Regulation and function of Staphylococcus aureus secreted proteases on biofilm integrity

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REGULATION AND FUNCTION OF *STAPHYLOCOCCUS AUREUS*
SECRETED PROTEASES ON BIOFILM INTEGRITY

by

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology in the Graduate College of The University of Iowa

August 2013

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Tim Yahr
To my lovely wife Tiara, for her constant encouragement and smiles
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ABSTRACT

*Staphylococcus aureus* is a known cause of chronic biofilm infections. Recent studies have demonstrated the importance of proteinaceous material in the biofilm matrix. *S. aureus* secretes at least ten proteases and there is growing evidence that these enzymes have self-cleavage roles that alter biofilm integrity. The goal of this dissertation is to characterize the function of secreted proteases with respect to *S. aureus* biofilms. In addition, we aimed to determine whether a known regulator of *S. aureus* proteases, the Regulator of toxins (Rot), is also a regulator of biofilms.

Studies presented in Chapter II utilize a mutation in Sigma factor B (SigB) to analyze the role of secreted proteases in biofilm formation. *S. aureus* strains with *sigB* mutations have enhanced protease activity and a biofilm negative phenotype, and this biofilm phenotype was conserved on human plasma coated surfaces. To identify the protease(s) responsible for the phenotype, inhibitor studies revealed that the addition of the cysteine protease inhibitor E-64, or Staphostatin inhibitors that specifically target the *S. aureus* cysteine proteases SspB or ScpA (Staphopains), could restore biofilm formation. Using gene deletion mutants, we identified that the combined mutation of *sspB* and *scpA* also restored Δ*sigB* biofilm formation. Together these findings indicated that Staphopain enzymes are responsible for the Δ*sigB* biofilm negative phenotype. To address the Staphopain inhibitory role in biofilms further, regulatory studies indicated that enzymes levels were maintained at a low level during biofilm formation and exogenous addition of purified Staphopains inhibited biofilm formation or disassembled established biofilms across multiple *S. aureus* strain lineages. Taken together, these findings suggest an unappreciated role of the Staphopains in biofilm maturation.

Studies in Chapter III examine the role of the transcriptional regulator rot in biofilm formation. Using both coated and uncoated surfaces in biofilm assays, Rot was
found to be essential for *S. aureus* biofilm formation. The expression of RNAIII, the major effector molecule of the *agr* quorum-sensing system, inversely correlated with intracellular Rot protein concentration. Rot protein levels were found to be high under biofilm conditions and modulation of *rot* expression demonstrated that intracellular levels of Rot modulate biofilm formation. Examination of protease expression, production, and activity revealed that production of secreted proteases are inhibited by Rot and electrophoretic mobility shift assays demonstrated that inhibition was due to the direct binding of Rot to protease promoters. Chemical and biochemical protease inhibitors restored the capacity to form a biofilm to Δ*rot* and follow-up mutation of protease genes confirmed these findings. Finally, Δ*rot* mutant is attenuated in a murine catheter model of infection. These findings suggest an essential role for Rot as an inhibitor of proteases to modulate biofilm formation, and demonstrate the importance of coordinated regulation during infection.
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CHAPTER I
INTRODUCTION

*Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive bacterium that frequently colonizes humans and other warm-blooded animals. Approximately 25% of the human population is persistently colonized by *S. aureus*, and the other 75% are intermittently or not colonized [1, 2]. Colonization primarily occurs in the anterior nares, and the throat, skin, axilla, perirectal area, and groin are potential secondary sites [3]. Though a human commensal, *S. aureus* acts as an opportunistic pathogen and carriage is associated with increased risk of subsequent infection [1, 4]. In the United States, infections associated with *S. aureus* have a mortality rate of 25% and hospitalizations are typically twice the length as normal stays and double the medical cost [5, 6]. *S. aureus* is one of the most common causes of healthcare and community-associated infections [7-9]. Healthcare-associated infections occur readily due to patients predisposing risk factors including surgery, indwelling medical devices and general immunocompromised nature. These infections are typically bacteremias and usually result from *S. aureus* transmission by hospital personnel who are transiently colonized [10].

A major concern in the treatment of *S. aureus* infections is the remarkable ability of this microorganism to acquire resistance to antibiotics. Antibiotic-resistant *S. aureus* infections first became problematic in the 1950's when the organism acquired a plasmid-encoded beta-lactamase, allowing for resistance to penicillin [11]. Shortly thereafter, penicillin-resistant *S. aureus* became pandemic [12]. In the late 1950's, methicillin was introduced to treat penicillin-resistant *S. aureus* and within a year cases of methicillin-resistant *S. aureus* (MRSA) were reported [13]. Over the last few decades, MRSA has spread worldwide and is now endemic in most hospitals or healthcare facilities of
industrialized countries. MRSA is a leading cause of death by an infectious agent in the United States [14, 15].

Over the past 15 years, community-associated MRSA (CA-MRSA) has emerged worldwide and is epidemic in the United States. These strains commonly cause skin and soft tissue infection but are also capable of causing invasive disease [10]. CA-MRSA strains are adept at causing disease independent of the hospital setting, and in immunocompetent individuals [10]. Of the CA-MRSA strains, the pulse field gel electrophoresis type USA300 is most common [16]. These strains are highly transmissible and considered hypervirulent when compared with healthcare-associated MRSA (HA-MRSA). CA-MRSA are more virulent in numerous models of infection and have an enhanced ability to evade killing by neutrophils [17, 18]. The mechanism of enhanced virulence is currently unknown, but several factors have been hypothesized to contribute including the Panton-Valentine leukocidin (PVL), type 1 arginine catabolic mobile elements (ACME), and the high levels ofagr-regulated virulence factors [17] (discussion of theagr quorum-sensing system can be found on pages 11 and 12). Due to the rapid emergence of USA300, and their high degree of virulence, these strains have gained increasing popularity as an active area of research. For these reasons, my work has focused on the study of USA300. More specifically, in Chapters II and III we examine biofilm formation by the USA300 strain LAC.

\textit{S. aureus} pathogenesis

\textit{S. aureus} causes a wide variety of both acute and chronic infection. Infection of the skin and soft tissue can result in furuncles, impetigo, and abscesses, and more severe invasive infections include osteomyelitis, endocarditis, necrotizing pneumonia, and bacteremia [19].
The potential for causing a diverse array of disease is due in part to this organism’s multitude of virulence factors that include both secreted proteins and adhesins. The secreted virulence factors are composed of hemolysins, toxins, proteases, and phenol-soluble modulins, which all play an important role in *S. aureus* dissemination and immune evasion. Structural or surface-associated virulence factors are typically members of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family that includes Protein A (Spa), Elastin-binding protein (Ebp), Collagen-binding protein, the fibronectin-binding proteins A and B (FnbpAB), and the clumping factors A and B (ClfAB). Many of these members are covalently linked to cell wall peptidoglycan by the enzyme Sortase A [20], and collectively they mediate attachment to host matrix proteins, such as collagen [21], fibronectin [22], and fibrinogen [23, 24]. MSCRAMMs are particularly important in the initiation of endovascular infections, bone and joint infections, and prosthetic device infections [25]. The virulence factors listed are just a sampling of those that contribute to the pathogenic potential of *S. aureus*.

**S. aureus biofilms**

Biofilm formation is an important contributing factor for the establishment of chronic infection by the opportunistic pathogen *S. aureus* [26]. *S. aureus* readily forms biofilms on host surfaces such as bone [27], cartilage, and heart valves [28], as well as on foreign body implants, including catheters and orthopedic devices [29].

The mature biofilm is composed of a community of cells encased in an extracellular matrix. This structure provides inherent resistance to the innate immune system and other antimicrobials and thereby promotes bacterial persistence [30, 31]. Several factors contribute to biofilm recalcitrance. For one, the extracellular matrix provides a protective barrier against antimicrobials, reducing their permeability into the biofilm. Components of the immune system such as macrophages [32] and large
molecule immunoglobulins and superoxides have limited capacity to invade biofilms. However, smaller antibacterial molecules can freely transverse the matrix material and still do not cause bacterial killing; therefore the importance of this mechanism is not fully understood [33]. Other contributors are the metabolic state of bacteria that reside within the biofilm and their gene expression profiles. For the most part these are dormant, non-growing cells that display gene expression patterns similar to that of stationary phase cultures [29, 30]. While this state promotes antibiotic resistance, the changes in gene expression can also actively modulate host immune functions by attenuating proinflammatory responses [32]. In addition, spread of antibiotic resistance mechanisms via gene transfer occurs more frequently within biofilms and biofilm conditions select for mutants with enhanced biofilm development properties. This is especially evident in the frequency of spontaneous agr mutants in biofilms [34].

The mechanism by which biofilms resist antimicrobials appears to be multifactorial. Due to biofilm recalcitrance, treatment options all limited and typically involve removing infected devices or tissues [35]. These strategies are not ideal for patient care and further research is needed to understand the molecular mechanisms of *S. aureus* biofilm development to improve therapeutic strategies for treating chronic infections.

**Stages of biofilm development**

*S. aureus* biofilm development occurs in several loosely defined stages (1-4) and is cyclical in nature, beginning and ending with free-floating cells that mimic a planktonic lifestyle and as such are susceptible to antimicrobials (Figure 1-1). In the first stage (1), individual planktonic cells attach to a substratum such as a foreign body or host tissue. Interestingly, foreign bodies become rapidly coated in human matrix proteins including fibrinogen, fibronectin, and collagen [36, 37]. *S. aureus* possesses numerous surface exposed MSCRAMMs as well as secreted proteins that contain binding domains.
for these matrix proteins [38]. While *S. aureus* can adhere to both biotic and abiotic surfaces, this organism preferentially binds these matrix proteins *in vivo* rather than directly to foreign bodies [39, 40].

Following an initial substrate attachment phase by individual cells, bacteria multiply to form microcolonies (2). While there is no exact definition for the microcolony step, it is generally characterized by small aggregates of cells that contain some matrix material. This stage is often considered an "intermediate" stage of biofilm formation that links the attachment step with the mature biofilm as seen *in vivo* [41]. Further development of microcolonies by continued growth and extrapolymeric substance (EPS) production results in extracellular surface structure and a mature biofilm (3). This stage is characterized by increased resistance to antimicrobials and an overall shift in gene expression patterns. Finally, an environmental change can bring about biofilm disassembly resulting in free-floating planktonic cells that are able to re-initiate the biofilm development process (4) [42]. During this stage, the biofilm matrix is typically targeted for degradation, resulting in the release of cells into the surrounding milieu. Disassembly of the biofilm restores bacterial susceptibility to active chemotherapies and is an active area of research interest [42, 43]. Determining how biofilms are degraded will lead to a better understanding of biofilm matrix constituents and could lead to increased treatment options.

**The biofilm extracellular matrix**

At the heart of *S. aureus* biofilm development is the complex extracellular matrix composed of proteinaceous material, polysaccharides, extracellular DNA (eDNA), and other bacterial components. Protein and eDNA are the primary matrix components in CA-MRSA including USA300 isolates (Figure 1-2) [44, 45]. Key roles for each component have been demonstrated through the use of exogenously added enzymes that
target matrix constituents [26]. There are growing reports that *S. aureus* modulates this complex biofilm matrix through self-targeting by its own secreted enzymes [42, 46-49]. We have demonstrated that increased expression of the chromosomally encoded nuclease prevents *S. aureus* biofilm formation [48] and that *agr*-dependent control of proteases can disperse biofilms [42, 43].

The underlying mechanism through which *S. aureus* proteases prevent biofilm formation or disassemble established biofilms is complicated because *S. aureus* secretes at least ten proteases and these enzymes have myriad targets. Many *S. aureus* surface proteins have an important role in biofilm formation including SasC [50], SasG [51, 52], FnbpAB [47], Protein A [53], ClfB [54], and Bap [55]. In addition several secreted proteins aid in biofilm formation, such as beta-toxin [56]. How these proteases target and destroy proteinaceous matrix components, and which protease and/or component remains most important in the biofilm formation/dispersal mechanisms remains unclear.

**S. aureus secreted proteases**

The proteases are organized into four distinct operons encoding a metalloprotease (Aur), seven serine proteases (SspA and SplA-F), and two cysteine proteases (Staphopains ScpA and SspB) (Figure 1-3A). The *ssp* and *scp* operons also encode inhibitors (Staphostatins) that prevent cysteine protease activity in the cytoplasm before secretion (Staphostatins are described below). While the six Spl enzymes are active upon secretion, the Aur, SspA, SspB, and ScpA proteases are produced as zymogens (Figure 1-3B). The Aur and ScpA zymogens auto-activate outside the cell [57, 58], and SspA and SspB activation relies on a proteolytic cascade in which Aur processes SspA [59] and subsequently SspA processes SspB [60]. Pro-SspB has some activity, however it is approximately 3-4 fold less active than mature SspB [61].
The secreted proteases degrade both "self" and "host" proteins. With regards to *S. aureus* proteins, deletion of all ten core proteases results in increased abundance of secreted and surface-associated virulence factors [62]. This phenotype may explain why mice infected with a protease-null strain have an increase in mortality [62]. The proteases may also play a role in nutrient acquisition as they are important for growth in peptide-rich environments as well as in serum, human blood, and in the presence of antimicrobial peptides (AMPs) [62].

**Metalloprotease**

*S. aureus* secretes a single metalloprotease, Aureolysin (Aur), whose coordination of zinc (Zn$^{2+}$) and calcium (Ca$^{2+}$) ions is necessary for activity [59, 63]. Aur is required to activate SspA by proteolytic cleavage and initiates the proteolytic cascade of activation (Figure 1-3) [59]. Aur preferentially cleaves proteins on the N-terminal side of bulky, aliphatic, hydrophobic residues. Aur also cleaves surface-exposed clumping factor ClfB [64], AgrD [65], complement protein C3 [66], the antimicrobial peptide LL-37 [67], and phenol-soluble modulins (PSMs) [68]. Increased stability of PSMs in an *aur* mutant leads to increased osteoblast cell death and bone destruction in a murine model of osteomyelitis [69].

**Serine proteases**

Serine proteases are defined by their catalytic triad that is composed of essential active site histidine, serine, and aspartate residues. *S. aureus* secretes seven serine proteases encoded within two separate operons, and all of these proteases are members of the trypsin family. The SspA protease is encoded in a polycistronic operon with the cysteine protease SspB, and the six Spl proteases (SplABCDEF) are encoded in a separate polycistronic operon. Spl proteases share 44-95% sequence identity and are 33-36% identical to SspA [70].
SspA, also known as V8 protease, is a glutamyl endopeptidase that has the distinction of being the first purified and characterized proteolytic enzyme of *S. aureus* [71]. SspA has narrow substrate specificity, preferring to cleave after glutamic residues but has some capacity to cleave after aspartate. This enzyme plays a key role in the activation of SspB by processing the pro-form of the enzyme [61]. In addition to cleaving pro-SspB, SspA also degrades fibronectin-binding MSCRAMMs [49] as well as Protein A [72]. SspA also cleaves host proteins such as the heavy chain of all human immunoglobulin classes [73]. Like other secreted proteases of pathogenic bacteria, SspA also de-regulates host derived proteolytic activity by cleaving α1-protease inhibitor which results in increased proteolysis by the host [74]. However, the host α2-macroglobulin protease inhibitor is able to restrict SspA activity. In terms of *S. aureus* pathogenesis, an *sspA* mutant was attenuated for virulence in three different mouse models of infection [75], and a similar result was seen by an independent group using a murine skin abscess model of infection [76].

