AUTOCATALYTIC ACTIVATION AND CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* CYSTEINE PROTEASE STAPHOPAIN A

by

Jessica Ip

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

ABSTRACT

Autocatalytic Activation and Characterization of *Staphylococcus aureus* Cysteine Protease Staphopain A

Jessica Ip, M.Sc., 2009

Department of Laboratory Medicine and Pathobiology

University of Toronto

Staphylococcus aureus secretes two cysteine proteases, Staphopain A (*scpA*) and Staphopain B (*sspB*). We hypothesized that ScpA will exhibit a distinct activation mechanism, and a different or complementary substrate specificity compared to SspB. A Cys>Ala active site substitution led to the accumulation of unprocessed 40-kDa proScpA, confirming that ScpA undergoes autocatalytic activation. A temporal analysis of ScpA expression revealed that activation was initiated by processing at Lys171 and Glu176, producing an intermediate that was rapidly converted to several isoforms of mature protease by processing after Thr202, Lys209, Thr214 and Asn216. Consistent with broad specificity, mature ScpA was sensitive to autocatalytic degradation. ScpA demonstrated activity towards elastin, fibrinogen and indicated evidence for binding to heparin. Elastinolytic activity was uniquely associated with strains belonging to CC30, and was correlated with ScpA expression. Therefore, although ScpA and SspB share both sequence and structural similarity, they exhibited very different substrate specificities and activation mechanisms.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my supervisor, Dr. Martin McGavin, who has shared with me a lot of his research insight and expertise. His leadership and encouragements have allowed me to successfully accomplish my work and I am thankful for the opportunity he has given me to be part of his lab.

I would also like to thank my committee members, Dr. Miles Johnston and Dr. Dean Rowe-Magnus for their guidance and valuable suggestions. Special thanks to Dr. Eric Yang from the Proteomics Core Facility at Sunnybrook for all his help and advice.

To the good friends I made in S-Wing, thanks for all the good laughs and memories. Your company made my days more enjoyable and I wish you all the best in your future endeavours. To the members of the McGavin Lab, Latha Jacob, Nicholas Nickerson, Carly Pigeon and Melinda Yeung, this was the best lab I have ever been part of and I am grateful for the time we had together and for all the encouragements and guidance you have given me.

To my loving parents, my brother Barry and sister Jasmine, thank you for believing in me and supporting me from the very beginning. To Silas, thank you for your utmost patience and encouragements through every step of the way. To the close friends who have walked beside me during the course of this work, thank you.

Most importantly, this thesis would not have been possible without the infinite grace and peace given to me everyday by my Lord and Saviour, Jesus Christ. Thank You for everything.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Agr	Accessory gene regulator
AIP	Autoinducing peptide
AMC	Aminomethyl coumarin
ATP	Adenosine tri-phosphate
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CA-MRSA	Community-Acquired Methicillin Resistant *Staphylococcus aureus*
$CaCl_2$	Calcium chloride
CAPD	Continuous Ambulatory Peritoneal Dialysis
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CC	Clonal Complex
CNBr	Cyanogen Bromide
$CuSO_4$	Copper Sulphate
dNTP	Deoxynucleotide Triphosphate
E. coli	*Escherichia coli*
Ebp	Elastin binding protein
ECM	Extracellular Matrix
EDTA	Ethylene diaminetetraacetic acid
ET	Exfoliative Toxin
Fg	Fibrinogen
Fn	Fibronectin
Fnbp	Fibrinogen binding protein
HA-MRSA	Hospital-Acquired Methicillin Resistant *Staphylococcus aureus*
HPLC	High Liquid Chromatography
IPTG	Isopropyl β-D Thiogaactopyranoside
LB	Luria Betrani
LC-MS/MS	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix molecules
NaCl	Sodium Chloride

OD	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*
P. gingivalis	*Porphorymonas gingivails*
PCR	Polymerase Chain Reaction
PHYRE	Protein Homology Recognition Engine
pNA	p-nitroanilide
PVDF	Polyvinylidene difluoride
PVL	Panton-Valentine Leukocidin
RPM	Revolutions Per Minute
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. pyogenes	*Streptococcus Pyogenes*
SarA	Staphylococcal Accessory Regulator
Scp	Staphylococcal cysteine protease
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPC	Staphylococcal Proteolytic Cascade
Ssp	Staphylococcal serine protease
SSSS	Staphylococcal Scalded Skin Syndrome
ST	Sequence Type
TCA	Trichloroacetic acid
TSB	Tryptic Soy Broth
TSST	Toxic Shock Syndrome Toxin
VRE	Vancomycin Resistant Enterococci

UNITS

Da – Dalton	µl – microlitre	nm – nanomole
°C – Degree Celcius	ml – milliliter	NIH – Normalized Index of Hemolysis
g – gram	min – minute	% – Percent
g – gravity	M – Molar	U – Enzyme Unit Activity
h – hour	mM – millimolar	V – Volt
µg – microgram	ng – nanogram	w/v – Weight/Volume

LIST OF FIGURES

LIST OF TABLES

PART I: INTRODUCTION

I. *Staphylococcus aureus*

(i) Identification

Staphylococcus aureus is a Gram-positive pathogen that was first described by Sir Alexander Ogston in the late 1800s when he discovered that a cluster-forming bacteria was involved in causing abscess formation in humans. Because of the clusters of cocci observed through the microscope, the name "*Staphylococcus*" was given based on the Greek term *staphyle*, which meant, "bunch of grapes" (171). The term "*aureus*" comes from the Latin meaning "golden" describing the golden-yellow pigmentation of the colonies when grown on a nutrient agar medium.

In addition to its unique golden pigmentation, *S. aureus* can be distinguished from other staphylococcal species by the production of coagulase, an enzyme that induces fibrin clot formation. All other members of the staphylococcal species unable to produce free coagulase are referred to as coagulase-negative staphylococci (207). *S. aureus* can also be identified by its growth in mannitol salt agar, by which colonies grow as a result of mannitol fermentation and a change in the pH of the medium. Furthermore, using deoxyribonucleases tests, the level of zone clearing of nutrient agar can differentiate the staphylococcal species (252).

(ii) Clinical Importance of Staphylococcal Infections

S. aureus is one of the most successful and persistent human pathogens to date. *S. aureus* typically colonizes the skin and mucosa, primarily in the anterior nares, but also in the vagina and perianal area (8). Approximately 30 to 50 percent of colonization occurs

in healthy people, with 10 to 20 percent persistently colonized (33, 162). An increased risk for infection is present for individuals colonized with *S. aureus* (251). *S. aureus* proved to be virulent as reported in Boston City Hospital in 1941, where the mortality rate was 82% in 122 patients associated with *S. aureus* bacteremia (225). During the period 1997 to 1999, *S. aureus* was reported as the leading cause of bloodstream, lower respiratory tract, and skin and soft tissue infections in the United States, Canada, Latin America, Europe, and the Western Pacific region (55).

Staphylococcal infections are initiated when there is a breach of the skin or mucosal barrier allowing for *S. aureus* to infect surrounding tissues or enter the bloodstream. *S. aureus* can cause a variety of diseases ranging from mild subcutaneous infections, to severe life threatening conditions such as osteomyelitis, endocarditis and sepsis (125).

Staphylococcal outbreaks among adults commonly occur in hospitals, particularly in burn or surgical units. Medical devices such as intravenous catheters and deep wounds in patients serve as a favourable growth environment for staphylococci (27, 48, 222). In the recent decades, there has been higher prevalence of nosocomial staphylococcal infections, partly as a result of increased use of intravascular devices (233).

Due to the persistence of *S. aureus* in the environment, outbreaks may occur as a result of exposure to environmental sources (48), or more commonly, through contact with the colonized hands of health care workers which allows for *S. aureus* to be readily transmitted (135). In addition, the spread of epidemic strains has been associated with the admission of infected patients from one hospital to another (24, 208).

(iii) Emergence of Antibiotic Resistant Strains of *S. aureus*

β-lactam antibiotics interferes with cell wall synthesis by inactivating the transpeptidases responsible for cross-linking peptidoglycan chains in the cell wall. As a result of compromised structure, cell wall-based metabolism and cell division, as well as the function of the cell wall, is weakened, therefore leading to autolysis (229).

S. aureus can acquire resistance by mutation, conjugation transduction or transformation although the latter two methods are less common (5). Prior to the introduction of antibiotics in the 1940s, invasive staphylococcal infections often resulted in mortality (225). When penicillin was introduced clinically in 1941, mortality rate was markedly reduced (232). Unexpectedly, in 1942, penicillin resistance in staphylococcal infections was reported (200) and by the late 1940s, penicillin was commonly used in hospitals and penicillin-resistant strains began to surface and exceed sensitive strains (18). Penicillin-resistance was due to the production of a serine protease, β-lactamase, which hydrolyzes pencillin's β-lactam ring (125).

Since penicillin was no longer an effective treatment for most *S. aureus* infections, semisynthetic β-lactamase-resistant penicillins, such as methicillin and oxacillin were developed to treat methicillin-sensitive *S. aureus.* However, within a few years of its introduction, the emergence of methicillin-resistant *S. aureus* (MRSA) strains were reported (178). From 1975 to 1991, hospitals in the United States reported an increase from 2.4% to 29% in the proportion of *S. aureus* strains resistant to methicillin. By 1991, 79% of hospitals were reporting MRSA cases (178) and by 2003, 59.5% of *S. aureus* strains isolated from intensive care units were methicillin resistant (3). Methicillin resistance is due to the acquisition of the *mec* gene that encodes for an altered penicillin-

binding protein, PBP2a (34). Cross-resistance is conferred since PBP2a has a lower affinity for most β-lactams compared to PBP2 found in methicillin-sensitive *S. aureus* (8).

Until recently, vancomycin has remained an effective treatment against most MRSA infections. There has been increasing concern about the potential for vancomycin-resistance in *S. aureus*. These concerns are based on the reports that clinical isolates of certain species of coagulase-negative staphylococci had developed resistance to vancomycin (214, 216). Furthermore, Gram-positive vancomycin-resistant enterococci (VRE) had developed and spread in many hospitals in the United States (152). The selective pressure for the development of resistant strains suggests it may only be a matter of time before vancomycin-resistant *S. aureus* is a threat to effective treatment for severe staphylococcal infections.

Although the persistence and high level of resistance of strains in the hospital setting have been a serious concern, community-acquired MRSA (CA-MRSA) has shown to be more virulent than hospital-acquired MRSA (HA-MRSA) causing severe and often fatal infections in healthy individuals (77). While the rate of HA-MRSA infections had increased to nearly 50% in the late 1990s, community-acquired strains had begun to surface in diverse community groups including American Indian and Alaska Natives (14), sports teams (2), prisoners (1), and children (110). By 2002, up to 20% of CA-MRSA isolates were obtained from healthy individuals who had no established risk factors (78). The emergence of hospital outbreaks compared to the subsequent delayed onset of disease in the community has suggested that hospital acquired strains may have been transmitted to the community (26). However, studies of the emergence of

community-acquired MRSA disease over the past decade reported that *S. aureus* isolates from the community and those that were hospital-acquired were distinct (153).

II. Virulence Strategy

The virulence strategy of *S. aureus* proceeds through five stages: 1) colonization, 2) local infection, 3) systemic dissemination and/or sepsis, 4) metastatic infection, and 5) toxinosis (8). Following colonization, infections are initiated by a breach of skin or mucosal barrier allowing the bacteria to spread to the local tissues or invade the blood stream. The capacity of *S. aureus* to express a variety of virulence factors has contributed to its success as a threatening pathogen. A brief list of those factors known to contribute to the establishment or maintenance of an infection is listed in Table 1.

The virulence of *S. aureus* is a result of coordinated activity of proteins that are expressed on the bacterial surface that bind to the extracellular matrix, as well as secreted toxins and enzymes. The expression of many virulence factors appears to be growth-phase dependent. Surface proteins are mostly produced during the exponential growth phase, while secreted proteins are produced during the stationary phase. This strategy of gene expression may have clinical implications whereby different stages of staphylococcal infections seem to require different types of virulence factors. Furthermore, whether *S. aureus* remains in the local tissues or continues to spread depends on the regulation of virulence factors by the complex networks of different staphylococcal global regulatory systems (125).

Table 1. Major virulence factors of *S. aureus* and their associated pathogenic mechanisms

Pathogenic Mechanisms	Virulence Factor
Adhesion to Host Cells	Fibronectin-binding proteins
	Fibrinogen-binding proteins
	Collagen-binding protein
	Elastin-binding protein
Immune Evasion	Microcapsules (Serotypes 5 and 8)
	Protein A
	Coagulase
	Fatty acid-metabolizing enzyme
	Leukocidin and/or γ-toxin
Tissue Invasion and Bacterial Dissemination	Proteases
	Nucleases
	Lipases
	Hyaluronate lyase
	Staphylokinase
	Panto-Valentine leukocidin
Induces Sepsis	Toxic shock syndrome toxin
	Enterotoxin
	Hemolysins (α-, β-, δ-, γ-)
Promotes Specific Toxinosis	Toxic shock syndrome toxin
	Enterotoxin
	Exfoliative toxin

The data for this table was compiled from References (8, 101, 125).

Based on their pathogenic mechanisms, virulence factors may be grouped into three functional categories which include factors that mediate adhesion of bacteria to host cells, factors that help the bacteria evade the host immune response and factors that promote tissue damage and bacterial dissemination (12). Selected virulence factors from these three categories will be discussed in further detail below.

(i) Colonization Factors

The ability to adhere to the extracellular matrix (ECM) allows *S. aureus* to colonize the host. As the initial step in the infection process, adherence of the *S. aureus* to the host ECM utilizes bacterial surface adhesins designated as MSCRAMMS (microbial surface components recognizing adhesive matrix molecules). MSCRAMMS must be localized to the bacterial cell surface and be able to bind with high affinity and specificity to a macromolecular ligand present within the ECM (180). Such ECM ligands include fibronectin, fibrin and collagen.

For most surface-associated proteins, which are covalently attached, a LPXTG motif is located at its C-terminal end, followed by hydrophobic residues and a positively charged tail (215). The LPXTG motifs are proteolytically cleaved by a membrane-bound transpeptidase referred to as sortase prior to anchoring the C-terminus of the protein to the cell wall (156).

a. Fibronectin-Binding Proteins

S. aureus possesses two related fibronectin (Fn) binding proteins, *fnbA* and *fnbB,* both of which are adjacent to each other but are transcribed separately. Most *S. aureus*

strains harbour both of these genes (183, 204), but some isolates may only carry a single *fnb* gene (146, 148, 183, 204, 228). FnbpA and FnbpB have similar protein structures and share almost identical D domains, which are located close to the cell-wall-spanning domain and considered as the primary domain responsible for interaction with Fn (71, 180, 224). In addition to binding to Fn, both FnbpA and FnbpB can interact with elastin (206) and FnbpA can bind to fibrinogen (Fg) (249).

It has been shown that lack of adherence and host-cell invasion of *S. aureus* strain Newman was caused by point mutations within the *fnb* genes which led to the loss of the cell wall anchor function of these proteins (84). In addition to host cells, it is possible for *S. aureus* to adhere to biomaterial surfaces such as intravenous catheters coated with host plasma and ECM proteins to cause a foreign body infection (246). Furthermore, fibronectin was shown to be an important ligand for bacterial attachment as blood clots formed *in vitro* without Fn inhibited attachment of *S. aureus* (199). The observation that Fn is present on epithelial and endothelial cell surfaces suggests that Fn may contribute to the colonization of *S. aureus* in CAPD (continuous ambulatory peritoneal dialysis) peritonitis, severe endocarditis and ruminant mastitis (74). In addition, a role for Fnbps in virulence is implicated in Fnbp mutants that reduced adherence of *S. aureus* to traumatized heart valve tissue in rats (119).

b. Fibrinogen-binding proteins

Present in high concentrations in the blood plasma, fibrinogen is a glycoprotein involved in coagulating blood as well as wound healing processes. Blood clots are formed when fibrinogen is proteolytically cleaved to yield fibrin (180). *S. aureus*

synthesizes two structurally similar but genetically distinct (73) fibrinogen (Fg) binding proteins also known as clumping factors ClfA and ClfB, which are encoded by genes *clfA* and *clfB*, respectively (136, 157). The Fg-binding domain is represented by the A domain which is anchored by the R domain to the cell wall (87). The region that is responsible for Fg-binding is located at the C-terminal end of the A domain in ClfA (88). The 340-kDa Fg is composed of six polypeptide chains (Aα, Bβ, γ). ClfA recognizes the C-terminal of the γ-chain (137) while ClfB binds to the Aα- and Bβ-chains (157).

Virulence of both clumping factors is demonstrated with its involvement in causing endocarditis in a rat infection model. In this model, ClfA was shown to be more effective than ClfB (67, 149). Endocarditis can be further developed by the effective attachment of *S. aureus* to platelet aggregates present on the surface of the heart valves (236). It has been suggested that the activation of human platelets and the adherence of *S. aureus* to these platelets is facilitated by the clumping factors (168, 223). Furthermore, ClfA has been associated with staphylococcal arthritis (106, 175) and has shown to protect *S. aureus* against macrophage phagocytosis, although, protection did not require the presence of Fg (177). ClfB has been implicated as a virulence factor that promotes nasal colonization through adhering cytokeratin 10 molecules on the surface of desquamated nasal epithelial cells and keratinocytes (169).

c. Collagen binding protein

Collagen is a major component of the ECM and studies have found that *S. aureus* is capable of adhering to collagen via a specific receptor (231, 237). *S. aureus* produces a gene *cna* which encodes for a collagen adhesin (Cna) that facilitates in attachment to

collageneous tissues. Cna is comprised of an A domain that is responsible for collagen-binding activity (181). Although only one gene has been identified in *S. aureus*, several different forms of the *cna* gene exists as the A domain is followed by varying numbers of B domains (80). Cna has been implicated in virulence since strains isolated from osteomyelitis and septic arthritis were able to adhere to collageneous tissues *in vitro* (74). Furthermore, Cna has been shown to sustain experimental endocarditis, but not necessarily initiate the infection (93).

d. Elastin binding protein

Elastin is a major component of the ECM and is important in maintaining the elasticity and function of tissues rich in elastin and elastin fibres such as lungs, skin and major blood vessels (154). In addition to Fnbps which demonstrates elastin-binding activity, *S. aureus* produces another surface adhesin, the elastin binding protein EbpS, that is also responsible for adhering to elastin (58). Distinct from other adhesins of *S. aureus* such as fibrinogen-, collagen- and fibronectin-binding proteins, EbpS is an integral protein with two transmembrane domains. The N-terminus of EbpS containing the elastin binding site is exposed to the extracellular milieu (58). The ability of EbpS to adhere to elastin may contribute to the bacterial colonization of *S. aureus*.

(ii) Immune Evasion Factors

When there is a breach of the skin or mucosal barrier, *S. aureus* is faced with the primary defence of the host. Once the infection has been initiated, a strong inflammatory response is stimulated thereby recruiting neutrophils and macrophages to the site of infection. The neutrophils and macrophages will attempt to phagocytose and remove invading *S. aureus* (72). However, *S. aureus* has been well adapted to employ several strategies to evade the host immune response.

a. Staphylococcal Protein A

One of the strategies *S. aureus* uses to evade phagocytic killing is by expressing surface-associated anti-opsonic proteins (72). Staphylococcal Protein A (Spa) is a surface protein known to bind to the Fc region of IgG (245). The interaction between Spa and the Fc region of IgG results in the coating of the bacterial surface with IgGs positioned in such a way that renders it unavailable for recognition by neutrophil Fc (72). Therefore, Spa may contribute to the antiphagocytic effects of *S. aureus*. This is supported by the observation that *S. aureus* strains deficient in Spa were more susceptible to phagocytosis by neutrophils *in vitro* (79) and resulted in attenuated virulence in several animal infection models (176, 179). Spa can also bind the von Willebrand factor, which is a multimeric serum glycoprotein implicated in mediating platelet adhesion in sites of endothelial damage (89, 209). It has also been shown that Spa can act as a receptor for TNFR1, which is a receptor for tumour-necrosis factor α, and stimulating an inflammatory response in airway epithelial cells (82).

b. Bacterial Capsules

Many strains of *S. aureus* express a capsular polysaccharide. Of the known 11 serotypes, serotype 5 and 8 have been found to be implicated in increased virulence in animal infection models (13, 126, 161, 243). The presence of the bacterial capsule was shown to reduce phagocytosis by neutrophils in the presence of normal serum opsonins. This suggests that encapsulated *S. aureus* strains are capable of evading opsonophagocytosis (161, 243).

(iii) Invasion Factors

As mentioned before, the expression of *S. aureus* virulence factors are growth-phase dependent. Surface proteins are mainly expressed during exponential growth phase, whereas the synthesis of various extracellular proteins occurs during stationary phase (125). It has been suggested that this transition may be clinically relevant, as it is believed that the expression of surface proteins allows for successful colonization during the early stages of infection while secreted proteins are important for bacterial survival and dissemination during the late stages of infection (174).

Once *S. aureus* invades the skin barrier, it secretes exotoxins and enzymes that can initiate various severe cutaneous and systemic infections. Some of these virulence factors include proteases, nucleases, lipases, hyaluronate lyase, staphylokinase and hemolysins (α-, β-, δ-, γ-) (101). An example of a systemic effect by toxins are the staphylococcal exfoliative toxins (ETs), including epidermolytic toxins A and B which have been shown to cause detachment in the epithelial layer, a feature of staphylococcal scalded skin syndrome (SSSS) (125). Also, Toxic Shock Syndrome Toxin (TSST-1) has

been linked to toxic shock syndrome, which is a potentially fatal illness and is characterized by high fever, rashes, peeling of the skin, hypotension and multisystem involvement (56). Moreover, Panton-Valentine leukocidin (PVL) toxin, a cytolytic toxin, has been associated causing furunculosis (boils) (194) and necrotizing pneumonia in young, otherwise healthy individuals (124, 257). Collectively, these diseases strongly support the importance of exotoxins in the virulence of *S. aureus*.

In addition to toxins, secreted proteases have also demonstrated an important role in the growth and dissemination of *S. aureus*. A detailed review of secreted proteases as virulence factors will be examined in Chapter III.

(iv) Regulation of Virulence Factors in *S. aureus*

The virulence of *S. aureus* is a result of coordinated expression of secreted and surface-associated proteins by specific global regulatory systems. These systems regulate the production of virulence factors at specific stages of growth and also respond to environmental stimuli. Two of the best-described regulators for virulence factors are Agr (accessory gene regulator) and sarA (staphylococcal accessory regulator).

The *agr* locus was identified by a transposon Tn*551* insertional mutant that caused a pleiotropic effect on extracellular and cell wall-associated proteins. Production of extracellular proteins were decreased whereas cell wall-associated proteins such as protein A and fibronectin-binding protein were increased (202). It has been found that *agr* can unregulate 104 genes and downregulate 34 genes in a cell density-dependent manner (65).

The *agr* locus consists of two divergent transcripts, RNAII and RNAIII, which are

transcribed by two distinct promoters, P2 and P3 respectively (184). The P2 transcript, referred to as RNAII, encodes AgrB, AgrD, AgrC and AgrA that together assemble a quorum sensing system (263). AgrB and AgrD work together to produce an autoinducing peptide (AIP) while AgrC and AgrA make up the a two-component signal transduction system which is sensitive to the signal, AIP (12).

During the exponential growth phase, *agrD* encodes a propeptide that undergoes maturation by the anchored membrane protein AgrB prior to secretion into the extracellular environment. AgrB is a 26-kDa membrane protein containing six transmembrane segments (105) while the processed AIP consists of seven to nine residues (104, 133). ArgC is a 46-kDa transmembrane receptor made from five transmembrane domains. Upon binding of AIP, AgrC is phosphorylated at the sensor of the system which is a conserved cytoplasmic histidine kinase present at the C-terminal end of the protein (104). Subsequently, a phosphate residue is transferred from AgrC to the response regulator, AgrA, thereby allowing AgrA to bind to the P2 and P3 promoters or interact with other factors to increase transcription from these promoters (29).

The P3 promoter initiates transcription of RNAIII, which has been shown to be a pleiotropic effector molecule of the *agr* system. Upon accumulation of AIP, which signals the AgrCA system, RNAIII upregulates the transcription for secreted proteins while downregulating surface-associated virulence factors (102, 165, 166). Therefore surface-associated virulence factors are typically expressed during early growth phase whereas secreted proteins are activated and transcribed during late exponential growth phase (12). RNAIII also encodes for δ-toxin gene (103) that when translated, appears to modulate the RNAIII regulatory function (16). The *agr* gene has demonstrated its

importance to virulence by *S. aureus* in animal models of infection including arthritis, subcutaneous abscesses, endocarditis, mastitis, and osteomyelitis. Models have shown greatly attenuated virulence as a result of *agr* mutants (45, 195).

Another well-characterized pleiotropic regulator of *S. aureus* is the staphylococcal accessory regulator, sarA. The *sarA* locus was identified locus by a transposon Tn917 insertional mutant that decreased fibrinogen binding in a clinical strain of *S. aureus*. Moreover, opposite to the phenotype of an *agr* mutant, surface-associated proteins were reduced to lower levels in the Tn917 insertional mutants than in the parent strain (39). SarA is transcribed from three distinct promoters, P1, P2 and P3, which all shares a common 3' end. Therefore, the 14.5-kDa SarA protein is a product of all three transcripts (19).

