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Staphylococcus aureus response to long chain antimicrobial fatty acids

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

STAPHYLOCOCCUS AUREUS RESPONSE TO LONG CHAIN ANTIMICROBIAL FATTY ACIDS

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ABSTRACT

Staphylococcus aureus is a common pathogen that has the ability to infect virtually every tissue and organ system of the body. Despite its propensity to cause invasive infection, *S. aureus* is also a commensal organism, asymptomatically colonizing ~25% of the population. Much research has gone into resolving this paradox, focusing on both human and bacterial factors that may contribute to colonization. Antimicrobial fatty acids present on the skin and in the nasal mucosa are important components of the innate immune system, and thus we undertook to further understand how *S. aureus* responds to these fatty acids, and how this response facilitates survival during colonization, or promotes the transition from colonization to infection. Using physiological concentrations of several different fatty acids present on the skin and in the nasal mucosa, we assessed the response of community acquired methicillin resistant *S. aureus* (CA-MRSA) to long chain fatty acids. 25μM of several unsaturated fatty acids including linoleic (C18:2), palmitoleic (C16:1), sapienic (C16:1), and linolenic (C18:3) added to the culture media was not growth inhibitory, yet caused a marked increase in the expression of the secreted proteases comprising the *Staphylococcal* proteolytic cascade (SPC). The increase in the expression of proteases was associated with the processing of the lipase glycerol ester hydrolase (Geh) into its mature form. This was not observed in a protease null mutant.

Growth in the presence of 50μM linoleic acid caused a ~12h lag phase, after which the bacteria resumed a normal growth rate. Cells recovered after growth in 50μM linoleic acid displayed permanent resistance to the same concentration of linoleic acid during subsequent growths, and these resistant strains each had single nucleotide

polymorphisms (SNP) present in their genomes, most commonly targeting the ribonuclease *cvfA*.

Keywords: Fatty acids, protease, *Staphylococcus aureus*, pathogenesis, virulence, community acquired methicillin resistant *Staphylococcus aureus*, lipase, glycerol ester hydrolase

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CHAPTER 2:

Induction of the Staphylococcal Proteolytic Cascade by unsaturated fatty acids in community-acquired methicillin resistant *Staphylococcus aureus*

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Yue Zhu contributed to this work with the construction of protease deficient mutants in strain Newman.

Dr. Martin McGavin and Dr. David Heinrichs contributed to the conceptualization of the research, and the writing and editing of this chapter.

CHAPTER 3:

Insights into the molecular events leading to the adaptation of *Staphylococcus aureus* to unsaturated fatty acids

Yue Zhu contributed to this work with the construction of protease deficient mutants.

Dr. Martin McGavin and Dr. David Heinrichs contributed to the conceptualization of the research, and the writing and editing of this chapter.

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LIST OF ABBREVIATIONS

Agr	Accessory gene regulator
AIP	Autoinducing peptide
AMP	Antimicrobial peptide
Aur	Aureolysin
BHI	Brain heart infusion medium
BSA	Bovine serum albumin
CA-MRSA	Community-acquired methicillin resistant *Staphylococcus aureus*
$CaCl_2$	Calcium chloride
CBP	Collagen binding protein
CC	Clonal complex
Cfu	Colony forming units
CHIPS	Chemotaxis inhibiting protein of *Staphylococcus aureus*
Clf	Clumping factor
Coa	Coagulase
CP	Capsular polysaccharide
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Erm	Erythromycin
Fn	Fibronectin
FnBp	Fibrinogen-binding protein
Geh	Glycerol ester hydrolase
HA-MRSA	Hospital-acquired methicillin resistant *Staphylococcus aureus*
HIV	Human Immunodeficiency Virus
Kan	Kanamycin
LA	Linoleic Acid
LB	Luria Bertani growth medium
MGE	Mobile genetic element

mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MSCRAAMs	Microbial surface components recognizing adhesive matrix molecules
Neo	Neomycin
O.D.	Optical density
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PVL	Panton-Valentine leukotoxin
RNA	Ribonucleic acid
RPM	Revolutions per minute
SAK	Staphylokinase
Sar	Staphylococcal accessory regulator
SCC	Staphylococcal cassette chromosome
SCIN	Staphylococcal complement inhibitor
Scp	Staphylococcal cysteine protease
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SigB	Sigma factor B (σB)
Spa	Staphylococcal protein A
SPC	Staphylococcal Proteolytic Cascade
Ssp	Staphylococcal serine protease
SSTI	Skin and soft tissue infection
ST	Sequence Type
TCA	Trichloroacetic acid
TSB	Tryptic soy broth
TSST	Toxic shock syndrome soxin
uFFA	Unsaturated free fatty acid

LIST OF UNITS

Da – Dalton

µg – microgram

mM – millimolar

°C – Degrees celsius

mg – milligram

ng – nanogram

F – Farad

µL – microlitre

nM – nanomole

g – gram

mL – millilitre

Ω – ohm

h – hour

min – minute

V – Volt

CHAPTER 1 – Literature Review

1.1 Clinical relevance of *Staphylococcus aureus*

The notoriety of *Staphylococcus aureus* as a pathogen began with its discovery in 1880 by Scottish surgeon Alexander Ogston, as the causative agent of post-operative suppurative infections (1). Although the advent of antibiotics has reduced the mortality rate since that time, particularly the serendipitous discovery of penicillin by Alexander Fleming in 1928, *S. aureus* is now the worldwide leading cause of skin and soft tissue infections (SSTI). In addition to SSTIs, *S. aureus* is capable of infecting virtually every tissue in the body, and is also the leading cause of infectious mortality in North America (2, 3). While the prognosis of a *S. aureus* infection is usually optimistic, the incidence of infection is so high that the overall number of fatalities per year from *S. aureus* is greater than that of HIV in North America. Furthermore, in non-fatal incidences, treatment of a *S. aureus* infection can be difficult, necessitating an extended stay in hospital, and thus causing a severe burden on the health care system (2).

The prevalence of *S. aureus* infection is in large part due to its ability to survive at the host pathogen interface. Approximately 25% of the population are persistent carriers of *S. aureus*, while up to 60% are transient carriers, predominantly in the anterior nares, but may also be colonized on the warm and moist areas of the skin, primarily the axillae and perineum (4, 5) . Importantly, patients with nosocomial *S. aureus* infections are often infected by the same strain that has colonized the skin or anterior nares (6)

1.2 Description of *S. aureus*

Staphylococci are Gram positive, spherical, and non-motile bacteria that are ~1μm in diameter. They are distinguishable from other micrococci based on their propensity to form "grape-like" clusters. Ogston observed the micrococci while examining the pus of post-operatively infected patients, and appropriately termed the organism "*Staphylococcus*", from the Greek term "Staphyle", meaning "bunches of grapes". The species *Staphylococcus "aureus"* was further defined by German surgeon Anton Rosenbach in 1884 after he observed the yellow pigmentation of the bacteria due to the pigment molecule staphyloxanthin(7) . *S. aureus* can also be distinguished from other staphylococci in simple laboratory tests *via* the detection of its production of coagulase, a protein which mediates the conversion of fibrinogen to fibrin through its interaction with prothrombin, and clumping factor, a cell surface protein that causes agglutination of the blood by direct binding of fibrinogen (8, 9).

1.3 *S. aureus* genome

S. aureus has a 60-70% mol A+T chromosome of 2.87 Mbp (10). Approximately 75% of the genome is composed of 'core' chromosome, and the additional 25% consists of more variable mobile genetic elements such as prophages, insertion sequences, pathogenicity islands and plasmids (11, 12) . In general, the core genome consists of housekeeping genes, and virulence factors that are common to most strains of *S. aureus*, which include the lipase Glycerol Ester Hydrolase (Geh), proteases aureolysin, SspA, and staphopains A and B, alpha-hemolysin, and coagulase. Much of the genetic variability between strains is derived from the accessory genome, existing in the form of mobile genetic elements. Further genetic variability comes from DNA loss and mutation to existing genes. Evolution through MGE's, DNA loss, and mutation to core chromosomal

genes has been crucial to the success of *S. aureus* as a pathogen, providing the bacteria novel virulence factors, antibiotic resistance genes, and tailoring some strains to become adept at survival in particular host niches (13). For example, methicillin resistant strains of *S. aureus* have acquired β - lactam resistance on the MGE known as the staphylococcal Cassette Chromosome SCCmec acquired via horizontal gene transfer from a coagulase-negative *Staphylococcus* species (14, 15) , and several superantigen genes are encoded on prophage genes and pathogenicity islands (11, 16, 17) . Niche adaptation in some strains may have occurred due to DNA loss, and accumulation of pseudogenes that allow for host colonization at the expense of virulence (18)

1.4 *S. aureus* paradigm of infection

A hypothetical infection by *S. aureus* may be divided into three stages; i) colonization/attachment ii) tissue invasion/abscess formation and iii) metastatic infection. In addition to these, the bacteria are also faced with the burden of immune evasion across all three stages. Staphylococcal pathogenesis is multifactorial, and the ability of *S. aureus* to infect virtually every tissue of the body is mediated by its large endowment of virulence factors. Virulence factors come in the form of secreted proteins, membrane bound proteins, and cell surface components.

First, colonization of the skin and anterior nares by *S. aureus* is a common precursor to infection, as demonstrated by the finding that patients with invasive infections are usually colonized by the same strain isolated from the site of infection (19, 20) . Furthermore, colonized individuals are four times more likely to suffer from infection. Infection follows colonization through a breach in the host-pathogen interface,

and attachment of the bacteria to host tissue or extracellular matrix through cell wall associated adhesion proteins, which include members of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family, which will be discussed in further detail in the next section. Examples of a breach in the host pathogen interface leading to infection include the inoculation of an open wound on the skin with colonizing bacteria, or an influenza-mediated damage in the mucosa of the respiratory tract, followed by infection with *S. aureus* resulting in pneumonia.

Tissue invasion and abscess formation is mediated by a range of secreted and cell surface factors that degrade host tissues (e.g. proteases), and seize nutrients via the breakdown of host factors (hemolysins, proteases, lipases). The formation of an abscess results from incoming neutrophils encapsulating the bacteria. Metastatic infection is a result of the escape of the bacteria from abscesses, through mechanisms which are not completely understood, but likely involves production of several secreted virulence factors, which include but are not limited to proteases, nuclease, lipase, leukotoxins, and small cytotoxins of the phenol soluble modulin family. This may result in septicemia and infection in almost any other tissue of the body. Throughout the course of infection, *S. aureus* may also secrete toxins that travel systemically resulting in toxinosis in the absence of metastatic infection, a classic example being toxic shock syndrome, caused by toxic shock syndrome toxin-1 (TSST-1) (21, 22) .

1.5 Attachment and adhesion factors

Attachment to host tissue components is a necessary step to *S. aureus* infection, and is mediated through cell wall associated virulence factors, in particular the microbial

surface components recognizing adhesive matrix molecules (MSCRAMMs) (23, 24) , fibronectin binding protein (FnBP), collagen binding protein (CBP), clumping factor (Clf) and extracellular fibrinogen binding protein. MSCRAMMs are anchored to the peptidoglycan by the enzyme Sortase A, which recognizes the LPXTG amino acid sequence near the C-terminus of peptidoglycan-bound proteins. Sortase A cleaves at the C-terminal end of the threonine residue, and catalyzes the formation of an amide bond between the threonine and an amino group on the peptidoglycan (24, 25) . The vast majority of peptidoglycan anchored proteins in *S. aureus* utilize sortase A and the LPXTG signal sequence, however *S. aureus* also maintains a second sortase enzyme, sortase B, which recognizes and anchors proteins with the signal sequence NPQTN. The only protein known to be anchored via this enzyme is the Iron-regulated Surface Determinant C (IsdC) protein (26)

As its name suggests, FnBP binds to fibronectin, a glycoprotein of the extracellular matrix that is typically bound to integrins, but may also be associated with collagen and fibrin. Clumping factor associated with the cell wall, and the secreted extracellular fibrinogen binding protein both directly bind fibrinogen and cause agglutination. Attachment and agglutination may also cause emboli, and diseases such as infective endocarditis, where *S. aureus* attaches and accumulates on cardiac valves resulting in inflammation of the cardiac tissue.

Attachment and agglutination allow for the assembly of high numbers of bacteria, which, as discussed later, is necessary for the expression of virulence factors responsible for the transition to invasive infection.

1.6 Tissue Invasion and metastatic infection

Tissue invasion and dissemination begins with the enzymatic breakdown and decrease in expression of the MSCRAMMs involved in attachment and adhesion, typically mediated by extracellular proteases (27, 28) . Host tissues are broken down by extracellular enzymes such as lipases, proteases, hemolysins, phospholipases, hyaluronidase, and nucleases. In addition to tissue invasion, these toxins also provide nutrients to the bacteria through the breakdown of host proteins, lipids and red blood cells. Abscess formation in host tissues follows, and the escape from abscesses further promotes metastatic infection and sepsis (29).

Alpha-toxin, which is considered to be the major lethal toxin of *S. aureus*, exerts its cytolytic pore forming activity on red blood cells, oligomerizing to form a β–barrel in the cytoplasmic membrane, thus lysing the cell and releasing heme to be scavenged by the bacteria (30-32) . Studies have suggested that alpha toxin may be a candidate vaccine antigen, as the virulence of some of the most prevalent and virulent strains of *S. aureus* lacking the gene for *hla* was attenuated in a murine infection model, and immunization of mice against alpha-toxin reduced the severity of disease in a skin wound infection model (33) . Other hemolysins encoded on the core genome of *S. aureus* include beta-toxin, gamma hemolysin, and delta toxin.

Leukocidins are also cytolytic pore forming toxins that cause lysis of neutrophils. These toxins typically consist of two components, S and F, which assemble in the membrane of white blood cells, forming a pore and leading to cell lysis (34) . The

significance of leukotoxins will be discussed further in the section on Community Acquired Methicillin Resistant *S. aureus* (CA-MRSA).