Currently, the *S. aureus* Spl proteases are poorly understood. SplA, SplB, and SplC have been structurally characterized and share homology to SspA [77, 78]. SplA and SplB both have very specific cleavage sites. However, no proteins within *S. aureus* contain the specific sequences identified for SplA and SplB suggesting the enzymes target the host. At this time, host targets have not yet been identified, although some proteins do contain the SplA cleavage site [77]. In terms of *S. aureus* pathogenesis, no role in virulence for an spl deletion mutant was observed using a intraperitoneal rat infection model [70].

**Staphopains and Staphostatins**

Cysteine proteases are characterized by a catalytic mechanism involving a nucleophilic cysteine thiol in a catalytic dyad. *S. aureus* secretes two cysteine proteases,
ScpA and SspB, which have been termed Staphopain A and Staphopain B, respectively. Each Staphopain belongs to the papain protease family and the mature form of the enzymes are each approximately 20 kDa in size and have nearly identical three-dimensional (3D) structures [79]. However, they are only 49% identical by sequence and are thought to have different substrate specificity [80]. Kalinska et al. determined that the Staphopains most efficiently cleave a P2-Gly/Ala(Ser) sequence motif, as described using Schechter and Berger nomenclature, and the P2 position distinguishes the specificity of Staphopain A (Leu) from Staphopain B (Phe/Tyr) [81]. The amino acid at the P1 position is also important as Staphopain B has a strong preference for arginine at P1 [61].

Staphopains are known to cleave several different host proteins. Fibrinogen and collagen are degraded at low nanomolar concentrations of enzyme, with Staphopain B being more efficient at cleavage [82]. Staphopain A is also capable of degrading elastin [83] and can cleave the complement receptor CXCR2 thereby inhibiting neutrophil activation and chemotaxis [84]. Staphopain B cleaves CD31 on neutrophils [85], CD11b on peripheral blood phagocytes [86], and also promotes the detachment of cultured keratinocytes by an unknown mechanism [61]. While these attributes suggest a role for these enzymes in pathogenesis, their function in vivo is unclear. The human plasma protein α2-macroglobulin is an inhibitor of both Staphopains, and other plasma proteins are known to inhibit activity. However, the Staphopains are not inhibited by host proteins such as kininogens or cystatins. Seven human cystatins have been investigated for Staphopain inhibition, and none were found to inhibit Staphopain A or B, rather the extracellular cystatins C, D and E/M were hydrolyzed by both Staphopains [87].

It has been hypothesized that Staphopain A may participate in S. aureus tissue invasion and destruction associated with Staphylococcal ulceration [83]. Staphopain B appears to have a role in pathogenesis as a sspB mutant is attenuated in a murine subcutaneous skin abscess model of infection [76]. Interestingly, less virulent coagulase-
negative Staphylococci (CNS), including *Staphylococcus epidermidis*, contain orthologs of *scpA* while they lack *sspB*. In fact, other than *Staphylococcus warneri*, each CNS has a monocistronic *ssp* operon, containing only *sspA* [88-90].

Encoded directly downstream of each Staphopain gene is a Staphopain inhibitory protein termed "Staphostatin". The Staphostatins share limited sequence identity (19%) [80] but have a similar 3D structure, resembling that of lipocalins [91]. Each Staphostatin interacts specifically with their cognate Staphopain encoded upstream, and together they form a stable non-covalent complex [92]. The interaction is highly specific and there is no cross inhibition with other cysteine proteases. Each complex forms a 1:1 stoichiometry of Staphopain:Staphostatin and the SspB::SspC complex has a half-life of about 24 hrs at 37°C. The Staphostatins have no secretory signal sequences and are only present in the cytoplasm. These interactions are presumed to be required to protect staphylococcal cytoplasmic proteins from being degraded by prematurely activated/folded prostaphopains [92].

**Protease-mediated biofilm disassembly**

The *S. aureus* biofilm matrix contains protein components that maintain biofilm integrity. The treatment of biofilms with proteases that have broad specificity, such as Proteinase K and Trypsin, leads to biofilm disassembly [42, 50]. As stated above, *S. aureus* secretes its own proteases and these enzymes have known self-cleavage roles, some of which target biofilm matrix constituents with identified roles in biofilm formation. There is preliminary evidence that the SspA (V8) serine protease might be important in biofilm remodeling [46, 47, 49], but the contribution of the other proteases is less clear. The primary goal of my project is to determine the role of extracellular proteases in modulating biofilm formation. In Chapter II, we aim to identify which
secreted proteases are deleterious to biofilm formation. In doing so, we provide evidence for new roles of Staphopains in modulating biofilm integrity.

**Regulation of biofilm development**

Global changes in *S. aureus* gene expression control biofilm remodeling. These changes are coordinated by a suite of regulators [93-95], and in part the staphylococcal accessory regulator (*sarA*), the stress response sigma factor B (*sigB*) and the accessory gene regulator (*agr*) quorum-sensing system are key players in this process.

SarA is a global regulator of virulence factors. This DNA-binding protein positively regulates the expression of factors that promote biofilm accumulation, such as the biofilm-associated protein (*bap*) and the ica locus, and negatively regulates exoenzymes that are detrimental to biofilms, including secreted proteases and nuclease. *sarA* expression is increased under biofilm conditions compared with planktonic growth and *sarA* mutants are unable to form biofilms [40, 96]. The *sarA* biofilm-negative phenotype is thought to be due to the over-expression of secreted proteases and nuclease as inhibiting these enzymes using chemical inhibitors restores *sarA* biofilm-forming capacity [97].

Sigma B is an alternate sigma factor that is necessary for survival in response to a variety of environmental stressors including high temperature, and oxidative or alkaline stress [98-100]. In addition, it has known roles in virulence, intracellular survival, and persistence within the host [98-101]. Loss of function mutations in *sigB* up-regulate the *agr* system and result in the inability of the strain to form a biofilm [102]. While the molecular and biochemical mechanisms behind these phenotypes are not completely understood, it is known that SigB is a negative regulator of secreted virulence factors [103, 104], which is largely due to the repressive effect exerted on the *agr* system [102]. In Chapter II we provide evidence that the Δ*sigB* biofilm negative phenotype is due to
overexpression of proteases. Furthermore, we utilize this mutant to identify which proteases are most important with respect to biofilm modulation.

In *S. aureus*, biofilm formation and disassembly is regulated by the accessory gene regulator (*agr*) quorum-sensing system [105]. Quorum-sensing is a common mechanism utilized by a variety of bacteria to respond to their environment and coordinate a group response. *S. aureus* is one such bacteria which monitors levels of "self" and responds to these levels with global changes in gene expression that influence biofilm formation. The *agr* system produces and senses an auto-inducing peptide (AIP) that regulates virulence gene expression in a population density dependent manner [106]. Under low *agr* expression conditions, cell surface protein expression is high while secreted enzyme expression is low. The opposite is true under high *agr* expression conditions [107]. Free-floating cells with low level *agr* expression attach to a substrate and multiply until a critical threshold of AIP is reached (in the low nM range). This results in increased expression of the major *agr* effector RNAIII, which leads to extracellular virulence factor expression and biofilm disassembly [42]. *agr* expression inversely correlates with levels of biofilm biomass such that induction or over-expression of the system can disassemble established biofilms or prevent them from forming. While the mechanism of biofilm disassembly is not fully understood, it is primarily thought to result from increased production of exo-enzymes, such as proteases and nucleases, that have deleterious effects on biofilm matrix constituents.

A major question that remains to be answered with respect to *agr*-mediated biofilm regulation is how the *agr* system effects transcription of exo-enzymes that directly impact biofilm matrix constituents. The primary effector molecule of the *agr* system is RNAIII and this 514 nucleotide regulatory RNA can impact protein expression both directly and indirectly. RNAIII directly interacts with the mRNA of target proteins thereby enabling or preventing its translation. This is the case with numerous virulence
factor transcripts including *hla* (Alpha-hemolysin), *coa* (Coagulase), and *spa* (Protein A) [108, 109]. Alternatively, RNAIII can indirectly regulate protein expression by modulating the translation of a transcriptional regulatory protein. One such protein is the **Repressor of toxins (Rot)**.

**The transcriptional regulator Rot**

Rot is a member of the SarA family of transcriptional regulators. This family contains at least ten members that share 20-45% identity and 45-65% similarity to SarA [110]. SarA family members are characterized by a DNA-binding domain containing a conserved KXRXXXDER motif. These proteins share homology to the MarR family of proteins involved in antibiotic resistance in Gram-negative bacteria [110].

Rot was identified during a screen in an *agr* mutant looking for transposons that restored protease and alpha-toxin production [111]. Rot promotes the expression of genes that encode for surface proteins and immunomodulators, such as Protein A and the superantigen-like proteins [112], and Rot represses the expression of exo-toxins and exo-enzymes [111, 113]. When the *agr* system is activated, RNAIII levels rise and prevent translation of Rot protein [108], relieving the Rot repressive effect. Based on the key role for proteases in *S. aureus* biofilm development, we hypothesized that inactivation of Rot would have biofilm phenotypes. In Chapter III, we examine the regulatory role of Rot in maintaining biofilm integrity.
Figure 1-1. Stages of *S. aureus* biofilm development. Free-floating *S. aureus* cells exhibit qualities seen during planktonic growth. (1) Individual cells attach to an abiotic or biotic surface via their MSCRAMMS. (2) Cells grow and divide, eventually developing microcolonies that contain biofilm matrix material. (3) Extensive production of matrix material leads to the establishment of a mature biofilm. These structures are characterized by their enhanced resistance to antimicrobials. Furthermore, biofilm cells are dormant and differentiated from planktonic cells by changes in their gene expression profiles. Under certain conditions, subsets of cells will detach from the biofilm via low level production of matrix altering enzymes. (4) Environmental conditions can alter the regulatory scheme of the bacterium resulting in high level exoprotein production which leads to destruction of the biofilm matrix and dispersal of cells from the biofilm. These cells revert back to a free-floating planktonic growth state and are again susceptible to antimicrobials.
Figure 1-2. *S. aureus* USA300 extracellular matrix composition. In CA-MRSA, the primary constituents of the biofilm matrix are protein and eDNA. Protein components include both surface-associated proteins such as MSCRAMMS and membrane-spanning proteins, as well as secreted proteins. In addition, cytoplasmic proteins have also been found in the biofilm matrix. These proteins are likely remnants from the cellular lysis that occurs during biofilm initiation and are essential for eDNA deposition and biofilm formation. The importance of various biofilm matrix components has in large part been identified through the use of exo-enzymes, such as proteases and nuclease, to disrupt mature biofilms. These enzymes are likely also involved in the natural development of biofilms as *agr*-mediated biofilm dispersal is due to overexpression of secreted proteases.
Figure 1-3. Schematic of *S. aureus* protease genes and activation cascades. A. Genomic organization of secreted protease operons based on the USA300 genome. Colors are as follows: red, metalloprotease; blue, serine proteases; green, cysteine proteases (Staphopains); orange, cysteine protease intracellular inhibitors (Staphostatins).

B. *S. aureus* proteolytic cascade of activation. The Aur, SspA, SspB and ScpA proteases are secreted as inactive zymogens (Pro-) that must undergo processing for full activity. Pro-Aur auto-activates allowing for processing of SspA. Upon SspA activation, SspA is able to process Pro-SspB. Like Pro-Aur, Pro-ScpA is able to auto-activate.
Figure 1-4. Model of regulation of *S. aureus* biofilm formation. Sigma B inhibits the *agr* system by an unknown mechanism. RNAIII, the primary *agr* effector molecule, binds to *rot* mRNA and prevents translation. Rot, a DNA-binding transcriptional regulator, directly binds protease gene promoters and inhibits transcription. Secreted proteases are destructive towards biofilms due to cleavage of biofilm matrix constituents.
CHAPTER II
STAPHOPAINS MODULATE
STAPHYLOCOCCUS AUREUS BIOFILM INTEGRITY

Introduction

The *S. aureus* biofilm matrix is a complex structure that provides protection to the cells that reside within. Numerous reports have underlined the importance of proteinaceous material in the *S. aureus* biofilm matrix [40, 43, 47, 52, 55, 97, 114]. These protein components are susceptible to cleavage by proteases, which can result in biofilm disassembly. *S. aureus* secretes at least ten of its own proteases and often these can cleave *S. aureus* adhesins [46, 47, 49].

Our lab previously demonstrated that the overproduction of extracellular proteases is a major determinant of the biofilm-forming capacity of *S. aureus*. Regulatory changes that increase these protease levels, such as activation of *agr* quorum-sensing or mutations in sigma factor B (*sigB*), result in biofilm inhibition or the dispersal of mature biofilms. Herein, we took advantage of a *sigB* mutation to uncover the most important proteases involved in controlling *S. aureus* biofilm phenotypes. All our biochemical and genetic studies led to the somewhat surprising observation that the cysteine proteases, also called Staphopains, are the key modulators of *S. aureus* biofilm development. Exogenous addition experiments confirmed these findings and demonstrate the important role of Staphopains in controlling *S. aureus* biofilm integrity.

The majority of the work in Chapter II has been accepted for publication in Mootz, JM, Malone, CL, Shaw, LN, and Horswill, AH. 2013. *Infect Immun*
Materials and Methods

Strains and growth conditions

The bacterial strains and plasmids used in this study are described in Table 2-1. *Escherichia coli* cultures were grown in Luria Bertani (LB) broth or on LB agar plates supplemented with 100 µg/ml ampicillin (Amp) or 50 µg/ml kanamycin (Kan) as required for plasmid maintenance. *S. aureus* cultures were grown in Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA). *S. aureus* chromosomal markers or plasmids were selected for, or maintained in, 10 µg/ml of chloramphenicol (Cam), erythromycin (Erm), or tetracycline (Tet), and 50 µg/ml kanamycin (Kan). Unless otherwise stated, all broth cultures were grown at 37°C with shaking at 200 RPM.

Recombinant DNA and genetic techniques

*S. aureus* chromosomal DNA was prepared using the Puregene DNA Purification System from Gentra Systems (Minneapolis, MN). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Table 2-2. Restriction modification enzymes were purchased from New England Biolabs (NEB) and used according to the manufacturer’s instructions. Plasmid construction was carried out in *E. coli* BW25141 or ER2566. Plasmids were first electroporated into *S. aureus* RN4220 as previously described [115] and subsequently transformed into select strains. As needed, chromosomal mutations were transduced into select strains using bacteriophage 80α [116]. Non-radioactive sequencing was performed at the University of Iowa DNA Facility.
**Plasmid construction**

**Protease promoter fusions**

The protease promoters were PCR amplified from AH1263 genomic DNA and cloned into the HindIII and KpnI sites of pCM11 [43] to generate reporters expressing sGFP. Oligonucleotides CLM369 and CLM370 (Table 2-2) were used to generate the *aur* promoter, resulting in plasmid pCM13. Oligonucleotides CLM359 and CLM360 were used to generate the *spl* promoter, resulting in pCM15. Oligonucleotides CLM362 and CLM375 were used to generate the *ssp* promoter, resulting in pCM16. Oligonucleotides CLM453 and CLM454 were used to generate the *scp* promoter, resulting in pCM35. Plasmid construction was confirmed by PCR and enzymatic digestion.

**MBP-Staphostatin fusions**

The Staphostatin encoding genes (*scpB* and *sspC*) were cloned into the EcoRI and PstI sites of pMAL-c2X (New England Biolabs) to generate MBP-Staphostatin protein fusions. pMAL-*scpB* was constructed by PCR amplification of *scpB* using oligonucleotides JMM007 and JMM008. To clone *sspC* into pMAL-c2X, JMM009 and JMM010 oligonucleotides were used. Plasmid construction was confirmed by PCR and enzymatic digestion.

