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Characterization of Staphylococcus aureus Lipase

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

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Characterization of *Staphylococcus aureus* Lipase

Thesis format: Monograph

By

Vithooshan Vijayakumaran

Graduate Program in Microbiology and Immunology

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

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

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ABSTRACT

USA300, a strain of community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA), has become prevalent in the community. Colonization of human skin requires mechanisms that allow this bacterium to overcome the innate immune defenses on the skin, including secretion of antimicrobial lipids. Antimicrobial lipids inhibit *S. aureus* growth and induce the staphylococcal proteolytic cascade, producing aureolysin (Aur) which processes the lipase glycerol ester hydrolase (Geh). Nearly all *S. aureus* strains secrete Geh, yet little information exists concerning its function. Using purified Aur and Geh we confirm that aureolysin processes proGeh to Geh. We then confirmed that *geh* was required for lipase activity and both forms of the purified enzyme had lipase activity. Finally we showed that optimal growth in trilinolein requires Aur, and might reflect a requirement for the proGeh form of the enzyme to convert trilinolein into toxic linoleic acid, despite that fact that both unprocessed and processed Geh catalyze the hydrolysis of trilinolein.

**Keywords:** Staphylococci, triglyceride, anti-microbial lipids, USA300, Geh, lipase
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**TABLE OF CONTENTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>List Abbreviations</td>
<td>viii</td>
</tr>
</tbody>
</table>

**Chapter 1 – INTRODUCTION**

1. **Staphylococcus aureus**
   1.1.1 Description
   1.1.2 *S. aureus* and Virulence
   1.1.3 MRSA
   1.1.4 USA300

1.2 Lipids
   1.2.1 Chemical and Physical Properties of Lipids
   1.2.2 Biological Role of Lipids
   1.2.3 Lipids in Infection and Immunity
   1.2.4 Resistance to Antimicrobial Fatty Acids

1.3 Staphylococcal Proteases
   1.3.1 Proteases
   1.3.2 The Staphylococcal Proteolytic Cascade

1.4 Lipases
   1.4.1 Microbial Lipases
   1.4.2 *S. aureus* Lipases

1.5 Rationale and Hypothesis

**Chapter 2 – MATERIALS AND METHODS**

2.1 Bacterial Strains and Growth Conditions

2.2 DNA Methodology
   2.2.1 Plasmid Isolation from *E. coli*
   2.2.2 Plasmid Isolation from *S. aureus*
   2.2.3 Isolation of Chromosomal DNA from *S. aureus*
   2.2.4 DNA Ligation
   2.2.5 Recombinant DNA Methodology
   2.2.6 Agarose Gel Electrophoresis
   2.2.7 Isolation of DNA Fragment from Agarose Gels
   2.2.8 Polymerase Chain Reaction (PCR)
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.9</td>
<td>DNA Sequencing</td>
<td>29</td>
</tr>
<tr>
<td>2.2.10</td>
<td>Computer Analysis</td>
<td>29</td>
</tr>
<tr>
<td>2.3</td>
<td>Transformation Methodologies</td>
<td>30</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Preparation of Transformation Competent <em>E. coli</em></td>
<td>30</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Transformation of CaCl$_2$ Competent <em>E. coli</em></td>
<td>30</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Preparation of Transformation Competent <em>S. aureus</em></td>
<td>31</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Transformation of Electrocompetent <em>S. aureus</em></td>
<td>31</td>
</tr>
<tr>
<td>2.4</td>
<td>Mutagenesis and DNA Cloning Methods - Mutagenesis of geh</td>
<td>31</td>
</tr>
<tr>
<td>2.5</td>
<td>Protein Methodology</td>
<td>33</td>
</tr>
<tr>
<td>2.5.1</td>
<td>TCA Precipitation, and Visualization of Secreted Proteins</td>
<td>33</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Purification of Aur and Geh</td>
<td>34</td>
</tr>
<tr>
<td>2.6</td>
<td>Assays</td>
<td>35</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Lipase Assays</td>
<td>35</td>
</tr>
<tr>
<td>2.6.2</td>
<td>Protease Assays</td>
<td>36</td>
</tr>
<tr>
<td>2.6.3</td>
<td>Aureolysin-Geh Processing</td>
<td>36</td>
</tr>
<tr>
<td>2.7</td>
<td>Influence of triglycerides on <em>S. aureus</em> growth</td>
<td>37</td>
</tr>
<tr>
<td>2.8</td>
<td>Lipid Extraction and Analysis by GC/MS</td>
<td>37</td>
</tr>
</tbody>
</table>

**Chapter 3 – RESULTS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Deletion of <em>geh</em> locus</td>
<td>40</td>
</tr>
<tr>
<td>3.2</td>
<td>Growth in Fatty Acid Results in Processing of Geh</td>
<td>42</td>
</tr>
<tr>
<td>3.3</td>
<td>Aureolysin is required for Processing of Geh</td>
<td>42</td>
</tr>
<tr>
<td>3.4</td>
<td>The USA300 <em>geh</em> mutant has no detectable lipase activity</td>
<td>47</td>
</tr>
<tr>
<td>3.5</td>
<td>Growth in Trilinolein Results in Inhibition of Growth</td>
<td>49</td>
</tr>
<tr>
<td>3.6</td>
<td>Aureolysin is required for optimal growth in trilinolein</td>
<td>51</td>
</tr>
<tr>
<td>3.7</td>
<td>GC/MS confirms that trilinolein is a substrate for proGeh and Geh</td>
<td>57</td>
</tr>
</tbody>
</table>

**Chapter 4 – DISCUSSION**

**REFERENCES**

**Curriculum Vitae**
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>10</td>
</tr>
<tr>
<td>Lipid categories</td>
<td></td>
</tr>
<tr>
<td>Table 2.</td>
<td>24</td>
</tr>
<tr>
<td>Bacterial strains used in this study</td>
<td></td>
</tr>
<tr>
<td>Table 3.</td>
<td>26</td>
</tr>
<tr>
<td>Plasmids used in this study</td>
<td></td>
</tr>
<tr>
<td>Table 4.</td>
<td>32</td>
</tr>
<tr>
<td>Oligonucleotides and their sequences</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>The arginine mobile genetic element</td>
</tr>
<tr>
<td>2</td>
<td>Structures of lipids</td>
</tr>
<tr>
<td>3</td>
<td>The staphylococcal proteolytic cascade</td>
</tr>
<tr>
<td>4</td>
<td>Generation of a Δgeh mutant in <em>S. aureus</em> strain USA300</td>
</tr>
<tr>
<td>5</td>
<td>Induction of Aur by linoleic acid results in processing of proGeh to Geh in <em>S. aureus</em> culture supernatants</td>
</tr>
<tr>
<td>6</td>
<td>Purification of Aur and proGeh</td>
</tr>
<tr>
<td>7</td>
<td>Visualization of proGeh processing by aureolysin</td>
</tr>
<tr>
<td>8</td>
<td><em>p</em>NPP lipase assay of culture supernatants and purified Geh</td>
</tr>
<tr>
<td>9</td>
<td>Grow curve analysis of USA300 wild-type, Δgeh and Δaur with trilinolein</td>
</tr>
<tr>
<td>10</td>
<td>Secreted protein profile of cells grown in 50 μM trilinolein</td>
</tr>
<tr>
<td>11</td>
<td>Comparison of cell-surface proteins of washed and unwashed cells and growth of washed cells in trilinolein</td>
</tr>
<tr>
<td>12</td>
<td>Delayed growth of Δgeh strain in trilinolein after re-suspension in supernatant from wild-type and Δaur</td>
</tr>
<tr>
<td>13</td>
<td>Impaired growth of Δgeh in trilinolein with addition of purified proGeh or Geh at inoculation</td>
</tr>
<tr>
<td>14</td>
<td>Gas chromatography-mass spectrometry confirms release of linoleic acid from trilinolein by both forms of Geh</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>Community associated methicillin resistant <em>S. aureus</em></td>
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<td>CoNS</td>
<td>Coagulase negative staphylococci</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Em</td>
<td>Erythromycin</td>
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<td>HA-MRSA</td>
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<tr>
<td>Km</td>
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<td>LB</td>
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<td>Molar</td>
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<td>Nanometer</td>
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<tr>
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</tr>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>TAE</td>
<td>Tris-acetate EDTA</td>
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<tr>
<td>TBE</td>
<td>Tris-borate EDTA</td>
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<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

1.1 *Staphylococcus aureus*

1.1.1 Description

*Staphylococcus aureus* is a Gram-positive bacterium where the individual cocci divide in two planes resulting in the formation of grape-like clusters, and for this reason the word Staphylococcus is derived from the Greek term *staphylē* (“bunches of grapes”). *S. aureus* is the most pathogenic of the staphylococci and was identified in the 19th century. *S. aureus* strains are characterized by a golden-yellow pigment, staphyloxanthin (*aureus*: “golden”). Additionally, *S. aureus* are the only staphylococci with the ability to produce the protein coagulase which allows for the conversion of fibrinogen to fibrin, allowing fibrin to coat the bacteria and thus protecting them from attacking host cells (59). *S. aureus* is a highly persistent organism which can survive in high salt conditions (up to 15% NaCl), dry environments and can survive over a wide pH range from 4.8 to 9.4 (15, 58). These survival mechanisms allow it to thrive in many harsh environments, ranging from the surfaces of medical equipment to the epidermal and mucosal layer of mammals.

1.1.2 *S. aureus* and Virulence

*Staphylococcus aureus* is a human commensal bacterium found to persistently colonize about 30% of the human population (73). While the organism is generally harmless to healthy individuals, *S. aureus* is a significant opportunistic pathogen and is the causative agent in over 10% of all nosocomial bacterial infections in the United States (25). Furthermore *S. aureus* infection can range from mild skin and soft tissue infections to more
severe conditions such as necrotizing pneumonia, osteomyelitis, toxic shock syndrome, bacteremia and infective endocarditis (56).

