP-containing CvfA from strain CS5 (CvfA(_{P280L}))</td>
<td>This study</td>
</tr>
<tr>
<td>pET28a (+)</td>
<td>Expression vector for (E. coli) carrying an N-terminal His tag, thrombin recognition sequence, and T7 terminator; (\text{Km}^\text{r})</td>
<td>Novagen</td>
</tr>
<tr>
<td>p-TM</td>
<td>pET28a(+) expressing wild-type CvfA lacking its transmembrane domain and with an N-terminal histidine tag</td>
<td>This study</td>
</tr>
<tr>
<td>pML3</td>
<td>pET28a(+) expressing full length wild-type CvfA with a C-terminal histidine tag</td>
<td>This study</td>
</tr>
<tr>
<td>pML4</td>
<td>BL21 containing pET28a(+) expressing full length CvfA(_{L269F}) with a C-terminal histidine tag</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(^a\)\(\text{Amp}^\text{r}, \text{Cm}^\text{r}, \text{and} \text{Km}^\text{r}\) designate resistance to ampicillin, chloramphenicol, and kanamycin, respectively
2.2.4 Plasmid construction

The construction of *S. aureus* complementation vectors was completed using the CvfA_clonF and CvfA_clonR primers to amplify *cvfA* from the appropriate *S. aureus* strain. Following digestion as described, the amplified fragments were ligated into pΔtetR. The pΔTetR plasmid was derived from pALC2073 and constructed by Jessica Sheldon via allelic replacement of the *tetR* gene through the use of the pKOR1 vector.

For the expression of CvfA lacking its transmembrane domain in *E. coli*, we used the noTM_F and noTM_R primers, amplified *cvfA*, digested, and ligated into pET28a(+).

We employed the FullCvfA_F and FullCvfA_R primers for cloning full length CvfA into pET28a(+) and this resulted in expression of CvfA with the addition of six histidines at its C-terminus. We later added a thrombin recognition sequence and an additional six histidine residues by insertional mutagenesis. Using the AddHis_F and AddHis_R primers, we amplified the existing plasmid. The resulting mixture was cleaned using a QIAquick PCR purification kit (Qiagen) and then digested with DpnI to degrade the methylated template DNA.

2.3 Transformation methodology

2.3.1 Preparation of transformation competent *E. coli* and *S. aureus*

*E. coli* DH5α and BL21 were made chemically competent by growing a 100 ml culture in LB broth to an OD$_{600}$ of 0.5, followed by a 20 minute incubation on ice. Cells were
pelleted and resuspended in 25 ml cold 0.1 M CaCl$_2$, 15% (v/v) glycerol. Following another 30 minute incubation on ice, cells were pelleted again and resuspended in 1 ml of cold 0.1 M CaCl$_2$, 15% (v/v) glycerol. Cells were stored in 100 µL aliquots at -80°C.

*Staphylococcus aureus* RN4220 or USA300 was made electrocompetent by growing a culture in 100 ml TSB at 37 °C until the OD$_{600}$ reached 0.3. The flask was put on ice for 10 minutes prior to pelleting cells and resuspending in 10 ml of cold 0.5 M sucrose. Cells were pelleted and resuspended in 1 ml cold 0.5 M sucrose twice and 60 µL aliquots were stored at -80°C.

### 2.3.2 Transformation of competent *E. coli* and *S. aureus*

Plasmid DNA (100-1000 ng) was added to competent *E. coli* cells that were thawed on ice, and this mixture was incubated on ice for 30 minutes. The cells were moved to 42°C for two minutes then back to ice for two minutes, after which 900 µL of LB broth was added and the mixture was incubated at 37 °C for 1-2 hours. Aliquots of 100 µL were plated on selective agar and incubated at 37 °C overnight to allow colony growth. All plasmid sequences were confirmed by Sanger sequencing which was completed by the London Regional Genomics Centre (LRGC) at the Robarts Research Institute.

Electrocompetent *S. aureus* cells were thawed on ice and 50-1000 ng of plasmid was added, followed by a 30 minute incubation on ice. Cells were transferred to a cold electroporation cuvette (2mm, Bio-Rad) and electroporated using a Bio-Rad Gene Pulser II at 200 ohms, 25 µF, and 2.5 kV. 940 µL of TSB was then added to the cuvette and cells were transferred to a microfuge tube. Following 2-4 hours at 37 °C, cells were plated on selective TSA and incubated overnight at 37 °C.
2.4 Selection of linoleic acid resistant mutants

We have previously shown that *S. aureus* undergoes a 10-12 hour lag phase when grown in TSB with 50 µM linoleic acid (141). To select for mutants with a genetically encoded resistance mechanism, we took the cells that had grown up after one round of growth in TSB with 50 µM linoleic acid, sub-cultured twice in TSB alone, then back into TSB containing 50 µM linoleic acid. All cultures were grown at 37°C and 220 rpm in 125 ml flasks containing 25 ml TSB with or without 50 µM linoleic acid. This was performed with seven separate cultures, and dilutions of each of the final cultures were plated to isolate single colonies. One colony from each of the biological replicates was selected and genomic DNA was isolated using the Invitrogen genomic DNA isolation kit following the manufacturer’s protocol.

2.5 Genome sequencing

Genomic DNA from the fatty acid resistant clones and from a wild-type USA300 strain were sent for processing and sequencing at the LRGC to identify potential resistance-conferring mutations. Samples were quantified, fragmented, and sample-specific adapters were ligated to the fragments. The appropriate size fragments were selected for and following several quality checks, the genomes were sequenced using the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA). Sequences were analyzed using CLC Bio Genomics Workbench (CLC Inc., Aarhus, Denmark) and mapped to the published genome of *S. aureus* subsp. *aureus* USA300_FPR3757. SNPs were identified using the neighbourhood quality standard algorithm (163) and SNPs that were present in both the wild-type strain as well as the fatty acid resistant strains were discounted.
2.6 Minimum inhibitory concentration and viability assays

To characterize the phenotypes and resistance profiles of the fatty acid resistant strains, MIC and viability assays were performed. A stock solution containing 5 mM fatty acid in TSB was made as described in section 2.1. For the MICs, 240 µL of this stock was added to a glass tube containing 1.76 ml TSB and vortexed, resulting in a final concentration of 600 µM fatty acid. Two-fold serial dilutions were then performed down to 37.5 µM. For viability assays, 125 ml flasks with 25 ml TSB were made 100 µM with respect to linoleic acid.

Biological triplicates of appropriate strains were grown overnight in TSB with chloramphenicol. All strains were sub-cultured into fresh TSB at an OD$_{600}$ of 0.01. For MICs, when cultures reached an OD$_{600}$ of 1, they were inoculated to an OD$_{600}$ of 0.01 into glass tubes containing 1 ml TSB with the indicated concentration of fatty acid. Culture growth was assessed at the 24 hour time point to determine the MIC. For viability assays, cultures were also harvested at an OD$_{600}$ of 1 but were inoculated into flasks at an OD$_{600}$ of 0.05. An aliquot of each culture was taken at the indicated time points and plated to determine CFUs.

