Hyaluronidase in Staphylococcus aureus physiology and pathogenesis

Carolyn Brook Ibberson

University of Iowa

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HYALURONIDASE IN STAPHYLOCOCCUS AUREUS PHYSIOLOGY AND PATHOGENESIS

by

Carolyn Brook Ibberson

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 2015

Thesis Supervisor: Associate Professor Alexander R. Horswill
This is to certify that the Ph.D. thesis of

Carolyn Brook Ibberson

has been approved by the Examining Committee for
the thesis requirement for the Doctor of Philosophy degree
in Microbiology at the August 2015 graduation.

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To my husband, family, and friends for their love and support.
You’ll miss the best things if you keep your eyes shut.

-Dr. Seuss
I Can Read With My Eyes Shut!
ACKNOWLEDGMENTS

I would like to first extend my deepest thanks to my family, friends, labmates, and professors who I have come to know over my years here. You have fostered an appreciation for science and have given me the encouragement I needed when challenges were presented. To my parents, thank you for teaching me to challenge ideas and encouraging me to pursue my interests. Thank you to the members of my thesis committee for all of your advice and suggestions about my project over the years. To Alex, thank you for allowing me to pursue the questions that interested me. I’d also like to thank you for your mentorship and encouragement throughout my graduate career. Thank you to the members of the Horswill lab, past and present. To Megan, thank you for your introduction to all things biofilm and your friendship during your time here and afterwards. To Jeff, thank you for teaching me to introduce rigor into my experiments and that more time points and conditions are always better. To Michael and Cherie, thank you for teaching me how the intricacies of staphylococcal genetics.

Lastly, I need to thank my wonderful husband, Jeremy. This is your Ph.D. as much as it is mine. You never doubted me and provided constant encouragement and support. Thank you.
ABSTRACT

*Staphylococcus aureus* encodes for a secreted hyaluronidase, *hysA*.

Hyaluronidases are bacterial enzymes that cleave hyaluronic acid (HA) at the $\beta$-1,4 glycosidic bond, yielding unsaturated disaccharides. Initially, little was known about the regulation of this enzyme as well as its roles in *S. aureus* physiology and pathogenesis. The goal of this dissertation was to determine the regulation of *hysA*, and to determine the biological and physiological roles of this enzyme.

Studies presented in Chapter II focus on determining the regulation of *hysA* and role of *hysA* in *S. aureus* pathogenesis. By screening the Nebraska Transposon Mutant Library (NTML) we identified 8 mutations that significantly altered HysA activity. Further analysis revealed that CodY directly represses *hysA* by binding to the CodY consensus binding sequence upstream of the *hysA* translational start site. Additionally, we found that a *hysA* mutant was attenuated in a neutropenic murine pneumonia model of infection and that there was reduced degradation of HA in the lungs of mice infected with the *hysA* mutant compared to wildtype by immunofluorescence.

Studies presented in Chapter III focus on examining if HA can be incorporated into the *S. aureus* biofilm matrix and if HysA is involved in biofilm dispersal. We show that HA is incorporated into the biofilm matrix both *in vitro* and *in vivo* by confocal microscopy and HA ELISA. Additionally, we found that HA can enhance biofilm formation of the *hysA* mutant as well as other staphylococcal species *in vitro*. We show that induction of *hysA* can prevent
biofilm formation in the presence of HA and that exogenous addition of purified HysA can disperse established HA containing biofilms. Finally, we found that a hysA mutant has reduced dissemination in an implant-associated infection model. Together these studies support our hypothesis that HA is incorporated into staphylococcal biofilms and that HysA is involved in dispersing S. aureus from the biofilm.
Staphylococcus aureus is a leading cause of bacterial infection in both hospital and community settings. The ability of S. aureus to cause a wide variety of infections is due in part to the large array of virulence factors it produces. The focus of my thesis was to determine the role of the secreted virulence factor, hyaluronidase, in S. aureus physiology and pathogenesis.

Hyaluronidases are bacterial enzymes that cleave hyaluronic acid, a main component of the mammalian extracellular matrix. My initial experiments investigated the regulation of the S. aureus hyaluronidase, encoded by the gene hysA. I found that hysA is repressed directly by the global regulator, CodY. In addition, we found that hysA was required for virulence in a mouse pneumonia model of infection.

Later studies investigated the role of hyaluronic acid in S. aureus biofilms. Biofilms are communities of cells encased in an extracellular matrix and attached to a surface. We found that S. aureus incorporates hyaluronic acid into the biofilm matrix both during infection and in the laboratory. Additionally, we found that HysA is involved in dispersing from biofilms and dissemination during infection.

Novel therapies for S. aureus infections must be developed as antibiotics lose efficacy. One option is the development of anti-virulence therapies that target virulence factors to weaken the bacteria to host defenses. My work shows that HysA is a versatile virulence factor that is involved in multiple aspects of S. aureus pathogenesis and lays groundwork for the development of treatments targeting hyaluronidase and similar spreading factors.
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CHAPTER I.
INTRODUCTION

*Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive, non-motile bacterium capable of causing a myriad of bacterial infections. The name is derived from its tendency to form grape-like clusters (“Staph”), spherical shape (“coccu”), and golden pigmentation (“aureus”) resulting from the production of staphyloxanthin [1]. *S. aureus* frequently colonizes the skin and mucosal surfaces of humans, but is most often harbored in the anterior nares [2]. Additionally, *S. aureus* causes a wide variety of infections including acute conditions such as skin and soft tissue infections, food poisoning, toxic shock syndrome, pneumonia, and sepsis as well as chronic biofilm-type infections such as osteomyelitis and implant-associated infections. *S. aureus* is also notorious for quickly gaining antibiotic resistance, often by horizontal transfer from other organisms [3].

*S. aureus lineages*

Many methods have been proposed to universally classify *S. aureus* lineages, but the most commonly accepted are pulse-field gel electrophoresis (PFGE), which assigns strains to established USA types, and multilocus sequence typing (MLST) which assigns strains to sequence type (ST) and clonal complex (CC) based on the sequence of seven housekeeping genes [4]. The USA type, clonal complex, and sequence type of many commonly used *S. aureus* isolates are described in Table 1-1. Historically, *methicillin-resistant S. aureus* (MRSA) has been mainly contained to nosocomial settings, particularly in intensive-care units (ICU) and long term care facilities [3, 5-7]. MRSA is
still one of the most common causes of bacterial nosocomial infection, accounting for 40-70% of the S. aureus infections in the ICU [8, 9]. Hospital-associated MRSA (HA-MRSA) infections are most commonly caused by strains of the USA100 and USA200 lineages [10-12]. These isolates typically colonize mucosal surfaces and produce low levels of cytolysins and produce toxic-shock syndrome toxin (TSST-1) at high levels [11]. More recently S. aureus has also emerged as a major cause of community-associated infections, which have been linked to the spread of an epidemic community associated methicillin resistant S. aureus (CA-MRSA) clone from the USA300 lineage [13-15]. In contrast to the HA-MRSA strains, CA-MRSA strains typically colonize the skin and produce Panton-Valentine leukocidin (PVL) and high levels of cytolysins [8, 10-12, 16].

S. aureus infections

Approximately 30% of the human population is persistently colonized asymptomatically [3, 7, 17-20], and up to 70% is transiently colonized [21] by S. aureus. Colonization by S. aureus is a major risk factor for infection with S. aureus [17-21]. Types of infections caused by S. aureus range from acute infections such as skin and soft tissue infections, pneumonia, food poisoning and sepsis, to more chronic infections such as osteomyelitis, implant-associated infections, and persistent bacteremia [22]. The success of S. aureus as a pathogen is due in large part to the extensive arsenal of virulence factors which include surface associated factors such as the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) and other adhesins, as well as secreted factors such as hemolysins, cytolysins, proteases, superantigens, and hyaluronidases [23, 24]. Many emerging MRSA isolates are
particularly known for causing tissue-destructive infections [3, 25], which is thought to be at least partially attributed to the large quantities of toxins and secreted enzymes produced by these isolates [26].

Another virulence factor of *S. aureus* is the ability to form a biofilm, particularly during chronic infections, such as osteomyelitis and implant-associated infections [27-29]. Implant associated infections are a growing problem and are a significant healthcare and economic burden [30-33]. Approximately 1 million nosocomial implant-associated infections occur each year in the United States alone [31, 33]. Staphylococci are the most common isolates, responsible for two-thirds of the infections of these devices. Treatment of this type of infection often requires long-term antibiotic therapy, and often ultimately results in surgical intervention [31].

---

*S. aureus* biofilms

A bacterial biofilm can be defined as a community of cells encased in an extracellular matrix. In the staphylococci, biofilm development (Fig. 1-1) is thought to occur in four stages: (1) attachment; (2) microcolony formation; (3) maturation; and (4) detachment. During the first stage, free-floating cells attach to an abiotic or biotic substratum such as a foreign body or host tissue. Following this phase, bacteria multiply to form microcolonies (Stage 2), which are defined as small aggregates of cells that contain some matrix material. It is often considered an intermediate stage of biofilm formation that links the attachment step with the mature biofilm, and it is sometimes not

---

1 Some of the sections in this chapter may also be found in Rosenthal CB, Mootz JM, Horswill AR. 2014. Staphylococcus aureus biofilm formation and inhibition *Antibiofilm Agents [Rumbaugh KP, Ahmad, I (eds.)], Springer Series on Biofilms* 8:233-255
considered a separate stage. However, it has been repeatedly observed through in vitro studies [34-36] and in clinical samples [37], and for these reasons we will consider the microcolony an independent stage. The continued growth of microcolonies and production of biofilm matrix components result in the significant accumulation of biomass and development of a mature biofilm (Stage 3). This stage has the characteristic surface structure often associated with bacterial biofilms, such as tower formation and water channels, and the cells display the maximal level of resistance to antimicrobials. Finally, mechanical and active mechanisms can trigger cellular detachment from the biofilm (Stage 4). During this stage, the biofilm matrix is typically targeted for degradation resulting in bacterial dissemination, which allows free-floating cells to reinitiate the biofilm development process at new sites. Detachment of the biofilm restores bacterial susceptibility to chemotherapies and is an active area of research interest [27, 38-40].

**Hyaluronic Acid**

Hyaluronic acid (HA) is a large glycosaminoglycan (GAG) composed of repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid (Fig. 1-2). Glycosaminoglycans are linear polysaccharides composed of disaccharides consisting of an amino sugar (N-acetylglucosamine, glucosamine that is variously N-substituted, or N-acetylgalactosamine) and a uronic acid (glucuronic acid or iduronic acid) or galactose [41]. In addition to HA, other members of the GAG family include chondroitin sulfate, dermatan sulfate, heparin, and keratan sulfate (Fig. 1-3)[41]. Although they share similarities, HA is distinct from the other GAGs in that it is non-sulfated, does not
contain a peptide in its backbone, and is synthesized in the plasma membrane [42, 43]. Additionally, the molecular weight of HA is typically much greater than the other GAGs, often reaching into the millions of Daltons [44]. Hyaluronic acid is a main component of the mammalian extracellular matrix and is abundant in the skin, synovial fluid, vitreous of the eye, skeletal tissues, umbilical cord, heart valves, lungs, kidneys, and brain [42]. The average adult human body contains 15 grams of HA, one third of which is turned over every day [45].

A main role of HA is to act as a physical barrier, controlling the migration of both host and invasive cells [42-44, 46]. One way HA accomplishes this is through its disproportionately high osmotic activity in relation to its molecular weight, allowing it to play a large role in water homeostasis. In addition, hydrogen bonding forms twists in the HA chains, creating hydrophobic patches which permit HA to nonspecifically interact with cell membranes and other lipid structures [42-44]. These properties give HA the ability to influence a large variety of cellular processes, as well as act as a main structural component of tissues [47].

In addition to the intrinsic properties of HA resulting from its unique chemical make up, HA plays a large role in many immune responses. It has been well established in the literature that HA has different roles depending on its molecular mass [42-44, 46]. High molecular weight HA inhibits endothelial cell growth, is anti-inflammatory, and immunosuppressive [42, 44, 46]. Additionally, there is increased production of high molecular weight HA at inflammation sites, corresponding to leukocyte adhesion and migration [46]. In contrast, low and mid molecular weight HA (20-450 kDa) has been shown to strongly promote inflammatory cytokines and adhesins, cell proliferation of
chondrocytes, endothelial cells and fibroblasts, as well as angiogenesis [43, 44, 46, 48, 49]. Even smaller HA oligomers (6-20 kDa) signal through the MyD88 pathway of Toll-like receptor (TLR) 2 and TLR 4 to activate the inflammatory response [43, 46, 49, 50], and are also immunostimulatory and potent activators of dendritic cells. Finally, HA oligomers of tetra and hexasaccharides, which are the primary products of degradation by vertebrate hyaluronidases, induce the expression of heat shock proteins and are anti-apoptotic [42, 44, 46, 49].

**Hyaluronidases**

In 1928 Duran-Reynals identified a factor present in testicular tissue and sperm, which he termed “spreading factor” for its ability to increase tissue permeability and enhance bacterial and viral infections [51, 52]. Multiple groups later demonstrated this spreading factor was due to the mucolytic enzyme hyaluronidase [53-59]. Hyaluronidases are a class of enzymes that primarily degrade HA, although some have a limited ability to also degrade chondroitin and chondroitin sulfate [47, 48]. There are three major groups of hyaluronidases, which differ in their enzymatic mechanism as well as the organisms by which they are produced. The first group, the mammalian hyaluronidases, are endo-β-N-acetyl-hexosaminidases that randomly hydrolyze the β-1,4 glycosidic bond [44, 47]. The mammalian enzymes can also hydrolyze the β-1,4 glycosidic bond of chondroitin and chondroitin-sulfate, albeit to a lesser extent [44]. Humans encode six hyaluronidase like genes, also called HYAL genes, which share about 40% identity with each other [44, 47, 48]. Each of the HYAL genes has a unique tissue distribution, although Hyal1 and Hyal2 are the major hyaluronidases expressed in human somatic tissues [44]. Mammalian
hyaluronidases have been implicated in a variety of host responses including HA metabolism and turnover, oncogenesis, and inducers of heat shock proteins [47]. In addition to their ability to cleave hyaluronic acid, mammalian hyaluronidases have been shown to be able to cross-link chains of HA, which may have a role in cell signaling [47].

The second group of hyaluronidases are produced by leeches and certain crustaceans, and cleave the β-1,3 glycosidic bond yielding mainly tetrasaccharides as the product of complete HA digestion. Little is known about their physiologic role of this group or of the mechanism of HA cleavage. Finally, the third group of hyaluronidases is the bacterial versions of these enzymes, which will be discussed in more detail in the next section. In addition to these three groups, hyaluronidases are also often founds in many types of animal venoms, including the venom of snakes [60], bees [61], stonefish [62], scorpions [63], spiders [64], and hornets [65]. Venom hyaluronidases cause local tissue damage, and the spreading property of the enzyme is thought to be a critical event in spreading the toxins from the injection site to systemic circulation [44, 60]. These enzymes are not well studied and their enzymatic mechanisms vary widely between species [44].

**Bacterial hyaluronidases**

In contrast to the mammalian hyaluronidases, bacterial hyaluronidases (a.k.a. hyaluronate lyases), cleave the β-1,4 glycosidic bond of hyaluronic acid by β-elimination in a processive manner [47]. This results in unsaturated disaccharides as the final product of complete digestion, with an unsaturation in the carbon ring of glucuronic acid (Fig. 1-4) [66]. Of the bacterial hyaluronidases, the most well studied enzymatically are those produced by *Streptococcus pneumoniae* and *Streptococcus agalactiae*, encoded by the
genes hyl and hylB respectively [67-70]. Both of these enzymes have been crystalized and their structures have been solved [71-74]. Figure 1-5A&B show the S. aureus hyaluronidase that has been threaded through the solved structure of S. agalactiae HylB, which is closely related to the S. aureus enzyme. These enzymes are composed of two large domains, the α-domain which is entirely α-helices and the β-domain which is almost entirely β-sheets. These domains are joined by a short linker to form a pocket where HA binds and is cleaved. The β-elimination mechanism of HA cleavage has been investigated in depth, and requires a tyrosine and a histidine in the catalytic site (Fig.1-5C), although the tyrosine seems to be more critical [69, 75]. These residues are conserved in all three enzymes as shown in the alignment in Figure 1-5D.

These enzymes have been implicated as important virulence factors since they were first characterized as “spreading factor” produced by “invasive” staphylococci in 1933 by Duran-Reynals [76]. This “spreading factor” was found to promote the spread of staphylococci, India Ink, and a non-infectious virus in a rabbit skin infection model. Later studies by multiple groups confirmed that this staphylococcal spreading factor was hyaluronidase [55, 58, 76]. However, this was somewhat contentious as Hobby et al. demonstrated that there was not complete overlap in the activities of hyaluronidase and spreading factor, causing them to propose that “spreading factor” is composed of multiple different components that include hyaluronidase [77].

For example, µ toxin, a hyaluronidase of Clostridium perfringens, was shown to increase the penetration of α-toxin in a gas gangrene model of infection [78]. Additionally, hyaluronidase mutants in Streptococcus pyogenes and Staphylococcus aureus have reduced tissue penetration in a murine skin infection model [23, 79]. In
addition, a number of organisms have been shown to utilize HA as a sole carbon source, and there are increasing reports showing the ability to catabolize HA is important during infection by these organisms [79-81]. A recent paper in *Streptococcus pneumoniae* identified a PTS transport system and a glucuronyl hydrolase that in addition to the hyaluronidase are essential for the ability to grow on hyaluronic acid [81]. The ability to catabolize HA may be another way that hyaluronidases contribute to virulence by allowing for access to an additional carbon source and increasing the fitness of the bacteria.