The DNA-binding protein sarA regulates target genes by binding to a conserved AT-rich sequences present in their promoter regions. sarA acts as an activator of the *agr* system by binding to the *agr* P2 and P3 promoter regions. This activates RNAII and RNAIII thereby regulating the production of *S. aureus* virulence factors (38, 150). Furthermore, sarA may regulate the synthesis of target proteins by binding to the conserved regions within the promoters of surface-associated as well as extracellular proteins (41, 65).

III. Role of Secreted Proteases in Virulence

Secreted proteases facilitate in the survival of bacteria and dissemination of infection (125). In particular, secreted staphylococcal proteins include metallo-, serine- and cysteine proteases (10, 61, 188, 205). With the exception of α_2-macroglobulin, these

proteases are not regulated by human plasma protease inhibitors. In fact, these staphylococcal proteases may interfere with host protease activity by proteolytically inactivating some of these host-derived inhibitors resulting in harmful effects on the host (128, 193). Proteases can modulate bacterial adhesion and cell surface proteins as the bacteria progresses through virulence factor expression (141). Besides toxins and other enzymes, proteases play an important role in host tissue destruction allowing *S. aureus* to acquire nutrients and spread into adjoining tissues (188, 244). Furthermore, proteases initiate the release of kinins causing pathogenic effects ranging from pain and edema, translocation of bacteria from the site of infection into circulation and septicemia (128). In addition to degradation of immunoglobulins and complement cascade proteins, proteases interact with coagulation and fibrinolysis pathways to allow the bacteria to defend against host immune response and promote dissemination (9, 63).

(i) The Staphylococcal Proteolytic Cascade

Amongst the virulence factors secreted by *S. aureus*, it has been found that the activity of three major proteases secreted by *S. aureus* work together in a Staphylococcal Proteolytic Cascade (SPC). This cascade is initiated by the rapid autocatalytic activation of a metalloprotease, aureolysin. Mature aureolysin subsequently activates a serine protease, SspA by processing its N-terminal propeptide. SspA proceeds to the last step of removing the N-terminal propeptide of the cysteine protease precursor SspB to yield an active protease (4, 5). SspA and SspB are encoded in a staphylococcal serine protease (*ssp*) operon along with SspC, of unknown function. The proteases in the *ssp* operon have been previously identified to be required for the *in vivo* growth and survival of *S. aureus*.

This was supported by a study using signature-tagged mutagenesis on *sspA* that had led to reduced virulence in three different infection models (47). However, in another study, a nonpolar inactivation of SspA resulted in a pleiotropic effect and did not show to be directly involved in the development of tissue abscess infections (205). In addition, aureolysin, in combination with α-hemolysin, sortase A, and functional *agr and* sig*B* regulators, were required for bacterial survival and evasion of human monocyte-derived macrophages. Furthermore, confocal microscopy demonstrated that Aur, SspA and SspB were produced following phagocytosis by human neutrophils (31). *S. aureus* also secretes another, although less characterized, cysteine protease called Staphopain A (ScpA) which shares 47% amino acid identity with SspB (81). ScpA combined with the activity of SspB can induce vascular leakage activity by targeting kininogens to release bradykinin, a potent vasodilator (100). Although it has been suggested that ScpA is not involved in the SPC pathway for activation, secreted proteases ScpA along with Aur, SspA and SspB undoubtedly plays a significant and complex role in the virulence strategy of *S. aureus*. These proteases and other similar cysteine proteases involved in virulence will be described in further detail in the following sections.

(ii) Aureolysin

Aureolysin belongs to the M4 family of metalloproteases (201) and was first purified and identified as an EDTA-sensitive protease from *S. aureus* strain V8 (11). Genetic analysis has shown that the *aur* gene is strongly conserved and is widespread amongst strains isolated from humans indicating an important role in bacterial growth and survival (213). Two allelic forms of the *aur* gene have been identified as aureolysin

type I and type II in strains isolated from humans and domestic animals (213, 239). Purification and biochemical analysis of both aureolysins from domestic animals have shown that the specific activity of aureolysin type II was two times higher than that of type I (239).

Aureolysin is structurally similar to zinc metalloprotease thermolysin (17) with a polypeptide chain of 301 amino acids folded into a β-pleated N-terminal domain and an α-helical C-terminal domain. Gene analysis has suggested that the aureolysin protease is produced in preproform (213). This 56 kDa preproAur is comprised of a signal peptide followed by an N-terminal propeptide, and a 33 kDa metallopeptidase domain containing a catalytic HExxH (159). Its similarity to thermolysin and other M4 metalloproteases suggests that Aur may undergo autocatalytic activation. A previous study had concluded that Aur maturation does not proceed autocatalytically (217). However, a more recent study has shown that proAur does proceed through rapid autocatalytic activation and is processed within the fungalysin-thermolysin-propeptide domain, an internal segment of the propeptide. This domain, which is conserved in other M4 metalloprotease, has proved to function as a chaperone as well as a modulator for the specificity of Aur autocatalytic activation (159).

Expression of Aur is upregulated by *agr* and is repressed by *sarA* (25, 35, 65). Aur exhibits specificity for peptide bonds on the N-terminal side of bulky hydrophobic residues. Aur can be inactivated by *o*-phenanthroline, reactivated by zinc ions and its structure can be stabilized with calcium (17, 59, 191). Aur functions as the initiator of the SPC pathway beginning with its rapid autocatalytic activation and subsequently activating SspA precursor (59, 160). *In vitro* studies have shown that Aur processes and

thereby inactivates human plasma protease inhibitors α_1-antichymotrypsin and α_1-proteinase inhibitor, a neutrophil elastase inhibitor. Processing of α_1-antichymotrypsin yields a molecule with potent chemotactic activity towards neutrophils (189). Compared to SspA, Aur inefficiently cleaves α_1-proteinase inhibitor suggesting that these two proteases may work in concert to deregulate neutrophil-derived proteolytic activity (193). In addition, Aur may be involved in coagulation activity induced by staphylococci as *in vitro* studies have suggested that a metalloprotease from *S. aureus* can directly activate prothrombin, thereby promoting plasma-clotting (250). Similar to SspA, Aur modifies cell surface proteins of *S. aureus*, which can potentially promote detachment of bacterial cells from colonized sites and facilitate in bacterial dissemination. In particular, Aur is responsible for the cleavage of the N-terminal domain surface-associated clumping factor, ClfB (134).

(iii) SspA

The staphylococcal serine protease (Ssp) SspA, also known as V8 protease, was the first purified and characterized secreted enzyme of *S. aureus* (61). From the glutamyl endopeptidase family, SspA purified from different strains have demonstrated structural heterogeneity (20, 210, 261). This heterogeneity was likely due to the predicted intrinsic region of disorder at the C-terminus of the protease comprised of tandemly repeated Pro-Asn/Asp-Asn tripeptides (60). Using sequence analysis of the *S. aureus* strain COL genome (http://www.tigr.org), the *sspA* was followed by an open reading frame encoding an extracellular cysteine protease, *sspB*. The *sspA* and *sspB* proteases are encoded on one operon which also included a third open reading frame, *sspC*, of unknown function. The

ssp operon expression is upregulated by *agr* (205) and strongly repressed by *sarA* (35). SspA is synthesized in preproform and is cleaved by signal peptidase to remove the signal peptide during secretion. proSspA is subsequently released for initial processing exclusively through autocatalytic intramolecular maturation, which was shown to be a necessary step for efficient processing to yield mature SspA by Aur (59, 160).

SspA exhibits a high specificity, with a preference for the carboxyl-side of glutamic acid residues (94). As a component of the SPC pathway, SspA is required for activating the 40-kDa SspB by proteolytic removal of the SspB propeptide (130). SspA may moderate adhesion functions of *S. aureus* by modifying surface proteins. Consistent with its preference for cleaving after glutamic acid residues, SspA degrades cell-surface fibronectin binding proteins (Fnbps), which have a high glutamic acid content, including the required motif involved in ligand binding (140, 141). Studies have shown that the stability of Fnbp was enhanced by supplementing cultures with α_2-macroglobulin (141) as well as inactivation of SspA (113).

In addition to efficiently inactivating α-proteinase inhibitor (193), SspA is able to generate kinins from high molecular weight kininogen which may assist in bacterial spread into the systemic circulation (128, 147). Furthermore, *in vitro* studies showed SspA may promote immune evasion by its ability to cleave the heavy chains of all classes of immunoglobulin (9, 196). Contrary to a study observing attenuated virulence after a transposon insertion into the *sspA* gene (47), a nonpolar allelic replacement mutation of *sspA* led to inactivation of SspA but did not result in attenuated virulence in tissue abscess model of infection. However, a pleiotropic effect was observed on the profile of secreted proteins including autolysin activity suggesting that SspA may be involved in

controlling autolytic activity. There was also an effect on the maturation of the SspB as noted by the accumulation of a 40 kDa protein in the culture supernatant, which was later identified to be proSspB (205).

(iv) SspB

As mentioned above, the *sspB* gene encoding the preproform of a cysteine protease is preceded by *sspA* and followed by *sspC* on the *ssp* operon. During activation, the propeptide of the 40 kDa proSspB is removed by SspA yielding a 20 kDa mature form of SspB, also known as Staphopain B. This was noted by the accumulation of proSspB in the culture supernatant when SspA was inactivated (205). With 30.52% GC content, SspB shares a 47% amino acid identity with another secreted cysteine protease of *S. aureus* Staphopain A (ScpA). Both staphopain genes were conserved among *S. aureus* strains suggesting an important role in the growth and survival of *S. aureus*. Although SspB exhibits some degree of polymorphism, there is minimal alteration in protein sequence and therefore is able to retain its function (81). The thiol histidine active site of SspB was identified to be LGHALAVVGNA (205).

SspB belongs to the papain family of cysteine proteases, which requires a bulky hydrophobic amino acid at the P2 site relative to the scissile bond (142, 143, 163, 235). SspB exhibits a narrow substrate specificity as it demonstrates a strong preference for arginine at the P1 site and a hydrophobic amino acid at the P2 site (130). SspB is stimulated by EDTA in the presence of cysteine and inhibited by a cysteine protease inhibitor, E-64. As suggested by the nonpolar inactivation of SspA which did not appear to contribute to the development of tissue abscess infection (205), virulence may have

been a result of downstream effects on SspB by the transposon insertion in the *sspA* gene (47) thereby supporting SspB as an important virulence determinant. This is supported by a study in a skin abscess mouse model whereby the insertional inactivation of *sspB* resulted in attenuated virulence (217).

SspB is unable to process resorufin-labeled casein, IgG, IgA, or serum albumin suggesting that SspB does not have a general role in protein degradation. However, SspB demonstrates a role in controlling adhesive functions by targeting extracellular matrix molecules. As such, fibronectin is degraded by SspB at $VR_{259}\downarrow A$, which mimics the cleavage site by urokinase plasminogen activator. Furthermore, SspB can process fibrinogen at $YR_{42}\downarrow A$, which is the same site that is cleaved by plasmin to initiate fibrinolysis. SspB is able to cleave single-chain kininogen yielding fragments similar in size to the heavy and light chains. Although bradykinin was not excised from the putative heavy chain, the conversion may increase attachment to cell surfaces and also facilitate in efficient conversion to active kallikrein, which ultimately can enhance vascular permeability thereby promoting the dissemination of infection. Moreover, SspB promotes the detachment of primary human keratinocytes suggesting a means for *S. aureus* to gain access to the ECM (130). SspB has also demonstrated a possible role for initiating chronic inflammation. This was suggested by the observation that SspB can mediate the activation of chemoattractant chemerin, which could result in the recruitment of plasmacytoid dendritic cells and macrophages to the site of infection (118). A possible strategy for *S. aureus* to evade the immune system was noted when phagocytosis by neutrophils and monocytes were reduced when treated with SspB (227).

(v) ScpA

S. aureus also secretes another, although less characterized, cysteine protease called Staphopain A (ScpA). The less virulent *Staphylococcus epidermidis* has orthologues of *scpA* and s*spA*, but the latter locus is monocistronic and therefore has no orthologue of s*spB* (64). Hence, the simultaneous existence of two staphopain operons in *S. aureus* distinguishes the proteolytic capacity of *S. aureus* from *S. epidermidis*, thereby suggesting a key role of staphopains in causing virulent infections. The presence of two staphopains does not suggest functional redundancy as each protease differs in substrate specificity and function (81). In addition, the *scpA* gene has been found in high protease-producing avian strains of *S. aureus* (238).

The *scpA* gene is encoded on the staphylococcal cysteine protease operon (*scpAB*) and is co-transcribed with a downstream specific inhibitor of ScpA called *scpB* (staphostatin A) (62). ScpA is secreted in preproenzyme form but its activation mechanism has yet to be elucidated. Similar to *sspB*, *scpA* is conserved and has a thiol histidine active site AGHAMAVVGNA (130). A study using strains with insertional inactivations of SspA, SspB or Aur that continue to yield mature ScpA suggested that it is not involved in the SPC pathway for activation (217). The ScpA orthologue of *S. epidermidis,* Ecp, efficiently cleaves substrates containing the Yaa-Glu↓Xaa sequence motif, where Yaa represents a large hydrophobic amino acid and Xaa represents any amino acid except for proline (172). In contrast, it has been suggested that ScpA exhibits a broader substrate specificity (25). Due to its broad specificity, its proteolytic activity may lead to degradation of *E. coli* host cell proteins. Therefore, a study had shown that

recombinant ScpA could only be expressed with its cognate inhibitor, ScpB, due to cytoplasmatic toxicity when expressed alone (254).

Ecp of *S. epidermidis*, which shows 75% identity with the sequence of ScpA, has been known to possess elastinolytic activity (172). Similarly, it has been proposed that ScpA may also possess elastinolytic properties (188) and therefore may play a role in the degradation of host connective tissue. Moreover, ScpA is able to hydrolyze cysteine protease inhibitors cystatins C and D by cleaving at hydrophobic residues (Val and Leu) in the P2 residue, thereby downregulating cystatins necessary for host protease-inhibitor balance (248). Although less efficient than SspA, ScpA is also able to process human α-proteinase inhibitor (193). In addition, ScpA combined with the activity of SspB can induce vascular leakage activity by targeting kininogens to release bradykinin, a potent enhancer of vascular permeability (100). Despite its activity on these substrates and limited activity on synthetic substrate Z-Phe-Leu-Glu-*p*NA, further investigation into the substrate preferences is needed to gain a deeper understanding of its target protein substrates.

(vi) Staphostatins

Several strategies have been implemented by microorganisms to protect the cells that produce them from the broad specificity of proteases, which may degrade host cell proteins. Mechanisms to control proteolytic activity of the enzymes include secreting proteases as inert zymogens and/or producing specific inhibitors (114). As such, staphostatins are a distinct, novel class of cysteine inhibitors found to be co-expressed with staphopains in *S. aureus*. Organization of the *ssp* operon and the *scp* operon show

that staphostatins *sspC* and *scpB* are encoded downstream of *sspB* and *scpA*, respectively. This organization suggested that *sspC* and *scpB* might function as inhibitors of papain-like cysteine proteases. However, despite the homology between SspB and ScpA, the inhibitors were found to be highly specific for the proteases encoded in the same operon and did not exhibit any cross-inhibition with other cysteine proteases (212).

Staphostatins form a 1:1 tight, non-covalent inhibitory complex with their respective proteases completely eliminating enzyme activity and preventing reaction with substrates and inhibitors (212). Staphostatins occludes the active site clefts of their corresponding proteases in a substrate-like orientation (70). Although they have the same overall folding pattern, SspC and ScpB show little amino acid sequence homology, with SspC sharing only 18.2% identity with ScpB (212). At the structural level, staphostatins form eight-stranded mixed β-barrels and SspC has been found to be most similar to lipocalins, which are serine protease inhibitors (211). Sequence analysis has predicted that SspC and ScpB are localized within the cell due to a lack of secretory signal sequences in SspC and ScpB (212). Therefore, staphostatins may serve as protectors of the cytoplasm proteins from premature activation of staphopains that would otherwise cause proteolytic degradation within the cell. Evidence of this was supported by a finding that ScpA cannot be expressed without its specific inhibitor ScpB. Expression of ScpA alone resulted in toxicity to the host cells (254).

IV. Other Similar Cysteine Proteases Involved in Virulence

(i) Streptococcal pyrogenic-erythrogenic exotoxin B (SpeB)

Streptococcus pyogenes is a Gram-positive pathogen responsible for causing 15 to

30% of all cases of pharyngitis in children and 5 to 10% in adults (22). *S. pyogenes* can infect skin and soft tissue which may lead to impetigo, erysipelas and cellulitis (23). In addition, it can cause high mortality by further disseminating into the tissues thereby eliciting necrotizing fasciitis as well as sepsis and toxic shock syndrome (167, 234). Furthermore, severe infections of *S. pyogenes* can include poststreptococcal glomerulonephritis, which may result in renal failure and acute rheumatic fever (49).

S. pyogenes produces several extracellular enzymes, many of which interact with the host immune system. SpeB, streptococcal pyrogenic-erythrogenic exotoxin B, is a cysteine protease and is one of the most characterized secreted proteins of *S. pyogenes* (43). It is secreted as a 40 kDa zymogen which subsequently undergoes autocatalytic activation involving several maturation intermediates to convert to the active 28 kDa mature protease (37, 57). The *speB* gene is highly conserved and is found in virtually all *S. pyogenes* strains (262). Although there is minimal sequence homology, the 3D crystal structure of the zymogen form reveals a similar folding pattern to papain (108).

SpeB is transcribed during early stationary phase and is downregulated by glucose and other nutrients in the culture medium (36). Positive regulators of *speB* expression include the global transcriptional regulator *mga* (186), the *speB*-specific *ropB* in the regulation of proteinase (Rop) loci (127) and pleiotropic effect locus (*pel*) (121). The possible contribution of the two-component system CsrR-CsrS in the repression of *speB* remains to be established (68, 90). The contribution of SpeB expression to virulence is controversial, as findings have shown that there is an inverse relationship between disease severity and SpeB expression (40). However, it has also been suggested that the decreased expression of SpeB may help *S. pyogenes* stabilize its M1 protein structure,

which is involved in resisting immune clearance (109). At the same time, there are still many pieces of evidence that support SpeB in pathogenesis in humans. For instance, it has been shown that SpeB is produced *in vivo* during infection of patients with severe invasive disease (85). Furthermore, SpeB production has been linked to a particular isolate of *S. pyogenes* involved in Toxic Shock Syndrome (TSS) patients (241).

SpeB has a substrate specificity similar to that of cysteine proteases of the papain family, which prefers a bulky hydrophobic amino acid at the P2 site relative to the scissile bond (163). SpeB activates matrix-metalloproteases, which may enhance tissue damage and bacterial invasion (32, 242). It can also release surface adhesins such as M proteins and Protein H (21) and can fragment fibronectin (112) suggesting a role in bacterial dissemination. SpeB has demonstrated a role in immune evasion by degrading IgG to reduce the ability of opsonizing IgG that can kill *S. pyogenes* in human blood (44). SpeB is also able to cleave IgA, IgM and IgD and degrade IgE (42). SpeB has also shown to release proinflammatory molecules. In particular, SpeB can activate cytokine interleukin-1β, which is a strong inflammatory mediator (111). As well, SpeB is able to release bradykinin from high molecular weight kininogen, which may be the reason for hypovolemic hypotension observed in sepsis caused by *S. pyogenes* (92).

(ii) Gingipains

Porphyromonas gingivalis is a Gram-negative pathogen that has been linked with the onset and development of human periodontitis, an inflammatory disease that results in the destruction of supporting tissues of the teeth (230). This disease has been estimated to affect around 30% of the adult population with severe cases ranging from 5-10% (173).

P. gingivalis has been shown to produce several proteases that are considered to be involved with virulence. Among these enzymes, there are three predominant cysteine proteases which have been suggested to contribute to *P. gingivalis* pathogenesis (190). These proteases are collectively referred to as gingipains and are designated as RgpA, RgpB and Kgp (50). The gingipains are cell-surface associated and/or secreted depending on the bacterial strain and environmental conditions (170). The gingipains contribute to pathogenesis by indirectly causing periodontitis through activation of host matrix metalloproteinases and by degrading important proteins of the immune system (187).

Gingipains R, RgpA and RgpB, are encoded by two individual genes *rgpA* and *rgpB*. The protein structures are strongly conserved as there are little coding sequence variations between strains (145). The 50 kDa RgpA polypeptide includes a profragment, followed by the protease domain, and a large hemagglutinin/adhesion domain at its carboxy terminus (182). Kgp also has the hemagglutinin/adhesion domain, which is responsible for hemagglutinating activity and specific binding to fibrinogen, fibronectin and laminin by gingipains (185). In contrast, RgpB lacks the sequence encoding hemagglutinin/adhesion domain (226).

Gingipains R have been found to exclusively cleave Arg-Xaa while Kgp is specific for Lys-Xaa peptide bonds (192). To date, only the activation mechanism for proRgpB has been elucidated. Three sequential autolytic steps are involved in the activation of proRgpB for full activity. The first cleavage occurs at Arg_{103} followed by removal of the remainder of the N-terminal propeptide, and lastly, removal of a C-terminal peptide to yield the mature protease (144). Although the structure of proRgpA and proKgp is more complex and may require many more proteolytic events to generate

the mature protease, it has been suggested that these proteases may undergo similar activation process as proRgpB (192). Interestingly, it has also been found that active RgpB is involved in efficient and correct maturation of Kgp by cleaving the C-terminal portion of Kgp (107).

Substrates for gingipains R include various plasma proteins, extracellular matrix components, cytokines and their receptors as well as other host cell surface proteins (187). Gingipains R are able to enhance vascular permeability whereas gingipain K does not harbour this ability. However, gingipain K can work in synergy with gingipains R to promote the release of bradykinin from high molecular weight kininogen, thereby mimicking the function of kallikrein. This action may potentially contribute to the gingival crevicular fluid production and edema formation at periodontitis sites (97). Furthermore, it has been found that gingipains R can accelerate human plasma clotting time and activate coagulation factors and prothrombin (95, 98, 99). Although all three gingipains can degrade fibrinogen (185), Kgp is more potent and specific than gingipains R (96). Furthermore, RgpB can degrade C3, the central factor in the complement system, therefore possibly allowing *P. gingivalis* to evade the host immune response (253). In addition, gingipains can up-regulate expression of matrix metalloproteinases (MMPs) in fibroblasts (52) and activate secreted latent MMPs (53), which may cause connective tissue damage in periodontal disease.

V. Activation Mechanism for Bacterial Proteases

Protease activity is regulated and controlled at specific times and cellular locations while needing to remain in a properly folded conformation. Therefore,

proteases require intramolecular chaperones, or propeptides to act as a folding catalyst as well as maintaining the precursor form of the protease as an inactive zymogen. Many bacterial proteases are synthesized in a preproform that consists of an N-terminal signal peptide required for secretion (pre), a propeptide domain (pro) and the mature protease (28).

Precursor activation is initiated in three major stages: i) proper folding of the precursor by its cognate propeptide (221, 264); ii) autoproteolytic cleavage of the peptide bond between the propeptide and the mature protein (120, 220); and iii) degradation of the propeptide and release of the active protease in a stable conformation (15). Autocatalytic processing of precursors can occur via an intramolecular and/or intermolecular processing mechanism. An intramolecular mechanism would be characterized by a zero-order reaction, where precursor-processing is independent of the precursor concentration. Therefore, each precursor protease is responsible for removing and degrading its own propeptide. In contrast, an intermolecular mechanism is characterized by a higher order reaction, where the precursor-processing rate is dependent on the precursor concentration (247). Thus, a precursor protease undergoes an intermolecular mechanism when a neighbouring active protease facilitates in the propeptide removal of the precursor.

Much of what we know about precursor activation has been based on the autocatalytic activation of prosubtilisin. This model can likely be applied to other proteases produced as precursors. The proposed model for activation of precursor proteases is illustrated in Figure 1 (256).