The expression of proteases and their role in *S. aureus* virulence will be discussed in further detail in a following section.

1.7 Immune Evasion

Both secreted and cell surface virulence factors are employed by *S. aureus* to evade the innate and adaptive immune systems. Examples of membrane associated immune evasion factors include carotenoid pigment, which resists extracellular reactive oxygen species produced in the oxidative burst of neutrophils (35) . Hydrogen peroxide is also detoxified by the enzyme catalase, which converts H_2O_2 to H_2O and O_2. Staphylococcal protein A (Spa) can be either membrane bound or secreted, and sequesters the Fc region of IgG antibodies, thereby preventing their opsonic binding to the cell surface (36, 37) . The 5 and 8 serotype capsular polysaccharide produced by *S. aureus* acts as a cloak to hide the bacterial antigens from the innate and adaptive immune systems, and clumping factor may also function in this capacity through the accumulation of fibrinogen at the cell surface (38-41) . Antimicrobial peptides of the innate immune system can be sensed by the antimicrobial peptide sensor (*aps*), causing an increase in the positive charge of the cell membrane via D-alanylation and lysine modification, thus repelling the positively charged AMP (42) . AMPs may also be degraded by extracellular proteases. Interference with the complement system is mediated through three proteins, Staphylokinase (SAK), chemotaxis inhibiting protein of *Staphylococcus aureus* (CHIPS), and staphylococcal complement inhibitor (SCIN) (43-45) . SAK can be either secreted or

cell membrane associated, and it converts the plasma protein plasminogen into the active form, plasmin (46) . Plasmin is an important human plasma enzyme that degrades other plasma proteins, most notably fibrin clots. However, plasmin may also degrade antibodies and AMPs, thus protecting the bacteria. CHIPS is a secreted protein that inhibits neutrophil chemotaxis by binding the C5a complement receptor and the N-formlyated peptide receptor located on the neutrophil cell surface. Both receptors, which are critical to attract neutrophils, are negated by CHIPS interference (47-49) . SCIN is also a secreted protein that interferes with the C3 convertase complex that forms on the cell surface of the bacteria.

More recently, small secreted molecules known as phenol soluble modulins (PSMs) have been the subject of debate regarding staphylococcal pathogenesis. PSMs are small peptides that polymerize to create pores in the cytoplasmic membranes of neutrophils, causing lysis(50, 51) .

A more detailed discussion on proposed mechanisms of resistance to antimicrobial fatty acids follows in a later section.

1.8 Regulation of virulence factors

The expression of virulence factors during the attachment and invasive infection stages of staphylococcal pathogenesis is vastly different, and the shift from colonization/attachment to invasive infection is governed by the regulation of these virulence factors. Regulation is umpired by a complex network of two component systems, sigma factors, and other transcription factors that respond to environmental and cellular signals.

The most established and well-studied of these systems is the Accessory Gene Regulator (Agr) two-component system, of which the main function is triggering the positive expression of virulence factors contributing to invasive infection (52) . The Agr system is activated upon sufficiently high bacterial cell density using quorum sensing (53). Briefly, the *agr* locus contains four genes, directed by two promoters, P2 and P3. Transcription of *agrBDCA* is controlled by promoter P2, while P3 controls transcription of RNAIII, which is the effector molecule for downstream gene regulation (54) . Autoinducing peptide AgrD is processed, modified and secreted by the transmembrane protein AgrB (55, 56) , and at sufficiently high cell density, the concentration of AgrD in the local environment also becomes sufficient to stimulate the sensor protein AgrC. AgrC in turn phosphorylates AgrA, which is then able to bind and activate P2 and P3 (57) . Activated P3 stimulates increase in the production of regulatory RNAIII, which causes upregulation of genes involved in invasive infection such as proteases, hemolysins, lipase, and superantigens. Also embedded within the RNAIII transcript is the mRNA encoding a small cytolytic toxin δ-hemolysin, which is a member of the phenol soluble modulin family. (54)

Although a major regulator of virulence in *S. aureus*, Agr is only one of several two component systems present in the genome, including *saeRS*, *srrAB*, and *arlRS* (58-60) . The regulon of TCS *saeRS* overlaps with that of *agr*, and is induced in response to RNAIII. The environmental signals contributing to *saeRS* expression are not as well understood, but it has been demonstrated that its expression may be reduced in response to low pH, and its regulon overlaps that of *agr*. The TCS encoded by *arl* induces

autolysis, while downregulating *agr* (61) . *srrAB* is also an antagonist of *agr*, and is induced in response to low O_2 (60).

In addition to TCS, *S. aureus* also employs transcription factors and alternative sigma factors in the regulation of its virulence. *S. aureus* has several transcription factors, most notably the Staphylococcal accessory regulator (Sar) family of helix-turn-helix DNA binding proteins. This family contains 6 members, SarA, R, S, T, U, and V, but, specific mention will be made to only SarA here. SarA represses the transcription of proteases, and also effects the expression of RNAIII, thus indirectly regulating the expression of Agr-regulated genes (62) . Alternative sigma factors are also involved in the regulation of gene expression in response to environmental stimuli, most nobably σ^B, which is a major alternative sigma factor responding to environmental stresses such as energy depletion, and oxidative stress. In general, σ^B causes a decrease in the expression of exoproteins, and an increase in many adhesion proteins. Importantly, σ^B increases the expression of SarA, thus heavily decreasing the expression of secreted proteases, and is also required for carotenoid pigment synthesis. σ^B mutants are thus completely deficient in pigment synthesis and are hyper-producers of secreted protease (63) .

1.9 Antibiotic resistance

The combination of the virulence and staggering rates of antibiotic resistance of *S. aureus* has led to its appointment to the list of so called "Superbugs". As stated earlier, the introduction of the beta lactam penicillin led to a large reduction in the mortality rate of *S. aureus*, due to its ability to bind and inhibit the transpeptidase (penicillin binding protein, PBP) that catalyzes the crosslinking of the peptidoglycan. However, two years

later penicillin resistant strains emerged, and today, more than 90% of *S. aurues* isolates are penicillin resistant (64). Penicillin resistance is mediated by a penicillinase, which breaks down the beta-lactam ring of penicillin, preventing it from inhibiting PBP (65) . To battle penicillin resistance, penicillinase-resistant antibiotics were produced, such as methicillin. However, methicillin resistant *Staphylococcus aureus* (MRSA) emerged in the United States and the United Kingdom in 1961, and as the name suggests, it is resistant to methicillin, and in fact all beta-lactam antibiotics. Beta-lactam resistance is mediated by the acquisition of an alternative PBP, PBP2a, that has less affinity for beta-lactam antibiotics, thus rendering the treatment ineffective. PBP2a is encoded on the gene *mecA*, present on a pathogenicity island known as the Staphylococcal Chromosomal Cassette (SCC*mec*), likely acquired via horizontal gene transfer from *Staphylococcus epidermidis* (66) . Thus far, there are 8 different types of SCC*mec* elements discovered in *S. aureus*, which differ based on size and antibiotic resistance genes (67) . Although all SCC*mec* elements harbour the *mecA* gene, certain SCC*mec* elements have other mobile genetic element insertions that contain non-beta-lactam antibiotic resistant genes, such as SCC*mec* II and III (14) . Additional antibiotic resistance genes provide for further protection against antibiotics, although this comes at a fitness cost, and thus these strains may be less aggressive, but much more difficult to treat. Hospital associated methicillin resistant (HA-MRSA) strains characteristically have SCC*mec* types II and III, which carry additional antibiotic resistance genes (68). Community-associated methicillin resistant (CA-MRSA) strains typically have been associated with type IV SCC*mec* elements, although types V and VII have been found as well. These SCCmec types lack additional non-beta-lactam antibiotic resistance genes and the mobile genetic insertion elements that are characteristic of SCCmec types of HA-MRSA, and it is likely that this

contributes to their increase virulence and propensity to infect healthy individuals, although renders the bacteria more susceptible to antibiotic treatment then their HA-MRSA counterparts (69) .

1.10 CA-MRSA and USA300

CA-MRSA, and strain USA300 in particular, are the most common cause of aggressive SSTI reporting to emergency rooms in North America (70-72) . In addition to the differences between CA-MRSA and HA-MRSA listed above, there are other factors that differentiate the two, and there is thus an ongoing debate in the literature that addresses these factors as possible causes of the increased virulence of CA-MRSA. These include higher production of virulence factors such as PSMs, the presence of the Panton Valentin Leukocidin (PVL) toxin, and in the case of USA300, the presence of the Arginine Catabolic Mobile Element (ACME) (73). Arguably, the most fiercely debated of these factors is the presence of the PVL encoding genes, *lukS-PV* and *lukF-PV* on a prophage. This is an obvious target because it is both a putative virulence factor, and is present in the vast majority of CA-MRSA strains, and absent in HA-MRSA strains. Literature has provided evidence both in favor of and against the notion that PVL is indeed the virulence factor that is responsible for the increased virulence of CA-MRSA. Specifically, *in vivo* infection models using rabbits have established that PVL is indeed necessary for virulence; however this has not been unequivocally supported in studies using murine models. It has been suggested that differences in sensitivity of leukocytes of different animals is the cause of this ambiguity (67, 73-77) .

The ACME is with a few exceptions, exclusively associated with USA300, and is physically linked to the SCCmec type IV element (78) . Present on this mobile genetic element is an arginine deiminase operon, and an oligopeptide permease operon, and it has been suggested that these may contribute to fitness of the organism. Specifically, the arginine deiminase pathway culminates in the release of an ATP molecule, and a basic NH_3 molecule that may neutralize the acidic environment of the skin, and thus promote colonization. Oligopeptide permeases allow for the uptake of small nutrients in the form of small polypeptides. Although ACME may theoretically increase the fitness of USA300, this has yet to be unambiguously demonstrated, and thus is not generally accepted as a major cause of the increased virulence seen in USA300 (28, 79, 80) . It is perhaps more likely that ACME contributes to enhanced survival on the skin, and thus increased pervasiveness in the community.

1.11 Staphylococcal proteolytic cascade

The Staphylococcal Proteolytic Cascade (SPC) consists of three secreted proteases, the metalloprotease Aureolysin (Aur), staphylococcal serine protease A (SspA), and cysteine protease Staphopain B (SspB), with SspA and SspB in an operon along with a cognate inhibitor to Staphopain B, Staphostatin B (SspC), collectively forming the *sspABC* operon. The cascade is that of sequential activation of the proteases, culminating in the activation of SspB. The cascade begins with the production and autocatalytic activation of Aureolysin, which then activates SspA, and activated SspA then activates SspB cysteine protease(28, 81-83) .

Aureolysin is an M4 family metalloprotease and its 56 kDa precursor form becomes active via the autocatalytic removal of its 23 kDa propeptide, yielding a 33 kDa active enzyme. Activity of this protease depends on a Zn^{2+} in the active site, coordinated by His_{352} (84). As with other M4 metalloprotease family members, aureolysin often acts as a proprotein convertase, which serves to process and activate other enzymes, and also has a broad substrate specificity, preferring a hydrophobic amino acid at the P1 position.

Although the activation of glutamyl endopeptidase SspA depends on aureolysin, proSspA is able to undergo initial steps of autocatalysis and these steps are necessary for the efficient final activation by Aur. A glutamine rich segment between propeptide residues 40 and 53 facilitates autocatalysis by the glutamyl endopeptidase, and preferred cleavage is at Gln_{43}. This allows for the release of mature SspA by cleavage at Leu_{56}, and then Val_{69} by aureolysin (82) . Mature SspA preferentially cleaves on the C-terminal side of glutamic acid residues, and controls adhesion of *S. aureus* by breaking down the MSCRAMM FnBP, thus contributing to invasive infection (83) , and also activates proSspB.

Mature Staphopain B cysteine protease represents the fruition of the SPC. Secreted as an inactive precursor, proSspB's activation depends on SspA cleavage on the C-terminal end of Glu_{220} on the propeptide of SspB (28) . Mature SspB performs a variety of functions, including the degradation of fibrinogen, collagen, the chemottractant chemerin(28, 85, 86) , thus contributing to virulence. Staphopain B cleaves with the preference for arginine at the P1 position, and a bulky amino acid at P2 (83) .

The proteases of the SPC, along with another cysteine protease outside of the SPC, Staphopain A (ScpA), constitute the major proteolytic repertoire of *S. aureus*. Although the list of the virulence capabilities of protease has not been exhausted here, cumulatively, proteases contribute to infection by i) Controlling adhesion to host tissues ii) Acting as proprotein convertases, iii) Degrading plasma molecules contributing to blood clotting, and iv) Degrading immune system molecules.

1.12 Glycerol Ester Hydrolase and role of lipases in virulence

The lipase Glycerol Ester Hydrolase (Geh) is expressed as a 72kDa precursor enzyme, and is processed into a 42kDa mature protein. Its annotated function is to break down triglycerides via the hydrolysis of the ester bonds between fatty acids and the glycerol molecule, and in this sense, it has been deemed to aid the bacteria in the breakdown of host tissues and the liberation of nutrients. Traditionally, Geh has been dismissed as a significant virulence factor, and *S. aureus* researchers have even used *geh* as the target gene for the insertion of plasmids and expression of exogenous genes. However, it was recently demonstrated that a lipase mutant was less virulent in a murine model, and was also less able to form biofilms (87) . Apart from this, there has been no significant research into the contribution of lipases to virulence in *S. aureus*, or how lipases may promote colonization.