**Staphylococcus aureus hyaluronidase**

*Staphylococcus aureus* is the only staphylococcal species that produces a hyaluronidase [82, 83], and the enzyme most closely related to those produced by *Streptococcus pneumoniae* and *Streptococcus agalactiae* [83]. Hyaluronidase, encoded by the gene *hysA*, is conserved across all clonal lineages of *S. aureus*. Additionally, USA200 (CC30) strains encode for a second hyaluronidase, *hysA2*, which shares 75.9% amino-acid identity across the length of the protein with *hysA*. Although there was much interest in hyaluronidase following its initial identification as a spreading factor, work on hyaluronidase stalled until the gene, *hysA*, was identified and sequenced [84]. Later work by Dr. Keith Holland’s lab found that a *hysA* mutant was attenuated in a murine skin infection model [23].

**Regulation of S. aureus hyaluronidase**

Very little work has been done to investigate the regulation of *hysA*. There have been multiple microarrays done in *S. aureus* to determine the regulon of global regulators and no consensus has been reached for potential regulators of *hysA* [85]
In targeted studies, there was some evidence that SarA may repress \textit{hysA} and that the \textit{agr} quorum-sensing system may activate \textit{hysA} gene expression [23, 86], although this was not shown conclusively.

As evidenced in the literature, the regulation of \textit{hysA} and the biological and physiological roles of this enzyme have not been well examined. In this dissertation I characterize \textit{S. aureus} hyaluronidase to better understand the contribution of \textit{hysA} in \textit{S. aureus} physiology and pathogenesis. In Chapter II, I focus on the regulation of \textit{hysA} and the role of \textit{hysA} during acute pneumonia. In Chapter III, studies were performed to understand the contribution of \textit{hysA} and hyaluronic acid to \textit{S. aureus} biofilm formation. As a whole, this work describes the regulation of \textit{hysA} and provides insight into the role of HysA during infection and during biofilm formation.
Table 1-1. Characteristics of common *S. aureus* lineages

<table>
<thead>
<tr>
<th>Common Stains</th>
<th>USA Type</th>
<th>MLST</th>
<th>Clonal Complex</th>
<th>MSSA or MRSA?</th>
<th>SCCmec Type</th>
<th>Agr Type</th>
<th>Hyaluronidases</th>
</tr>
</thead>
<tbody>
<tr>
<td>N315</td>
<td>USA100</td>
<td>ST5</td>
<td>CC5</td>
<td>MRSA</td>
<td>II</td>
<td>II</td>
<td>hysA</td>
</tr>
<tr>
<td>Mu50</td>
<td>USA100</td>
<td>ST5</td>
<td>CC1</td>
<td>MRSA</td>
<td>II</td>
<td>II</td>
<td>hysA</td>
</tr>
<tr>
<td>MN8</td>
<td>USA200</td>
<td>ST36</td>
<td>CC30</td>
<td>MRSA</td>
<td>II</td>
<td>III</td>
<td>hysA1, hysA2</td>
</tr>
<tr>
<td>MRSA252</td>
<td>USA200</td>
<td>ST36</td>
<td>CC30</td>
<td>MRSA</td>
<td>II</td>
<td>III</td>
<td>hysA1, hysA2</td>
</tr>
<tr>
<td>UAMS-1</td>
<td>USA200</td>
<td>ST36</td>
<td>CC30</td>
<td>MRSA</td>
<td>II</td>
<td>III</td>
<td>hysA1, hysA2</td>
</tr>
<tr>
<td>EMRSA16</td>
<td>USA200</td>
<td>ST36</td>
<td>CC30</td>
<td>MRSA</td>
<td>II</td>
<td>III</td>
<td>hysA1, hysA2</td>
</tr>
<tr>
<td>LAC</td>
<td>USA300</td>
<td>ST8</td>
<td>CC8</td>
<td>MRSA</td>
<td>IV</td>
<td>I</td>
<td>hysA</td>
</tr>
<tr>
<td>Col</td>
<td>USA300</td>
<td>ST250</td>
<td>CC8</td>
<td>HA-MRSA</td>
<td>I</td>
<td>I</td>
<td>hysA</td>
</tr>
<tr>
<td>NCTC8325</td>
<td>ST8</td>
<td>CC8</td>
<td>MSSA</td>
<td>N/A</td>
<td>I</td>
<td>N/A</td>
<td>hysA</td>
</tr>
<tr>
<td>Newman</td>
<td>ST8</td>
<td>CC8</td>
<td>MSSA</td>
<td>N/A</td>
<td>I</td>
<td>N/A</td>
<td>hysA</td>
</tr>
<tr>
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<td>USA400</td>
<td>ST1</td>
<td>CC1</td>
<td>CA-MRSA</td>
<td>IV</td>
<td>III</td>
<td>hysA</td>
</tr>
</tbody>
</table>
Figure 1-1 Model of *S. aureus* biofilm development.
Stage 1, a subpopulation of planktonic cells lyse and adhere to a conditioned surface using a combination of surface adhesins, eDNA, and PIA. Stage 2, the attached cells grow to a microcolony that begins to display some morphological features of a biofilm. Stage 3, the biomass accumulates and the structure matures into an established biofilm that displays the expected characteristics, such as tower formation and antimicrobial resistance. Stage 4, active (quorum-sensing) or mechanical mechanisms lead to biofilm detachment and a return to the planktonic state. Multiple factors are involved in the detachment phase, such as proteases and PSMs.
Figure 1-2 Structure of hyaluronic acid
The structure of hyaluronic acid (HA) is shown. HA is composed of repeating disaccharide units of N-acetylglucosamine (green) and glucuronic acid (red), which are linked by alternating β-1,3 (black), β-1,4 (blue) glycosidic bonds.
Figure 1-3 Structures of the glycosaminoglycans
The carbohydrate sequences of the five types of glycosaminoglycan chains. Possible sulfation presence and location (2S, 4S, or 6S) are indicated.
Figure 1-4 Cleavage of hyaluronic acid by bacterial hyaluronidases
Hyaluronic acid is cleaved by bacterial hyaluronidases by β-elimination at the β-1,4 glycosidic bond to yield unsaturated disaccharides as the final product of complete digestion. This is in contrast to the mammalian enzymes which hydrolyze the β-1,4 glycosidic bond which does not lead to an unsaturation.
Figure 1-5 Structure and alignment of HysA

(A&B) HysA was threaded through the solved S. agalactiae HylB structure. HysA is composed of two large domains, the α-domain (orange) and the β-domain (blue), which are joined by a short linker. HA is shown (red and green) and the catalytic residues are highlighted (magenta). (C) Zoomed in view of the catalytic cleft shows the His296 is in an a nucleophilic attacking position to C5 in glucuronic acid and Tyr306 is H-bonded to the glycosidic oxygen atom. His296 extracts the acidic C5 proton on glucuronic acid, resulting in a double bond between C4 and C5 Tyr306 then provides a proton to the glycosidic oxygen and the linkage is broken. (D) Alignment of the catalytic pocket of hyaluronidases from S. aureus, S. pneumoniae, and S. agalactiae. The catalytic residues are shown in red.
CHAPTER II.

STAPHYLOCOCCUS AUREUS HYALURONIDASE IS A CODY-REGULATED VIRULENCE FACTOR²

Introduction

*Staphylococcus aureus* is a leading cause of both hospital and community associated infections in the developed world, accounting for $14.5 billion dollars in healthcare costs annually in the United States alone [87]. These infections range from mild skin and soft tissue infections to potentially fatal infections such as pneumonia, endocarditis, osteomyelitis, sepsis, and toxic-shock syndrome [22]. Additionally, the increasing prevalence of methicillin-resistant *S. aureus* strains in both the hospital and community has exacerbated the problem [13, 16, 25]. *S. aureus* is a successful pathogen due to its extensive arsenal of virulence factors that consist of both surface associated proteins, such as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), as well as secreted proteins including hemolysins, immunomodulators, and a number of exo-enzymes [16, 22]. Many emerging MRSA isolates are known for causing tissue-destructive infections [3, 25], and it has been proposed that the excessive damage is due, in part, to the large quantities of toxins and secreted enzymes produced by these new isolates [26].

Our studies have focused on the exo-enzyme hyaluronidase (also called hyaluronate lyase) encoded by the gene *hysA* [82]. Hyaluronidases are bacterial enzymes

² The majority of the work found in Chapter II can be found in Ibberson, CB, Jones, CL, Singh, S, Wise, MC, Hart, ME, Zurawski, DV, and Horswill, AR. Infection and Immunity. 2014; 82(10): 4253-64.
that cleave the β-1,4 glycosidic bond of hyaluronic acid (HA), a high molecular weight polymer composed of repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid [43, 83]. Hyaluronic acid is synthesized and secreted from the plasma membrane of mammalian cells, and it is abundant in the skin, skeletal tissue, umbilical cord, lungs, heart valves, brain, and a number of other tissues [43, 46, 83]. Hyaluronic acid also possesses a multitude of functions for the host including providing structure to tissues, water homeostasis, cell proliferation, and as an immune regulator [43, 46]. Many of these tissues with high HA concentrations are frequently infected with S. aureus [22].

Cleavage of HA by bacterial hyaluronidases occurs by β-elimination of the β-1,4 glycosidic bond and results in unsaturated disaccharides as the product of complete digestion [83]. In contrast, the eukaryotic hyaluronidases hydrolyze the β-1,4 glycosidic bond, producing mostly tetra- and hexasaccharides as end products. For a number of Gram-positive organisms, hyaluronidases have been shown to be essential virulence factors for their ability to disseminate cells and virulence factors through tissue [83]. For example μ-toxin, a hyaluronidase, of Clostridium perfringens was found to significantly increase the ability of α-toxin to penetrate tissue [78]. Similarly, a mutation in the hyaluronidase of Streptococcus pyogenes showed reduced tissue penetration when compared to wild-type in a murine skin abscess model [79]. Additionally, there have been a number of structural studies on the hyaluronidases from Streptococcus pneumoniae and Streptococcus agalactiae [69, 75, 88], and the S. aureus enzyme is reported to have a similar structure to these streptococcal enzymes [83].

Reports of S. aureus hyaluronidase date back to pioneering 1933 studies performed by Duran-Reynals [76], who identified a “spreading factor” found in the spent
media of “invasive” *S. aureus* strains. This spreading factor increased lesion size in a rabbit skin infection model as measured by the spread of India Ink [76], and it could increase the size of lesions caused by other bacterial pathogens. Subsequent reports concluded that the enzyme hyaluronidase was responsible for the spreading factor activity [55, 57, 58, 89], but Hobby et al. suggested that other agents may contribute to the phenomena [77]. A number of follow-up studies investigated the enzymatic properties and expression of *S. aureus* hyaluronidase [90-92], but in the intervening years, progress stagnated until the *hysA* gene was identified and sequenced [84]. More recently, HysA enzyme was detected in proteomic studies [86] and shown to be required for increased wound size in a murine skin infection model [23]. Little is known about *hysA* regulation, although SarA is thought to repress *hysA* gene expression and there is some evidence that the *agr* quorum-sensing system induces expression [23, 93].

To expand our knowledge of HysA in *S. aureus*, we proceeded to investigate the regulation and the contribution of this enzyme to pathogenicity. We identified genes involved in the regulation of *hysA* utilizing a transposon mutagenesis library, and the most prominent of these was the global regulator CodY. We characterized the role of HysA in the pathogenesis of *S. aureus* infection using a murine pulmonary infection model, and we present evidence that HysA promotes virulence through the breakdown of hyaluronic acid during infection.
Materials and Methods

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are described in Table 2-1. All S. aureus strains were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA) with the appropriate antibiotics for plasmid maintenance or selection (ampicillin 100 µg/mL, chloramphenicol 10 µg/mL, erythromycin 10 µg/mL, tetracycline 0.625 µg/mL) at 37°C with shaking at 220 rpm with a flask to volume ratio of approximately 5:1 unless otherwise specified. Hyaluronic acid sodium salt purified from Streptococcus equi was purchased from Sigma-Aldrich. Plasmid DNA was purified from Escherichia coli and electroporated into S. aureus RN4220 as previously described [94]. Plasmids and transposon insertions were moved from S. aureus RN4220 or S. aureus JE2 with bacteriophage Φ11 to other S. aureus strains by transduction. All of the oligonucleotides used for this study can be found in Table 2-2.

The ΔhysA mutant was constructed using the pJB38-hysA, modified pKOR1 system, as described [95]. Briefly, 600-bp flanking regions upstream and downstream of hysA were amplified and joined by overlap PCR extension using genomic DNA from AH1263 as the template and oligonucleotides CBR12-CBR13 to generate the upstream fragment, and CBR14-CBR15 to generate the downstream fragment. The outermost primers (CBR12 and CBR15) were engineered to include SbfI and XmaI sites at their 5’ ends, respectively, and the inner primers (CBR13 and CBR14) were similarly engineered with XhoI and MluI sites at their 5’ ends to aid in the overlap extension. PCR products were ligated with T4 DNA Ligase (Invitrogen) into pJB38 at the ShfI and XmaI sites and electroporated into E. coli strain BW25141. Plasmid DNA was purified from this strain,
isolated using a High Speed Plasmid Mini-kit (IBI Scientific), and electroporated into RN4220. The plasmid construct was isolated from *S. aureus* RN4220 and transduced into AH1263 using bacteriophage Φ11. The *hysA* mutation was constructed on the chromosome as previously outlined in the pJB38 method [95]. The final colonies were screened for plasmid loss by antibiotic susceptibility and confirmed by PCR.

The complementing plasmid, pHysA (pCR01), was created by amplifying the *hysA* promoter region (~600 bp upstream of the putative translational start site) through the stop codon with the flanking primers CBR35 and CBR29. Amplified DNA containing the *hysA* coding sequence and the promoter region was then restriction digested with *Bam*HI-HF and *Nhe*I-HF (New England Biolabs) and ligated into linearized pSKerm-MCS plasmid DNA at the *Bam*HI and *Nhe*I sites. Ligated DNA was electroporated into *E. coli* strain BW25141 using standard molecular techniques.

To create the HysA overexpression construct pCR03, the *hysA* gene without the encoded signal sequence was amplified from AH1263 genomic DNA using primers CBR41 and CBR42, which contain *Nhe*I and *Xho*I sites at their 5’ ends, respectively. The resulting PCR product was ligated into pET28a linearized with *Nhe*I and *Xho*I and electroporated into *E. coli* strain ER2566 to generate strain AH2856.

To mutate the CodY binding box upstream of *hysA*, plasmids pCR04, pCR05, and pCR06 were constructed using overlap PCR to engineer the six nucleotide changes. A 5’ region introducing the mutations into the CodY box upstream of *hysA* was amplified using CBR35 and either CBR83, CBR85, or CBR87. A corresponding 3’ region using homologous primers to introduce the same mutations and amplify the remainder of the
hysA promoter region and hysA was generated using CBR29 and either CBR82, CBR84 or CBR86. The 5’ and 3’ fragments were mixed at a ratio of 1:1 in the combinations as follows: CBR35-CBR83:CBR82-CBR82, CBR35-CBR85:CBR84-CBR29, and CBR35-CBR87:CBR29-CBR86. Overlap PCR was done using the mixed fragments as a template and CBR29 and CBR35 to amplify hysA and the promoter region containing the mutated CodY boxes. pSKermMCS and the PCR product inserts were restriction digested with BamHI-HF and NheI-HF and ligated using T4 DNA Ligase. Ligations were transformed into E. coli strain DH5α as previously described. The resulting plasmids were confirmed by sequencing.

The spa mutant was generated using the same protocol as described above for deleting hysA. Flanking regions of spa (SAUSA300_0113) were amplified with the primer pairs spa_delA_EcoRI and spa_delB; and spa_delC and spa_delD_SalI. The products were fused using overlap extension PCR with the primers spa_delA_EcoRI and spa_delD_SalI. The resulting fragment was digested with EcoRI and SalI and ligated into pJB38. The spa deletion was confirmed by PCR with primers spa_upstream and spa_downstream. The spa codY double mutation construct was generated by transducing the codY::ΦNS cassette into S. aureus strain LAC Δspa with bacteriophage Φ11. To generate strain AH3207, the pTet marker switching plasmid was used to replace the erythromycin cassette of the codY::ΦNS transposon insertion in S. aureus strain AH2946 with a tetracycline cassette as described by Bose et al. [96]. This construct was then transduced by bacteriophage Φ11 into the spa deletion strain that also harbored the hysA::ΦNS transposon insertion.
**Protein purification**

AH2856 was grown overnight at 37°C in LB containing kanamycin (50 µg/mL), and subcultured into 1 L of fresh LB medium at a ratio of 1:250. The culture was grown at 30°C to mid-logarithmic phase, IPTG was added to 1 mM final concentration, and the culture was allowed to grow for an additional 4 h. Cells were harvested by centrifugation, washed once with water, pelleted by centrifugation, and frozen at -20°C. Cells were mechanically lysed using a Microfluidics Microfluidizer model LV1 (Newton, MA) at 25,000 psi and running the samples through the machine twice. Cell lysate was clarified by centrifugation for 20 min at 15,000 × g at 4 °C. Protein was purified using Fractogel His-Bind Resin (Novagen) as per the manufacturer’s instructions, dialyzed into PBS, and concentrated using Millipore Centrifugal Filter Unit (30,000 molecular weight cut-off), and brought to 10 mg/ml in 1X PBS. The protein suspension was frozen in 10% glycerol at -20°C until used.