Virulence factors provide bacteria with mechanisms that aid in their in vivo growth and enhance their ability to cause disease. *S. aureus* possesses a vast array of virulence factors which contribute to its success as a pathogen. *S. aureus* possesses a plethora of surface-associated virulence factors which aid in adherence followed by the establishment of infection (15). Furthermore, some of these surface-associated proteins also provide an immune evasion mechanism by modulating the host immune response. Cell surface virulence factors can include cell wall anchored proteins or polysaccharides. Polysaccharides such as teichoic acid on the *S. aureus* cell wall aid in adhesion to endothelial surfaces while other cell surface components such as peptidoglycan and lipoteichoic acids can cause neutrophil infiltration and septic shock (45, 50, 95, 96). *S. aureus* also produces several pore forming toxins which create pores in the cell membrane resulting in lysis through osmotic imbalance. Two of these pore forming toxins are known as α-toxin and β-toxin which are capable of destroying erythrocytes (15). Destruction of erythrocytes is a key nutritional strategy that provides *S. aureus* with a source of heme-iron for uptake. Additionally *S. aureus* secretes a variety of toxins and exoenzymes capable of causing damage to host tissue. For example, *S. aureus* produces the toxic shock syndrome toxin-1 (TSST-1), a known superantigen, which through the activation of T cells induce a massive cytokine storm resulting in fevers, rashes, hypotension, multi-organ failure and death (15, 92). Exoenzymes such as proteases, lipases and nuclease are some of the less well-understood virulence factors of *S. aureus*, and the relevance of both proteases and lipases will be further explored throughout this study.
1.1.3 Methicillin Resistant *Staphylococcus aureus*

Administration of penicillin was the effective traditional treatment for *S. aureus* infections and could significantly reduce mortality rates in patients (27). However, due to the use of penicillin, antibiotic-resistant strains of *S. aureus* emerged and widespread penicillin resistance around the world had occurred by the 1950s (15). Even with the development of next generation antibiotics, both resistant hospital-acquired and community-acquired strains emerged. The emergence of resistance to beta-lactam antibiotics such as cephalosporins, carbapenems, cephamycins and monobactams prompted development of methicillin. Unfortunately, by 1961, just 2 years since the introduction of the drug to penicillin resistant *S. aureus*, the first methicillin resistant *S. aureus* (MRSA) were reported. Over the next several decades MRSA spread around the world, and is now endemic in most healthcare facilities in developed countries. Methicillin and beta-lactam class antibiotic resistance is encoded on a genomic island called the staphylococcal cassette chromosome *mec* (SCC*mec*), which also encodes tetracycline resistance (38). A major concern is the increase of multidrug resistant strains of MRSA, which have evolved resistance to other classes of antibiotics including rifampin, fluoroquinolones, aminoglycosides, tetracyclines and chloramphenicol. Additionally, the emergence of MRSA strains resistant to vancomycin, the treatment of last resort for some MRSA strains, leaves the possibility of emerging *S. aureus* infections that would be untreatable with current antibiotics (16, 27).

In contrast to healthcare-associated MRSA (HA-MRSA), which often infect individuals who are at high risk or already ill, community-associated MRSA (CA-MRSA) strains can infect healthy individuals. This, in combination with the unusually severe disease caused by these strains, suggests that CA-MRSA possess greater virulence potential than traditional strains (16). CA-MRSA is transmitted by direct contact, usually skin-to-skin, with
an infected individual and this often occurs among military settings, locker rooms and among children in daycare centers where there is a combination of close body contact and low personal hygiene. In particular the CA-MRSA strain USA300 has become a leading cause of visits to hospital emergency departments as it is easily transferred to household contacts compared to other *S. aureus* strains (24, 63).

### 1.1.4 USA300

The USA300 strain is a particularly hypervirulent strain of CA-MRSA which has become endemic within the community. USA300 clones show a high level of genetic similarity, with different isolates being distinguished only by a few single nucleotide polymorphisms (71). In a brief time span this strain has become the most frequent cause of skin and soft tissue infections in emergency rooms in the United States, being responsible for 97% of MRSA and 58% of all reported skin infections (67). Since the appearance of USA300 the percentage of MRSA carriers has doubled (31). It can be inferred that the increased incidence of USA300 infections can, in part, be attributed to an increased frequency of colonization.

In addition to its exceptional transmission and colonization from host to host, the USA300 strain has been associated with highly invasive diseases such as necrotizing pneumonia, severe septicaemia and necrotizing fasciitis (28, 30, 64). USA300 was found to be more resistant to killing by human polymorphonuclear leukocytes and more effective at causing host cell lysis. Mouse bacteraemia models show that it is significantly more virulent than other MRSA strains and is more highly invasive of major organs (94).

Like other CA-MRSA, USA300 carries the type IV SCC*mec* cassette, which lacks additional resistance genes other than *mecA*, thereby eliminating the fitness cost imposed by
This cassette when there is a lack of antibiotic stress (19). However, the extraordinary success of USA300 as a pathogen and its ability to overtake other CA-MRSA clones can be attributed to the presence of unique genetic elements that enhance colonization and transmission. Exclusive to the USA300 strain is the 31-kb arginine catabolic mobile element (ACME) (Figure 1) which is a putative pathogenicity island found adjacent to SCCmec (19).

In USA300, ACME is thought have been acquired from *S. epidermidis*, likely through horizontal gene transfer. However, ACME has also been identified among other coagulase-negative staphylococci (CoNS) such as *S. haemolyticus* and *S. capitis* in which it has a more genetically diverse organization than in *S. aureus* (83). ACME encodes two major gene clusters which include an arginine deiminase (*arc*), a spermine/spermidine acetyl-transferase (*speG*) and oligopeptide permease (*opp*) operon, both of which are homologs of genes that are accepted virulence factors (18).

Most pathogens often have difficulty colonizing the harsh environment of the human skin, yet USA300 is able to colonize this niche at a higher rate than other strains. One attribute of skin that deters infections is the acidic pH (4.2-5.9). However, the presence of Arc encoded by ACME has been found to enhance the acid tolerance of USA300, including to, for example, exogenous lactic acid, the major organic acid present on human skin (1, 62, 93). The ACME Arc encoded arginine deiminase pathway converts L-arginine to carbon dioxide, ATP and ammonia, which counteracts the acidity of the environment (18, 19, 66, 83, 93).

Polyamines are aliphatic compounds produced by many organisms that exert a variety of effects on cellular physiology. Polyamines include putrescine, spermidine and spermine
Figure 1. The Arginine mobile genetic element. The 31-kb ACME is unique to the USA300 strain of *S. aureus* and is thought to confer upon this strain an enhanced ability to colonize the human skin.
and are all compounds synthesized from L-arginine. However, *S. aureus* is unable to produce polyamines and exogenous polyamines actually inhibit *S. aureus* growth, and are bactericidal at concentrations found in humans. USA300 is a notable exception in that it exhibits complete resistance to high levels of exogenous polyamines due to the presence of a spermine/spermidine acetyl-transferase system encoded on ACME (41).

Additionally ACME encodes a putative oligopeptide permease operon, Opp-3. Opp operons in Gram-positive and Gram-negative bacteria possess a range of functions including peptide uptake, quorum sensing, chemotaxis, binding of serum components and expression of virulence determinants (74). *In vivo* models have shown that disruption of Opp-1 and Opp-2, which are found on the core genome have been shown to result in major growth defects and attenuated virulence (12).

USA300 also possesses several other newly acquired genes such as the enterotoxin K and Q (*sek2* and *seq2*) in the unique SaPI5 pathogenicity island, and these contribute to pathogenesis by binding T-cells (18). USA300 also possesses Panton-Valentine leukocidin (PVL), a pore forming toxin which causes necrosis and apoptosis in leukocytes. PVL is a bi-component toxin encoded by the *lukS-PV* and *lukF-PV* genes (12). Furthermore, research indicates a high level of correlation between the production of PVL and severe skin and soft tissue infections, necrotizing pneumonia and fasciitis (14, 29). Levels of PVL sufficient to result in rapid neutrophil lysis can be directly detected in abscesses on human skin (4, 5). In rabbit models, USA300 strains lacking PVL displayed attenuated virulence in pneumonia, osteomyelitis and skin abscess models (13, 17, 48). Additionally, the expression of several virulence genes such as α-toxin (Hla), a potent pore forming toxin, δ-toxin (*hld*) and α-type phenol soluble modulin (PSMs) is also elevated in the USA300 strain (7, 40, 53).
Consequently, the success of USA300 as a pathogen can be attributed to the amalgamation of an array of factors.

1.2 Lipids

1.2.1 Chemical and Physical Properties of Lipids

Lipids are an integral part of the physiology and pathophysiology of biological systems. Although there is no widely accepted definition of the term lipid, a broad definition would be that lipids are hydrophobic or amphipathic molecules that are created either entirely or partly from carbanion-based condensations of thioesters or by carbocation-based condensations of isoprene units (26). Although there are several categorization systems for lipids (Table 1), they can broadly be classified as “simple” and “complex” lipids. Simple lipids yield two products upon hydrolysis while complex lipids yield three or more products. A lipid is saturated if all available spaces where hydrogen bonding to carbon can occur are occupied, while an unsaturated lipid has at least one double bond between carbon atoms. As a result, unsaturated lipids tend to have lower melting points and are more vulnerable to lipid peroxidation (51). In this study we will focus on free fatty acids and triglycerides such as those seen in Figure 2.
<table>
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<tr>
<th>Category</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acyls</td>
<td>dodecanoic acid</td>
</tr>
<tr>
<td>Glycerolipids</td>
<td>1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycerol</td>
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<td></td>
<td>1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>Glycerophospholipids</td>
<td>N-(tetradecanoyl)-sphing-4-enine</td>
</tr>
<tr>
<td>Sterol lipids</td>
<td>cholest-5-en-3β-ol</td>
</tr>
<tr>
<td>Prenol lipids</td>
<td>2E,6E-farnesol</td>
</tr>
<tr>
<td></td>
<td>UDP-3-O-(3R-hydroxy-tetradecanoyl)-αD-N-acetylglucosamine</td>
</tr>
<tr>
<td>Saccharolipids</td>
<td>aflatoxin B₁</td>
</tr>
</tbody>
</table>
Figure 2. Structures of lipids. A) Linoleic acid and B) trilinolein which is composed of three linoleic acid molecules esterified to a glycerol backbone.
1.2.2 Biological Role of Lipids

Lipids have several important roles in biological systems, which include energy storage, signaling, and as one of the structural components of the membrane (26, 90). The biological membrane is essentially a lipid bilayer, the formation of which is energetically favoured when glycerophospholipids are put in an aqueous environment, arranging themselves so that the polar head groups face the aqueous side and the hydrophobic fatty acid tails face each other (97). Recently, it has been shown that lipid signaling is important for communication in cells. Some of these signaling lipids are involved in regulation of cell growth, apoptosis, protein activation, and calcium mobilization (36, 80). One of the most important and well understood functions of lipids is for energy storage in animals and plants, especially in the form of triglycerides (8).