1.13 Resistance to antimicrobial fatty acids

In addition to other innate defenses at the host-pathogen interface such as low pH, high NaCl concentration and antimicrobial peptides, long chain fatty acids are abundant on the skin and in nasal secretions, and have been reported to be potent antimicrobials. Fatty

acids exist on the skin in sebum, which is a waxy substance secreted by the sebaceous glands and consists of ~28% free fatty acids, with the rest being composed of triglycerides, wax esters and squalene (88). In addition to the skin, there are also fatty acids present at μM concentrations in nasal secretions, and mM concentrations in an abscess (89, 90). Fatty acids have been identified as important components of innate skin defense, and patients with a deficient production of Sapienic acid (C16:1Δ6) on the skin were more prone to develop atopic dermatitis (91) , an inflammatory condition that is highly associated with *S. aureus* colonization. Fatty acids on the skin and in the nasal mucosa include myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), sapienic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2). These fatty acids are present in varying concentrations from individual to individual (89).

Some bacteria are able to utilize fatty acids as a means of energy; however, this requires that the fatty acid be broken down into two-carbon acetyl-CoA molecules by beta-oxidation for entrance into the TCA cycle. According the annotated genome sequence, *S. aureus* lacks the enzymes necessary for beta-oxidation, and thus it is unlikely that *S. aureus* is able to break down fatty acids using this method (11, 78). In addition to utilizing fatty acids, some bacteria also produce and secrete fatty acids. Importantly, *Lactobacillli* of the nasal microbiota have been shown to produce conjugated linoleic acid, an isomer of linoleic acid (92-94), and Importantly, *S. aureus* must also compete with the nasal microbiota for colonization, including several phyla of bacteria, including Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria. As an example of this competition, it has been shown that there is an inverse correlation of the amount of *Staphylococcaceae* and members of the *Actinobacteria* family (92). The differences in the

profile of microbiota between individuals may, in part, account for the differences in the composition of fatty acids of the nasal fluid, and these differences in the composition of the microbiota and fatty acid profile of the skin an nasal passage must be taken into consideration when putting this research into context.

Literature thus far indicating mechanisms of resistance to fatty acids have typically found that the absence of a cell wall anchored element causes increased sensitivity to fatty acids, and thus implicates that element in resistance. The heme coordinating surface protein IsdA, *S. aureus* surface protein F (SasG), and teichoic acids have all been implicated in resistance to fatty acids (95-97). While the mechanism of resistance had not been demonstrated, it has been suggested that this is due to the physical presence of the cell surface proteins acting as a barrier to fatty acids.

The most demonstrable and specific known mechanism of resistance to fatty acids is the Fatty Acid Modifying Enzyme (FAME). The FAME is an enzymatic activity that can be detected in 80% of *S. aureus* strains, and is characterized by the esterification of a fatty acid to a free hydroxyl, usually on a cholesterol molecule, thus detoxifying the fatty acid (98, 99) . In spite of the knowledge of FAME's existence since the 1980's, the enzyme itself has never been isolated, nor has the genetic component been identified. The enzymatic activity of this enzyme is to perform an esterification reaction, optimally at a low pH (5.5-6); both properties opposing those of traditional lipases which have optimal esterase activity at a high pH (99-101) . In keeping with traditional virulence factors, and similar to Geh, FAME activity seems to be dependent on induction by Agr (102), and it has been shown that FAME producing strains are more virulent in a murine model than strains in which FAME is not detected (99) .

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HYPOTHESIS and OBJECTIVES:

Exposure of *Staphylococcus aureus* to long chain antimicrobial fatty acids will invoke an adaptive response that ultimately contributes to persistence on the skin and in the anterior nares, and promotes the shift from colonization to infection.

Objective 1: Assess the growth of clinically relevant strains of *S. aureus* in the presence of physiological concentrations of fatty acids present on the skin and in the nasal mucosa.

Objective 2: Determine the effect of fatty acids on virulence factor production by *S. aureus*

Objective 3: Determine the significance of differentially expressed virulence factors and the mechanism of *S. aureus* resistance to fatty acids.

CHAPTER 2

Induction of the Staphylococcal Proteolytic Cascade by unsaturated fatty acids in community-acquired methicillin resistant *Staphylococcus aureus*

Work presented in this chapter has been published in PLoS ONE

2.1 INTRODUCTION

Staphylococcus aureus has a dichotomous relationship with humans. It is a ubiquitous commensal that persistently colonizes 25%-30% of the human population (1), yet it also has a broad arsenal of virulence factors (2), which enable it to be a leading cause of infections, ranging from relatively mild skin and soft tissue infections, to severe and life-threatening conditions such as necrotizing pneumonia, osteomyelitis, and infective endocarditis (2,3). The preferred site of colonization is the anterior nares, and infections are typically a consequence of autologous nasal carriage (4). Significant effort has therefore been directed towards identifying host and microbial factors that determine the carriage or non-carriage status (1,5,6,7,8,9,10,11,12), and in this context, our study is based on two broad assumptions. First, in order to maintain a persistent carrier status, *S. aureus* must endure innate defense mechanisms of the skin and mucosal surfaces, and from this, it follows that highly invasive pandemic strains of *S. aureus* should possess effective means of responding to these innate defense mechanisms.

The USA300 strain of community acquired methicillin resistant *S. aureus* (CA-MRSA) is well suited for testing these tenets of virulence and transmission. For approximately 30 years after the emergence of methicillin resistance, MRSA were restricted to the hospital environment, and these hospital-associated MRSA evolved by acquiring resistance to multiple antimicrobial agents (13,14,15,16). However, beginning in the 1990's, the epidemiology of MRSA colonization and infection has undergone a paradigm shift with the rapid emergence and pandemic community transmission of the USA300 strain of CA-MRSA, which is known for causing aggressive skin and soft tissue infections that can progress to fatal complications if not rapidly treated (17,18). USA300

is now the leading cause of visits to hospital emergency departments in North America, for treatment of skin infections (19), and is displacing less virulent HA-MRSA, potentially aided by its ability to establish asymptomatic nasal carriage in health care workers (20). USA300 is more easily transmitted to household contacts compared to other *S. aureus* genetic backgrounds (21), and this study which surveyed the inguinal area in addition to the anterior nares, would have missed 51% of MRSA colonized persons if it had been conducted on a nares-only basis (21), which supports the contention that the rampant community transmission of USA300 could be due in part to a superior ability to persist on skin surfaces. A portion of this success is attributed to the arginine catabolism mobile element ACME, which uniquely confers resistance of USA300 to antimicrobial polyamines (22), and has been proposed to facilitate persistence on skin through catabolism of arginine with concomitant release of ammonia to neutralize acidic pH (23,24,25).

Another barrier to persistence of bacteria on skin is the antimicrobial properties of sebum (26,27), which is a liquid phase lipid mixture secreted from the sebaceous glands, consisting of approximately 28% free fatty acids, 32% triglycerides, 25% wax esters, and 11% squalene (28). In sebum triglycerides and free fatty acids, the major component is sapienic acid (C16:1Δ6), which is an isomer of palmitoleic acid (C16:1Δ9), and exhibits the primary antimicrobial activity (27). The importance of sapienic acid as an innate defense mechanism is evident in atopic dermatitis, where the skin is deficient in this fatty acid (29), and there is a near 100% recovery of *S. aureus* from the skin of atopic dermatitis patients. *S. aureus* is also exposed to antimicrobial fatty acids in colonization of the anterior nares, where palmitoleic (sapienic) and linoleic acid (C18:2) were

identified as the major unsaturated free fatty acids (uFFA) in human nasal secretions (30). Linoleic acid also accumulates to high levels within *S. aureus* abscesses (31), and abscess formation is a hallmark of *S. aureus* infection of the skin and soft tissues.

Although exposure to antimicrobial fatty acids would be one of the first signals encountered by *S. aureus* during colonization of the skin or anterior nares, studies that have assessed the response of CA-MRSA to host-specific environmental signals have focused on stresses related to growth in blood, or phagocytosis by neutrophils (32,33). Therefore, the goal of this study was to evaluate how growth and production of secreted virulence factors by USA300 is influenced by exposure to physiologic levels of uFFA. Herein, we describe the robust induction of the staphylococcal proteolytic cascade pathway (SPC) in response to subinhibitory concentrations of uFFA in USA300 and other strains of CA-MRSA, and this response was also evident to varying degrees in clinical MSSA. The staphylococcal proteolytic cascade is comprised of a metalloprotease Aureolysin, which is needed to activate the SspA serine protease, which in turn activates the SspB cysteine protease that is co-expressed with SspA in the *sspABC* operon (34,35,36,37,38). We discuss the implications of this environmental signal-response pathway, and its potential impact on colonization, transmission, and the aggressive nature of skin and soft tissue infections caused by CA-MRSA.

2.2 MATERIALS AND METHODS

2.2.1 Strains and growth conditions

Bacterial strains and plasmids used in this study are defined in Table 2.1. Cultures were maintained as frozen stocks (-80ºC) in 20% glycerol, and streaked on TSB agar when required. TSB was supplemented, when necessary, with 10µg/mL erythromycin or 2µg/mL tetracycline for propagation of strains bearing resistance markers.

Table 2.1 *S. aureus* strains and plasmids used in this study

Strain	Description	Source
RN4220	Restriction deficient lab strain	(42)
DU5969	RN4220*aur::lacZ*	(48)
USA300 LAC	Hypervirulent pandemic CA-MRSA Los Angeles county clone, clonal complex CC8 *spa* t008	(77,78) Barry Kreiswirth
USA300	USA300 LAC cured of antibiotic resistance plasmid	This study
USA300*aur::lacZ*	*aur::lacZ* from DU5969 transduced into USA300	This study
USA300*sspABC*	Replacement of *sspABC* in USA300 with Tc^r cassette, using pMJ232	This study
USA300*aur*	Transduction of *aur::lacZ* into USA300	This study
USA300*sspABCaur*	Transduction of *aur::lacZ* into USA300*sspABC*	This study
USA400	CA-MRSA fatal pediatric bacteremia, CC1 *spa* t127	(79)
MSSA476	CA-MSSA closely related to USA400; pediatric osteomyelitis	(75)
Newman	MSSA clinical isolate; routinely used in virulence studies, CC8 *spa* t008	(80,81)
WBG10049	Southwest Pacific Clone of CA-MRSA, CC30 *spa* t019	(82)
MRSA252	HA-MRSA; CC30, *spa* t016	(83)
UAMS-1	MSSA osteomyelitis, CC30 *spa* t033	(84)
SRI-138	MSSA dermatitis CC45 *spa* t065	(85)
SRI-109	MSSA dermatitis; CC45 *spa* t015	(85)
PED1-75	MSSA pediatric dermatitis; CC5 *spa* t002	(85)
SRI-116	MSSA dermatitis; CC1 *spa* t7404	(85)
SRI-142	MSSA dermatitis; CC1 *spa* t4938	(85)
PED2-1	CA-MRSA pediatric dermatitis; CC97 *spa* t7398	(85)
PED1-53	MSSA pediatric dermatitis; CC8 *spa* t008	(85)
L528	MSSA infective endocarditis; CC30 *spa* t033	(52)
PED1-37	MSSA pediatric dermatitis; CC398 *spa* t937	(85)

Table 2.1 Continued…..

Plasmid	Description	Source
pMAD	Shuttle vector for construction of mutations in Gram-positive bacteria	(41)
pDG1514	Source of Tc^r cassette	(86)
pMJ232	pMAD containing Tc^r cassette from pDG1514, flanked by *Bam*HI-(*sspA*-5P)-*Mlu*I and *EcoR*I-(*sspC*-3P)-*Bgl*II	This study

2.2.2 Generation of plasmid-cured USA300

USA300 LAC was a generous gift from Dr. B. Kreiswirth. To facilitate mutagenesis in the USA300 genetic background, USA300 LAC was cured of the 27-kb plasmid (39), yielding USA300, which is sensitive to erythromycin, kanamycin and neomycin, using the method previously described (40). The plasmid cured USA300 LAC is referred to as USA300 throughout.

Construction of USA300Δ*sspABC*

The *sspABC* operon encodes the SspA serine protease and Staphopain B cysteine protease SspB. For construction of USA300Δ*sspABC::tc*, three DNA segments were assembled in pMAD, consisting of a 702-nt *sspA* 5'-flanking segment *Bam*HI-(*sspA*-5P)-*Mlu*I, a 2.1kb *Pst*I-[illegible]

cgc***ggatcc***CGGTAAAGGATTTGTAAGGATTTCC-3') and *sspA*-5PR (5'-gcg***acgcgt***TTGCTGCTGGAGAACTCACAAGTG-3'), where the lower case residues in bold italics represent added *Bam*HI and *Mlu*I restriction sites. Similarly, the 760 nt *sspC*-3P flanking segment was amplified with primers *sspC*-3PF (5'-ccc***gaattc***CAATTTCTCACCAGCTCG-3') and *sspC*-3PR (5'-gga***agatct***GTAGGTGAAGACCAAATCCCTCG-3'), incorporating respective *Eco*RI and *Bgl*II sites. After plasmid assembly in *E. coli* DH5α, the resulting plasmid pMJ232 was transferred into *S. aureus* Newman via electroporation, using *S. aureus* RN4220 as an intermediate host. Construction of the *ΔsspABC::tc* deletion mutation was conducted following protocols established for use with pMAD (41). The *ΔsspABC::tc* deletion was confirmed by PCR with two primer pairs; one which flanks the external boundaries of the 2.6-kb deletion, and the second which anneals within the deleted segment, and yields a product only with wild type genomic DNA. The *ΔsspABC::tc* mutation was then transferred from strain Newman to USA300 using a phage ϕ85 transducing lysate.

2.2.3 Molecular biology protocols

Protocols for plasmid construction in *E. coli* DH5α and genetic manipulation of *S. aureus*, including isolation of plasmid and genomic DNA, electroporation, and phage transduction have been described previously (35,36),(42). Restriction enzymes and DNA ligase were purchased from New England BioLabs, and AmpliTaq Gold DNA polymerase was purchased from Life Technologies. DNA amplification was conducted using a PTC-100 Thermal Controller (MJ Research). The integrity of cloned PCR

products was confirmed by sequencing of plasmid constructs at the London Regional Genomics facility of the Robarts Research Institute.