**Transposon library screen**

The Nebraska Transposon Mutant Library (NTML) was grown overnight in TSB in 96-well microtiter plates. Cultures were stamped onto hyaluronic acid (HA) plates (TSB, 1% agarose, 0.4 mg/mL HA, 1% w/v BSA) prepared as described [83] and incubated at 37°C for 4 h. Acetic acid (10%) was added to the plates to visualize zones of clearing. Plates were scored for their hyaluronidase activity, and mutations that increased or decreased activity were rescreened in the same manner. Mutations that affected activity in both screens were quantitatively assessed for hyaluronidase activity by growing each strain overnight in TSB, sub-culturing to an OD₆₀₀ = 1.0, and dispensing the diluted culture as five aliquots (4 µL) onto HA plates. After 6 h of incubation, plates
were flooded with 10% acetic acid and zones resulting from HA cleavage were enumerated with a caliper.

**Hyaluronidase activity assay**

The hyaluronidase activity assay was performed as previously described [23] with some modifications. Briefly, *S. aureus* strains were grown overnight in TSB at 37°C with shaking, sub-cultured 1:1000, and grown at 37°C for the time specified. Spent culture media was isolated with Spin-X filters (0.22 µm) and frozen at -20°C until assayed. Hyaluronic acid (100 µL at 1 mg/mL) was mixed with 50 µL of spent media and allowed to react at 37°C for 15 min. The reaction was stopped by adding 25 µL of potassium tetraborate solution (0.8 M, pH 9.1), vortexed, and boiled for 3 minutes. In parallel, 12.5 µL of spent media was added to 31.25 µL of T=0 Stop Solution (hyaluronic acid 0.8 mg/mL, 0.8 M potassium tetraborate, pH 9.1), vortexed, and boiled for 3 min. The samples were dispensed into a 96-well microtiter plate in quadruplicate, and freshly prepared DMAB solution (10% (w/v) ρ-dimethylaminobenzaldehyde, 12.5% (v/v) 10 M HCl (Sigma-Aldrich), and 87.5% (v/v) glacial acetic acid (Fisher Scientific) was added to each well. The plate was incubated at 37°C for 20 min to allow the color reaction to take place. A TECAN Infinite M200 plate reader was used to measure the absorbance at 590 nm. Hyaluronidase specific activity is expressed as $10^3 \times \mu$mol N-acetylglucosamine (Sigma Aldrich) released ml⁻¹ min⁻¹ per OD₆₀₀ unit and is calculated by the equation:

$$10^3 \times 3 \times (1/15) \times (1/m) \times \Delta A_{590} \times (1/OD_{600})$$

where $\Delta A_{590}$ represents the difference in absorbance between the $t_{15}$ and $t_0$ readings for each sample, $m$ represents the slope of the standard curve of NAG, OD₆₀₀ is the optical
density of the culture when the sample was taken, 3 is the dilution factor of the sample tested in substrate, and 1/15 is the reciprocal of the reaction time allowed at 37°C. The assay was performed in triplicate for each condition tested.

For the RNAIII impact on hyaluronidase activity, strains with pALC2073 or pALC2073-RNAIII were grown overnight in TSB at 37°C and were sub-cultured 1:1000 into fresh TSB with anhydrotetracycline (aTc) at various concentrations. Cultures were grown for 8 hr, cells pelleted, and spent media was filtered and assayed for activity.

Limiting to rich media assays

Chemically defined medium (CDM) was made as described by Pohl et al. [97] except the group 6 vitamin solution was replaced with nicotinamide (500 µg/L), thiamine (500 µg/L), pantothenate (500 µg/L), and biotin (500 µg/L), final concentrations. Single amino acids were omitted or added in excess as indicated in the figure legends. The medium was buffered to pH 7.0. S. aureus strain LAC was grown overnight in TSB and subcultured 1:1000 into CDM or CDM lacking isoleucine. Bacteria were grown for 5 h at which point cells were pelleted, washed once with 1X PBS, and suspended into fresh CDM. For those cells grown in CDM lacking isoleucine, they were now suspended in fresh CDM containing 5X branched chain amino acids (isoleucine, valine, leucine). Cells were grown in the new medium for an additional two hours. Growth was monitored spectrophotometrically at 600 nm throughout by measuring the optical density of 100 µL of culture in a 96-well microtiter plate and a TECAN infinite M200 plate reader. Additionally, 200 µL of each culture was removed at the designated time points indicated in the figure legend, and spent media was isolated using 0.22 µm Spin-X Centrifuge Tube Filters (Costar). Spent media were used to determine hyaluronidase activity and the
presence or absence of PVL (see below).

**Protein Immunoblot for PVL**

Culture spent media isolated from cells grown in CDM, CDM lacking isoleucine, or CDM with 5X BCAA as described above (5 µL for 4 hr, or 1 µL for other time-points) were brought to a total volume of 10 µL in SDS-loading buffer containing 1% β-mercaptoethanol. Proteins were resolved by PAGE using Tricine/SDS gels containing 12% polyacrylamide and 170 kV. Proteins were transferred (160 mAmps for 1 h) onto nitrocellulose membranes and blocked overnight at 4°C in 0.1% Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% skim milk. The membrane was probed for using PVL, Luk-S polyclonal antibody (IBT Bioservices, Gaithersburg, MD) at 1:10,000 in TBST containing 5% skim milk for 2 hr at room temperature and then washed three times for 10 min each with TBST. Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied at 1:20,000 in blocking buffer at room temperature for one hr. Membranes were washed three times for 10 min each with 0.1% TBST and SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) was added to detect proteins followed by exposure to Classic X-Ray Film (Research Products International Corporation). Bands were quantified using ImageJ software.

**Protein Immunoblot Blot for HysA**

*S. aureus* strains AH3052, AH3134, and AH3207 were grown overnight in TSB at 37°C with shaking at 220 rpm. Strains were subcultured at a ratio of 1:100 into fresh TSB and grown for 8 hours at 37°C with shaking at 220 rpm. Cultures were normalized to an OD$_{600}$ = 3.0 with TSB, spent media were isolated by filtration using a 0.22 µm
Millex-GS syringe filter (Millipore), concentrated using Amicon Ultra 10,000 MWCO Centrifugal Filters (Millipore) and frozen at -20°C. The 10 µL samples were brought to a total volume of 15 µL in SDS-loading buffer containing 1% β-mercaptoethanol and proteins were resolved by PAGE using Tricine/SDS gels containing 9% polyacrylamide and 170 kV. Proteins were transferred (160 mAmperes for 1 h) to nitrocellulose membranes and blocked overnight at 4°C in blocking buffer (0.1% TBST containing 5% skim milk). A rabbit polyclonal antibody was generated by Pacific Immunology to HysA that was purified as described above. HysA was probed for used at a dilution of 1:40,000 with the anti-HysA rabbit serum for 1 h at room temperature in blocking buffer. The membrane was then washed three times for 10 min each in 0.1% TBST. Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG was applied at 1:20,000 in blocking buffer at room temperature for 1 h. Membranes were washed three times for 10 min each with 0.1% TBST and SuperSignal West Pico chemiluminescent substrate was added to detect proteins followed by exposure to Classic X-Ray Film.

**Neutropenic murine pneumonia model**

Female Balb/c mice were purchased from National Cancer Institute (Frederick, MD). All animals were housed under specific pathogen-free conditions in filter-top cages at Walter Reed Army Institute of Research (WRAIR) Animal Facility and provided sterile food and water *ad libitum*. Six to eight week old mice were injected intraperitoneally with 150 mg/kg of cyclophosphamide (Baxter) four days prior to infection and 100 mg/kg one day prior to infection as previously described [98]. Mice were anesthetized with isoflurane and infected intranasally with 1.0 x 10^7 CFU of *S.*

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3 These experiments were performed at Walter Reed Army Institute of Research in the laboratory of Dr. Daniel Zurawski
*aureus* suspended in 25 µL of PBS. At 48 hours post-infection, mice were euthanized and lungs were extracted, homogenized, and serial diluted before lung suspensions were plated onto TSA. Colony-forming units were enumerated to assess bacterial burden. For survival studies, infected mice were monitored twice a day for signs of morbidity. Following sacrifice, paraffin-embedded lung samples from infected and uninfected mice were sectioned and stained with hematoxylin and eosin (H&E). All procedures were performed in accordance with a protocol approved by the WRAIR Institutional Animal Care and Use Committee (protocol # IB02-10). All animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals* [99].

**Immunohistochemistry**

The lungs of PBS-treated and infected mice were harvested at 48 h post-infection, fixed in 4.0% paraformaldehyde and cut into 5 µm sections. As a control, uninfected lung samples were pretreated with 100 Turbidity Reducing Units (TRU) of *Streptomyces hyalurolyticus* hyaluronidase (Sigma) for 2 h at 60°C [100]. Hyaluronic acid was labeled as previously described [101]. Briefly, samples were incubated in 0.2 % BSA for 2 h at 4 °C to block nonspecific binding. Following incubation, samples were treated with 2 µg/mL of biotinylated hyaluronic acid binding protein (EMD Biosciences) for 16 h at 4 °C. FITC-conjugated streptavidin (Invitrogen) was used to visualize hyaluronic acid. The presence of hyaluronic acid in lung sections was quantified by measuring the mean fluorescent intensity of 40 fields per section. Images were acquired with an Olympus

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4 This experiment was performed at Walter Reed Army Institute of Research in the laboratory of Dr. Daniel Zurawski.
AX80 microscope and analyzed with Image Pro 7.0 software.

**Statistical analysis**

Statistical significance of all data was analyzed using the Student’s t-test with Graphpad Prism 6 software (Graphpad, La Jolla, CA) except for survival studies. Survival studies were analyzed by Mantel-Cox test \( p = 0.0022 \).

**Results**

**Initial characterization of S. aureus hysA**

The gene encoding hyaluronidase \((hysA)\) is conserved across all \(S. aureus\) clonal lineages [83], and only one hyaluronidase is encoded on the genome of CA-MRSA USA300 [102]. The \(hysA\) gene \((SAUSA300_2161)\) is located near a number of genes of unknown function, and is transcribed by itself in the opposite direction of neighboring genes (Fig. 2-1A). To begin our studies on \(hysA\), we created a markerless deletion construct in the strain AH1263 [103], an erythromycin-sensitive version of the USA300 isolate LAC (hereafter called LAC-WT). The \(\Delta hysA\) knockout mutant was confirmed by PCR and lack of enzymatic cleavage of HA as detected by an agar plate assay (Fig. 2-1B). We also verified the absence of hyaluronidase activity in the knockout mutant using a quantitative, spectrophotometric assay (Fig. 2-1C). The HysA activity was complemented to LAC-WT levels by providing \(hysA\) on a plasmid (Fig. 2-1), demonstrating the phenotype was due to the mutant construction and not secondary or polar effects.
**Screen of NTML for modulators of hyaluronidase activity**

The regulation of *hysA* has received only limited attention, although there are reports for the potential involvement of global regulators *sarA* and *agr* [23, 93]. To identify genetic loci that contribute to *hysA* regulation, we screened 1,952 mutants from the Nebraska Transposon Mutant Library (NTML) [104] for hyaluronidase activity using a HA plate assay. Screening of the NTML resulted in the identification of 8 *bursa aurealis* transposon insertions that significantly altered hyaluronidase activity. To determine the relative extent that each mutation affected activity, five replicates of each mutant were spotted on HA plates and zone size was enumerated with a caliper (Fig. 2-2A). NTML wild-type (WT) strain JE2 resulted in an average zone size of 1.01 cm (±0.02), while a mutation in *hysA* resulted in no detectable zone of clearing. Multiple strains with insertions in the sigma-factor B operon (*sigB, rsbU, and rsbW*), and strains with insertions in *sarA, hslU, and codY* resulted in elevated hyaluronidase activity. Interestingly, insertion into the putative tRNA deaminase, *SAUSA300_0543*, gave a reduced zone of clearing of HA (0.76 ± 0.03 cm) and was the only mutation in the library besides *hysA* to have a smaller zone size when compared to WT zone size.

To quantitatively measure the effect of the identified transposon insertions on hyaluronidase activity, we performed a spectrophotometric specific activity assay on spent media of 8-hour cultures (Fig. 2-2B). In support of the HA plate assay, the *codY* transposon mutant was found to increase activity 12-fold compared to WT. Additionally, multiple insertions in the *sigB* operon, as well as one in *hslU* increased activity approximately 3-fold, and an insertion in *sarA* resulted in a 10-fold increase. The transposon insertion in *SAUSA300_0543* had one third of the activity of WT (Fig. 2-2B),
similar to the reduction in activity observed in the HA plate assay. As expected, there was no activity observed in this assay by the hysA transposon mutant.

**Role of the agr system in hysA regulation**

Despite previous reports of agr quorum-sensing involvement in hysA regulation [23], we did not find any insertions in the agr locus to significantly alter hyaluronidase activity in our screen. We hypothesized that perhaps this was due to the previous study using S. aureus strain 8325-4, which has a known mutation in rsbU that results in a defect in SigB activity. As shown in Fig. 2-2, we found multiple mutations in the sigB operon, including a mutation in rsbU, that increase hyaluronidase activity. Thus a role for agr could be masked in the USA300 background that has an intact rsbU gene. To investigate this question, we shifted our studies to the USA300 LAC (LAC-WT) background, and we quantified HysA activity in LAC-WT as well as agr, sigB, and agr sigB double mutant strains. We found that HysA activity was unchanged in an agr mutant and increased significantly in a sigB mutant. Comparing the sigB agr double mutant to the sigB mutant (Fig. 2-3A), there is a significant decrease in activity when agr is deactivated. This observation is in agreement with published reports [23], and we also made similar observations in the USA400 MW2 background (Fig. 2-3C&D), indicating this effect occurs across multiple S. aureus strain types.

CodY is reported to regulate the agr-system [105, 106], and we hypothesized that some hysA regulation might be occurring indirectly through the known CodY - agr interactions. To address this question, we initially confirmed the CodY effect was consistent in the LAC background by crossing the transposon insertion into LAC-WT, and we observed that HysA activity increased 16-fold in the codY mutant over LAC-WT
Next, we quantified HysA activity in LAC-WT, as well as *agr, codY*, and *agr codY* double mutant strains (Fig. 2-3B). As anticipated, HysA activity was unchanged in an *agr* mutant and increased in a *codY* mutant. However, HysA activity was significantly decreased in the *agr codY* double mutant when compared to the *codY* mutant (Fig. 2-3B). We speculated that the unnaturally high levels of RNAIII in the *codY* mutant could be distorting HysA production. To test this question, we transformed USA300 WT with a plasmid that expresses RNAIII under the control of a tetracycline inducible promoter. In the absence of inducer, there was essentially no difference in HysA activity between this strain and an empty vector control (Fig. 2-3E). However, HysA activity increased with induction of RNAIII expression compared to the control (Fig. 2-3E).

Collectively, this data led us to suggest the model depicted in Figure 3D for CodY and *agr*-mediated regulation of HysA.

**CodY directly regulates hysA**

In considering the regulatory model (Fig. 2-3F), it still remained possible that CodY directly regulated *hysA* gene expression. The CodY regulon has been previously investigated [105-107], and *hysA* was not identified as a target in any of these studies. We inspected the sequence upstream of *hysA* for the presence of the previously published CodY consensus binding box [105], and we found a well-conserved CodY binding box 202 base pairs upstream of the putative translational start site (Fig. 2-4A), with one nucleic acid change from a cytosine to a thymine in the region between the inverted repeat. The presence of this box lead us to predict that CodY may interact with the *hysA* putative promoter region. To confirm that we were not being misled by the hyaluronidase activity assays, we tested to see if the HysA protein levels increased...
accordingly in a *codY* mutant. We used an antibody developed to HysA and found enzyme levels were increased in a *codY* mutant compared to the WT strain by immunoblot (Fig. 2-4B). To eliminate background binding in the immunoblot, Protein A was inactivated in each of these strains. The band corresponding to HysA was not detected in a *codY hysA* double-mutant, demonstrating the antibody was specific and suggesting the increase in hyaluronidase activity is due to elevated HysA levels. Also of note, HysA was not stable in spent media and we frequently observed lower molecular weight breakdown products by immunoblot (Fig. 2-4B)

To further test the possibility that CodY represses HysA production, we took advantage of the CodY response to the availability of branched chain amino acids (BCAA). CodY gains a high affinity for target promoters when BCAA are available, leading to repression of its targets. When BCAA are limiting, CodY loses this affinity, such as when cells are in stationary phase or under starvation, resulting in relieved repression of CodY targets [108, 109]. We grew LAC-WT in chemically defined media (CDM) lacking isoleucine, or in CDM with standard levels of BCAA, until the cells began to enter stationary phase (Fig. 2-5A). If *hysA* is under CodY control, we would anticipate increased HysA activity in the cells grown without isoleucine compared to those grown in CDM with standard levels of BCAA. In support of this hypothesis, there was more than a 2.5-fold increase in hyaluronidase activity when BCAA were limiting, as compared to bacteria grown in CDM at both 4 and 5 hours (Fig. 2-5B).

We also tested the impact of high levels of BCAA on CodY repression. The cells grown in media lacking isoleucine were switched to media that contained excess BCAA. If *hysA* is under CodY control, we would anticipate the excess BCAA would bind to
CodY and repress expression. As predicted, when BCAA were supplied in excess, HysA dropped over 5-fold compared to cells grown in CDM (Fig. 5B). These activity changes were not due to differences in growth (Fig. 2-5A). As a control, samples were taken from each time point and immunoblots were performed for Panton-Valentine Leukocidin (PVL) (Fig. 2-5C & D), which is known to be regulated by CodY in USA300 lineages [110]. The trends in PVL production mirrored those of HysA at all the conditions tested, suggesting they are regulated in a similar manner.