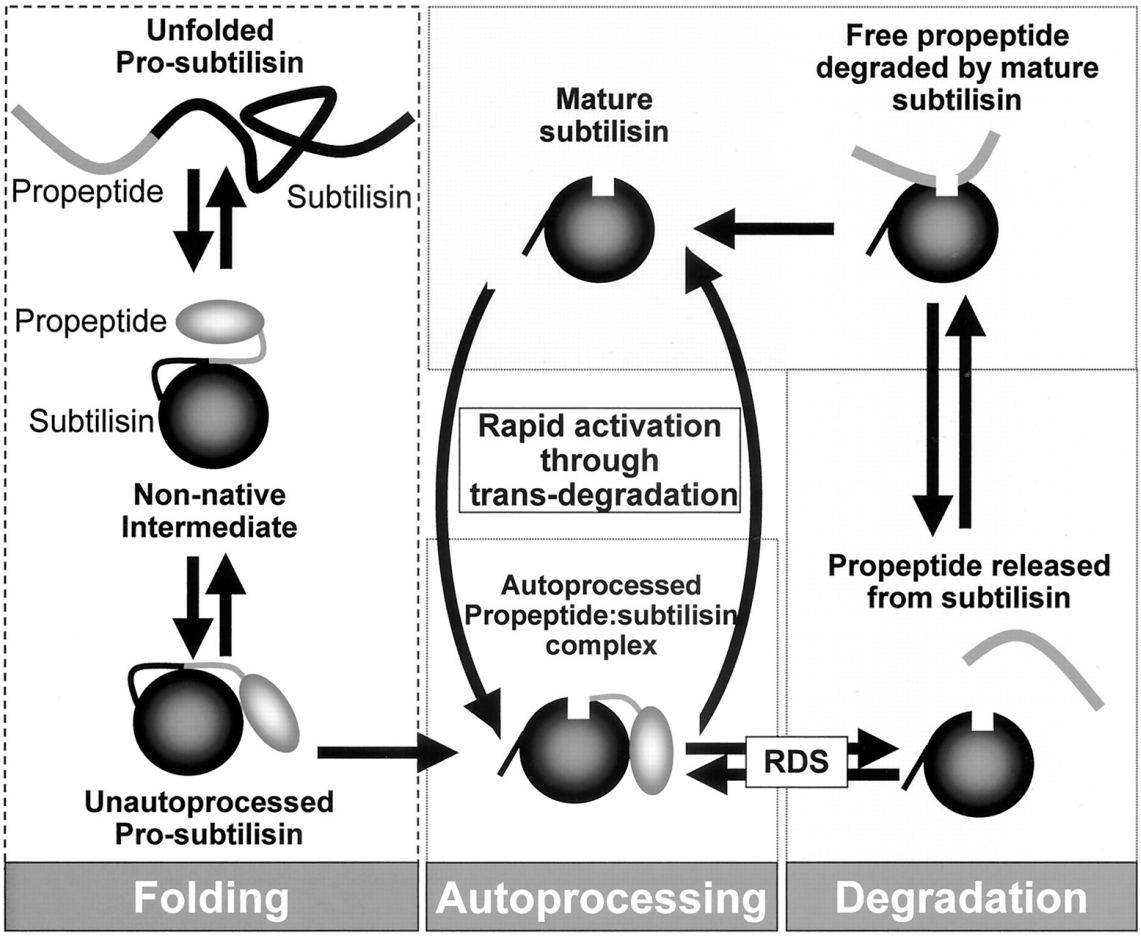


Figure 1. An example of an activation model for precursor proteases.

When folded in a correct orientation, prosubtilisin undergoes maturation by the autoprocessing of the propeptide of pro-subtilisin. The propeptide is subsequently released and then degraded by mature subtilisin. The event where the protease domain is freed from its cognate propeptide has been found to be the rate-determining step of the maturation reaction. Once activated, mature subtilisin proceeds to activate neighbouring precursors (256).

VI. Hypothesis and Objectives

Staphylococcus aureus secretes two closely related cysteine proteases, Staphopain A (ScpA) and Staphopain B (SspB). The presence of two staphopain operons in *S. aureus* distinguishes it from the less virulent *S. epidermidis*, which has the *scp* operon, but no equivalent of the *ssp* operon. From the SPC pathway, we know that SspA activates SspB by removing the N-terminal propeptide of proSspB. In contrast, little is known regarding the activation mechanism of ScpA as it does not appear to be involved in this pathway.

The ScpA orthologue of *S. epidermidis,* Ecp, exhibits specificity towards the Yaa-Glu↓Xaa sequence motif, where Yaa represents a large hydrophobic amino acid and Xaa represents any amino acid except for proline (172). In addition, SspB has been shown to be specific for cleaving after Arg at the P1 site of the scissile bond. In contrast, previous work suggests that ScpA may not share similar specific substrate specificities. The specificity difference seen amongst these proteases warrants for further investigation into the substrate specificity of ScpA.

Although ScpA has been shown to be involved in vascular leakage, very little is known about its contributions in clinical isolates of *S. aureus* to facilitate in causing infections. As outlined in the literature review, clinical isolates of *S. aureus* including hyper-virulent strains, and community acquired MRSA demonstrate various pathogenic mechanisms. Therefore, more work is needed to determine the specific role and function of ScpA as a virulence factor in these clinical isolates, which may be capable of causing severe infections.

Hypothesis: Although Staphopain A is structurally similar to SspB, it is not involved in the SPC pathway and therefore will exhibit a distinct activation mechanism and a different or complementary substrate specificity.

Objectives:

1) To elucidate the activation mechanism for ScpA

2) To further characterize the substrate specificity of ScpA and identify new substrates

3) To determine the role of ScpA in *S. aureus* clinical isolates

PART II: MATERIALS AND METHODS

I. Buffers and Media

Alkaline phosphatase (AP) Developing buffer	0.5 mM $MgCl_2$, 0.1 M Tris, pH 9.5
Antibody Blocking buffer	3% w/v BSA and 0.02% NaN_3 in PBS
Antibody Dilution buffer	0.1% w/v BSA, 0.05% Tween20, 0.02% w/v NaN_3 in PBS
Brain Heart Infusion (BHI) agar plates	15 g agar in 1 L of BHI Broth media
BHI Broth media	37 g of BHI powder in 1 L of ddH_2O
Coomassie Stain	2.5 g Coomassie Brilliant Blue, 500 ml Methanol, 100 ml Acetic acid in 1 L ddH2O
Coomassie destain	40% Methanol, 10% Acetic Acid in 500 mL ddH_2O
Elastin agar plates	1.5 g agar, 0.3 g elastin bovine neck ligament, 0.25 g yeast extract, 0.1 g glucose, 0.441 g hydrated trisodium citrate, 1 g sodium caseinate, 2 mM cysteine, 20 mM $CaCl_2$ in 100 ml ddH_2O
Fibrin agar plates	1.2 % human fibrinogen in 0.1 M sodium phosphate buffer, pH 7.4, thrombin solution (100 NIH U/ml), 1% (w/v) agarose solution, 10 mM cysteine
LB Broth media	25 g of LB powder in 1 L of ddH_2O
Loading Dye for DNA	0.05% w/v Bromophenol blue, 40% w/v sucrose, 0.1 M EDTA, pH 8.0, 0.5% w/v SDS
PBS buffer	8 g NaCl, 0.2 g KCl, 0.24 g KH_2PO_4, 1.44 g Na_2HPO_4 in 1 L of ddH_2O
Reducing Buffer (3X)	0.64 g SDS, 1 mL 2 M Tris (pH 6.8), 2.12 mL ddH_2O, 3.2 mL glycerol, 0.08 mL 0.5% Bromophenol blue, 1.6 mL β-

	mercaptoethanol
SDS-PAGE Running Buffer	15 g Tris, 72 g glycine, 5 g SDS, in 5 L of ddH_2O
SDS-PAGE Blotting Transfer Buffer	3.03 g Tris, 14.4 g glycine, 200 mL methanol in 1 L of ddH_2O
TAE buffer	4.84 g Tris, 1.14 mL glacial acetic acid, 2 mL 0.5 M EDTA, per litre of ddH_2O (pH 8.0)
Trypticase Soy Broth (TSB) (soybean casein digest broth) agar plates	15 g agar in 1 L of TSB Broth media
TSB Broth media	30 g of TSB powder per litre of ddH_2O
Wash Buffer for Western Blots	0.05% Tween20, 0.02% NaN_3 in PBS

II. Bacterial Strains and Growth Conditions

Bacterial strains and recombinant plasmids used in this study are listed in Table 2. *S. aureus* and *E.coli* stock cultures were maintained in Brain Heart Infusion (BHI) (Difco Laboratories) and Luria-Bertani (LB) media (Invitrogen) respectively, at -80°C in 80% glycerol (Biobasic). *S. aureus* and *E.coli* strains were streaked and grown on BHI or LB medium containing 1.5% agar (Becton-Dickinson) and were supplemented with antibiotics (ampicillin, 100 μg/ml; erythromycin, 10 μg/ml; chloramphenicol, 10 μg/ml; kanamycin, 25 μg/ml and tetracycline, 50 μg/ml) as required. Bacteria on solid agar were grown lid side down in a 37°C stationary incubator. For liquid cultures, bacterial strains were grown in BHI broth, Tryptic Soy Broth (TSB) or LB media and supplemented with antibiotics as required. Bacterial strains were cultured with shaking at 250 rpm in a 37 °C shaker-incubator. When a specific cell concentration was required, the OD_{600} of an overnight culture was measured and sub-cultured into the appropriate media such that an initial OD_{600} of 0.1 was obtained.

Table 2. Bacterial strains and recombinant plasmids used in this study.

Strain or Plasmid	Description	Source
S. aureus:		
RN6390	Derivative of NCTC 8325 with increased expression of secreted proteins	(166)
RN4220	Entry level host strain for plasmids, restriction deficient strain	(164)
RN6390 Pspac::*ssp*ABC	A conditional inactivation mutant of the *ssp* operon	(158)
E.coli:		
DH5α	Host strain for construction of recombinant plasmids	Invitrogen
DH10B	Host strain for expression of recombinant proteins	Gibco BRL
XL-Blue	Entry level host strain for site-directed mutagenesis of plasmids	Stratagene
M15 pREP	Host strain for expression of recombinant proteins	Qiagen
CC30 Isolates:		
WBG10049	Australia, 1999	
BK12003	New York; Queen's; 1990's MSSA	
BK5969	San Francisco; Contemporary	
M1015	Australia, 1962	
BK9950	Alabama, 1964 (Early TSST Strain)	
M809/BK22029	Australia, 1961	
Seattle 1945 (ATCC25923)	Seattle, 1945	
L516	Winnipeg, 1994: MSSA	
MN-8/BK21203	TSST High Producer	

L528	Manitoba, MSSA
BK21339	France; Necrotizing Pneumonia; PVL MSSA
BK13271	NJ MSSA
ATCC49775	V8 Strain, PVL+
PM7 (MRSA252)	Contemporary MRSA, epidemic

pRN5548a	pRN5548 lacking blaZ promoter fragment	(130)
pUC18	*E.coli* cloning vector	(259)
pQE30	*E.coli* expression vector incorporating N-terminal 6His tag	Qiagen
pBAD24	High expression vector with arabinose-inducible promoter	(86)
ScpAB-pRN5548	Expression of ScpAB from native promoter	This study
ScpAB $C_{238}A$	ScpAB-pRN5548 modified with mutagenic primers ScpABala-F and ScpABala-R	This study
ScpAB $L_{174}A$	ScpAB-pRN5548 modified with mutagenic primers ScpAB-Leu-Ala-F and ScpAB-Leu-Ala-R	This study
6His_proScpAB $C_{238}A$	ScpA active site mutant with ScpB cloned into pQE30 expression vector	This study
6His_proSspB	proSspB active site mutant cloned into pQE30 expression vector	(158)
ScpAB_6His	ScpAB with 6His-tag incorporated to the C-terminus and cloned into pBAD24	This study

III. Molecular Biology Techniques

(i) Isolation of Plasmid DNA

Plasmid DNA was isolated from *S. aureus* and *E.coli* using GenElute Plasmid Miniprep Kit (Sigma) according to manufacturer's protocol. Briefly, single colonies were inoculated into BHI for *S. aureus* or LB for *E.coli* and cultured in a 37 °C shaker-incubator overnight. Overnight bacterial cells (6 ml) were harvested via centrifugation in microcentrifuge tubes (12, 000x*g*, 1 min). Subsequently, bacterial pellets were resuspended with 200 μl of Resuspension Solution containing RNase A. For plasmid isolation from *S. aureus* only, 20 μg/ml of lysostaphin was added to the resuspension and incubated on a 37 °C heat block for 30 minutes prior to proceeding to the lysis step. To lyse the resuspended cells, 200 μl of the Lysis Buffer was added and gently mixed by inversion until the mixture became clear and viscous. Cell debris was precipitated by adding and mixing with 350 μl of Neutralization/Binding buffer. Cell debris was pelleted by centrifugation (12, 000x*g*, for 10 minutes) and the cleared lysate was transferred, washed and eluted through a GenElute Miniprep Binding Column. All buffers that were used were provided by the GenElute Plasmid Miniprep Kit (Sigma).

(ii) Polymerase Chain Reaction and Recombinant DNA Procedures

S. aureus genomic DNA was isolated using the DNeasy Tissue kit (Qiagen) according to manufacturer's protocol. PCR was performed with the Expand High Fidelity PCR system (Roche Applied Science). Primers used for PCR in this study are described in Table 3. The PCR reaction was performed in a 50 μl volume, containing 0.1 ng of

Table 3. Oligonucleotides used for PCR and plasmid modification.

Primer	Sequence[a]	Coordinates[b]
6390_ScpAB_F	ggatccCTACGCTTCTTTCAAAATCG	(-)223/(-)204
StpAB-R	gaattcTTATGACTTATGCTTAATGAAAGTC	2385-2361
ScpABala-F[c]	CTCAAGGTAACAATGGTTGGGCGGCAGGCTATACGATGTCT	692-732
ScpABala-R[c]	AGACATCGTATAGCCTGCCGCCCAACCATTGTTACCTTGAG	732-692
pScpA-F	ggatccGAGAGCAATTCAAATATCAAAGC	76-98
pScpA-R	ctgcagTTATGACTTATGCTTAATGAAAGTC	2385-2361
BspHI-ScpAB-F	tcatgaACGCTGAGAGCAATTCAAATATCAAAGC	931-959
ScpABHisR	ctgcag**TCA**ATGGTGATGGTGATGATGTGACTTATGCTTAATGAAAGTCATTCTAGG	2382-2353
ScpAB-Leu-Ala-F	CTAAAAACACCACGTGCAGAAGATAAAAAG	1369-1398
ScpAB-Leu-Ala-R	CTTTTTATCTTCTGCACGTGGTGTTTTTAG	1398-1369

[a]Restriction endonuclease sites are in lower case, stop codons are in bold, and nucleotide changes for site-directed mutagenesis are underlined.
[b]The coordinates are numbered with +1 corresponding to the first nucleotide in the ATG initiation codon of the *scpA* gene SAOUHSC_02127 to the end of the *scpB* gene SAOUHSC_02129 from the *S. aureus* NCTC 8325 genome sequence (AF309515). Nucleotides that are 5' of the initiation codon are numbered with negative integers.
[c] This set of primers were obtained from reference (254).

plasmid DNA or 10 ng of genomic DNA with 200 μM of deoxynucleotide triphosphates (Roche Applied Science), 0.3 μM of each forward and reverse primers, 1x concentration of Expand High Fidelity buffer with $MgCl_2$ and 2.6U of Expand High Fidelity DNA polymerase. DNA manipulations using restriction enzymes, T4 DNA Ligase (New England BioLabs), Calf Intestine Alkaline Phosphatase (Roche Applied Science) were used according to manufacturer's protocol. PCR products and digested plasmids used in cloning were purified using QIAquick PCR Purification kit (Qiagen) or PureLink Quick Gel Extraction Kit (Invitrogen). Plasmid constructs with cloned DNA fragments were submitted to the Center for Applied Genomics at the Toronto Hospital for Sick Children for verification of the expected nucleotide sequences.

(iii) DNA Manipulations

a. Construction of ScpAB Expression Vector

A derivative of plasmid pRN5548 (130), lacking the *bla*Z promoter fragment, was used as the vector to construct the ScpAB expression vector. We designed a forward primer 6390_ScpAB_F to anneal upstream of the *scpA* native promoter and a reverse primer StpAB-R with *BamHI* and *EcoRI* restriction sites, respectively, in order to amplify the *scpAB* genes. Thermocycling conditions were as follows: 95°C for 2 min, followed by 30 cycles of amplification: 95°C for 30 s, a specific annealing temperature 54°C for 1 min, and 72°C for a set amount of time 2 min with a final extension at 72°C for 10 min. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and digested with *BamHI* and *EcoRI* for 2 hours in 37°C and ligated with T4 DNA ligase to pRN5548a which was previously digested with *BamHI* and *EcoRI* and dephosphorylated

with alkaline phosphatase. Ligation reactions were conducted in a 16°C waterbath overnight. After initial transformation of RN4220 by electroporation, the resulting ScpAB-pRN5548 expression construct was electroporated into strain RN6390 Pspac::*sspA*BC.

b. Construction of ScpA mutants in S. aureus

To construct ScpA mutants, the 1.7 kb ScpAB cloned fragment was excised from the ScpAB-pRN5548 expression vector and ligated into pUC18 and transformed into DH5α for site-directed mutagenesis. Mutagenic primers were designed with the desired mutation. For amplification of ScpA active site mutant (ScpABC$_{238}$A) with the Cys$_{238}$ residue change to Ala, mutagenic primers ScpABala-F and ScpABala-R were designed. For amplification of ScpA with Leu$_{174}$ changed to Ala (ScpAB L$_{174}$A), ScpAB-Leu-Ala-F and ScpAB-Leu-Ala-R mutagenic primers were designed. Site-directed mutagenesis was performed using the QuikChange II-E Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's protocol. Briefly, the ScpAB-pUC18 plasmid was used as the DNA template with the appropriate primers for amplification using *PfuUltra* HF DNA polymerase (2.5 U). The mutagenized plasmids were transformed into XL1-Blue *E.coli* competent cells. Once the plasmids were verified to have the desired mutation, the inserts were excised from these plasmids and cloned back into pRN5548a and electroporated into RN4220, then into RN6390 Pspac::*sspA*BC.

c. Construction of 6His_proScpAB C$_{238}$A

To construct a 6His-tag to the N-terminal end of proScpA, primers pScpA-F and

pScpA-R were used to clone ScpAB C_{238}A into *BamHI* and *PstI* sites of pQE30, which is an expression vector that incorporates a N-terminal 6His-tag. The vector ScpAB C_{238}A was used as the DNA template to ensure that proScpA would not undergo autocatalytic activation. Thermal cycling conditions were as described above except with a specific annealing temperature 52 °C for 1 min. Constructs with the cloned fragment were subsequently transformed into M15 *E.coli* competent cells.

d. Construction of ScpAB_6His

To construct a 6His-tag to the C-terminal end of ScpAB, primers BspHI-ScpAB-F and ScpABHisR were used. The forward primers were designed with *BspHI* restriction sites while the reverse primers were designed with *PstI* restriction sites. Since the multiple cloning site of pBAD24 did not include *BspHI*, DNA inserts were digested with *BspHI* and *PstI* but ligated to pBAD24 digested with *NcoI* and *PstI*. This allowed for an appropriate frameshift so that ultimately the ScpA could be expressed without its signal peptide and with N-terminal ends beginning with MNA. Thermal cycling conditions using RN6390 genomic DNA were as described above except with a specific annealing temperature 63.6°C for 1 min. The mutant construct was subsequently transformed into DH10B *E.coli* competent cells.

(iv) Transformation procedures and Electroporation Conditions

Following the ligation reactions of DNA inserts to its appropriate vectors, the ligation reaction was subjected to ethanol precipitation. Briefly, in a 620 μl volume, 20 μl ($1/10^{th}$ of total volume) of 3 M sodium acetate, pH 5.2 and 400 μl (2x total volume) of

100% ice cold ethanol were mixed into the ligation reaction and stored in -20 °C conditions. After 3 hours, the microcentrifuge tube was centrifuged (14, 000xg, 10 minutes) to collect the DNA pellet. Subsequently, the DNA pellet was washed with 70% ethanol and centrifuged again for an additional 5 minutes (14, 000x*g*). After decanting the wash solution, the DNA pellet was air-dried and resuspended in 50 μl of *E. coli* or 80 μl of *S. aureus* electrocompetent cells on ice.

For *E. coli* transformations, the electroporation was carried out in pre-chilled 0.1 cm gap electroporation cuvettes (Bio-Rad Laboratories) using the Micropulser (Biorad). Immediately following the electroporation, the suspension was supplemented with SOC medium and incubated at 37°C with shaking at 250 rpm for 1 hour. Subsequently, the cells were plated on LB agar with the required antibiotics. For *S.aureus* transformations, the same procedures are followed with 0.2 cm gap electroporation cuvettes (Bio-Rad Laboratories) and the Gene Pulser (Bio-Rad) apparatus. Also, the suspension was supplemented with BHI medium and plated on BHI agar with the required antibiotics.

IV. Protease Expression and Purification

For purification of mature ScpA and the propeptides of the ScpAB $L_{174}A$ mutant, an overnight culture *S. aureus* RN6390 Pspac::*ssp*ABC with ScpAB-pRN5548 and *S. aureus* RN6390 Pspac::*ssp*ABC with ScpAB $L_{174}A$ expression vector, respectively, was subcultured into TSB to achieve an initial OD_{600} of 0.1. After 6 hours of growth, protein in the culture supernatant was precipitated with 80% saturation of ammonium sulfate and 1mM EDTA and collected by centrifugation (5500 rpm, 60 mins, 4 °C). The protein pellet was dissolved and dialyzed in 20 mM sodium phosphate, pH 7.4 then syringe-

filtered through a 0.20 μM filter before applying the supernatant to a HiTrap SP HP ion exchange column (Amersham Biosciences) connected to a AKTA FPLC system (Amersham Biosciences). Proteins were eluted over a linear NaCl gradient with the elution buffer 50 mM sodium phosphate, pH 7.4 and 1.0M NaCl. 2 ml fractions were collected and fractions containing proteins were resolved using SDS-PAGE. The fractions with protease activity from the mature ScpA purification were pooled and subjected to size exclusion chromatography using the HiLoad 16/60 Superdex 75 column as a final purification step to yield purified mature ScpA in 1 ml fractions.

To determine the interaction of heparin and ScpA, similar expression and purification procedures as described above were used. Ammonium sulfate precipitated and dialyzed culture supernatant of *S. aureus* RN6390 Pspac::*ssp*ABC with ScpAB-pRN5548 was applied to a HiTrap Heparin HP column and eluted in 10 mM sodium phosphate, pH 6.5 and 1M NaCl.

For purification of 6His_proScpAB C_{238}A, *E. coli* M15 pREP with proScpAB C_{238}A cloned into pQE30 was induced with 2mM IPTG for 4 hours and purified from the soluble fraction of cell lysate using the HiTrap HP Chelating affinity column. Proteins were eluted over a linear imidazole gradient with the elution buffer 20 mM sodium phosphate, pH 7.4, 500 mM NaCl and 500 mM imidazole, pH 7.4. 2 ml fractions were collected and pooled and subject to size exclusion chromatography to resolve the proteins that co-purified with 6His_proScpAB C_{238}A. For purification of proScpAB_6His, *E. coli* DH10B with proScpAB_6His cloned into pBAD24 was induced with 0.02% arabinose for 4 hours and followed the same purification procedures for HiTrap HP Chelating affinity column as described above.

All protein concentrations were determined with a BCA assay. Briefly, proteins were mixed with BCA solution and 4% $CuSO_4$ in a microtiter plate in triplicates and incubated for 30 minutes at 37°C. Purified proteins were measured in dilutions of 1:1, 1:2 and 1:3 against a BSA protein standard curve. Absorbances were measured at 560 nm.

V. Enzymes Assays

(i) Chromogenic peptide assay for ScpA activity

ScpA protease activity was measured by monitoring the release of p-nitroaniline (pNA) from chromogenic substrate Z-Phe-Leu-Glu-pNA (Bachem). Assays were conducted in triplicate wells of a microtiter plate in a 100 µl volume containing 100 mM sodium phosphate, pH 7.4, 2 mM cysteine, 5 mM EDTA and 0.4 mM substrate. Absorbances were read at specified time points using a microplate reader (BioTek) with a 405 nm filter.

(ii) Fluorogenic substrate assay for ScpA activity

ScpA protease activity was measured by monitoring the release of AMC fluorophore from fluorogenic substrate Ac-Arg-Ser-Leu-Lys-AMC. Assays were conducted in triplicate wells of a microtiter plate in a 100 µl volume containing 100 mM sodium phosphate, pH 7.4, 2 mM cysteine, 0.2 mM substrate and absorbances were read at specified time points with a FLX800 luminescence spectrometer (360 nm excitation, 460 nm emission) (BioTek).

(iii) Fibrin Plate Method

Fibrin degradation was monitored using the fibrin plate method as described in (115). Briefly, plasminogen-free fibrin plates were made by using a fibrinogen solution containing 1.2 % human fibrinogen in 0.1 M sodium phosphate buffer, pH 7.4, thrombin solution (100 NIH U/ml), 1% (w/v) agarose solution and 10 mM cysteine in a petri dish. The plates were allowed to stand for 30 minutes to allow for fibrin clots to form. Subsequently, holes were made using the tip of an autoclaved Pasteur pipette and 0.05 nmol of protein was dispensed into each hole. Zones of fibrin clearing following incubation at 37 °C indicated fibrinolytic activity.

(iv) Elastin Degradation Assay

Elastin degradation was monitored using elastin agar containing 1.5 g agar, 0.3 g elastin bovine neck ligament, 0.25 g yeast extract, 0.1 g glucose, 0.441 g hydrated trisodium citrate, 1 g sodium caseinate, 2 mM cysteine, 20 mM $CaCl_2$ in 100 ml ddH_2O. The agar medium was autoclaved and poured into petri dishes to allow the elastin agar to solidify. Subcultures for the strains of interest were grown starting from OD_{600} of 0.1 for 2 hours. After 2 hours of growth, the OD_{600} was measured and 5 μl of culture were dispensed onto the elastin agar plates and incubated in 37 °C for 4 days. Zones of elastin clearing indicated elastinolytic activity.

(v) Autodegradation Assay

To demonstrate that ScpA, in contrast to SspB, is capable of autodegradation, 2 μg of ScpA and SspB were incubated in 100 mM sodium phosphate, pH 7.4, 2 mM

cysteine, and 5 mM EDTA for 6 and 18 hours in a 37 °C incubator. 28 μM of cysteine inhibitor E64 added to control samples to inhibit cysteine protease activity. Following incubation, samples were resolved with 12% SDS-PAGE.