2.7 Uptake of $^{14}$C-linoleic acid

For further characterization of the fatty acid resistant strains, radioactive uptake assays were performed similar to that described in a previous report (159). Strains were grown in triplicate in 25 ml TSB from an OD$_{600}$ of 0.01 to approximately 0.5 at which point a 1 ml aliquot was taken and supplemented with 0.2 µCi/mL of $^{14}$C-linoleic acid. A 200 µL
Aliquot from each sample was removed at 3, 5, 10, and 15 minute time points and the samples were placed on 0.45 μm pore-size filter discs connected to a vacuum manifold. The cells were washed twice with 4 ml of buffer (0.1 M Na-phosphate pH 7, 1% Triton X-100) and placed in scintillation vials. The discs were allowed to dry and then 4 ml of Cytoscient scintillation cocktail (Fisher Scientific) was added. 14C-linoleic acid within cells was then measured using a Beckman LS 6500 scintillation system. Data were normalized by total protein content based on OD_{600}.

2.8 RNA sequencing

Given that CvfA is an RNase, an RNA sequencing assay was performed to identify transcripts that are impacted by the L269F mutation. Six biological replicates of wild-type USA300 and CS1 were diluted to an OD_{600} of 0.01 in 25 ml TSB with 15 μM linoleic acid and grown to an OD_{600} of one. Three OD units were pelleted and washed with 750 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA). After centrifugation, the pellet was resuspended in 100 μL of 500 μg/mL lysostaphin in TE buffer and samples were frozen overnight at -80°C. Upon thawing, the samples were incubated for 1 hour at 37°C. The samples were then processed to obtain RNA using the Aurum™ Total RNA Mini Kit (Bio-rad). The kit was used according to the manufacturer’s protocol with the addition of a second DNase I treatment (Roche), and the samples were stored at -80°C. The RNA quality and quantity was checked on an agarose gel and using a Nanodrop (Thermo Scientific) prior to being sent to the LRGC for analysis on the Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). The four highest quality samples for both USA300 and CS1 were chosen to continue. For removal of rRNA, we employed
the MICROBExpress™ Kit (Life Technologies) and followed the manufacturer’s protocol. The mRNA samples were sent for quality analysis on the BioAnalyzer by the LRGC and the top three samples of each were selected to be sequenced. Samples were processed by the LRGC according to the TruSeq Stranded mRNA Library Prep Kit and protocol (Illumina) and sequenced using the MiSeq (Illumina). Samples were fragmented, converted to cDNA, and adapters were ligated to allow samples to be pooled and sequenced on the same flow cell.

Data was obtained in the form of fastq files and analyzed using CLC Bio Genomics Workbench (CLC Inc., Aarhus, Denmark) following the user manual for RNAseq analysis. Briefly, the fastq files were mapped to the USA300_FPR3757 genome and expression values were normalized by RPKM (reads per kilobase of transcript per million mapped reads). The normalized values of the three wild-type samples were compared to those of the three CS1 samples and a t-test was used to determine p-values.

### 2.9 Secreted protein profiles

Upon evaluation of the RNA sequencing data, several virulence factors were identified as being down-regulated and to follow up on this observation, we performed a TCA precipitation of culture supernatants to look for differences in secreted protein profiles. Strains were grown overnight in TSB and diluted to OD$_{600} = 0.01$ in 25 ml TSB with 15 µM linoleic acid. Cultures were grown for 18 hours at which point the OD$_{600}$ was determined and the cultures were centrifuged at 3,000 × g and 4°C for 30 minutes. Three OD units worth of supernatant were removed and an equal volume of cold 20% trichloroacetic acid was added and mixed, and samples were stored at 4°C overnight to
precipitate proteins. Samples were spun at 13,000 rpm and 4°C for five minutes, the supernatant was discarded, and the pellet was washed three times with cold 70% ethanol. Following the final wash, the supernatant was discarded and the pellets were left to air dry at 37°C for one hour. The samples were resuspended in 20 µL of Laemmli sample buffer and run on an 11% SDS polyacrylamide gel at 100 volts for 90 minutes, after which they were stained with Coomassie brilliant Blue R-250.

2.10 Hemolysin production

To evaluate hemolysis differences, strains were streaked on tryptic soy agar with or without chloamphenicol, as required, and incubated overnight at 37°C. Single colonies from these plates were picked and streaked on Trypticase™ Soy Agar w/5% sheep blood (Becton, Dickinson and Company, Sparks, MD) and incubated overnight at 37°C followed by 4°C for 4 hours.

2.11 Subcutaneous infection model

A subcutaneous abscess model was chosen to evaluate the impact of the CvfA<sub>L269F</sub> mutation in vivo. Cultures of USA300 or CS1 were grown to an OD<sub>600</sub> = 1 in TSB and sub-cultured into 25 ml TSB at an OD<sub>600</sub> of 0.02. Cultures were grown until the OD<sub>600</sub> was approximately 2.5 and then spun at 3,000 × g for 10 minutes and washed twice with 25 ml sterile phosphate-buffered saline (PBS). Cultures were diluted to an OD<sub>600</sub> of 0.40 in PBS and dilutions were plated to determine CFUs.

Six-week-old BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in microisolator cages. Twenty mice were anesthetized
with isoflurane, their right flanks shaved and remaining hair removed with Nair™ cream. They were injected with 50 µL of the bacterial suspension containing 4-7 x 10^6 CFUs of USA300 or CS1 under the skin of the flank. Each mouse was injected with one strain of bacteria in one flank only. Images of the resulting abscesses were taken daily with a size marker in frame for measurement. The images of the abscesses were measured using FIJI (164). All animal protocols were approved by the University of Western Ontario's Animal Use Subcommittee.

2.12 Protein expression and purification

To evaluate the impact of the L268F mutation on protein function, the protein was expressed with or without its transmembrane domain in *E. coli* to be purified and tested. *E. coli* BL21 cells containing the vector control or plasmids expressing CvfA were grown overnight in LB with kanamycin. They were diluted to an OD_{600} of 0.01 in 500 ml LB + kanamycin and grown to an OD_{600} of 0.6-0.8. An aliquot corresponding to 1 OD unit was taken and cells from that aliquot were pelleted and resuspended in 100 µL 0.9% saline to serve as the uninduced control. Additionally, the culture was made 0.4 mM with respect to IPTG to induce expression off of the vector overnight at room temperature with shaking. The next day, the OD_{600} was measured and another aliquot corresponding to 1 OD unit was taken to serve as the induced control. Efficient induction was evaluated by visualization of the controls on a polyacrylamide gel stained with Coomassie brilliant Blue R-250.

The culture post-induction was pelleted at 5,000 rpm and 4°C for 20 minutes and then resuspended in 15 ml of phosphate buffer (100 mM Na-phosphate pH 7.4, 250 mM NaCl,
10% glycerol). Samples were passed through a cell disrupter (Constant Systems Inc.) at 30 lb/in² and spun at 3,000 × g and 4 °C for 20 minutes to pellet any insoluble material.