Finally, to assess whether CodY was acting to directly regulate \textit{hysA}, we constructed plasmids with CodY binding box mutations in the \textit{hysA} promoter region (Fig. 2-4C). As our testing system, we used the LAC Δ\textit{hysA} mutant with plasmid pHysA, which complements to near WT levels of hyaluronidase activity (Fig. 2-1C). Using pHysA as a backbone, we introduced six nucleotide substitutions to the putative CodY box upstream of \textit{hysA}, and these were based on previous studies in \textit{Bacillus subtilis} where this number of changes was sufficient to disrupt CodY based repression [2]. These constructs were tested by transforming them into the LAC Δ\textit{hysA} mutant or Δ\textit{hysA codY} double mutant and measuring HysA activity from plasmid expression. We first introduced “A-to-C” and “T-to-C” changes into the first half-site of the box in constructs pCR04 and pCR05 (Fig. 2-4C). In testing these constructs, the CodY-based repression of HysA activity was reduced markedly to approximately 2-fold (Fig. 2-4D). We made additional, independent nucleotide changes in the second box half-site in construct pCR06 (Fig. 2-4C), and CodY based repression of HysA activity was completely eliminated (Fig. 2-4D). These results provide strong support that CodY directly regulates \textit{hysA} expression through binding to the identified box in the promoter region.
The **hysA mutant is attenuated in a neutropenic murine pneumonia model**

To determine whether HysA is required for virulence, neutropenic Balb/c mice were infected intranasally with $1 \times 10^7$ CFU of either LAC-WT or the ΔhysA mutant. After 4 days of infection, ~80% of mice infected with LAC-WT succumbed to infection, which is in striking contrast to only ~20% death in mice infected with the ΔhysA mutant (Fig. 2-6A). A 4-log reduction in bacterial burden in the lungs at 48 hours post-infection was observed with the ΔhysA mutant compared to LAC-WT (Fig. 2-6B), suggesting the virulence defect was likely caused by an inability of the mutant to replicate in the lungs of infected mice.

The hyaluronidase activity of multiple Gram-positive pathogens has been shown to cause severe tissue damage **in vivo** [23, 78, 79]. Therefore, we investigated whether loss of virulence of the ΔhysA mutant strain was associated with reduced tissue damage in the lung. Histological analysis showed that the lungs of mice infected with the ΔhysA mutant exhibited less pathology and influx of alveolar macrophages than did LAC-WT infected mice (Fig. 2-6C). To determine whether hyaluronidase activity contributed to virulence of *S. aureus in vivo*, HA was fluorescently labeled and quantified in the lungs of infected mice. There was a significant increase in HA (P<0.0001) present in the lungs of ΔhysA mutant-infected mice compared to LAC-WT infected mice (Fig. 2-7A & B). In fact, the level of HA present in the lungs of wild-type infected mice is similar to that found in the lungs of mice treated with purified hyaluronidase from *Streptomyces hyalurolyticus* (Fig. 2-7C). Taken together, these findings show that hysA is an important determinant for causing a *S. aureus* infection.
Discussion

Hyaluronidases are thought to act as “spreading factors”, allowing for dissemination of pathogens throughout mammalian tissue during infection [83]. In fact, the earliest studies on spreading factors described it as an agent that increases lesion size when combined with various bacterial and viral pathogens [57, 76]. In this report, we investigated the regulation and in vivo function of HysA during S. aureus infection using a CA-MRSA USA300 strain, and we found that HysA is indeed an important factor for causing an infection.

Through our screen of the NTML, we identified 8 mutations that significantly altered HysA activity, including sarA, multiple mutations in the sigB operon, and an uncharacterized nucleoside deaminase encoded by gene SAUSA300_0543. The deaminase was the only target identified that is required for full expression of HysA. Surprisingly, we did not identify mutations in the agr locus during the screen, although it was reported that that agr is required for HysA production [23]. Considering the previous studies were done with a strain with a natural mutation in rsbU, which is known to mimic a sigB mutant and elevate agr function [111], we investigated this question. When an agr deletion was introduced into a sigB mutant, we observed a drop in HysA activity (Fig. 3A), supporting the previous studies. Thus when the agr system is hyper-activated, HysA levels increase, and by removing the agr locus these levels drop, which supports the previously identified agr-dependent regulation. We speculated that this agr role only occurred at unnaturally high levels of RNAIII, and we confirmed this by expressing RNAIII from a plasmid and boosting HysA output. Thus, agr has a role in HysA production, but it only seems to be important in situations where RNAIII is
elevated beyond normal WT levels.

Interestingly, our screen of the NTML revealed that a mutation in the global regulator CodY resulted in increased HysA activity. CodY responds to intracellular concentrations of branched chain amino-acids in *S. aureus* to control target gene expression [97, 105, 106, 109]. We initially speculated that the CodY regulation might be indirect through the *agr* system, and indeed the introduction of an *agr* deletion reduced HysA production in a *codY* mutant (Fig. 2-3B). However, we observed that the *hysA* promoter region had a near perfect CodY binding box (Fig. 2-4A), and importantly nucleotide changes to the conserved positions of the box eliminated CodY-based repression (Fig. 2-4C & D). Thus, there are indirect and direct mechanisms of CodY-dependent regulation occurring to coordinate HysA production in *S. aureus*.

CodY-dependent regulation suggests that HysA has a potential nutrient scavenging function. HA is a major component of the viscous substance produced by connective tissues, and it acts as a host defense mechanism by creating a protective barrier that impedes penetration of bacteria and bacterial products into deeper tissues [42]. Sequestration of essential nutrients, such as iron [112] and tryptophan [113], is another mechanism of bacterial infection prevention that works through generation of nutrient-limited environments. Bacterial pathogens can rapidly adapt to these hostile situations by regulating the expression of genes important for virulence and metabolism [114], and our findings suggest that *S. aureus* uses CodY to regulate HysA in this manner. HA can be broken down by hyaluronidases produced by a variety of bacterial pathogens, such as *Streptococcus pyogenes, Streptococcus pneumoniae,* and *Mycobacterium tuberculosis* [83], and metabolized as a source of carbon [80, 81, 115].
At this time, it has not been clearly demonstrated that *S. aureus* can use HA as a carbon source *in vivo*, or whether HysA is involved in this growth mechanism, both questions are in need of further examination. However, it is possible that the reduction in bacterial burden in a ΔhysA mutant during pulmonary (Fig. 2-6B) and soft-tissue infections [23] can be explained by an inability of *S. aureus* to utilize an alternative carbon source under nutrition-limited conditions *in vivo*.

This finding is consistent with previous work showing that CodY regulates other virulence factors to act as a link between metabolism and virulence [97]. For example, the hemolysins (α and β), as well as the secreted proteases SspA and SspB, have been shown to be regulated directly by CodY. HysA was required for the breakdown of HA *in vivo* and this correlated with a loss in structural integrity and enhanced necrosis of pulmonary tissues, allowing the pathogen access to deeper tissues. In addition to HysA, *S. aureus* possesses other mechanisms for penetrating the host. One example is a secreted cysteine protease, Staphopain A, which is able to induce vascular leakage during a guinea pig infection [116], and this effect can be augmented by the addition of another secreted cysteine protease, Staphopain B. Additionally, lipases have been observed to enhance *S. aureus* tissue penetration [117]. Taken together these results indicate that HysA is part of a class of *S. aureus* secreted proteins that act in coordinated fashion as spreading factors by breaking down host tissues to facilitate bacterial dissemination [77]. Interestingly, the target for each enzyme differs despite the functional redundancy of these proteins. Considering the dynamic range of infections caused by *S. aureus*, these findings lead us to speculate that these spreading factors may be a part of a fine-tuned virulence mechanism utilized by this pathogen to non-specifically breakdown host barriers and
promote dissemination from any site of infection.

Our studies give new insights into the regulation and role of the *S. aureus* virulence factor HysA. This work identified an important link between metabolism and virulence by illustrating a relationship between the metabolic regulator CodY and the virulence factor HysA. By therapeutic targeting of HysA and other spreading factors, it might be possible to prevent localized infections from evolving into more complicated, systemic disease.
### Table 2-1 Bacterial strains and plasmids used in Chapter II

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Figure 2-1. Generation and initial characterization of the CA-MRSA USA300 ΔhysA mutant and complemented strains.

(A) Location of hysA on the CA-MRSA USA300 LAC chromosome. Numbers indicate SAUSA300 locus numbers. The hysA gene is surrounded by genes with unknown function and is transcribed by itself on the chromosome. (B and C) HA plate and hyaluronidase (HL) specific activity assays of LAC-WT, LAC ΔhysA, and complemented (pHysA) strains. As shown, HysA activity is abolished for the ΔhysA mutant and restored to WT levels when it is complemented in both assays.
Figure 2-2. Transposon mutagenesis library screen for altered HysA activity.
(A) Summary of results of screen for HysA activity on HA agarose plates. Values of HysA activity are reported as diameters (cm) of zones of clearing, measured with a caliper, and represent the mean standard deviations of five technical replicates for each strain plated on HA agarose. Pictures are representative images of each strain. (B) Hyaluronidase specific activity assay of strains containing mutations that significantly altered HysA activity on HA agarose plates. The data are presented as the fold changes compared to the activity of USA300 WT strain JE2. Values represent the means and standard deviations of four technical replications for three independent biological determinations. Statistical significance (****, P <0.0001) was determined by Student’s t test.
Figure 2-3. Role of the *agr* system in *hysA* gene regulation.

Cultures of various LAC or MW2 strains were grown for 8 h, spent medium was prepared, and the hyaluronidase specific activity in each sample was measured. (A&C) To assess the *agr* and SigB interaction, activity was measured in the WT, *agr* and *sigB* single mutant, and *agr sigB* double mutant strains and plotted. (B&D) To assess the *agr* and CodY interaction, activity was measured in the WT, *agr* and *codY* single mutant, and *codY agr* double mutant strains. For panels A and B, statistical significance (****, P < 0.0001) was determined by Student’s t-test. (E) Hyaluronidase specific activity for the WT containing the empty vector (black bars) or RNAIII under the control of a tetracycline-inducible promoter (gray bars). Student’s t-test was used to determine statistical significance (**, P < 0.01; ****, P < 0.0001). (F) Model for SigB and CodY regulation of HysA through the *agr* system.
Figure 2-4. The CodY binding box is required for *hysA* regulation.

(A) Location of the putative CodY binding box in the *hysA* promoter region. An alignment of the consensus CodY binding box and the proposed *hysA* box is shown. (B) Immunoblotting for HysA performed with a LAC-WT strain. Spent medium was concentrated 20- or 50-fold from the WT and the *codY* and *codY* *hysA* mutant strains, as indicated, and immunoblotted for HysA. In each strain, a Δspa deletion was engineered to remove background antibody binding. (C) Mutations generated in the CodY box in plasmids pCR04, pCR05, and pCR06. (D) Hyaluronidase specific activity assay at 8 h for the WT (LAC Δ*hysA* with one of the indicated plasmid constructs) or a *codY* mutant (the LAC Δ*hysA* *codY* double mutant with one of the indicated plasmid constructs) containing the p*HysA*, pCR04, pCR05, or pCR06 construct. Statistical analysis was performed using a Student’s t-test (****, P < 0.0001).
Figure 2-5. Effect of nutrient availability on hysA regulation.
(A) Growth curve of LAC-WT grown in CDM or in CDM lacking isoleucine for the first 5 h. Cells were harvested by centrifugation, washed 1 time in PBS, and suspended in either fresh CDM or CDM containing 5X BCAAs. Arrows, times when samples were taken or where medium was switched, as indicated. (B) Hyaluronidase specific activity of samples taken at the time points indicated in panel A. Statistical analysis was performed using Student’s t test (****, P < 0.0001). (C) Immunoblot for the LukS subunit of PVL at the time points indicated in panel A. (D) Quantification of LukS immunoblot using ImageJ software, post, samples taken at time points after the medium switch.
Figure 2-6. HysA is required for USA300 virulence.
Mice were intranasally inoculated with $1.0 \times 10^7$ CFU of LAC-WT, its ΔhysA mutant strain, or PBS. (A) Mice (n = 27) were monitored daily for signs of morbidity and mortality. **, $P = 0.0022$, Mantel-Cox test. (B) Groups of mice (n = 21) were euthanized after 48 h, and the bacterial burden in the lungs was quantified by CFU enumeration. **, $P = 0.0095$. (C) Histopathology of the lungs from mice treated with PBS or infected with either LAC-WT or the ΔhysA mutant. Black arrowheads, areas shown in the enlarged images in the insets. Data are representative of those from three independent experiments.
Figure 2-7. HysA is required for the breakdown of hyaluronic acid in vivo.

(A and B) Micrographs of immunofluorescently labeled HA in the lungs of mice inoculated with $1.0 \times 10^7$ CFU of either strain LAC-WT or its $\Delta$hysA mutant strain. (C) The mean fluorescence intensity was quantified for each sample (40 fields per section). A sample treated with hyaluronidase from Streptomyces hyalurolyticus (Strep Hys) was also included as a control. NS, not significant; ****, $P < 0.0001$. 
CHAPTER III.

HYALURONAN IS AN IMPORTANT COMPONENT OF THE EXTRACELLULAR MATRIX OF STAPHYLOCOCCUS AUREUS BIOFILM INFECTIONS

Introduction

*Staphylococcus aureus* is a Gram-positive pathogen and a leading cause of bacterial infections both in the hospital and in the community. Many of the infections caused by *S. aureus* are chronic biofilm-like infections, such as those associated with implants and conditions such as osteomyelitis. Approximately one million nosocomial indwelling device associated infections occur annually in the United States [31]. Staphylococci are the most common isolates, accounting for nearly two-thirds of this type of infection [33]. Treatment of these infections requires long courses of antibiotics and additional surgeries, which often leads to removal of the infected device [30, 31, 33]. The recalcitrance of biofilm infections to treatment is thought to be in part due to reduced permeability of antimicrobial agents through the extracellular biofilm matrix [124, 125].

The main components of the biofilm matrix are exopolysaccharides, proteins, and extracellular DNA [122, 126, 127]. However, little work has been done to determine if *S. aureus* can incorporate host matrix materials into the biofilm matrix *in vivo*. *S. aureus* has been shown to extensively bind host matrix molecules such as fibrin, fibronectin, and fibrinogen [128-131], and there are increasing reports of bacteria incorporating host glycosaminoglycans into the extracellular biofilm matrix. For example heparin has been shown to enhance *S. aureus* biofilm formation and to be incorporated into the *S. aureus*
biofilm matrix in vitro [35]. Additionally, hyaluronic acid (HA) has been shown to promote biofilm formation in Gram-positive pathogens, including *Streptococcus pneumoniae* [132] and *Streptococcus intermedius* [133].

Hyaluronic acid (HA) is an extremely large glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid, which are linked by alternating β-1,3, β-1,4 glycosidic bonds. HA is a main component of the mammalian extracellular matrix and is abundant in the skin, synovial fluid, skeletal tissues, vitreous of the eye, heart valves, lungs, kidneys, and brain [42]. HA has been shown to have a number of host functions, which largely depend on the molecular weight [42, 43, 46]. High molecular weight HA (HMW-HA, >450 kDa) is anti-inflammatory and immunosuppressive and there is increased production of HMW-HA at inflammation sites [42, 43, 46]. In contrast, low and mid molecular weight HA (20-450 kDa) strongly promotes inflammatory cytokines, adhesins, and cell proliferation of chondrocytes, endothelial cells, and fibroblasts [46, 49, 50]. Finally, HA oligomers (6-20 kDa) activate dendritic cells, and are immunostimulatory and inflammatory [43, 46, 49, 50].

Bacterial hyaluronidases cleave HA at the β-1,4 glycosidic bond which yields unsaturated disaccharides as a final product of complete digestion. *S. aureus* produces a hyaluronidase, encoded by the gene *hysA*, that is conserved across all *S. aureus* lineages. Our group and others have shown that HysA is a virulence factor in a variety of infection models [23, 134], which is thought to be due to its role as a spreading factor. However, it has become increasingly clear that hyaluronidases act as more than just spreading factors. A number of bacteria, including *S. pneumoniae* and *S. pyogenes*, have been found to catabolize HA in a hyaluronidase dependent manner [79, 81]. Additionally, hyaluronidase
addition to HA containing biofilms led to biofilm dispersal in S. intermedius leading the authors to predict that hyaluronidase may be important for detachment from biofilms [133].

Due to the high abundance of HA in mammalian tissues we hypothesized that S. aureus may incorporate HA into the biofilm matrix during infection. Additionally, we predicted that HysA therefore might be involved in biofilm dispersal. Here we show that S. aureus incorporates hyaluronic acid into the biofilm matrix both in vivo and in vitro. Additionally, we find that HysA can disperse established biofilms in vitro. Finally, we find that a hysA mutant has a dissemination defect in a murine implant-associated infection model. Together, this work provides strong evidence that HA is an important component of the biofilm matrix during infection and that HysA is involved in dispersing the biofilm and disseminating to new infection sites.

**Materials and Methods**

**Bacterial Strains, Plasmids and Culture Conditions**

Bacterial strains and plasmids used in this study are described in Table 3-1. All S. aureus strains were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA) with the appropriate antibiotics for plasmid maintenance or selection (chloramphenicol, 10 µg/mL) at 37°C with shaking at 220 rpm, unless otherwise specified. Hyaluronic acid sodium salt purified from Streptococcus equi was purchased from Sigma-Aldrich. Plasmid DNA was purified from Escherichia coli and electroporated into S. aureus RN4220 as previously described [94]. Plasmids were moved from RN4220 with
bacteriophage Φ11 to other S. aureus strains by transduction. All of the oligonucleotides
used for this study can be found in Table 3-2.

The mCherry gene sequence was codon optimized for S. aureus strain USA300
using OPTIMIZER [135], and the sod RBS was then added upstream of the start codon.
The optimized sequence was synthesized by IDT and then amplified by PCR using the
primers HC281 and HC285. The PCR product was digested with KpnI and EcoRI before
ligating into pCM29 digested with the same enzymes. The resulting plasmid, pHc47,
expresses optimized mCherry under the control of the sarA promoter.