(vi) Intramolecular Cleavage Assay

To determine the type of autocatalytic processing mechanism utilized by ScpA, 10 μg of purified precursor ScpA C_{238}A was treated with purified mature ScpA in a 10:1 molar ratio. The 30 μl reaction contained the proteases in 100 mM sodium phosphate, pH 7.4, 2 mM cysteine and 5 mM EDTA. The reactions were incubated in 37 °C and were stopped with 28 μM E64 after 2 hours. Following incubation, samples were resolved on 12% SDS-PAGE.

VI. SDS-PAGE

To determine the profiles of secreted proteins, culture supernatants were precipitated with an equal volume of ice-cold 20% (w/v) trichloroacetic acid (TCA). Protein from the culture supernatant was collected and ethanol-washed via centrifugation. The protein pellet was air-dried and resuspended in 1x SDS loading buffer and boiled for 5 minutes. Proteins were subject to SDS-PAGE and a broad range protein marker (New England Biolabs) was used as the molecular weight standard. Protein gels were visualized by staining with Coomassie Brilliant Blue R-250.

VII. Mass Spectrometry and Mass Analysis

Coomassie-stained proteins were carefully excised from the protein gel and subjected to in-gel digestion using trypsin (Promega). The processed samples were submitted to the Proteomics Core Facility of the Toronto Angiogenesis Research Center at Sunnybrook Health Sciences Centre. Peptides were analyzed by LC-MS/MS using Agilent 1100 nanoflow HPLC with Agilent XCT-Plus ion trap (Agilent Technologies).

Mass analysis of the ScpA propeptide was determined by dialyzing the protein sample in 0.1% acetic acid. Following dialysis, the sample was submitted to Proteomics Core Facility of the Toronto Angiogenesis Research Center at Sunnybrook Health Sciences Centre and processed using time of flight mass spectrometry (QStar-XL, Applied Biosystems/MDS Sciex) and analyzed using BioAnalyst 1.1.5 software package (Applied Biosystems/MDS Sciex).

VIII. N-Terminal Sequencing

Three OD_{600} units of culture supernatant were TCA precipitated and resolved using SDS-PAGE. Purified proteins did not require TCA precipitation but were resolved by SDS-PAGE as well. Proteins were transferred to a PVDF membrane in 1x CAPS buffer and quickly stained with 0.1% Coomassie Blue R-250 dissolved in 40% methanol solution to visualize the proteins. After 5 minutes of staining, the membrane was destained with 50% methanol for 10 minutes and allowed to dry. The membrane was submitted to the Advanced Protein Technology Center at the Hospital for Sick Children for N-terminal sequencing.

Another method that was used was to determine the N-terminal sequence of peptides was by a modification of the procedures of McDonald *et al.* (138, 139). Proteins were separated by SDS-PAGE and excised from the gel. Free amino groups in the excised protein bands were acetylated before proteolysis by trypsin. After trypsin digest, the primary amino groups in the sample were subjected to coupling with CNBr-activated Sepharose (GE Healthcare). Peptides with the acetylated primary amino group were eluted through the matrix and submitted for LC-MS/MS at the Proteomics Core Facility of the Toronto Angiogenesis Research Center at Sunnybrook Health Sciences Centre.

PART III: RESULTS

I. ScpA Undergoes Autocatalytic Activation

In contrast to SspB, which is activated by the SspA serine protease as part of the SPC pathway, we hypothesized that ScpA will exhibit a distinct activation mechanism. To confirm this hypothesis, the gene encoding preproScpA (*scpA)* and its cognate inhibitor, ScpB (*scpB)*, was first cloned into plasmid pRN5548a and expressed in *S. aureus* RN6390 Pspac::*ssp*ABC, which is a strain harbouring an insertional disruption of the *ssp*ABC operon. Subsequently, a ScpA mutant with an active site substitution $C_{238}A$ was constructed and expressed in *S. aureus* RN6390 Pspac::*ssp*ABC. Consistent with autocatalytic activation, an unprocessed 40 kDa proScpA, which was not present in the recombinant ScpA control, accumulated in the culture supernatant after 6 hours of growth (Figure 2). After 18 hours of growth, the 40 kDa proScpA was no longer evident (data not shown). The absence of the 40 kDa proScpA during 18 hours of growth suggested that if proScpA is not activated immediately, it would eventually be degraded. Therefore, this data suggests that ScpA uniquely undergoes an autocatalytic maturation mechanism, which is distinct from SspB and the SPC pathway.

II. Identification of Intermediates in ScpA Activation

A temporal analysis of ScpA activation was conducted by growing *S. aureus* RN6390 Pspac::*ssp*ABC expressing recombinant ScpA to various time points. The

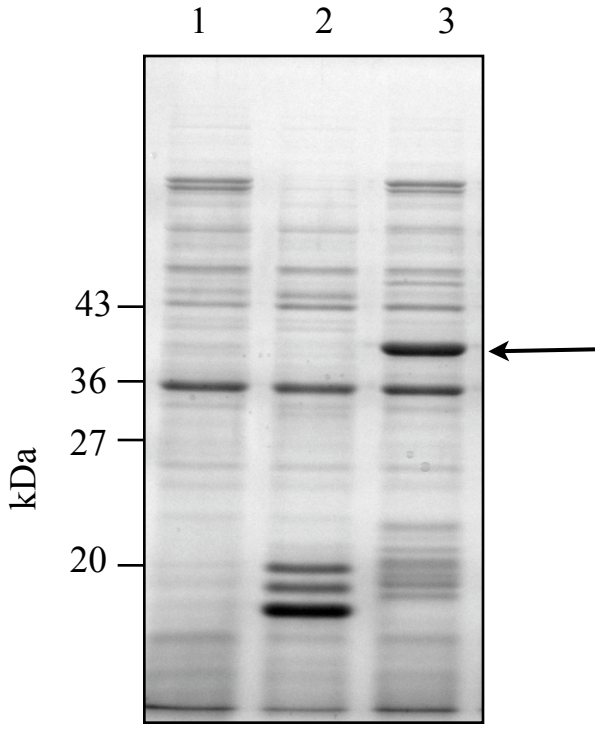


Figure 2. ScpA undergoes autocatalytic activation.

preproScpA with its cognate inhibitor, ScpB was expressed in *S. aureus* RN6390 P*spac*::*ssp*ABC (Lane 2) against a RN6390 P*spac*::*ssp*ABC control (Lane 1). An active site substitution of ScpAB C_{238}A led to the expression and accumulation of unprocessed 40 kDa precursor ScpA in the culture supernatant (Lane 3). Cultures of respective strains were grown for 6 hours in TSB media and supernatants (1.0 OD units) were resolved on SDS-PAGE. The arrow indicates the 40 kDa proScpA.

culture supernatants of the various time points were subjected to TCA precipitation followed by SDS-PAGE. At 4 hours of growth, a faint 22 kDa intermediate appeared along with a 20 kDa and a 17 kDa intermediate. At 5 hours of growth, these intermediates became more prominent. After 6 hours of growth, proceeding through the three intermediates, was a 19 kDa intermediate (Figure 3). At 18 hours of growth, the intermediates appear to have gradually degraded (data not shown). Compared to the RN6390 Pspac::*ssp*ABC control, the 40 kDa proScpA was not apparent at any time point in the culture supernatant of RN6390 Pspac::*ssp*ABC expressing recombinant ScpA (data not shown).

To identify the polypeptides, N-terminal sequencing of the three prominent intermediates ranging from 17-20 kDa in the 6 hour culture supernatant were determined. The analysis showed that the lowest of the three bands (17 kDa) corresponded to the ScpA propeptide having been cleaved at the predicted signal peptidase site ANA$\downarrow_{26}$ESNSN. Furthermore, the N-terminal analysis for the first stable intermediate of ScpA, with a molecular mass of 20 kDa, indicated processing at VT$\downarrow_{203}$MPTSQ. This stable isoform was further processed at FK$\downarrow_{210}$SNNYT to yield the 19 kDa ScpA intermediate. The N-terminal sequence of the 22 kDa intermediate present in the 4 hour culture supernatant could not be determined due to the presence of more than one indistinguishable band within that mass size. Consistent with the papain family of proteases, a trend of bulky hydrophobic amino acid was revealed in all the P2 residues at the processing sites relative to the scissile bond. Moreover, ScpA demonstrated a preference for polar amino acids at the P1 residues. Therefore, while ScpA and SspB are both members of the papain family and require a hydrophobic amino acid in the P2

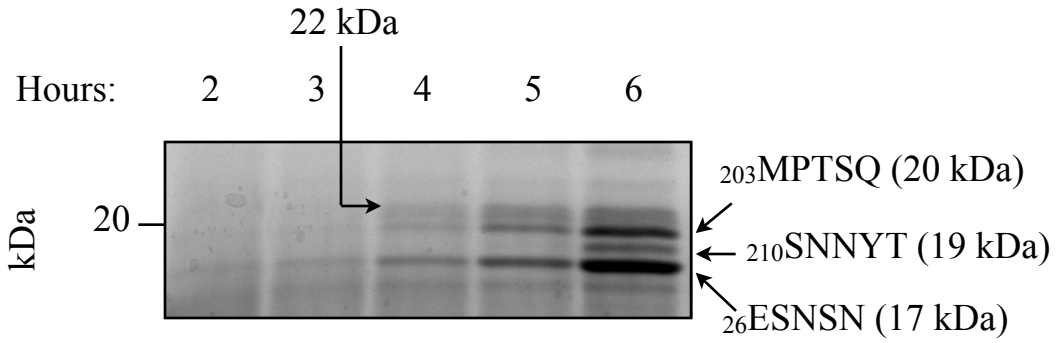


Figure 3. Temporal analysis of proScpA activation.

Culture supernatants (1.0 OD units) of RN6390 P*spac*::*ssp*ABC expressing recombinant ScpA were sampled at 2-6 hours of growth. A 22 kDa transient intermediate appears with the 20 kDa stable intermediate processed at $_{203}$MPTSQ and the 17 kDa propeptide in as early as 4 hours of growth. At 6 hours, an additional intermediate is processed at $_{210}$SNNYT.

position, ScpA has a broader specificity by recognizing substrates with a polar amino acid at P1, while SspB is restricted to arginine.

III. Purification and Activity Assay for Mature ScpA

Subjecting the 6 hour culture supernatant of RN6390 Pspac::*ssp*ABC expressing recombinant ScpA to cation exchange chromatography, a 19 kDa polypeptide was eluted over a broad peak of several fractions and showed protease activity when assayed with a known ScpA chromogenic substrate, Z-Phe-Leu-Glu-*p*NA. This confirmed that the 19 kDa polypeptide corresponded to active ScpA. In addition, fractions containing a 17 kDa polypeptide with the 19 kDa polypeptide (Figure 4, lanes 3-5) and the 17 kDa polypeptide alone (Figure 4, lane 6-8) were also eluted but showed minimal protease activity. The fractions with specific protease activity were pooled and subjected to size exclusion chromatography yielding a purified mature ScpA, which migrated as a doublet on SDS-PAGE (Figure 4, lane 9). Mass spectrometry analysis using a modified procedure by McDonald *et al.* (138, 139) of the upper mass of the purified ScpA doublet proved to be consistent with the N-terminal sequence of the 19 kDa band seen in the culture supernatant of RN6390 Pspac::*ssp*ABC expressing recombinant ScpA with an N-terminal sequence of FK$\downarrow_{210}$SNNYT. Furthermore, when N-terminal sequence of the lower mass was determined using this method, several N-terminal peptides were recovered. However, only two of the four peptides recovered were consistent with the size of the lower mass, which was approximately 18 kDa. Therefore, the N-terminal sequences for the lower mass peptide suggested a combination of peptides processed at YT$\downarrow_{215}$YNEQY

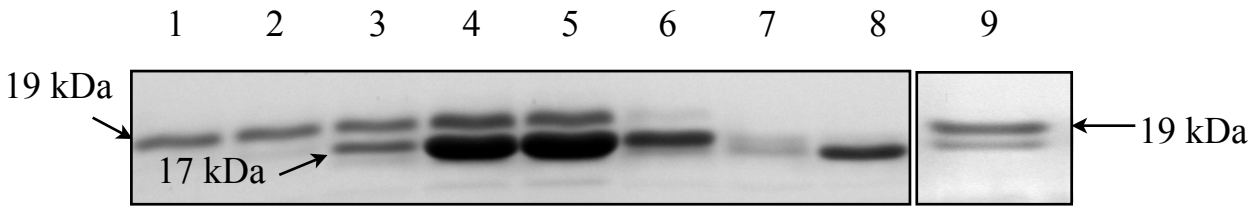


Figure 4. Purification of ScpA using cation exchange chromatography.

Culture supernatants were subject to ammonium sulfate precipitation and subsequently purified in 50 mM sodium phosphate, pH 7.4 over a linear NaCl gradient using cation exchange chromatography. Purified ScpA (Lane 1, 2) eluted over a broad peak (only two of several fractions shown in this figure). Subsequently, purified ScpA and propeptide eluted together (Lane 3-6) and lastly propeptide alone was eluted at the end of an increasing NaCl gradient (Lane 7-8). Samples with ScpA protease activity were subject to size exclusion chromatography, yielding purified mature ScpA, which migrated as a doublet (1 μg, Lane 9). The upper mass of purified mature ScpA had an N-terminal sequence of $_{210}$SNNYT while the lower mass had a combination of polypeptides with N-terminal sequences $_{215}$YNEQY and $_{217}$EQYIN.

and YN↓$_{217}$EQYIN indicating that purified ScpA underwent additional, slower processing after purification.

IV. ScpA is Activated via an Intramolecular Mechanism

To determine whether the autocatalysis of proScpA occured via an intra- or intermolecular mechanism, ScpAB C_{238}A from *S. aureus* RN6390 Pspac::*ssp*ABC was purified using cation exchange chromatography and subjected to mature ScpA at a 10:1 molar ratio of ScpAB C_{238}A:mature ScpA. There was some evidence of processing but the products did not distinctly correspond to intermediates involved in maturation. Therefore, autocatalytic processing of proScpA occurs mostly via an intramolecular mechanism (data not shown).

V. Determining the Initial Processing Site

To gain insight into the initial processing site for ScpA maturation, Protein Homology Recognition Engine (PHYRE) was used to visualize a potential initiation site. A modeling of proScpA on the PHYRE showed that Leu_{174} is optimally oriented toward the catalytic Cys and His residues. Since the main specificity determinant of the papain family is a hydrophobic amino acid in the P2 position relative to the scissile bond, Leu_{174} is in an ideal position such that autocatalytic processing could be initiated at LE↓$_{176}$DKKLK. To confirm this, the mass value of the propeptide byproduct from cation exchange chromatography of RN6390 Pspac::*ssp*ABC expressing recombinant ScpA was determined. With this value, the C-terminus sequence of the propeptide could be inferred to reveal the N-terminus of the initial processing site involved in ScpA maturation.

Although the predicted site for signal peptidase cleavage is ANA↓$_{26}$ESNSN, it may be possible that, through autocatalytic processing, the propeptide is shortened to ↓$_{33}$AKDKK. In this case, it was hypothesized that the theoretical mass values of two major species for the propeptide with an N-terminus of ↓$_{33}$AKDKK would be 16572.97 Da and 15976.61 Da. Consistent with the theoretical masses, the measured mass values for the purified propeptide were 16573.3 Da and 15977.14 Da. Therefore, these values suggested that processing occurred mostly at LE↓$_{176}$DKKLK and some at LK↓$_{171}$TPRLE, respectively. Furthermore, a minor species with a measured mass value of 17346.64 Da was consistent with a propeptide with N-terminus ESNSN and processing at LE↓$_{176}$DKKLKwith a predicted mass value of 17345.99 Da (Table 4). Collectively, these results verify that initial processing mostly occurs at LE↓$_{176}$DKKLK (Figure 5).

It was hypothesized that if LE↓$_{176}$DKKLK were the site of initiation, then a mutation of that site would lead to an accumulation of proScpA in the culture supernatant. To prove that autocatalytic processing was initiated at LE↓$_{176}$DKKLK, site-directed mutagenesis was performed on *scpA* to mutate residue Leu$_{174}$ to Ala. Unexpectedly, when the ScpAB L$_{174}$A construct was expressed, there was no apparent accumulation of proScpA. Instead, a more prominent 22 and 20 kDa polypeptide and a less prominent 17 kDa and 19 kDa polypeptides were expressed compared to expression patterns for recombinant ScpA. At 18 hours of growth, the expression patterns of the ScpAB L$_{174}$A mutant were similar to that of the ScpAB L$_{174}$A mutant strain grown for 6 hours with the exception of a slight increase in the intensity of bands present at about 17-19 kDa (Figure 6).

Table 4. Mass values for N-terminal propeptide of ScpA

N-terminal propeptide of ScpA (RN6390 Pspac::*ssp*ABC; 6 hr)	Mass		Area	
	Theoretical	Observed	Absolute[a]	Relative
	m/Z			
$_{33}$AKDKK//LKTPRLE$_{176}$	16572.97 Da	16573.3 Da	4284078	100
$_{33}$AKDKK//KHKAKLV$_{171}$	15976.61 Da	15977.14 Da	1052951	25
$_{26}$ESNSN// LKTPRLE$_{176}$	17345.99 Da	17346.64 Da	263782	6

[a]Absolute area is the total intensity (counts/s) integrated over the *m/Z* range of the ions contributing to the mass information for each isoform collected over 3 minutes.

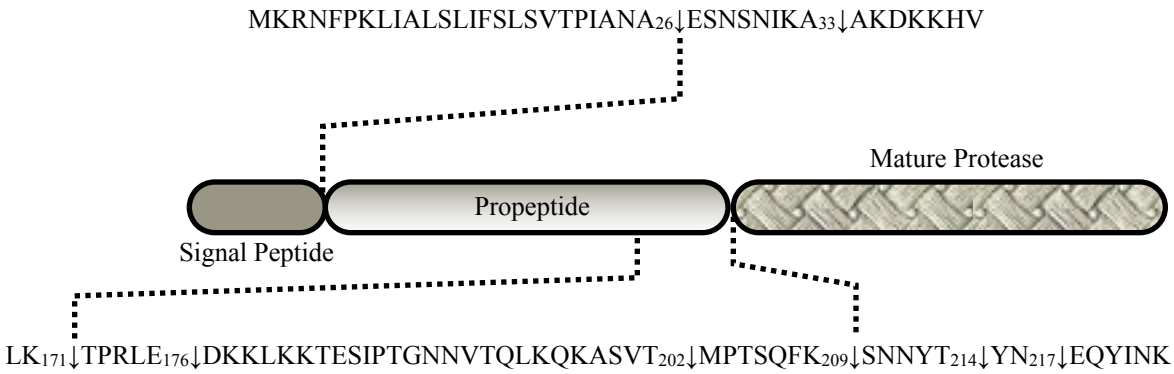


Figure 5. **Schematic diagram of proScpA activation.**

The signal peptide is shaded in gray, the N-terminal propeptide is shaded in a gray gradient and the mature protease is represented by the crosshatched pattern. The experimentally determined initial processing is indicated by $\downarrow_{176}$DKKLK. The $\downarrow_{26}$ESNSN segment indicates the N-terminus of the propeptide and the $\downarrow_{210}$SNNYT segment indicates the N-terminus of mature ScpA. Additional details are provided under “Results” and “Discussion”.

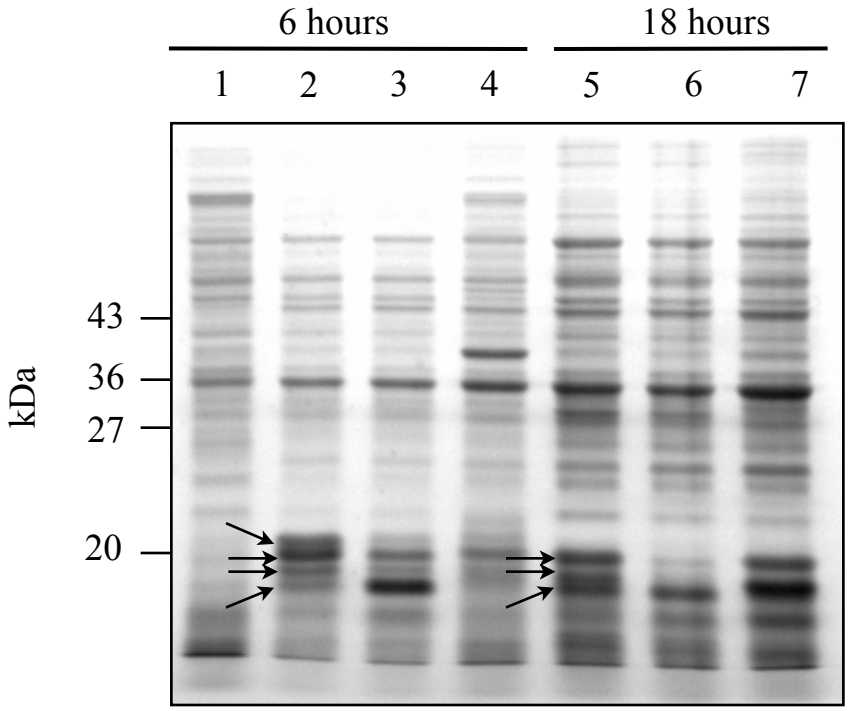


Figure 6. SDS-PAGE analysis of ScpA $L_{174}A$ mutant.

Site-directed mutagenesis was performed on recombinant ScpA to confirm that the initial processing site occurred at LE↓$_{176}$DKKLK. Lane 1, RN6390; lanes 2 and 5, ScpAB $L_{174}A$ mutant; lane 3 and 6, recombinant ScpA; lanes 4 and 7, ScpAB $C_{238}A$ mutant. Cultures were grown for 6 and 18 hours, and supernatants (1.0 OD units) of respective strains were subjected to SDS-PAGE analysis.

It is possible that the substitution did not fully inhibit processing at LE↓$_{176}$DKKLK but instead, may have slowed down the processing and diverted the mechanism to process alternative sites of preference such as VT↓$_{203}$MPTSQ and of FK↓$_{210}$SNNYT resulting in a similar expression pattern compared to recombinant ScpA. Furthermore, this would lead to propeptides of various masses depending on the number of alternative sites in the mechanism. To investigate this, the 6 hour culture supernatant of the Leu$_{174}$→Ala mutant was subjected to cation exchange chromatography. As a result, a number of polypeptides of different masses presumed to be the propeptide were eluted over a linear gradient and resolved using SDS-PAGE alongside the propeptide purified from recombinant ScpA expressed in *S. aureus* RN6390 Pspac::*ssp*ABC. The SDS-PAGE gel showed that the propeptides purified from the ScpAB L$_{174}$A mutant culture supernatant differed slightly in masses compared to the propeptide from recombinant ScpA (Figure 7). This suggested that the Leu$_{174}$→Ala mutation might not have been completely effective in inhibiting initiation at that particular site. As a result, the activation mechanism appeared to be slower and diverted to other sites of preference.

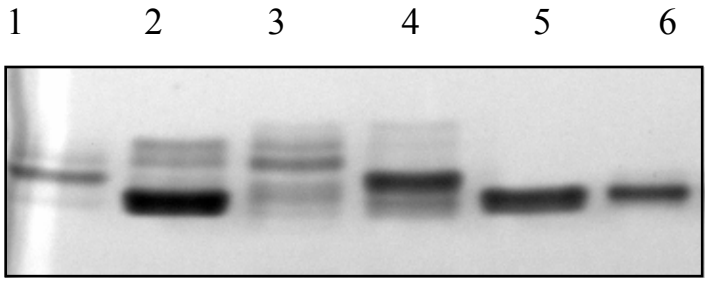


Figure 7. Propeptides purified from *S. aureus* RN6390 Pspac::*ssp*ABC ScpA L_{174}A using cation exchange chromatography.

Culture supernatants were subject to ammonium sulfate precipitation, dialysed and subsequently purified in 50 mM sodium phosphate, pH 7.4 over an increasing NaCl gradient using cation exchange chromatography. Purified propeptides of various masses were eluted (Lanes 1-5). Lane 6 is the propeptide purified from the culture supernatant of *S. aureus* RN6390 Pspac::*ssp*ABC ScpA.

VI. ScpA is Sensitive to Autodegradation

As suggested in the 18 hour culture supernatant of *S. aureus* RN6390 Pspac::*ssp*ABC expressing recombinant ScpA, ScpA may be sensitive to degradation over time. To confirm this finding, purified mature ScpA and SspB were incubated at 37 °C for 6 and 18 hours. After 6 and 18 hours of incubation, ScpA appears highly sensitive to autodegradation since in samples with added cysteine protease inhibitor, E64, there was no evidence of autodegradation. SspB remained stable throughout the incubation. Therefore, this showed that ScpA was sensitive to autocatalytic degradation while SspB was stable under the same conditions (Figure 8).