2.12.1 Inclusion body Purification

The construct producing CvfA lacking its transmembrane domain pelleted as inclusion bodies. This pellet was washed four times with 25 ml of PBS + 1% Triton X-100 and spins were performed at 3,000 × g and 4°C for 30 minutes. Another four washes were performed using 25 ml PBS and increasing concentrations of NaCl: 0 mM, 150 mM, 300 mM, and 500 mM NaCl. The resulting pellet was denatured in 25 ml of 8 M urea in PBS and left overnight at 4°C with stirring.

An ultracentrifugation was performed at 25,000 rpm and 4°C for 45 minutes to pellet anything that did not solubilise after the overnight treatment in urea. The solution was diluted ten-fold in PBS to decrease the concentration of urea to 0.8 M and left overnight at 4°C with stirring. The next day, the protein sample was dialyzed against 2 L of PBS and the buffer was exchanged 4 times over 48 hours to rid the protein-containing solution of all remaining urea. The dialyzed product was collected by centrifugation at 3,000 × g and 4°C for 30 minutes resulting in a soluble fraction and a small amount of precipitate. The supernatant was concentrated using Amicon Ultra 30k centrifugal filters (EMD millipore), visualized on a polyacrylamide gel, and the concentration was determined via Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate).
2.12.2 Membrane purification

The constructs producing full length CvfA were expected to localize to the membrane. Following cell lysis and the low speed spin to pellet any insoluble material, membrane-bound CvfA from BL21 + pML3 and BL21 + pML4 as well as the vector control, were found in the supernatant. The supernatants were carried through to an ultracentrifugation at 50,000 \( \times \) g and 4°C for 45 minutes after which CvfA was found in the pelleted membrane fraction. The membranes were resuspended in 1 mL of phosphate buffer (100 mM Na-phosphate pH 7.4, 250 mM NaCl, 10% glycerol) containing 2% n-dodecyl \( \beta \)-D-maltoside (DDM). The samples were sonicated four times for 30s each and the insoluble fraction was pelleted with an ultracentrifugation at 100,000 \( \times \) g and 4°C for 30 minutes. The supernatant was saved and the pellet was resuspended in 1 ml of phosphate buffer containing 2% octyl-\( \beta \)-D-glucopyranoside (OG). The samples were sonicated and centrifuged again as described and the pellet was resuspended in hepes buffer (50 mM hepes-KOH pH 8, 20% glycerol) containing 2% tween-20. Following another sonication and centrifugation, the result was three solubilised membrane fractions in three different detergents. The samples were visualized by PAGE and protein concentrations determined via Bradford assay.

2.13 Phosphodiesterase activity assay

To test for activity of both wild-type CvfA and CvfA\(_{L269F}\), a phosphodiesterase assay was employed and adapted from a previous report (165). A 2X-stock buffer was made (100 mM hepes-KOH pH 9, 1.2 M KCl, 2 mM MnCl\(_2\)) and 100 µL was aliquoted into each well of a 96 well plate. Protein solutions were diluted to 0.4 µg/µL and 50 µL was added
to each well, resulting in 20 µg of protein in each well. 50 µL of protein buffer (50 mM hepes-KOH pH 8, 20% glycerol, 2% Tween-20) was added into the blank wells instead of the protein solution and a separate blank well was used for each concentration of bis-4-NPP. Additionally, protein samples were treated with DEPC to a final concentration of 1% in 50 µL to demonstrate that phosphodiesterase activity could be abolished. A 60 mM bis-4-NPP stock solution was made and the appropriate amount was added to each well to make 0, 1, 3, 5, 10, and 15 mM solutions and water was added to each well to achieve a final volume of 200 µL. The plate was incubated at 37°C and the A_{405 nm} was read at 3 hours. The enzyme activity was calculated using an extinction coefficient for 4-nitrophenyl phosphate of 17,800 M⁻¹ cm⁻¹.

### 2.14 Pulldown assay

Finally, to examine whether the L269F mutation in CvfA impacts protein interactions, a pulldown assay was performed with purified CvfA and whole cell lysate. USA300 whole cell lysate was prepared by growing a 500 ml culture to an OD_{600} of 1-1.5, pelleting the cells, and resuspending in 15 ml of phosphate buffer (50 mM Na-phosphate, pH 8) with 40 mM imidazole and one cOmplete™ mini EDTA-free protease inhibitor cocktail tablet (Roche). Cells were lysed with multiple passages through a cell disrupter (Constant Systems Inc.) at 30 lb/in² and any insoluble material and unbroken cells were pelleted by centrifugation at 3,000 × g and 4°C for 20 minutes. The supernatant was collected as the whole cell lysate and the protein concentration determined via Bradford assay.

DDM-solubilised protein samples were mixed with 1 mg of whole cell lysate and the final concentration of DDM was adjusted to 1% and imidazole was kept at 40 mM. After
a 20 minute incubation with rotation at room temperature, this mixture was added to 25 µL (1 mg) of Dynabeads TALON (Invitrogen). Following a second incubation with rotation for 20 minutes at room temperature, the tubes were placed on a magnet to separate the beads. The supernatant was collected as the unbound fraction and the beads were washed three times with 200 µL of buffer (50 mM Na-phosphate pH 8, 50 mM imidazole + 1% DDM). The suspension was transferred to a clean tube during the second wash. Proteins were eluted from the beads with the addition of 20 µL laemmli buffer containing 1 M imidazole and a 15 minute incubation with rotation. As negative and positive controls, whole cell lysate and a DDM-solubilised protein sample were each incubated with the beads separately. Washes and the elution were completed as described above and all samples were visualized by SDS-PAGE.
Chapter 3

3 Results

Our skin is rich with antimicrobial compounds and yet there are many microbes making up the normal flora found there, of which *Staphylococcus aureus* is a common component. In the following work, we selected for mutants of *S. aureus* with increased resistance to antibacterial free fatty acids. We obtained and characterized mutants with genetically-encoded mechanisms of resistance and began the elucidation of the molecular mechanism underpinning the observed increased resistance.

3.1 Adaptation of *S. aureus* to growth in linoleic acid

Our lab has previously demonstrated that *S. aureus* growth is not inhibited in 25 µM linoleic acid, while 50 µM produces a 10-12 hour lag phase, and 100 µM is inhibitory (141). The growth after a significant lag phase in 50 µM linoleic acid suggested the presence of a single colony that was capable of growth in this media and required 10 hours before a detectable level of growth had occurred. It was hypothesized that the surviving bacteria possessed either a genetic mutation or an epigenetic change that allowed growth. To evaluate this hypothesis, *S. aureus* was grown in 50 µM linoleic acid and, following the lag phase, the cells that had grown to stationary phase were cultured twice in rich media alone, and then subsequently back into media containing 50 µM linoleic acid. During the second round of growth in linoleic acid-containing media, there was no longer a lag phase observed and these cells were able to grow immediately.
(Figure 1). This suggested the presence of a heritable genetic change rather than an epigenetic one as the phenotype was maintained through successive growth cycles lacking linoleic acid.
Figure 1. *Staphylococcus aureus* USA300 growth in successive cycles of linoleic acid. *S. aureus* was grown in rich media containing 50 μM linoleic acid (open squares) or in rich media alone (open circles). Cells that had grown up in linoleic acid were cultured twice in rich media alone and then back into media containing 50 μM linoleic acid (open triangles). The mean of three replicates ± standard error of the mean is plotted. Data used with permission from Ben Arsic.
3.2 Identification of SNPs in cvfA