1.2.3 Lipids in Infection and Immunity

The sebaceous glands on the skin secrete several factors that are growth inhibitory to many invading pathogens. An innate immune mechanism used to hinder bacterial persistence is the secretion of sebum (65, 98), a liquid concoction of lipids composed of 28% free fatty acids, 32% triglycerides, 25% wax esters and 11% squalene (88). Sapienic acid (C16:1Δ6), is the major constituent of sebum triglycerides and fatty acids (98), and S. aureus is also significantly exposed to linoleic acid (C18:2) from nasal secretions, during colonization of the anterior nares (20). Previous studies have shown that at physiological concentrations, linoleic acid (50 μM) is inhibitory to the growth of USA300 and results in an extended 12 hour lag phase in vitro (2). Fatty acids have been reported to interfere with cell growth by altering cell permeability, uncoupling oxidative phosphorylation or by blocking electron transport (9, 10, 33, 35, 46, 47, 87).
Generally lipid extracts from animal and microbial sources contain roughly 60 to 80% phosphates and glycolipids with the remainder consisting of neutral lipids (44). However lipid extracts from staphylococcal abscess homogenates revealed the composition to be 90% neutral lipids and 10% glycolipids and phosphatides, and of the neutral lipids about 40% were free fatty acids, including linoleic acid which is found to accumulate to high levels in tissue abscesses which are characteristic of *S. aureus* skin and soft tissue infection (22). This raises the question of how *S. aureus* can colonize and persist on human skin, mucosal membranes, and within abscesses, despite the abundance of antimicrobial lipids such as free fatty acids, monoglycerides and triglycerides, found in these environments (55).

### 1.2.4 Resistance to Antimicrobial Fatty Acids

While some bacteria can use fatty acids as a means of energy, this only occurs when the fatty acid is broken down into two-carbon acetyl-CoA molecules through the β-oxidation process so that they can enter the TCA cycle. However, *S. aureus* lacks the enzyme required for β-oxidation (18, 72) and consequently is unlikely to have the ability to break down fatty acids in this way. In addition to using fatty acid for energy, some bacteria secrete fatty acids such as linoleic acid. For example, *Lactobacilli* which are a part of the nasal microbiota, secrete linoleic acid, which has been shown to inhibit *S. aureus* growth (2). Since *S. aureus* is also part of this niche, it competes with the nasal microbiota for colonization, including several bacterial phyla such as Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria. Evidence of bacterial competition was seen from studies indicating an inverse correlation between the members of Actinobacteria family and the amount of *Staphylococcaceae* present (52). Differences in the fatty acid profile of nasal secretions may be dependent on the profile of microbiota found in different individuals.
One proposed mechanism of fatty acid resistance is the Fatty Acid Modifying Enzyme (FAME). It has been shown that *S. aureus* uses FAME, an extracellular enzyme, to counteract the staphylocidal activity of free fatty acids and monoglycerides and has been detected in 80% of *S. aureus* strains (55). Contrary to typical lipases, FAME has been shown to have optimal activity at a pH ranging from 5.5 to 6.0, which is similar to the acidic pH of human skin, which can range from pH 4.2 to 5.9 (18, 42). When fatty acids were incubated with FAME in the presence of ethanol or cholesterol, fatty acid esters were produced. Thus FAME acts by esterifying free fatty acids directly or transferring the acyl group of monoglycerides to short chain alcohols and cholesterol (57). While FAME has been detected experimentally, neither the FAME protein nor its corresponding gene has yet been identified (18, 42, 68). Interestingly, staphylococcal FAME is strongly inhibited by triglycerides, which are found in large pools in abscesses. However, studies show that all strains with FAME activity also exhibit lipase activity. In order to evade the issue of FAME inhibition, it is believed that *S. aureus* employs a lipase which releases the fatty acids from their glyceride backbone so that FAME can esterify the free fatty acids (55). In murine models, strains possessing FAME activity exhibit greater virulence than strains in which FAME has not been detected (68).

Other suggested mechanisms of fatty acid resistance implicate a cell wall component as a requirement for fatty acid resistance. This hypothesis is based on literature suggesting that the lack of a cell wall anchored component causes increasing sensitivity to fatty acids. Some of the components implicated include teichoic acids, surface protein G (SasG) and the heme coordinating surface protein IsdA (11). While the exact mechanism by which these
elements confer resistance has yet to be established, the implication is that they prevent fatty acids from seeping into the cell by acting as a barrier.

1.3 Staphylococcal Proteases

1.3.1 Proteases

Proteases are enzymes, which induce protein catabolism by hydrolyzing the peptide bonds that hold together the amino acids that form a polypeptide. Studies have shown that staphylococcal proteases are also key virulence determinants, as they have been known to cleave and degrade a variety of important host proteins including elastin, plasma proteinase inhibitor and the heavy chains of all human immunoglobulin classes (75-78). Furthermore, more recent studies suggest that proteases may be involved in the conversion of *S. aureus* from an adhesive to invasive state through the degradation of cell surface proteins such as fibronectin binding protein (43, 61, 79, 91).

1.3.2 The Staphylococcal Proteolytic Cascade

*S. aureus* encodes four major extracellular proteases, including a metalloproteinase (aureolysin, Aur), a serine glutamyl endopeptidase (serine protease, SspA) and two cysteine proteases, staphopain A (ScpA) and staphopain B (SspB) (3). SspA and SspB are co-transcribed in the *sspABC* operon, which also includes a third open reading frame *sspC*, encoding a cytoplasmic inhibitor of SspB (60). The Aur, SspA, and SspB proteases are expressed as proenzymes, which are subsequently activated in what is known as the Staphylococcal proteolytic cascade pathway, Figure 3 (2).
Figure 3. **The staphylococcal proteolytic cascade.** Induction of the staphylococcal proteolytic cascade results in the autocatalytic activation of aureolysin to its mature form, which in turn results in the maturation of SspA and culminates with the activation of SspB.
Aureolysin is a secreted protease of *S. aureus* belonging to the M4 metalloproteinase family of enzymes, which include a number of enzymes which are acknowledged virulence factors. Aureolysin cleaves several host proteins, including peptides involved in immune escape. *In vitro* experiments have shown that aureolysin cleaves some plasma proteinase inhibitors and activates prothrombin in human plasma (69).

Aureolysin is regulated by *agr* (accessory gene regulator) and *sarA* (Staphylococcal accessory regulator), both of which are known virulence regulators in *S. aureus* despite the lack of understanding on the importance of aureolysin in virulence. Transcription of extracellular proteases is generally repressed by *sarA* and stimulated by *agr* (54). The *agr* gene system results in production of RNA III, which stimulates the expression of several exoprotein genes. While the exact mechanism of RNA III dependent regulation is unknown, it is believed to interact with other regulatory proteins rather than direct binding to the promoter of target genes. During regulation of *agr*, RNA III is believed to neutralize a repressor of *aur*, the Rot protein expressed by the *rot* gene, a global gene regulator. However, a direct repressor of *aur* is SarA, the product of *sarA*, which binds the *aur* promoter (54, 82).

When the gene encoding aureolysin was characterized, it was revealed that the product would be about 509 amino acids in length, whereas the crystal structure of the protein revealed only 301 amino acids, suggesting that aureolysin undergoes post-translational processing. The enzyme in its unprocessed form is expressed as a 56 kDa propeptide which undergoes autocatalysis resulting in the removal of a 23 kDa propeptide to yield the 33 kDa active mature enzyme (69). As seen with other members of the M4 family of metallopeptidases, aureolysin acts as a proprotein convertase and processes and activates other enzymes. The activity of this protease depends on both a zinc ion in the active site, as well as three calcium ions, and its activity is susceptible to the addition of metal ion chelators.
such as EDTA. Aureolysin is required for the progression of the Staphylococcal proteolytic cascade, and inactivation of aureolysin results in a culture which lacks proteolytic activity (69).

Activation of the serine glutamyl endopeptidase is dependent on aureolysin, although it is necessary for proSspA to first undergo a series of initial autocatalytic steps in order for efficient final activation by aureolysin to occur. Autocatalysis is enabled by the presence of a glutamine rich region in the propeptide, and subsequent release of the mature enzyme occurs from cleavage at Leu$_{56}$ followed by Val$_{69}$ by aureolysin. The mature form of SspA is involved in the moderation of *S. aureus* adhesion to fibronectin, as it degrades cellsurface fibronectin binding proteins and consequently contributes to invasive infection (70). Furthermore, the mature SspA is also responsible for the activation of proSspB.

The final product of the staphylococcal proteolytic pathway is the mature form of the Staphopain B cysteine protease. It has been established that SspC helps to maintain the SspB precursor as an inactive zymogen (60). The activation of proSspB is dependent upon SspA cleavage of the C-terminal end of the propeptide of SspB. The mature SspB protease has a variety of roles, one of which is moderating the adhesive functions of the cell, and it has been shown to preferentially cleave fibronectin to release the N-terminal portion.

The proteases of *S. aureus* staphylococcal cascade contribute to the virulence of this pathogen by degrading plasma molecules, degrading molecules of the immune system enabling immune evasion, controlling adhesion, and activating other enzymes.
1.4 Lipases

1.4.1 Microbial Lipases

Lipases are enzymes found in animals, plants and microorganisms and have become important for industrial usage. They are a class of hydrolases and, more specifically, esterases which catalyze the hydrolysis of acylglycerol into glycerol and free fatty acids in a lipid-water interface. Lipases can also catalyze the hydrolysis and transesterification of other esters in addition to the synthesis of esters. Lipases are differentiated from esterases as they are capable of degrading both emulsions and monomeric substrate where esterases only degrade the latter (39). Thus lipase activity is dependent on the presence of an interface, such that lipases were further defined as carboxylesterases acting on emulsified substrates (39).