VII. proScpA Differs in Stability and Susceptibility to Intermolecular Processing

Similarly, an attempt to purify 6His_proScpAB C_{238}A demonstrated a significant difference in purification and stability between ScpA and SspB. When expressed as an N-terminal 6xHis-tagged protein in the cytoplasm of *E.coli* without its cognate inhibitor, the 40 kDa 6His_proScpAB C_{238}A recovered in poor yield in the soluble fraction of the cell lysate after metal affinity chromatography. In addition, 6His_proScpAB C_{238}A co-purified with 60- and 20-kDa polypeptides, which were later identified by trypsin digestion and mass spectrometry as GroE chaperone and the N-terminal propeptide of 6His_proScpAB C_{238}A, respectively. Upon size exclusion chromatography, the 6His_proScpAB C_{238}A eluted in complex with the heptameric GroE in the void volume followed by some monomeric 6His_proScpAB C_{238}A and the N-terminal propeptide

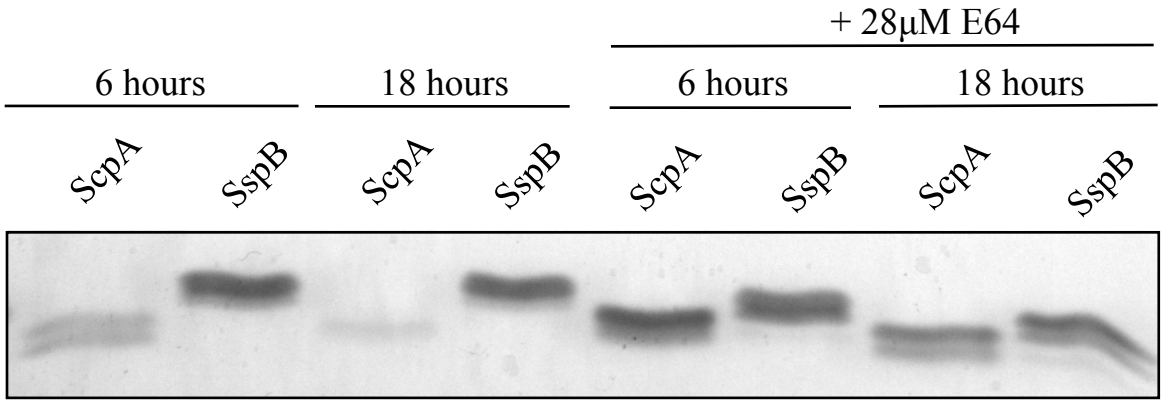


Figure 8. ScpA is sensitive to autodegradation over time.

2 μg of ScpA and SspB were incubated for 6 or 18 hours as indicated at 37°C. ScpA was substantially degraded after 18 hours while the sample with a cysteine protease inhibitor, E64, remained intact.

(Figure 9). The propeptide with the N-terminal 6xHis-tag appeared to have been processed from the mature protease by cytoplasmic proteases of *E.coli*, which may include ATP dependent GroE chaperone. In contrast, purifying 6His_proSspB using metal affinity chromatography resulted in a good and pure yield of stable 6His_proSspB. Therefore, although proScpA and proSspB are structurally similar, they differ in stability and susceptibility to intermolecular processing by other proteases.

VIII. Staphostatin A (ScpB) Captures ScpA During Autoactivation in *E.coli* Cytoplasm

Since ScpA is capable of autocatalytic activation, it may be possible that ScpA is rapidly auto-activated in the cytoplasm of *E.coli.* Therefore, it may be necessary for Staphostatin A (ScpB) to capture and complex with ScpA in order to prevent premature activation and guard cytoplasmic proteins of *S. aureus* from degradation. By resolving the complex, one can determine the N-terminal sequence for an earlier intermediate of ScpA at the point at which it becomes activated and is subsequently complexed with ScpB. Therefore, *scpAB* with a 6xHis-tag constructed at the C-terminus of *scpB* was expressed in *E.coli* using an arabinose-inducible promoter. The cell lysate was subjected to metal affinity chromatography and the protein samples were resolved with SDS-PAGE. From the lysate, although the 40kDa proScpA was not evident, ScpB-6His staphostatin co-eluted with a protein that was slightly larger than mature ScpA. This protein was subsequently identified to be an earlier intermediate of ScpA with an N-terminal sequence of LK$\downarrow_{197}$QKASV. Therefore, proScpA underwent autocatalytic

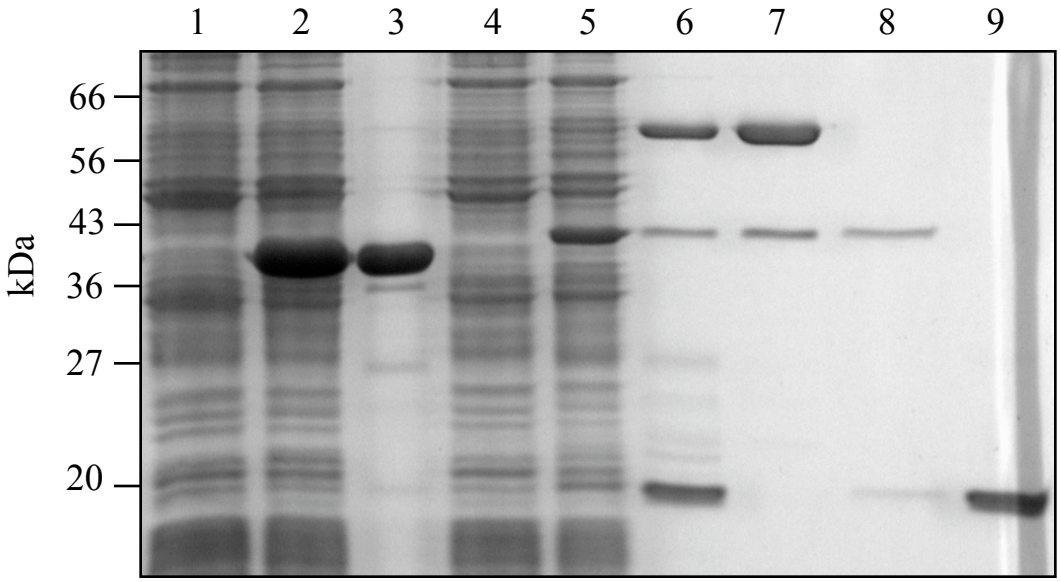


Figure 9. proScpA differs in stability and susceptibility to intermolecular processing.

E.coli strains of expressing 6His_proSspB (uninduced lane 1, induced lane 2) and 6His proScpA (uninduced, lane 4, induced lane 5) were purified using metal affinity chromatography. An abundant and pure yield of 6His_proSspB was purified (lane 3) while a much less stable 6His_proScpAB $C_{238}A$ was co-eluted with GroE (60 kDa) and N-terminal propeptide of ScpA (20 kDa) (lane 6). Upon size exclusion chromatography, the 6His_proScpAB $C_{238}A$ eluted in complex with GroE (lane 7) followed by monomeric 6His_proScpAB $C_{238}A$ (lane 8) and the N-terminal propeptide (lane 9).

activation in the cytoplasm of *E.coli* yielding an earlier activation intermediate, which was subsequently captured by ScpB-6His. This confirms a possible role for ScpB in protecting *S. aureus* by capturing ScpA. Furthermore, the processing site may serve as one of the alternative sites of preference for the activation of ScpA (Figure 10).

IX. ScpA Binds Heparin-Sepharose Column

The interaction of heparan sulfate and heparin with proteins regulates a wide range of biological processes (7). In particular, a study with *Trypanosoma cruzi* cruzipain has shown that heparan sulfate, a heparin-like glycosaminoglycan, was able to modulate kinin release through the activity of cruizpain (123). Since a study by Imamura *et al*. (100) has shown that ScpA, augmented by SspB, acts directly on kininogen to release bradykinin, it was hypothesized that heparin and other sulfated glycoasminoglycans such as heparan sulfate could also interact with ScpA to modulate its activity on kininogen. To determine whether ScpA had any affinity for heparin, the culture supernatant of RN6390 Pspac::*ssp*ABC expressing recombinant ScpA was subject to heparin-Sepharose chromatography. ScpA was eluted from the heparin-Sepharose column at 1.0M NaCl suggesting that ScpA is able to bind to heparin and that the binding is mediated mainly by electrostatic interactions.

X. Specificity of ScpA for Protein Substrates

An overview of the maturation mechanism confirms that ScpA has a broader substrate specificity compared to SspB. ScpA has been shown to hydrolyze cysteine

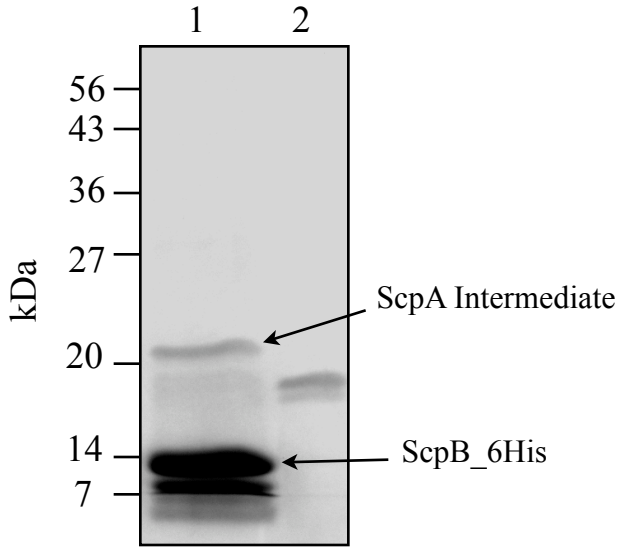


Figure 10. ScpB captures ScpA during autoactivation in *E.coli* cytoplasm.

A ScpA intermediate was co-purified with ScpB_6His using metal affinity chromatography (lane 1) with a linear gradient of 2M imidazole. A mature ScpA standard (1 μg) was loaded in lane 2. N-terminal sequencing identified the ScpA intermediate as being processed at LK↓$_{197}$QKASV.

protease inhibitors cystatins C and D and targeting kininogens to release bradykinin, but despite its activity on these substrates and limited activity on synthetic substrate Z-Phe-Leu-Glu-*p*NA, further investigation into the substrate preferences is needed to gain a deeper understanding of its target protein substrates. Since the use of synthetic substrate Z-Phe-Leu-Glu-*p*NA was only effective to a limited extent, we tested ScpA with another synthetic substrate with properties Ac-Arg-Ser-Leu-Lys-AMC but the activity of ScpA was minimal and less efficient (data not shown).

XI. ScpA Exhibits a Limited Capacity to Degrade Fibrinogen

Since the orthologue of ScpA in *S. epidermidis* as well as SspB from *S. aureus* have exhibited effective fibrinolytic activity, ScpA was also tested against fibrinogen using a fibrin plate. 0.05 nmol of protease was dispensed into small wells of the plate and incubated overnight at 37°C. Although not as efficient as the plasmin control, ScpA demonstrated slight activity on fibrinogen suggesting that ScpA may have a limited capacity to act as an anticoagulant and interfere with fibrin clots (data not shown).

XII. ScpA can Effectively Degrade Elastin

Studies of Ecp in *S. epidermidis* also demonstrate effective elastinolytic activity (172). To determine whether ScpA in *S. aureus* can degrade elastin, *S. aureus* RN6390 Pspac::*ssp*ABC expressing recombinant ScpA was grown on elastin agar. After 4 days of incubation in 37°C, RN6390 Pspac::*ssp*ABC expressing recombinant ScpA effectively

cleared the elastin fibres, but cells expressing ScpAB $C_{238}A$ could not, confirming that ScpA has elastinolytic activity (Figure 11A). Furthermore, this activity was unique to ScpA as the growth of RN6390 and RN6390 expressing recombinant ScpA showed that RN6390 alone could not degrade elastin. Furthermore, the results indicated that ScpA does not synergistically degrade elastin more rapidly with proteases from the *ssp* operon (Figure 11B).

When RN6390 Pspac::*ssp*ABC expressing ScpAB $L_{174}A$ was grown on the elastin agar, more extensive clearing of the elastin fibres were observed compared to RN6390 Pspac::*ssp*ABC expressing recombinant ScpA. Therefore by creating ScpAB $L_{174}A$, which may have led to slower processing and diversion of maturation mechanism to other sites of preference, this allowed ScpA to be more stable thereby allowing for more effective elastinolytic activity (Figure 11C). Therefore, these results provides some evidence that ScpA possesses elastinolytic activity and may play a role in host tissue invasion and destruction.

XIII. Prevalence of ScpA in CC30 strains

Degradation of elastin by ScpA may be implicated in staphylococcal infection of the lungs as well as staphylococcal pneumonia. Therefore, to investigate the elastinolytic activity of *S. aureus* clinical isolates, we assayed clinical isolates of diverse genetic backgrounds for clearing of elastin agar and found that this activity was uniquely associated with strains belonging to clonal complex CC30. In particular, WBG10049, a community-acquired MRSA in the CC30 clonal complex had potent elastinolytic activity.

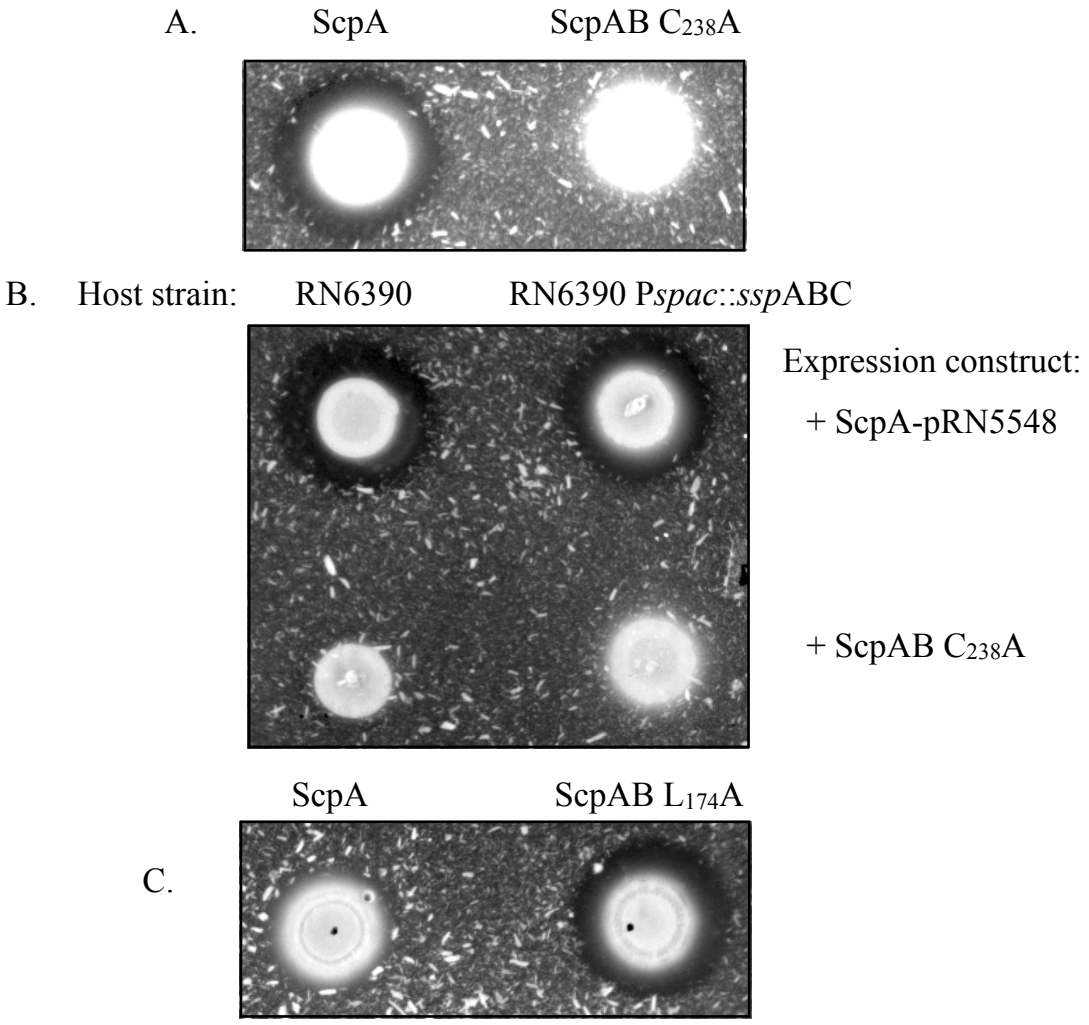


Figure 11. ScpA can Effectively Degrade Elastin.

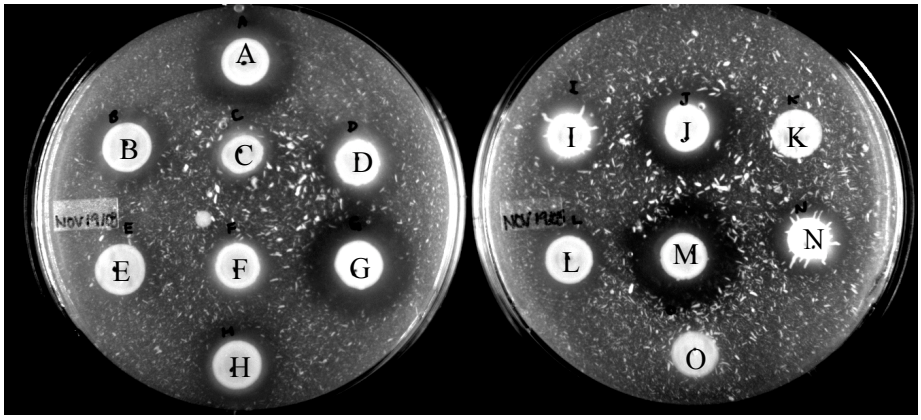
Strains were grown for 2 hours to approximately 1.0 OD units and was dispensed onto the elastin agar and incubated for 4 days at 37 °C. Radial clearing zones were used to determine elastinolytic activity.

A. RN6390 P*spac*::*ssp*ABC expressing recombinant ScpA effectively cleared the elastin fibres (Left), but cells expressing ScpA C_{238}A could not (Right).

B. Elastinolytic activity of RN6390 expressing recombinant ScpA (Left) did not differ significantly compared to RN6390 P*spac*::*ssp*ABC expressing recombinant ScpA (Right).

C. Elastin fibres were cleared more rapidly by RN6390 P*spac*::*ssp*ABC expressing ScpA L_{174}A (Right) compared to RN6390 P*spac*::*ssp*ABC expressing recombinant ScpA (Left).

Strains BK14038, ATCC25923 and L528, all of which are CC30, also demonstrated very effective clearing of elastin (Figure 12). When the protein profile from several CC30 isolates was assessed by SDS-PAGE, all the strains tested expressed a cluster of polypeptides that resembled the expression and maturation patterns of ScpA (Figure 13). Mass spectrometry analysis on the 17 kDa polypeptide of two of the strains confirmed that the expression patterns were in fact that of ScpA. These results are further supported by previous findings from our lab that showed the secretome analysis on L516, which belongs to the CC30 clonal complex, also identified expression of ScpA in the culture supernatant. Therefore, among clinical isolates, ScpA is a distinct trait of CC30 strains.



CC30 ISOLATES

CODE	STRAIN
A	WBG10049
B	BK12003
C	BK5969
D	M1015
E	BK9950
F	M809/BK22029
G	SEATTLE 1945 (ATCC25923)
H	L516
I	MN-8 (BK21203)
J	L528
K	BK21339
L	BK13271
M	BK14038
N	ATCC49775
O	PM7/MRSA252

Figure 12. **Elastinolytic activity is uniquely associated with the CC30 clonal complex.**

CC30 strains were grown for 2 hours to approximately 1.0 OD units and was dispensed onto the elastin agar and incubated for 4 days at 37 °C. Radial clearing zones were used to determine elastinolytic activity.

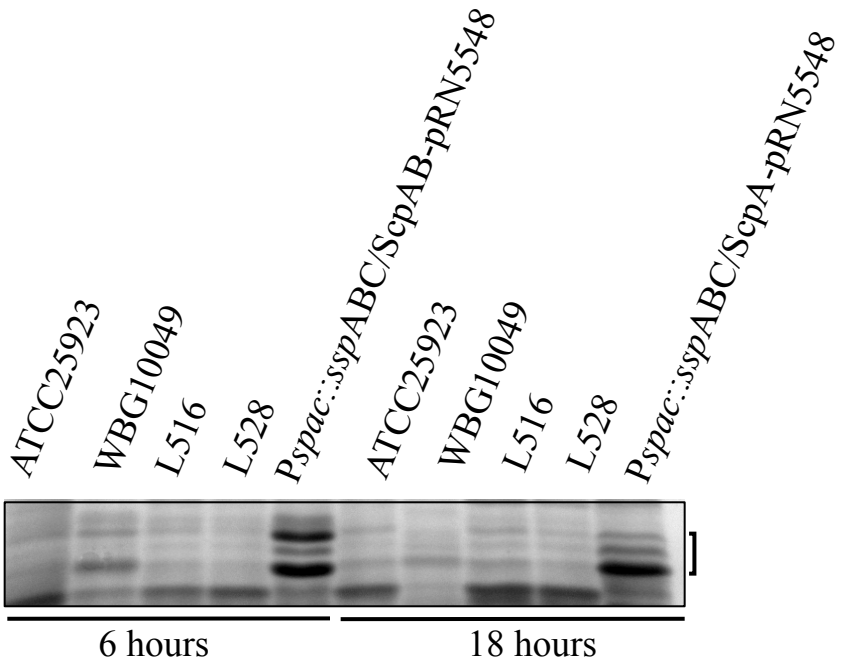


Figure 13. Expression of ScpA is uniquely associated with the CC30 lineage of *S. aureus*.

CC30 strains were grown for 6 and the supernatants (3.0 OD units) were TCA precipitated and analyzed using SDS-PAGE**.** The three polypeptides bracketed indicate the pattern of polypeptides expressed by recombinant ScpA.

PART IV: DISCUSSION

I. ScpA Undergoes a Stepwise Autocatalytic Activation

ScpA is one of two known cysteine proteases secreted by *S. aureus*. While it has been suggested that ScpA may not be involved in the SPC pathway for activation (217), the exact activation mechanism had not yet been elucidated. To determine the activation mechanism for ScpA, we created an active site mutant by substituting the active site cysteine for alanine. Expression of this active site mutant led to the accumulation of proScpA indicating that ScpA undergoes autocatalytic activation. This further confirmed that ScpA maturation occurs independent of the SPC pathway which involves the other major proteases secreted by *S. aureus*, Aur, SspA and SspB. Autocatalytic activation is commonly observed in similar cysteine proteases including SpeB from *S. pyogenes* (57), RgpB from *P. gingivalis* (144), and Interpain A (InpA) from *Prevotella intermedia* (129). However, unlike propapain (247), proSpeB (57) and procathepsin B (197), proScpA could only be processed intramolecularly as treatment of inactive mutant proScpA with mature ScpA did not yield similar maturation intermediates as observed in the culture supernatant of RN6390 Pspac::*ssp*ABC expressing ScpA. In contrast to the maturation of papain, a temporal analysis of ScpA showed that the autocatalytic maturation of proScpA occurred through several intermediates. It has been shown that the maturation of SpeB also proceeds through a multi-step process during maturation (57). Furthermore, there was no accumulation of proScpA observed after 18 hours of growth suggesting that if proScpA is not activated immediately, it will be degraded. This behaviour is similar to

autocatalytically activated proAureolysin, whereby after renaturation, the unprocessed proAureolysin is not accumulated and is degraded (159).

A modeling of proScpA on the PHYRE demonstrated that Leu_{174} was optimally positioned toward the active site of ScpA. We confirmed this prediction using mass analysis to determine the C-terminal sequence of the cleaved propeptide which allowed us to deduce the initial processing site. To further show that this was the initial processing site, we substituted the P2 residue Leu_{174} with Ala in attempt to prevent the first step in the activation mechanism. Instead of observing an accumulation of the proScpA, polypeptides similar to but not exactly the same as the wild type expression of ScpA were detected in the culture supernatant. This indicated that the substitution did not completely inhibit processing at the initial site but rather, had slowed down and diverted the processing to other sites of preference. This was supported by the varied sizes of propeptides that were purified from the $L_{174}A$ mutant demonstrating that as a result of the mutation, initiation of the activation mechanism had occurred in other sites. We conclude that although $L_{174}A$ mutant was capable of initiating processing, it was much less efficient and occurred in more than one site. However, the $LE_{175}\downarrow$ cleavage site proved to be critical for efficient maturation and the C-terminal sequences of the propeptide of wildtype ScpA showed that it was strongly preferred as the first step in autocatalytic activation.

II. Staphostatin A (ScpB) Captures ScpA During Autoactivation in *E.coli* Cytoplasm

To determine the point at which ScpA becomes activated and subsequently complexed with ScpB, we expressed *scpA* and cognate inhibitor *scpB* using an arabinose-inducible promoter. A 6xHis-tag was constructed on the C-terminal end of *scpB* to perform pull down assays. From the *E. coli* cell lysate, it appeared that ScpB co-eluted with an earlier intermediate of ScpA. This demonstrated that proScpA underwent autocatalytic activation in the cytoplasm of *E.coli* but was quickly captured by ScpB. The captured ScpA intermediate could likely represent an earlier intermediate in the activation process. Since ScpA exhibits broad substrate specificity, this data is in agreement with the concept that broad specificity proteases may require an inhibitor to protect the cells that produce them from premature activation. An inhibitor would serve to prevent detrimental effects such as proteolysis of cytoplasmic proteins in the cells (114). Evidence of this was noted in Wladyka *et al.* where they showed that ScpA was toxic for *E. coli* when expressed without its cognate inhibitor. Furthermore, they found that production of ScpA and ScpB was abrogated when there was a mutation in the reactive center of ScpB (254).