2.2.4 TCA precipitation of proteins, SDS-PAGE, Western blotting and mass spectrometry

For SDS-PAGE analyses, proteins in the cell-free culture supernatant were precipitated by mixing with an equal volume of ice-cold 20% TCA, washed in ice cold 70% ethanol, then air dried and dissolved in SDS-PAGE reducing buffer as described previously (37). The culture density (OD_{600}) was determined prior to preparation of cell-free culture supernatant, and for analysis of secreted protein profiles, TCA precipitated protein derived from 2.0 OD_{600} units of culture was applied to each lane of a 12% acrylamide gel. Identification of Coomassie-Blue stained proteins was conducted at the London Regional Proteomics Centre at Western University. Protein bands were excised using an Ettan™ Spot Picker, and processed for mass spectrometry using a Waters MASSPrep Automated Digestor as described (43). Processed samples were spotted on MALDI plates and analyzed on an Applied Biosystems 4700 Proteomics Analyzer. Data were acquired and processed using 4000 Series Explorer and Data Explorer (Applied Biosystems), and the peptide fingerprints were compared to the NCBInr database for Gram-positive bacteria, using the MASCOT search engine.

For Western blotting, a volume of cell-free culture supernatant corresponding to 0.02 to 0.05 OD_{600} units was mixed directly with SDS-PAGE reducing buffer, and applied to 12% polyacrylamide gels. Rabbit polyclonal antisera specific for SspA and Aur proteases was used as described previously (36,37), and rabbit polyclonal antiserum

specific for Hla was purchased from Sigma. Blots were developed with donkey anti-rabbit IgG IR800 conjugate (Rockland Immunochemicals Inc.), and images were captured using an Odyssey infrared imager from LiCor Biosciences.

2.2.5 Influence of fatty acids on growth of *S. aureus*

Sapienic acid (cis-6-Hexadecenoic acid; 16:1ω10) was purchased from Matreya LLC. Palmitic acid (hexadecanoic acid; 16:0), palmitoleic acid (cis-9-hexadecenoic acid; 16:1), stearic acid (octadecanoic acid; 18:0), oleic acid (cis-9-octadecenoic acid; 18:1), linoleic acid (cis, cis-9,12-octadecadienoic acid; 18:2), linolenic acid (cis,cis,cis-9,12,15-octadecatrienoic acid; 18:3) and glycerol monolaurate were all purchased from Sigma. Prior to supplementing TSB media, fatty acids were first mixed with an equal volume of DMSO, and then diluted in TSB to a working stock concentration of 5mM.

For growth analyses, bacteria from single colonies on TSB agar were inoculated into culture tubes containing 3mL of antibiotic free TSB, and grown overnight at 37ºC on an orbital shaker, followed by measurement of OD_{600}. A 25mL volume of TSB, supplemented with fatty acid where indicated, was then inoculated to achieve a starting OD_{600} of 0.01, and the cultures were grown 37ºC on an orbital shaker incubator, set at 180rpm. Measurements of OD_{600} were taken at hourly intervals, and at set time points, samples were withdrawn for recovery of cell-free culture supernatant. All growth analyses were conducted in triplicate, from three separate cultures.

2.2.6 Protease and β-galactosidase assays

Total protease activity in cell free culture supernatant was assayed with FITC-casein substrate (Sigma Type II). Prior to assay, the supernatant samples were normalized

by dilution with sterile water as needed, to adjust for minor differences in cell density of the stationary phase cultures at time of harvest. Triplicate aliquots of the normalized culture supernatant (490µL) were mixed with 460µL of incubation buffer (40mM Tris-HCl pH 7.4, 300mM NaCl, 20mM $CaCl_2$, and 2mM L-cysteine) and 50µL of 0.2% w/v FITC-casein. Blanks were prepared using 490µL of sterile culture supernatant. The samples were incubated at 37°C in the dark for 2h. Trichloroacetic acid was then added to 4% w/v to stop the reaction, and the samples were centrifuged at maximum speed for 15m to pellet undigested casein. The supernatant was then mixed with an equal volume of 0.5 M Tris-HCl, pH 8.5, and after transfer to Optilux black clear bottom microtitre plates (BD Falcon), fluorescence was quantified on a Cary Eclipse Fluorometer using excitation at 485nm and emission at 535nm.

For β-galactosidase reporter assays, USA300*aur::lacZ* was cultured in TSB or TSB supplemented with palmitic or palmitoleic acid, and 1mL aliquots were withdrawn at 5, 6, 7 and 8h of growth. After washing in 1mL of ice cold PBS, the cells were resuspended in 1mL of PBS, and transferred to Lysing Matrix B tubes containing 0.5ml of 0.1mm silica beads (MP Biomedicals). The tubes were placed in a FastPrep120 homogenizer (Savant Biosystems) for 20s at a setting of 6.5. After centrifugation, a 20µl aliquot of each cell-lysate was added to triplicate wells of Optilux microtitre plates containing 50µM fluorescein-di-β-D-galactopyranoside (AnaSpec) in 150µL of 0.1M sodium phosphate buffer pH 7.3, 1mM $MgCl_2$, and 45mM β-mercaptoethanol. After incubation in the dark for 30 min, the assay was terminated by adding 50µL of 0.2M Na_2CO_3 to each well, and fluorescence was read using the Varian Cary Eclipse Fluorescence Spectrophotometer (Excitation/Emission = 490nm/520nm).

2.3 RESULTS

2.3.1 Influence of fatty acids on growth of *S. aureus* USA300

A recent study reported that the median concentrations of palmitoleic acid (C16:1) and linoleic acid (C18:2) were 48μM and 16μM respectively in human nasal secretions (30). Moreover, Kenny *et al.* reported that 10μM linoleic acid (LA) was not growth inhibitory, whereas 100μM LA inhibited growth of *S. aureus* strain MRSA 252 (44). We therefore chose this range of concentrations to assess the influence of LA and other saturated and unsaturated fatty acids on growth of USA300, a clinically important strain notorious for its ability to cause serious skin and soft tissue infections.

Growth of USA300 was not influenced by up to 200μM saturated stearic acid (C18:0; data not shown), or by 25μM linoleic acid (Fig. 2.1A). However there was a sharp boundary between sub-inhibitory and growth inhibitory concentrations of linoleic acid (LA), and a concentration of 50μM consistently promoted a 12h lag phase, followed by unimpeded exponential growth. When viable cell counts were measured, there was a slight loss of viability over the first 8h of incubation in TSB containing 50μM LA, after which the culture began to recover, with initiation of exponential growth at 12h (Fig. 2.1B). As reported by Kenny *et al.* (44), there was no growth at 100μM LA (data not shown), and this was also noted with linolenic, palmitoleic, and sapienic acid (Fig. 2.1D, E and F). The growth inhibition of sapienic acid closely resembled linoleic acid, with a 50μM concentration promoting an extended lag phase followed by unimpeded exponential growth (Fig. 2.1F). The only exception to growth inhibition by uFFA was oleic acid, which was not inhibitory up to 200μM, and this is consistent with data from

Parsons *et al.*, where *S. aureus* was cultured with 500µM oleic acid, albeit with use of BSA as a carrier (45).

Saturated C16:0 palmitic acid was not inhibitory up to 200µM (Fig. 2.1G), confirming that antimicrobial activity is restricted to uFFA. A possible exception is lauric acid C12:0, which has been described as an antimicrobial component of human sebum (46). We therefore tested glycerol monolaurate as a source of C12:0, and found that growth was not affected by up to 100µM (data not shown), whereas 250µM permitted growth into late exponential phase, after which there was a rapid decline in cell viability (Fig. 2.1H). We conclude that unsaturated sapienic, palmitoleic and linoleic acid are the most effective inhibitors of *S. aureus* growth of the compounds studied, and the threshold between sub-inhibitory and growth inhibitory concentration occurs between 25µM and 50µM, which approximates the median concentrations of linoleic and palmitoleic acid in human nasal secretions.

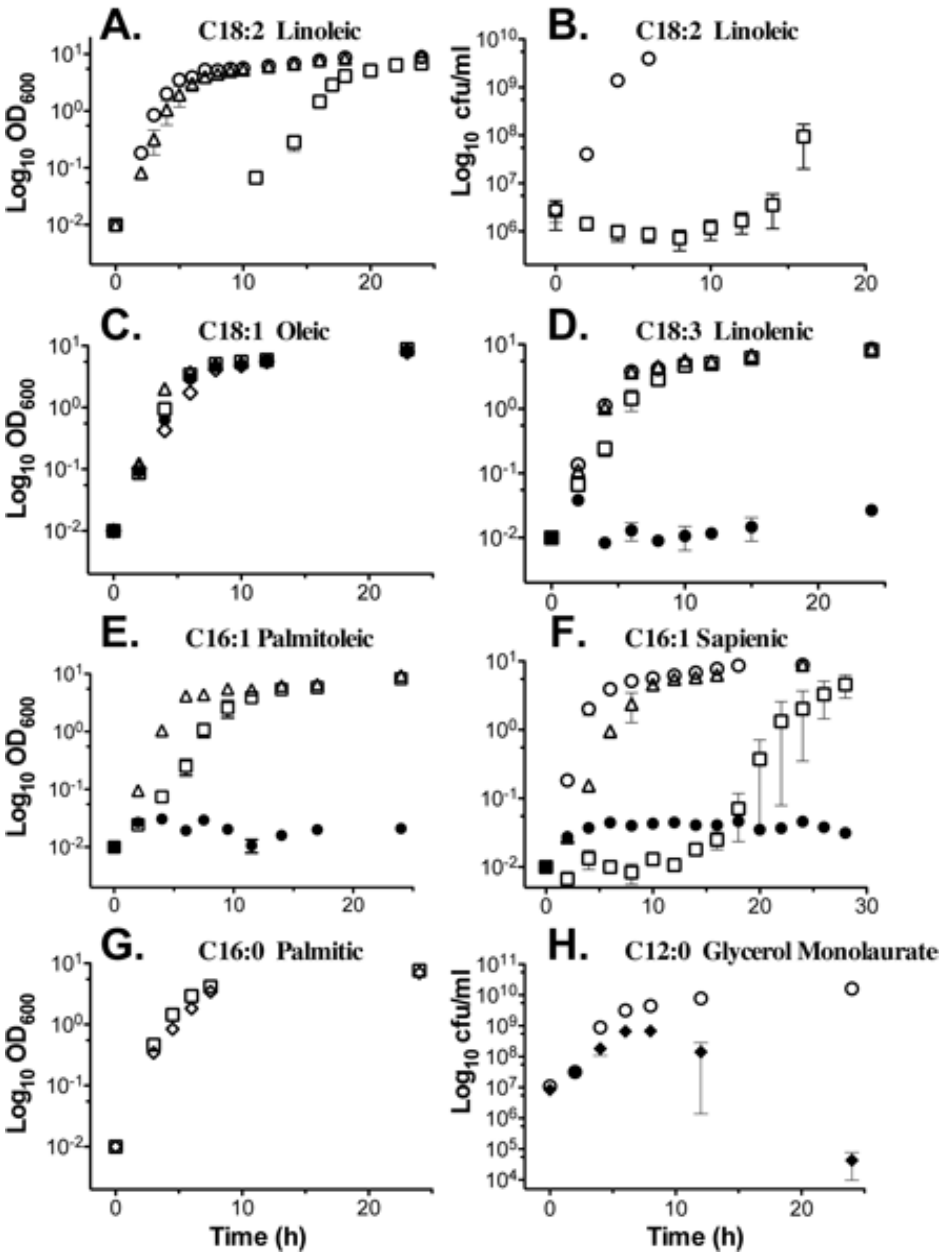

Figure 2.1: Influence of fatty acids on growth of USA300. Each point represents the mean of OD_{600} (A, C, D-G) or cfu/ml determination (B, H) from triplicate flasks of USA300 grown in TSB supplemented with the indicated amount of fatty acid; (○), TSB only; (△), 25μM; (□), 50μM; (●), 100μM; (◇), 200μM; (◆), 250μM. Lauric acid (C12:0) was provided in the form of triacylglycerol-monolaurate. Y-axes, OD_{600} or cfu/ml; X-axis, growth time (h). Error bars represent the Standard Error of the Mean.

2.3.2 Expression of secreted proteins is altered by uFFA

As a means to understand the response of *S. aureus* to the various FAs, we examined protein expression profiles. While no significant changes were readily detected in whole cell lysate (data not shown), we readily observed that the profile of secreted proteins produced by USA300 was profoundly altered after growth to stationary phase in the presence of unsaturated sapienic (C16:1Δ6), palmitoleic (C16:1Δ9), linoleic (C18:2), oleic (18:1), and linolenic (C18:3) fatty acids (Fig. 2.2), whereas 100μM saturated palmitic acid (C16:0) or stearic acid (C18:0) had no influence on growth or profile of secreted proteins compared to TSB alone (Fig. 2.2A and C). These changes were clearly evident even at 25μM concentrations of uFFA that did not alter growth kinetics. As determined by mass spectrometry analyses of selected proteins, the most significant change upon exposure to uFFA was the appearance of a new protein corresponding to the SspA serine protease, which is co-expressed with the SspB cysteine protease in the staphylococcal serine protease operon *sspABC* (38), and this was especially evident with the C16:1 fatty acids, and 50μM linolenic acid. This was typically accompanied by loss or diminished production of a 72kDa precursor isoform of glycerol ester hydrolase, together with accumulation of 40kDa mature Geh.

A Western blot confirmed that production of SspA was up-regulated by the C16:1 fatty acids, whereas saturated C16:0 had no effect, with SspA remaining in its slightly larger precursor isoform (Fig. 2.2B). Conversely, production of Hla was not influenced by either saturated or unsaturated fatty acids. From these data, we conclude that uFFA primarily influence the production of secreted proteases, concomitant with maturation of proGeh.