Lipases can be classified into three groups, based on their substrate specificity. One group of lipases hydrolyses only the primary ester bond of a glyceride, although recently lipases with low activity towards hydrolyzing the second ester bond have also been discovered. Another group of lipases exhibit high preference for certain fatty acids, for example lipase B from *Geotrichum candidum* is specific for fatty acids with a double bond between C9 and C10 (85). However no bacterial lipase belongs to this group. The final group of lipases, which includes the lipases from *S. aureus*, have no positional or fatty acid specificity (39). Bacterial lipases vary greatly in size, and although the overall homology of lipases is relatively low, all share a similar three dimensional fold known as the $\alpha/\beta$ hydrolase, and the region of highest conservation is the active site containing a Ser-His-Asp catalytic triad (81). In most lipase structures, the active site is covered by surface loops and helical structures, making it inaccessible. The active site becomes exposed upon interaction with micelles and substrate molecules (81).
1.4.2 *S. aureus* Lipase

Lipolytic activity in staphylococci was discovered as early as 1901. Lipolytic activity by *S. aureus* is known to release large amounts of fatty acids, for example linoleic acid in human plasma. The lipases responsible for these reactions are extracellular lipases which are secreted into the medium (32). The Glycerol Ester Hydrolase lipase of *S. aureus*, is secreted as a 72 kDa precursor enzyme (proGeh), which is then processed into mature 42 kDa form (Geh). Its function, as the name suggests is to catalyze the hydrolysis of the ester bonds between glycerol and fatty acids, which form triglycerides and this is believed to aid the bacteria by contributing to the breakdown of host tissue, subsequently liberating nutrients. The lipolytic activity of this lipase is sensitive to metal ion chelators such as EDTA, as the enzyme requires calcium to function (32).

Geh has been implicated in virulence, and has been shown to interfere with the host granulocyte function, and increase survival of the bacteria against the host defense by inactivating bactericidal lipids (37). *S. aureus* strains recovered from deep infections show significantly more lipase activity than those obtained from superficial abscesses. Furthermore, during biofilm formation the lipase encoding genes were found to be induced and were found to be up-regulated during biofilm formation in *S. aureus*. Furthermore, lipase inhibitors have been shown to reduce biofilm formation in *S. aureus*. Additionally, mutation of the lipase genes results in reduced peritoneal abscess formation (37). Other than this, however, there has not been any significant research regarding the contribution of Geh to *S. aureus* virulence or colonization, especially in relation to persistence on human skin.
1.5 Rationale and Hypothesis

The goal of this research was to elucidate a mechanism involving lipid metabolism in \textit{S. aureus}. Previous research indicates that when grown in the presence of linoleic acid, the lag phase of growth of USA300 was severely prolonged. Additionally, robust induction of the Staphylococcal proteolytic cascade was also observed, resulting in processing of proGeh to Geh, due to the presumed proteolytic activity of aureolysin. As a result, \textit{we hypothesize that lipid metabolism is a key factor contributing to the persistence of USA300 on human skin and its dissemination within the community}. The \textit{first objective} of this study was to purify both proGeh and aureolysin, and confirm that aureolysin was required for the processing of proGeh. The \textit{second objective} was to determine the effect of triglycerides on growth of USA300 and mutants deficient for proGeh (and consequently the mature Geh) and aureolysin. This was a follow-up on the multitude of events seen during growth in linoleic acid, including the effects on the proteolytic cascade, lipase maturation and overall growth.

From these experiments it was determined that trilinolein resulted in an extended lag phase in wild-type and aureolysin deficient strains, and the lag phase was especially prolonged in aureolysin deficient strains. Conversely, trilinolein had no effect on Geh deficient strains. Since the aureolysin mutant, which produces only proGeh, had a longer lag phase than the wild-type, the \textit{third objective} was to determine which factors caused the lag in growth and characterize their activity on trilinolein.
MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are described in Table 2. MRSA isolate pulsed-field gel electrophoresis type USA300 LAC that had been cured of the erythromycin resistance plasmid was used in all experiments as the wild-type (WT) strain. Unless otherwise indicated, both *E. coli* and *S. aureus* were cultured at 37°C and stored at -80°C in 20% glycerol. *E. coli* strains were grown in Luria Bertani (LB) medium. *S. aureus* strains were grown in tryptic soy broth (TSB). For strains carrying resistance genes, antibiotics were used at the following concentrations: chloramphenicol (10 μg/mL) and erythromycin (3 μg/mL) for growth of *S. aureus* strains; ampicillin (100 μg/mL) and kanamycin (40 μg/mL) for growth of *E. coli* strains. Solid media were obtained by the addition of 2% (w/v) Bactor agar (Difco). Water for preparation of growth media and solutions was obtained by passage through a Milli-Q water filtration system (Millipore Corp.).
**Table 2. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Descriptiona</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
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<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA300 LAC</td>
<td>Community-acquired MRSA; WT strain, cured of resistance plasmid</td>
<td>(2)</td>
</tr>
<tr>
<td>RN4220</td>
<td>rK⁻ mK⁺; capable of accepting foreign DNA</td>
<td>(49)</td>
</tr>
<tr>
<td>H2660</td>
<td>USA300Δgeh</td>
<td>This Study</td>
</tr>
<tr>
<td>H2789</td>
<td>USA300Δaur::Erm; Ermf</td>
<td>(2)</td>
</tr>
<tr>
<td>RN6390ΔsarA</td>
<td>RN6390ΔsarA</td>
<td>(70)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Fϕ80dlacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (rK⁻ mK⁺) supE44 relA1 deoR Δ(lacZYA-argF)U169phoA</td>
<td>Promega</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td><em>E. coli</em> B (DE3)[F dcm ompT hsdR (rB⁻ mB⁻)]</td>
<td>(89)</td>
</tr>
</tbody>
</table>

*a Abbreviations: Ermf designates resistance to Erythromycin*
2.2 DNA methodology

2.2.1 Plasmid isolation from *E. coli*

All plasmids used in this study are listed in Table 3. Plasmid DNA was prepared from *E. coli* using the E.Z.N.A. Plasmid Miniprep Kit (Omega Biotek) according to the manufacturer’s instructions. Briefly, approximately 5 mL of stationary phase culture of *E. coli* were pelleted via centrifugation and resuspended in 250 μL of SolutionI/RNase (50mM Tris, pH 8.0, 20 mM EDTA, 100 μg/mL of RNaseA). Cells were lysed by adding 250 μl of Solution II (200 mM NaOH, 1% (w/v) SDS), gently inverting the tubes followed by incubation at room temperature for 2-5 minutes. To neutralize the solution, 350 μl of Solution III (guanidine hydrochloride with acetic acid) was added to the lysate and was immediately inverted several times until a precipitate formed. The insoluble material was subsequently centrifuged for 10 minutes at 13000 rpm to form a pellet. The resulting cleared supernatant was aspirated into a HiBind DNA Miniprep Column (I) and was centrifuged at 13000 rpm for 1 minute. 500 μl of Buffer HB was added to wash the column and ensure that residual protein contaminations are removed. 700 μl of DNA Wash Buffer diluted with absolute ethanol was added to the column and centrifuged for 1 minute at 13000 rpm. The column was subsequently centrifuged for 2 minutes at 13000 rpm to dry the column matrix. Plasmid DNA was then eluted from the column into a fresh microcentrifuge tube by addition of 30 μl to 100 μl of dH2O followed by centrifugation at 13000 rpm for 1 minute.
Table 3. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Descriptiona</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET28a(+)</td>
<td>T7 expression vector carrying N terminal His Tag/thrombin/T7 tag; Km(^r)</td>
<td>Novagen</td>
</tr>
<tr>
<td>pVVgeh</td>
<td>pET28a(+) containing geh; Km(^r)</td>
<td>This Study</td>
</tr>
</tbody>
</table>

\(^a\) Abbreviations: Km\(^r\) designates resistance to Kanamycin
2.2.2 Plasmid isolation from S. aureus

Plasmid DNA isolation from S. aureus followed the same protocol as described for E. coli but with a few modifications. The harvested cells were incubated at 37°C for 30-60 minutes in a 250 mL mixture of Solution I containing lystostaphin (Sigma) (1 mL Solution I added to 50 μg of lysostaphin) in lieu of RNase A solution, prior to addition of Solution II.

2.2.3 Isolation of chromosomal DNA from S. aureus

Chromosomal DNA was obtained from S. aureus by pelleting 500 μl of overnight stationary phase cells grown in TSB culture. 200 μl of STE (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5) was added to the cells along with 50 μg/ml of lysostaphin dissolved in 20 μL of STE in order to facilitate cell lysis. The cell suspension was incubated at 37°C for 1 hour. 20 μl of 10% SDS and 20 μl of Proteinase K (New England Biolabs) were added and incubated overnight at 55°C. Subsequently, 80 μl of 5M NaCl was added and mixed by inversion. 320 μl of a 25:24:1 phenol : chloroform : isoamyl alcohol (IAA) (Invitrogen) was added and was allowed to sit at room temperature for 30 minutes. The aqueous layer was removed after the mixture was spun at 12000 rpm for 10 minutes. Addition of 300 μl of 24:1 chloroform:IAA was added and then the mixture was spun at 12000 rpm for 10 minutes. The aqueous layer was subsequently removed, and 400 μl of isopropanol was added until the DNA formed a visible mass while gently inverting. The mixture was allowed to sit for 10 minutes at room temperature, and was then spun at 12000 rpm for 5 minutes. The pellet was washed with 70% ethanol, dried, and resuspended in 100 μl of dH20.
2.2.4 Restriction Enzyme Digests

Restriction enzymes were purchased from Life Technologies, MBI Fermentas, New England Biolabs, or Roche Diagnostics. Reactions were typically carried out in 30-40 μl volumes over a 1-2 hour incubation at the appropriate temperature (typically 37°C). Digested DNA was subsequently cleaned using a QIAquick PCR purification kit (QIAgen) as described by the manufacturer.

2.2.5 DNA ligations

DNA fragments were ligated in a 20 μl reaction volume using a 10:1 ratio of insert to vector DNA. Reactions were carried out using the T4 DNA ligase Rapid Ligation Kit (Roche Diagnostics) in accordance with the manufacturer’s recommendations.