Likewise, Shaw *et al.* has shown that an isogenic *sspC* mutant of *S. aureus* 8325-4 exhibited significant changes in phenotype as a result of the mutation. Defective growth, a less structured peptidoglycan layer within the cell envelope, and complete lack of exoproteins secreted into the growth media were some of many changes observed in the mutant phenotype (218). However, experiments conducted by Nickerson (158) using

sspC mutant constructs in RN6390 (similar strain to 8325-4) did not result in the same phenotype suggesting that SspC was not essential for cell viability. Although the reason for this discrepancy is not obvious, it is clear that the role of staphostatins SspC and ScpB do differ in terms of its importance in protease activation. It is possible that this may be due to the different activation mechanisms of each protease. We can conclude that our results show that ScpA interacts with ScpB forming a possible enzyme-inhibitor complex suggesting that ScpB may serve to guard ScpA from non-specific degradation by prematurely activated ScpA. Understanding the staphopain A-staphostatin A interactions provides further insight into regulation of virulence and survival of *S. aureus*.

III. ScpA Binds Heparin-Sepharose Column

To determine the affinity of ScpA for heparin, ScpA was purified using a heparin-Sepharose column and eluted in a linear NaCl gradient. Binding of cysteine proteases with basement membranes have been demonstrated in papain and cathepsin B which are able to bind to laminin of the basement membrane (51) suggesting a role in degradation of extracellular matrix constituents. Several reports (7, 122, 123, 155) have also shown that cysteine proteases can interact with another membrane component called glycosaminoglycans. Among the glycosaminoglycans are heparin and heparin-like glycosaminoglycans, which are mostly present at the cell surface or extracellular matrix (258). The interaction of glycosaminoglycans with cysteine proteases can regulate a variety of biological processes including hemostasis, inflammation, angiogenesis, cell adhesion and others (7). Lysosomal cysteine protease, Cathepsin B, has been implicated in several diseases involving tissue remodelling, including degradation of extracellular

matrix proteins and inflammation. A study has shown that heparin and heparan sulfate are able to bind to cathepsin B suggesting that heparan sulfate may serve as an important binding site for cysteine proteases at the basement membrane (6). Furthermore, it has previously been noted that heparan sulfate is a critical molecule for the initial binding of *Trypanosoma cruzi* to host cell surfaces (91). Therefore, the ability of ScpA to bind to the heparin-Sepharose column are preliminary results suggesting that ScpA may be capable of binding to heparin in the extracellular matrix of host cell surfaces.

A study with *Trypanosoma cruzi* cysteine protease cruzipain has shown that heparan sulfate was able to modulate kinin release through the activity of cruizpain (123). The plasma kallikrein-kinin system plays an important role in inflammation. Vasoactive peptides referred to as kinins are excised and released from kininogens. Once liberated, these peptides can contribute to edema formation, vasodilation, and pain (54). In human plasma, *S. aureus* has been shown to trigger the release of bradykinin (132), which induces vascular leakage leading to hypotension (203). A study by Imamura *et al*. (100) has shown that ScpA, augmented by SspB, acts directly on kininogen to release bradykinin. Since heparan sulfate was able to modulate kinin release through cruzipain, it is plausible that heparin and heparan sulfate may also interact with ScpA to modulate its activity on kininogen and therefore promote the dissemination of infection.

IV. ScpA Exhibits a Limited Capacity to Degrade Fibrinogen

ScpA was tested against fibrinogen using a fibrin plate. Although not as efficient as the plasmin control, ScpA demonstrated slight activity on fibrinogen suggesting that ScpA has a limited capacity to act as an anticoagulant and interfere with fibrin clots.

Degradation of fibrinogen is also observed in several cysteine proteases including SspB from *S. aureus* (130), EcpA from *S. epidermidis* (172) and SpeB from *S. pyogenes* (131). Fibrinogen is a key factor in blood clotting whereby fibrinogen is converted to fibrin by thrombin to prevent blood loss during an injury. Furthermore, fibrin/fibrinogen can serve as a substrate for microbial adhesion in sites of vascular injury (180). Therefore, ScpA may be involved in wound infections and may be involved in compromising the structural integrity of fibrin clots. This may contribute to further bacterial dissemination by *S. aureus*. In particular, it may play a role in causing infective endocarditis which can be further developed by the effective interaction of *S. aureus* to platelet-fibrin aggregates present on the surface of the heart valves (236).

V. ScpA can Effectively Degrade Elastin

Results from *S. aureus* RN6390 Pspac::*ssp*ABC expressing recombinant ScpA grown on elastin agar plates demonstrated that ScpA was capable of degrading elastin. A study by Potempa *et al*. (188) had previously suggested that a cysteine protease of *S. aureus* exhibited elastinolytic activity. However, the molecular weight of the cysteine protease that was identified and purified in this study was well below the actual molecular weight of ScpA (19 kDa). It is possible that the cysteine protease isolated from the V-8 strain may correspond to ScpA, however, this study did not provide definitive evidence that this was the case. By comparing the elastinolytic activity of ScpA and an inactive ScpA mutant, our results showed that ScpA can effectively degrade elastin.

Therefore, this finding is unique and meaningful towards further characterization of this cysteine protease, ScpA.

Degradation of elastin, a major structural component of the extracellular matrix in tissues, occurs in several other bacteria including *Pseudomonas aeruginosa* (151) and *Flavobacterium menningosepticum* (83). From these bacteria, it has been shown that the bacterial pathogenicity is directly linked to the production of elastase. Elastin is also abundantly present in the lungs, skin, and major blood vessels. Therefore, since *S. aureus* commonly infects tissues that are abundant in elastin, the production of ScpA by *S. aureus* may serve to be an important component for the success of an infection by degrading surrounding tissues. In particular, the elastinolytic activity of *P. aeruginosa* has been shown to contribute to the destruction of lung connective tissue in cystic fibrosis (30). It would be interesting to investigate whether elastinolytic activity of *S. aureus,* particularly by the activity of ScpA, would play a similar role in the destruction of connective tissues in cystic fibrosis.

Elastinolytic activity has been previously identified in several different cysteine proteases including papain (46) and cathepsins L (116), K (198), S (219), V (260) and Ecp of *S. epidermidis* (172). Interestingly, a gingipain complex consisting of lipopolysaccharide, Rgp and Kgp present in *P. gingivalis* significantly degraded elastin. Monomeric Rgp and Kgp indicated lower levels of elastinolytic activity compared to the complex form, therefore, demonstrating a way for proteases of *P. gingivalis* to work together in complex to effectively degrade elastin (240). In *S. aureus,* such a synergistic effect for rapidly degrading elastin was not observed amongst the staphylococcal proteases. Therefore, the comparison of elastinolytic activity in RN6390 expressing the

recombinant ScpA with RN6390 Pspac::*ssp*ABC expressing the same construct indicated that proteases in the *ssp* operon were not involved with ScpA in elastin degradation and that this activity was unique to ScpA. When RN6390 Pspac::*ssp*ABC expressing ScpAB $L_{174}A$ was grown on the elastin agar, the elastin fibres were cleared more rapidly than RN6390 Pspac::*ssp*ABC expressing recombinant ScpA. Genetic analysis of SspB showed that the site equivalent to Leu_{174} is not processed in SspB during maturation. Since SspB is a much more stable protease than ScpA, this may account for the enhanced stability and therefore more extensive clearing of the elastin agar observed in the ScpA (Leu_{174}→Ala) mutant.

VI. Prevalence of ScpA in CC30 Clinical Isolates

Several clinical isolates of diverse genetic backgrounds were assayed for elastinolytic activity. Of these genetic backgrounds, only strains belonging to CC30 demonstrated the ability to degrade elastin. *S. aureus* strains belong to clonal complexes, which are groups of sequence types (ST). For these STs to be part of a clonal complex, they must share at least five of seven identical alleles with at least one other ST within the group. Major disease causing strains (69) and MRSA (66) belong to several clonal complexes, including CC30 which consists of STs such as ST30 and ST36. Studies in different countries and continents have indicated that strains belonging to the CC30 complex are the most prevalent strains in nasal carriages (4, 69, 75, 117). Furthermore, CC30 strains have been associated with increased infection severity and frequent hematogenous complications (66). In addition, a great percentage of persistent bacteremia, which is common in patients with endovascular infection (76), was

frequently associated with CC30 isolates (255). Without a doubt, CC30 strains can cause serious staphylococcal infections. Therefore, its ability to degrade elastin may have a contributing role in the invasiveness and dissemination of *S. aureus* strains belonging to the CC30 complex.

In addition to identifying elastinolytic activity in CC30 strains, a secretome analysis on L516, a CC30 strain involved in complicated nosocomial bacteremia, identified expression of ScpA in the culture supernatant. We examined other strains in the CC30 genetic background and found that of the strains tested, all expressed a cluster of polypeptides that resembled the expression and maturation patterns of ScpA. Mass spectrometry of two of the strains confirmed that the patterns did in fact correspond to ScpA. Therefore, this correlation between ScpA and CC30 may suggest a pathogenic role in the degradation of elastin in host tissues.

Interestingly, it has been noted that the transition from a particular historic ST30 strain to contemporary ST30 clinical isolates has been associated with expression of ScpA, loss of expression of the *ssp* operon, and co-acquisition of TSST and Orf011. Our findings support this noted transition, as some of the strains that we tested are contemporary ST30 strains that demonstrate elastinolytic activity suggesting the expression of ScpA.

PART V: CONCLUSION AND FUTURE DIRECTIONS

Our studies have found that ScpA undergoes a rapid activation mechanism distinct from the well-characterized SPC pathway. An active site mutation resulted in the

accumulation of the ScpA precursor in the culture supernatant proving that ScpA is activated autocatalytically. The intramolecular autocatalytic maturation of precursor ScpA proceeded through several intermediates, most of which were characterized using using N-terminal sequencing and mass spectrometry. Comparable to the papain family of proteases, ScpA demonstrated a strong preference for hydrophobic amino acids at the P2 site. As with many papain-like proteases, the specificity for the P1 site is often less defined. Our results show that ScpA exhibits a broad specificity by recognizing substrates with a polar amino acid at the P1 site. Consistent with a broad substrate specificity and in contrast to SspB, ScpA was sensitive to autodegradation when incubated over a period of time. Furthermore, in agreement with the concept that broad specificity proteases may require an inhibitor to protect the cells that produce them, pull down assays indicated that ScpA becomes complexed with its cognate inhibitor, ScpB, within the cytoplasm of *E. coli*. ScpA demonstrated an affinity for heparin and also the ability to degrade fibrinogen and elastin, all of which are components of the ECM. In addition, analysis of clinical isolates showed that elastinolytic activity and the expression of ScpA was uniquely associated with CC30 strains suggesting a role in the destruction of host tissues. In conclusion, the results from this work provide us with new insight into the activation mechanism and further characterization of ScpA. These characteristics of ScpA differ significantly from that of SspB indicating that the presence of two cysteine proteases in *S. aureus* both uniquely contribute to the pathogenesis of *S. aureus*. Our findings on ScpA will allow us to better understand the specific role and function of ScpA in the virulence strategy of *S. aureus*.

Since ScpA is rapidly activated, all the maturation intermediates may not have been visually identified in the SDS-PAGE of the culture supernatant. Therefore, it would be worthwhile to produce ScpA antibodies and use Western blotting to detect the intermediates involved in the activation mechanism. These antibodies would allow us to affirmatively identify the number of intermediates required for full activity as well as characterize the cleavage sites. An alternative to producing antibodies would be to synthesize DCG-04, which is a labeling probe based on E-64, to identify papain family of cysteine proteases.

Our results and interpretations suggest that LE↓$_{176}$DKKLK is the initial processing site for ScpA activation, however, further experiments are needed to confirm this hypothesis. Since, the alanine in the Leu$_{174}$>Ala change did not appear to be an effective substitution, using another non-hydrophobic amino acid could potentially inhibit activation. As the mass analysis to determine the C-terminus of the ScpA propeptide eluded to initial processing occurring mostly at LE↓$_{176}$DKKLK and maybe LK↓$_{171}$TPRLE, it is possible that ScpA activation may be initiated earlier. Therefore, another site to investigate would be that of LK↓$_{171}$TPRLE.

Preliminary results showing that ScpA can interact with heparin opens up many opportunities to explore the impact of this interaction. Further studies would include investigating whether heparan sulfate proteoglycans would be able to regulate ScpA activity. As such, it would be interesting to examine the potential for heparan sulfate to modulate kinin release by ScpA and other ScpA functions.

Lastly, to gain insight into the *in vivo* effects of ScpA, creating deletion mutants of ScpA in various CC30 strains would allow us to observe any changes in the virulence of these strains in murine models.

PART VI: REFERENCE LIST

1. 2003. From the Centers for Disease Control and Prevention. Public health dispatch: outbreaks of community-associated methicillin-resistant Staphylococcus aureus skin infections--Los Angeles County, California, 2002-2003. JAMA **289:**1377.
2. 2003. Methicillin-resistant Staphylococcus aureus infections among competitive sports participants - Colorado, Indiana, Pennsylvania, and Los Angeles County, 2000-2003. Conn Med **67:**549-51.
3. 2004. National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004, issued October 2004. Am J Infect Control **32:**470-85.
4. **Aires de Sousa, M., T. Conceicao, C. Simas, and H. de Lencastre.** 2005. Comparison of genetic backgrounds of methicillin-resistant and -susceptible Staphylococcus aureus isolates from Portuguese hospitals and the community. J Clin Microbiol **43:**5150-7.
5. **al-Masaudi, S. B., M. J. Day, and A. D. Russell.** 1991. Antimicrobial resistance and gene transfer in Staphylococcus aureus. J Appl Bacteriol **70:**279-90.
6. **Almeida, P. C., I. L. Nantes, J. R. Chagas, C. C. Rizzi, A. Faljoni-Alario, E. Carmona, L. Juliano, H. B. Nader, and I. L. Tersariol.** 2001. Cathepsin B activity regulation. Heparin-like glycosaminogylcans protect human cathepsin B from alkaline pH-induced inactivation. J Biol Chem **276:**944-51.
7. **Almeida, P. C., I. L. Nantes, C. C. Rizzi, W. A. Judice, J. R. Chagas, L. Juliano, H. B. Nader, and I. L. Tersariol.** 1999. Cysteine proteinase activity regulation. A possible role of heparin and heparin-like glycosaminoglycans. J Biol Chem **274:**30433-8.
8. **Archer, G. L.** 1998. Staphylococcus aureus: a well-armed pathogen. Clin Infect Dis **26:**1179-81.
9. **Arvidson, S.** 2000. Extracellular enzymes, p. 379-385, Gram-positive pathogens. American Society for Microbiology, Washington, D.C.
10. **Arvidson, S.** 1973. Studies on extracellular proteolytic enzymes from Staphylococcus aureus. II. Isolation and characterization of an EDTA-sensitive protease. Biochim Biophys Acta **302:**149-57.
11. **Arvidson, S., T. Holme, and B. Lindholm.** 1973. Studies on extracellular proteolytic enzymes from Staphylococcus aureus. I. Purification and characterization of one neutral and one alkaline protease. Biochim Biophys Acta **302:**135-48.
12. **Arvidson, S., and K. Tegmark.** 2001. Regulation of virulence determinants in Staphylococcus aureus. Int J Med Microbiol **291:**159-70.

13. **Baddour, L. M., C. Lowrance, A. Albus, J. H. Lowrance, S. K. Anderson, and J. C. Lee.** 1992. Staphylococcus aureus microcapsule expression attenuates bacterial virulence in a rat model of experimental endocarditis. J Infect Dis **165:**749-53.

14. **Baggett, H. C., T. W. Hennessy, K. Rudolph, D. Bruden, A. Reasonover, A. Parkinson, R. Sparks, R. M. Donlan, P. Martinez, K. Mongkolrattanothai, and J. C. Butler.** 2004. Community-onset methicillin-resistant Staphylococcus aureus associated with antibiotic use and the cytotoxin Panton-Valentine leukocidin during a furunculosis outbreak in rural Alaska. J Infect Dis **189:**1565-73.

15. **Baker, D., J. L. Sohl, and D. A. Agard.** 1992. A protein-folding reaction under kinetic control. Nature **356:**263-5.

16. **Balaban, N., and R. P. Novick.** 1995. Translation of RNAIII, the Staphylococcus aureus agr regulatory RNA molecule, can be activated by a 3'-end deletion. FEMS Microbiol Lett **133:**155-61.

17. **Banbula, A., J. Potempa, J. Travis, C. Fernandez-Catalan, K. Mann, R. Huber, W. Bode, and F. Medrano.** 1998. Amino-acid sequence and three-dimensional structure of the Staphylococcus aureus metalloproteinase at 1.72 A resolution. Structure **6:**1185-93.

18. **Barber, M., and M. Rozwadowska-Dowzenko.** 1948. Infection by penicillin-resistant staphylococci. Lancet **2:**641-4.

19. **Bayer, M. G., J. H. Heinrichs, and A. L. Cheung.** 1996. The molecular architecture of the sar locus in Staphylococcus aureus. J Bacteriol **178:**4563-70.

20. **Beaudet, R., S. A. Saheb, and G. R. Drapeau.** 1974. Structural heterogeneity of the protease isolated from several strains of Staphylococcus aureus. J Biol Chem **249:**6468-71.

21. **Berge, A., and L. Bjorck.** 1995. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. J Biol Chem **270:**9862-7.

22. **Bisno, A. L.** 2001. Acute pharyngitis. N Engl J Med **344:**205-11.

23. **Bisno, A. L., and D. L. Stevens.** 1996. Streptococcal infections of skin and soft tissues. N Engl J Med **334:**240-5.

24. **Bitar, C. M., C. G. Mayhall, V. A. Lamb, T. J. Bradshaw, A. C. Spadora, and H. P. Dalton.** 1987. Outbreak due to methicillin- and rifampin-resistant Staphylococcus aureus: epidemiology and eradication of the resistant strain from the hospital. Infect Control **8:**15-23.

25. **Bjoorklind, A., and H. Jornvall.** 1974. Substrate specificity of three different extracellular proteolytic enzymes from Staphylococcus aureus. Biochim Biophys Acta **370:**524-9.

26. **Boyce, J. M.** 1998. Are the epidemiology and microbiology of methicillin-resistant Staphylococcus aureus changing? JAMA **279:**623-4.

27. **Boyce, J. M., M. Landry, T. R. Deetz, and H. L. DuPont.** 1981. Epidemiologic studies of an outbreak of nosocomial methicillin-resistant Staphylococcus aureus infections. Infect Control **2:**110-6.

28. **Braun, P., and J. Tommassen.** 1998. Function of bacterial propeptides. Trends Microbiol **6:**6-8.

29. **Bronner, S., H. Monteil, and G. Prevost.** 2004. Regulation of virulence determinants in Staphylococcus aureus: complexity and applications. FEMS Microbiol Rev **28:**183-200.

30. **Bruce, M. C., L. Poncz, J. D. Klinger, R. C. Stern, J. F. Tomashefski, Jr., and D. G. Dearborn.** 1985. Biochemical and pathologic evidence for proteolytic destruction of lung connective tissue in cystic fibrosis. Am Rev Respir Dis **132:**529-35.

31. **Burlak, C., C. H. Hammer, M.-A. Robinson, A. R. Whitney, M. J. McGavin, B. N. Kreiswirth, and F. R. Deleo.** 2007. Global analysis of community-associated methicillin-resistant Staphylococcus aureus exoproteins reveals molecules produced in vitro and during infection. Cellular Microbiology **9:**1172-90.

32. **Burns, E. H., Jr., A. M. Marciel, and J. M. Musser.** 1996. Activation of a 66-kilodalton human endothelial cell matrix metalloprotease by Streptococcus pyogenes extracellular cysteine protease. Infect Immun **64:**4744-50.

33. **Casewell, M. W., and R. L. Hill.** 1986. The carrier state: methicillin-resistant Staphylococcus aureus. J Antimicrob Chemother **18 Suppl A:**1-12.

34. **Chambers, H. F.** 1997. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev **10:**781-91.

35. **Chan, P. F., and S. J. Foster.** 1998. Role of SarA in virulence determinant production and environmental signal transduction in Staphylococcus aureus. J Bacteriol **180:**6232-41.

36. **Chaussee, M. S., E. R. Phillips, and J. J. Ferretti.** 1997. Temporal production of streptococcal erythrogenic toxin B (streptococcal cysteine proteinase) in response to nutrient depletion. Infect Immun **65:**1956-9.

37. **Chen, C. Y., S. C. Luo, C. F. Kuo, Y. S. Lin, J. J. Wu, M. T. Lin, C. C. Liu, W. Y. Jeng, and W. J. Chuang.** 2003. Maturation processing and characterization of streptopain. J Biol Chem **278:**17336-43.

38. **Cheung, A. L., M. G. Bayer, and J. H. Heinrichs.** 1997. sar Genetic determinants necessary for transcription of RNAII and RNAIII in the agr locus of Staphylococcus aureus. J Bacteriol **179:**3963-71.

39. **Cheung, A. L., J. M. Koomey, C. A. Butler, S. J. Projan, and V. A. Fischetti.** 1992. Regulation of exoprotein expression in Staphylococcus aureus by a locus (sar) distinct from agr. Proc Natl Acad Sci U S A **89:**6462-6.

40. **Chiang-Ni, C., and J. J. Wu.** 2008. Effects of streptococcal pyrogenic exotoxin B on pathogenesis of Streptococcus pyogenes. J Formos Med Assoc **107:**677-85.

41. **Chien, Y., A. C. Manna, S. J. Projan, and A. L. Cheung.** 1999. SarA, a global regulator of virulence determinants in Staphylococcus aureus, binds to a conserved motif essential for sar-dependent gene regulation. J Biol Chem **274:**37169-76.

42. **Collin, M., and A. Olsen.** 2001. Effect of SpeB and EndoS from Streptococcus pyogenes on human immunoglobulins. Infect Immun **69:**7187-9.

43. **Collin, M., and A. Olsen.** 2003. Extracellular enzymes with immunomodulating activities: variations on a theme in Streptococcus pyogenes. Infect Immun **71:**2983-92.

44. **Collin, M., M. D. Svensson, A. G. Sjoholm, J. C. Jensenius, U. Sjobring, and A. Olsen.** 2002. EndoS and SpeB from Streptococcus pyogenes inhibit immunoglobulin-mediated opsonophagocytosis. Infect Immun **70:**6646-51.

45. **Collins, V. L., and A. Tarkowski.** 2000. Animal models of experimental *Staphylococcus aureus* infection., p. 422-30. *In* V. A. Fischetti, R. P. Novick, J. J. Feretti, D. A. Portnoy, and J. I. Rood (ed.), Gram-Positive Pathogens. ASM Press, Washington.

46. **Coulson, W. F.** 1971. The effect of proteolytic enzymes on the tensile strength of whole aorta and isolated aortic elastin. Biochim Biophys Acta **237:**378-86.

47. **Coulter, S. N., W. R. Schwan, E. Y. Ng, M. H. Langhorne, H. D. Ritchie, S. Westbrock-Wadman, W. O. Hufnagle, K. R. Folger, A. S. Bayer, and C. K. Stover.** 1998. Staphylococcus aureus genetic loci impacting growth and survival in multiple infection environments. Mol Microbiol **30:**393-404.

48. **Crossley, K., B. Landesman, and D. Zaske.** 1979. An outbreak of infections caused by strains of Staphylococcus aureus resistant to methicillin and aminoglycosides. II. Epidemiologic studies. J Infect Dis **139:**280-7.

49. **Cunningham, M. W.** 2000. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev **13:**470-511.

50. **Curtis, M. A., H. K. Kuramitsu, M. Lantz, F. L. Macrina, K. Nakayama, J. Potempa, E. C. Reynolds, and J. Aduse-Opoku.** 1999. Molecular genetics and nomenclature of proteases of Porphyromonas gingivalis. J Periodontal Res **34:**464-72.

51. **Dalet-Fumeron, V., L. Boudjennah, and M. Pagano.** 1998. Binding of the cysteine proteinases papain and cathepsin B-like to coated laminin: use of synthetic peptides from laminin and from the laminin binding region of the beta 1

integrin subunit to characterize the binding site. Arch Biochem Biophys **358:**283-90.

52. **DeCarlo, A. A., H. E. Grenett, G. J. Harber, L. J. Windsor, M. K. Bodden, B. Birkedal-Hansen, and H. Birkedal-Hansen.** 1998. Induction of matrix metalloproteinases and a collagen-degrading phenotype in fibroblasts and epithelial cells by secreted Porphyromonas gingivalis proteinase. J Periodontal Res **33:**408-20.

53. **DeCarlo, A. A., Jr., L. J. Windsor, M. K. Bodden, G. J. Harber, B. Birkedal-Hansen, and H. Birkedal-Hansen.** 1997. Activation and novel processing of matrix metalloproteinases by a thiol-proteinase from the oral anaerobe Porphyromonas gingivalis. J Dent Res **76:**1260-70.

54. **DeLa Cadena, R. A., and R. W. Colman.** 1991. Structure and functions of human kininogens. Trends Pharmacol Sci **12:**272-5.

55. **Diekema, D. J., M. A. Pfaller, F. J. Schmitz, J. Smayevsky, J. Bell, R. N. Jones, and M. Beach.** 2001. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clin Infect Dis **32 Suppl 2:**S114-32.

56. **Dinges, M. M., P. M. Orwin, and P. M. Schlievert.** 2000. Exotoxins of Staphylococcus aureus. Clin Microbiol Rev **13:**16-34.

57. **Doran, J. D., M. Nomizu, S. Takebe, R. Menard, D. Griffith, and E. Ziomek.** 1999. Autocatalytic processing of the streptococcal cysteine protease zymogen: processing mechanism and characterization of the autoproteolytic cleavage sites. Eur J Biochem **263:**145-51.