To create a tetracycline inducible hysA (pRMC2-HysA) the hysA gene was
amplified from AH1263 genomic DNA using primers CBR63 and CBR64, which contain
SacI and KpnI sites at their 5’ ends, respectively. The resulting PCR product was ligated
into pRMC2 linearized with KpnI and SacI and electroporated into E. coli
strain BW25141.

To generate the HysA catalytically inactive (Y306A) overexpression construct
(pET28a-Y306A HysA) hysA DNA was amplified by around the world PCR from
purified plasmid DNA (pCR03) using primers CBR49 and CBR50. Amplified DNA was
digested with DpnI for 1 hour at 37°C and transformed into E. coli strain ER2566 using
standard molecular techniques.

**Murine Catheter Infection Model**

10-12 week male and female C57/BL6 mice are anesthetized and the flanks were
shaved. 18-g IV catheters were cut to 1 cm, UV sterilized, and incubated in C57/BL6
serum for 1 hour at 37°C. S. aureus WT or hysA mutant were grown overnight at 37°C with shaking at 225 rpm in TSB, subcultured 1:1000 into fresh media, and grown to an optical density at 600 nm (OD_{600}) equal to 1.0. 1 mL of culture from each strain was pelleted and resuspended into 1X DPBS +/-+. The catheters were removed from the serum and added to the washed S. aureus cells or as a negative control into PBS alone, and incubated at 37°C for 1 hour. Catheters were resheathed onto the 18-g needles, inserted subcutaneously into the flank of the mouse, and the insertion site was sealed with Dermabond. Mice were weighed and monitored once daily for 6 days, when mice were sacrificed and catheters were harvested for analysis of bacterial burdens and histology.

HA ELISA of Infected Catheters

Catheters were colonized and implanted subcutaneously in the right flank of male C57/Bl6 mice as described above. After 6 days, catheters were harvested into 1 mL PBS to perform a HA detection assay using a Hyaluronan Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Catheters were sonicated for 10 min in a water bath sonicator and then vortexed for 30 seconds to release HA. Samples were diluted 1:100 with the supplied calibrator buffer, and the assay was performed per the manufacturer’s instructions. Concentrations were determined by calculation from a standard curve of hyaluronic acid.

Synovial fluid aggregates

Synovial fluid was isolated from wildtype newborn pigs and centrifuged to
remove any host cells. *S. aureus* strains were grown overnight in TSB at 37°C with shaking at 220 rpm. Cultures were normalized by optical density and 1 mL was pelleted and cells were resuspended into 1 mL of phosphate buffered saline (PBS). 200 µL of PBS or 10% synovial fluid, diluted with PBS, was added to each well of a 48-well microtiter plate. 20 µL *S. aureus* washed cells were added to each well. Plate was incubated for 1 hour at 37°C with shaking at 220 rpm. To image aggregates ethidium bromide (2.5 µg/mL) was added to each well and the plate was incubated for 30 min at 37°C with shaking at 220 rpm. Cells were visualized by ultraviolet (UV) light and imaged with a BioRad GelDoc 2000.

**Preparation of Biofilms for SEM**

Catheters were colonized with the *S. aureus* wildtype strain LAC and implanted subcutaneously in the right flank of male C57/Bl6 mice as described above. After 6 days, catheters were harvested, transferred to a 48-well microtiter plate and fixed with 2.5% gluteraldehyde overnight at 4°C. The synovial fluid biofilms were formed in a 48-well polystyrene microtiter plate with the *S. aureus* wildtype strain LAC as described above. After 1 hr of incubation in 10% porcine synovial fluid biofilms were transferred to a fresh well and fixed overnight at 4°C with 2.5% gluteraldehyde. All biofilms were then prepared for SEM in the same manner. In brief biofilms were washed three times for 15 min with 0.1 M sodium cacodylate buffer to remove fixative, and treated with 1% osmium tetroxide for 1.5 hrs. Samples were washed three times with buffer followed by one wash with double distilled water. Increasing concentrations of ethanol were then for 15 min each to dehydrate the samples. Biofilms were incubated with 95% ethanol for 30
min and 100% ethanol twice for 30 min each. Samples were cross-linked with a 1:1 mixture of hexamethyldisilizane (HMDS) and 100% ethanol for 15 min followed by HMDS alone twice for 15 min each. HMDS was removed, and samples were allowed to air dry in a tissue culture hood overnight. Coverslips were mounted on stubs and coated with gold particles using an Emitech K550 sputter coater. Images were captured with a Hitachi S-4800 scanning electron microscope with Super ExB filter technology.

**Microtiter Biofilms**

Bacterial strains were grown overnight in TSB and sub-cultured 1:500 into biofilm media (BHI + 0.4% glucose) or biofilm media containing HA as indicated in the figure legends. Wells of a 48-well microtiter plate (Corning Costar 3548) were inoculated and plates were incubated at 37°C with shaking at 500 rpm in a Stuart microtitre plate incubator for 18 hours in quadruplicate. The media was aspirated, the wells were washed once with water, and stained with 0.1% Crystal Violet. The wells were washed again with water and stained biomass was solubilized with 1 mL of isopropanol for 20 min. The absorbance was measured using a Tecan Infinite M200 plate reader between 595-615 nm as indicated in the figure legends.

The following modifications for those biofilms formed with the tetracycline inducible plasmid (pRMC2 and pRMC2-<i>hysA</i>). For induction at time zero, strains were grown overnight in TSB in the presence of chloramphenicol (10 µg/mL) to maintain plasmid. The next day, strains were subcultured 1:500 into biofilm media (BHI + 0.4% glucose) that contained 2 mg/mL HA, chloramphenicol, and anhydrotetracycline at the
concentrations indicated in the figure legend. Biofilms were grown in a 48-well microtiter plate for 18 hours at 37°C with shaking at 500 rpm in a Stuart microtitre plate incubator, when biomass was determined with the microtiter biofilm assay as described above. For the biofilms that were induced at 18 hours, strains were also grown overnight in TSB in the presence of chloramphenicol. The next day, strains were subcultured 1:500 into biofilm media containing HA and chloramphenicol as indicated above. Strains were inoculated into 48-well microtiter plates and incubated at 37°C with shaking at 500 rpm in a Stuart microtitre plate incubator. After 18 hours, anhydrotetracycline was added as indicated in the figure legend and the microtiter plate was incubated for an additional 4 hours. Biomass was then determined with the microtiter biofilm assay as described above.

**Confocal Microscopy of HA Biofilms**

12 mm circular coverslips were added to each well of a 24-well microtiter plate and sterilized with ultraviolet light for 1 hour. *S. aureus* strain LACΔhysA that contained a plasmid that constitutively expresses mCherry1A (pHC47) was grown overnight in TSB and subcultured 1:500 into BHI + 0.4 % glucose that contained 2 mg/ml HA that was 20% labeled with fluorescein isothiocyanate (HA-FITC; Creative PEGworks, Chapel Hill, NC). 1 mL was added to each well of the 24-well microtiter plate that contained the coverslips and the plate was incubated statically for 18 hrs at 37°C. Coverslips were removed, added to fresh wells, and fixed for 15 min at room temperature with 4% paraformaldehyde. Coverslips were then washed with PBS to remove fixative, and mounted onto glass slides with Prolong Diamond Antifade Mountant (Molecular Probes).
mounting media overnight at 4°C. Biofilms were imaged with a Leica SP3confocal microscope.

**Confocal Microscopy of Synovial Fluid Aggregates**

Synovial fluid aggregates were formed as described using *S. aureus* WT and hysA mutant strains (AH3743 and AH3744) that lack the ica locus and express sGFP constitutively from a plasmid (pCM29). Aggregates were fixed with 4% paraformaldehyde for 10 min at room temperature and washed 3 times with PBS to remove excess fixative. Hyaluronic acid was stained with wheat germ agglutinin (WGA) at room temperature for 15 min and aggregates were washed with PBS to remove excess stain. Aggregates were transferred to a microscope slide and imaged by confocal laser scanning microscopy using a Leica SP3microscope. Z-stacks were assembled using FIJI Image J software.

**Protein Purification**

AH2856 and AH3053 were grown overnight at 37°C in LB medium containing kanamycin (50 µg/mL) and subcultured into 1 liter of fresh LB medium at a ratio of 1:250. Cultures were grown at 30°C to mid-logarithmic phase, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a 1 mM final concentration, and the culture was allowed to grow for an additional 4 hrs. Cells were harvested by centrifugation, washed with water, pelleted by centrifugation, and frozen at -20°C. Cells were mechanically lysed with a Microfluidics microfluidizer (model LV1; Newton, MA) at 25,000 lb/in² and
running the samples through the machine twice. Cell lysate was clarified by centrifugation at 15,000 x g for 20 min at 4°C. Proteins were purified using Fractogel His-bind resin (Novagen) per the manufacturer’s instructions, dialyzed into phosphate-buffered saline (PBS), concentrated using Millipore centrifugal filter units (30,000 molecular-weight cutoff), and brought to 3 mg/mL in 1 X PBS. The protein suspensions were frozen at -20° in 10% glycerol until use.

**Statistical Analysis**

Statistical significance of all data was determined using a Student t test with GraphPad Prism (version 6) software (GraphPad, La Jolla, CA) unless otherwise noted in figure legends.

**Results**

**HA is present in S. aureus biofilm infections**

We hypothesized that HA may enhance *S. aureus* biofilms through incorporation into the biofilm matrix during infection. To test this question, we used a murine implant-associated infection model and challenged the mice with a community-associated methicillin-resistant *S. aureus* (CA-MRSA) USA300 wildtype (WT) strain, LAC, or an isogenic *hysA* deletion. After 6 days, mice were sacrificed, and catheters were prepared for scanning electron microscopy (SEM). We found that a large biofilm forms in the lumen of the catheter during infection (Figure 3-1A), with an exceptional amount of extracellular matrix material (Figure 3-1B). We next measured the HA concentration of
the biofilm formed in the catheter lumen with an ELISA. There was a significant increase in the concentration of HA of the catheters infected with the \textit{hysA} mutant, which cannot cleave HA, compared to the WT infected and to the uninfected control catheters (Fig. 3-1C).

We predicted HA might also be present in other types of biofilm infection. \textit{S. aureus} was recently found to form macroscopic biofilm-like aggregates in synovial fluid (SF) of joint infections [136]. The highest concentrations of HA in any tissues are in SF with concentrations of 1.5-3.6 mg/ml in humans [43]. Therefore, we hypothesized that HA may also be involved in the formation of these SF aggregates. To test this hypothesis, we isolated SF from newborn pigs and were able to replicate the formation of biofilm-like aggregates with the porcine SF (Fig. 3-1D). We next examined the architecture of the synovial fluid aggregates (Fig. 3-1E) SEM and we were able to determine that the large aggregates of cells are tightly encased in a polymeric rope-like substance similar to a biofilm. Using a HA specific ELISA, we found that HA is also present at high concentrations in porcine SF, between 2-4 mg/ml (Figure 3-1F) similar to concentrations found in humans.

\textit{The presence of HA enhances staphylococcal biofilms in vitro}

Hyaluronic acid has been shown to enhance biofilm formation in other organisms \textit{in vitro} [132, 133]. Therefore, we assessed biofilm formation using a microtiter plate assay in the presence and absence of HA. Figure 3-2A shows that there was no significant difference in biofilm formation with or without HA for the WT LAC strain in the
conditions tested. However, there was a significant increase in biofilm formation in the
*hySA* mutant in the presence of HA. This finding led us to predict that the hyaluronidase
enzyme may cleave HA in the biofilm matrix and limit the accumulation of biomass.

We speculated that other staphylococcal species might also incorporate HA into
the biofilm matrix and promote biomass accumulation. Only *S. aureus* secretes a
hyaluronidase enzyme (Fig. 3-2C), suggesting other staphylococci may behave similarly
to the *S. aureus hySA* mutant during biofilm formation in the presence of HA. As
predicted, *Staphylococcus epidermidis*, *S. lugdunensis*, *S. saprophyticus*, *S. xylosus*, *S.
capitis*, and *S. caprae* all formed significantly larger biofilms in the presence of HA
compared to our standard biofilm media (Fig. 3-2B). Interestingly, there was no
difference in biofilm formation between the two conditions for *S. carnosus* (Fig. 3-2B).
This may indicate that *S. carnosus* may lack a factor to incorporate HA into the biofilm
matrix.

### Visualization of hyaluronic acid in the biofilm matrix

After our observations of the HA-induced biofilm enhancement, we used confocal
microscopy to visualize the localization of HA in the biofilm matrix. For this assessment,
we used *S. aureus* wildtype and *hySA* mutant strains containing a plasmid that
constitutively expresses the red fluorescent protein mCherry1A (RFP). We then used
biofilm media that contained FITC labeled HA to form biofilms on coverslips. We found
that there was a high degree of localization of HA with the *S. aureus* biofilm by confocal
microscopy (Fig. 3-3). Upon higher magnification HA appeared to be tightly associated
with the *S. aureus* cells in the biofilm in the extracellular matrix (Fig. 3-3).

We next assessed the localization of HA in SF aggregates. We formed SF aggregates in microtiter plates with WT and *hysA* mutant *S. aureus* strains that constitutively express GFP on a plasmid and that have deletions in the *ica* polysaccharide intracellular adhesion (PIA) synthesis locus (Fig. 3-5). We next used wheat germ agglutinin, which binds β-1,4 linked N-acetylglucosamine, to stain HA in the synovial fluid aggregates. We found there was a high degree of staining with WGA within the SF aggregates (Fig. 3-4), which indicates that HA is likely incorporated into the SF biofilm-like aggregates. However, it should be noted that it is possible that WGA is also staining glycosylated *S. aureus* surface proteins.

**HysA disperses biofilms that contain HA**

We hypothesized that secreted HysA enzyme may be involved in dispersal from HA containing biofilms. To test this hypothesis, we pre-formed biofilms in the presence of HA with the *hysA* mutant for 18 hours. We then treated the biofilms purified HysA, or a purified catalytically inactive version of HysA (Y306A), for 3 hours before measuring biomass. We found that the active purified enzyme significantly reduced biomass compared to the no treatment control (Fig. 3-6A). Additionally, there was no difference in biomass between the biofilms treated with the catalytically inactive HysA compared to the no treatment control (Fig. 3-6A). Our observations indicated that HysA enzyme activity is involved in dispersal from HA containing biofilms. We further assessed the function of HysA in biofilm dispersal using strains engineered to express *hysA* under the
control of a tetracycline inducible promoter. With increasing concentrations of inducer, the expression of HysA prevented HA-containing biofilm formation compared to the empty vector control (Fig. 3-6B). Additionally, we found that induction of \textit{hysA} in an established HA-containing biofilm resulted in biofilm dispersal (Fig. 3-6C) when compared to the control.

We extended these studies to SF aggregate formation and assessed the impact of HysA activity. We predicted that HysA might be able to prevent the formation of SF aggregates, similar to its ability to prevent biofilm formation following induction. To test this, we pre-digested SF with purified HysA and tested the ability of the \textit{S. aureus hysA} mutant to form aggregates. We found that \textit{S. aureus} formed significantly smaller aggregates (Fig. 3-4), providing evidence for the role of HA in the formation of these biofilm aggregates and therefore HysA in dispersal from these structures.

\textbf{\textit{S. aureus uses HysA to escape from a biofilm infection}}

Based on the \textit{in vitro} impact of HysA on biofilms, we assessed the contribution of HysA to \textit{S. aureus} dissemination from a biofilm infection. For this test, we challenged mice with WT or an isogenic \textit{hysA} deletion using a murine implant-associated infection model. In this model, catheters are coated with murine serum and \textit{S. aureus} cells are allowed to adhere prior to subcutaneous implantation in the flank of the mouse. After 6 days, mice were sacrificed and the bacterial burdens were determined for the catheters, surrounding tissue, and kidneys. We found that there was no difference in bacterial burden between the wildtype and \textit{hysA} mutant on the catheters themselves (Fig. 3-7B).
However, there was a significant reduction in bacterial in for the *hysA* mutant in the tissue surrounding the catheters as well as in the kidneys when compared to the wildtype (Fig. 3-7 C&D) indicating there was reduced dissemination from the site of infection for the *hysA* mutant. Additionally, the mice that were infected with the wildtype lost significantly more weight than those infected with the *hysA* mutant or the PBS control (Fig. 3-7A), consistent with increased systemic disease.

**Discussion**

In this report we set out to determine if *S. aureus* can incorporate the mammalian extracellular glycosaminoglycan hyaluronic acid into the biofilm matrix. Using a HA specific ELISA (Fig. 3-1) and confocal microscopy (Fig. 3-3 & 3-4), we were able to demonstrate that HA is incorporated into the biofilm matrix of *S. aureus* both *in vivo* and *in vitro*. Hyaluronic acid is an extremely large negatively charged polymer, which are characteristics of other bacterial biofilm matrix components such as extracellular DNA (eDNA) and exopolysaccharide. How these matrix materials are incorporated into the matrix is still an area of ongoing research. One possibility is bacterial proteins bind these polymers to the surface of the cell. Heparin and eDNA-binding proteins have been identified in a number of bacteria including *S. aureus* [137-142], lending support to this idea. Due to the tight association of HA with *S. aureus* biofilms demonstrated by confocal microscopy (Fig. 3-3), we hypothesize that *S. aureus* may also have HA binding proteins to incorporate HA into the biofilm matrix. An extensive number of eukaryotic proteins have been found to bind and coat HA *in vivo* [143]. Many of these proteins, including RHAMM, CD44, and aggrecan, contain the LINK module, which is
responsible for interacting with HA [143]. Additionally, we found that HA enhanced biofilm formation in other staphylococci (Fig. 3-2B), which may indicate that HA binding proteins may be conserved across a number of species. Future studies will work to identify HA binding proteins and characterize their role in HA containing biofilms.