2.2.6 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation and analysis of DNA fragments. Agarose gels (0.8% w/v) were prepared using 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA) to which either 1.5 μg/ml of ethidium bromide of 2 μl of SYBR Safe DNA gel stain (Invitrogen) was added. DNA samples to be run were mixed with loading buffer (5% glycerol, 0.04% bromophenol blue, 0.04% xylene cyanol, 10 mM EDTA, pH 7.5) prior to being loaded in the gel. Electrophoresis was typically carried out at 110 V for 20-25 minutes. The 1 kb-Plus ladder (Invitrogen) was used as a standard reference marker for estimation of DNA fragment size. Following electrophoresis, DNA fragments were visualized using a gel doc (Bio-Rad).
2.2.7 **Isolation of DNA fragments from agarose gels**

Desired DNA fragments were visualized under long-wave UV light (365 nm) and excised from agarose gels following electrophoresis. DNA was isolated using the QIAquick Gel Extraction Kit (QIAGEN) using a protocol as described by the manufacturer.

2.2.8 **Polymerase chain reaction (PCR)**

PCR reactions were carried out in 50 μl reactions containing: DNA template, 1x PCR buffer, 200 uM dNTP mix (Roche Diagnostics), 12 pM of forward and reverse primers, and 0.5 units of Taq DNA polymerase. PCR reactions were also carried using KAPA HiFi HotStart PCR Kits (Kapa Biosystems). Briefly, 25 μl reactions containing template DNA, 1X Kapa HF Buffer (2.0 mM Mg 2+ at 1X), 300 μM of dNTPs, 300 nM each of forward and reverse primer and 0.5 units of KAPA HiFI HotStart DNA Polymerase. PCRs were performed using the GeneAmp PCR system (Perkin Elmer), DNA engine Gradient Cycler (Bio-rad) or the MJ Mini Personal Thermal Cycler (Bio-rad). Oligonucleotide primers were obtained from Integrated DNA Technologies.

2.2.9 **DNA sequencing**

DNA sequencing was performed at the DNA Sequencing Facility of the Robarts Research Institute (London, Ontario, Canada), with sequencing reactions prepared according to their guidelines.

2.2.10 **Computer Analyses**

DNA sequence analysis, sequence alignments, and oligonucleotides primer design were carried out using Vector NTI Suite 7 software package (Informax, Inc., Bethesda,
Blast searches were performed using tools available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/).

2.3 Transformation and transduction methodologies

2.3.1 Preparation of transformation competent E. coli

E. coli DH5a or E. coli BL21 DE3 CaCl₂ competent cells were prepared as follows. An overnight, stationary phase culture of DH5a was diluted 1:100 into 500 ml of fresh LB and grown to an OD₆₀₀ of approximately 0.5 and placed on ice for 30 minutes. The cells were then harvested via centrifugation and resuspended in 100 ml of ice cold 100 mM CaCl₂ plus 15% glycerol and incubated on ice for 30 minutes. Cells were again collected by centrifugation, resuspended in 4 ml of CaCl₂ plus 15% glycerol, and stored as 100 μl aliquots at -80°C.

2.3.2 Transformation of CaCl₂ competent E. coli

To transform CaCl₂ competent E. coli DH5a or BL21, purified plasmid DNA or ligation mixtures were added to an aliquot of competent cells and kept on ice for 45 minutes, after which cells were subjected to a heat shock treatment at 42°C for 2 minutes immediately followed by a 2 minute incubation on ice. An 800-μl aliquot of LB broth was added to the tube, mixed, and the cells were permitted to recover for 1 hour at 37°C before being plated on selective media and incubated overnight.
2.3.3 Preparation of transformation competent \textit{S. aureus}

Strains of \textit{S. aureus} were made competent for transformation via electroporation as follows. An overnight culture of \textit{S. aureus} was diluted 1:100 into 100 mL of fresh TSB and grown to an OD$_{600}$ of approximately 0.3. Cells were then harvested via centrifugation and resuspended in 500 mM sucrose. Cells were subsequently washed three times with ice cold 500 mM sucrose. After the final wash, cells were resuspended in 1 mL of 500 mM sucrose and stored as 80 μl aliquots at -80°C.

2.3.4 Transformation of electrocompetent \textit{S. aureus}

Electrocompetent \textit{S. aureus} were transformed using purified plasmid DNA (typically 5 μl from an EZNA miniprep). DNA was added to a tube of competent cells and allowed to incubate on ice for 30 minutes before being transferred to an ice-cold electroporation cuvette (2mm, Bio-Rad) for electroporation. Following incubation, electroporation was performed using a Bio-Rad Gene Pulser II with setting of 2.5 V, 200 mA, and 25 Ω. Ice cold TSB (800 μl) was immediately added to pulsed cells, which were then recovered for a minimum of 1 hours at 37°C before being plated on appropriate selective media.

2.4 Mutagenesis and DNA cloning methods - mutagenesis of \textit{geh}

A \textit{S. aureus} USA300 \textit{geh} deletion mutant was constructed using the pKOR-1 plasmid (6). Sequences flanking the \textit{geh} locus of USA300 FPR3757 were PCR amplified using specific primers (Table 4) to produce the upstream and downstream arms. A PCR amplicon of the joined DNA fragments was recombined into the temperature sensitive pKOR1 vector, resulting in the pKOR-1Δ\textit{geh} plasmid. The resultant plasmid was first passaged through \textit{S. aureus} RN4220 before being introduced into USA300 by electroporation. The in-frame
Table 4.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Descriptiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>geh 5'F</td>
<td>GGGGACAAGTTTGTACAAAAAAGCAGGCTAACA TAGGGCATAAAGTGGAC</td>
</tr>
<tr>
<td>geh 5'R</td>
<td>CGCTAACACTGACACCACG</td>
</tr>
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<td></td>
<td>Generation of <em>S. aureus</em> <em>geh</em> allele, 5’ arm</td>
</tr>
<tr>
<td>geh 3'F</td>
<td>/5Phos/GGTATCTGGCAAGTTAAACC</td>
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<tr>
<td>geh 3'R</td>
<td>GGGGACCACTTTGTACAAGAAAGCTGGGTCTTGCACAAACTCATTTCACC</td>
</tr>
<tr>
<td></td>
<td>Generation of <em>S. aureus</em> <em>geh</em> allele, 3’ arm</td>
</tr>
<tr>
<td>geh 5'F-NdeI</td>
<td>TTTTCATATGTTAAGGAGGCAAG</td>
</tr>
<tr>
<td>geh 3'R-EcoRI</td>
<td>TTTGAATTCCAGCAGATTTACATAGC</td>
</tr>
<tr>
<td></td>
<td>Cloning of <em>S. aureus</em> <em>geh</em></td>
</tr>
</tbody>
</table>

a Restriction sites in sequences are underlined.
allelic replacement of the *geh* locus was achieved using the method previously described (6). The correct deletion of codons 25 to 631, of the *geh* gene, was confirmed by PCR and DNA sequence analysis.

2.5 **Protein methodology**

2.5.1 **TCA Precipitation, and visualization of secreted proteins**

Proteins in the cell-free culture supernatant were precipitated by mixing supernatant with an equal volume of ice-cold 20% trichloroacetic acid, washed in ice cold 70% ethanol, then air dried and dissolved in SDS-PAGE reducing buffer (70). Density of the culture (OD<sub>600</sub>) was determined before preparation of cell-free culture supernatant, and for the analysis of secreted protein profiles, TCA precipitated protein derived from 5.0 OD<sub>600</sub> units of culture was loaded into the lanes of an 12% acrylamide gel. The gels were stained with Coomassie Brilliant Blue R-250 to visualize the protein bands.

Protein bands were excised using an Ettan™ Spot Picker, and processed for mass spectrometry using a Waters MASSPrep Automated Digestor. Processed protein samples were spotted on MALDI plates and analysis was done using an Applied Biosystems 4700 Proteomics Analyzer. Data were acquired and processed using the 4000 Series Explorer and Data Explorer (Applied Biosystems), and using the MASCOT search engine, the peptide fingerprints were compared to the NCBInr database for Gram-positive bacteria.
2.5.2 Purification of Aur and Geh

For purification of aureolysin, 1.5 liters of RN6390ΔsarA was grown overnight in TSB at 37°C in an orbital shaker, set at 200 rpm and the cell-free supernatant was collected. The supernatant was precipitated by adding ammonium sulphate up to 85% saturation. The precipitate was then resuspended in binding buffer (20 mM Tris-HCl pH 7.4, 5 mM CaCl₂) and loaded on a sepharose packed 5 mL HiTrap column equilibrated with binding buffer. The protein was eluted over a gradient of 0%-80% elution buffer (20 mM Tris pH 7.4, 500 mM NaCl, 5 mM CaCl₂). Protein fractions were collected and dialysed against 20 mM Tris pH 7.4 with 5 mM CaCl₂. Protein purity was confirmed using SDS-PAGE, and protease activity was confirmed with a protease assay (described above).

The full length of the gene encoding Geh was PCR-amplified from S. aureus strain USA300 using forward and reverse primers containing NdeI and EcoRI restriction sites, respectively. The amplicon was digested with NdeI and EcoRI and cloned into NdeI-EcoRI-digested pET28a(+) (Novagen), which incorporates a thrombin-cleavable His₆ tag at the N-terminus of the encoded recombinant protein. The pVVgeh (pET28 with Geh) construct was introduced into E. coli BL21 (DE3). Cells were grown in LB media containing 50 μg/mL kanamycin at 37°C in an orbital shaker, set at 200 rpm. When the culture reached an OD₆₀₀ of 0.6, isopropyl β-D-1-thiogalactopyranoside was added to a concentration of 0.5 mM. The culture was further incubated for another 16 hours at 25°C, after which cells were collected via centrifugation. The cell pellet was re-suspended in 30 mL Buffer A (10 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole). The cells were lysed using a cell disrupter (Constant Systems Inc.) at 30 psi after which 10 μM E64, a cysteine protease inhibitor, was added to the lysate, and further cell debris was removed by centrifugation (3000 rpm for 15 minutes).
Any remaining cellular debris was removed by ultracentrifugation (50000 rpm for 60 minutes) and the soluble lysate was applied to a nickel-loaded 1-ml HisTrap column (GE Healthcare) equilibrated with buffer A. The His6-tagged protein was eluted from the column with a gradient of 0%–80% buffer B (10 mM Tris-HCl pH 8.0, 300 mM NaCl, 500 mM imidazole) using an ÄKTA FPLC (GE Healthcare). Protein fractions were collected and dialysed against 10 mM Tris pH 8.0. Protein purity was confirmed using SDS-PAGE. Lipase activity was confirmed using the pNPP based assay as described previously (34) and the protein was quantified using a Bradford Assay.