In an attempt to confirm that a genetic change was associated with this linoleic acid resistant phenotype and to identify this mutation, the genomes of seven linoleic acid-resistant clones were sequenced using an Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA). Two of these clones, CS1 and CS2, had distinct SNPs in the same gene, cvfA. Intrigued by this finding, 10 additional independent cultures of USA300 were grown, as above, to select for additional resistant clones. Anticipating that additional SNPs may be identified in cvfA, the cvfA gene was amplified from each and sent for traditional Sanger sequencing. This resulted in the identification of three more independently isolated clones with SNPs in cvfA (CS3, CS4, and CS5). In total, five linoleic acid resistant S. aureus mutants have been identified with SNPs in this gene (Table 4).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene</th>
<th>SNP(^a)</th>
<th>Codon Change</th>
<th>Amino Acid Substitution</th>
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<td>CS1</td>
<td>USA300_1179</td>
<td>1298394 C→T</td>
<td>CTT→TTT</td>
<td>Leu269Phe</td>
</tr>
<tr>
<td>CS2</td>
<td>USA300_1179</td>
<td>1298586 C→A</td>
<td>CAA→AAA</td>
<td>Gln333Lys</td>
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<td>CS3</td>
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<td>CS5</td>
<td>USA300_1179</td>
<td>1298428 C→T</td>
<td>CCA→CTA</td>
<td>Pro280Leu</td>
</tr>
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</table>

\(^a\)SNP, single nucleotide polymorphism. Number refers to the nucleotide position in the genome of USA300_FPR3757.
The cvfA gene is also referred to as RNase Y as it is the homolog of RNase Y in Bacillus subtilis and the functional equivalent of RNase E in Escherichia coli (166). It was first identified in S. aureus in 2005 when researchers obtained a knockout mutant of this gene and observed attenuated virulence in a silkworm infection model, naming the gene conserved virulence factor A (cvfA) (167). CvfA has been shown to act as part of the degradosome complex in S. aureus, which is a multi-protein complex responsible for bulk mRNA degradation. In particular, CvfA is known to dimerize, and interact directly with CshA and Enolase, and indirectly with RNases J1 and J2, PNPase, Pfk, and RnpA (168). CvfA itself is a membrane-bound RNase with mapped domains for dimerization, RNA-binding, and catalytic activity, however binding sites for protein partners are currently unknown (Figure 2).

The five mutated amino acid positions identified in linoleic acid resistant clones are outlined in Figure 2. All of these mutations are located in the same region of CvfA, largely in a domain of unknown function, with outliers on the edges of the RNA-binding and catalytic domains. Given that the region within CvfA that is responsible for facilitating protein-protein interactions is unknown, we hypothesized that SNPs in the cvfA gene engender S. aureus with increased resistance to unsaturated free fatty acids through altered interactions with degradosome partners (see section 3.7 for the biochemical characterization of the CvfA protein).
Figure 2. CvfA domain architecture and location of SNPs. The CvfA protein is a membrane protein with one transmembrane domain (TM), a coiled-coil domain to allow dimerization, a KH domain for RNA binding and an HDc domain responsible for its catalytic activity as a phosphodiesterase. The 5 mutated amino acid positions identified in linoleic acid resistant clones are indicated and located in a region with unknown function.
3.3 Fatty acid resistant phenotype is specific to linoleic and arachidonic acids

Although mutations in cvfA were confirmed in each of the 5 linoleic acid resistant clones, these SNPs needed to be confirmed as the cause of increased resistance. Minimum inhibitory concentration (MIC) assays were performed to characterize these clones and identify their FFA resistance profiles. We observed a greater than 4-fold increase in the MIC of linoleic and arachidonic acids for all five cvfA mutants (Figure 3). For linoleic acid, the MIC increased from 150 µM for wild-type USA300 to greater than 600 µM for the SNP-containing strains, with 600 µM being the highest concentration tested. Similarly for arachidonic acid, there was a 4-fold increase in MIC from 75 µM to 600 µM. The cvfA SNP-containing strains did not have an increased MIC to other unsaturated FFAs tested.
Figure 3. Minimum inhibitory concentration of various fatty acids on wild-type *S. aureus* USA300 and *cvfA* SNP mutants (CS1-5). Exponential phase cultures were inoculated at an OD$_{600}$ of 0.01 into glass tubes containing 1 ml TSB with the indicated amount of fatty acid and growth was assessed after 24 hours. MIC assays were performed using linoleic (A), arachidonic (B), sapienic (C), palmitoleic (D), and linolenic (E) acids and repeated in triplicate.
To confirm that these SNPs were alone responsible for the increased linoleic acid resistance, the wild-type or SNP-containing cvfA gene was cloned into a plasmid that was introduced into wild-type USA300. This cross-complementation approach was taken in lieu of traditional complementation of a gene knockout strain because our attempts to knock out cvfA from the chromosome were unsuccessful.

Strain USA300 expressing the cvfA genes from CS1, CS3, and CS5 fully recapitulated the resistance phenotype for both linoleic and arachidonic acids (Figure 4). The strain cross-complemented with cvfA from CS4 had a full increase in MIC of arachidonic acid and a slight increase for linoleic acid, and the strain cross-complemented with cvfA from CS2 had a moderate increase in MIC for both fatty acids. These MICs illustrated that the SNPs in cvfA are indeed responsible for increased resistance to linoleic and arachidonic acids. The SNPs in cvfA in strains CS2 and CS4 may require elimination of wild-type cvfA from the genome to appreciate their full effect.
Figure 4. The MIC of linoleic and arachidonic acids on cross-complemented strains. MIC assays were performed as described previously using linoleic and arachidonic acids with the addition of chloramphenicol in the overnight pre-cultures. USA300 + pALC is the empty vector control while the other strains are cross-complemented with the indicated cvfA gene (wild-type or SNP-containing). Experiments were performed using biological triplicates.
To gain an appreciation of the immediate impact of FFAs on wild-type or SNP-containing bacteria, we performed a killing assay using 100 µM linoleic acid. All strains were initially impacted by the presence of linoleic acid however CS1 was significantly better able to withstand the toxicity of linoleic acid compared to wild-type USA300 (Figure 5a). Viability of USA300 cross-complemented with wild-type cvfA was below detectable limits throughout the experiment and USA300 + pCS1 was impacted by the linoleic acid to a greater degree than CS1; however, we eventually saw recovery of this strain by ten hours (Figure 5b). The increased sensitivity observed in USA300 + pCS1 compared to CS1 may be explained by the presence of wild-type cvfA in addition to SNP-containing cvfA. These data, along with the MICs, demonstrate that SNPs in cvfA protect S. aureus against toxic and inhibitory concentrations of linoleic acid.
Figure 5. The viability of *S. aureus* strains grown in 100 µM linoleic acid over 5 hours. Exponential phase cultures of wild-type and CS1 (A) or strains cross-complemented with wild-type or SNP-containing *cvfA* (B) were inoculated to an OD$_{600}$ of 0.05 into 25 ml TSB containing 100 µM linoleic acid and an aliquot was removed every hour and plated to determine colony forming units (CFUs). The mean of 3 replicates ± standard deviation is plotted, with significant differences determined via an unpaired, one-tailed student’s $t$ test, ** $p < 0.01$, *** $p < 0.001$. LOD = limit of detection.
3.4 Uptake of $^{14}$C-linoleic acid shows less accumulation of fatty acid over time in strains containing a SNP in cvfA