*S. aureus* has recently been found to form biofilm-like aggregates in SF [136]. We found that *S. aureus* can form these aggregates in porcine SF and that HA is incorporated into the matrix (Fig. 3-1 & 3-4). Additionally, we show that pre-digestion of the SF with purified HysA can prevent aggregate development in SF. One question that remains is how these aggregates form if *S. aureus* hyaluronidase is being produced *in vivo*. In previous studies, we found that *in vitro* HysA is produced at very low levels [134]. One explanation is that HysA is produced at levels that are inadequate to prevent HA-containing aggregate development. Another explanation is that once incorporated into the aggregate, HA is protected from digestion by hyaluronidases by proteins. Although never characterized, studies in the 1940s identified a hyaluronidase inhibitor in serum that was likely proteinaceous [144-146]. It is possible that a similar mammalian inhibitory protein exists in synovial fluid. Similarly, eDNA is thought to be protected from nuclease digestion in biofilms in other organisms [147, 148]. Future studies will work to delineate between these two possibilities and further characterize SF biofilm aggregates and their role during joint infections.

Finally, we found that *S. aureus* HysA can disperse HA containing biofilms *in vitro* and that an *hysA* mutant has reduced dissemination *in vivo*. Historically, hyaluronidases have been thought to be important for virulence due to their role as spreading factors [23, 55, 67, 77, 83]. Therefore, *S. aureus* may have gained a
hyaluronidase to disseminate to new tissues during infection. Additionally, this would allow *S. aureus* a mechanism to escape from biofilms following incorporation of HA into the biofilm matrix. Biofilms are often nutrient-limiting environments [124, 125, 149], and dissemination would allow *S. aureus* to access new infection sites and carbon sources. Additionally, pathogens such as *S. pneumoniae*, *S. pyogenes*, and *Mycobacterium tuberculosis* that produce a hyaluronidase have been shown to utilize HA as a sole carbon source in a hyaluronidase dependent manner [79, 81]. It is possible that cleavage of HA during dissemination from biofilms similarly liberates HA as an additional carbon source for *S. aureus*. In previous studies we found that the global regulator CodY directly represses *hysA* [134]. CodY responds to nutrient levels in the cell and is often thought to be a link between metabolism and virulence. By regulating *hysA*, CodY may simultaneously be allowing *S. aureus* to disseminate from a biofilm and increase virulence and provide an additional nutrient source. However, the ability of *S. aureus* to catabolize HA has not been demonstrated definitively [150].

HA is added to a number of cosmetics [151] and used in the treatment of multiple conditions including osteoarthritis [152] and cataracts [153]. Heparin is also used in a number of medical devices including catheter lock solutions [154]. There is growing evidence that these polymers can enhance biofilm formation in a number of bacterial species [35, 132, 133]. Biofilms have reduced susceptibility to host defenses and increased antibiotic resistance [124, 125, 155]. Additionally, biofilm infections often result in long courses of antibiotic treatment [31], increased length of hospital stays, and reduced patient outcomes. Therefore, the full risks of addition of glycosaminoglycans to these products and their relationship to biofilm formation must be assessed. Future
biofilm studies should work to consider the importance of host molecules in biofilm formation.
### Table 3-1 Bacterial strains and plasmids used in Chapter III

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<th>Description</th>
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Figure 3-1. HA is present at high concentrations in biofilms in vivo and in synovial fluid. SEM of a biofilm formed in the lumen of a catheter from a murine implant associated infection model and removed for imaging after 6 days (A). A large biofilm formed in the lumen with an exceptional amount of extracellular matrix material (B). In this model, there is increased HA in the biofilm after 6 days (C). S. aureus forms biofilms in porcine synovial fluid (D), and by SEM the S. aureus cells are encased in a matrix composed of long polymers in the SF biofilms (E). Quantification of HA in the SF reveals that HA is present at high concentrations in porcine SF (F). Statistical analysis was performed using a Student’s T-test (**= P-value <0.01)
Figure 3-2. HA enhances staphylococcal biofilms in vitro.
HA enhances biofilm formation of LACΔhysA in vitro (A). HA also enhances biofilm formation of other staphylococcal species in vitro (B). Only S. aureus WT produces a hyaluronidase as shown on HA agarose (C).
**Figure 3-3.** HA is incorporated into the *S. aureus* biofilm matrix. LACΔ*hysA* that constitutively expresses mCherry1A was grown on coverslips in biofilm media that contained 2 mg/ml of 20% FITC labeled HA and imaged at 60X magnification by confocal laser scanning microscopy.
Figure 3-4. HA is incorporated into the matrix of SF aggregates. SF biofilms were formed over 1 hour with S. aureus strains LAC (WT) and LACΔhysA that constitutively express GFP from a plasmid. Biofilms were stained with WGA to stain HA and washed before imaging by confocal laser scanning microscopy. Additionally, prior to incubation with S. aureus SF was predigested with purified HysA and biofilms were unable to form (right).
Fig. 3-5. IVIS of wells used for confocal of synovial fluid aggregates
Prior to processing for imaging, the ability of *S. aureus* to form aggregates in each condition was measured by assessing sGFP with the IVIS live animal imaging system.
Figure 3-6. HysA can disperse HA containing biofilms. Purified HysA can disperse established biofilms in a microtiter plate, but a catalytically inactive site directed mutant HysA (Y306A) cannot (A). Induction of HysA can prevent biofilm formation in the presence of HA (B) and can disperse established biofilms (C).
Figure 3-7. *hysA* mutant has reduced dissemination in an implant associated infection model. Mice infected with the *S. aureus* WT lose significantly more weight than those infected with LACΔ*hysA* or the PBS negative control (A). There are similar bacterial burdens on the catheters 6 dpi between the WT and *hysA* mutant. However, there is a significant reduction in bacterial burden for the *hysA* mutant compared to WT in the tissue surrounding the catheters (C) and in the kidneys (D) 6 dpi.
CHAPTER IV.
GENERAL DISCUSSION AND FUTURE DIRECTIONS

The overall goal of the work in this dissertation was to define the roles of hysA in S. aureus disease and physiology. Historically, hyaluronidases have been thought to be important for virulence mainly for their role as a “spreading factor” cleaving HA in the host extracellular matrix. However, through the studies presented in this dissertation we found that HysA plays a broader role in S. aureus physiology. Hyaluronidases have typically been evaluated using skin infection models to determine their role in virulence. However, in Chapter II we found that hysA is also required for full virulence of S. aureus in a murine pneumonia model of infection (Figure 2-6). In Chapter III, we found that HA is incorporated into the S. aureus biofilm matrix and that hysA mutant had reduced dissemination in a murine biofilm infection model. Additionally, in Appendix I we found that S. aureus can catabolize HA in a HysA-dependent manner. Together, these studies indicate that HA and HysA are involved in many facets of S. aureus physiology, from dissemination during infection, to catabolism, to biofilm formation and dispersal.

Complicating the matter, S. aureus strains from clonal complex 30 (CC30, USA200; Table 1-1) produce a second hyaluronidase, hysA2. This enzyme shares 75.9% amino acid identity with hysA, but is located distally on the chromosome. The activity and relative contribution of this second enzyme to virulence remains unknown. Additionally, no studies have been performed to date to investigate the regulation of the second enzyme. The remainder of this chapter will focus on discussing the remaining questions regarding S. aureus hysA and to propose future studies to address these concerns.
Regulation of \textit{hysA}

Prior to this work, very little was known about the regulation of \textit{hysA}. There were some observations that the SarA and Agr global regulatory systems modulated HysA activity, but the mechanisms of these interactions were not defined [23]. The goal of Chapter II was to determine how \textit{hysA} is regulated. Through these studies, we found evidence that the regulation of \textit{hysA} is likely complex, involving multiple global regulatory systems (Fig. 4-1). I confirmed previous observations that SarA represses \textit{hysA} (Fig. 2-2), and elucidated the mechanism by which the Agr-system activates \textit{hysA} (Fig. 2-3). Additionally, I found that the global regulator, CodY, directly represses \textit{hysA} likely through binding to the conserved CodY binding sequence in the \textit{hysA} promoter to block transcription (Fig. 2-2, 2-4, 2-5). However, a number of questions remain regarding the regulation of \textit{hysA}. Unlike some of the other spreading factors and hemolysins, \textit{hysA} is produced at very low levels \textit{in vitro} (Fig. 2-4). Nearly all of the regulators identified in Chapter II work to repress \textit{hysA} expression. However, it is unclear how these regulators interact to control \textit{hysA} expression. Finally, although we found the Agr system activates \textit{hysA} in some situations, no true activator has been identified. Future studies into \textit{hysA} regulation should investigate how these global regulatory systems work together to control \textit{hysA} expression.

Future Directions on the Regulation of \textit{hysA}

In Chapter II, we found that CodY represses \textit{hysA} through interaction with to the CodY Binding Box in the region upstream of the translational start site (Fig. 2-4). However, it is unknown where this binding occurs in relation to the transcriptional start site. To determine this, the \textit{hysA} transcriptional start site should be mapped by 5’ RACE.
Additionally, we found that both SarA and CodY strongly repress hysA activity (Fig. 2-2). However, it has not been demonstrated whether SarA also binds to the hysA promoter to block transcription. To demonstrate if SarA binds to the hysA promoter, electromobility shift assays (EMSAs) could be performed. Finally, it is unknown if both of these regulators can simultaneously repress hysA or if there is a hierarchy to their binding. DNA footprinting assays could be performed to determine where the CodY and SarA binding sites are in relation to each other in the hysA promoter region. Additionally, to determine whether there is ordered binding to the hysA promoter between CodY and SarA, competitive binding of both proteins to the hysA promoter could be assessed by EMSA.

Although we found the Agr system activates hysA in some situations, no true activator has been identified. It is possible that we have not identified an activator for hysA because during our studies the signal is missing for activation. Bacterial cleavage of HA results in unsaturated disaccharides (Fig. 1-4) as the product of complete digestion, while mammalian enzymatic efficiency drops below 20 kDa [44, 47]. HA in the media has been found to induce expression of the hyaluronidase of Streptococcus intermedius [133]. Therefore, it is possible that the degradation products produced by digestion of HA by these different enzymes then work as a signal to the bacteria that they are inside the host. Future studies on hysA should investigate if these breakdown products act as a signal in S. aureus to turn on an activator and induce hysA expression. qRT-PCR could be performed to determine if transcript levels of hysA increase in the presence of the products of HA enzymatic digestion.
Future directions on the role of CodY in regulating bacterial hyaluronidases

CodY has often been described as a link between metabolism and virulence. In Chapter II we found that \( hysA \) is required for virulence of \( S. \) \textit{aureus}, and in Appendix I we found that \( S. \) \textit{aureus} can catabolize HA in a \( hysA \) dependent manner. Therefore, \( hysA \) appears to have a dual role during infection as both a spreading factor and for nutrient scavenging. Through regulation of \( hysA \), CodY can directly control metabolism and virulence simultaneously allowing for access to new infection sites as well as allowing for the availability of a new carbon source. \( S. \) \textit{aureus} is the only staphylococcal species that encodes for a hyaluronidase and \( hysA \) has significant homology to \( hyl \) and \( hylB \) from \( S. \) \textit{pneumoniae} and \( S. \) \textit{agalactiae}, respectively. Therefore it is likely that \( hysA \) was acquired through horizontal gene transfer from the streptococci. CodY is a conserved global regulator across a wide variety of Gram-positive organisms, including \( S. \) \textit{agalactiae} and \( S. \) \textit{pneumoniae}. It is possible that the CodY binding box and therefore regulation of \( hysA \) by CodY were co-acquired. Future studies into CodY regulation of hyaluronidases should determine if CodY regulates \( hylB \) and \( hyl \) in \( S. \) \textit{agalactiae} and \( S. \) \textit{pneumoniae}, respectively. One way to assess this would to create \( codY \) deletion constructs in these organisms and compare hyaluronidase activity between wildtype and a \( codY \) mutant using the hyaluronidase specific activity assay described in Chapter II.

Future directions on the regulation of \( hysA2 \)

All sequenced \( S. \) \textit{aureus} strains contain \( hysA \), but isolates from clonal complex 30 (CC30, USA200; Table 1-1) produce an additional hyaluronidase, \( hysA2 \). Although these enzymes share significant homology (75.9% identity), they are located distally on the chromosome and share little conservation in their promoter regions. The lack of
conservation in the promoter regions may indicate there is differential regulation of $hysA$ and $hysA2$. To identify potential regulators of $hysA2$, mutations in global regulators should be constructed in the $hysA$ deletion background in the $S. aureus$ CC30 strain MRSA252. Potential candidates should include $codY$, $sarA$, $sigB$, and $agr$. Additionally, as it is possible that there is differential regulation of $hysA2$ compared to $hysA$, the ability of the 15 nonessential $S. aureus$ two-component systems to modulate the activity of $hysA2$ should be assessed. The regulation of $hysA2$ could be investigated through a screen on HA-agarose plates, similar to the screen of the Nebraska Transposon Mutant Library performed in Chapter II.

**Role of HysA in $S. aureus$ pathogenesis**

Another goal of the studies carried out in this work was to determine the role of $hysA$ in $S. aureus$ pathogenesis. HA production is upregulated upon injury, which is in part thought to be a host defense from invading pathogens. Therefore, hyaluronidases historically haven been considered important for virulence predominantly for their role as a “spreading factor” by cleaving HA in the extracellular matrix and allowing for bacterial spread [47, 55, 56, 59, 79, 83]. Investigations into the role of hyaluronidases during infection have therefore typically been limited to skin infection models [23, 56, 79, 83]. In Chapter II we found that a $hysA$ mutant was attenuated in a murine neutropenic pneumonia model of infection. Additionally, we showed that there was reduced lung pathology of the $hysA$ mutant at 48 hours post infection and that HA levels were decreased in the lungs in the wildtype strain compared to the $hysA$ mutant and negative control infected mice by immunofluorescence. Finally, in Chapter III we found that a
A recent study in *S. agalactiae* found that a *hylB* mutant had reduced resistance to macrophage killing compared to the wildtype and complemented strains [162]. To address the possibility that the *hysA* mutant has increased susceptibility to host defenses future studies should evaluate the survival of the *hysA* mutant in blood compared to the wildtype. Additionally, susceptibility to killing by macrophages could be assessed, similar to the studies performed by Wang et al. [162]. In Chapter III, we found that in a biofilm-like infection the *hysA* mutant has a reduced ability to disseminate during a biofilm implant-associated infection. However, it is unclear at which point during infection the *hysA* mutant is defective for dissemination. HA production is upregulated during wound healing, in part to prevent bacterial spread [42, 44, 83]. The drug 4-methylumbelliferone is known to prevent HA synthesis *in vivo* [163]. Therefore, one way to assess when HysA is required for dissemination would be treating mice with 4-methylumbelliferone to block HA synthesis at early, mid, and late time-points during the implant-associated infection model to determine at what point the *hysA* mutant is rescued.
the dissemination defect. Finally, to assess growth differences in vivo, growth of the hysA mutant could be compared to the wildtype using a constitutive chromosomal luciferase expressing strain and monitoring the luciferase signal over time during infection with the Xenogen IVIS 200 whole animal live imaging system (Caliper). I have created wildtype and hysA mutant strains that constitutively express the lux operon of Photorhabdus luminescens on the chromosome [164], which could be utilized for these studies. Finally, bacterial counts of infected tissues could be performed at multiple time-points during infection to support the IVIS data.

Another possible role for HysA during infection is to modulate the host immune response. Full length HA is known to be immunosuppressive, whereas HA degradation products are known to be proinflammatory through interactions with Toll-like Receptor 2 (TLR2) and Toll-like Receptor 4 (TLR4) [50]. However, the products of HA digestion by bacterial hyaluronidases are too small to bind and signal through TLR2 and TLR4 [42, 44, 46, 49]. Therefore, these products may no longer be immunostimulatory. In support, a recent study found that HylB of S. agalactiae reduces proinflammatory cytokine production by macrophages and during infection [162]. Therefore, the role of hysA in modulating the immune response during infection should be assessed in future studies.

Role of HysA in S. aureus Biofilms

Prior to this work, little was known about the role of host extracellular matrix polymers such as hyaluronic acid in S. aureus biofilms. There was some evidence that a similar molecule, heparin, enhanced S. aureus biofilm formation [35]. Additionally, HA
was found to promote biofilm formation in *S. intermedius* and hyaluronidase was found to disperse *S. intermedius* from HA containing biofilms [133]. The goals of Chapter III were to determine if *S. aureus* can incorporate HA into the biofilm matrix and if HysA was involved in biofilm dispersal. We found that HA is incorporated into the *S. aureus* biofilm matrix both in vivo (Fig. 3-1) and in vitro (Fig. 3-3 & 3-4). Additionally, we found that *S. aureus* could form biofilm aggregates in SF (Fig. 3-1D) and that predigestion of the SF with HysA prevented SF biofilm formation (Fig. 3-4). Finally, we found that HysA could disperse HA containing biofilms when added exogenously (Fig. 3-6A) and that induction of HysA could both prevent biofilm formation and disperse established biofilms formed in the presence of HA (Fig. 3-6B&C). Additionally, we found that a *hysA* mutant displayed reduced dissemination in a murine implant-associated infection model. However, many questions remain about how biofilms form and incorporate HA in vivo and when and where *hysA* is expressed within the biofilm.