2.6 Assays

2.6.1 Lipase Assay

Lipase activity was assayed with para-nitrophenyl palmitate (pNPP) substrate (Sigma). For assessment of lipase activity in cell-free supernatants, cultures were grown for 18 hours and filtered across a 0.22 micron filter. Supernatant was concentrated using Amicon 10 K centrifugal filters by centrifugation for 30 minutes 3000 rpm. 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. Solution A (795 μM pNPP substrate in isopropanol) was added to solution B (0.005 % Triton X-100, 50 mM Tris-HCl pH 8.0, 1 mg/mL gum arabic) at a 1:9 ratio. Aliquots of the normalized supernatant were added to the assay buffer (Solution A and B). For measurement of activity of purified proGeh or mature Geh (as described previously), aliquots of enzyme were added to the assay buffer. Samples were incubated for 30 minutes at 37°C in the dark and absorbance was quantified at 410 nm using a Varian Cary 50 spectrophotometer.
2.6.2 Protease assay

Total protease activity of culture supernatants, as well as purified aureolysin, was assayed with FITC-casein substrate (Sigma). Before the assay, the supernatant samples were normalized by dilution with sterile water to adjust for minor differences in cell density of the stationary phase cultures at time of harvest. For purified aureolysin, aliquots of the protease were mixed in sterile water to a volume of 490 μL. These samples were mixed with 460 μL of incubation buffer (40 mM Tris-HCl pH 7.4, 300 mM NaCl, 20 mM CaCl2, and 2 mM L-cysteine) and 50 μL of 0.2% w/v FITC-casein. EDTA was used as an aureolysin inhibitor. The samples were incubated at 37°C for 2 h in the dark. Trichloroacetic acid was then added to 4% w/v to stop the reaction, and the samples were centrifuged at 15000 rpm for 15 minutes 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 Biotech plate reader using excitation at 485 nm and emission at 535 nm.

2.6.3 Aureolysin-Geh processing

The rate of Geh processing by aureolysin was determined to optimize the minimum quantity of aureolysin required to produce mature Geh. Briefly, 3 μg of Geh was first incubated at 37°C for 3 hours in lipase buffer (50 mM Tris HCl pH 8.0) containing varying amounts (1 - 0.001 μg) of aureolysin. Additionally, a suitable time frame for the reaction was determined by incubation of 3 μg of Geh at 37°C in lipase buffer (50 mM Tris HCl pH 8.0) with 0.01 μg of aureolysin. Samples were removed from the incubator at varying time points (5-90 minutes). Reactions were stopped with the addition of SDS-PAGE reducing buffer,
followed by heating for 5 minutes at 95°C. Assessments of the levels of proGeh and mature Geh were made visually following SDS-PAGE.

2.7 Influence of triglycerides on *S. aureus* growth

Trilinolein was purchased from TCI America. Prior to supplementing TSB media, the triglyceride was first mixed with an equal volume of DMSO, and then diluted in TSB to a working stock concentration of 5 mM. Cell-free supernatant was obtained from cultures grown for 18 hours in 25 mL volume TSB at 37°C on an orbital shaker incubator. Purified proGeh was prepared by incubation at 37°C in lipase buffer (50 mM Tris HCl pH 8.0) whereas mature Geh was prepared by incubation of proGeh with aureolysin (0.002 μg) in lipase buffer for 3 hours at 37°C. Washed cells were prepared by removing the cell free supernatant from precultured cells, and washed in saline twice before re-suspension in fresh TSB media.

For growth analyses, bacteria from single colonies on TSB agar were inoculated into culture tubes containing 5 mL of antibiotic free TSB, and grown overnight at 37°C on an orbital shaker, followed by measurement of OD$_{600}$. A 25-mL volume of TSB, supplemented with triglyceride, supernatant (100 μL) and/or purified protein (2.7 μg), was then inoculated to achieve a starting OD$_{600}$ of 0.01, and the cultures were grown at 37°C on an orbital shaker incubator, set at 200 rpm. Measurements of OD$_{600}$ were taken at set time points. All growth analyses were conducted in at least triplicate, from individual cultures.

2.8 Lipid extraction and analysis by GC/MS

The substrate trilinolein was tested against the two forms of Geh, proGeh and mature Geh. For analysis of proGeh, 3 μg of proGeh was incubated in lipase buffer (50 mM Tris-
HCL pH 8.0) for 3 hours at 37°C. To obtain mature Geh, 3 μg of proGeh was incubated in lipase buffer (50 mM Tris-HCL pH 8.0) with 0.002 μg of aureolysin for 3 hours at 37°C. Post incubation, trilinolein was added to a final concentration of 5 μM in a total 50 μL final reaction volume and incubated at 37°C for 60 minutes.

Fatty acids were then extracted two consecutive times with 500 μL of hexane, and the hexane extracts were then pooled together and dried under a constant stream of nitrogen gas. Dried samples were trimethylsilylated with 50 μL of pyridine and 50 μL of Obis(trimethylsilyl)trifluoroacetamide (BSFTA) + 1% Trimethylchlorosilane (TMS) (Sigma), and incubated at 70°C for 40 minutes.

Fatty acids were chromatographed as their TMS-esters on an Agilent 7890 GC equipped with a CP-Sil 5 column (0.25 mm x 30 m) and a flame ionization detector (FID). Samples (1 μL) were injected in splitless mode onto the column and eluted with a temperature gradient as follows: initial temp. 80°C held for 2 min, followed by a ramp up to 220°C (40°C/min) and then a ramp up to 300°C (15°C/min). The final temperature was held for 4.2 min for a total run time of 15 min. Nitrogen (N2) was used as a carrier gas at 2.0 mL/min. The injector and FID temperatures were 270°C and 300°C, respectively.
3.1 Deletion of the *geh* locus

As stated previously, our laboratories' previous work showed that unsaturated fatty acids, including linoleic acid, induced robust expression of the proteolytic cascade. One result of this was the processing of proGeh (pro glycerol ester hydrolase; lipase; SAUSA300_0320) to its mature form. One of the main goals of this study was to determine if this processing had any role to play in the biological response of *S. aureus* to the presence of fatty acids. The *geh* gene is 2,073 bp and encodes a protein containing 690 amino acids with a molecular mass of 72 kDa. In order to assess the importance of Geh during growth in the presence of antimicrobial lipids, a *geh* in-frame deletion was constructed as described in the Materials and Methods section. PCR amplification across the *geh* locus of wildtype and mutant confirmed the deletion in the mutant strain (Figure 4).
Figure 4. Generation of a Δgeh mutant in S. aureus strain USA300. PCR amplification across the geh open reading frame confirms the deletion in the mutant strain. The PCR amplicon was sequenced for final confirmation. Strain H2660 is the number given to the Δgeh mutant.
3.2 Growth in fatty acid results in processing of Geh

As stated above, previously, our laboratories showed that when grown in 50 μM linoleic acid, USA300 exhibited an extended lag phase in addition to the robust expression of the staphylococcal proteolytic cascade (2). This proteolytic cascade begins with the expression of the metalloprotease aureolysin and culminates with the activation of the cysteine protease SspB. Additionally under these conditions, the 72 kDa precursor form of Geh is processed into its mature 42 kDa form, but this processing is absent in an aureolysin mutant (2). The previous data was shown using MS to identify proteins of interest. We confirmed this phenotype and, using the geh and aur mutants, proved that indeed Geh was the protein being processed in an Aur-dependent fashion (Figure 5). Moreover, when we followed the kinetics of growth in the presence of linoleic acid, both the USA300Δgeh mutant and the USA300Δaur mutant displayed the same 12 hour lag period as wild type USA300 (data not shown), indicating that neither geh nor aur are required for growth in inhibitory concentrations of linoleic acid, nor are they directly responsible for the delay in growth in response to linoleic acid.

3.3 Purification of Aur and Geh, and confirmation of their activity

In a mutant lacking the aur gene, proGeh is among several proteins which remain unprocessed, as they require the proteolytic activity of the metalloprotease aureolysin. aureolysin is a zinc and calcium ion-dependent metalloprotease which initiates the staphylococcal proteolytic cascade. Therefore, to conduct a detailed analyses of the role of Aureolysin in processing of proGeh, we first needed to purify the two enzymes. As previously published by the McGavin laboratory (70), Aur was purified from the supernatant
Figure 5. Induction of Aur by linoleic acid results in processing of proGeh to Geh in *S. aureus* culture supernatants. SDS-PAGE of the culture supernatants of wild type and mutant USA300 strains grown in TSB or TSB supplemented with 50 μM linoleic acid for 18 hours. Proteins in the cell free culture supernatant were precipitated in ice-cold TCA, and after solubilization in SDS-PAGE reducing buffer, protein equivalent to 3.5 OD600 units of culture supernatant was loaded in each lane. Note that while the cultures grown in the presence of LA took longer to reach stationary phase, the cultures eventually reach the equivalent biomass as those grown in the absence of LA.
of an RN6390ΔsarA strain, a strain which overexpresses Aur in the culture supernatant, due to inactivation of sarA, which is a repressor of protease expression.

Aur was purified as described in the Materials and Methods (Figure 6A) and activity was confirmed using a FITC-labeled casein substrate, Figure 6B, where cleavage of the casein by Aur results in a measureable increase in fluorescence.

Next a recombinant geh was cloned into the pET28a vector and overexpressed and purified from E. coli BL21 by passage through a nickel affinity column. The purified protein runs at 72 kDa in an SDS-PAGE gel, although numerous other bands were also observed (Figure 6). These were confirmed via mass spectrometry to be degradation products of proGeh, in addition to small quantities of the mature 42 kDa Geh. After several attempts to obtain highly purified 72 kDa proGeh, we concluded that the protein was simply prone to degradation, as has been observed previously (86), such that the small number of degradation products present were not going to be easily removed.