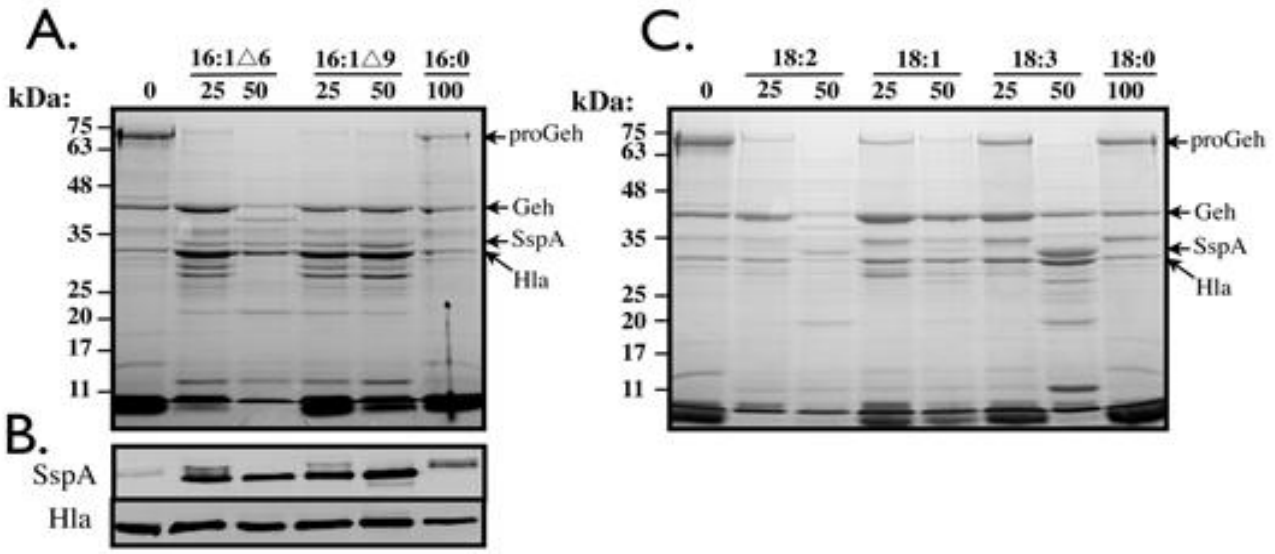

Figure 2.2 Influence of fatty acids on the protein production of *S. aureus*: SDS-PAGE of secreted proteins (A, C) and Western blot for detection of SspA and Hla (B), in culture supernatant of USA300 after growth for 18-24h in the presence of C16 (A) or C18 (C) fatty acids. Cultures were grown with the indicated amounts of C16:1△6 (sapienic acid), C16:1△9 (palmitoleic acid), C16:0 (palmitic acid), C18:2 (linoleic acid), C18:1 (oleic acid), C18:3 (linolenic acid) or C18:0 (stearic acid) fatty acids. Proteins in the cell-free culture supernatant were precipitated in ice-cold TCA, and after solubilization in SDS-PAGE reducing buffer, protein equivalent to 2.0 OD_{600} units of culture supernatant was loaded in each lane (A and C). For Western blot (C), 0.02 OD_{600} units of cell free culture supernatant were subjected directly to SDS-PAGE, prior to detection with specific antisera (see Materials and Methods).

2.3.3 The Staphylococcal proteolytic cascade (SPC) is induced by UFFa

The SPC is initiated by autocatalytic activation of the metalloprotease Aureolysin (36). However, we have observed that mature Aureolysin is unstable, and its secretion and maturation precedes the appearance of proSspA and proSspB in culture supernatant (36). We therefore cultured USA300 and isogenic *aur* or *sspABC* derivatives in TSB for 8h, to assess the influence of linoleic acid at an earlier time point (Fig. 2.3). In terms of the impact on secreted proteins, our data clearly establish that the primary effect of linoleic acid is to induce protease expression, as evident from the appearance of a new protein corresponding to Aur in USA300*sspABC*, and induction of SspA in USA300*aur*, while both proteins are produced in wild type USA300 (Fig. 2.3A). The only other obvious changes were alterations in the relative amounts of 72kDa proGeh in USA300 and USA300*sspABC* after growth with linoleic acid, but this was less evident in USA300*aur*. This is consistent with a role for Aur in processing of proGeh, as reported for orthologous metalloprotease and lipase proteins in *S. hyicus* (47).

Enhanced production of secreted protease was confirmed by assay of total protease activity in culture supernatant (Fig. 2.3B), which was significantly increased when USA300 was cultured with 25μM LA. Protease activity was also enhanced, although to a lesser extent, when USA300*sspABC* was cultured with 25μM LA, and this activity was inhibited with EDTA, confirming that metalloprotease is induced by LA. No increase in activity was evident when USA300*sspABC-aur* was cultured with LA, even though *scpA* encoding the Staphopain A cysteine protease was not targeted for disruption. Therefore, it appears that the influence of uFFA is specific to *aur* and the *sspABC* genes that comprise the staphylococcal proteolytic cascade (SPC), but does not affect

Staphopain A (ScpA), which does not comprise part of the staphylococcal proteolytic cascade, because it undergoes autocatalytic activation independently of other protease functions (35).

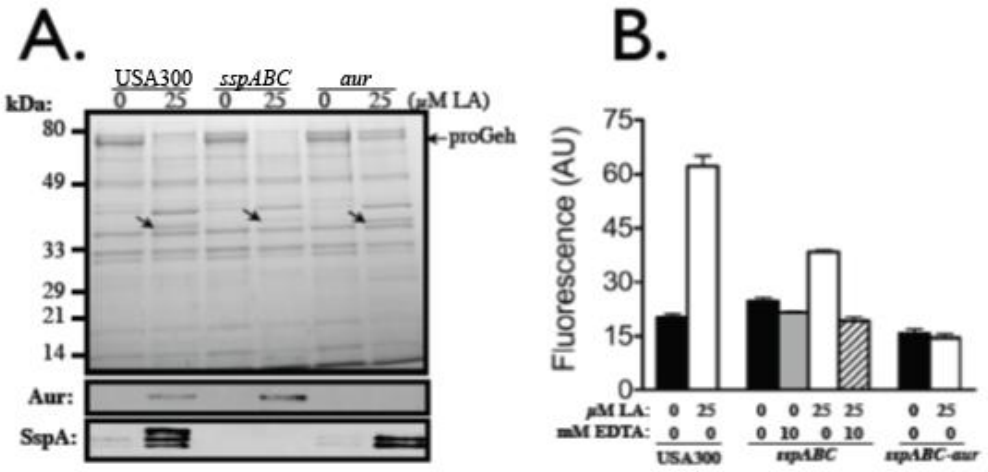

Figure 2.3 Growth in fatty acids results in increased production of the proteases of the SPC: SDS-PAGE and Western blot analyses of secreted proteins produced by USA300 and isogenic variants after 8h of growth in TSB, or TSB supplemented with 25μM linoleic acid (A), and assay of total protease activity in culture supernatant (B). For (A), protein loading was 2.0 OD_{600} units for Coomassie staining, and 0.02 OD_{600} units for Western blots, which were developed with primary antibody specific for Aur, and SspA as indicated. Arrows on the Coomassie stained gel indicate the selective induction of secreted proteases in response to linoleic acid. The arrow on the right margin indicates the position of proGeh. In (B), total protease activity in 8h culture supernatant of USA300 and isogenic variants was determined with FITC-casein substrate. Cultures were grown with 25μM linoleic acid as indicated, and assay buffer was supplemented with 10mM EDTA where indicated, to inhibit metalloprotease. Data are reported as fluorescence emission at 535nm (ε_{535}), measured in arbitrary fluorescence units. Error bars represent the Standard Error of the Mean.

To confirm that these changes occur at the transcriptional level, we took advantage of the *aur::lacZ* fusion created by inactivation of *aur*, which places *lacZ* under transcriptional control of the *aur* promoter (48). We cultured USA300*aur* in either TSB, or TSB supplemented with 25μM palmitic (C16:0) or palmitoleic acid (C16:1), taking samples between 5 and 8h, which as shown in Fig. 2.1E and 2.1G, corresponds to the transition between exponential growth and post-exponential phase. Assay of β-galactosidase activity in the total cell lysates revealed a clear induction after 8h when USA300*aur* was cultured with 25μM palmitoleic acid (Fig. 2.4). This experiment was repeated with replicates of three cell lysates for each growth condition, and after 8h of growth, palmitoleic acid promoted significantly greater β-galactosidase activity compared to palmitic acid ($p = 0.037$) or TSB alone ($p = 0.004$), and there was no significant difference comparing growth in TSB alone versus TSB supplemented with palmitic acid ($p = 0.090$). Cumulatively, these data establish that uFFA induce expression of secreted proteases comprising the staphylococcal proteolytic cascade.

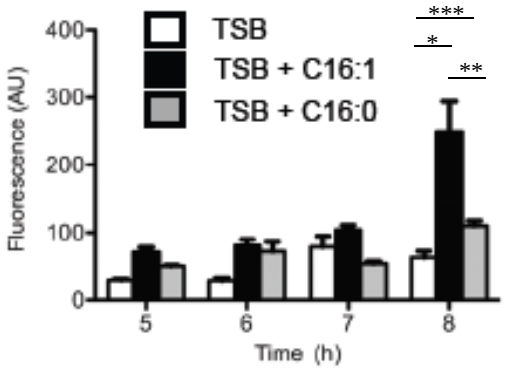

Figure 2.4 Expression of aureolysin is induced at the transcriptional level: β-galactosidase reporter gene assay in cell lysate of USA300*aur* after growth for 5-8h in TSB, or TSB supplemented with 25μM palmitic (C16:0) or palmitoleic (C16:1) acid. Error bars represent the Standard Error of the mean. * $p < 0.01$, **$p < 0.05$, *** $p > 0.05$, by Student's T test.

2.3.4 Induction of the Staphylococcal Proteolytic Cascade in response to uFFA is characteristic of *S. aureus* clinical isolates

To evaluate the response of other *S. aureus* strains to LA, we first tested another strain of CA-MRSA known as USA400, and a closely related strain of community acquired MSSA known as MSSA476, which are genetically distinct from USA300. These and other strains used for this analysis are defined in Table 2.1. After growth in TSB containing 25μM LA, the culture supernatants of USA300, USA400 and MSSA476 each exhibited accumulation of a protein corresponding to SspA (Fig. 2.5A) as confirmed by Western blot (Fig. 2.5B), together with a marked decrease in proGeh, concomitant with appearance of mature Geh (Fig. 2.5A). Production of SspA by USA300 and MSSA476 appeared to exceed that of USA400, and on several repetitions, USA300 always exhibited more robust production of SspA in response to LA, compared to USA400. Consequently, there are strain dependent differences in induction of the SPC in response to uFFA. We therefore tested additional strains to assess variation in SspA production in response to uFFA (Fig. 2.5C and D).

Once again, induction of SspA in USA400 was less robust compared to USA300, although the overall impact on the profile of secreted proteins was similar, as noted from the marked reduction in accumulation of proGeh concomitant with induction of SspA (Fig. 2.5C). Strain WBG10049, representing the Southwest Pacific Clone of CA-MRSA, also exhibited robust induction of SspA (Fig.2.5C and 2.5D) concomitant with disappearance of proGeh, but this characteristic response was not readily observed in MSSA strains Newman and UAMS-1, or in HA-MRSA strain MRSA 252, which have been used as model pathogens to address virulence and gene expression in *S. aureus*.

Although these latter strains did not respond to LA, expression of secreted virulence factors in Newman is affected by a unique polymorphism in the *saeRS* regulatory locus (49), while UAMS-1 and MRSA 252 belong to a distinct clade within clonal complex CC30 characterized by altered signaling of the accessory gene regulator *agr* (50,51,52). We therefore tested an additional panel of *S. aureus* isolates obtained from the skin of adult or pediatric atopic dermatitis patients, and strain L528, which is genetically related to UAMS-1, and derived from a patient with infective endocarditis (Table 2.1).

To varying degrees, with the exception of Newman, which was included as a non-responsive control, each strain exhibited enhanced production of SspA when cultured in TSB supplemented with 25μM LA. In some of these, including SRI-109, PED1-75, PED2-1, and PED1-53, there was some production of SspA in TSB alone, which was slightly larger than the more abundant isoforms that were produced when cultured with 25μM LA. This likely reflects our finding that SspA is secreted as an inactive precursor proSspA, and then undergoes a stepwise maturation that requires processing of the N-terminal propeptide by Aureolysin. One of these strains, PED2-1, is a CA-MRSA that corresponds to clonal complex CC97, while the others are MSSA (Table 2.1). In consideration of these data, we conclude that variable induction of the staphylococcal proteolytic cascade pathway in response to uFFA is a characteristic trait of *S. aureus* clinical isolates, and that this response is particularly robust in the USA300 strain of CA-MRSA, which is known for causing aggressive skin and soft tissue infections.