**sspB::pSMUT insertion mutation plasmid**

For *sspB* mutation, a 446 bp internal fragment of *sspB* was PCR generated using primers OL-5505 and OL-5506. This fragment was cloned into pSMUT (gift from Dr Simon Foster, University of Sheffield), a derivative of pMUTIN [117] where the *lacZ* gene was removed, and the multiple cloning site improved.
Strain construction

To build Δaur, ΔsspAB, and ΔscpA mutants, we used the pKOR1 knockout protocol [118] to integrate pKOR1-Δaur [119], pCM34 [120] or pCM39 [120] into AH1263. Double and triple mutations were built as needed and listed in Table 2-1. For ΔsspB, sspB::pSMUT was used to transform strain RN4220 with selection for Erm resistance. RN4220 sspB null-isolates were confirmed by Southern blot, and the mutation was moved by phage transduction into other strains as needed. To inactivate the spl operon, the Δspl::erm mutation [121] was moved by phage transduction. To create mutant combinations in a ΔsigB background, we transformed pKOR1-ΔsigB [102] into select protease mutant strains and carried out the knockout protocol. pKOR1-ΔsigB was also transformed into AH1292 [48] to create ΔsigB Δagr::tetM (designated AH2032). All mutations were confirmed using PCR. The sspAB, scpA, and sspB::pSMUT mutants were confirmed by immunoblot (information provided below). The lack of SspA and SspB was also confirmed by reduced protease activity using FRET and Bz-Pro-Phe-Arg-pNA substrates, and the lack of ScpA using elastin agar (substrate and agar information provided below).

Hemolysis assay

As a measure of α-toxin production, qualitative hemolysis was monitored using rabbit blood agar. Rabbit blood plates contained 5% rabbit blood (v/v) in TSA. Defibrinated rabbit blood was purchased from Hemostat Laboratories (Dixon, CA).

Protease reporter assay

S. aureus reporter strains were grown overnight in TSB supplemented with Erm and sub-cultured in 25 ml to an OD600 of 0.025. 200 µl samples were collected for 48 hr throughout growth and measured for fluorescence (excitation 490 nm, emission 520 nm) in a microtiter plate (Corning) using a Tecan Infinity 200M plate reader. In Figure 2-1A,
the results from the 24 hr. time point are shown as they are most representative of the differences observed throughout the time course.

**Protease activity assays**

Protease activity was monitored using milk agar, elastin agar, and two unique peptide substrates. To prepare samples for measurement of protease activity, cultures were grown overnight in TSB at 37°C. For milk plate assays, 2 µl of overnight culture was placed onto dried milk plates. Milk plates consisted of 5% non-fat dry milk and 3% Bacto agar. Elastin plate assays were conducted as previously described [122]. Elastin from bovine neck ligament was purchased from Elastin Products CO., INC (Owensville, Missouri).

A Fluorescence Resonance Energy Transfer (FRET) assay was developed to examine protease activity. The FRET substrate (5-FAM-Lys-Lys-Ala-Ala-Glu-Ala-Ser-Lys-(QXL520)-OH; AnaSpec, Fremont, CA), was based on a known SspA peptide substrate [123]. The substrate was resuspended to 50 µM using 20 mM Tris pH 7.4. For measurement of protease activity, overnight cultures were sub-cultured to an OD₆₀₀ of 0.1 and allowed to grow for an additional 24 hr, and spent media was filtered through 0.22 µm Costar Spin-X centrifuge tube filters (Corning, NY). For the FRET assay, filtered media samples were buffered in Tris pH 7.4 (25 µl of 20 mM Tris pH 7.4 was added to 175 µl filtered media). To start the reaction, 175 µl buffered media was mixed with 25 µl of FRET substrate in a microtiter plate, and fluorescence measurements (excitation 490 nm, emission 520 nm) were obtained at 37°C in a Tecan Infinity 200M plate reader. To test the specificity of the FRET substrate, we used a combination of purified enzymes and protease mutational analyses. Purified Aureolysin was purchased from BioCentrum (Krakow, Poland), and purified SspA was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Our findings suggest that the FRET substrate is cleaved
primarily by SspA, but also by Aur and ScpA to a lesser extent, but not by SspB or the Spl proteases (data not shown).

SspB protease activity was measured using the synthetic chromogenic substrate Bz-Pro-Phe-Arg-pNA (Bachem, Torrance, CA). A 10 mM stock solution of Bz-Pro-Phe-Arg-pNA was prepared in 100 mM HEPES, pH 6.4, containing 25% methanol. Spent media activity was assayed in a similar manner to that described by Massimi et al [60]. Briefly, reaction mixtures consisted of 2 mM Bz-Pro-Phe-Arg-pNA, 20 mM EDTA and 10 mM CaCl₂ in 100 mM HEPES, pH 6.4. The mixture was pre-incubated 15 min at 37°C in a microtiter plate, and then mixed in a 1:1 volume with spent media. Absorbance at 405 nm was taken at regular time intervals in a Tecan Infinity 200M plate reader.

**Construction of mature length, inactive sspB and scpA expression plasmids**

To generate proteins for antibody production, SspB and ScpA were mutated to change the active site cysteine to an alanine [C237A in ScpA and C243A in SspB] with overlap extension PCR. The oligonucleotides for scpA were CLM551/CLM550 and CLM549/CLM552 and genomic DNA from LAC (AH1263) was used as template. The two PCR products were mixed and used as template for a second PCR with CLM551 and CLM552. This product was digested by NheI and EcoRI and ligated into pET28a digested by the same enzymes, and the resulting plasmid, pCM48, encoded a full length, 6xHis ScpA C237A protein. To construct inactive SspB protein, oligonucleotides CLM546/CLM544 and CLM545/CLM 547 were used pair-wise for PCR on genomic DNA from LAC. The purified PCR products were mixed and used as template for a second round of PCR with CLM546 and CLM547. The resulting PCR product was digested by SpeI and EcoRI, and cloned into pET28a digested by NheI and EcoRI. The cloned full length, 6xHis SspB C243A expressing plasmid is designated pCM49.
Next, the mature length (processed form) plasmids were generated. To create a mature length ScpA C237A protein, oligonucleotide CLM559 and CLM552 were used for PCR with pCM48 as template. The resulting PCR product was digested with NheI and EcoRI, ligated to pET28a digested by the same enzymes, and designated pCM51. The mature, SspB C243A protein was cloned using oligonucleotides CLM548 and CLM547 for PCR and pCM49 as template. After digestion by SpeI and EcoRI, the PCR product was ligated to pET28a digested by NheI and EcoRI and designated pCM50. Expression of pCM51 and pCM50 was tested in the *E. coli* strain ER2566 grown in LB Kan at 37°C. Cells were grown to OD\(_{600}\) of 0.6, induced with 1 mM IPTG, and harvested after 2½ hr incubation. The ScpA protein migrates at about 21 kDa and the SspB protein at about 22 kDa. However, the ScpA protein was not soluble in our tests, similar as previously reported [124].

**Purification and refolding of inactive Staphopain A**

The *scpA* expression plasmid was electroporated into *E. coli* BL21(DE3) cells. Cultures (900 ml) were grown with Kan at 37°C to an OD\(_{600}\) about 0.6, induced with 1 mM IPTG, grown another 4 hr, and harvested. Pellets were lysed with BugBuster (Novagen) diluted to 1X in 100 mM sodium phosphate pH 8. DNA was sheared by passing the lysate through a 26 gauge needle seven times. Centrifugation at 20K × g, 30 min at room temperature gave a pellet of inclusion bodies. This pellet was resuspended in 6 M guanidine hydrochloride, 100 mM sodium phosphate pH 8 (buffer A) by stirring overnight at room temperature. After centrifugation, the supernatant was mixed with 5 mL of prewashed His-Select HF Nickel Affinity (Sigma) resin and washed batch-wise. Washes were done with buffer A until OD\(_{260}\) absorbing material was negligible and with buffer B (6 M guanidine hydrochloride, 100 mM sodium phosphate pH 6.2). Elution was performed using buffer C (6 M guanidine hydrochloride, 100 mM sodium phosphate pH
Fractions containing Staphopain A were pooled and concentrated with Amicon Ultra 10K MWCO (Millipore). To refold, 5 ml of concentrated eluant was added dropwise to 500 ml of cold refolding buffer (50 mM sodium phosphate, 150 mM sodium chloride, 1 mM EDTA, 0.5 M arginine pH 6.0, 30% glycerol, 1 mM CHAPS) and stirred at 4°C for 64 hr. The refolded Staphopain A solution was centrifuged at 18K rpm, 15 min, 4°C. The supernatant was concentrated to 22 ml using an Amicon cell with a YM 10 membrane (10K MWCO). The concentrated Staphopain A was dialysed overnight in PBS. Centrifugation (10K × g, 30 minutes, 4°C) removed any precipitate. The protein was further concentrated to about 0.1 mg/ml and sent for production of chicken antibodies to ScpA (Pacific Immunology).

**Purification of inactive Staphopain B protein**

2 Liters of *E. coli* ER2566 containing pCM50 were grown with LB Kan at 37°C with shaking to an OD$_{600}$ of about 0.6. Cultures were induced with IPTG at 1 mM, grown another 6 hr and centrifuged. Pellets were lysed with BugBuster diluted in equilibration buffer (50 mM sodium phosphate, 0.3 M sodium chloride, 10 mM imidazole pH 8.0). Lysates were passed through a 26 gauge needle to shear DNA and centrifuged at 16K × g, 20 min, room temperature. The cleared lysate was loaded onto a pre-equilibrated 5 ml His-Select HF Nickel Affinity resin column. The column was washed with equilibration buffer until protein concentration in the effluent was negligible. Elution buffer (50 mM sodium phosphate, 0.3 M sodium chloride, 250 mM imidazole, pH 8.0) was applied to the column, and fractions containing the 22 kDa SspB protein were pooled and dialysed versus PBS at 4°C. Pure SspB protein at 2.4 mg/ml (by BioRad protein assay) was sent for production of chicken antibodies (Pacific Immunology).
**Purification of antibodies**

Purification of Staphopain B antibodies from chicken egg yolks was performed using the Pierce Chicken IgY Purification Kit (Thermo Scientific) according to the manufacturer’s instructions. ScpA antibodies were purified from chicken egg yolks according to Ko *et al.* [125]. The yolk was passed through cheesecloth to remove membrane, and ten volumes of water were added and the pH adjusted to 5.0. The mixture was stirred for 2 hr at 4°C and allowed to sit at 4°C overnight. Centrifugation at 10K × g for 30 min at 4°C removed most of the lipids. The pH of the supernatant was adjusted to 4 with hydrochloric acid and 0.01% activated charcoal was added to remove the last of the lipids. Charcoal was removed by filtration through Whatman no. 1 filter paper. The pH was adjusted to 7 by addition of sodium hydroxide and ammonium sulfate was slowly added to 50% at 4°C. After one hour, the mixture was centrifuged at 10K × g for 30 minutes at 4°C. The pellet was resuspended and dialysed in PBS. A subsequent ammonium sulfate precipitation (50%) yielded higher purity antibodies.

**Western blotting**

Purified protein or Trichloroacetic acid (TCA) precipitated spent supernatant samples were mixed with SDS-PAGE loading buffer and 5 µL of each sample was electrophoresed on a 12% polyacrylamide gel. The proteins were transferred to Immobilon-P PVDF membranes (Millipore) using a Protean II device (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked overnight at 4°C with 5% milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.0, with 137 mM NaCl) containing 0.1% Tween 20 (TBST). Primary antibodies were diluted in 5% milk in TBST and incubated with the membranes at room temperature for 2 hr. Membranes were rinsed 3 times in TBST and washed with agitation for 15 min followed by two more washes for 5 min. Secondary HRP-conjugated goat anti-chicken IgG was diluted 1:20,000 in 5% milk in
TBST and incubated at room temperature for 1 hr. The membrane was rinsed and washed again as above. SuperSignal West Pico chemiluminescent substrate was added for 5 min at room temperature followed by exposure to X-ray film.

**Biofilm assays**

*Microtiter plate biofilms*

Biofilm formation was assessed using a plasma-coated static microtiter plate assay as previously described [126]. Our protocol was slightly modified from the published version in that we eluted the crystal violet immediately following the second set of triplicate washes. Biofilm biomass was completely resuspended by scraping attached biomass off the bottom of wells, followed by thorough pipetting. Absorbance was measured in a Tecan Infinity 200M plate reader at 595 nm. If the absorbance value fell out of the linear range, samples were instead measured at 600 or 605 nm, thereby allowing all samples to be read.

All biofilm additives were added at time zero at concentrations that did not inhibit growth (data not shown). Antibiotics were used to maintain plasmids as necessary. Protein expression from pALC2073 and pALC2109 was induced with 50 ng/ml anhydrotetracycline. For the experiment involving inhibition of WT biofilm formation by Δ*sigB* spent supernatant, an overnight culture of Δ*sigB* grown in TSB was filtered through a 0.22 µm Costar Spin-X centrifuge tube filter (Corning) to collect spent supernatant. 1,10-Phenanthroline monohydrate (1,10-PA), 3,4-Dichloro-isocoumarin (DIC) and E-64 were purchased from Sigma (St. Louis, MO) and each used at a concentration of 10 µM. Purified MBP, MBP-ScpB and MBP-SspC were used at a final concentration of 400 nM. Pure ScpA and SspB (BioCentrum) were used at final concentrations of 50 or 250 nM.
Flow cell biofilms

Flow cell biofilm formation was assessed as previously described [48] with one exception. To mimic the plasma-coated surfaces used in the microtiter assays, we coated plastic coverslips in human plasma. Rinzle Plastic Coverslips from Electron Microscopy Sciences (Hatfield, PA) were glued to flow cell chambers and UV-sterilized for 4 hours. Once sterilized, 20% human plasma was injected into each flow cell chamber and moved to 4°C for 24 hours, allowing for plasma protein attachment. Following attachment, flow cell chambers were washed 3 times with 1X PBS before fresh culture was used to inoculate the flow chambers.

Purification and testing of MBP fusion proteins

Purification of MBP fusion proteins from pMAL-scpB and pMAL-sspC on amylose resin was performed according to the manufacturer’s instructions (New England Biolabs). For lysis of cells containing induced proteins, we incubated harvested cells in 1X Novagen Bug Buster (EMD Chemicals, San Diego, CA) for 2 hr at room temperature. Eluted fractions were analyzed via SDS-PAGE and concentrated with an Amicon Ultra-15 with a 10K cut-off (Millipore). Protein concentration was measured using the Bio-Rad protein assay (Hercules, CA). Activity of MBP-ScpB was measured by inhibition of protease activity of strain AH2383 (ΔsigB Δaur ΔsspAB Δspl::erm) against the FRET substrate. Activity of MBP-SspC was measured by inhibition of protease activity of the ΔsigB mutant against Bz-Pro-Phe-Arg-pNA.