58. **Downer, R., F. Roche, P. W. Park, R. P. Mecham, and T. J. Foster.** 2002. The elastin-binding protein of Staphylococcus aureus (EbpS) is expressed at the cell surface as an integral membrane protein and not as a cell wall-associated protein. J Biol Chem **277:**243-50.

59. **Drapeau, G. R.** 1978. Role of metalloprotease in activation of the precursor of staphylococcal protease. J Bacteriol **136:**607-13.

60. **Drapeau, G. R.** 1978. Unusual COOH-terminal structure of staphylococcal protease. J Biol Chem **253:**5899-901.

61. **Drapeau, G. R., Y. Boily, and J. Houmard.** 1972. Purification and properties of an extracellular protease of Staphylococcus aureus. J Biol Chem **247:**6720-6.

62. **Dubin, G.** 2003. Defense against own arms: staphylococcal cysteine proteases and their inhibitors. Acta Biochimica Polonica **50:**715-24

63. **Dubin, G.** 2002. Extracellular proteases of Staphylococcus spp. Biological Chemistry **383:**1075-1086

64. **Dubin, G., D. Chmiel, P. Mak, M. Rakwalska, M. Rzychon, and A. Dubin.** 2001. Molecular cloning and biochemical characterisation of proteases from Staphylococcus epidermidis. Biological Chemistry **382:**1575-82

65. **Dunman, P. M., E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes, and S. J. Projan.** 2001. Transcription profiling-based identification of Staphylococcus aureus genes regulated by the agr and/or sarA loci. J Bacteriol **183:**7341-53.

66. **Enright, M. C., D. A. Robinson, G. Randle, E. J. Feil, H. Grundmann, and B. G. Spratt.** 2002. The evolutionary history of methicillin-resistant Staphylococcus aureus (MRSA). Proc Natl Acad Sci U S A **99:**7687-92.

67. **Entenza, J. M., T. J. Foster, D. Ni Eidhin, P. Vaudaux, P. Francioli, and P. Moreillon.** 2000. Contribution of clumping factor B to pathogenesis of experimental endocarditis due to Staphylococcus aureus. Infect Immun **68:**5443-6.

68. **Federle, M. J., K. S. McIver, and J. R. Scott.** 1999. A response regulator that represses transcription of several virulence operons in the group A streptococcus. J Bacteriol **181:**3649-57.

69. **Feil, E. J., J. E. Cooper, H. Grundmann, D. A. Robinson, M. C. Enright, T. Berendt, S. J. Peacock, J. M. Smith, M. Murphy, B. G. Spratt, C. E. Moore, and N. P. Day.** 2003. How clonal is Staphylococcus aureus? J Bacteriol **185:**3307-16.

70. **Filipek, R., J. Potempa, and M. Bochtler.** 2005. A comparison of staphostatin B with standard mechanism serine protease inhibitors. The Journal of Biological Chemistry **280:**14669-74

71. **Flock, J. I., G. Froman, K. Jonsson, B. Guss, C. Signas, B. Nilsson, G. Raucci, M. Hook, T. Wadstrom, and M. Lindberg.** 1987. Cloning and expression of the gene for a fibronectin-binding protein from Staphylococcus aureus. EMBO J **6:**2351-7.

72. **Foster, T. J.** 2005. Immune evasion by staphylococci. Nat Rev Microbiol **3:**948-58.

73. **Foster, T. J., and M. Hook.** 1998. Surface protein adhesins of Staphylococcus aureus. Trends Microbiol **6:**484-8.

74. **Foster, T. J., and D. McDevitt.** 1994. Surface-associated proteins of Staphylococcus aureus: their possible roles in virulence. FEMS Microbiol Lett **118:**199-205.

75. **Fowler, V. G., Jr., C. L. Nelson, L. M. McIntyre, B. N. Kreiswirth, A. Monk, G. L. Archer, J. Federspiel, S. Naidich, B. Remortel, T. Rude, P. Brown, L. B. Reller, G. R. Corey, and S. R. Gill.** 2007. Potential associations between hematogenous complications and bacterial genotype in Staphylococcus aureus infection. J Infect Dis **196:**738-47.

76. **Fowler, V. G., Jr., G. Sakoulas, L. M. McIntyre, V. G. Meka, R. D. Arbeit, C. H. Cabell, M. E. Stryjewski, G. M. Eliopoulos, L. B. Reller, G. R. Corey, T. Jones, N. Lucindo, M. R. Yeaman, and A. S. Bayer.** 2004. Persistent bacteremia due to methicillin-resistant Staphylococcus aureus infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein. J Infect Dis **190:**1140-9.

77. **Francis, J. S., M. C. Doherty, U. Lopatin, C. P. Johnston, G. Sinha, T. Ross, M. Cai, N. N. Hansel, T. Perl, J. R. Ticehurst, K. Carroll, D. L. Thomas, E. Nuermberger, and J. G. Bartlett.** 2005. Severe community-onset pneumonia in healthy adults caused by methicillin-resistant Staphylococcus aureus carrying the Panton-Valentine leukocidin genes. Clin Infect Dis **40:**100-7.

78. **Fridkin, S. K., J. C. Hageman, M. Morrison, L. T. Sanza, K. Como-Sabetti, J. A. Jernigan, K. Harriman, L. H. Harrison, R. Lynfield, and M. M. Farley.** 2005. Methicillin-resistant Staphylococcus aureus disease in three communities. N Engl J Med **352:**1436-44.

79. **Gemmell, C., R. Tree, A. Patel, M. O'Reilly, and T. J. Foster.** 1991. Susceptibility to opsonophagocytosis of protein A, α-haemolysin and β-toxin deficient mutatns of *Staphylococcus aureus* isolated by allele-replacement. Zentralbl. Bakteriol. **21:**273-77.

80. **Gillaspy, A. F., J. M. Patti, F. L. Pratt, Jr., J. J. Iandolo, and M. S. Smeltzer.** 1997. The Staphylococcus aureus collagen adhesin-encoding gene (cna) is within a discrete genetic element. Gene **196:**239-48.

81. **Golonka, E., R. Filipek, A. Sabat, A. Sinczak, and J. Potempa.** 2004. Genetic characterization of staphopain genes in Staphylococcus aureus. Biol Chem **385:**1059-67.

82. **Gomez, M. I., A. Lee, B. Reddy, A. Muir, G. Soong, A. Pitt, A. Cheung, and A. Prince.** 2004. Staphylococcus aureus protein A induces airway epithelial inflammatory responses by activating TNFR1. Nat Med **10:**842-8.

83. **Grimwood, B. G., T. H. Plummer, Jr., and A. L. Tarentino.** 1994. Purification and characterization of a neutral zinc endopeptidase secreted by Flavobacterium meningosepticum. Arch Biochem Biophys **311:**127-32.

84. **Grundmeier, M., M. Hussain, P. Becker, C. Heilmann, G. Peters, and B. Sinha.** 2004. Truncation of fibronectin-binding proteins in Staphylococcus aureus strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. Infect Immun **72:**7155-63.

85. **Gubba, S., D. E. Low, and J. M. Musser.** 1998. Expression and characterization of group A Streptococcus extracellular cysteine protease recombinant mutant proteins and documentation of seroconversion during human invasive disease episodes. Infect Immun **66:**765-70.

86. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol **177:**4121-30.

87. **Hartford, O., P. Francois, P. Vaudaux, and T. J. Foster.** 1997. The dipeptide repeat region of the fibrinogen-binding protein (clumping factor) is required for functional expression of the fibrinogen-binding domain on the Staphylococcus aureus cell surface. Mol Microbiol **25:**1065-76.

88. **Hartford, O. M., E. R. Wann, M. Hook, and T. J. Foster.** 2001. Identification of residues in the Staphylococcus aureus fibrinogen-binding MSCRAMM clumping factor A (ClfA) that are important for ligand binding. J Biol Chem **276:**2466-73.

89. **Hartleib, J., N. Kohler, R. B. Dickinson, G. S. Chhatwal, J. J. Sixma, O. M. Hartford, T. J. Foster, G. Peters, B. E. Kehrel, and M. Herrmann.** 2000. Protein A is the von Willebrand factor binding protein on Staphylococcus aureus. Blood **96:**2149-56.

90. **Heath, A., V. J. DiRita, N. L. Barg, and N. C. Engleberg.** 1999. A two-component regulatory system, CsrR-CsrS, represses expression of three Streptococcus pyogenes virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. Infect Immun **67:**5298-305.

91. **Herrera, E. M., M. Ming, E. Ortega-Barria, and M. E. Pereira.** 1994. Mediation of Trypanosoma cruzi invasion by heparan sulfate receptors on host cells and penetrin counter-receptors on the trypanosomes. Mol Biochem Parasitol **65:**73-83.

92. **Herwald, H., M. Collin, W. Muller-Esterl, and L. Bjorck.** 1996. Streptococcal cysteine proteinase releases kinins: a virulence mechanism. J Exp Med **184:**665-73.

93. **Hienz, S. A., T. Schennings, A. Heimdahl, and J. I. Flock.** 1996. Collagen binding of Staphylococcus aureus is a virulence factor in experimental endocarditis. J Infect Dis **174:**83-8.

94. **Houmard, J., and G. R. Drapeau.** 1972. Staphylococcal protease: a proteolytic enzyme specific for glutamoyl bonds. Proc Natl Acad Sci U S A **69:**3506-9.

95. **Imamura, T., A. Banbula, P. J. Pereira, J. Travis, and J. Potempa.** 2001. Activation of human prothrombin by arginine-specific cysteine proteinases (Gingipains R) from porphyromonas gingivalis. J Biol Chem **276:**18984-91.

96. **Imamura, T., J. Potempa, R. N. Pike, J. N. Moore, M. H. Barton, and J. Travis.** 1995. Effect of free and vesicle-bound cysteine proteinases of Porphyromonas gingivalis on plasma clot formation: implications for bleeding tendency at periodontitis sites. Infect Immun **63:**4877-82.

97. **Imamura, T., J. Potempa, R. N. Pike, and J. Travis.** 1995. Dependence of vascular permeability enhancement on cysteine proteinases in vesicles of Porphyromonas gingivalis. Infect Immun **63:**1999-2003.

98. **Imamura, T., J. Potempa, S. Tanase, and J. Travis.** 1997. Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from Porphyromonas gingivalis. J Biol Chem **272:**16062-7.

99. **Imamura, T., S. Tanase, T. Hamamoto, J. Potempa, and J. Travis.** 2001. Activation of blood coagulation factor IX by gingipains R, arginine-specific cysteine proteinases from Porphyromonas gingivalis. Biochem J **353:**325-31.

100. **Imamura, T., S. Tanase, G. Szmyd, A. Kozik, J. Travis, and J. Potempa.** 2005. Induction of vascular leakage through release of bradykinin and a novel kinin by cysteine proteinases from Staphylococcus aureus. J Exp Med **201:**1669-76.

101. **Iwatsuki, K., O. Yamasaki, S. Morizane, and T. Oono.** 2006. Staphylococcal cutaneous infections: invasion, evasion and aggression. J Dermatol Sci **42:**203-14.

102. **Janzon, L., and S. Arvidson.** 1990. The role of the delta-lysin gene (hld) in the regulation of virulence genes by the accessory gene regulator (agr) in Staphylococcus aureus. EMBO J **9:**1391-9.

103. **Janzon, L., S. Lofdahl, and S. Arvidson.** 1989. Identification and nucleotide sequence of the delta-lysin gene, hld, adjacent to the accessory gene regulator (agr) of Staphylococcus aureus. Mol Gen Genet **219:**480-5.

104. **Ji, G., R. Beavis, and R. P. Novick.** 1997. Bacterial interference caused by autoinducing peptide variants. Science **276:**2027-30.

105. **Ji, G., R. C. Beavis, and R. P. Novick.** 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci U S A **92:**12055-9.

106. **Josefsson, E., O. Hartford, L. O'Brien, J. M. Patti, and T. Foster.** 2001. Protection against experimental Staphylococcus aureus arthritis by vaccination with clumping factor A, a novel virulence determinant. J Infect Dis **184:**1572-80.

107. **Kadowaki, T., K. Nakayama, F. Yoshimura, K. Okamoto, N. Abe, and K. Yamamoto.** 1998. Arg-gingipain acts as a major processing enzyme for various cell surface proteins in Porphyromonas gingivalis. J Biol Chem **273:**29072-6.

108. **Kagawa, T. F., J. C. Cooney, H. M. Baker, S. McSweeney, M. Liu, S. Gubba, J. M. Musser, and E. N. Baker.** 2000. Crystal structure of the zymogen form of the group A Streptococcus virulence factor SpeB: an integrin-binding cysteine protease. Proc Natl Acad Sci U S A **97:**2235-40.

109. **Kansal, R. G., A. McGeer, D. E. Low, A. Norrby-Teglund, and M. Kotb.** 2000. Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpeB, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. Infect Immun **68:**6362-9.

110. **Kaplan, S. L., K. G. Hulten, B. E. Gonzalez, W. A. Hammerman, L. Lamberth, J. Versalovic, and E. O. Mason, Jr.** 2005. Three-year surveillance

of community-acquired Staphylococcus aureus infections in children. Clin Infect Dis **40:**1785-91.

111. **Kapur, V., M. W. Majesky, L. L. Li, R. A. Black, and J. M. Musser.** 1993. Cleavage of interleukin 1 beta (IL-1 beta) precursor to produce active IL-1 beta by a conserved extracellular cysteine protease from Streptococcus pyogenes. Proc Natl Acad Sci U S A **90:**7676-80.

112. **Kapur, V., S. Topouzis, M. W. Majesky, L. L. Li, M. R. Hamrick, R. J. Hamill, J. M. Patti, and J. M. Musser.** 1993. A conserved Streptococcus pyogenes extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. Microb Pathog **15:**327-46.

113. **Karlsson, A., P. Saravia-Otten, K. Tegmark, E. Morfeldt, and S. Arvidson.** 2001. Decreased amounts of cell wall-associated protein A and fibronectin-binding proteins in Staphylococcus aureus sarA mutants due to up-regulation of extracellular proteases. Infection and Immunity **69:**4742-8

114. **Khan, A. R., and M. N. James.** 1998. Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Protein Sci **7:**815-36.

115. **Kim, W., K. Choi, Y. Kim, H. Park, J. Choi, Y. Lee, H. Oh, I. Kwon, and S. Lee.** 1996. Purification and characterization of a fibrinolytic enzyme produced from Bacillus sp. strain CK 11-4 screened from Chungkook-Jang. Appl Environ Microbiol **62:**2482-8.

116. **Kirschke, H., A. J. Barrett, and N. D. Rawlings.** 1995. Proteinases 1: lysosomal cysteine proteinases. Protein Profile **2:**1581-643.

117. **Kuehnert, M. J., D. Kruszon-Moran, H. A. Hill, G. McQuillan, S. K. McAllister, G. Fosheim, L. K. McDougal, J. Chaitram, B. Jensen, S. K. Fridkin, G. Killgore, and F. C. Tenover.** 2006. Prevalence of Staphylococcus aureus nasal colonization in the United States, 2001-2002. J Infect Dis **193:**172-9.

118. **Kulig, P., B. A. Zabel, G. Dubin, S. J. Allen, T. Ohyama, J. Potempa, T. M. Handel, E. C. Butcher, and J. Cichy.** 2007. Staphylococcus aureus-derived staphopain B, a potent cysteine protease activator of plasma chemerin. J Immunol **178:**3713-20.

119. **Kuypers, J. M., and R. A. Proctor.** 1989. Reduced adherence to traumatized rat heart valves by a low-fibronectin-binding mutant of Staphylococcus aureus. Infect Immun **57:**2306-12.

120. **Li, Y., and M. Inouye.** 1994. Autoprocessing of prothiolsubtilisin E in which active-site serine 221 is altered to cysteine. J Biol Chem **269:**4169-74.

121. **Li, Z., D. D. Sledjeski, B. Kreikemeyer, A. Podbielski, and M. D. Boyle.** 1999. Identification of pel, a Streptococcus pyogenes locus that affects both surface and secreted proteins. J Bacteriol **181:**6019-27.

122. **Li, Z., Y. Yasuda, W. Li, M. Bogyo, N. Katz, R. E. Gordon, G. B. Fields, and D. Bromme.** 2004. Regulation of collagenase activities of human cathepsins by glycosaminoglycans. J Biol Chem **279:**5470-9.

123. **Lima, A. P., P. C. Almeida, I. L. Tersariol, V. Schmitz, A. H. Schmaier, L. Juliano, I. Y. Hirata, W. Muller-Esterl, J. R. Chagas, and J. Scharfstein.** 2002. Heparan sulfate modulates kinin release by Trypanosoma cruzi through the activity of cruzipain. J Biol Chem **277:**5875-81.

124. **Lina, G., Y. Piemont, F. Godail-Gamot, M. Bes, M. O. Peter, V. Gauduchon, F. Vandenesch, and J. Etienne.** 1999. Involvement of Panton-Valentine leukocidin-producing Staphylococcus aureus in primary skin infections and pneumonia. Clin Infect Dis **29:**1128-32.

125. **Lowy, F. D.** 1998. Staphylococcus aureus infections. The New England Journal of Medicine **339:**520-32.

126. **Luong, T. T., and C. Y. Lee.** 2002. Overproduction of type 8 capsular polysaccharide augments Staphylococcus aureus virulence. Infect Immun **70:**3389-95.

127. **Madden, J. C., N. Ruiz, and M. Caparon.** 2001. Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in gram-positive bacteria. Cell **104:**143-52.

128. **Maeda, H., and T. Yamamoto.** 1996. Pathogenic mechanisms induced by microbial proteases in microbial infections. Biol Chem Hoppe Seyler **377:**217-26.

129. **Mallorqui-Fernandez, N., S. P. Manandhar, G. Mallorqui-Fernandez, I. Uson, K. Wawrzonek, T. Kantyka, M. Sola, I. B. Thogersen, J. J. Enghild, J. Potempa, and F. X. Gomis-Ruth.** 2008. A new autocatalytic activation mechanism for cysteine proteases revealed by Prevotella intermedia interpain A. J Biol Chem **283:**2871-82.

130. **Massimi, I., E. Park, K. Rice, W. Muller-Esterl, D. Sauder, and M. J. McGavin.** 2002. Identification of a novel maturation mechanism and restricted substrate specificity for the SspB cysteine protease of Staphylococcus aureus. The Journal of Biological Chemistry **277:**41770-7.

131. **Matsuka, Y. V., S. Pillai, S. Gubba, J. M. Musser, and S. B. Olmsted.** 1999. Fibrinogen cleavage by the Streptococcus pyogenes extracellular cysteine protease and generation of antibodies that inhibit enzyme proteolytic activity. Infect Immun **67:**4326-33.

132. **Mattsson, E., H. Herwald, H. Cramer, K. Persson, U. Sjobring, and L. Bjorck.** 2001. Staphylococcus aureus induces release of bradykinin in human plasma. Infect Immun **69:**3877-82.

133. **Mayville, P., G. Ji, R. Beavis, H. Yang, M. Goger, R. P. Novick, and T. W. Muir.** 1999. Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence. Proc Natl Acad Sci U S A **96:**1218-23.

134. **McAleese, F. M., E. J. Walsh, M. Sieprawska, J. Potempa, and T. J. Foster.** 2001. Loss of clumping factor B fibrinogen binding activity by Staphylococcus aureus involves cessation of transcription, shedding and cleavage by metalloprotease. J Biol Chem **276:**29969-78.

135. **McBryde, E. S., L. C. Bradley, M. Whitby, and D. L. McElwain.** 2004. An investigation of contact transmission of methicillin-resistant Staphylococcus aureus. J Hosp Infect **58:**104-8.

136. **McDevitt, D., P. Francois, P. Vaudaux, and T. J. Foster.** 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of Staphylococcus aureus. Mol Microbiol **11:**237-48.

137. **McDevitt, D., T. Nanavaty, K. House-Pompeo, E. Bell, N. Turner, L. McIntire, T. Foster, and M. Hook.** 1997. Characterization of the interaction between the Staphylococcus aureus clumping factor (ClfA) and fibrinogen. Eur J Biochem **247:**416-24.

138. **McDonald, L., and R. J. Beynon.** 2006. Positional proteomics: preparation of amino-terminal peptides as a strategy for proteome simplification and characterization. Nat Protoc **1:**1790-8.

139. **McDonald, L., D. H. Robertson, J. L. Hurst, and R. J. Beynon.** 2005. Positional proteomics: selective recovery and analysis of N-terminal proteolytic peptides. Nat Methods **2:**955-7.

140. **McGavin, M. H., D. Krajewska-Pietrasik, C. Ryden, and M. Hook.** 1993. Identification of a Staphylococcus aureus extracellular matrix-binding protein with broad specificity. Infect Immun **61:**2479-85.

141. **McGavin, M. J., C. Zahradka, K. Rice, and J. E. Scott.** 1997. Modification of the Staphylococcus aureus fibronectin binding phenotype by V8 protease. Infect Immun **65:**2621-8.

142. **McGrath, M. E.** 1999. The lysosomal cysteine proteases. Annu Rev Biophys Biomol Struct **28:**181-204.

143. **Menard, R., E. Carmona, C. Plouffe, D. Bromme, Y. Konishi, J. Lefebvre, and A. C. Storer.** 1993. The specificity of the S1' subsite of cysteine proteases. FEBS Lett **328:**107-10.

144. **Mikolajczyk, J., K. M. Boatright, H. R. Stennicke, T. Nazif, J. Potempa, M. Bogyo, and G. S. Salvesen.** 2003. Sequential autolytic processing activates the zymogen of Arg-gingipain. J Biol Chem **278:**10458-64.

145. **Mikolajczyk-Pawlinska, J., T. Kordula, N. Pavloff, P. A. Pemberton, W. C. Chen, J. Travis, and J. Potempa.** 1998. Genetic variation of Porphyromonas gingivalis genes encoding gingipains, cysteine proteinases with arginine or lysine specificity. Biol Chem **379:**205-11.

146. **Minhas, T., H. A. Ludlam, M. Wilks, and S. Tabaqchali.** 1995. Detection by PCR and analysis of the distribution of a fibronectin-binding protein gene (fbn) among staphylococcal isolates. J Med Microbiol **42:**96-101.

147. **Molla, A., T. Yamamoto, T. Akaike, S. Miyoshi, and H. Maeda.** 1989. Activation of hageman factor and prekallikrein and generation of kinin by various microbial proteinases. J Biol Chem **264:**10589-94.

148. **Mongodin, E., O. Bajolet, J. Cutrona, N. Bonnet, F. Dupuit, E. Puchelle, and S. de Bentzmann.** 2002. Fibronectin-binding proteins of Staphylococcus aureus are involved in adherence to human airway epithelium. Infect Immun **70:**620-30.

149. **Moreillon, P., J. M. Entenza, P. Francioli, D. McDevitt, T. J. Foster, P. Francois, and P. Vaudaux.** 1995. Role of Staphylococcus aureus coagulase and clumping factor in pathogenesis of experimental endocarditis. Infect Immun **63:**4738-43.

150. **Morfeldt, E., K. Tegmark, and S. Arvidson.** 1996. Transcriptional control of the agr-dependent virulence gene regulator, RNAIII, in Staphylococcus aureus. Mol Microbiol **21:**1227-37.

151. **Morihara, K., and H. Tsuzuki.** 1977. Production of protease and elastase by Pseudomonas aeruginosa strains isolated from patients. Infect Immun **15:**679-85.

152. **Morris, J. G., Jr., D. K. Shay, J. N. Hebden, R. J. McCarter, Jr., B. E. Perdue, W. Jarvis, J. A. Johnson, T. C. Dowling, L. B. Polish, and R. S. Schwalbe.** 1995. Enterococci resistant to multiple antimicrobial agents, including vancomycin. Establishment of endemicity in a university medical center. Ann Intern Med **123:**250-9.

153. **Naimi, T. S., K. H. LeDell, K. Como-Sabetti, S. M. Borchardt, D. J. Boxrud, J. Etienne, S. K. Johnson, F. Vandenesch, S. Fridkin, C. O'Boyle, R. N. Danila, and R. Lynfield.** 2003. Comparison of community- and health care-associated methicillin-resistant Staphylococcus aureus infection. JAMA **290:**2976-84.

154. **Nakakido, M., Y. Tanaka, and K. Tsumoto.** 2007. The N-terminal domain of elastin-binding protein of Staphylococcus aureus changes its secondary structure in a membrane-mimetic environment. J Biochem **142:**131-4.

155. **Nascimento, F. D., C. C. Rizzi, I. L. Nantes, I. Stefe, B. Turk, A. K. Carmona, H. B. Nader, L. Juliano, and I. L. Tersariol.** 2005. Cathepsin X binds to cell surface heparan sulfate proteoglycans. Arch Biochem Biophys **436:**323-32.

156. **Navarre, W. W., and O. Schneewind.** 1994. Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. Mol Microbiol **14:**115-21.

157. **Ni Eidhin, D., S. Perkins, P. Francois, P. Vaudaux, M. Hook, and T. J. Foster.** 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of Staphylococcus aureus. Mol Microbiol **30:**245-57.

158. **Nickerson, N. N.** 2009. Activation of Secreted Protease Virulence Factors of Staphylococcus aureus is controlled by a Novel Cascade Pathway. University of Toronto, Toronto.