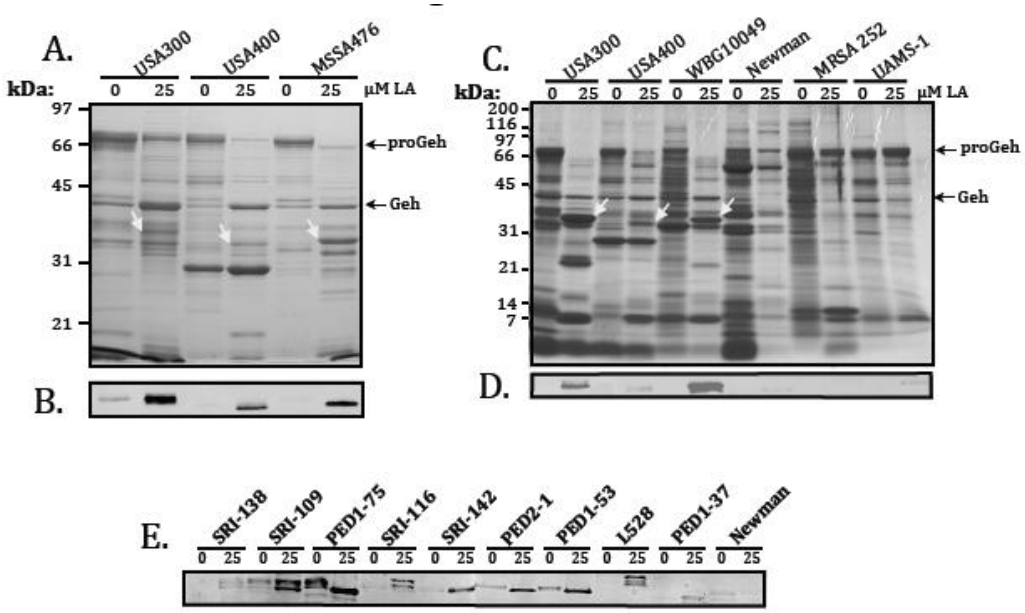

Figure 2.5 Response of different strains of *S. aureus* to linoleic acid: SDS-PAGE and Coomassie staining (A and C), or Western blot for detection of SspA (B, D and E), in cultures of *S. aureus* grown in TSB containing 0 or 25µM linoleic acid (LA) as indicated. Protein loading was 2.0 OD_{600} units for SDS-PAGE, and 0.02 OD_{600} units for Western blot. The *S. aureus* strains are as defined in Table 2.1. Black arrows and labels on the right margins of panels A and C indicate the location of 72kDa glycerol ester hydrolase precursor (proGeh) and mature lipase (Geh). White arrows in Panels A and C point to SspA protein that is induced in response to 25µM LA. The SspA protein exhibits some expected variation in size, being comprised of 327 amino acids in USA400 (MW_0932), 336 amino acids in USA300 (SAUSA300_0951), and 357 amino acids in MRSA252 (SAR_1022) and other CC30 strains, due to variation in a C-terminal disordered segment comprised of tripeptide repeats. Different isomers produced by the same strain as shown on Western blot (2.5E), as explained in the text, are also attributed to varying degrees of processing of the N-terminal propeptide of the SspA precursor, proSspA.

2.4 DISCUSSION

Our previous work defined the staphylococcal proteolytic cascade, comprised of a metalloprotease Aureolysin, which undergoes rapid autocatalytic activation, and is then needed to activate the SspA serine protease, which in turn is required to activate the SspB cysteine protease that is co-expressed with SspA in the *sspABC* operon (34,35,36,37,38). Cumulatively, the activities of these proteases are consistent with multiple functions related to modulation of adhesion, colonization, tissue invasion and immune evasion, which include degradation of complement and antimicrobial peptides (53,54), processing of phenol soluble modulins (55), degradation of microbial adhesion proteins (48,56) and their tissue ligands (34,57), and processing of kininogen to promote enhanced vascular permeability (34,58). The major findings of our present study are that (i), the SPC is induced by antimicrobial unsaturated long chain fatty acids uFFA, in four different genetic backgrounds of CA-MRSA; (ii), amongst CA-MRSA there is variable induction of the SPC in response to uFFA, with USA300 consistently exhibiting a more robust induction of SspA relative to USA400; and (iii) this response was also manifested to varying degrees by clinical MSSA, including pediatric osteomyelitis (MSSA476), infective endocarditis (L528), and several strains recovered from the skin of adult and pediatric atopic dermatitis patients.

With respect to *S. aureus* persistence on skin, Sapienic acid (C16:1Δ6), which is an isomer of palmitoleic acid (C16:1Δ9), is the major fatty acid component of human sebum, and separate studies reported minimum inhibitory concentrations of 10-20µg/ml, and 30µg/ml respectively, for *S. aureus* (27,46). Another study reported the median concentration of palmitoleic acid in human nasal secretions as 12µg/ml (30), which

corresponds to 48μM. Based on these considerations and our present data, it is apparent that physiologic concentrations of palmitoleic, sapienic and linoleic acid are sufficient to induce the SPC in *S. aureus*, and this is unique to unsaturated fatty acids. Although *S. aureus* does not have the capacity for β-oxidation of fatty acids, exogenous unsaturated fatty acids are transported across the cytoplasmic membrane through as yet unknown mechanisms, and then either directly incorporated into membrane phospholipid, or alternately, can be extended by the Type II fatty acid synthase machinery, prior to incorporation into phospholipid (45,59). Unsaturated fatty acids have reduced packing density in membranes, leading to increased membrane fluidity, and our data may reflect a mechanism for sensing changes in membrane fluidity.

It is unclear whether induction of the SPC by unsaturated fatty acids represents an innate immune function of the epithelial barrier to infection, or whether this promotes colonization and virulence. Several studies have implicated a role for secreted proteases of *S. aureus* in promoting biofilm dispersal (60,61,62), and it has been suggested that the biofilm mode of growth promotes nasal carriage of *S. aureus* (63). Specifically, an extracellular serine protease Esp of *S. epidermidis*, which is orthologous to SspA, promoted *S. aureus* biofilm dispersal *in vitro*, and eradicated *S. aureus* nasal carriage in human subjects when administered intranasally. Although it has been debated whether this was due to biofilm dispersal, or degradation of adhesion proteins and their epithelial ligands (64), it supports the contention that nasal carriage could be regulated through induction of the SPC by unsaturated fatty acid in nasal secretions. One study with a limited number of subjects, revealed a wide variance in the level of palmitoleic/sapienic acid in nasal secretions, ranging from 1.8 to 27μg/ml (30), which corresponds to 7.1μM

to 106μM. Therefore, it is reasonable to speculate that individuals with higher sapienic acid content would not carry *S. aureus*, due to a combination of antimicrobial activity and induction of the SPC, which would promote biofilm dispersal and interfere with microbial adhesion.

Alternatively, induction of the SPC by uFFA could facilitate the initiation and maintenance of a stable carriage relationship. Importantly, the phenol soluble module (PSM) family of peptides produced by *S. aureus* exhibit antimicrobial activity towards *Streptococcus pyogenes*, which may comprise a mechanism of interference with competing colonizing pathogens, and the PSMα peptides appear to require proteolytic processing by Aureolysin to activate their antimicrobial properties (55,65). Moreover, individuals who are colonized by *S. aureus* also have elevated levels of host-derived antimicrobial α- and β-defensins in their nasal secretions (6). The antimicrobial peptide dermcidin, which is secreted from eccrine sweat glands, can induce expression of the SepA metalloprotease of *S. epidermidis*, and antimicrobial peptides also trigger protease production in *S. aureus* (66). In this situation, signaling is mediated through the antimicrobial peptide sensor *aps* (67,68), which also responds to glycopeptides (69,70), and triggers a global response that leads to modification of membrane lipids and cell wall teichoic acids, which cumulatively promote resistance to antimicrobial peptides. Therefore, induction of the SPC by uFFA could promote colonization, through degradation of host-derived antimicrobial peptides, concomitant with activation of the antimicrobial properties of PSMα1 and PSMα2, which function to eliminate competing pathogens.

In this context, the capacity for uFFA to induce production of secreted proteases appears to exceed that of other environmental signals, and to our knowledge this represents the only environmental stimulus identified thus far, that can lead to accumulation of SspA serine protease as one of the major secreted proteins. Transcriptional profiling studies have evaluated the transcriptome of USA400 CA-MRSA in response to neutrophil microbicides, including azurophile granule proteins, HOCl, and hydrogen peroxide, and although transcription of certain toxin genes, most notably encoding γ-hemolysin, was strongly induced by these signals, there was no major influence on transcription of *sspA* or *aur* (33), and similar observations were noted in assessing the transcriptome of USA300 in response to growth in blood (32). Therefore, our data allude to a novel signaling pathway that may selectively induce the SPC, which has an important role in modulating adhesion, colonization and invasion (34,38,48,53,56,57,71).

Induction of the SPC was particularly robust in the USA300 strain of CA-MRSA, and in WBG10049 representing the Southwest Pacific clone of CA-MRSA, both of which cause aggressive skin and soft tissue infections, whereas induction of SspA was evident, but consistently less robust in USA400. Intriguingly, USA400 appears to be associated with septicemia, and disruption of the *saeRS* regulator in USA400 strongly attenuated virulence in a bacteremia model, but had no significant influence on virulence in a subcutaneous abscess model (72). These observations support the contention that the expression and role of subsets of virulence factors is strongly influenced by the site of infection. In this respect, it is noteworthy that *S. aureus* Newman has been used to evaluate the genetic requirements for kidney abscess development (73,74), establishing

the importance of Coagulase and vonWillebrand Factor binding protein in promoting the formation of a fibrin pseudocapsule that impedes migration of neutrophils into the microbe dense core of the abscess (73). However we, and others, have defined a role for secreted proteases in degrading fibrin, and cell surface fibrinogen binding proteins (34,48,57), and abscess tissue has high levels of uFFA, of which the major bactericidal activity is attributed to linoleic acid (31). Therefore, we must consider that the ability of strain Newman to form large, well defined abscesses could be due in part to its failure to induce protease expression in response to uFFA, as we have demonstrated in this study.

MRSA 252, which is a successful epidemic strain of HA-MRSA (75), also failed to induce the SPC in response to linoleic acid. Strikingly, MRSA 252 was used to evaluate changes in the transcriptome in response to linoleic acid (44), but our data show that it is not representative of *S. aureus*, in terms of its response to uFFA. MRSA 252 belongs to a distinct clade within clonal complex CC30, characterized by accumulation of pseudogenes, and single nucleotide polymorphisms that attenuate virulence, and/or promote enhanced intrinsic resistance to antimicrobial agents (50,52,76). Conversely, WBG10049 is a hypervirulent CA-MRSA that belongs to a separate CC30 clade, and as with USA300, it exhibited robust induction of the SPC in response to linoleic acid.

In summary, we conclude that induction of the SPC in response to uFFA is another factor that should now be considered in addressing the aggressive nature of skin and soft tissue infection caused by some strains of CA-MRSA, and as a general virulence mechanism for most strains of *S. aureus*. Future work will focus on the mechanistic details of induction, including evaluation of the USA300 transcriptome in response to

linoleic acid, evaluation of protease null mutants in bacteremia and subcutaneous wound infection models, and the significance of lipase processing by proteolytic activity.

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CHAPTER 3

Insights into the molecular events leading to the adaptation of *Staphylococcus aureus* to unsaturated fatty acids

3.1 INTRODUCTION

Staphylococcus aureus is a major human pathogen owing to its ubiquitous presence, antibiotic resistance, and its ability to infect virtually every tissue of the body

(1). Much research has gone into resolving the paradox that is inherent in the ability of *S. aureus* to exist both as a commensal, asymptomatically colonizing ~25% of the population (2), and an aggressive pathogen and a leading cause of infectious morbidity and mortality worldwide (3). Community acquired methicillin resistant *S. aureus* strain USA300, in particular, causes very aggressive skin and soft tissue infections (SSTI) and is the leading cause of visit to emergency departments in North America for the treatment of SSTIs (4-6).

Colonization provides opportunity for infection, illustrated in the finding that patients with nosocomial *S. aureus* infection are typically infected by the same strain they are colonized with (7). Therefore, it is important to elucidate how signals encountered during colonization contribute to the survival of bacteria or the shift from colonization to infection. Colonization normally occurs in the anterior nares, but *S. aureus* may also colonize the warm, moist areas of the skin such as the axillae and perineum. Fatty acids are potent antimicrobials that are present on both the skin, through sebum, and in nasal secretions (8, 9), and thus we reason that in order to colonize the skin and anterior nares, *S. aureus* must have means of resisting their antimicrobial effects. Based upon results garnered in chapter 2, we undertook to further understand the response and resistance mechanisms of *S. aureus* to unsaturated fatty acids, using linoleic acid as a model fatty acid, on the basis of it being a major antimicrobial component of human nasal secretions (10), and also accumulating to millimolar concentrations within an abscess (11-13).

Herein, we show that growth at subinhibitory concentrations of linoleic acid is associated with the processing of the secreted lipase Glycerol Ester Hydrolase (Geh) into its mature form, and that this processing is dependent on the metalloprotease aureolysin,

which is upregulated in response to linoleic acid. Although the inability to process lipase has no consequence on growth of USA300 in media containing linoleic acid, as determined from growth of an aureolysin- deficient mutant, we have isolated USA300 variants with reduced susceptibility to linoleic acid, and have identified common single nucleotide polymorphisms (SNP) among these strains that may indicate candidate genes involved in fatty acid resistance. The research in this section contributes towards resolving the paradox that is inherent in the ability of *S. aureus* to exist as both a pathogen that is a leading cause of invasive skin and soft tissue infections, as well as a commensal organism that colonizes humans asymptomatically.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are defined in Table 3.1. Cultures were maintained as frozen stocks (-80ºC) in 20% glycerol, and streaked on TSB agar when required. TSB was supplemented, when necessary, with 10µg/mL erythromycin or 2µg/mL tetracycline for propagation of strains bearing resistance markers.