Results

SigB and agr-dependent regulation of protease expression

We previously reported that sigB mutants are defective in biofilm formation in multiple different strains, and the agr system and extracellular proteases are linked to this
phenotype [102]. To investigate this interconnection in more detail, we evaluated *sigB*, *agr*, and protease regulation using a community-associated methicillin-resistant *S. aureus* (CA-MRSA) isolate of the USA300 group (strain LAC, see Table 2-1), hereafter called LAC-WT. We developed a series of reporter constructs that fused the promoter for each protease transcript (*aur*, *sspABC*, *scpAB*, and *splABCDE*) to a gene encoding a modified green fluorescent protein (sGFP) [127]. The reporter plasmids were transformed into LAC-WT, and isogenic Δ*sigB* and Δ*agr* mutants, and changes in protease gene expression were monitored throughout a time course. Our results show that expression from each of the four protease operon promoters is markedly upregulated in a Δ*sigB* knockout and downregulated in Δ*agr* as compared to its isogenic parent (Figure 2-1A). The changes in gene expression coincide with those seen in overall protease activity (Figure 2-1B), as measured using a FRET based substrate assay (see Materials and Methods). The Δ*agr* mutation was also epistatic to Δ*sigB* in the double mutant (Figure 2-1B), demonstrating that *agr* acts downstream of SigB and supporting previous observations [102]. The high protease level in the Δ*sigB* mutant inhibits the establishment of a biofilm, presumably by cleaving surface and secreted matrix proteins. While the molecular details of some of these cascade steps are not fully established, we can take advantage of the Δ*sigB* mutation to gain insight on the protease(s) responsible for the biofilm phenotype.

**Δ*sigB* mutant is deficient in biofilm formation on human plasma due to a secreted factor**

Throughout this work, we used developed biofilm assays with human plasma coated surfaces to investigate the biofilm roles of *S. aureus* proteases. The use of conditioned surfaces has gained popularity due to the observations that implant materials are coated with host matrix proteins [36, 37, 39, 126], and *S. aureus* attaches to these...
proteins in vivo rather than directly to abiotic materials [40, 97]. Notably, the ΔsigB mutant is defective in biofilm formation on the plasma-coated surface (Figure 2-2A), and this phenotype can be complemented. As with the protease phenotype, introduction of the Δagr mutation repaired the biofilm deficiency, confirming that the agr system acts downstream of SigB in this biofilm development pathway.

Several factors including proteases and nuclease could act downstream of SigB to inhibit biofilm formation. To determine which factors are biofilm inhibitory, we initially examined whether secreted factors were responsible for the phenotype. We collected overnight spent media from a ΔsigB mutant and added varying amounts to LAC-WT biofilms at time zero. As shown in Figure 2-2B, low levels of ΔsigB spent media inhibit biofilm formation in a concentration dependent manner. Growth of the organism is not negatively affected across the concentrations tested (data not shown). These data suggested that something is released into the extracellular environment by the ΔsigB mutant that causes biofilm disruption. Knowing that S. aureus secreted nuclease could inhibit biofilm formation on an abiotic substratum [48], we tested whether this was also the case in the plasma-coated assay by inactivating the nuc gene in the ΔsigB mutant background. The ΔsigB nuc double mutant displayed a similar level of biofilm capacity compared with ΔsigB on a plasma-coated surface, suggesting that additional factors were involved (data not shown).

**Cysteine protease inhibition restores biofilm capacity to a ΔsigB mutant**

Considering the agr mutation was epistatic to SigB (Figure 2-2A), and the secreted nuclease (Nuc) was not involved in these phenotypes, we focused attention on the secreted proteases. Initially, we attempted to restore ΔsigB biofilm formation by the exogenous addition of chemical protease inhibitors. Three inhibitors were tested: 1,10-
phenanthroline (1,10-PA), a metal ion chelator that blocks Aur metalloprotease activity [119]; 3,4-dichloroisocoumarin (DIC), a serine protease irreversible inhibitor that blocks SspA (V8) activity and potentially that of the six Spl proteases A-F (data not shown); and E-64, a cysteine protease inhibitor that blocks activity of both Staphopain proteases [83].

Using plasma-coated biofilm assays with the ΔsigB mutant (Figure 2-2C), 1,10-PA had no effect and DIC had an intermediate effect. In contrast, E-64 greatly restored biofilm formation, suggesting that the cysteine proteases Staphopain A (ScpA) and Staphopain B (SspB) are contributors to the ΔsigB biofilm-negative phenotype.

**Staphopain inhibition restores biofilm capacity to a ΔsigB mutant**

The challenge of using protease inhibitors like E-64 is that they can alter the function of other fundamental cellular processes in *S. aureus*, such as the activity of the critical enzyme sortase [128], which is required for proper presentation of matrix binding proteins and biofilm formation [47]. Downstream of each Staphopain gene on the chromosome is a co-transcribed gene encoding a cytoplasmic inhibitor (Staphostatin) with specificity to its cognate Staphopain (Figure 1-4A). The Staphostatins are thought to protect the cell against pre-mature activation of Staphopain precursors before protein export by forming a 1:1 protein complex [129]. Due to their unique target specificity, the Staphostatins can also be used as tools to selectively inactivate the Staphopains in a complex mixture, unlike the non-specific effects that are associated with E-64.

We affinity purified the Staphostatins ScpB and SspC (called Staphostatin A and Staphostatin B, respectively, to indicate the cysteine protease target). To test the functionality and specificity of the purified statins, we measured the inhibition of ScpA and SspB protease activity using spent media from *S. aureus* strains producing the enzymes (see Materials and Methods). As shown in Figure 2-3A, only Staphostatin A
could inhibit ScpA protease activity in a complex mixture at concentrations as low as 200 nM, and similarly, only Staphostatin B could inhibit Staphopain B at low concentrations (Figure 2-3B). Therefore, each Staphostatin protein inhibitor was functional and acted in a selective manner against its respective Staphopain. When tested in a biofilm assay, 400 nM of Staphostatin A and Staphostatin B were each individually able to partially restore biofilm formation of the ΔsigB mutant (Figure 2-3C). When added together, ΔsigB biofilm formation was completely restored, reaching the same level as E-64 inhibition and that of a double staphopain knockout in the ΔsigB mutant (see below). These data further suggest that the Staphopains (ScpA and SspB) contribute to the ΔsigB mutant biofilm phenotype.

**Mutation of sspB and scpA restore ΔsigB biofilm formation**

To follow-up on the inhibitor observations, we took a genetic approach and constructed protease mutations in the ΔsigB background. First, we made single deletion mutations in the *aur, sspAB, scpA*, and *spl* operons. Next we made combinations of protease mutations including a complete extracellular protease knockout, herein referred to as Δprotease. All mutations were confirmed by PCR, and wherever possible immunoblot, protease activity assays, and/or antibiotic resistance testing were also utilized as confirmation (see Materials and Methods). To test for the occurrence of spontaneous *agr* mutations, hemolysis on blood agar was visualized for each mutant and levels were similar to LAC-WT or ΔsigB for each constructed strain (data not shown).

The complete extracellular protease knockout in a *sigB* mutant (ΔsigB Δprotease) was able to form a biofilm with capacity similar to that of wild-type, confirming that extracellular proteases have an inhibitory effect on biofilm formation (Figure 2-4A). Taking our studies further, we tested each protease knockout individually, and in
combination, in the sigB mutant background. While introduction of the Δspl operon mutation was unable to restore biofilm formation, single deletion mutations in Δaur, ΔsspAB, or ΔscpA showed partial restoration. Using mutant combinations, only strains containing deletions of both the ΔsspAB operon and ΔscpA greatly restored biofilm formation (Figure 2-4A and data not shown). To elucidate the roles of SspA and SspB, we constructed a sspB single mutant in the ΔsigB ΔscpA strain and confirmed that SspA was retained via protease activity assay and immunoblot (data not shown). Although SspA (V8) has a demonstrated role in biofilm remodeling [47], the introduction of both Staphopain single mutations into the ΔsigB background completely restored biofilm formation (Figure 2-4A), suggesting that SspA was not a significant factor in this assay.

Staphopains have been implicated in cleavage of fibrinogen, a human plasma protein capable of binding S. aureus adhesins (61). It is possible that the Staphopains cleave plasma proteins and this is the explanation for the biofilm phenotypes in our assays. To assess this question, we repeated our microtiter plate experiments using uncoated microtiter wells. As shown in Figure 2-4B, the overall quantity of biofilm biomass is reduced in uncoated wells as compared to plasma coating, which supports previous observations (43). Importantly, while ΔsigB has significantly less biomass than LAC-WT, inactivation of Staphopain A or B in the ΔsigB background partially restored biofilm formation, and removal of both Staphopains restored biofilm capacity to WT levels (Figure 2-4B). Taken together, and in conjunction with the inhibitor data, our findings suggest that SspB and ScpA are important determinants of biofilm formation.

To confirm these observations, we repeated experiments using flow cell biofilm assays. Unlike typical flow cell experiments, the substratum was coated with human plasma protein to maintain consistency with other assays in this study. The flow biofilms were post-stained with SYTO-9 to detect biomass and images were obtained with confocal microscopy. A no-bacteria control experiment revealed that the hydrophobic
nature of the dye has some, albeit limited, background binding to the plasma proteins on conditioned surfaces (Figure 2-4C). LAC-WT forms a robust biofilm in this assay (Figure 2-4D), and the ΔsigB mutant is defective (Figure 2-4E). Some small clumps of ΔsigB mutant cells do attach to the plasma proteins, giving a punctate staining appearance, but the level of biofilm accumulation observed with the LAC-WT strain is not achieved with the ΔsigB mutant. Finally, introduction of the double cysteine protease mutations (ΔsspB and ΔscpA) into the ΔsigB mutant background restored biofilm capacity to WT level, supporting the observations made with the microtiter assay (Figure 2-4A).

**Staphopains are repressed under biofilm forming conditions**

Knowing that Staphopain inhibition restores biofilm formation to a ΔsigB mutant, we reasoned that *S. aureus* maintains low Staphopain levels in order to facilitate establishment of the biofilm. To address this question, LAC-WT and strains with either a ΔsigB or a ΔsigB ΔsspB ΔscpA triple mutation were grown under three different conditions: i) TSB, to mimic planktonic growth; ii) biofilm media (TSB with 3% NaCl and 0.5% glucose [126]), to simulate regulatory conditions during biofilm initiation; and iii) biofilm forming conditions, where biomass was prepared using the plasma-coated microtiter assay. Cells in each condition were grown for 24 hours and the amount of supernatant assayed were normalized to a cellular OD$_{600}$ of 1.5. Staphopain levels were assessed using an immunoblot approach with anti-ScpA and anti-SspB antibodies prepared in this work.

For ScpA, the active, processed form of the protease was not detectable in LAC-WT cultures grown under any of the conditions tested, while this form was detectable in a ΔsigB mutant (Figure 2-5A). Levels of ScpA (various processed forms) in a ΔsigB
mutant were markedly higher in the TSB grown cultures compared to those grown in biofilm media or the biofilm biomass. In our experience, the series of processed bands in immunoblots is typical as the zymogen is cleaved at various positions to generate the active form of the Staphopain. As a control, a ΔsigB ΔsspB ΔscpA triple mutant was tested and ScpA was not detectable in any condition. In LAC-WT, the fact that ScpA was not detectable at 24 hr even in the TSB grown condition matched previous reports [84]. To confirm that the anti-ScpA antibodies were functioning properly, an immunoblot time course was performed (Figure 2-6A), and indeed ScpA levels are high in late-logarithmic growth and early stationary phase, with the ScpA degrading by 24 hr as previously observed (Figure 2-B) [84].

The SspB enzyme is more stable and facilitated a better assessment of Staphopain regulation within a biofilm (Figure 2-5B). Processed SspB levels were high in a ΔsigB mutant, especially in the TSB grown condition, and all of these levels were substantially elevated over LAC-WT. Similar to the ScpA immunoblot, processed SspB in the biofilm or the biofilm growth media was not observed in LAC-WT, except for trace levels of the SspB zymogen in each growth condition. However, LAC-WT levels of SspB were elevated in the TSB grown culture, a striking contrast from the biofilm grown conditions. To confirm functionality of the SspB antibody, a time course was performed and the SspB zymogen was detected at all time points (Figure 2-6C), except in sspB mutant strains. Processed SspB was detectable only in LAC-WT by 24 hr, albeit at low levels. In all the SspB immunoblots, the sspB::pSMUT mutation results in an antibody-detectable, truncated form of the protein. The truncation is absent from TSB grown cultures, possibly suggesting that it is degraded under high protease conditions, and importantly, SspB protease assays confirm the absence of activity (data not shown) in sspB::pSMUT mutant strains. Altogether, these experiments demonstrate that
Staphopain accumulation is repressed during biofilm growth and that these proteases are overproduced in SigB-defective strains.

**Exogenous addition of Staphopains inhibit biofilm formation**

Based on our observations that Staphopain overproduction is biofilm inhibitory, we tested whether the exogenous addition of purified Staphopains could inhibit LAC-WT biofilm formation. Since Staphopain A is produced in the 50-200 nM range by LAC-WT *S. aureus* when grown in TSB [84], and ΔsigB produces even more proteases, we used similar levels in our experiments. As shown in Figure 2-7A, pure ScpA was able to significantly inhibit biofilm formation at low enzyme concentrations (50 nM) and more dramatically at higher concentrations (250 nM). Addition of SspB was not as effective, with only higher concentrations of the enzyme (250 nM) inhibiting biofilm formation. Supporting these observations, when both SspB and ScpA were simultaneously added at 50 nM, biofilm formation was completely prevented. A strain lacking all the proteases (ΔsigB Δprotease) was also tested and addition of both SspB and ScpA also prevented biofilm formation. Taken together, these findings support our observations that the Staphopains have a biofilm inhibitory role.

Due to our previous reports on protease-mediated biofilm dispersal [42, 43], we tested the ability of the Staphopains to disassemble established biofilms. We allowed a LAC-WT biofilm to form and achieve full biomass, and at this time point (12 hr), the biofilm was subjected to either ScpA, SspB, or both enzymes, at a concentration of 250 nM. ScpA protease was able to disperse the established biofilm (Figure 2-7B), but SspB was less effective. The addition of both SspB and ScpA together also dispersed the biofilm, but the inhibition level was similar to that of ScpA alone. These findings suggest that ScpA is more proficient at dispersing an established biofilm.
Staphopain A prevents biofilm formation across
*S. aureus* lineages

The *S. aureus* biofilm matrix is a complicated meshwork of cellular components and the importance of each component likely depends on both the strain examined and the environment in which the biofilm is growing. In this work, we demonstrated the role of Staphopain-susceptible protein components in biofilm formation by the USA300 isolate LAC-WT. To assess the generality of the observations, we examined the susceptibility of biofilms to Staphopains in a variety of strain backgrounds. Since Staphopain A was able to both prevent and disassemble LAC-WT biofilms at low concentrations of enzyme, we focused on this enzyme in the strain assessment. A single clinical isolate from each USA PFGE type 100-800 was selected and assessed for biofilm formation in the plasma-coated microtiter assay. USA100, USA200 (MN8), USA300 (LAC), USA400 (MW2), USA500, USA600, USA700 and USA800 were each able to form a biofilm in this assay. As before, we used 250 nM ScpA for the biofilm treatments and this prevented biofilm formation in all strain types tested (Figure 2-8). These findings demonstrate that ScpA enzyme has a conserved ability to inhibit biofilm formation across a broad range of *S. aureus* strains.