159. **Nickerson, N. N., V. Joag, and M. J. McGavin.** 2008. Rapid autocatalytic activation of the M4 metalloprotease aureolysin is controlled by a conserved N-terminal fungalysin-thermolysin-propeptide domain. Molecular Microbiology **69:**1530-43

160. **Nickerson, N. N., L. Prasad, L. Jacob, L. T. Delbaere, and M. J. McGavin.** 2007. Activation of the SspA serine protease zymogen of Staphylococcus aureus proceeds through unique variations of a trypsinogen-like mechanism and is dependent on both autocatalytic and metalloprotease-specific processing. The Journal of Biological Chemistry **282:**34129-38.

161. **Nilsson, I. M., J. C. Lee, T. Bremell, C. Ryden, and A. Tarkowski.** 1997. The role of staphylococcal polysaccharide microcapsule expression in septicemia and septic arthritis. Infect Immun **65:**4216-21.

162. **Noble, W. C., H. A. Valkenburg, and C. H. Wolters.** 1967. Carriage of Staphylococcus aureus in random samples of a normal population. J Hyg (Lond) **65:**567-73.

163. **Nomizu, M., G. Pietrzynski, T. Kato, P. Lachance, R. Menard, and E. Ziomek.** 2001. Substrate specificity of the streptococcal cysteine protease. J Biol Chem **276:**44551-6.

164. **Novick, R. P.** 1991. Genetic systems in staphylococci. Methods Enzymol **204:**587-636.

165. **Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S. Moghazeh.** 1995. The agr P2 operon: an autocatalytic sensory transduction system in Staphylococcus aureus. Mol Gen Genet **248:**446-58.

166. **Novick, R. P., H. F. Ross, S. J. Projan, J. Kornblum, B. Kreiswirth, and S. Moghazeh.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J **12:**3967-75.

167. **Nowak, R.** 1994. Flesh-eating bacteria: not new, but still worrisome. Science **264:**1665.

168. **O'Brien, L., S. W. Kerrigan, G. Kaw, M. Hogan, J. Penades, D. Litt, D. J. Fitzgerald, T. J. Foster, and D. Cox.** 2002. Multiple mechanisms for the activation of human platelet aggregation by Staphylococcus aureus: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. Mol Microbiol **44:**1033-44.

169. **O'Brien, L. M., E. J. Walsh, R. C. Massey, S. J. Peacock, and T. J. Foster.** 2002. Staphylococcus aureus clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. Cell Microbiol **4:**759-70.

170. **O'Brien-Simpson, N. M., R. A. Paolini, B. Hoffmann, N. Slakeski, S. G. Dashper, and E. C. Reynolds.** 2001. Role of RgpA, RgpB, and Kgp proteinases in virulence of Porphyromonas gingivalis W50 in a murine lesion model. Infect Immun **69:**7527-34.

171. **Ogston, A.** 1984. Classics in infectious diseases. "On abscesses". Alexander Ogston (1844-1929). Rev Infect Dis **6:**122-8.

172. **Oleksy, A., E. Golonka, A. Banbula, G. Szmyd, J. Moon, M. Kubica, D. Greenbaum, M. Bogyo, T. J. Foster, J. Travis, and J. Potempa.** 2004. Growth phase-dependent production of a cell wall-associated elastinolytic cysteine proteinase by Staphylococcus epidermidis. Biol Chem **385:**525-35.

173. **Oliver, R. C., L. J. Brown, and H. Loe.** 1998. Periodontal diseases in the United States population. J Periodontol **69:**269-78.

174. **Otto, M.** 2001. Staphylococcus aureus and Staphylococcus epidermidis peptide pheromones produced by the accessory gene regulator agr system. Peptides **22:**1603-8.

175. **Palmqvist, N., T. Foster, J. R. Fitzgerald, E. Josefsson, and A. Tarkowski.** 2005. Fibronectin-binding proteins and fibrinogen-binding clumping factors play distinct roles in staphylococcal arthritis and systemic inflammation. J Infect Dis **191:**791-8.

176. **Palmqvist, N., T. Foster, A. Tarkowski, and E. Josefsson.** 2002. Protein A is a virulence factor in Staphylococcus aureus arthritis and septic death. Microb Pathog **33:**239-49.

177. **Palmqvist, N., J. M. Patti, A. Tarkowski, and E. Josefsson.** 2004. Expression of staphylococcal clumping factor A impedes macrophage phagocytosis. Microbes Infect **6:**188-95.

178. **Panlilio, A. L., D. H. Culver, R. P. Gaynes, S. Banerjee, T. S. Henderson, J. S. Tolson, and W. J. Martone.** 1992. Methicillin-resistant Staphylococcus aureus in U.S. hospitals, 1975-1991. Infect Control Hosp Epidemiol **13:**582-6.

179. **Patel, A. H., P. Nowlan, E. D. Weavers, and T. Foster.** 1987. Virulence of protein A-deficient and alpha-toxin-deficient mutants of Staphylococcus aureus isolated by allele replacement. Infect Immun **55:**3103-10.

180. **Patti, J. M., B. L. Allen, M. J. McGavin, and M. Hook.** 1994. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol **48:**585-617.

181. **Patti, J. M., J. O. Boles, and M. Hook.** 1993. Identification and biochemical characterization of the ligand binding domain of the collagen adhesin from Staphylococcus aureus. Biochemistry **32:**11428-35.

182. **Pavloff, N., J. Potempa, R. N. Pike, V. Prochazka, M. C. Kiefer, J. Travis, and P. J. Barr.** 1995. Molecular cloning and structural characterization of the

Arg-gingipain proteinase of Porphyromonas gingivalis. Biosynthesis as a proteinase-adhesin polyprotein. J Biol Chem **270:**1007-10.

183. **Peacock, S. J., N. P. Day, M. G. Thomas, A. R. Berendt, and T. J. Foster.** 2000. Clinical isolates of Staphylococcus aureus exhibit diversity in fnb genes and adhesion to human fibronectin. J Infect **41:**23-31.

184. **Peng, H. L., R. P. Novick, B. Kreiswirth, J. Kornblum, and P. Schlievert.** 1988. Cloning, characterization, and sequencing of an accessory gene regulator (agr) in Staphylococcus aureus. J Bacteriol **170:**4365-72.

185. **Pike, R. N., J. Potempa, W. McGraw, T. H. Coetzer, and J. Travis.** 1996. Characterization of the binding activities of proteinase-adhesin complexes from Porphyromonas gingivalis. J Bacteriol **178:**2876-82.

186. **Podbielski, A., B. Pohl, M. Woischnik, C. Korner, K. H. Schmidt, E. Rozdzinski, and B. A. Leonard.** 1996. Molecular characterization of group A streptococcal (GAS) oligopeptide permease (opp) and its effect on cysteine protease production. Mol Microbiol **21:**1087-99.

187. **Potempa, J., A. Banbula, and J. Travis.** 2000. Role of bacterial proteinases in matrix destruction and modulation of host responses. Periodontol 2000 **24:**153-92.

188. **Potempa, J., A. Dubin, G. Korzus, and J. Travis.** 1988. Degradation of elastin by a cysteine proteinase from Staphylococcus aureus. The Journal of Biological Chemistry **263:**2664-7.

189. **Potempa, J., D. Fedak, A. Dubin, A. Mast, and J. Travis.** 1991. Proteolytic inactivation of alpha-1-anti-chymotrypsin. Sites of cleavage and generation of chemotactic activity. J Biol Chem **266:**21482-7.

190. **Potempa, J., R. Pike, and J. Travis.** 1995. The multiple forms of trypsin-like activity present in various strains of Porphyromonas gingivalis are due to the presence of either Arg-gingipain or Lys-gingipain. Infect Immun **63:**1176-82.

191. **Potempa, J., Z. Porwit-Bobr, and J. Travis.** 1989. Stabilization vs. degradation of Staphylococcus aureus metalloproteinase. Biochim Biophys Acta **993:**301-4.

192. **Potempa, J., A. Sroka, T. Imamura, and J. Travis.** 2003. Gingipains, the major cysteine proteinases and virulence factors of Porphyromonas gingivalis: structure, function and assembly of multidomain protein complexes. Curr Protein Pept Sci **4:**397-407.

193. **Potempa, J., W. Watorek, and J. Travis.** 1986. The inactivation of human plasma alpha 1-proteinase inhibitor by proteinases from Staphylococcus aureus. J Biol Chem **261:**14330-4.

194. **Prevost, G., B. Cribier, P. Couppie, P. Petiau, G. Supersac, V. Finck-Barbancon, H. Monteil, and Y. Piemont.** 1995. Panton-Valentine leucocidin and gamma-hemolysin from Staphylococcus aureus ATCC 49775 are encoded by

distinct genetic loci and have different biological activities. Infect Immun **63:**4121-9.

195. **Projan, S. J., J. Kornblum, B. Kreiswirth, S. L. Moghazeh, W. Eisner, and R. P. Novick.** 1989. Nucleotide sequence: the beta-hemolysin gene of Staphylococcus aureus. Nucleic Acids Res **17:**3305.

196. **Prokesova, L., B. Potuznikova, J. Potempa, J. Zikan, J. Radl, L. Hachova, K. Baran, Z. Porwit-Bobr, and C. John.** 1992. Cleavage of human immunoglobulins by serine proteinase from Staphylococcus aureus. Immunol Lett **31:**259-65.

197. **Pungercar, J. R., D. Caglic, M. Sajid, M. Dolinar, O. Vasiljeva, U. Pozgan, D. Turk, M. Bogyo, V. Turk, and B. Turk.** 2009. Autocatalytic processing of procathepsin B is triggered by proenzyme activity. FEBS J **276:**660-8.

198. **Punturieri, A., S. Filippov, E. Allen, I. Caras, R. Murray, V. Reddy, and S. J. Weiss.** 2000. Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages. J Exp Med **192:**789-99.

199. **Raja, R. H., G. Raucci, and M. Hook.** 1990. Peptide analogs to a fibronectin receptor inhibit attachment of Staphylococcus aureus to fibronectin-containing substrates. Infect Immun **58:**2593-8.

200. **Rammelkamp, C., and T. Maxon.** 1942. Resistance of *Staphylococcus aureus* to the action of penicillin. Proc. Soc. Exp. Biol. **51:**386-389.

201. **Rawlings, N. D., and A. J. Barrett.** 1995. Evolutionary families of metallopeptidases. Methods Enzymol **248:**183-228.

202. **Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss, and R. P. Novick.** 1986. Regulation of exoprotein gene expression in Staphylococcus aureus by agar. Mol Gen Genet **202:**58-61.

203. **Regoli, D., and J. Barabe.** 1980. Pharmacology of bradykinin and related kinins. Pharmacol Rev **32:**1-46.

204. **Rice, K., M. Huesca, D. Vaz, and M. J. McGavin.** 2001. Variance in fibronectin binding and fnb locus polymorphisms in Staphylococcus aureus: identification of antigenic variation in a fibronectin binding protein adhesin of the epidemic CMRSA-1 strain of methicillin-resistant S. aureus. Infect Immun **69:**3791-9.

205. **Rice, K., R. Peralta, D. Bast, J. de Azavedo, and M. J. McGavin.** 2001. Description of staphylococcus serine protease (ssp) operon in Staphylococcus aureus and nonpolar inactivation of sspA-encoded serine protease. Infection and Immunity **69:**159-69

206. **Roche, F. M., R. Downer, F. Keane, P. Speziale, P. W. Park, and T. J. Foster.** 2004. The N-terminal A domain of fibronectin-binding proteins A and B promotes adhesion of Staphylococcus aureus to elastin. J Biol Chem **279:**38433-40.

207. **Rogers, K. L., P. D. Fey, and M. E. Rupp.** 2009. Coagulase-negative staphylococcal infections. Infect Dis Clin North Am **23:**73-98.

208. **Roman, R. S., J. Smith, M. Walker, S. Byrne, K. Ramotar, B. Dyck, A. Kabani, and L. E. Nicolle.** 1997. Rapid geographic spread of a methicillin-resistant Staphylococcus aureus strain. Clin Infect Dis **25:**698-705.

209. **Ruggeri, Z. M., L. De Marco, L. Gatti, R. Bader, and R. R. Montgomery.** 1983. Platelets have more than one binding site for von Willebrand factor. J Clin Invest **72:**1-12.

210. **Ryden, A. C., L. Ryden, and L. Philipson.** 1974. Isolation and properties of a staphylococcal protease, preferentially cleaving glutamoyl-peptide bonds. Eur J Biochem **44:**105-14.

211. **Rzychon, M., R. Filipek, A. Sabat, K. Kosowska, A. Dubin, J. Potempa, and M. Bochtler.** 2003. Staphostatins resemble lipocalins, not cystatins in fold. Protein Sci **12:**2252-6.

212. **Rzychon, M., A. Sabat, K. Kosowska, J. Potempa, and A. Dubin.** 2003. Staphostatins: an expanding new group of proteinase inhibitors with a unique specificity for the regulation of staphopains, Staphylococcus spp. cysteine proteinases. Mol Microbiol **49:**1051-66.

213. **Sabat, A., K. Kosowska, K. Poulsen, A. Kasprowicz, A. Sekowska, B. van Den Burg, J. Travis, and J. Potempa.** 2000. Two allelic forms of the aureolysin gene (aur) within Staphylococcus aureus. Infect Immun **68:**973-6.

214. **Sanyal, D., A. P. Johnson, R. C. George, B. D. Cookson, and A. J. Williams.** 1991. Peritonitis due to vancomycin-resistant Staphylococcus epidermidis. Lancet **337:**54.

215. **Schneewind, O., D. Mihaylova-Petkov, and P. Model.** 1993. Cell wall sorting signals in surface proteins of gram-positive bacteria. EMBO J **12:**4803-11.

216. **Schwalbe, R. S., J. T. Stapleton, and P. H. Gilligan.** 1987. Emergence of vancomycin resistance in coagulase-negative staphylococci. N Engl J Med **316:**927-31.

217. **Shaw, L., E. Golonka, J. Potempa, and S. J. Foster.** 2004. The role and regulation of the extracellular proteases of Staphylococcus aureus. Microbiology **150:**217-28

218. **Shaw, L. N., E. Golonka, G. Szmyd, S. J. Foster, J. Travis, and J. Potempa.** 2005. Cytoplasmic control of premature activation of a secreted protease zymogen: deletion of staphostatin B (SspC) in Staphylococcus aureus 8325-4 yields a profound pleiotropic phenotype. J Bacteriol **187:**1751-62.

219. **Shi, G. P., J. S. Munger, J. P. Meara, D. H. Rich, and H. A. Chapman.** 1992. Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease. J Biol Chem **267:**7258-62.

220. **Shinde, U., and M. Inouye.** 1995. Folding mediated by an intramolecular chaperone: autoprocessing pathway of the precursor resolved via a substrate assisted catalysis mechanism. J Mol Biol **247:**390-5.

221. **Shinde, U. P., J. J. Liu, and M. Inouye.** 1997. Protein memory through altered folding mediated by intramolecular chaperones. Nature **389:**520-2.

222. **Shooter, R. A., M. A. Smith, J. D. Griffiths, M. E. Brown, R. E. Williams, J. E. Rippon, and M. P. Jevons.** 1958. Spread of staphylococci in a surgical ward. Br Med J **1:**607-13.

223. **Siboo, I. R., A. L. Cheung, A. S. Bayer, and P. M. Sullam.** 2001. Clumping factor A mediates binding of Staphylococcus aureus to human platelets. Infect Immun **69:**3120-7.

224. **Signas, C., G. Raucci, K. Jonsson, P. E. Lindgren, G. M. Anantharamaiah, M. Hook, and M. Lindberg.** 1989. Nucleotide sequence of the gene for a fibronectin-binding protein from Staphylococcus aureus: use of this peptide sequence in the synthesis of biologically active peptides. Proc Natl Acad Sci U S A **86:**699-703.

225. **Skinner, D., and C. S. Keefer.** 1941. Significance of bacteremia caused by *Staphylococcus aureus*: A study of one hundred and twenty-two cases and a review of the literature concerned with experimental infection in animals. Arch Intern Med **68:**851-875.

226. **Slakeski, N., P. S. Bhogal, N. M. O'Brien-Simpson, and E. C. Reynolds.** 1998. Characterization of a second cell-associated Arg-specific cysteine proteinase of Porphyromonas gingivalis and identification of an adhesin-binding motif involved in association of the prtR and prtK proteinases and adhesins into large complexes. Microbiology **144 (Pt 6):**1583-92.

227. **Smagur, J., K. Guzik, M. Bzowska, M. Kuzak, M. Zarebski, T. Kantyka, M. Walski, B. Gajkowska, and J. Potempa.** 2009. Staphylococcal cysteine protease staphopain B (SspB) induces rapid engulfment of human neutrophils and monocytes by macrophages. Biol Chem **390:**361-71.

228. **Smeltzer, M. S., A. F. Gillaspy, F. L. Pratt, Jr., M. D. Thames, and J. J. Iandolo.** 1997. Prevalence and chromosomal map location of Staphylococcus aureus adhesin genes. Gene **196:**249-59.

229. **Smith, T. L., and W. R. Jarvis.** 1999. Antimicrobial resistance in Staphylococcus aureus. Microbes Infect **1:**795-805.

230. **Socransky, S. S., A. D. Haffajee, M. A. Cugini, C. Smith, and R. L. Kent, Jr.** 1998. Microbial complexes in subgingival plaque. J Clin Periodontol **25:**134-44.

231. **Speziale, P., G. Raucci, L. Visai, L. M. Switalski, R. Timpl, and M. Hook.** 1986. Binding of collagen to Staphylococcus aureus Cowan 1. J Bacteriol **167:**77-81.

232. **Spink, W. W., and W. H. Hall.** 1945. Penicillin therapy at the University of Minnesota Hospitals: 1942-1944. Ann Intern Med **22:**510-525.

233. **Steinberg, J. P., C. C. Clark, and B. O. Hackman.** 1996. Nosocomial and community-acquired Staphylococcus aureus bacteremias from 1980 to 1993: impact of intravascular devices and methicillin resistance. Clin Infect Dis **23:**255-9.

234. **Stevens, D. L.** 1992. Invasive group A streptococcus infections. Clin Infect Dis **14:**2-11.

235. **Storer, A. C., and R. Menard.** 1994. Catalytic mechanism in papain family of cysteine peptidases. Methods Enzymol **244:**486-500.

236. **Sullam, P. M., A. S. Bayer, W. M. Foss, and A. L. Cheung.** 1996. Diminished platelet binding in vitro by Staphylococcus aureus is associated with reduced virulence in a rabbit model of infective endocarditis. Infect Immun **64:**4915-21.

237. **Switalski, L. M., P. Speziale, and M. Hook.** 1989. Isolation and characterization of a putative collagen receptor from Staphylococcus aureus strain Cowan 1. J Biol Chem **264:**21080-6.

238. **Takeuchi, S., K. Matsunaga, S. Inubushi, H. Higuchi, K. Imaizumi, and T. Kaidoh.** 2002. Structural gene and strain specificity of a novel cysteine protease produced by Staphylococcus aureus isolated from a diseased chicken. Vet Microbiol **89:**201-10.

239. **Takeuchi, S., M. Saito, K. Imaizumi, T. Kaidoh, H. Higuchi, and S. Inubushi.** 2002. Genetic and enzymatic analyses of metalloprotease (aureolysin) from Staphylococcus aureus isolated from domestic animals. Vet Microbiol **84:**135-42.

240. **Takii, R., T. Kadowaki, A. Baba, T. Tsukuba, and K. Yamamoto.** 2005. A functional virulence complex composed of gingipains, adhesins, and lipopolysaccharide shows high affinity to host cells and matrix proteins and escapes recognition by host immune systems. Infect Immun **73:**883-93.

241. **Talkington, D. F., B. Schwartz, C. M. Black, J. K. Todd, J. Elliott, R. F. Breiman, and R. R. Facklam.** 1993. Association of phenotypic and genotypic characteristics of invasive Streptococcus pyogenes isolates with clinical components of streptococcal toxic shock syndrome. Infect Immun **61:**3369-74.

242. **Tamura, F., R. Nakagawa, T. Akuta, S. Okamoto, S. Hamada, H. Maeda, S. Kawabata, and T. Akaike.** 2004. Proapoptotic effect of proteolytic activation of matrix metalloproteinases by Streptococcus pyogenes thiol proteinase (Streptococcus pyrogenic exotoxin B). Infect Immun **72:**4836-47.

243. **Thakker, M., J. S. Park, V. Carey, and J. C. Lee.** 1998. Staphylococcus aureus serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model. Infect Immun **66:**5183-9.

244. **Travis, J., J. Potempa, and H. Maeda.** 1995. Are bacterial proteinases pathogenic factors? Trends Microbiol **3:**405-7.

245. **Uhlen, M., B. Guss, B. Nilsson, S. Gatenbeck, L. Philipson, and M. Lindberg.** 1984. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. J Biol Chem **259:**1695-702.

246. **Vaudaux, P., D. Pittet, A. Haeberli, P. G. Lerch, J. J. Morgenthaler, R. A. Proctor, F. A. Waldvogel, and D. P. Lew.** 1993. Fibronectin is more active than fibrin or fibrinogen in promoting Staphylococcus aureus adherence to inserted intravascular catheters. J Infect Dis **167:**633-41.

247. **Vernet, T., H. E. Khouri, P. Laflamme, D. C. Tessier, R. Musil, B. J. Gour-Salin, A. C. Storer, and D. Y. Thomas.** 1991. Processing of the papain precursor. Purification of the zymogen and characterization of its mechanism of processing. J Biol Chem **266:**21451-7.

248. **Vincents, B., P. Onnerfjord, M. Gruca, J. Potempa, and M. Abrahamson.** 2007. Down-regulation of human extracellular cysteine protease inhibitors by the secreted staphylococcal cysteine proteases, staphopain A and B. Biol Chem **388:**437-46.

249. **Wann, E. R., S. Gurusiddappa, and M. Hook.** 2000. The fibronectin-binding MSCRAMM FnbpA of Staphylococcus aureus is a bifunctional protein that also binds to fibrinogen. J Biol Chem **275:**13863-71.

250. **Wegrzynowicz, Z., P. B. Heczko, G. R. Drapeau, J. Jeljaszewicz, and G. Pulverer.** 1980. Prothrombin activation by a metalloprotease from Staphylococcus aureus. J Clin Microbiol **12:**138-9.

251. **Wenzel, R. P., and T. M. Perl.** 1995. The significance of nasal carriage of Staphylococcus aureus and the incidence of postoperative wound infection. J Hosp Infect **31:**13-24.

252. **Wilkinson, B., K. Crossley, and G. Archer (ed.).** 1997. The Staphylococci in Human Disease. Churchhill Livingstone, New York.

253. **Wingrove, J. A., R. G. DiScipio, Z. Chen, J. Potempa, J. Travis, and T. E. Hugli.** 1992. Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from Porphyromonas (Bacteroides) gingivalis. J Biol Chem **267:**18902-7.

254. **Wladyka, B., K. Puzia, and A. Dubin.** 2005. Efficient co-expression of a recombinant staphopain A and its inhibitor staphostatin A in Escherichia coli. Biochem J **385:**181-7.

255. **Xiong, Y. Q., V. G. Fowler, M. R. Yeaman, F. Perdreau-Remington, B. N. Kreiswirth, and A. S. Bayer.** 2009. Phenotypic and genotypic characteristics of persistent methicillin-resistant Staphylococcus aureus bacteremia in vitro and in an experimental endocarditis model. J Infect Dis **199:**201-8.

256. **Yabuta, Y., H. Takagi, M. Inouye, and U. Shinde.** 2001. Folding pathway mediated by an intramolecular chaperone: propeptide release modulates activation precision of pro-subtilisin. J Biol Chem **276:**44427-34.

257. **Yamasaki, O., J. Kaneko, S. Morizane, H. Akiyama, J. Arata, S. Narita, J. Chiba, Y. Kamio, and K. Iwatsuki.** 2005. The association between Staphylococcus aureus strains carrying panton-valentine leukocidin genes and the development of deep-seated follicular infection. Clin Infect Dis **40:**381-5.

258. **Yanagishita, M., and V. C. Hascall.** 1992. Cell surface heparan sulfate proteoglycans. J Biol Chem **267:**9451-4.

259. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-19.

260. **Yasuda, Y., Z. Li, D. Greenbaum, M. Bogyo, E. Weber, and D. Bromme.** 2004. Cathepsin V, a novel and potent elastolytic activity expressed in activated macrophages. J Biol Chem **279:**36761-70.

261. **Yoshikawa, K., H. Tsuzuki, T. Fujiwara, E. Nakamura, H. Iwamoto, K. Matsumoto, M. Shin, N. Yoshida, and H. Teraoka.** 1992. Purification, characterization and gene cloning of a novel glutamic acid-specific endopeptidase from Staphylococcus aureus ATCC 12600. Biochim Biophys Acta **1121:**221-8.

262. **Yu, C. E., and J. J. Ferretti.** 1991. Frequency of the erythrogenic toxin B and C genes (speB and speC) among clinical isolates of group A streptococci. Infect Immun **59:**211-5.

263. **Zhang, L., L. Gray, R. P. Novick, and G. Ji.** 2002. Transmembrane topology of AgrB, the protein involved in the post-translational modification of AgrD in Staphylococcus aureus. J Biol Chem **277:**34736-42.

264. **Zhu, X. L., Y. Ohta, F. Jordan, and M. Inouye.** 1989. Pro-sequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. Nature **339:**483-4.