We have shown that SNPs in cvfA result in a higher tolerance to the presence of linoleic acid during growth, however we do not know the fate of that linoleic acid. It has been shown that S. aureus incorporates exogenous fatty acids into its membrane (169, 170). Therefore, we sought to evaluate whether CS1 is better able to tolerate the accumulation of this fatty acid in its membrane compared to wild-type, or if there is some other mechanism occurring, such as reduced entry of the fatty acid or increased efflux. S. aureus was incubated with $^{14}$C-linoleic acid, then applied to filter paper and washed to allow the detection of linoleic acid accumulated on or in the cells. We observed a decrease in accumulation of $^{14}$C-linoleic acid in CS1 compared to wild-type USA300 and this difference was exacerbated when USA300 expressed CS1 CvfA from a plasmid (Figure 6).
Figure 6. Uptake of $^{14}$C-linoleic acid in strains containing a SNP in cvfA. USA300 and CS1 (A) were grown to stationary phase overnight in TSB while USA300 cross-complemented with pWT or pCS1 (B) were grown in TSB containing chloramphenicol. All pre-cultures were diluted to an OD$_{600}$ of 0.01 in fresh TSB and grown to an OD$_{600}$ of approximately 0.5 at which point 0.2 µCi/mL of $^{14}$C-linoleic acid was added to the cultures and 200 µL aliquots were removed at the indicated time points for analysis. The mean of 4 replicates ± standard deviation is shown, with significant differences determined via an unpaired, one-tailed student’s $t$ test, * $p < 0.05$, ** $p < 0.01$. 
3.5 Virulence factor production is impaired in CS1

Given that cvfA is an RNase and we aim to identify a mechanism by which the SNPs in this gene lead to increased FFA resistance, we performed an RNA-sequencing assay comparing wild-type USA300 and CS1 grown in a sub-inhibitory concentration of linoleic acid. Hundreds of transcripts were altered in the CS1 strain, and a few select hits are shown in Table 5. Of particular interest are SAUSA300_1121, SAUSA300_1122 (plsX), SAUSA300_0227 (fadD), and SAUSA300_0229. SAUSA300_1121 is annotated as a fatty acid biosynthesis transcriptional regulator, while fadD and SAUSA300_0229 (annotated as a putative acyl-coA transferase) may be involved in fatty acid processing. The plsX gene was recently identified as being involved in the incorporation of exogenous fatty acids in S. aureus phospholipids (170). Additionally, we identified many downregulated genes in CS1 that are involved in virulence, including saeR, saeS, members of the ESAT-6 secretion system, and various hemolysins. Additional virulence factors were also downregulated, and those shown in Table 5 are a representative selection.
Table 5. Summary of RNA sequencing results

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Gene name</th>
<th>Fold-change</th>
<th>P-value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAUSA300_1749</td>
<td>-</td>
<td>13.54</td>
<td>0.009</td>
<td>Hypothetical protein, most highly upregulated gene</td>
</tr>
<tr>
<td>SAUSA300_1180</td>
<td>-</td>
<td>6.34</td>
<td>0.001</td>
<td>Gene located downstream of cvfA and divergently transcribed</td>
</tr>
<tr>
<td>SAUSA300_0268</td>
<td>-</td>
<td>5.38</td>
<td>0.038</td>
<td>Putative drug transporter</td>
</tr>
<tr>
<td>SAUSA300_1121</td>
<td>-</td>
<td>3.99</td>
<td>0.003</td>
<td>Fatty acid biosynthesis transcriptional regulator</td>
</tr>
<tr>
<td>SAUSA300_1179</td>
<td>cvfA</td>
<td>3.10</td>
<td>0.005</td>
<td>Phosphodiesterase (RNase Y)</td>
</tr>
<tr>
<td>SAUSA300_2127</td>
<td>-</td>
<td>3.05</td>
<td>0.004</td>
<td>Multidrug resistance efflux pump, SepA</td>
</tr>
<tr>
<td>SAUSA300_1122</td>
<td>plsX</td>
<td>2.61</td>
<td>0.002</td>
<td>Putative glycerol-3-phosphate acyltransferase</td>
</tr>
<tr>
<td>SAUSA300_0227</td>
<td>fadD</td>
<td>-2.83</td>
<td>0.012</td>
<td>Acyl-coA dehydrogenase</td>
</tr>
<tr>
<td>SAUSA300_0229</td>
<td>-</td>
<td>-3.56</td>
<td>0.003</td>
<td>Putative acyl-CoA transferase, FadX</td>
</tr>
<tr>
<td>SAUSA300_0691</td>
<td>saeR</td>
<td>-4.70</td>
<td>0.048</td>
<td>DNA-binding response regulator</td>
</tr>
<tr>
<td>SAUSA300_0690</td>
<td>saeS</td>
<td>-4.74</td>
<td>0.033</td>
<td>Sensor histidine kinase</td>
</tr>
<tr>
<td>SAUSA300_0283</td>
<td>essC</td>
<td>-7.28</td>
<td>0.001</td>
<td>Member of the ESAT-6 (type 7) secretion system</td>
</tr>
<tr>
<td>SAUSA300_1058</td>
<td>hla</td>
<td>-10.75</td>
<td>0.058</td>
<td>Alpha-hemolysin</td>
</tr>
<tr>
<td>SAUSA300_0304</td>
<td>-</td>
<td>-11.49</td>
<td>0.006</td>
<td>Hypothetical membrane protein</td>
</tr>
<tr>
<td>SAUSA300_0409</td>
<td>-</td>
<td>-11.91</td>
<td>0.051</td>
<td>Hypothetical protein, most highly downregulated gene</td>
</tr>
</tbody>
</table>
Given that several virulence factors were downregulated and that cyfA is known to process the *sae* T1 transcript (49), we wanted to further characterize this phenotype. We began by looking for differences in secreted proteins between wild-type and SNP-containing *S. aureus* (Figure 7). Strains were grown in sub-inhibitory linoleic acid for 18 hours and then culture supernatants were harvested and normalized by OD$_{600}$ before being subjected to trichloroacetic acid to precipitate proteins. We saw a moderate global decrease in secreted protein production across the SNP-containing strains, with two proteins in particular being largely impacted (indicated by arrows). Further analysis would be required to identify these proteins.
Figure 7. Secreted protein profiles of USA300 and 5 strains containing SNPs in cvfA. Stationary phase cultures were normalized by OD$_{600}$ and centrifuged to pellet bacteria while the supernatants were subjected to 20% trichloroacetic acid to precipitate secreted proteins which were visualized by PAGE. Arrows indicate two proteins in wild-type USA300 that appear to be most affected in the SNP-containing strains.
Additionally, we streaked single colonies of each mutant as well as the wild-type and CS1 cross-complemented strains on a blood agar plate to look for differences in hemolysin production (Figure 8). The cvfA SNPs do not appear to have a significant impact on hemolysis, despite the observed down-regulation of hla (encoding alpha hemolysin) from the RNA-sequencing assay. Interestingly, when USA300 is cross-complemented back with the wild-type cvfA gene (USA300 + pWT), there is a significant decrease in beta-hemolysis.
Figure 8. Visualization of the impact of SNPs in cvfA on hemolysin production. Single colonies of the indicated strains were streaked on 5% sheep’s blood agar and incubated at 37°C for 16 hours followed by 4°C for 4 hours.
3.6 Increased survival *in vitro* in the presence of linoleic acid does not correlate with increased survival *in vivo*