**Discussion**

The *S. aureus* biofilm matrix is complex and has been the focus of numerous studies in recent years [26]. Of interest for this work, there are growing reports that proteins within the biofilm matrix are critical for *S. aureus* biofilm structure, with primary evidence coming from the ability of exogenously added proteases to inhibit biofilms [45, 130, 131], and the findings that secreted proteases of *S. aureus* also self-inhibit biofilms [42, 43, 46, 47]. To date, studies have pointed at a key role for SspA (V8) protease in cleaving important surface adhesins, such as FnbpAB [47, 49], and
preventing biofilm formation, thus making V8 the only known protease of *S. aureus* with biofilm inhibitory functions. *S. epidermidis* Esp protease is a V8 homologue and there is a recent report that it can destroy *S. aureus* biofilms [132], further supporting the anti-biofilm activity of V8-like enzymes. In this report we investigated the contribution of proteases to *S. aureus* biofilm phenotypes and all of our findings pointed to the Staphopain cysteine proteases as being the most important modulators of biofilm integrity.

Considering published findings, why did our studies identify Staphopains and not V8 protease? One possibility is that the assays used to identify *S. aureus* protease-biofilm interconnections vary between reports. In this study, we focused on plasma-coated surfaces, while the other published examples tested V8 activity against biofilms grown directly on an abiotic surface [46, 47, 132]. Additionally, some of the properties of V8 make it an unusual choice to be the dominant enzyme involved in biofilm remodeling. V8 has narrow substrate specificity, cutting specifically after glutamate residues [133], and the zymogen also has to be activated by Aur to be functional [59]; both of those properties limit the utility of V8 as a biofilm modulator. In contrast, the other major proteases (Aur, ScpA, SspB) have broader target specificity and both Aur and ScpA self-activate [57, 58, 80]. ScpA in particular seems like an obvious candidate to control biofilm matrix structure. The enzyme is one of the first proteases produced and activated in the extracellular environment and reaches high local concentrations [84], all properties that would be advantageous for controlling biofilm integrity. The fact that cysteine proteases are inhibitory toward a bacterial biofilm is also not unique. In Group A *Streptococcus* (GAS), the prominent SpeB protease (Streptopain) has been linked to biofilm formation [134]. When SpeB levels are high, such as in *srv* mutants, GAS biofilm formation is eliminated unless the cysteine protease is inhibited genetically or biochemically, all findings that share striking parallels to our observations with *S. aureus*.
What are the Staphopains cutting that negatively impact *S. aureus* biofilms? The targets of the proteases will be important to understanding how the biofilm matrix retains structure. Presumably, FnbpAB are a potential target since they are produced by most *S. aureus* strains, have a key role in biofilm formation, and also are known to be targeted by proteases [47, 135]. It is also possible that the Staphopains are targeting surface adhesins that bind human matrix proteins, and potentially even the matrix proteins themselves. Fibrinogen is likely an important handle for binding and initiation of *S. aureus* biofilm formation, and the Staphopains are known to cleave fibrinogen [82], more specifically SspB cleaves the Aα-chain under physiologically relevant conditions. However, our results indicate that the Staphopains can inhibit biofilm formation independent of fibrinogen cleavage (Figure 2-4B). This finding suggests that Staphopains are capable of cleaving *S. aureus* biofilm matrix proteins, which results in a biofilm phenotype in overexpressed conditions. Thus, for the plasma-coated surfaces, the significance of fibrinogen cleavage in the context of a *S. aureus* biofilm remains unclear. It should also be noted that the biofilm dispersing functions of the Staphopains differ from the biofilm inhibition functions (Figure 2-7), suggesting some of the targets might be varied depending on the developmental stage of the biofilm. Taken together, the limited information on targets and the interconnection between the proteases is an area in need of further investigation.

Altogether, our studies have uncovered an unexpected new role for the *S. aureus* Staphopain proteases in controlling biofilm development. In conditions where the Staphopains are up-regulated, such as *sigB* mutants, the proteases reach levels that prevent biofilm formation, presumably due to the cleavage of surface structures or secreted proteins in the biofilm matrix. ScpA exogenous addition experiments, and other overall properties of this enzyme, suggest it might be the more significant player in biofilm remodeling, although SspB clearly has an important role. Our observations were
consistent across strains suggesting this is a general trend among *S. aureus* biofilms. Uncovering new strategies to up-regulate the Staphopains could be an innovative approach to treating biofilm infections.
Table 2-1 Strains and plasmids used in Chapter II.

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Figure 2-1. Mutations in agr are epistatic to sigB for protease expression. A. Transcriptional regulation of protease operons by agr and sigB. Promoters for each protease transcript (aur, ssp, spl, scp) were cloned in front of sGFP and transformed into WT, Δagr and ΔsigB strains. The constructed strains were grown 24 hr in TSB and GFP fluorescence intensity was determined. B. Protease activity in global regulatory mutants. Cell-free overnight culture supernates were incubated with the protease susceptible FRET substrate 5-FAM-Lys-Lys-Ala-Ala-Glu-Ala-Ser-Lys (QXL520)-OH. Rate of change in fluorescence over a 60 min incubation period is shown. Colors as are follows: blue, WT; black, ΔsigB; green, ΔsigB Δagr.
Figure 2-2. *S. aureus sigB* mutants form a protein-dependent biofilm on plasma-coated surfaces. **A.** Biofilm formation by strain LAC (WT) and regulatory mutants in plasma-coated biofilm assay. Bacterial cultures were grown overnight and inoculated 1:200 in biofilm media over microtiter wells pre-coated in 20% human plasma. Static biofilm cultures were grown for 24 hr. Biomass was calculated by staining washed biofilms with crystal violet and measuring absorbance of solubilized cultures. **B.** WT biofilm levels upon addition of exogenous Δ*sigB* spent media. Increasing amounts cell-free spent media were added at time zero to WT microtiter plate cultures and grown for 24 hrs. **C.** Δ*sigB* biofilm formation in the presence of protease inhibitors at sub-inhibitory concentrations [10 µM] of either the metalloprotease inhibitor 1,10-phenanthroline, serine protease inhibitor 3,4-Dichloroisocoumarin (DIC) or cysteine protease inhibitor E-64 (**P<0.01, ***P<0.001 relative to Δ*sigB* strain as determined by paired T-test).
Figure 2-3. Staphostatin specificity and restoration of ΔsigB biofilm phenotype.  

A. Specificity of ScpB Staphostatin against ScpA activity.  Overnight cultures of ΔsigB Δaur ΔsspAB Δspl were incubated with the FRET substrate for 60 min. Increasing concentrations of MBP-ScpB or MBP-SspC were incubated with culture supernate and the FRET substrate at time zero.  % ΔsigB activity was measured by comparing the rate of fluorescent change at each Statin concentration to the rate of fluorescent change of ΔsigB ((Slope of ΔsigB Δaur ΔsspAB Δspl + MBP-Statin) / (Slope of ΔsigB Δaur ΔsspAB Δspl)) x 100. Figure legend shown in Figure 2-3B.  

B. Specificity of SspC Staphostatin against SspB activity. The activity of MBP-ScpB and MBP-SspC against SspB were measured using the SspB-specific substrate Bz-Pro-Phe-Arg-pNA. Overnight cultures of ΔsigB were incubated with this substrate for 60 min. % ΔsigB activity was measured as above using the ΔsigB strain.  

C. ΔsigB biofilm formation in the presence of MBP-Staphostatin fusion proteins. ΔsigB biofilm formation was assessed following treatment of cultures with MBP [250 nM], MBP-Staphostatins [250 nM] or E-64 [10 µM] at time zero (**P<0.01, ***P<0.001 relative to ΔsigB strain as determined by paired T-test).
Figure 2-4. Deletion of the Staphopain genes restores biofilm formation to a ΔsigB mutant. A. Biofilm forming capacity of protease mutants in ΔsigB background. The sigB deletion plasmid was transformed into single, double, triple and quadruple protease mutants, and the resulting strains were tested for biofilm formation in the static plasma-coated microtiter plate assay. B. Biofilm forming capacity of Staphopain mutants on an uncoated surface. Mutant combinations were tested for biofilm formation using identical experimental conditions as those in (A) except that microtiter wells were not pre-coated in human plasma (for A and B, *P<0.05, **P<0.01, ***P<0.001 relative to ΔsigB strain as determined by paired T-test). Red bars indicate cysteine protease mutant strains. C-F. Plasma-coated coverslip biofilms grown in flow cells. As an additional assay, flow cell biofilm formation was assessed using plastic coverslips that were UV-sterilized and treated overnight with 20% human plasma. Flow cell biofilms contained C) no cells, D) WT, E) ΔsigB, and F) ΔsigB ΔsspB ΔscpA strains. Biofilms were post-stained with SYTO-9 and imaged with confocal microscopy. Representative top-down images of flow cell biofilm are shown. Experiments were performed in triplicate.
Figure 2-5. **Staphopain levels are repressed during biofilm formation.** WT, ΔsigB and ΔsigB ΔscpA ΔsspB strains were grown overnight and sub-cultured in either TSB, biofilm media or under static biofilm conditions. Cultures were grown for 24 hr and cell-free spent media was collected and normalized to cell suspension OD$_{600}$ of 1.5. Protein in spent media were precipitated with TCA and prepared for electrophoresis. **A.** Immunoblot detection of ScpA. **B.** Immunoblot detection of SspB.
Figure 2-6. **Immunoblot detection of ScpA and SspB during *S. aureus* growth.**

**A.** Collection of supernates during growth time course. WT, ΔscpA, ΔsspB, ΔsigB and ΔsigB ΔsspB ΔscpA strains were inoculated in TSB at an initial OD_{600} of 0.05 and grown for 24 hr. 1 ml samples were collected at the indicated time points and filtered for cell-free spent media. Optical density measurements at the absorbance of 600 nm are shown.

**B.** Immunoblot detection of processed forms of ScpA in culture spent media at collected time points.

**C.** Immunoblot detection of unprocessed and processed forms of SspB in culture spent media at collected time points. The *sspB::pSMUT* construct leaves a remnant of SspB in the culture that can be detected with antibody. For B and C, the lane numbers underneath the blots correspond to the strains numbered in the panel A legend.
A

**Absorbance (600 nm)**

Time (h)

- WT (1)
- ΔscpA (2)
- ΔsspB (3)
- ΔsigB (4)
- ΔsigB ΔsspB ΔscpA (5)

B  Staphopain A

4 hr  6 hr  12 hr  24 hr

- ScpA processed
- ScpA fragments

C  Staphopain B

4 hr  6 hr  12 hr  24 hr

- SspB zymogen
- pSMUT remnant?
- SspB processed
Figure 2-7. Staphopain addition inhibits biofilm formation and disperses established biofilms.  A. Staphopain inhibitory capacity of LAC (WT) biofilm formation.  Purified Staphopains [nM] were added at time zero to WT or ΔsigB Δprotease strains and biofilm formation was assessed.  B. Staphopain disassembly of established WT biofilms. Purified Staphopains were added to WT biofilm cultures after 12 hr and biomass was assessed 12 hr later (24 hr from time zero). Concentrations of 250 nM ScpA, 250 nM SspB, or 250 nM of each were used (*P<0.05, ***P<0.001 relative to untreated control as determined by paired T-test).
Figure 2-8. ScpA inhibition of biofilm formation is conserved across *S. aureus* strains. Strains representing the PFGE types USA100-800 were selected to examine the capacity to form a biofilm in the presence of 250 nM ScpA. White bars, Untreated; Black Bars, ScpA treated. All ScpA treatments were significant relative to untreated control (*P* <0.001) as determined by paired T-test.
CHAPTER III
ROT IS ESSENTIAL FOR BIOFILM FORMATION
IN STAPHYLOCOCCUS AUREUS

Introduction

The pathways that regulate S. aureus biofilm formation are an intricate network of overlapping circuits. It is generally appreciated that inactivation of the sarA and sigB global regulators restricts biofilm formation [102, 142], while inactivation of the agr quorum-sensing system has the opposite effect [42, 43]. In recent years, it has become accepted that a major factor dictating these biofilm phenotypes are the array of extracellular enzymes produced by S. aureus strains, with most reports indicating that the proteases have an important role [40, 42, 46, 102, 114, 143, 144]. However, for the sigB and agr systems, the regulation of protease transcription and activity is not direct and instead requires intermediate players. The most important intermediary that has been identified is the Repressor of toxins (Rot). Rot positively regulates the expression of several surface proteins including Protein A while negatively regulating the expression of exo-enzymes, such as the secreted proteases [111, 113]. Genetic analysis through epistasis studies suggests that Rot functions downstream of the agr system and represses toxin and exo-enzyme production [111, 145]. When the agr system is activated, RNAIII actively prevents translation of Rot protein [108], relieving the Rot repressive effect.

Since Rot is a protease repressor, and proteases have a key role in S. aureus biofilm development, we hypothesized that inactivation of Rot would result in a biofilm-

The work presented in Chapter III was done in collaboration with the laboratories of Dr. Victor Torres (NYU) and Dr. Tammy Kielian (UNMC). As a result, several sections from the materials and methods portion of this chapter are absent. These will become available in a future manuscript.
negative phenotype. Indeed, in this work we demonstrate that a rot mutant is defective in biofilm formation using multiple different assays. We find that extracellular protease activity is elevated in a rot mutant, and genetic and biochemical inhibition of the proteases repairs the biofilm phenotype. The rot defective phenotype is conserved in a murine model of catheter biofilm formation.

**Materials and Methods**

**Strains and growth conditions**

*S. aureus* cultures were grown in Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA). The bacterial strains and plasmids used in this study are described in Table 3-1. *Escherichia coli* cultures were grown in Luria Bertani (LB) broth or on LB agar plates supplemented with 100 µg/ml ampicillin (Amp) as required for plasmid maintenance. *S. aureus* chromosomal markers or plasmids were selected for, or maintained in, 10 µg/ml of chloramphenicol (Cam), erythromycin (Erm) or tetracycline (Tet), and 100 µg/ml spectinomycin (Spec). Unless otherwise stated, all broth cultures were grown at 37°C with shaking at 200 RPM.

**Recombinant DNA and genetic techniques**

Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction modification enzymes were purchased from New England Biolabs (NEB) and used according to the manufacturer’s instructions. Plasmids were first electroporated into *S. aureus* RN4220 as previously described [115] and subsequently transformed into select strains. As needed, chromosomal mutations were transduced into select strains using bacteriophage 80α [116]. Non-radioactive sequencing was performed at the University of Iowa DNA Facility to confirm constructs.
**Plasmid construction**

*rot complementation plasmid empty vector*

An empty vector control with a new multiple cloning site (MCS) was developed from the *rot* complementation plasmid. Oligonucleotides JMM032 5’ TATGTAAGGTACCACGCGTGAGCTCTCTAGAC 3’ and JMM033 5’ TCGAGTCTAGAGAGCTCACGCGTGGTACCTTACA 3’ were allowed to anneal and ligated into NdeI and XhoI digested pOS1 plgt *rot* resulting in pJM01. The new MCS contains restriction sites for NdeI, KpnI, MluI, SacI, MbaI and XhoI. Plasmid construction was confirmed by PCR and enzymatic digestion.

**Strain construction**

All subsequent *rot* mutant strains were developed by phage transduction of Δ*rot::spec* from phage lysate of AH2184. Mutations in *rot* were made in clinical isolates of the USA types 100, 400, 500, 700 and 800. Transduction of Δ*rot::spec* into AH1292 resulted in the double mutant Δ*rot::spec Δagr::tet* (designated AH2544). Protease mutant strains were previously constructed [144]. For construction of *rot/protease* mutant combinations, Δ*rot::spec* was transduced into select protease mutants. Clones were selected for on TSA with Spec and mutations were confirmed using PCR.