Processing of proGeh by aureolysin was determined by incubation of the two proteins for various times, as well as at several ratios of Aur to proGeh. The samples were then visualized on SDS-PAGE gels for the presence of the 38 kDa Aur band, the disappearance of the 72 kDa proGeh band and an increasing abundance of the 42-kDa band representing mature Geh (Figure 7). The result showed that Aur is highly active on proGeh, with only 0.01 μg of aureolysin being needed for complete processing of 3 μg of proGeh in 30 minutes. Furthermore, the addition of metal ion chelators such as EDTA inhibit the activity of the metalloprotease Aur, as expected, resulting in loss of activity on FITC-casein (Figure 6B) and on proGeh (data not shown).
Figure 6. Purification of Aur and proGeh. A) SDS-PAGE showing purified aureolysin (38 kDa) and purified proGeh (72 kDa). 3 μg of protein were added to each lane. Lack of stability of proGeh enzyme resulted in a mixture of degradation products of various sizes, including the mature 42 kDa Geh, during the purification steps. B) Protease assay using FITC-labeled casein as a substrate for Aur. EDTA, which chelates metal ions such as Zn\(^+\) and Ca\(^{2+}\) which are required for functional Aur, was added to the Aur sample where indicated. Data is reported as fluorescence emission at 535 nm measured in arbitrary fluorescence units.
Figure 7. Visualization of proGeh processing by Aur. SDS-PAGE gel showing processing of Geh by Aur. Lanes 1 and 2 contain 3 μg of pure Aur and proGeh, respectively. Lanes 3-7 contain 3 μg of pure Geh incubated with ratios of pure Aur:proGeh, as indicated, for 30 minutes at 37°C. The reaction was stopped with the addition of SDS-PAGE buffer followed by heating the samples at 95°C.
3.4 The USA300 *geh* mutant has no detectable lipase activity

In addition to *geh*, other putative lipase genes exist within *S. aureus* USA300, including *lip* and the putative lysophospholipases SAUSA300_1194 and its homologue SAUSA300_0070 encoded on the ACME cassette. However, the function of these genes and their products are not well characterized. The *lip* gene encodes a putative triacylglycerol lipase; however this gene has not yet been characterized in the literature. The presence of SAUSA300_0070 on the ACME cassette suggests it may have a function in skin colonization as do some of the other genes present in the ACME cassette, such as *speG*, and the *arc* genes.

In order to determine which of these lipases was needed for lipase activity in *S. aureus* we used a *para*-nitrophenyl palmitate (*pNPP*) substrate-based colourimetric assay (34). In *pNPP*, palmitate (C16:0) a saturated long chain fatty acid, is esterified to a *para*-nitrophenol. The enzymatic hydrolysis of the ester bond between the fatty acid from *para*-nitrophenol results in the liberation of the *para*-nitrophenol group which can be measured colourimetrically at 410 nm.

To determine whether *geh* was required for lipase activity, the supernatants of the Δ*geh* and Δ*aur* mutants were analyzed using the *pNPP* lipase assay. The wild type supernatant contained both pro*Geh* and mature *Geh*, while the supernatant from the Δ*aur* strain possessed only pro*Geh*, and the Δ*geh* contained neither. As shown in Figure 8A, the most activity is seen in the supernatant of the Δ*aur* mutant while the supernatant of the Δ*geh* mutant was completely devoid of any measureable *para*-nitrophenol. The higher level of lipase activity measured in the Δ*aur* strain may be caused by a variety of effects including potential upregulation of pro*Geh* production in this mutant or due to the pro*Geh* having a higher substrate preference for *pNPP* than mature *Geh*. To rule out the latter possibility, using the *pNPP* assay we also showed that both purified pro*Geh* and *Geh* possess similar
Figure 8. *p*NPP lipase assay of culture supernatants and purified Geh. Lipase activity was assayed from culture supernatants of USA300 isogenic mutants grown for 18 hours in TSB (A), and from both purified proGeh and Geh (B). For testing lipase activity in culture supernatants, $OD_{600}$ 0.2 unit equivalents of cultures supernatant was used. For pure proteins, 0.9 μg of protein was used.
levels of lipase activity on pNPP as a substrate (Figure 8B). Therefore we conclude that both proGeh and Geh have similar activities on a saturated long chain fatty acid ester (palmitate) and that lipase activity in USA300 is dependent on the presence of geh.

3.5 Growth in trilinolein results in inhibition of growth

The literature suggests that substantial quantities of unsaturated triglycerides are produced in abscesses (22, 84). Since 50 μM free linoleic acid caused a 12 hour lag phase, and as triglycerides are the major substrate for lipases, we opted to observe the effects on growth in trilinolein, a triacylglycerol composed of three linoleic acid molecules esterified to glycerol. A 50 μM concentration of trilinolein was chosen, since it was theorized that even if one free fatty acid was released from each molecule of trilinolein, it would result in a concentration of free fatty acid that would delay the growth of USA300.

To assess the role of a long chain unsaturated triglyceride, such as trilinolein on the growth of USA300, 50 μM of trilinolein was added to cultures in TSB. In TSB, USA300 typically takes about 7-8 hours incubation at 37°C to reach stationary phase from a starting inoculum of OD$_{600}$ 0.01. However, in the presence of trilinolein the wild-type USA300 had a 6 hour extended lag phase and stationary phase was reached about 12-14 hours from inoculation (Figure 9). In contrast, the Δgeh mutant, which possesses no active lipases (see above) showed no such growth delay. In stark contrast to that result, the aureolysin mutant, in which there is an excess of proGeh due to lack of Aur for maturation into Geh, shows a very long lag phase lasting 10-12 hours and reaching stationary phase at around 18 hours from inoculation. The results suggest that Geh or some factor in the supernatant dependent on Geh activity, or a combination of both was likely involved in the breakdown of trilinolein, leading to the liberation of linoleic acid, and ultimately causing a delay in growth. Initially, the
Figure 9. Grow curve analysis of USA300 wild-type, Δgeh and Δaur with trilinolein.

During growth in trilinolein (50 μM), a USA300Δgeh mutant grows without delay, however the wild-type strain exhibits an extended lag phase. The USA300Δaur mutant produces and an even longer lag phase than the wild-type. Cultures were grown in triplicate in flasks containing 25 mL TSB and trilinolein (50 μM). Cultures were inoculated to a starting OD₆₀₀ of 0.01. Y-axis, OD₆₀₀; X-axis, growth time (hours). Error bars represent the Standard Error of the Mean. N=6, * p<0.05 for USA300 vs. USA300Δgeh, ** p<0.01 for USA300 vs. USA300Δgeh, *** p<0.001 for USA300 vs. USA300Δgeh, + p<0.05 for USA300 vs. USA300Δaur, ++ p<0.01 for USA300 vs. USA300Δaur, +++ p<0.001 for USA300 vs. USA300Δaur.
increased lag phase observed in the Δaur mutant suggested that the proGeh, which accumulates in this mutant, was more active on trilinolein compared to mature Geh. To determine the secreted protein profile of these strains, samples were grown in sub-inhibitory concentration of trilinolein (25 μM) and the cell-free supernatant was TCA precipitated and visualized by SDS-PAGE (Figure 10). Analysis of secreted proteins of these strains indicates that Geh is secreted and processed in the wild-type and confirms the lack of Geh in either form in the Δgeh strain. It further showed us that the Δaur mutant produced very minute amounts of proGeh. It is possible that Geh, in addition to some other factor in the supernatant is required for optimal growth in trilinolein.

3.6 Aureolysin is required for optimal growth in Trilinolein

We were interested to elucidate the mechanism for why the aur mutant displayed a prolonged growth delay in the presence of trilinolein. We first decided to determine whether it was the result of a factor that was on the cell surface or whether it was found in the supernatant. Cells from the pre-culture were pelleted and the supernatant removed. These cells were then washed with saline to ensure any cell surface bound proteins were removed. A SDS-PAGE of unwashed and washed cells showed no difference in the protein profiles (Figure 11). Washed cells were then inoculated into TSB containing trilinolein and the resultant growth curve can be seen in Figure 11B. Washed wild-type and Δaur cells grow at the same rate as the Δgeh strain and showed no extended lag phase. This suggests that the factor implicated in causing the growth lag was present in the supernatant and not cell-surface associated.

Since the Δgeh mutant showed no lag during growth in trilinolein, we wanted to determine the factor in the supernatant that caused the lag. To do this, Δgeh preculture cells
Figure 10. Secreted protein profile of cells grown in 50 μM trilinolein. SDS-PAGE of the culture supernatants of wild type and mutant USA300 strains grown in TSB or TSB supplemented with 25 μM trilinolein for 18 hours. Proteins in the cell free culture supernatant were precipitated in ice-cold TCA, and after solubilization in SDS-PAGE reducing buffer, protein equivalent to 5.0 OD600 units was loaded in each lane.
Figure 11. Comparison of cell-surface proteins of washed and unwashed cells and
growth of washed cells in trilinolein. A) SDS-PAGE of cell surface proteins of USA300
strains grown in TSB for 18 hours. Samples were standardized to OD\textsubscript{600} 2.0 of cells.
Unwashed cells were pelleted and re-suspended in SDS-PAGE buffer while washed cells
were first washed with saline before being re-suspended in SDS-PAGE buffer. Cells were
then boiled and pelleted and the sample above the pellet was loaded in the gel. B) Pre-
cultured cells from all strains washed before inoculation into TSB containing trilinolein grow
without an extended lag phase.
were pelleted and their supernatants were removed. The cell pellet was then re-suspended in cell-free supernatant of a wild-type or Δaur culture grown to stationary phase in TSB before being inoculated into TSB media containing trilinolein. As seen in Figure 12, both the wild-type and Δaur supernatant induced an extended growth lag in the Δgeh strain, where none was previously observed. This confirmed the presence of a factor in these supernatants, which was responsible for the growth lag. Furthermore, fractionation of the wild-type supernatant using a 30 kDa amicon centrifugation filter, allowed us to determine that the factor in question was greater than 30 kDa, as cells re-suspended in the supernatant filtrate (molecules < 30kDa) showed no growth lag, in contrast to the lag observed in cultures inoculated with cells re-suspended in media containing a small amount of the retentate (molecules>30 kDa) (Figure 12).