Although CS1 appeared to have altered virulence factor expression, this only moderately extended to virulence factor production, as evidenced in Figure 7 and Figure 8. Also, given that CS1 is significantly more resistant to linoleic and arachidonic acids *in vitro* and that linoleic acid is known to accumulate to high levels in staphylococcal abscesses (2), we performed a subcutaneous murine skin abscess model comparing abscess formation between wild-type *S. aureus* and CS1 (Figure 9). The course of infection was monitored over 10 days, with the peak of infection, as determined by abscess size, occurring at 24 hours. There was no statistically significant difference at any time point; however there was a consistent trend toward decreased virulence in mice infected with CS1 compared to those infected with wild-type USA300.
Figure 9. A subcutaneous murine infection model comparing wild-type USA300 and CS1. 4-7 x 10^6 colony forming units of *S. aureus* were injected under the skin of the flank of each Balb/c mouse. The course of infection over 10 days is shown with abscess area having been measured by tracing images of the abscess using the computer program FIJI. The mean of 10 replicates with standard error of the mean is shown.
3.7 Biochemical characterization of CvfA

3.7.1 Purification of CvfA lacking its transmembrane domain

We have shown that USA300 containing a SNP in cvfA is substantially more resistant to linoleic and arachidonic acids in vitro. Given that the identified SNPs all localize within the same region of the gene that codes for a domain with no known function, and also that the region within CvfA that is responsible for binding other proteins is unknown, we sought to test whether these SNPs were impacting interactions with protein partners. We chose to use CvfA<sub>L269F</sub> as the prototype for the following biochemical assays because when USA300 was cross-complemented with this SNP-containing gene, it produced a robust increase in MIC of the two FFAs and also because we have the full genome sequence for the strain in which this SNP was initially identified (CS1).

To begin protein analysis, we cloned the wild-type and SNP-containing genes into pET28a for expression in E. coli, using a cloning strategy that resulted in the removal of the transmembrane domain (Figure 10a). Expression of cvfA was induced with IPTG, cells were lysed, and following a low speed centrifugation to pellet insoluble material, CvfA was found in the pellet, indicating that it had likely formed inclusion bodies (Figure 10b). The inclusion bodies were resuspended in 8 M urea and left overnight to solubilise. Following dilution to 0.8 M urea, the solution was dialysed thereby removing the remaining urea and allowing the protein to re-fold (Figure 11). This resulted in soluble CvfA protein that was mostly free of contaminating proteins.
Figure 10. CvfA was cloned lacking its transmembrane domain and with an N-terminal histidine tag (A). Upon expression in *E. coli* (B), CvfA can be visualized at approximately 58 kDa in induced whole cell lysates. Following lysis and centrifugation, CvfA was located in the pellet.
Figure 11. Soluble CvfA was obtained from inclusion bodies via denaturation using urea. Inclusion bodies were washed and then solubilised in 8 M urea overnight. Following dilution to 0.8 M urea overnight, all remaining urea was dialysed out of the solution over two days, resulting in soluble, refolded CvfA.
Renatured CvfA was used in a bead-based pulldown assay to evaluate protein-protein interactions. *S. aureus* whole cell lysate was either added alone to cobalt-coated magnetic beads as a negative control or first incubated with renatured CvfA. Upon elution from the beads, samples were visualised for examination of differences in proteins present. When CvfA was included, it was eluted from the beads; however there are no other obvious proteins present in this sample (Figure 12). Additionally, we tested the activity of the protein through a phosphodiesterase assay and found that the protein was inactive (data not shown), possibly indicating mis-folding of the protein upon renaturation.
Figure 12. An *in vitro* pulldown assay using re-natured wild-type CvfA and *S. aureus* whole cell lysate. CvfA was incubated with *S. aureus* whole cell lysate and added to cobalt-coated magnetic beads. Following two washes, bound protein was eluted in 1 M imidazole and visualized using PAGE. As a negative control, whole cell lysate was incubated with the beads alone and the eluate was visualized.
3.7.2 Purification of full length CvfA from *E. coli* membranes

Given the unsuccessful attempts with renatured CvfA lacking its transmembrane domain, we cloned full length CvfA, including its transmembrane domain, for expression in *E. coli* with the assumption that the protein would be present in the membrane, and detergent extraction would be necessary for its purification (Figure 13a). Post-expression, we first evaluated the solubility of CvfA in various detergents. We identified that CvfA was solubilised to varying degrees in n-dodecyl β-D-maltoside (DDM), octyl-β-D-glucopyranoside (OG) and tween-20. Moreover, when they were used in sequential fashion, from DDM to OG, to tween-20, the tween-20 fraction contained what appeared to be pure CvfA (Figure 13b). Unfortunately, however, we found that CvfA present in this Tween-20-solubilised fraction was incapable of binding to cobalt-coated magnetic beads (data not shown).
Figure 13. Full length WT and SNP-containing CvfA genes were cloned with a C-terminal histidine tag into the pET28a E. coli expression vector (A). Upon expression, CvfA is visualized at approximately 61 kDa (B). Following lysis, a low speed centrifugation to pellet insoluble material, and an ultracentrifugation to pellet the membrane, CvfA was found in the membrane fraction, as predicted. A series of membrane solubilisations using DDM, OG, and Tween-20 are shown (B). E. coli containing the vector control (pET28a) was also purified in the same manner (C).
3.7.3 CvfA characterization

Given that the Tween-20-solubilised protein fraction was unable to interact with cobalt-coated beads, we were unable to perform the pulldown assay as we could not pull the protein out of solution; however, we were able to perform a phosphodiesterase assay to look for differences in activity between wild-type and SNP-containing CvfA. The phosphodiesterase assay used a synthetic substrate, bis-4-nitrophenyl phosphate, and measured cleavage of this substrate by quantifying the release of product, which absorbs light at 405 nm. This assay demonstrated that CvfA containing the L269F mutation does not have altered activity compared to wild-type (Figure 14), with both proteins having a $K_m$ of approximately 3 mM (Table 6).
**Figure 14.** Phosphodiesterase activity of Tween-20 solubilised WT or SNP-containing CvfA and the vector control (pET). Samples were treated with DEPC to inactivate RNases as a control. Production of 4-nitrophenol (4-NP) by cleavage of bis-4-NPP was measured by absorbance at 405 nm. The mean ± standard deviation of three technical replicates is plotted.