**Purification and testing of MBP fusion proteins**

Plasmid constructs were previously generated for expression of MBP-Staphostatin fusion proteins [144]. Purification of MBP-ScpB and MBP-SspC was performed according to the manufacturer’s instructions (NEB). Proteins were purified over amylose resin and eluted fractions analyzed via SDS-PAGE and concentrated using an Amicon Ultra-15 with a 10K cut-off (Millipore). Protein concentration was measured using the Bio-Rad protein assay (Hercules, CA). Activity of MBP-ScpB and MBP-SspC was measured as previously described [144].
Biofilm assays

Microtiter plate biofilms

Biofilm formation was assessed on both coated and uncoated surfaces. Plasma-coated static microtiter plate assays were performed as previously described [144]. Briefly, lyophilized human plasma (Sigma) was resuspended to 20% in Carbonate-Bicarbonate buffer, filtered through a 0.22 µm membrane (Millipore), and added to the wells of a 96-well microtiter plate (Corning). Coated plates were incubated overnight at 4°C before the plasma was removed by aspiration. Immediately following aspiration, bacteria diluted 1:200 in biofilm media (TSB + 3% NaCl + 0.5% glucose) were added to coated wells and statically incubated for 24 hours at 37°C. Spent supernatant was removed, and biofilms were washed 3 times in 1X PBS, fixed with 100% ethanol, stained with crystal violet, and again washed 3 times with PBS. Biomass was assessed by resuspending biofilms in isopropanol and measuring absorbance at 595 nm using a Tecan Infinity 200M plate reader. Uncoated microtiter plate biofilm assays were performed as previously described [48].

All biofilm additives were added at time zero at concentrations that did not inhibit growth (data not shown). Antibiotics were used to maintain plasmids as necessary. pOS1-phrtAB rot was induced with 0, 0.5, 2 and 5 µM hemin (Sigma). 1,10-Phenanthroline monohydrate (1,10-PA), 3,4-Dichloro-isocoumarin (DIC), and E-64 (Sigma) were each used at a concentration of 10 µM. Purified MBP, MBP-ScpB, and MBP-SspC were used at a final concentration of 400 nM.

Flow cell biofilms

Flow cell biofilm formation was assessed as previously described [48]. Bacteria were grown in 2% TSB supplemented with 0.2% glucose in flow cell chambers for 48 hours. When required for plasmid maintenance, 5 µg/ml Cam was added to the growth
media. Biofilms were post-stained with SYTO-9 to detect biomass and confocal laser scanning microscopy (CLSM) was performed on a Nikon Eclipse E600 microscope using the Radiance 2100 image capturing system (Biorad). Image acquisition was performed with the Laser Sharp 2000 software (Zeiss) and images were processed using Velocity software (Improvision).

**RNAIII reporter assay**

A *S. aureus* LAC reporter strain containing pAmiAgrP3 (*agrP3-lux*) [146] was grown overnight in TSB supplemented with Cam and sub-cultured in 25 ml to an OD\textsubscript{600} of 0.1. 200 µl samples were collected throughout a 24 hr. time course and measured for luminescence in a microtiter plate (Corning) using a Tecan Infinity 200M plate reader.

**Protein preparation and western blotting**

*S. aureus* Rot protein was derived from whole cell bacterial lysates. To determine relative quantities of Rot protein in bacterial cultures grown under differing conditions, cells were collected after 24 hr growth and standardized to an OD\textsubscript{600} of 1.5. For cells collected from mature biofilms, spent supernatant was removed from microtiter wells and the biofilm was subsequently resuspended in 200 µl of fresh biofilm media. Multiple biofilm cultures from separate wells were pooled prior to measuring light absorbance. Protein was collected from whole cell lysates by Trichloroacetic acid (TCA) precipitation. Similarly, spent supernatants were collected from 24 hr. cultures and TCA precipitated to analyze protease protein content.

TCA precipitated samples were mixed with SDS-PAGE loading buffer and 5 µl of each sample was electrophoresed on a 12% polyacrylamide gel. The proteins were transferred to Immobilon-P PVDF membranes (Millipore) using a Protean II device (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked overnight at 4°C with 5% milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.0, with 137 mM NaCl) containing
0.1% Tween 20 (TBST). Primary antibodies were diluted in 5% milk in TBST and incubated with the membranes at room temperature for 2 hr. Membranes were rinsed 3 times in TBST and washed with agitation for 15 min, followed by two washes for 5 min. Secondary HRP-conjugated goat anti-chicken IgG (or goat anti-rabbit) was diluted 1:20,000 in 5% milk in TBST and incubated at room temperature for 1 hr. The membrane was rinsed and washed again as above. SuperSignal West Pico chemiluminescent substrate was added for 5 min at room temperature followed by exposure to X-ray film. For quantification of Rot protein, the X-ray film was scanned and densitometry analysis performed using ImageJ software. Polyclonal rabbit anti-Aur and anti-SspA were generously provided by Dr. Martin McGavin and used at 1:2,500 or 1:5,000, respectively. Polyclonal chicken anti-SspB was generated as previously described [144] and used at concentrations of 1:2,000.

**Protease activity assays**

Protease activity was monitored using milk agar, and three unique peptide substrates. To prepare samples for measurement of protease activity, cultures were grown overnight in TSB at 37°C. For milk plate assays, 2 µl of overnight culture was placed onto dried milk plates. Milk plates consisted of 5% non-fat dry milk and 3% Bacto agar. To prepare samples to measure protease activity using peptide substrates, overnight cultures were sub-cultured to an OD$_{600}$ of 0.1 and allowed to grow for an additional 24 hrs. Spent media was collected throughout the time course and filtered through 0.22 µm Costar Spin-X centrifuge tube filters (Corning, NY). Fluorescence measurements (excitation 490 nm, emission 520 nm) were obtained at 37°C in a Tecan Infinity 200M plate reader.

A Fluorescence Resonance Energy Transfer (FRET) assay was used to examine protease activity. The FRET substrate (5-FAM-Lys-Lys-Ala-Ala-Glu-Ala-Ser-Lys-
(QXL520)-OH; AnaSpec, Fremont, CA) is based on a known SspA peptide substrate but is also cleaved to a lesser extent by Aur and ScpA [144]. Similarly, a FRET substrate assay was developed to measure ScpA activity. The FRET substrate (5-FAM-Lys-Leu-Leu-Asp-Ala-Ala-Pro-Lys(QXL-520)-OH; AnaSpec) is based on the known ScpA cleavage substrate CXCR2 [84]. The substrate was resuspended to 50 µM using 20 mM Tris pH 7.4. Spent media samples were mixed 1:1 with the FRET substrate and fluorescence measurements (excitation 490 nm, emission 520 nm) were obtained at 37°C in a Tecan Infinity 200M plate reader. Substrate specificity was measured using a combination of purified enzymes and protease mutational analyses. Purified Aur, SspB and ScpA were purchased from BioCentrum (Krakow, Poland), and purified SspA was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Our results suggest that among the S. aureus secreted proteases only ScpA readily cleaves this substrate (data not shown). SspB protease activity was measured using the synthetic chromogenic substrate Bz-Pro-Phe-Arg-pNA (Bachem, Torrance, CA) as described [61].

**Mouse model of S. aureus catheter-associated biofilm infection**

S. aureus catheter-associated biofilm infections were established as previously described [126, 147]. Briefly, male C57BL/6 mice (6-8 weeks of age) were obtained from Charles River Laboratories (Frederick, MD), anesthetized with tribromoethanol (Avertin) and the skin was shaved and cleaned with povidone-iodine. A small s.c. incision was made in the flank and a blunt probe was used to create a pocket for insertion of a sterile 14-gauge teflon i.v. catheter 1 cm in length (Excel International, St. Petersburg, FL). Incisions were sealed using Vetbond Tissue Adhesive (3M, St. Paul, MN), whereupon $10^3$ cfu of USA300 LAC, or its isogenic mutants USA300 LAC $\Delta$rot::spec were slowly injected through the skin, directly into the catheter lumen in a volume of 20 µl sterile
PBS. Beginning on day 1 post-infection, animals received antibiotic treatment (rifampicin and daptomycin; 0.125 and 0.25 mg/kg, respectively) twice per day until animals were sacrificed at the appropriate time points for determination of bacterial burdens.

**Recovery of catheters and surrounding tissues for S. aureus enumeration**

Mice were euthanized at days 3 and 7 post-infection with an overdose of inhaled isoflurane. Catheters were removed and sonicated in 1 ml PBS to dissociate bacteria from the catheter surface. The heart, kidney, and tissues surrounding the infected catheters were collected, weighed, and disrupted in 500 µl homogenization buffer (PBS supplemented with 100 µl RNasin and a protease inhibitor tablet [Roche Diagnostics, Indianapolis, IN]) using a Bullet Blender (Next Advance, Averill Park, NY). Bacterial titers associated with catheters and surrounding tissues were quantified on tryptic soy agar plates supplemented with 5% sheep blood (HemoStat Laboratories, Dixon, CA) and expressed as Log_{10} cfu per milliliter for catheters or Log_{10} cfu per gram wet tissue weight.

**Statistical analysis**

Significant differences between experimental groups were determined using Student’s *t*-test with Welch’s correction for unequal variances (GraphPad Prism 4.02, GraphPad Software, Inc., La Jolla, CA). For all analyses, a *p*-value of less than 0.05 was considered statistically significant.

**Results**

**Rot is essential for biofilm formation**

For our studies, we constructed a Δrot mutant in the community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain LAC background. This clinical isolate is of the pulse-field gel electrophoresis (PFGE)-type USA300, the predominant CA-
MRSA strain in the United States, and USA300 strains are known causes of chronic biofilm infections [148, 149]. We also constructed a complementation plasmid with the rot gene constitutively expressed using the lgt promoter [150]. The LAC-WT, Δrot mutant, and complemented strains were compared in both plasma-coated and uncoated biofilm experiments. The Δrot mutant displayed a marked defect in biofilm capacity on both a plasma-coated surface (Figure 3-1A) and uncoated surface (Figure 3-1B). In each case, biofilm formation could be restored through complementation of rot. To confirm our observations, the biofilm experiments were repeated in a flow cell assay. Biofilms were grown for 2 days on uncoated coverslips and post-stained with SYTO-9, and to visualize biomass, the biofilms were imaged with confocal laser scanning microscopy (CLSM). Similar to as in the microtiter assays, WT S. aureus formed a thick, confluent biofilm (Figure 3-1C). In contrast, the isogenic Δrot mutant was unable to form a biofilm (Figure 3-1D), and biofilm formation could be restored to WT levels upon rot complementation (Figure 3-1E). Taken together, a S. aureus Δrot mutant is unable to form a biofilm in standard in vitro assays.

**Rot expression modulates biofilm formation**

We hypothesized that intracellular levels of Rot would be increased under conditions that favored biofilm formation. Glucose is usually added to media to promote S. aureus biofilm formation, and it is well established that the large pH decrease from glucose metabolism represses the agr quorum-sensing system [42, 151]. With reduced agr function and RNAIII levels, it seems probable that Rot production would be increased. To test this question, we grew an agr P3-lux reporter strain in TSB for 24 hours and collected samples throughout the time course. As shown in Figure 3-2A, agr gene expression peaked in late log/early stationary phase and rapidly decreased during stationary phase. In addition, Rot protein concentration inversely correlated with RNAIII
expression, which taken along with published data suggests that RNAIII repression of Rot translation is the major regulator of Rot production.

To test whether Rot levels increased under biofilm growth, we grew LAC-WT, $\Delta agr$, and $\Delta rot$ strains under three separate conditions. Rot production in a biofilm was assessed with either growth in biofilm-promoting media ("TSBg" TSB supplemented with 0.5% glucose and 3% NaCl) or the direct collection of biofilm biomass. For comparison, Rot levels were compared under planktonic conditions in TSB broth culture. In each condition, cells were grown for 24 hours and normalized, and Rot levels were determined with immunoblot and quantified by densitometry. Under planktonic conditions, low levels of Rot were detected in LAC-WT as compared with an $\Delta agr$ mutant, indicating that the $agr$ system was active as anticipated and Rot translation was repressed (Figure 3-2B). In contrast, when $S. aureus$ was grown in biofilm media, or when a biofilm was established, Rot protein concentration was high in both the LAC-WT and $\Delta agr$ mutant strains. Thus, as the $agr$ system is repressed to form a biofilm, Rot levels increase accordingly.

To determine whether the intracellular levels of Rot can be altered to modulate biofilm capacity, a hemin-inducible rot expression plasmid was constructed and transformed into a $\Delta rot$ strain. Biofilm formation was assessed under varying sub-inhibitory concentrations of hemin. At low hemin concentrations, the $\Delta rot$ mutant was unable to form a biofilm as expected compared to LAC-WT (Figure 3-2C). At high hemin levels, the biofilm capacity increased and exceeded WT levels. Altogether, these data suggest that the intracellular concentration of Rot modulates biofilm formation.

**Protease gene expression is upregulated in $\Delta rot$**

To define the mechanism by which Rot regulates biofilm formation, we used microarray technology to examine gene expression in WT compared with isogenic $\Delta agr$
and $\Delta rot$ strains. In general, $rot$ and $agr$ had an opposite regulatory scheme (Torres lab unpublished data). As such, transcripts encoding secreted proteins were highly upregulated in a $rot$ mutant and downregulated in an $agr$ mutant. The opposite was seen with numerous cell-associated adhesins, as has been observed in previous studies [113].

Expression from secreted protease operons was up-regulated in the $\Delta rot$ mutant. Protease upregulation could explain the lack of biofilm formation by this strain. To confirm our microarray results, we constructed protease transcriptional reporter fusions using green fluorescent protein (GFP) to monitor transcription. The resulting constructs were transformed into WT, $\Delta agr$, and $\Delta rot$ strains, and growth and fluorescence was monitored throughout a time course. As shown in Figure 3-3, expression from all four promoter elements ($P_{aur}$, $P_{ssp}$, $P_{spl}$, $P_{scp}$) was increased in $\Delta rot$. However, it appears that there may be differences in the amount of Rot-mediated transcription across protease operons, as the $scp$ promoter was only slightly upregulated in $\Delta rot$ compared with WT. Overall, these data show that secreted protease gene expression is inhibited by Rot.

**Production and activity of secreted proteases is increased in $\Delta rot$**

To expand our studies of Rot regulation, we examined global differences in protein concentration between WT, $\Delta agr$, and $\Delta rot$ strains using 2D-gel electrophoresis. Similar to our microarray analysis, compared with WT, there was an increase in the amount of exoproteins produced by $\Delta rot$ (Torres lab unpublished data) and in the abundance of secreted proteases compared with WT (Torres lab unpublished data). To follow-up on these studies, we examined Aur, SspA, and SspB protease production in WT, $\Delta rot$, and the complemented strain by immunoblot (Figure 3-4A-C). In each case, protease concentration was higher in the $\Delta rot$ mutant compared with WT and completely absent in a complemented strain. Interestingly, the fully processed form of SspB, which
is 3-4 times more active than the pro-form, can only be observed in spent media from \( \Delta \text{rot} \) (Figure 3-4C) [61]. Furthermore, SspA production was examined across \( S. \text{aureus} \) strain lineages encompassing USA PFGE types 100, 400, 500, 700 and 800. While the levels of SspA varied across strain type, in each case the concentration of SspA was increased in \( \Delta \text{rot} \) compared with their WT parent strains (Figure 3-4D).