Based on this we surmised that proGeh/Geh was the factor or one of the factors responsible for the extended lag phase, likely due to processing of trilinolein resulting in the liberation of linoleic acid. To confirm this, at the time of inoculation we added a small amount of purified proGeh and Geh to Δgeh cultures inoculated into TSB containing 50 μM trilinolein and, as expected, an extended lag phase occurred (Figure 13). This suggests that Geh is at least in part responsible for the extended lag phase observed in trilinolein. Additionally, the longer lag phase observed in the Δaur strain compared to wild-type USA300 suggests that aureolysin is required for optimal growth in the presence of trilinolein.
Figure 12. Delayed growth of Δgeh strain in trilinolein after re-suspension in supernatant from wild-type and Δaur. USA300Δgeh mutant experiences a delayed lag phase in 50 μM trilinolein when cells are re-suspended in supernatant from other strains as indicated. OD$_{600}$ 0.01 of cells were inoculated into 25 mLs of media with 100 μL of supernatant from the indicated strains.
Figure 13. Impaired growth of Δgeh in trilinolein with addition of purified proGeh or Geh at inoculation. USA300Δgeh mutant incurs a delayed lag when purified proGeh or mature Geh are added during inoculation. OD$_{600}$ 0.01 of cells were inoculated with 1 μg proGeh or Geh and 0.002 μg Aur.
3.7 **Gas chromatography confirms that trilinolein is a substrate for proGeh and Geh**

Gas chromatography was used to confirm if both proGeh and Geh could release free linoleic acid from trilinolein. The trilinolein was added to a mixture containing purified proGeh or mature Geh. The lipid fraction was extracted and analyzed using gas chromatography. The results revealed that trilinolein was processed by both proGeh and Geh resulting in the production of linoleic acid, seen as the peak occurring around 7.7 minutes (Figure 14).
Figure 14. Gas chromatography-mass spectrometry confirms release of linoleic acid from trilinolein by both forms of Geh. Gas chromatography of purified proGeh and Geh incubated with trilinolein. The production of linoleic acid (7.7 minutes) was used as a determinant of lipolytic activity.
DISCUSSION

The superior transmissibility and colonization ability of the USA300 strain in addition to its ability to survive within the host make it one of the most successful human pathogens. Several mechanisms of innate immunity exist, preventing pathogens such as *S. aureus* from establishing an infection. One of the mechanisms of innate defense is the presence of anti-microbial fatty acids and lipids due to the secretion of sebum on the skin and lipid production in wound environments (22, 55, 65, 98). Several free fatty acids such as palmitoleic acid (C16:1Δ9), sapienic acid (C16:1Δ6) and linoleic acid are capable of inhibiting the growth of *S. aureus*, and have been shown to produce an extended lag during growth and induce the robust expression of the staphylococcal proteolytic cascade (2).

As reviewed within the introduction, previous research suggested that aureolysin, a zinc and calcium dependent secreted metalloprotease which initiates the staphylococcal proteolytic cascade, processed the maturation of proGeh (72kDa), the lipase of *S. aureus*, into the mature Geh (42 kDa) (2). Arsic *et al.* reported that this accumulation of proGeh was not present in a USA300ΔsspABC strain but was observed in a Δaur strain (2). When the secreted protein profile of the Δaur mutant was compared to that from a wild-type USA300, the accumulation of proGeh was very apparent. We further confirmed this function of aureolysin using purified aureolysin and proGeh. However, we found that obtaining pure proGeh proved difficult as the protein was prone to degradation, and as a result multiple degradation products, as confirmed by mass spectrometry, can be seen in Figure 6. The production of only mature Geh upon incubation of proGeh with aureolysin confirmed that aureolysin was responsible for the processing of Geh. Additionally, an aureolysin titering assay indicates that aureolysin has very high activity on proGeh, even when the amount of
proGeh to aureolysin was titred to 1:1500. However, the purpose for the processing of the lipase has yet to be determined.

The exact role and importance of Geh in *S. aureus* has not been determined, but there is some literature attempting to characterize this enzyme. However, part of the reason for the lack of data on proGeh is due to difficulty with purification of this form. What can be taken from the literature is that Geh is a calcium dependent lipase, which has optimal activity at the higher pH ranges of 8.0-9.0, (21, 32). In addition to *geh* however, USA300 possesses other putative lipase genes such as *lip* and the putative lysophospholipases 0070 and 1194. Using a pNPP assay, we found that in USA300, deletion of *geh* resulted in a lack of detectable lipase activity, indicating that these genes do not directly contribute to secreted lipase activity.

*S. aureus* encounters a large range of lipids both during skin colonization and during deep tissue infections. In staphylococcal abscesses, the production of neutral lipids is highly up-regulated (23). This includes triglycerides such as tripalmitolein, triolein and trilinolein. Previous studies have shown that when staphylococcal abscess homogenates are mixed with culture filtrate, there is a reduction in the quantity of triglycerides and a consequent increase in free fatty acids. Additionally, these activated culture homogenates had increased staphylocidal activity and implicated the presence of a secreted lipase for this activation. Furthermore, leucocyte lipases are also believed to contribute to the liberation of free fatty acid within abscess environments (21-23). We assessed the growth of a strain which could only produce the proGeh (*Δaur*) and a strain deficient for both forms of the enzyme (*Δgeh*) in the presence of the long chain unsaturated triglyceride, trilinolein. The cause of this phenotype could have been that only strains containing *geh* produced an extended lag phase due to the hydrolysis of trilinolein by Geh, resulting in the release of growth inhibitory linoleic acid.
However we needed to confirm that the factor responsible for growth lag we observed was present in the supernatant instead on the cell surface. Removal of the cell-free supernatant followed by washing cells before they were sub-cultured abolished the lag phenotype seen in wild-type and Δaur strains during growth in trilinolein. This, in combination with a lack of a difference between the protein profiles of cell surface associated proteins of washed and unwashed cells, suggests that the culprit responsible for the lag phenotype is present in the supernatant, in agreement with the possibility that Geh is responsible for the liberation of linoleic acid from trilinolein.

The growth lag seen in the wild-type and Δaur strains could be replicated in the Δgeh mutant if cell-free culture supernatant from the wild-type or Δaur strain was added to the media containing trilinolein at the time of inoculation. When the wild-type supernatant was separated into retentate and filtrate fractions, the lack of the extended lag phase due to addition of the filtrate fraction meant that the molecule causing the extended lag was greater than 30 kDa, increasing the likelihood that either proGeh or Geh were the culprits in question. The extended lag phase observed when either purified proGeh or Geh was added to a Δgeh culture during inoculation confirmed that these lipases were indeed capable of causing an extended lag when grown in trilinolein (see Figure 13).

The presence of lipase activity in Δaur supernatant as shown by the pNPP assay indicated that the unprocessed form of Geh had lipase activity. Interestingly the addition of proGeh to Δgeh cultures in trilinolein produced a longer lag phase than when only the processed enzyme was added. In addition to this, the lag phase observed in the Δaur mutant, that is incapable of processing proGeh or inducing the Staphylococcal proteolytic cascade, was nearly twice as long as that observed in the wild-type strain. This lead to the possibility that proGeh and mature Geh could have different substrate specificities, more specifically
that trilinolein was a substrate for proGeh but not Geh. Furthermore, the literature suggests that the mature form of *S. aureus* lipase, in contrast to traditional lipases which preferentially cleave long chain triglycerides, was shown to be more active on short to medium chain triglycerides such as tributyrin (86). This further supported the notion that the two forms of Geh could have differential substrate specificities.

Gas chromatography was used to confirm the activity of Geh on trilinolein, and to confirm whether the different forms of the enzyme bound to different substrates. While the gas chromatography confirmed that linoleic acid was produced when proGeh and Geh were incubated with trilinolein, it also showed no significant difference between either forms of the lipase for trilinolein. However, before concrete conclusions can be drawn from these studies, these experiments need to be performed again in an effort to make them as quantitative as possible.

The secretion of Geh into an environment containing triglycerides is paradoxical if it results in the release of growth inhibitory free fatty acids, which add to the already existing pool of these compounds on the skin or in a wound environment. It is unlikely that *S. aureus* would have evolved to maintain conserved expression of Geh, if it did not confer a substantial benefit, and *S. aureus* does not have the metabolic capacity to generate energy through β-oxidation of fatty acids (72). Recall that one of the predicted mechanisms of resistance to free fatty acids is the existence of the fatty acid modifying enzyme (FAME) which is believed to esterify free fatty acids to primary and secondary alcohols or cholesterol to form fatty acid esters. In fact 80% of staphylococcal strains which produce lipase were also found to produce FAME (57). Interestingly, FAME is strongly inhibited by long chain unsaturated triglycerides and, thus, Geh may work in concert with FAME to alter the lipid composition of the bacterial environment (55).
The 3 hour longer duration of the extended lag phase of cultures containing \( \Delta \text{aaur} \) supernatant cultured in trilinolein compared to cultures containing wild-type supernatant, in addition to the longer lag phase observed in \( \Delta \text{aaur} \) strains than in wild-type strains, indicates that aureolysin is also required for optimal growth in trilinolein. Since autocatalysis and activation of aureolysin is required for the subsequent and step-wise initiation of the Staphylococcal proteolytic cascade, a mutant unable to express aureolysin is likely to suffer from pleiotropic effects. This is because aureolysin is involved in the cleavage of several cell surface associated factors in addition to activation of zymogens including itself, proGeh and SspA. Additionally, a trickle-down effect due to lack of processing of SspA and SspB protease substrates would also occur causing several possible phenotypes.

In summary, we confirm that Geh is required for lipase activity in \( S. \text{aureus} \) strain USA300 and that both the precursor and mature forms of Geh are capable of processing long chain unsaturated triglycerides, such as trilinolein, that are present in the skin and within wounds. Additionally further characterization of both proGeh and Geh substrate specificities on acylglycerols of various chain lengths and saturation can be determined by gas chromatography. Degradation products of these reactions, including the products from trilinolein can also be assessed to determine whether this lipase has positional and fatty acid chain length specificity. Moreover, in the presence of triglycerides we conclude that aureolysin is required for optimal growth and future work can focus on the mechanism by which lipid metabolism is regulated by the synergistic activity of these enzymes and how they facilitate survival of USA300 in wound environments and during colonization.
REFERENCES


through unique variations of a trypsinogen-like mechanism and is dependent on both autocatalytic and metalloprotease-specific processing. J Biol Chem 282:34129-38.


# Curriculum Vitae

<table>
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