Table 3.1 *S. aureus* strains used in this study

Strain	Description	Source
RN4220	Restriction deficient lab strain	(14)
DU5969	RN4220*aur::lacZ*; transcriptional fusion of *lacZ* to *aur* promoter	(15)
USA300 LAC	Hypervirulent pandemic CA-MRSA Los Angeles county clone; clonal complex 8	(16) Barry Kreiswirth
USA300	USA300 LAC cured of antibiotic resistance plasmid	This study
USA300*aur::lacZ*	Transduction of *aur::lacZ* from DU5969 donor	This study
USA300*sspABC*	Replacement of *sspABC* operon in USA300 with Tc^r cassette, using pMJ232	This study
USA300*aur*	Transduction of *aur::lacZ* into USA300	This study
USA300*sspABCaur*	Transduction of *aur::lacZ* into USA300*sspABC*	This study
pMAD	Shuttle vector for the construction of mutations in Gram positive bacteria	(17)
pDG1514	Source of Tc^r cassette	(18)
pMJ232	pMAD containing Tc^r cassette from pDG1514, flanked by *Bam*HI-[*sspA*-5P]-*Mlu*I and *EcoR*I-[*sspC*-3P]-*Bgl*II	This study

3.2.2 Growth of *S. aureus* in fatty acids

Linoleic acid (cis, cis-9,12-octadecadienoic acid; 18:2), was purchased from Sigma. Prior to supplementing TSB media, fatty acids were first mixed with an equal volume of DMSO, and then diluted in TSB to a working stock concentration of 5 mM. For growth analyses, bacteria from single colonies on TSB agar were inoculated into culture tubes containing 3 mL of antibiotic free TSB, and grown overnight at 37ºC on an orbital shaker, followed by measurement of OD_{600}. A 25mL volume of TSB, supplemented with fatty acid where indicated, was then inoculated to achieve a starting OD_{600} of 0.01, and the cultures were grown at 37ºC in an orbital shaker incubator, set at 180 rpm. Measurements of OD_{600} and samples of cell free supernatant were taken at 18 hours of growth.

3.2.3 Linoleic acid adaptation and genome sequencing

USA300 (LAC, cured) was cultured into 3mL TSB and grown at 37°C overnight in an orbital incubator shaking at 250 rpm. OD_{600} was determined, and the bacteria were subcultured into 25mL TSB, or TSB supplemented with 50μM linoleic acid to a starting inoculum corresponding to an OD_{600} of 0.01 in a 125mL Erlenmeyer flask. The bacteria were then grown at 37°C in an orbital shaker at 180 rpm until stationary phase, where the OD_{600} was determined. The bacteria were then subcultured into 25mL of TSB or TSB containing 50μM linoleic acid, and grown to stationary phase. Cells were then plated on TSB agar, and single colonies were selected for preparation of stock cultures.

For genome sequencing, genomic DNA from stock cultures was isolated using an Invitrogen PureLink™ genomic DNA preparation kit. Samples of genomic DNA were sent to the London Regional Genomics Center for genomic sequencing using Ion Torrent Next-Generation sequencing. Pure genomic DNA from each of the control and adapted

clones were sent to the sequencing facility, where unique bar codes (Ion Xpress Barcode Adapter kit) for each clone were incorporated into the DNA, and sequencing was performed on an Ion Torrent 318 chip. After sequencing, reads from the same bar codes were aligned to the reference genome of USA300 FPR3757. SNPs were identified using the CLC Genomics Workbench software.

3.2.4 TCA precipitation of proteins, SDS-PAGE, Western blotting and mass spectrometry:

For SDS-PAGE analyses, proteins in the cell-free culture supernatant were precipitated by mixing with an equal volume of ice-cold 20% TCA, washed in ice cold 70% ethanol, then air dried and dissolved in SDS-PAGE reducing buffer. The culture density (OD_{600}) was determined prior to preparation of cell-free culture supernatant, and for analysis of secreted protein profiles, TCA precipitated protein derived from 2.0 OD_{600} units of culture was applied to each lane of a 12% acrylamide gel. Identification of Coomassie-Blue stained proteins was conducted at the London Regional Proteomics Centre at Western University. Protein bands were excised using an Ettan™ Spot Picker, and processed for mass spectrometry using a Waters MASSPrep Automated Digestor. Processed samples were spotted on MALDI plates and analyzed on an Applied Biosystems 4700 Proteomics Analyzer. Data were acquired and processed using 4000 Series Explorer and Data Explorer (Applied Biosystems), and the peptide fingerprints were compared to the NCBInr database for Gram-positive bacteria, using the MASCOT search engine.

For Western blotting, a volume of cell-free culture supernatant corresponding to 0.02 OD_{600} units was mixed directly with SDS-PAGE reducing buffer, and applied to

10% polyacrylamide gels. Rabbit polyclonal antisera specific for SspA and Aur proteases was used, and rabbit polyclonal antiserum specific for Hla was purchased from Sigma. Blots were developed with donkey anti-rabbit IgG IR800 conjugate (Rockland Immunochemicals Inc.), and images were captured using an Odyssey infrared imager from LiCor Biosciences.

3.2.5 Gas Chromatography Mass Spectrometry sample preparation

1.0 mL of culture supernatant was collected for GC/MS analysis. The supernatants were acidified (pH < 2.0) with concentrated H_2SO_4. Fatty acids were then extracted three consecutive times with 750µL of hexane, and the hexane extracts were then pooled together and dried under a constant stream of nitrogen gas. Dried samples were trimethylsilylated with 25µL of pyridine and 25µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSFTA) + 1% Trimethylchlorosilane (TMS) (Sigma), and incubated at 70°C for 40 minutes.

3.3 RESULTS

3.3.1 Metalloprotease aureolysin processes the Glycerol Ester Hydrolase (Geh) in response to growth in linoleic acid

It has been suggested in the literature that lipases are proteolytically processed into their mature form (19). The response to linoleic acid includes the upregulation of three proteases, SspA, SspB and aureolysin, as well as the processing of proGeh, as discussed earlier. Using isogenic protease mutants for the three proteases, we determined that USA300*aur* is the only single protease mutant defective for Geh maturation, and that USA300*sspABC* retains the ability to process lipase in the presence of linoleic acid (Fig 3.1). We thus conclude that aureolysin is required for the processing of Geh. Arrows on the right of the SDS-PAGE gel indicate proteins identified by mass spectrometry. Both proSspB and mature Geh were detected in the same region (~40kDa) in the protein profile of wild-type USA300, and in the USA300*sspABC* mutant grown in the presence of 25μM linoleic acid, only the mature Geh band protein was identified, while the mutant devoid of all SPC proteases does not produce a protein band in the region corresponding to proSspB or mature Geh, as expected.

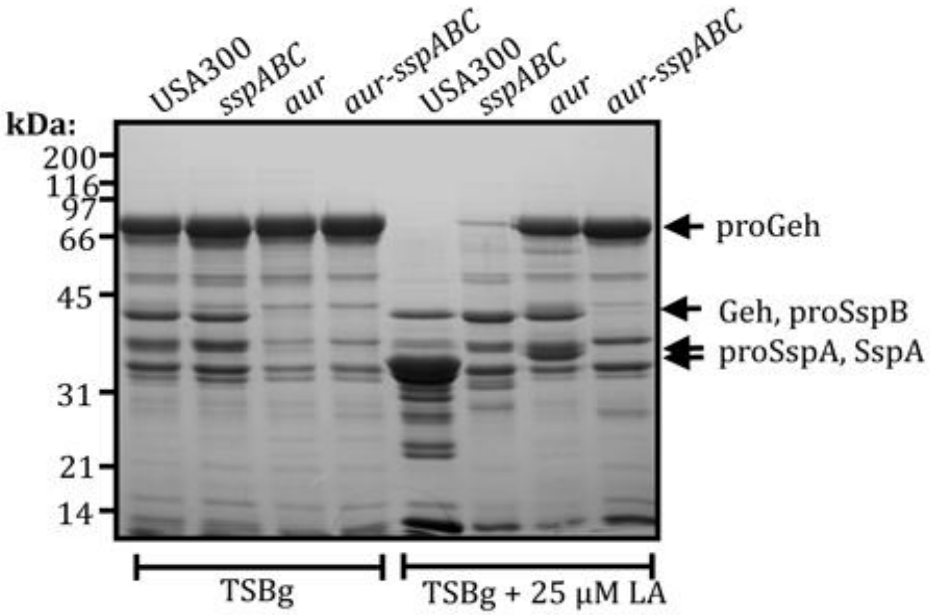


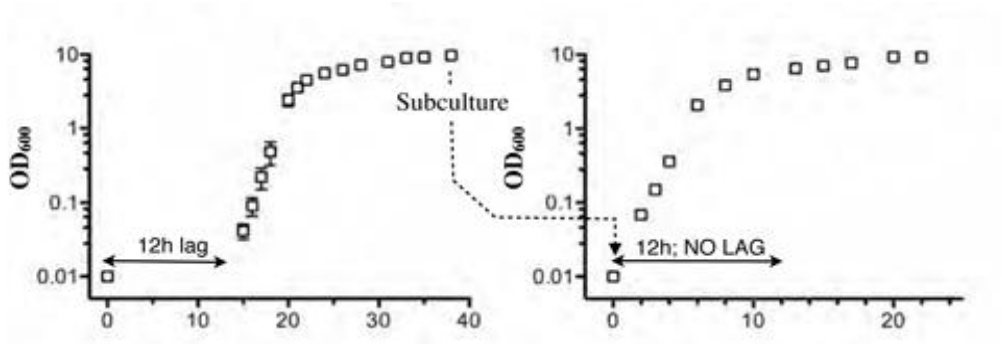
Figure 3.1: Aureolysin is required for lipase processing

SDS-PAGE of TCA precipitated protein from 18 hour culture supernatants of USA300 (LAC, cured) and isogenic protease mutants grown in TSB (left) or TSB supplemented with 25µM linoleic acid. Arrows indicate proteins identified by mass spectrometry.

3.3.2 USA300 adapts to growth in linoleic acid

As noted in the previous chapter, USA300 undergoes a ~12 hour lag phase when grown in the presence of 50μM linoleic acid, which is followed by of the resumption of exponential growth at a rate equivalent to that of growth in TSB alone. This observation is suggestive of an adaptive response by the bacteria, or selection of a naturally resistant mutant pre-existent in the culture. To advance upon these findings, we took stationary phase cultures that had grown in the presence of 50μM linoleic acid, and subcultured them back into 50μM linoleic acid again, and observed immediate induction of exponential growth, as opposed to a long lag phase (Fig 3.2 A, B). Importantly, these cells maintain their resistance to linoleic acid, even after passage through media lacking linoleic acid, and being frozen at -80^{o}C. This alludes to a genetic adaptation, as opposed to an intrinsic response that is only manifested in the presence of linoleic acid. Despite permanent resistance to linoleic acid, the adapted cells did not exhibit any major changes in production of secreted virulence factors, and maintained their ability to induce protease expression in response to linoleic acid (Fig 3.2C), suggesting that adaptation to linoleic acid had likely not caused a mutation that is strongly influencing a major global regulator of virulence factors.

A B



C

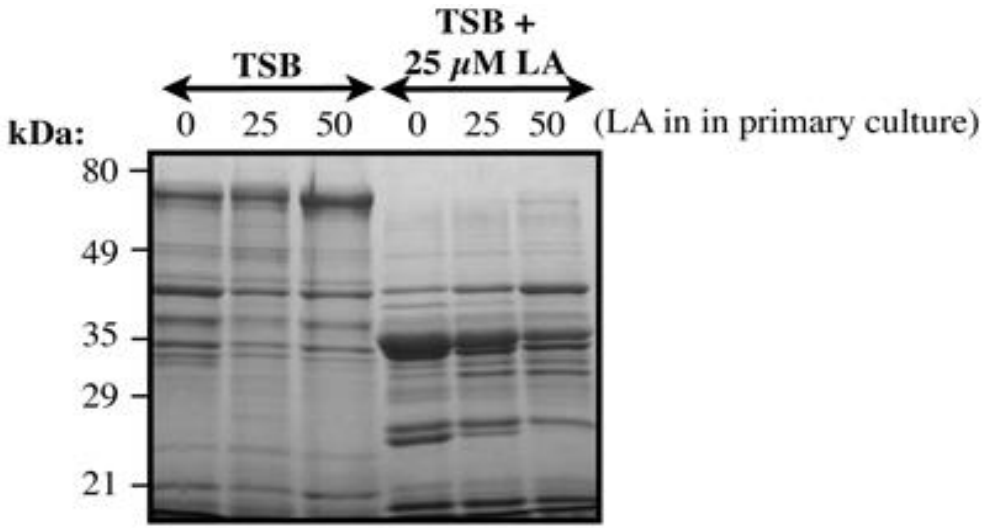


Figure 3.2: USA300 adapts to growth in linoleic acid. A) Growth of USA300 (LAC, cured) in TSB supplemented with 50μM linoleic acid, and subcultured into B) 50μM linoleic acid again. C) SDS-PAGE of the culture supernatants of wild type or cells passaged at the indicated concentration of linoleic acid, and then grown in TSB or TSB supplemented with 25μM linoleic acid again for 18 hours.

3.3.3 Linoleic acid adapted cells contain SNPs in common genes

Based on the phenotype of the linoleic acid adapted cells, we isolated genomic DNA from a single colony obtained from each of seven independently adapted cultures, along with two control cultures that had not been exposed to linoleic acid, and sent them to the London Regional Genomics facility at Robarts Research Institute for sequence determination on the Ion Torrent Next-Generation sequencing platform. Upon SNP analysis of the sequencing data, there were SNP mutations in all seven adapted cells that differed from the control cells, all resulting in amino acid substitutions. Importantly, five of the seven strains contained SNPs in two genes, highlighting these as candidates for linoleic acid resistance. Table 3.2 lists the SNPs identified in the seven adapted strains and the corresponding amino acid substitutions. With SNPs in three separate isolates, USA300_1179 is a ribonuclease gene, *cvfA*, which may be involved in mRNA degradation and subsequently may control post-transcriptional modification of gene expression. Interestingly, USA300_1126 is also a ribonuclease, *rnc*, which is involved in dsRNA degradation. US300_2490 is a member of the TetR family of transcriptional repressors, and two of the isolates contained SNPs causing the same amino acid substitutions.