**Table 6.** $K_m$ of wild-type and SNP-containing CvfA

<table>
<thead>
<tr>
<th>CvfA</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CvfA$_{WT}$</td>
<td>3.09</td>
</tr>
<tr>
<td>CvfA$_{L269F}$</td>
<td>3.08</td>
</tr>
</tbody>
</table>
We found that both the Tween-20 and OG-solubilised protein fractions were unable to bind cobalt, however the DDM-solubilised fraction was able to bind, allowing us to attempt the pulldown assay. Both Tween-20 and DDM are non-ionic detergents and are thus considered mild detergents not likely to denature proteins. Tween-20 however, is known to form very large micelles and this may interfere with the ability of CvfA to interact with other compounds. We employed the DDM-solubilised fraction in a series of pulldown assays, with the controls consisting of *S. aureus* lysate incubated with cobalt-coated beads or the protein fraction alone with beads. The protein fraction, both wild-type and SNP-containing, was also incubated with whole cell lysate and applied to the beads in an attempt to pull out interacting proteins from the lysate. After three washes with 50 mM imidazole, the samples were eluted in 1 M imidazole to remove any bound proteins. Unfortunately, there was significant background banding despite the use of 40 mM imidazole during binding. Regardless, there were no easily identifiable bands that were present in the experimental lanes (1 and 2) that were not also present in the control lanes (3 and 4) (Figure 15). As such, we have not yet been able to determine whether the L269F mutation in CvfA impacts protein partner interactions.
**Figure 15.** The eluates of 4 pulldown assays were visualized by PAGE. Either SNP-containing (CvfA<sub>L269F</sub>) or wild-type CvfA was incubated with *S.aureus* whole cell lysate and cobalt-coated magnetic beads were used to pull out CvfA and any interacting protein partners. Proteins were eluted from the beads using 1 M imidazole. Lysate alone and CvfA alone were used as negative and positive controls, respectively.
4 Discussion

In this work, we have selected for *S. aureus* strains that demonstrate increased resistance to linoleic and arachidonic acids. Further, we have identified single nucleotide polymorphisms (SNPs) in the RNase CvfA that are directly linked to this phenotype. The *cvfA* gene is the homolog of RNase Y in *B. subtilis* and the functional equivalent of RNase E in *E. coli*. As such, CvfA is thought to act as the central component of the degradosome, much like RNase Y and RNase E. Our multiple attempts to generate a *cvfA* knockout in USA300 met with failure. This, combined with the fact that a saturating transposon library in USA300, generated out of Nebraska (171), did not contain an insertion in *cvfA*, led us to conclude that this is an essential gene in USA300. It is widely regarded that the CvfA homolog in *B. subtilis*, RNase Y, is essential.

Interestingly, however, there have been previous reports of knockout *cvfA* mutants in different strains of *S. aureus* (49, 167), and it would be interesting to determine if some sort of suppressor mutations have occurred in these genetic backgrounds that allow for the loss of CvfA function.

The reason why the SNPs we have selected for result in a resistance profile that is specific to linoleic and arachidonic acids is currently unknown. The human body is not able to synthesize linoleic acid and as such, it is an essential fatty acid. Arachidonic acid is synthesized from linoleic acid. The structures of these fatty acids are shown in Figure 16 and they are both polyunsaturated fatty acids with double bonds in the *cis* orientation. The similarities, however, end there – linoleic acid has an 18 carbon chain length and two degrees of unsaturation, and arachidonic acid has a 20 carbon chain length and four
degrees of unsaturation. They are connected in that arachidonic acid is synthesized from linoleic acid in mammals but how that relates to *S. aureus* inhibition is unclear. The specificity toward linoleic and arachidonic acid has been documented in another recent example of *S. aureus* FFA resistance (159).

This previous report described a DNA-binding transcriptional regulator and efflux pump that provides *S. aureus* with protection against linoleic and arachidonic acids. It is tempting to speculate that our *cvfA* mutants are causing the efflux pump, *farE*, to be upregulated or that they are altering expression of the transcriptional regulator, *farR*. However, we did not identify either of these genes as being differentially regulated in our RNA-sequencing assay. Additionally, the resistance phenotype described is inducible whereas resistance in *cvfA* mutants does not require induction by sub-MIC concentrations of fatty acid.
Figure 16. The structures of linoleic and arachidonic acid. Both fatty acids are polyunsaturated with double bonds in the cis orientation.
Of particular interest in the RNA-sequencing dataset was the finding that *plsX* was upregulated. Recently, a Δ*plsX* mutant in *S. aureus* strain RN4220 was reported to be a fatty acid auxotroph (170). In anticipation of this phenotype, researchers supplemented the media with exogenous saturated fatty acids in order to obtain this knockout mutant. The upregulation of *plsX* in our *cvfA* SNP mutant, CS1, may be important in processing these exogenous FFAs for incorporation into *S. aureus* phospholipid. Additionally, several putative efflux pumps or drug transporters were also upregulated. Future work to narrow down an intracellular mechanism would involve creating knockout mutants of these genes and testing for altered levels of FFA resistance.

Another interesting finding from the RNA-sequencing data was that many virulence factors were downregulated. *CvfA* has previously been implicated in *S. aureus* virulence, in particular, in the processing of the *sae* transcript (49, 167). *CvfA* was shown to cleave the *sae* T1 transcript into the predominant T2 transcript, which results in transcript stabilisation (49). Another group showed that processing by *CvfA* stabilises some transcripts by protecting them from degradation by PNPase (172). Of course, this is not always the case as *cvfA* is responsible for the destabilisation of other transcripts (49). Evidently, understanding the full scope of *cvfA* activity and its regulation of RNA will require further investigation.

Apart from the regulation of the *saePQRS* operon, *cvfA* was shown to be involved in the regulation of other virulence factors (49) and we have observed a similar role in this study. In addition to the downregulation of *sae* transcripts, we found many proteins of the ESAT-6 secretion system (Ess) to be downregulated. The Ess pathway has been extensively studied in *Mycobacterium tuberculosis* and in 2005, homologs to the Ess
proteins were identified in *S. aureus* (173). EsxA and EsxB, two members of the Ess, are exoproteins and mutants defective in their production or secretion were found to be attenuated in a murine model of infection (173). EssC and EssB are membrane proteins involved in the translocation of EsxA and EsxB into the extracellular milieu (173, 174). Interestingly, it was also shown that the proteins of the Ess are negatively regulated by the *sae*PQRS system and as such, if the *sae* operon is downregulated, one would expect transcription of the Ess proteins to be increased (175). However, in our cvfA mutant, we found both Sae and Ess proteins to be downregulated and as such, CvfA is likely playing a direct role in the downregulation of the Ess pathway rather than an indirect role through *sae*.