The increased production of secreted proteases suggests higher levels of protease activity in spent media. As a broad measure of overall protease activity, we utilized a fluorescent-resonance energy transfer (FRET)-based substrate that is susceptible to cleavage by the proteases Aur, SspA, and ScpA [144]. Spent media from a \( \Delta \text{rot} \) mutant showed greater protease activity than LAC-WT and \( \Delta \text{rot} \) complemented strain (Figure 3-5A). Similar results were obtained using milk agar assays (data not shown). Based on our previous studies, the Staphopain enzymes are important modulators of \( S. \text{aureus} \) biofilm formation [144]. Staphopain A (ScpA) levels were measured using a specific FRET substrate based on the CXCR2 cleavage site [84], and surprisingly we observed only a minor increase in ScpA activity from \( \Delta \text{rot} \) spent media (Figure 3-5B). In contrast, Staphopain B (SspB) showed a more pronounced increase in a \( \Delta \text{rot} \) mutant versus LAC-WT using a specific pNA substrate (Figure 3-5C). Taken altogether, our results suggest that secreted protease expression, production, and activity is increased in \( \Delta \text{rot} \), and of the Staphopain enzymes, SspB upregulation is more pronounced.

**Inhibition of proteases restores biofilm capacity to \( \Delta \text{rot} \) mutants**

We hypothesized that it was the upregulation of secreted proteases in a \( \Delta \text{rot} \) mutant that was detrimental to biofilm formation. To test this hypothesis, protease inhibitors were assessed for their capacity to restore \( \Delta \text{rot} \) biofilm formation. The chemical inhibitors selected for our studies were metalloprotease inhibitor 1,10-
Phenanthroline (PA), serine protease inhibitor Dichloroisoucoumarin (DIC), and cysteine protease inhibitor E-64. In addition, the Staphostatin inhibitory proteins were purified and they selectively inhibit the Staphopain cysteine proteases [144]. Each inhibitor was added at sub-inhibitory concentrations to a Δrot mutant at time zero and biofilm formation was assessed after 24 hours. As shown in Figure 3-6A, only the general cysteine protease inhibitor E-64 and the SspC fusion protein were able to significantly restore biofilm forming capacity. This suggests that the Staphopains, and in particular SspB, are detrimental to biofilm formation in the Δrot mutant.

As a companion genetic approach, we tested whether protease mutations introduced into the Δrot background could restore biofilm capacity. We transduced Δrot into a collection of protease mutant strains that includes both individual and combination mutants in protease operons. As shown in Figure 3-6B, mutation of the aur, sspB, and scpA genes, but not the spl operon, significantly restored Δrot mutant biofilm formation (Figure 3-6B). Mutation of both staphopain genes (scpA and sspB), or inactivation of all protease genes, significantly restored Δrot biofilm formation to levels of biomass similar to that of the sspB mutant (Figure 3-6B). Thus, genetic and biochemical protease inhibition experiments indicate that proteases are the predominant players in the Δrot biofilm phenotype.

**Rot binds secreted protease promoters**

Since Rot is a DNA-binding global regulator of virulence factors, we hypothesized that it directly binds protease gene promoters to inhibit gene expression. To test this question, we PCR amplified the promoter regions of each of the four secreted protease operons and tested the ability of purified Rot protein to interact with these regions in electrophoretic mobility shift assays (EMSAs). As shown in Figure 3-7, the addition of increasing concentrations of Rot to labeled promoter DNA resulted in a shift
product compared with a no protein control. These interactions were specific in that increasing amounts of unlabeled promoter DNA could prevent formation of the shift product. Similar concentrations of cDNA (control DNA) were unable to compete with Rot binding to labeled protease promoters. These data suggest that Rot directly binds secreted protease promoters, and when combined with our expression data, that this binding inhibits protease gene expression.

**Δrot is attenuated in a mouse model of catheter infection**

Based on our results, we speculated that Δrot would be attenuated in a mouse model of biofilm infection. To test whether this was the case, we utilized an established model of *S. aureus* catheter-associated biofilm infection in which 1 cm catheters are inserted into the flank of C57BL/6 mice and $10^3$ CFUs of WT or Δrot were injected into the catheter lumen [32, 126]. Beginning 24 hours post-infection, animals receive antibiotic treatment twice daily for the duration of the experiment. Animals were sacrificed three and seven days post-infection and bacterial burden in the catheter and surrounding tissue was quantified. Compared with WT, Δrot had significantly lower bacterial burden in both the catheter and surrounding tissue at both time points examined (Figure 3-8).

**Discussion**

Since the establishment of *S. aureus* chronic infection is often due to biofilm formation, there is increasing interest in how regulatory factors coordinate the biofilm developmental process. Studies examining *S. aureus* biofilm regulation have pointed to several key regulators including *sarA, sigB,* and *agr* [93-95]. Often these regulators modulate each other's expression to impact biofilm formation, as is the case with SigB inhibition of *agr* function [102]. The *agr* system induces expression of secreted proteases
to inhibit biofilms, however, the molecular details of this cascade are not fully elucidated [42]. Based on previous studies [108, 109, 111, 113], the agr effector molecule RNAIII acts through a regulatory intermediate called Rot, whose translation is inhibited by RNAIII and is a known regulator of proteases.

In our current studies, we determined that rot was essential for biofilm formation (Figure 3-1) and that Rot intracellular levels modulate the biofilm forming capacity of S. aureus (Figure 3-2C). These levels appear to be dictated in large part by the agr system. We tracked RNAIII expression and found that levels inversely correlated with Rot protein concentration, as was expected (Figure 3-2A). Interestingly, when S. aureus cells were collected from a biofilm or when cells were grown under conditions that promote biofilm formation, such as in media supplemented with glucose, Rot levels in these cells were higher than with cells grown under planktonic conditions (Figure 3-2B). These findings mimic those of the essential biofilm regulator SarA, which is present at higher concentrations during biofilm growth [96]. In contrast, the agr system is significantly down-regulated in biofilms and this repressive effect is thought to be due to the sharp drop in extracellular pH that results from the supplementation of biofilm media with glucose [42, 151]. Our results fit with these previous studies in that mutation of agr increased Rot levels when cultures were grown in TSB but had no effect during biofilm conditions, in which agr activity would already be highly suppressed (Figure 3-2B). The mechanism of how agr is suppressed by pH is currently unknown and is in need of further investigation.

We took a comprehensive look at Rot regulation of proteases. First we observed increased quantities of protease transcripts in a rot mutant during our microarray studies (Torres lab unpublished data). Follow-up analysis using sGFP fusions to protease promoters clarified that Rot regulates each of the four secreted protease operons (Figure 3-3). In addition, we found that protease production and activity levels were higher in the
rot mutant and that Rot directly binds all four secreted protease promoters (Figures 3-4; 3-5; 3-7). The fact that Rot binds protease promoters is not surprising as previous work by Oscarsson et al. has shown that Rot binds the aur and ssp promoters [152]. These findings are significant in that they provide a direct link between the agr system and protease expression, such that agr activation results in RNAIII expression, RNAIII prevents Rot translation, and Rot directly represses protease gene expression (see Figure 1-4).

Rot inhibition of protease expression appears to be necessary for biofilm formation. Our studies found that biofilm formation could be restored to a Δrot mutant using the chemical protease inhibitor E-64, the biochemical inhibitor Staphostatin B (SspC), or through protease mutations (Figure 3-6). The chemical and biochemical inhibitor tests seem to point toward the cysteine proteases, and in particular Staphopain B (SspB), as being the biofilm inhibitory component. However, mutations in any secreted protease operon other than spl were able to significantly increase the biomass produced by Δrot mutants. It is difficult to determine the role of Aur and SspA in biofilm formation using mutational analyses since Aur is important for activation of SspA and SspA subsequently activates SspB [59, 61]. Therefore, deletion of aur results in a decrease in SspA and SspB activity while a deletion in SspA would also result in decreased SspB activity. Because inhibition of SspB, the last protease in the proteolytic cascade of activation, results in biofilm phenotypes we suspect that at least this protease is important in modulating biofilm formation. However, we cannot rule out Aur and SspA as also having biofilm roles independent of SspB activation.

Finally, we also observed that Δrot was attenuated in a murine catheter model of infection. Future studies will aim to determine whether this phenotype is due to the inability of the strain to form a biofilm. Rot is also an important regulator of immunomodulatory proteins [112] and these may also be important in protecting the
organism during catheter infection. The studies presented within identify exciting new roles for the transcriptional regulator Rot in maintenance of biofilm integrity.
### Table 3-1. Strains and plasmids used in Chapter III

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#### Plasmids

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Figure 3-1. Rot is essential for S. aureus biofilm formation. Biofilm formation by strain LAC (WT), Δrot mutant, and complemented strains in microtiter (A, B) and flow cell (C, D, E) biofilm assays. A. Biofilms on plasma-coated surface. B. Biofilms on uncoated surface. For A and B, color scheme is as follows: white, WT; red, Δrot; black, Δrot Comp (***P<0.001 relative to WT strain as determined by paired T-test). Flow cell biofilms contained C) WT, D) Δrot, and E) Δrot complemented strains. Biofilms were post-stained with SYTO-9 and imaged with confocal microscopy. Representative top-down images and three-dimensional image reconstructions from a z series are shown. Experiments were performed in triplicate.
A. Plasma-coated

B. Uncoated

C. WT

D. Δrot

E. Δrot / Comp
Figure 3-2. Rot expression is essential for biofilm formation. A. LAC (WT) containing the *agr* reporter plasmid pAmiAgrP3 was grown for 24 hours and samples were collected throughout a time course. Culture samples were standardized and whole cell bacterial lysates were used for immunoblot of Rot protein using an Anti-Rot antibody. Optical density measurements at 600 nm (black), luminescence (blue), and arbitrary units of Rot protein levels (red) as measured using densitometry are displayed. B. WT, Δ*agr* and Δ*rot* strains were grown overnight and sub-cultured in either TSB, biofilm media (TSBg) or under static biofilm conditions. Cultures were grown for 24 hr and cell cultures were collected and normalized an OD$_{600}$ of 1.5. Protein in whole cell lysates were precipitated with TCA for immunoblot of Rot protein. Arbitrary levels of Rot protein in each sample was measured using densitometry. C. LAC (WT) and Δ*rot* strains containing the hemin-inducible *rot* expression plasmid were analyzed for biofilm formation in the presence of 0, 0.5, 2, or 5 µM hemin. Color scheme is as follows: black, WT; red, Δ*rot*; blue, Δ*rot* P$_{{h}_{em}}$-rot (***P<0.001 relative to Δ*rot* strain as determined by paired T-test).
Figure 3-3. Transcription of secreted proteases is upregulated in Δrot.

Transcriptional regulation of protease operons by agr and rot. Promoters for each protease transcript (aur, ssp, spl, scp) were cloned in front of sGFP and transformed into WT, Δagr and Δrot strains. The constructed strains were grown 24 hr in TSB and GFP fluorescence intensity was determined.
Figure 3-4. Production of secreted proteases is increased in Δrot. A-C. Protein content from overnight cultures of WT, Δrot and Δrot containing the complementation plasmid pOS1 plgt rot (Δrot Comp) was examined by immunoblot using antibodies against Aur (A), SspA (B), and SspB (C). D. Immunoblot detection of SspA in spent media from parent or Δrot strains of the USA types 100, 300, 400, 500, 700 and 800.
Figure 3-5. Protease activity in spent media is higher in Δrot. Spent media samples were collected from WT, Δrot and Δrot Comp cultures grown in TSB throughout a 24 hour time-course. A. Overall protease activity was measured using 5-FAM-Lys-Ala-Ala-Glu-Ala-Ser-Lys-(QXL520)-OH, a FRET substrate that can be degraded by Aur, SspA and ScpA (Mootz et al. 2013). Activity against the substrate at 5 hours from the initial culture inoculation is shown. B. ScpA protease activity was measured using 5-FAM-Lys-Leu-Leu-Asp-Ala-Ala-Pro-Lys(QXL-520)-OH, a FRET substrate that is degraded specifically by the ScpA protease among S. aureus secreted proteases (data not shown). Activity against the substrate at 5 hours from the initial inoculation is shown. C. SspB protease activity from culture supernatants collected 24 hours from initial inoculation against the synthetic chromogenic substrate Bz-Pro-Phe-Arg-pNA.
Figure 3-6. Biochemical and genetic inhibition of Staphopains restores rot mutant biofilm capacity. Biofilm formation by strain LAC (WT), Δrot, and rot protease combination mutants were performed in a plasma-coated biofilm assay. A. Δrot biofilm formation was assessed in the presence of protease inhibitors at sub-inhibitory concentrations [10 µM] of either the metalloprotease inhibitor 1,10-phenanthroline, serine protease inhibitor 3,4-Dichloroisocoumarin (DIC) or cysteine protease inhibitor E-64. Δrot biofilm formation was also assessed in the presence of Staphostatin SspC or ScpB at 250 nM concentration. Red colored bars indicate conditions that include an inhibitor of SspB (*P<0.05, **P<0.01 relative to Δrot strain as determined by paired T-test). B. Biofilm formation of WT, Δrot and Δrot protease mutants was similarly assessed. Red colored bars indicate strains with sspB mutations (***P<0.001 relative to Δrot strain as determined by paired T-test).
Figure 3-7. Rot protein binds protease promoters. Protease promoter elements (Paur, PsplABCDEF, Pssp, and Pscp) were PCR amplified, and labeled, and incubated with varying concentrations of purified Rot protein (A) or with unlabeled competitor or cDNA (B). A. Promoter elements were shifted (black arrow) in an EMSA when in the presence of high concentration of Rot, and were not shifted at low concentrations (gray arrow). B. Unlabeled competitor DNA incubated in excess inhibited Rot binding to labeled promoter elements while control DNA (cDNA) was unable to inhibit similar Rot binding.
Figure 3-8. Rot is essential for biofilm formation in a murine catheter model of infection. 10³ cfu's of either WT or Δrot S. aureus were injected into the lumen of catheters inserted into the flanks of C57BL/6 mice to assess *in vivo* biofilm infection. Mice were sacrificed 3 (A) and 7 (B) days post-infection and bacterial burden in the catheter and surrounding tissue was assessed. Statistical analysis was performed using a Student’s *t*-test with Welch’s correction for unequal variances. For all analyses, a *p*-value of less than 0.05 was considered statistically significant.
CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

*S. aureus* is a common cause of numerous biofilm infections including osteomyelitis, endocarditis, and foreign body implant infections. These infections are particularly difficult to deal with due to their chronic nature and recalcitrance to antibiotics. Although the bacterial biofilm developmental process has been investigated in detail, the overall mechanism by which *S. aureus* forms and disassembles a biofilm is still in need of additional study. The overall goal of my research was to characterize the function of the secreted proteases in *S. aureus* biofilm development. In addition, we aimed to determine how these enzymes were regulated, with a focus on understanding how the *agr* system regulates protease gene expression and modulates biofilm integrity.

**S. aureus proteases and biofilms**

The aim of the studies presented in Chapter II was to determine the role of *S. aureus* secreted proteases in biofilm formation and disassembly. The *S. aureus* biofilm matrix is critical for maintaining biofilm integrity and is primarily composed of protein and eDNA. Previous work had shown that *S. aureus* biofilm development is controlled in large part by the *agr* quorum-sensing system, an inducer of proteases, and that *agr*-dependent biofilm disassembly could be prevented by inhibition of secreted proteases [42]. Furthermore, the alternative Sigma factor B (SigB) is essential for biofilm formation due to its repressive effect on the *agr* system and is also an inhibitor of protease gene expression [102]. We initially hypothesized that the ΔsigB biofilm-negative phenotype is dependent upon secreted proteases. Indeed, very low concentrations of ΔsigB spent supernatant could inhibit biofilm formation without deleterious growth effects on *S. aureus* (Figure 2-2B). The creation of strain AH1985 (Table 2-1), a complete knock-out of secreted proteases in the ΔsigB background, allowed us to examine whether the ΔsigB biofilm-negative phenotype was indeed
dependent upon proteases. This strain formed a biofilm with biomass similar to WT levels, suggesting that the sigB phenotype is in fact due to protease overproduction (Figure 2-4A). With this knowledge at hand, the ΔsigB mutant could be used to address the question of which proteases were responsible for the biofilm negative phenotype. Using chemical and biochemical inhibitors, as well as constructing protease mutations in the sigB mutant background, we demonstrated the Staphopain enzymes are inhibitors of S. aureus biofilm formation.