Table 3.2: Single Nucleotide Polymorphisms in linoleic adapted strains of USA300

Clone	Gene Number	Nucleotide Substitution	Amino Acid Substitution	Coverage/Frequency
1	USA300_1179	1298394 C>T	$Leu_{269}Phe$	65x/100%
2	USA300_1179	1298586 C>A	$Gln_{333}Lys$	14x/100%
3	USA300_1179	1298427 C>T	$Pro_{280}Ser$	24x/100%
4	USA300_2057	2220605 T>C	$Glu_{160}Gly$	31x/100%
5	USA300_1126	1232458 T>C	$Ser_{207}Pro$	14x/100%
6	USA300_2477	2676139 G>A	$His_{82}Tyr$	19x/100%
6	USA300_2490	2692995 C>T	$His_{121}Tyr$	31x/100%
7	USA300_2490	2692995 C>T	$His_{121}Tyr$	18x/100%

3.3.4 Fate of linoleic acid in culture media

To determine if a change in the concentration or composition of linoleic acid was coinciding with the end of lag phase growth in 50μM linoleic acid, the concentration of linoleic acid in culture media throughout the lag phase was assessed using gas chromatography-mass spectrometry (GCMS). From samples taken throughout the lag phase and in stationary phase, it was determined that linoleic acid concentrations remain high, up to the beginning of exponential phase growth, but no linoleic acid was detected in the stationary phase culture. This indicates that the initiation of exponential growth is not reliant upon a decrease in the linoleic acid concentration, and also that the linoleic acid is being removed from, or modified in the culture media during exponential phase growth (Fig 3.3).

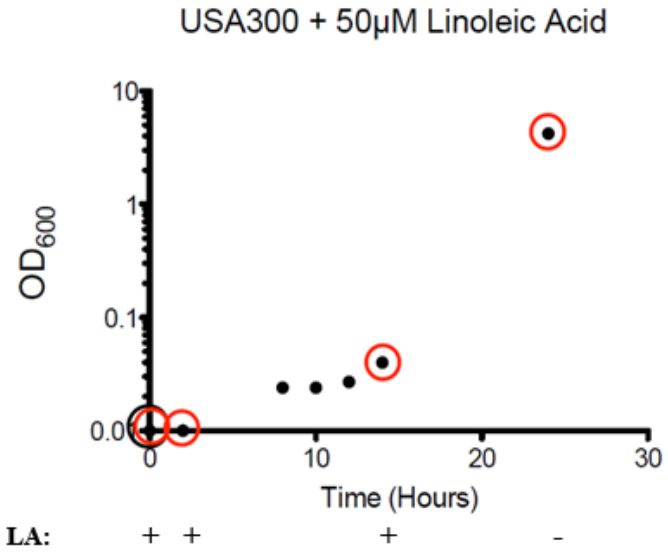


Figure 3.3: Linoleic acid disappears from culture media during exponential phase growth of USA300. Growth curve of USA300 (LAC, cured) in TSB supplemented with 50µM linoleic acid. Red circles indicated time points at which samples for GCMS were taken. The legend below represents the presence (+) or absence (-) of linoleic acid in culture media.

3.4 DISCUSSION

In this chapter, we have demonstrated that aureolysin is responsible for processing proGeh into its mature form, that USA300 adapts to growth in the presence of inhibitory concentrations of linoleic acid, and that this adaptation appears to be facilitated by SNPs in a limited number of genes, which are predicted to cause amino acid substitutions in the encoded gene products. We have also determined that linoleic acid is being modified or removed from the culture medium during growth of bacteria. Together, these results add evidence to this research that is leading towards our ultimate goal of determining how *S. aureus* is able to grow in the presence of antimicrobial fatty acids, and determining the significance of the response of *S. aureus* to fatty acids. To that end, the observation that proGeh is activated by aureolysin upon linoleic acid stimulation is intriguing. Literature has suggested that proGeh and mature Geh do not significantly differ in their ability to hydrolyze lipids (20), but these conclusions were drawn from analysis of SDS-PAGE zymogram assays, in which the propeptide of an inactive precursor may be incorrectly positioned, and thus the enzyme may be active in this assay. Demonstration of this effect can be made with precursor SspB, wherein a zymogram assay the proenzyme appears to be active, but has much reduced activity in biochemical assays using proper buffers and pure protein (21). Despite this controversy, we are unable to report any difference in the lipolytic activity of whole supernatants lacking the aureolysin convertase, and thus lacking the active lipase. However, there are additional lipases in the USA300 genome that may be performing a compensatory function. Another possibility is that the activity of mature Geh differs from its proprotein in terms of the reaction it catalyzes. Enzymes in general have a tendency to be able to perform the reverse reaction, typically when placed

in an environment that is sub-optimal. Interestingly, the activity of Geh is optimal at a high pH, and has almost no lipase activity at the pH found on the skin (19, 20, 22). FAME, however, has a low optimal pH and almost no FAME activity at the pH optimal for a lipase (23). Since FAME and Geh perform the reverse reaction of one another, one must consider the possibility that Geh is performing its reverse reaction by esterifying fatty acids to cholesterol molecules in low pH environments.

In addition to the induction of expression of secreted proteases, concomittant with maturation of lipase precursor, we have also demonstrated adaptation at the chromosomal level in response to linoleic acid. Two fields of view exist with regards to evolution of this kind. The classical Darwinian evolution would suggest that there are naturally occurring mutations that exist within the population of bacteria, and only the cells with mutations conferring linoleic acid resistance will survive under this selective pressure. Under this view, we are selecting for naturally occurring variants that were present in the original inoculum, and the observed lag phase comprises the time required for these few bacteria to multiply and surpass the cell density of the starting inoculum. The other view is that the bacteria are able to alter their genome in response to linoleic acid, and that this alteration allows them to survive. In support of this theory, literature has demonstrated that the production of reactive oxygen species in response to antibiotic treatment causes random mutations in chromosomal DNA, and certain random mutations confer resistance to the antibiotic (24). Although we have no direct evidence of either theory, the genes targeted in the linoleic acid resistant mutants will provide clues to the mechanism of resistance to and antimicrobial mechanism of fatty acids. Six of eight SNPs were occurring in genes that may control transcriptional and post-transcriptional regulation.

USA300_2490 is of the TetR family of transcriptional repressors, and thus a gain or loss of function in that gene as a result of a SNP would have downstream consequences on genes that are regulated by TetR. Likewise, if the ribonucleases CvfA and Rnc are involved in mRNA stability, a shift in the expression of genes could be occurring post-transcriptionally as a result of SNP induced alteration in the function of these ribonucleases. Although this theory is merely speculation at this juncture, further experiments will include a transcriptome analysis in response to linoleic acid to identify mRNAs that are being affected in response to linoleic acid.

Determination of linoleic acid concentration in culture media throughout growth is a necessary step in elucidating the Staphylococcal response to fatty acids, because it provides further knowledge into how the bacteria are physically coping with the lipid. The finding that linoleic acid concentration remains constant throughout the lag phase, up to the initiation of exponential growth is important, because it demonstrates that exponential growth is initiated in the presence of an inhibitory concentration of linoleic acid, further emphasizing our adaptation theory. It is also important because it shows that the lipid is not being spontaneously broken down in culture media. Literature has suggested that although *S. aureus* does not synthesize its own unsaturated fatty acids (25), it is capable of incorporating endogenous fatty acids into is phospholipid bilayer (26, 27). Therefore, next steps in this experiment will be to determine the fatty acid composition of the bacterial cell membrane during exponential growth in medium containing supplementary fatty acid, and to determine if the membrane composition is altered in cells that have adapted to growth at an inhibitory linoleic acid concentration.

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CHAPTER 4 - GENERAL DISCUSSION AND FUTURE DIRECTIONS

The main findings of this research on the *Staphylococcus aureus* response to antimicrobial fatty acids can be roughly divided into two main observations; i) The upregulation of the staphylococcal proteolytic cascade upon exposure to unsaturated fatty acids, and ii) Adaptation to linoleic acid. Based on the data in chapters 2 and 3, these two observations are seemingly independent of each other; meaning that even non-adapted strains display increased protease expression upon exposure to subinhibitory concentrations of fatty acid, and regardless of whether the strain has adapted to growth in linoleic acid or not, presence of the fatty acid in culture media is still essential to induce increased expression of the SPC. In addition, we report that the upregulation of the SPC, specifically aureolysin, results in proteolytic processing of the glycerol ester hydrolase into its mature form, and that this response is characteristic of hypervirulent community acquired strains, and not present in some strains that are commonly used to study Staphylococcal pathogenesis.

Induction of the SPC and processing of proGeh may be regarded as a dynamic adaptive response to fatty acids, as it only occurs in the presence of fatty acids, and the expression of proteases returns to normal upon growth in media without fatty acid. GCMS data from chapter 3 are also indicative of an adaptive response because it suggests that the bacteria are removing fatty acid from the media by an as of yet unknown mechanism. Together, we can conclude that USA300 responds to fatty acids by increasing the expression of the SPC, maturation of Geh and removal of fatty acids from

culture media. As opposed to this dynamic adaptive response, it is unlikely that the SNPs discovered after growth in fatty acids represent an adaptive response to fatty acids. If this were the case, one would expect that these SNPs would be found in nature, occurring in bacteria isolated from areas of high fatty acid concentration such as the anterior nares, and an abscess. Rather, these SNPs that allow growth in high concentrations of fatty acids were likely selected for, and may enlighten us further about the mechanism of antimicrobial activity of the fatty acid, and how the bacteria respond to the fatty acid. More experiments will have to be conducted on these SNPs in order to speculate on the mechanism of resistance in these adapted strains. Specifically, to determine whether SNPs are causing loss- or gain-of-function mutations in each gene, SNP containing genes will be cloned into wild type USA300, and in turn, wild type genes will be cloned into mutated USA300 strains. In each case, resistance to fatty acids will be determined. More adaptation experiments will also be done to determine the frequency, and thus importance, of each SNP in conferring resistance to fatty acids.

An important avenue to pursue will also be the fate of linoleic acid in culture media. In addition to the knowledge that fatty acids are removed from the media, determining the mechanism of this removal, and destination of the fatty acid beyond that point will be illuminating. As stated in chapter 3, we expect to observe that the fatty acids are being incorporated into the phospholipid bilayer of the bacteria, and determination of the bacterial membrane lipid composition during growth in fatty acids will be done using GCMS.

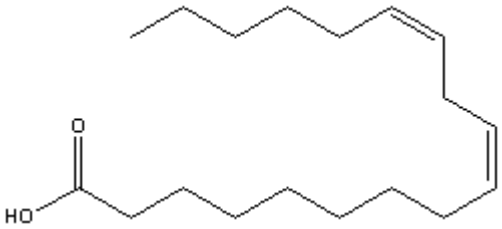
Finally, it will be informative to determine the consequence of the processing of proGeh discussed in both chapters 2 and 3. We have no evidence at this point as to

whether or not processing is necessary to confer or alter activity of the lipase, and thus the next step will be to purify both the precursor and mature forms. Further, one wonders whether this maturation confers increased resistance to fatty acids, or more likely, increased survival in areas of a more complex lipid composition such as sebum, and within an abscess. Survival assays using wild type USA300, and proGeh maturation deficient USA300*aur* in full human sebum, or in skin colonization models, would help to further elucidate the significance of this marked response to fatty acids.

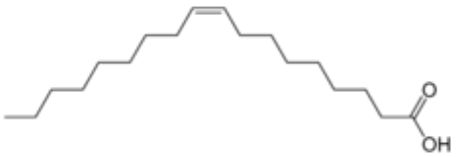
The work presented here consists mainly of descriptive data demonstrating the response of community acquired methicillin resistant *S. aureus* to long chain fatty acids. Because the data presented represents novel observations and branches in several different directions, this work provides a strong base for future research in each avenue, which will consist of elucidating the mechanism behind, and consequence of, each of the responses presented here. The impact of CA-MRSA currently as a pathogen, as well as the threat it presents as an emerging pathogen is incontrovertible, and therein lies the significance of this research.

APPENDIX

Structures of fatty acids



Linoleic acid (C18:2)



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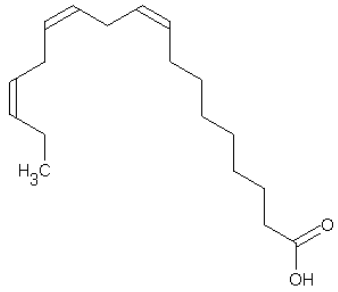
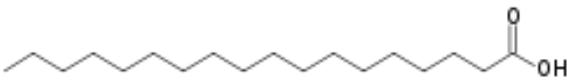
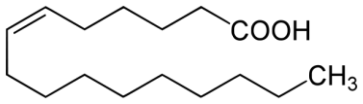


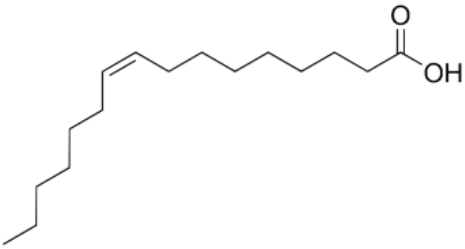
Linolenic acid (C18:3)



Stearic acid (C18:0)



Sapienic acid (C16:1Δ6)



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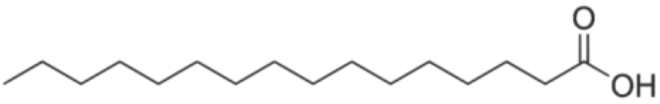

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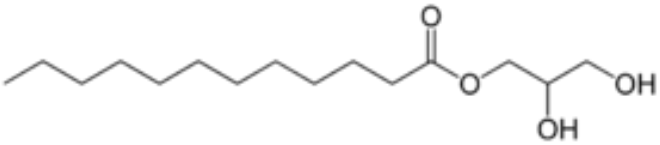
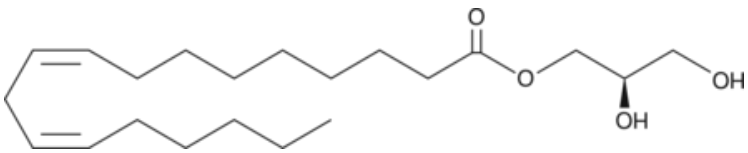


Glycerol monolaurate



1-Linoleoyl glycerol

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