**Future Directions into the role of hyaluronan in *S. aureus* Biofilms**

Although we found that HA is incorporated into the biofilm matrix, the mechanism by which this occurs is unknown. One possibility is that HA binding proteins exist to attach HA to the cell. Extracellular DNA (eDNA) binding proteins have been identified in *S. aureus* [137, 138], and *S. aureus* is notorious for its ability to bind to other host matrix molecules such as fibrinogen [142, 165]. There are a number of well defined eukaryotic HA binding proteins, including RHAMM, CD44, and aggrecan, which often contain the LINK module [143, 166][153, 171]. One method to identify HA binding proteins would be to use eukaryotic HA binding domains to search against the *S. aureus* genome for sequence and domain conservation. Systematic deletion mutations could then
be created in homologous proteins in the \( hysA \) mutant background. These double mutants could then be screened for the inability of HA to enhance biofilm formation. One possible shortcoming of this method is that HA binding proteins may not be identified in the screen due to possible functional redundancy. Another method to identify HA binding proteins would then be to fractionate \( S. aureus \) proteins into the secreted and membrane fractions and then to run the fractions over a HA column using streptavidin agarose and biotinylated HA. In addition to the mechanism of incorporation, the length of HA required for incorporation into the biofilm matrix is unknown. Previous studies by Izano et al. found that extracellular DNA (eDNA) needs to be >10 kb to be incorporated into the \( S. aureus \) biofilm matrix and prevent detachment [167]. It is possible that there is a similar length requirement for HA incorporation. To test this, HA of defined lengths can be purchased and the ability of the different length polymers to enhance biofilm formation in the \( \Delta hysA \) mutant could be assessed.

**Future directions on synovial fluid biofilms**

Synovial fluid is mainly composed of hyaluronic acid, fibrinogen, and serum proteins at low concentrations [168]. We were able to replicate the formation of large (>1 mm) biofilm aggregates in a synthetic form of synovial fluid (Fig. 4-2), which was composed of HA (2 mg/mL), human fibrinogen (175 µg/mL), and human serum (5%). These biofilm aggregates appear to be very similar to those formed in synovial fluid by gross and microscopic observation. Asymptomatic human synovial fluid is rarely isolated for scientific testing and the composition of human SF is largely dependent on disease state. How these SF biofilms relate to human disease progression is poorly understood. Therefore, this synthetic synovial fluid could provide a useful tool to further
our understanding of joint infections caused by *S. aureus*. Future studies should work to characterize the ability of synthetic SF to mimic biological SF. In addition work should be done to determine the overall role of SF biofilms in the development of joint infections such as septic arthritis and osteomyelitis.

**Future directions on the role of HysA in *S. aureus* biofilms**

Our finding that induction of *hysA* leads to dispersal of HA-containing biofilms hints that *hysA* may be expressed at very low levels in the biofilm. To examine this further, luciferase reporters could be constructed to monitor hysA expression in vivo using the IVIS live animal imaging system. Additionally, a *hysA* transcriptional reporter to sGFP could be used in the BioFlux system (Fluxion Biosciences Inc., San Francisco, CA) with our collaborators to determine *hysA* expression over time during biofilm formation. The BioFlux system allows for culture of biofilms in 96-well plate format with continuous media flow, as in a flow cell, and a camera mounted as part of the system continuously monitors expression of reporters. This would allow for a time course of hysA expression throughout biofilm formation.

Another possibility for why HA is not degraded by HysA in the biofilm *in vivo* is that perhaps the HA is protected by proteins. In Chapter III, we found that predigestion of SF with HysA could prevent biofilm aggregate formation. However, it was necessary to digest the HA with a high concentration of enzyme (10 µg/mL) overnight to degrade HA significantly. Dastgheyb et al. found that proteinase K could disperse SF biofilms, indicating that proteins contribute to SF biofilm formation [136]. Future studies should work to determine if HA is protected from HysA digestion *in vivo*. 
Overall Model

In this work I have demonstrated a number of roles and functions for the *S. aureus* hyaluronidase, *hysA*. Through these findings we developed the following model for the roles of HysA in *S. aureus* physiology (Fig. 4-3): 1) Expression of *hysA* is tightly controlled by a complex regulatory network. 2) Upon production, HysA is secreted from *S. aureus* cells. 3) HysA can then degrade host hyaluronic acid, which can result in either a new growth substrate (4) or allow *S. aureus* to disseminate to a new infection site (5). Although hyaluronidase has typically thought to be involved in virulence for its role as a spreading factor, we provide evidence that hyaluronidase also allows for the availability of an additional carbon source and dispersal from HA containing biofilms. *S. aureus* has evolved an elegant mechanism to utilize a host molecule to fulfill its needs. HA is highly abundant in mammalian tissues and production is upregulated upon injury [42, 44]. The complex *hysA* regulatory system allows *S. aureus* to tightly control *hysA* expression to adapt the role of HysA to the surroundings to take advantage of this host defense. During biofilm formation *hysA* levels can be kept low to prevent *hysA* degradation. However, *hysA* expression can also be quickly turned on to allow for spread to a new niche or to allow for HA catabolism upon carbon limitation. Collectively, these studies lend new insights into an intricate interaction between bacteria and host.
Figure 4-1. Regulation of *hysA* expression in *S. aureus*

We found that CodY directly represses *hysA*, and the Agr-system activates *hysA* under some conditions. Other groups have found SarA represses *hysA* and our findings support that model. Finally, we found the SigB system represses *hysA*, however it is unknown whether this is direct or indirect.
Figure 4-2. Development of synthetic synovial fluid for studying SF biofilms

(A) We developed a synthetic synovial fluid composed of hyaluronic acid (HA, 2.5 mg/ml), human serum (HS, 5%), and human fibrinogen (HF, 175 µg/ml). (B) Size of the aggregates was quantified with GraphPad ImageJ Software (v6). Large biofilm aggregates (>1 mM) are only observed in the presence of all three.
Figure 4-3. Model for the overall role of hysA in *S. aureus* physiology.

(1) *hysA* expression is tightly controlled by a complex regulatory network. (2) Upon production, HysA is secreted. (3) HysA can then cleave host hyaluronic acid, allowing for growth on the disaccharides (4) or dissemination to new infection sites (5).
APPENDIX A.

STAPHYLOCOCCUS AUREUS CAN GROW ON HYALURONIC ACID AS A SOLE CARBON SOURCE

Introduction

Hyaluronic acid is a mammalian glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid. It is highly abundant in host tissues, causing many to hypothesize that bacteria may utilize hyaluronic acid as a carbon source during infection [23, 79, 81, 83]. In support of this, multiple Gram-positive pathogens that produce a hyaluronidase have been shown to utilize hyaluronic acid as a sole carbon source [79, 81]. Additionally, Marion et al. found that *Streptococcus pneumoniae* is able to catabolize hyaluronic acid from multiple sources, and that this is dependent on a PTS transport system and an unsaturated glucuronyl hydrolase [81].

The goal of this work was to determine whether *Staphylococcus aureus* can catabolize hyaluronic acid (HA) as a sole carbon source and to identify genes involved in this process. We found that *S. aureus* can catabolize both full length HA as well as HA disaccharides. Additionally, we observed *S. aureus* is able to catabolize N-acetylglucosamine, but not D-glucuronic acid, which may indicate that *S. aureus* may lack a transporter or enzyme to degrade glucuronic acid. By screening the Nebraska Transposon Mutant Library (NTML), we found 13 genes that may be involved in HA catabolism. However, none of these mutations are in proteins that one would predict to be involved in the breakdown of HA. Future studies will work to characterize these mutants and to identify genes that may not have been identified in the screen due to redundancies,
limitations of the screening method, or absence from the NTML.

**Materials and Methods**

*Bacterial Strains, Plasmids, and Culture Conditions*

All bacterial strains and plasmids used in this work are described in Table A-1. All *S. aureus* strains were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA) at 37 °C with shaking at 220 rpm and a volume-to-flask ratio of 1:5 unless otherwise specified. Hyaluronic acid sodium salt from *Streptococcus equi* (Catalog #53747) was purchased from Sigma-Aldrich. N-acetylglucosamine (Catalog #A8625) and D-glucuronic (Catalog #G5269) were purchased from Sigma-Aldrich.

*Growth on Full Length Hyaluronic Acid*

Hyaluronic acid (HA) was prepared to 10 mg/mL in H₂O and filter sterilized. HA solution was dialyzed overnight in H₂O with 10,000 Molecular weight cut off (MWCO) dialysis tubing, changing the H₂O every 6 hours. The HA solution was concentrated back to the original volume using Amicon Ultra Centrifugal Filters with a 10,000 MWCO. *S. aureus* strains were grown overnight in 5 mL TSB at 37°C with shaking at 225 rpm, sub-cultured 1:100 into fresh TSB, and grown for 2 hours. 1 ml of culture was pelleted, washed once with H₂O, and resuspended into 100 μL 1X PBS. Carbon Limiting Media (CLM) was prepared as described in Olson et al. [123], and was supplemented with glucose (0.2%), hyaluronic acid (5 mg/mL), or no added carbon. 180 μL of media was added to each well in triplicate for each strain. 20 μL of washed cells were added and the plate was incubated at 37°C with shaking at 1000 rpm in a Stuart Microtitre Plate Shaker.
for 24 hours. Growth was monitored by OD$_{600}$ over time with a TECAN Infinite M200 plate reader.

**Growth on HA Disaccharides**

HA was prepared to 10 mg/mL as described. 10 µL of purified HysA (8 mg/mL) was added to the HA and allowed to digest for 48 hours at 37°C. Digested HA was passed across an Amicon Ultra Centrifugal Filters with a 3,000 MWCO and the flow through was collected. *S. aureus* strains were grown as described above. CLM was supplemented with glucose (6 mM), HA Disaccharides as noted in the figure legends, or no added carbon and 180 µL was added to each well of a 96-well microtiter plate. Growth was monitored overtime by OD$_{600}$ readings using a TECAN Infinite M200 plate reader.

**Screen for Genes Important for Growth on HA**

The Nebraska Transposon Mutant Library (NTML) [104] was inoculated into 200 µL TSB and grown overnight at 37°C with shaking at 1000 rpm in a Stuart Microtitre Plate Shaker. Each well was then subcultured 1:100 into CLM supplemented with 6 mM HA Disaccharides. Growth for each well was monitored over time using a TECAN Infinite M200 plate reader. Strains that were identified as putative hits were rescreened in the same manner, but in both CLM supplemented with 6 mM glucose and CLM supplemented with 6 mM HA disaccharides.

**Results**

*Staphylococcus aureus can grow on hyaluronic acid as a sole carbon source*

Although it has been established that some streptococci that produce a
hyaluronidase can utilize hyaluronic acid (HA) as a sole carbon source, this has not been shown in other Gram-positive pathogens such as *S. aureus*. Using a carbon limited media (CLM), we found that there is a significant boost in growth in CLM supplemented with high molecular weight HA (HMW-HA) for the wildtype strain, JE2, compared to CLM that was not supplemented with an additional carbon source (Fig. A-1). However, a similar boost was not observed in the isogenic *hysA* mutant (Fig. A-1), which may indicate that cleavage of HA is required in order to be an available carbon source. While we observed a boost in growth in the wildtype strain grown with CLM supplemented with HMW-HA compared to the unsupplemented control, the increase was modest (Fig. A-1). We hypothesized that this was due to an insufficient amount of HysA being produced to cleave HA enough to sustain growth. Therefore, we predigested HA with purified HysA to generate the final end product 3-(4-deoxy-β-D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine (Fig. A-2), referred to hereafter as HA Disaccharides. We then assessed the ability of *S. aureus* to grow in CLM supplemented with glucose, HA Disaccharides, or no added carbon. Figure A-3 shows that we saw a large boost in growth for the cells grown in CLM + HA Disaccharides when compared to the no added carbon control, and this effect was dose-dependent. In fact there was similar growth on CLM supplemented with glucose and CLM supplemented with HA Disaccharides. Additionally, there was no difference in growth on HA Disaccharides between the wildtype and a *hysA* mutant, as expected (Fig. A-4).

*S. aureus* can catabolize *N*-acetylglucosamine but not *D*-glucuronic acid

We next wanted to determine if *S. aureus* could grow on the monosaccharide components of HA, *N*-acetylglucosamine and *D*-glucuronic acid. Wildtype *S. aureus* was
grown in CLM supplemented with either N-acetylglucosamine, D-glucuronic acid, glucose, or no added carbon. We found that *S. aureus* is able to grow on N-acetylglucosamine but not glucuronic acid (Fig. A-5). This is similar to findings in other Gram-positive bacteria, such as *S. pneumoniae* [81] and *S. pyogenes* [79], and may indicate that these bacteria are unable to transport glucuronic acid alone.

**Results of NTML screen for growth on HA**

*S. pneumoniae* has been shown to use a PTS transport system and a glucuronyl hydrolase to transport and digest HA disaccharides during catabolism of HA [81]. Since we found that *S. aureus* can grow on HA, we hypothesized there might be a similar proteins involved in HA catabolism in *S. aureus*. Unfortunately neither the PTS transporter nor the hydrolase has significant homology to any *Staphylococcus aureus* proteins. Therefore, to identify potential genes involved in HA catabolism, we screened the NTML for growth on HA Disaccharides, with the assumption that those that could not grow on the HA Disaccharides were potentially involved in catabolism of HA. In our initial screen of the NTML, we identified 99 putative hits, which are described in Table A-2. Many of these genes were in mutations in general metabolic pathways. For example there were 10 mutations in the purine biosynthesis pathway that were identified as putative hits. To narrow down the possible genes involved in HA catabolism, we decided to re-screen the 99 putative hits for growth in CLM supplemented with HA Disaccharides or glucose to try to identify genes that are specific to growth on HA Disaccharides. The rescreen narrowed the pool to 13 transposon insertions that could not grow in CLM supplemented with HA Disaccharides, which are described in Table A-3.
**Discussion**

Here we investigated if *S. aureus* can utilize HA as a sole carbon source and worked to identify the genes required for HA catabolism. Hyaluronidase has long been thought to contribute to *S. aureus* virulence by cleaving host tissues and allowing for spread of bacteria and secreted proteins. Another mechanism by which hyaluronidase may contribute to virulence however may be by allowing for the availability of an additional carbon source. In the studies in Chapter II, we found CodY directly represses *hysA*. CodY has often been described in the literature as a link between metabolism and virulence [97, 169]. This is due to the ability of CodY to respond to nutrients such as branched chain amino acids, as well as its regulon, which consists of both virulence factors and metabolic genes. In this study, we found that *S. aureus* can utilize HA as a sole carbon source using a chemically defined media, and the catabolism of HA depends on secretion of HysA. Therefore, HysA may provide another link between metabolism and virulence. Increasing our understanding of the interactions between metabolism and virulence could give new insights into the pathogenesis of *S. aureus*. Additionally, virulence factors that also play a role in metabolism, such as HysA, could provide a novel target for therapeutics by targeting two systems simultaneously.

*S. aureus* can grow with high efficiency on HA Disaccharides, reaching cell densities higher than equivalent concentrations of glucose (Fig. A-3). This growth occurs independently of the HysA enzyme. This may indicate that HA is available *in vivo* and that *S. aureus* has evolved to exploit the availability of HA during infection. In a murine pneumonia model of infection, our collaborators found that there is a 4-log reduction in bacterial burden for a *hysA* mutant compared with WT 48-hours post-infection, which
lends support to the idea that a hysA mutant is attenuated in part because of reduced fitness in vivo [59]. However, this has not been shown definitively. Future studies will work to dissect the two potential roles of HysA in vivo, spreading and increasing the availability of nutrients.

PTS transporters are often thought to be highly specific to particular sugars. During these studies we found that S. aureus can catabolize N-acetylglucosamine but not glucuronic acid, the monosaccharides that comprise HA. Many pathogens, including S. aureus, have a N-acetylglucosamine specific PTS transport system. This transporter is included in the NTML, but was not required for HA catabolism in our screen. It is possible, that N-acetylglucosamine, HA Disaccharides, and D-glucuronic acid would each require a separate transporter. Therefore, there may be support for a model where the disaccharide is transported in and then the β-1,3 glycosidic bond is cleaved in the cytoplasm (Fig. A-6). This is similar to findings in S. pneumoniae where a PTS transport system transports in the disaccharide and then an unsaturated glucuronyl hydrolase cleaves to the monosaccharides [81]. During cleavage by HysA, glucuronic acid in HA becomes 4-deoxy-β-D-gluc-4-enuronosyl. Therefore, an alternate explanation is that S. aureus cannot transport and grow on D-glucuronic acid, but can transport and grow on 4-deoxy-β-D-gluc-4-enuronosyl.

By screening the NTML for growth on HA disaccharides, we hoped to identify proteins with similar functions in S. aureus. We identified mutations in 13 genes that could not grow on HA but could grow on glucose (Table A-3), indicating potential involvement in HA catabolism. Unfortunately, none of these genes have predicted functions that would obviously be involved in catabolism of HA such as in a putative
PTS transporter or a sugar hydrolase. Unfortunately, our screen did not lead to mutations that are likely to be responsible for these actions. The NTML only comprises 85% of the *S. aureus* genome, so it is possible that the proteins responsible for transport and cleavage of HA disaccharides are not in the library. Additionally, it is possible that there are multiple proteins that function redundantly to catabolize HA, which would not have been distinguished in our screen. Future studies will work to address these possibilities and identify the remaining proteins involved in HA catabolism.
### Table A-1 Bacterial strains and plasmids used in Appendix A

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Figure A-1. Growth of *Staphylococcus aureus* on hyaluronic acid polymer. Growth of *S. aureus* WT strain JE2 (blue) or a *hysA* Mariner transposon mutant (*hysA::tn*, green) over time on high molecular weight hyaluronic acid (HA) polymer compared to growth in carbon limiting media with no added carbon (No Carbon).
Figure A-2 Structure of 3-(4-deoxy-β-D-gluc-4-enuronosyl)-N-acetyl-D-glucosamine
The final product of complete digestion of HA with HysA
Figure A-3 Growth of *Staphylococcus aureus* on HA Disaccharides

Growth of wildtype *S. aureus* strain JE2 on hyaluronic acid that has been digested with HysA to disaccharides (Digested HA) at various concentrations. Growth is compared to carbon limiting media alone (No Carbon), or carbon limiting media that has been supplemented with glucose.
Figure A-4 Comparison of growth of wildtype and \textit{hysA} mutant on HA Disaccharides. Growth of the wildtype strain JE2 and the \textit{hysA} Mariner transposon mutant (\textit{hysA::tn}) in carbon limiting media that has not been supplemented with carbon (No Carbon, dotted lines), supplemented with glucose (gray and black solid lines), or that has been supplemented with various concentrations of HA that has been digested to disaccharides (colored lines). Statistical analysis was performed using a Student’s T-test with GraphPad ImageJ software with the final data point for each curve (27 hrs). ***=P-value<0.001, **=P-value<0.01, *=P-value<0.05.
Figure A-5. *Staphylococcus aureus* can grow on N-acetylglucosamine but not D-glucuronic acid.