While the RNA levels of many virulence factors were altered, we wanted to know if that extended to the protein levels as well. We visualised levels of secreted virulence factors in our cvfA SNP mutants and saw a slight global decrease in virulence factor production along with marked decreases in specific proteins (Figure 7). Further evaluation of these proteins would be required for their identification. In addition, we visualised the production of hemolysins by plating strains on a blood agar plate and didn’t find a significant impact on hemolysis in the SNP-containing strains; however, the wild-type strain overexpressing wild-type cvfA had a marked reduction in hemolysis (Figure 8). This strain also showed additional signs of distress, such as slowed growth and increased pigmentation. This showed us that the increased FFA resistance in cvfA SNP-containing strains is not simply due to increased expression of the protein and that high levels of wild-type CvfA negatively impact the bacteria.
Given the observed downregulation of several virulence factors in the CS1 clone, it was not surprising to see a trend to decreased virulence in our mouse skin abscess model (Figure 9). The assay will need to be repeated but also the use of a different in vivo model may provide more informative results. For example, we could employ a nasal colonisation model to evaluate FFA survival and a bacteremia model to evaluate virulence potential.

With a picture of the intracellular and in vivo effects of these cvfA SNPs forming, we focused on characterizing their biochemical effects. Unfortunately, expression of CvfA lacking its transmembrane domain resulted in the formation of inclusion bodies and upon refolding, we were unable to identify any interacting protein partners (Figure 12). Although the refolded protein was inactive, protein-binding domains are often unstructured until the interaction forms and thus we thought that the refolded protein would still form interactions with other proteins. It is possible that the protein re-folded in such a way that the residues required for interactions are hidden or blocked.

Given our inability to test for protein interactions with the refolded protein, we retained the transmembrane domain and assessed the effectiveness of various detergents on solubilising CvfA. Because we were unable to further purify CvfA in the Tween-20-solubilised samples due to an inability to interact with cobalt-coated beads, we also purified the vector control in the same manner to control for background proteins and used these samples in the phosphodiesterase assay. As expected, we found that this SNP did not alter the Km of the protein (Table 6) and that the vector control did not have phosphodiesterase activity, indicating that the activity in our CvfA-containing samples was indeed coming from CvfA (Figure 14).
The Km of 3 mM is relatively high indicating low affinity for this substrate; however it is possible that the detergent may interfere with activity. Tween-20 is known for forming very large micelles in solution and this may be the reason CvfA will not bind the cobalt beads in this detergent. It is also possible that the large Tween-20 micelle size impairs the ability of CvfA to access the substrate in the phosphodiesterase assay. Although the Km was high, this assay demonstrated that the L269F mutation in CvfA does not impact protein activity \textit{in vitro}.

The OG-solubilised protein fraction was also unable to be purified with cobalt beads. We were able to bind a small amount of CvfA to the beads when the concentration of OG was decreased from 2\% to less than 1\%; however this reduction in concentration often resulted in protein precipitation. This left the DDM-solubilised sample, in which CvfA was successfully able to bind the cobalt beads for further purification. We performed the pulldown assay using CvfA and \textit{S. aureus} whole cell lysates to identify any proteins with which CvfA interacts; however, we were not able to identify any proteins that were not also found when lysate or CvfA alone were incubated with the beads (Figure 15). Attempts to reduce background proteins were made by increasing the amount of imidazole during binding, however this did not improve the outcome. Unfortunately, we have not determined whether the identified mutations in CvfA impact protein-protein interactions.

4.1 Future Directions

There are many additional ways to address the pulldown assay. For example, one of the protein partners that CvfA is known to interact with could be tagged and the pulldown
assay performed in the same manner. Enolase is known to interact with CvfA and could be tagged, purified, and used in a pulldown assay with whole cell lysates containing either wild-type or mutant CvfA to evaluate the interaction between these proteins. Theoretically, this may be a simpler approach as enolase and the other proteins of the degradosome are soluble, cytoplasmic proteins as opposed to the membrane-bound CvfA. Additionally, the two proteins that CvfA has been shown to interact with, CshA and Enolase, could be separately purified and binding could be evaluated in vitro. This would likely be the approach that would give us the most definitive results for these proteins; however, it is possible that CvfA interacts with other proteins and this approach would preclude their identification. Finally, CvfA could be overexpressed directly in S. aureus cells with a tag to allow its purification. With the addition of a crosslinking reagent, we could pull out CvfA and any interacting partners directly from S. aureus. This approach would have the advantage of being performed directly within the native environment in which these proteins would normally interact and would therefore produce the most applicable results.

Apart from the pulldown assay, it would be interesting to purify the other SNP-containing proteins and determine their impact on protein activity. CvfA from CS1 (CvfA_{L269F}) was found to have no impact on phosphodiesterase activity, however, SNPs in cvfA from CS2 and CS4 cluster close to the catalytic domain of the protein. USA300 + pCS2 and USA300 + pCS4 also have a lower linoleic acid MIC compared to the other cross-complemented strains. This observation led us to question whether the five SNPs are all impacting the same aspect of protein function. It is possible that the SNPs in CS1, CS3, and CS5 may impact protein-protein interactions or RNA binding while the SNPs in CS2
and CS4 could have an impact on protein activity. CvfA from these strains would be cloned and purified from *E. coli* membranes and phosphodiesterase activity tested, as described for CS1.

Moreover, to gain a more complete understanding of the impact of the amino acid changes, we could mutate the identified amino acids to alanine and test for increased fatty acid resistance. This would help us determine whether it is the loss of amino acid side chain resulting in increased resistance or the gain of a specific side chain. We could also select for additional resistance-conferring SNPs in this gene and determine whether the mutations occur randomly throughout this uncharacterized domain or in a select few positions.

In addition to the characterization of the biochemical effect of the SNPs, another priority would be the elucidation of their intracellular impact. Following confirmation of RNA-seq results, a knockout mutant of *plsX* could be created and evaluated for its impact on linoleic acid resistance. If *plsX* is found to have an effect, an *in vitro* binding assay with purified wild-type and SNP-containing CvfA and *plsX* RNA would allow the determination of whether the SNP is impacting cleavage/degradation of *plsX*.

Finally, we are going to determine the fatty acid phospholipid profiles of USA300 and CS1 to look for changes in the composition of the membrane fatty acids. It has previously been shown that when *S. aureus* is grown in the presence of oleic acid, a nontoxic fatty acid, oleic acid is elongated by two carbons through bacterial type 2 fatty acid synthesis machinery and incorporated into the membrane (151). If the same thing occurs when *S. aureus* is grown in linoleic acid, the result would be eicosadienoic acid, a rare
polyunsaturated fatty acid, being incorporated into the membrane (176). The impact of this is unknown and could represent a potential mechanism of toxicity of linoleic acid in USA300. If there are differences in phospholipid fatty acid composition between USA300 and CS1, this may also implicate plsX or related proteins involved in the incorporation of exogenous fatty acids into the membrane as being involved in the increased fatty acid resistance of CS1.

4.2 Conclusion

Overall, we have described the characterization of *S. aureus* mutants that are highly resistant to linoleic and arachidonic acids, but not other unsaturated FFAs. These mutants have SNPs in *cvfA*, all within a narrow region of the gene that codes for a domain with unknown function. The *cvfA* gene encodes an RNase that is a central component of the degradosome within *S. aureus* and participates in the complex regulation of many different genes, particularly those involved in virulence. The protein is anchored in the membrane resulting in difficulties with protein purification; however we have successfully showed that the protein encoding a SNP variant, CvfA1269F, does not have altered activity compared to wild-type. Additional studies are required to determine whether the SNPs impact protein-protein interactions or any other aspect of protein function, such as RNA-binding.
References


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