The roles of proteases and nuclease in modulating biofilms

One interesting feature of the ΔsigB experiments is that our group had previously identified secreted nuclease as a biofilm inhibitor in fractionated ΔsigB spent supernatant [48]. Although nuclease is overproduced in a ΔsigB mutation, the studies in Chapter II demonstrate that additional biofilm inhibitory factors are produced in this mutant. Early studies using uncoated flow cell biofilm assays showed that while some level of biofilm was restored to a ΔsigB Δnuc double mutant compared with ΔsigB, these biofilms lacked thickness and were patchy in appearance [48]. When a ΔsigB Δnuc mutant was tested in the plasma-coated biofilm assay, biomass levels were similar to that of ΔsigB, suggesting nuclease was not the cause of the biofilm negative phenotype in this assay (data not shown).

So why weren't secreted proteases found in the fractionation experiment? One potential answer is that both Staphopain A and Staphopain B have to be present to inhibit biofilm formation. The ΔsigB spent media was fractionated using cation-exchange chromatography. However, the Staphopains have quite different isoelectric points (Staphopain A, pI of 8.65, Staphopain B, pI of 4.57), and Staphopain B probably did not even stick to the column. Additionally, Staphopain A is not stable, and will be
completely inactive by 24 hrs. in spent media [84]. Coupling these synergy and stability issues together, it is not surprising that Staphopains were difficult to identify by fractionation.

**Staphopain concentrations within biofilms**

In Chapter II, we show that either Staphopain A or Staphopain B can inhibit biofilms at concentrations of 250 nM (Figure 2-7A). Even though the Staphopains were separated during ΔsigB fractionation, either enzyme added individually could have been identified as being biofilm inhibitory. This discrepancy could be explained if the concentration of Staphopain produced by ΔsigB was low enough that both enzymes would be required for biofilm inhibition. After all, our experiments suggest that these enzymes act in concert, such that when both are present the overall concentration required to inhibit biofilms is lower than either would be on its own (Figure 2-7A).

Though WT strains of *S. aureus* produce up to 250 nM Staphopain A during late log phase [84], this enzyme is quickly degraded to the extent that activity is completely lost 24 hours from the initial inoculation of TSB (Figure 2-6B) [84]. While Staphopain B appears to be more stable (Figure 2-6C), the concentration of mature active enzyme produced by WT cells has not been determined. ΔsigB produces more of each protease, however the amount of enzyme activity retained after 24 hours growth, followed by fractionation of spent media was not examined.

Although either Staphopain can inhibit biofilm formation at high concentrations, the amounts and activity of the Staphopains produced in biofilms still requires further attention. Our results showed that the amount of each enzyme is significantly repressed under biofilm growth conditions (Figure 2-5). Our experiments attempting to restore ΔsigB biofilm formation using biochemical inhibitors and protease mutations showed that when activity of only one of the Staphopains was inhibited, biofilm formation could still
occur (Figure 2-3C; 2-4A; 2-4B). To address the question of whether a single Staphopain could prevent biofilm formation, we initially developed a Staphopain A over-expression construct and tested for the impact on biofilm formation. Unexpectedly, the over-expression of Staphopain A had a minimal effect on biofilm capacity. As a second approach, a protease combination knockout strain that lacked Aur, SspAB, and Spl proteases, but retained Staphopain A, all in a ΔsigB mutant background, was constructed. Using activity assays, we determined that the levels of Staphopain A were even higher in this strain than the plasmid overexpression construct (data not shown). Biofilm capacity in this ΔsigB mutant, Staphopain A positive, strain was slightly reduced, although not to the levels of the ΔsigB mutant alone.

We attempted similar approaches with Staphopain B to investigate the contribution of this protease to biofilm phenotypes. The challenge with Staphopain B is that the enzyme is produced as a zymogen that needs to be processed by the Aur-SspA cascade to become fully active (Figure 1-3). We generated multiple Staphopain B constructs in which we removed the zymogen inhibitory domain by fusing the signal sequence to the C-terminal protease domain and expressed them in S. aureus. Although protein was produced by these constructs, none of them were active as measured by pNA substrate cleavage (data not shown). Together with the Staphopain A over-expression experiments, we were unable to show that enough of either protease is produced and active under biofilm conditions to completely abolish biofilm formation by itself.

The role of biofilm assays and growth conditions

Differences in surface chemistry in biofilm assays might also explain why proteases were not identified in the ΔsigB fractionation experiments. The nuclease experiments were all completed on abiotic surfaces, and for the protease studies, we utilized a microtiter plate assay in which wells are pre-coated in human plasma. These
types of assays have become increasingly popular due to the observation that foreign body implants quickly become coated in human matrix proteins [36, 37, 39, 126] and *S. aureus* prefers attaching to these proteins *in vivo* rather than the abiotic surface [40, 97]. Biofilm phenotypes seen with regulatory mutants in other biofilm assays could be recapitulated using this assay (Figure 2-2A).

In the plasma-coated biofilm assay, *S. aureus* proteases might be cleaving host proteins that are used as attachment factors, resulting in the loss of biofilm formation. Both Staphopain A and B degrade human fibrinogen, a major component of human plasma bound by *S. aureus* [82]. However, when repeating the same assay without plasma coating, the Staphopain proteases are still inhibitory to biofilms (Figure 2-4B).

Other than the surface coating, microtiter biofilm assays used herein and in previous reports differ in several growth conditions. Cells were grown in BHI or dilute TSB supplemented with 0.4% glucose for the nuclease experiments, while the protease microtiter experiments utilized BHI supplemented with 0.5% glucose and 3% NaCl [48, 144]. The type of media used can effect expression of both secreted enzymes, including proteases and surface adhesins. The high salt conditions used in the media for plasma coated biofilms could potentially repress the SaeRS two-component system, a positive regulator of nuclease expression [153]. Additionally, the inoculations used in each assay differed in that an initial inoculation of 1:1000 of an overnight culture was used in the nuclease experiments compared with 1:200 for the plasma coated experiments [48, 144]. The concentration of the initial inoculum has been shown to effect the 3D structure of the biofilm and therefore could impact biofilm matrix components [154]. Finally, the abiotic experiments were done with shaking for approximately 16 hours in 24 well microtiter plates while the bacteria in the plasma coated assays were grown statically for 24 hours in 96 well plates. Changes in oxygenation, sheer force, and the allotted time for biofilm accumulation could all influence biofilm composition [154].
Future directions for identifying Staphopain targets

The *S. aureus* biofilm matrix is made up of protein components whose integrity is essential for cellular encasement within the biofilm. Proteins that are generally localized to the cell surface, the extracellular environment, or the cytoplasm have all been identified in biofilm matrices. Due to the cellular lysis that occurs during biofilm formation [154, 155], it is difficult to distinguish between proteins important for matrix integrity and those found there by happenstance. To further complicate the matter, many adhesins have redundant roles and therefore the importance of a specific protein may be missed during targeted deletion or degradation. Adhesin redundancy might suggest that different proteins will be vital to maintaining biofilm integrity under different environmental conditions.

Even with these complications, many surface proteins have been identified with *S. aureus* biofilm roles. Of the adhesins that are covalently attached to the cell wall by Sortase A, SasC [50], SasG [51, 52], FnbpAB [47], Protein A [53], and ClfB [54] each have biofilm roles. Interestingly, the secreted proteases Aur and SspA cleave FnbpA and ClfB, respectively [49, 64]. SspA also inhibits biofilm formation *in vitro* [47], but does not appear to play a major role in our plasma-coated biofilm assays (Figure 2-2C).

*S. aureus* produces a large repertoire of surface-exposed proteins whose expression is likely dependent upon environmental conditions. To examine the entire protein content on the surface of cells at a given time ("surfacome"), many studies have taken a cell shaving approach in which bacteria are incubated with proteases to cleave proteins from the cell surface and subsequently identify them through mass spectrometry [156-158]. Due to the vast amount of biofilm matrix proteins, and redundancy observed between proteins, we plan to take a similar unbiased approach to identify proteins with biofilm roles that are targeted and degraded by Staphopains (see Figure 4-1). Large quantities of biofilm biomass will be collected and incubated with either Staphopain A,
Staphopain B, or both enzymes. Staphopain-treated biofilm cultures will be spun down and filtered to remove cellular debris and the processed proteins in the extracellular supernatants will be analyzed. Proteins present in the extracellular milieu of treated cultures, but absent in mock treated samples, will be further examined for their importance in biofilm formation using biofilm assays. Our initial attempts at cell shaving have been promising and have identified a sub-set of proteins that have roles in adhesion and that share a common binding domain. We are currently in the process of following up on these exciting results.

**Future directions for the role of Staphopains in S. aureus biofilm infection**

The experiments described in this work have shown that Staphopains are detrimental to biofilm integrity in vitro. However, no studies have examined their role during in vivo biofilm infections. It would be interesting to assess the role of Staphopains in the murine catheter biofilm infection model utilized in Chapter III. Using this model, a direct comparison could be made between WT, ΔsigB, and ΔsigB ΔscpA ΔsspB. Other potential experiments would be to treat established catheter biofilms with AIP and antibiotics to test whether biofilms could be disassembled in vivo. If this approach is successful, we could mix purified Staphostatins with the AIP/antibiotic treatment and examine whether biofilm disassembly is prevented. Finally, to examine the potential for Staphopain treatment of biofilms as a therapy option, we could treat biofilm infections with antibiotics alone, antibiotics with Staphopains, or Staphopains alone and examine disease outcome and catheter bacterial burdens. Staphopains have known virulence roles and therefore measuring multiple parameters of disease would be necessary.
Regulation of *S. aureus* biofilm formation

Biofilm development by *S. aureus* is a highly complex process, involving multiple stages that are tightly controlled by numerous regulatory pathways. In Chapter III we provide evidence that the transcriptional regulator Rot is an important modulator of biofilm formation. Rot is essential for the formation of *S. aureus* biofilms in multiple assays, and the concentration of Rot correlated with the amount of biomass produced. In addition, Rot was produced at higher levels during growth in a biofilm than when compared with planktonic growth. These biofilm attributes share striking similarity to another important regulator of biofilm formation, *sarA*. Rot and SarA are both members of the Sar family of transcriptional regulators \[111]\), and like SarA, Rot was also found to inhibit numerous secreted virulence factors including the secreted proteases (Figures 3-3; 3-4; 3-5). Inhibition of these enzymes is direct as Rot bound each of the four distinct secreted protease promoters (Figure 3-7). Importantly, inhibition of Δ*rot* protease activity through the use of chemical and molecular inhibitors, as well as protease gene deletions, highlighted the importance of these enzymes in the biofilm negative phenotype (Figure 3-6). Finally, *rot* was also identified as an important regulator *in vivo*, as the Δ*rot* mutant was attenuated in a murine catheter model of infection.

*Future directions for the placement of Rot in the biofilm regulatory pathway*

Our studies have led us to place Rot downstream of *sigB* and *agr* in the regulatory pathway responsible for controlling secreted protease expression for maintenance of biofilm integrity (Figure 1-4). While epistasis experiments have shown that *agr* acts downstream of *sigB* and is responsible for the *sigB* biofilm phenotype \[102]\), similar experiments need to be conducted to place *rot* in this scheme. RNAIII interacts with Rot by inhibiting its translation, thereby effecting protease expression \[108, 109]\). However,
it is possible that there are additional regulatory circuits downstream of RNAIII. An additional regulatory protein, SarT, is repressed by the agr system [159] and sarT mRNA may form a duplex with RNAIII [109]. SarT can be linked to regulation of secreted proteases through the sarS gene. SarT binds the sarS promoter element inducing expression of sarS [159] and SarS directly inhibits sspA [152]. Therefore, examination of the Δagr- Δrot double mutant in both protease and biofilm assays would address the question of a linear pathway through Rot, or whether there are other players in this process.

One of the most interesting aspects of agr regulation of biofilm development is that induction of the system with AIP can result in biofilm disassembly [42]. Presumably, this would be due to inhibition of Rot translation and subsequent protease de-repression. To follow-up on any agr-rot epistasis experiments, it would be interesting to try Rot depletion experiments in flow cell biofilm assays. Using the hemin-inducible rot construct (pOS1 phrtAB rot, see Table 3-1), we could run flow cell biofilm experiments with sub-inhibitory concentrations of hemin added to the growth medium. After mature biofilm formation, we could replace the growth media such that hemin is no longer present. This loss of Rot would result in protease de-repression and could result in biofilm disassembly.

**Future directions for the role of Rot in S. aureus biofilms**

Studies on the agr system have demonstrated mixed roles with respect to biofilm formation across S. aureus strain types. For example, agr mutations result in either no effect or a positive effect on biofilm formation [45, 142, 160]. These observations would suggest that the role of Rot in biofilm formation may also depend on the S. aureus strain type examined. Here, we utilized the USA300 strain LAC for all of our biofilm
experiments with rot. The USA300 lineage has high agr expression compared with typical S. aureus isolates [17], which would suggest that the amount of Rot protein is already comparably low in this strain. By mutating rot, we still were able to see a drastic change in production of proteases and the levels of biofilm formed (see Chapter III). One might expect that in strains with higher levels of Rot, these phenotypes may be even more pronounced, however those studies have not yet taken place. The production of SspA protease was examined across USA strain lineages in both parent and rot mutants, and in each case SspA production was higher whenever rot was absent (Figure 3-4D). In addition, our studies have shown that biofilms of USA strain lineages 100-800 are all susceptible to Staphopain A protease and are therefore dependent on protein for biofilm matrix integrity (Figure 2-8). Both Staphopain and biofilm levels could be examined across a collection of clinical isolates to address how conserved the Rot phenotype is in S. aureus.
**Figure 4-1. A cell shaving approach to identify Staphopain targets.** *S. aureus* LAC Δprotease (AH1919) strain will be grown in drip-flow reactors with biofilm-inducing media to collect large quantities of biofilm. Biomass will be collected and mechanically disrupted, spun down, and washed multiple times to release loosely bound material. Cells will be treated with 250 nM Staphopain, incubated for 30 minutes at 37°C, and reactions will be quenched with 5X E-64. Treated cells will be pelleted and supernatant filtered to collect cleaved protein components present in the supernatant. Proteins in the complex mixture will be separated by reverse-phase high-performance liquid chromatography and analyzed by mass spectrometry.
Collect biofilm biomass
Remove loosely bound debris
Protease treatment
FASP method
RP-HPLC ESI/MS/MS Database Search
Repeat with controls
Identify protease specific targets
REFERENCES


57. Nickerson N, Ip J, Passos DT, McGavin MJ: **Comparison of Staphopain A (ScpA) and B (SspB) precursor activation mechanisms reveals unique secretion kinetics of proSspB (Staphopain B), and a different interaction with its cognate Staphostatin, SspC.** *Mol Microbiol* 2010, 75(1):161-177.