Growth of *S. aureus* wildtype strain LAC in carbon limiting media (CLM) that has been supplemented with 6mM of N-acetylglucosamine (blue) or D-glucuronic acid (green). Growth is compared to growth in CLM that has not been supplemented with an additional source (grey) or to growth in CLM supplemented with 6 mM glucose (black). Statistical analysis was performed with a Student’s T-test with GraphPad ImageJ software at 10 hr and 12 hr in comparison with the no carbon control. ****=P-value<0.0001.
Figure A-6. Proposed model for *S. aureus* catabolism of hyaluronic acid

*S. aureus* hyaluronidase, HysA, cleaves host HA in the extracellular matrix to produce HA Disaccharides. Disaccharides are then transported in by an unknown transporter. The β-1,3 glycosidic bond is then cleaved by an unknown enzyme to allow the monosaccharides to enter central metabolism. It is currently unknown if cleavage of the β-1,3 glycosidic bond occurs intracellularly or extracellularly.
APPENDIX B.

EXAMINATION OF NATURAL PRODUCT INHIBITORS OF

STAPHYLOCOCCUS AUREUS HYALURONIDASE

Introduction

*Staphylococcus aureus* is notorious for quickly acquiring antibiotic resistance through horizontal gene transfer [3, 25]. Penicillinase-producing *S. aureus* emerged in the mid-1940’s, only a few years after the introduction of penicillin in the clinic. Penicillin-resistant *S. aureus* then became pandemic in the 1950’s and 1960’s [3, 25]. A similar trend was seen with methicillin; only 2 years after its introduction in 1959 methicillin-resistant *S. aureus* (MRSA) was reported and MRSA strains are now endemic in hospitals [3, 25].

One concept to combat antibiotic resistance that has gained some traction is the idea of anti-virulence agents as an alternative to antibiotics. Anti-virulence agents inhibit the production of disease-causing virulence factors but are not bacteriostatic or bactericidal [170]. Since these agents do not interfere directly with growth, they are thought to apply less selective pressure to the development of resistance [170]. One potential target for the development of an anti-virulence agent is the *S. aureus* enzyme hyaluronidase, encoded by the gene *hysA*. Hyaluronidases have been well established as virulence factors for a number of Gram-positive organisms [23, 67, 79, 83]. These enzymes are typically thought to be involved in pathogenesis through their ability to degrade host extracellular matrix material by digestion of hyaluronic acid [53, 56, 57, 67, 76-79, 82, 83, 93, 134, 171]. Additionally, in the studies in Chapter II, we found that *S.*
*aureus* hyaluronidase is required for virulence, supporting its role as a virulence factor.

Little work has been done to identify hyaluronidase inhibitors, particularly inhibitors of the bacterial hyaluronidases. Research to identify hyaluronidase inhibitors has typically focused on targeting the mammalian hyaluronidases, which are upregulated in some types of cancer [146]. These inhibitors are then tested against the bacterial enzymes, and are often found to be ineffective. This is likely due to the fact that mammalian and bacterial enzymes have little similarity. Mammalian enzymes randomly hydrolyze the β-1,4 glycosidic bond, while bacterial enzymes cleave the β-1,4 glycosidic bond by β-elimination in a processive manner. The most potent inhibitors of the bacterial hyaluronidases that have been identified are vitamin C and vitamin C derivatives [172, 173]. The best of these is L-ascorbic acid 6-hexadecanoate (Vcpal), which was reported to have an IC$_{50}$ of 4.2 µM against *S. agalactiae* hyaluronidase [172]. However, its ability to inhibit the closely related *S. pneumoniae* hyaluronidase was greatly reduced (IC$_{50}$=100.0 µM) [172], which may indicate that it would have limited efficacy for broad application as a bacterial hyaluronidase inhibitor.

To identify novel inhibitors targeting bacterial hyaluronidases, we screened 34 pure compounds that were isolated from fungi for inhibition of purified HysA using a HA agarose plate assay. This led to the identification of four closely related compounds that significantly inhibited HysA with this assay. Additionally, we screened 34 whole fungal extracts that were isolated from fungi associated with *Anemopsis californica* for inhibition of purified HysA using a HA-PAGE assay. This led to the identification of multiple extract fractions that showed a significant ability to inhibit HysA across multiple assays.
Materials and Methods

Materials

Hyaluronic acid sodium salt from Streptococcus equis was purchased from Sigma-Aldrich (Catalog #53747). Alcian Blue 8GX was purchased from Sigma-Aldrich (Catalog #A3157).

Purification of HysA

AH2856 was grown overnight at 37°C in LB containing kanamycin (50 µg/mL), and subcultured into 1 L of fresh LB medium at a ratio of 1:250. The culture was grown at 30°C to mid-logarithmic phase, IPTG was added to 1 mM final concentration, and the culture was allowed to grow for an additional 4 h. Cells were harvested by centrifugation, washed once with water, pelleted by centrifugation, and frozen at -20°C. Cells were mechanically lysed using a Microfluidics Microfluidizer model LV1 (Newton, MA) at 25,000 psi and running the samples through the machine twice. Cell lysate was clarified by centrifugation for 20 min at 15,000 × g at 4°C. Protein was purified using Fractogel His-Bind Resin (Novagen) in the presence of 1 mM Tris(hydroxypropyl)phosphine (THP) as per the manufacturer’s instructions, dialyzed into PBS containing 1 mM THP, and concentrated using Millipore Centrifugal Filter Unit (30,000 molecular weight cut-off), and brought to 3 mg/ml in 1X PBS + 10% glycerol + 1 mM THP. The protein suspension was frozen at -20°C until used.

HA-PAGE Assays

For testing of fungal extracts, 10 µL of purified HysA (1.25 and 0.625 µg/ml) was added to 0.5 ml eppendorf tubes and pre-incubated with 0.5 µL fungal extracts (25 µg/ml
final concentration) for 15 minutes at 37°C. 10 µL of hyaluronic acid (5 mg/ml in ddH₂O) was added to the reaction mixtures. Control reactions of HA alone, HA + HysA, and HA + HysA + DMSO were prepared in parallel. Reactions were incubated at 37°C for 2 hours. 5 µL of 2 M sucrose was added to each sample, and reactions were loaded onto a 15% polyacrylamide gel in 1X TBE and electrophoresed at 300V for 45 min at 4°C. Gel was washed 3X with water followed by staining with 0.005% Alcian Blue in 2% acetic acid for 30 minutes in the dark. Gels were destained with 2% acetic acid and pictures were taken with a Canon PowerShot ELPH 330HS Camera.

**HA Agarose Plate Assays**

Purified HysA (1.25 µg/mL) was incubated with fungal extract (50 µg/mL) for 15 min at 37°C. HysA was incubated with an equivalent amount of DMSO as a negative control in parallel. 4 µL of reaction mixture was spotted onto HA agarose plates (TSB, 1% agarose, 0.4 mg/ml HA, 1% w/v BSA). Plates were incubated at 37°C for 2 hours and then flooded with 10% acetic acid to visualize zones of clearing.

**Hyaluronidase Specific Activity Assay**

The hyaluronidase activity assay was performed as previously described [23] with some modifications. Hyaluronic acid (100 µL at 1 mg/mL) was preincubated with compounds at the doses indicated in the figure legend for 15 minutes at 37°C. Purified HysA (50 µL at 5 ng/mL) was then added to the HA mixture and allowed to react at 37°C for 15 min. The reaction was stopped by adding 25 µL of potassium tetraborate solution (0.8 M, pH 9.1), vortexed, and boiled for 3 minutes. In parallel, 12.5 µL of spent media was added to 31.25 µL of T=0 Stop Solution (hyaluronic acid 0.8 mg/mL, 0.8 M potassium tetraborate, pH 9.1), vortexed, and boiled for 3 min. The samples were
dispensed into a 96-well microtiter plate in quadruplicate, and freshly prepared DMAB solution (10% (w/v) \( \rho \)-dimethylaminobenzaldehyde, 12.5% (v/v) 10 M HCl (Sigma-Aldrich), and 87.5% (v/v) glacial acetic acid (Fisher Scientific) was added to each well. The plate was incubated at 37°C for 20 min to allow the color reaction to take place. A TECAN Infinite M200 plate reader was used to measure the absorbance at 590 nm. Hyaluronidase specific activity is expressed as \( 10^3 \times \mu \text{mol N-acetylglucosamine (Sigma Aldrich)} \) released ml\(^{-1}\) min\(^{-1}\) per OD\(_{600}\) unit and is calculated by the equation:

\[
10^3 \times 3 \times (1/15) \times (1/m) \times \Delta A_{590} \times (1/\text{OD}_{600})
\]

where \( \Delta A_{590} \) represents the difference in absorbance between the \( t_{15} \) and \( t_0 \) readings for each sample, \( m \) represents the slope of the standard curve of NAG, OD\(_{600}\) is the optical density of the culture when the sample was taken, 3 is the dilution factor of the sample tested in substrate, and 1/15 is the reciprocal of the reaction time allowed at 37°C. The assay was performed in triplicate for each condition tested.

**Software and Statistical Analysis**

Comparison of zone of clearing was performed using ImageJ64 software. Briefly, the pixel density of three areas on the plate without clearing were averaged to determine background. Then the average pixel density of the DMSO control was compared to each compound, in triplicate. Statistical analysis was performed using a Student’s T-test by GraphPad Prism 6 software.

**Results and Discussion**

**Development of assay to evaluate HysA inhibitors**

In order to evaluate potential inhibitors of HysA, we first needed to develop an
appropriate assay. There are a number of hyaluronidase activity assays that vary in the level of complexity, sensitivity, and the ability to quantify. We decided that a polyacrylamide gel electrophoresis based assay (HA-PAGE) [174] would provide the best combination of simplicity, ability to quantify, and sensitivity. To determine the appropriate concentration of enzyme to test a two-fold dilution series of purified enzyme was prepared and allowed to digest hyaluronic acid for 4 hours. We determined from this experiment that a concentration of enzyme between 0.625-1.25 µg/ml would be most appropriate (Fig. B-1) to use for inhibition studies because it provided a sufficient amount of digestion to identify inhibition without digesting to the extent that the products ran off the gel. In addition to the HA-PAGE assay, we also optimized a HA-agarose plate assay to test for HysA inhibitors due to its lower limit of detection of hyaluronidase activity. For ease of comparison across the two assays, we decided test the ability of inhibitors to inhibit 1 µg/mL of HysA.

Characterization of Fungal Extracts for HysA Inhibition

Thirty-four fungal fractions isolated from fungi associated with the plant *Anemopsis californica* from the laboratory of Dr. Nadja Cech at UNC-Greensboro were then obtained to test for HysA inhibition using the HA-PAGE assay. Nine of the thirty-four extracts tested showed inhibition of HysA using this assay (Fig. B-2). The nine potential inhibitory extracts were then tested for HysA inhibition using a HA agarose plate assay (Fig. B-3 A&B). Two extracts, G152 and G154, showed almost complete inhibition of HysA in both of these assays. The Cech lab then performed an initial fractionation of extracts G121, G153, and G154 for further testing to identify inhibitory fractions. All of the whole extracts were still inhibitory by HA-PAGE and the
hyaluronidase specific activity assay (Fig. B-8). Additionally, G121-2, G121-4, G153-1, G153-3, and G154-3 were able to inhibit HysA to the greatest extent in both assays (Fig. B-8). Future studies will work to further fractionate each of the inhibitory fractions to identify pure active compounds. Once pure inhibitory compounds are identified, the IC$_{50}$ will be determined for each potential inhibitor. The Cech lab has developed a mass spectrometry based hyaluronidase assay, which would be one way that the IC$_{50}$ could be determined that would give a high degree of sensitivity and specificity.

**Examination of Pure Natural Compounds for HysA Inhibition**

Thirty-four pure compounds were isolated from fungi and given to us by Dr. Nicholas Oberlies laboratory at UNC-Greensboro to screen for HysA inhibition. To assess inhibition, a HA-agarose plate assay was utilized and compounds were assessed in triplicate. All of the compounds tested inhibited HysA to some degree compared to the DMSO control (Fig. B-4), and identified 14 compounds that significantly reduced HysA activity in this assay (Fig. B-4). Significant inhibition was defined as a zone of inhibition that was less than 20% of the DMSO control as determined by pixel density using GraphPad ImageJ software. Ten of these compounds were chosen to be re-tested based on their structure, availability, and ability to inhibit HysA in the initial screen (Fig. B-5). From this re-screen, four compounds (compounds 3, 6, 11, 15) were found to decrease HysA activity to the greatest extent (Fig. B-6). Additionally, these four compounds showed a high degree of structural similarity (Fig. B-5). Due to their high degree of inhibition against purified HysA, we next tested compounds 3 and 15 for HysA inhibition using our hyaluronidase specific activity assay. Using this assay, there was only modest inhibition of HysA when compared to the DMSO control (Fig. B-7).
The compound screen identified two compounds, Clearanol C (Compound 15) and (R)-3,4-Dihydro-4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methylene-isochromen-1-one (Compound 3), that warrant further testing (Fig. B-5). Both of these compounds showed nearly complete inhibition of HysA using the HA-agarose assay (Fig. B-4 & B-6). However, there was only limited inhibition using the hyaluronidase specific activity assay (Fig. B-7). The hyaluronidase specific activity assay is much more complex and requires a secondary chemical reaction to produce the chromagen that allows for measurement of activity. Therefore, the abilities of these compounds to inhibit HysA in the different assays may be attributed to the differences in assay complexity and ability to measure inhibition by each assay. The Cech lab has worked to develop a mass spectrometry based hyaluronidase assay, which gives the benefit of simplicity and high sensitivity. However, this mass spec assay has not yet been tested with the inhibitors reported here. Future studies should utilize this assay to further characterize the ability of Compounds 3 and 15 to inhibit HysA.

Conclusions

Hyaluronidase is an intriguing target for the development of an antivirulence therapy. We demonstrated in Chapter II that HysA is required for *S. aureus* virulence, and we demonstrated in Appendix I that *S. aureus* can catabolize HA in a HysA dependent manner. By inhibiting HysA one could potentially limit *S. aureus* dissemination by preventing the role of HysA as a “spreading factor” and prevent *S. aureus* from catabolizing HA in conjunction. This could lead to the development of an effective therapy by targeting multiple facets of pathogenesis. Additionally, the ability to inhibit the closely related *S. agalactiae* and *S. pneumoniae* enzymes should be
investigated to determine if a bacterial hyaluronidase inhibitor could have broad application across a number of pathogens. Future studies should work on further development of candidate fractions and compounds for the creation of an effective HysA inhibitor.
Figure B-1. Development of a HA-PAGE based inhibition assay for HysA
Two fold dilutions of HysA were incubated with HA and electrophoresed in a 15% polyacrylamide gel and stained with Alcian Blue 8GX to visualize hyaluronic acid cleavage products.
**Figure B-2.** Screen of whole fungal extracts for HysA inhibition by HA-PAGE
34 whole fungal extracts were tested for inhibition of HysA at two concentrations (black 1.25 µg/ml, blue 0.625 µg/ml of HysA) by HA-PAGE. The red boxes note the extracts with the greatest ability to inhibit HysA at both concentrations.
Figure B-3. Secondary screen of candidate whole fungal extracts by HA-agarose
Nine whole fungal extracts that were able to significantly inhibit HysA in the HA-PAGE assay were tested for inhibition of 1 µg/mL of HysA using a HA-Agarose plate assay. All extracts were tested in triplicate and compared to the DMSO vehicle control.
Figure B-4. Initial screen of pure compounds for inhibition of *hysA*
Quantification using ImageJ of the initial screen of thirty-four pure fungal compounds isolated by the Oberlies lab and tested for HysA inhibition using a HA-agarose plate assay. All compounds were tested in triplicate against 1 µg/ml Hysa and were compared to five replicates of the DMSO vehicle control.
Figure B-5. Structures of best inhibitory compounds of purified HysA. Structures of the best pure compound inhibitors of HysA isolated by the Oberlies lab from fungi.
Figure B-6. Secondary screen of inhibitory compounds by HA-agarose
Pure compounds that initially inhibited HysA by HA-agarose were rescreened again using the HA-agarose assay. Blue indicates those compounds that are candidates for further testing for their ability to reduce HysA activity to less than 20% of the DMSO control.
Figure B-7. Hyaluronidase specific activity assay for inhibition of HysA by Compounds 3 and 15

The ability of Compounds 3 and 15 to inhibit purified HysA (0.05 µg/ml) was determined using the hyaluronidase specific activity and were compared to the negative DMSO control.
Figure B-8. Inhibition of HysA by fungal extract fractions
The ability of the fungal extract fractions purified by the Cech lab to inhibit HysA were determined by HA-PAGE (A) and the hyaluronidase specific activity assay (B).
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