Characterization of Protein A and Sortase A in *Staphylococcus pseudintermedius*: Potential Targets for Novel Therapeutic Approaches

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DEDICATION

To all my loved ones

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This humble work of dissertation has been made possible by the countless blessings of the Almighty.

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ABSTRACT

Staphylococcus pseudintermedius is an opportunistic pathogen in dogs and is the most frequent cause of canine pyoderma. The recent emergence of methicillin resistance and multidrug resistance has made it increasingly difficult to treat infections using conventional antibiotics. There is an urgent need for alternative strategies to prevent *S. pseudintermedius* infections. The development of a successful vaccine against *S. pseudintermedius* is complicated by several virulence and immune evasion factors. The objective of this project was to identify new targets for treatment and prophylaxis of methicillin-resistant and multidrug-resistant *S. pseudintermedius* infection. This was achieved using two strategies (a) understanding the roles of Protein A and Sortase A during pathogenesis, along with exploring Sortase A inhibition as an alternate treatment strategy (b) analyzing the surface-associated and secreted proteins in *S. pseudintermedius* to identify novel vaccine targets.

In this study, a 3-D homology model for Sortase A was generated using molecular dynamic simulation. Using this model, drug databases were screened *in silico* and potential inhibitors of Sortase A were identified and tested in cell-free and functional whole-cell assays. Four promising compounds were identified using this strategy. Sortase A anchors proteins to the bacterial surface. These include potent virulence and immune evasion factors such as Protein A. Both cell wall-associated and secreted Protein A in *S. pseudintermedius* was characterized and its role in immune evasion was examined. Since Sortase A inhibition alone may not be an effective treatment option, the entire secretome and the surface-associated proteins in *S. pseudintermedius* were analyzed by mass spectrometry. Eight orthologous proteins across several *S. pseudintermedius* isolates were identified that could serve as potential vaccine targets. Ultimately, Sortase A inhibition along with a multicomponent vaccine containing a non-toxigenic version

of Protein A and other potential B-cell/T-cell epitopes may be effective against *S. pseudintermedius* infection.

This study is an important step in (i) understanding the nature of immunoglobulin binding onto Protein A and (ii) the inhibition of Sortase A as an alternate treatment strategy in *S.pseudintermedius,* and may ultimately pave the way towards the development of a successful vaccine against *S. pseudintermedius* infection.

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LIST OF ABBREVIATIONS

ATCC - American Type Culture Collection

Clf - clumping factor

CWA - cell wall-associated proteins

DMSO - dimethyl sulfoxide

DTT - dithiothreitol

ECM - extracellular matrix proteins

EDTA - ethylenediaminetetraaceticacid

ELISA - enzyme-linked immunosorbent assay

EMEM - Eagle's minimum essential medium

Fab - fragment antigen binding

Fc - fragment crystallizable

FITC - fluorescein isothiocyanate

FnBP - fibronectin binding protein

FRET - Fluorescent Resonance Energy Transfer

HBSS - Hank's balanced salt solution

HGT - horizontal gene transfer

HRP - horse-radish peroxidase

IACUC – Institutional Animal Care and Use Committee

IgBD - immunoglobulin binding domains

IgG - immunoglobulin G

LPXTG - motif in cell wall-associated proteins with the amino acid composition leucine-proline-any amino acid-threonine-glycine

MALDI-TOF MS - matrix assisted LASER desorption ionization-time of flight mass spectrometry

MD - molecular dynamic simulation

MLST - multilocus sequence typing

MOE - molecular operating environment

MRSA - methicillin-resistant *Staphylococcus aureus*

MRSP - methicillin-resistant *Staphylococcus pseudintermedius*

MSCRAMM - microbial surface components recognizing adhesive matrix molecules

MTSET - (2-sulfonatoethyl) methane-thiosulfonate

MWCO - molecular weight cut-off

NCI - National Cancer Institute

OrthoMCL - orthologous Markov Clustering Algorithm

PBP-2A - penicillin-binding protein 2A

PBS - phosphate buffered saline

PCR - polymerase chain reaction

POPI - prediction of peptide immunogenicity

RBC - red blood cells

SAg - superantigen

SCC - Staphylococcal cassette chromosome

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SIG - *Staphylococcus intermedius* group

Spa - Staphylococcal Protein A

Sps - *Staphylococcus pseudintermedius* surface proteins

Srt A - Sortase A

ST - sequence type

TMB - tetramethylbenzidine

TSB - trypticase soy broth

INTRODUCTION

Introduction and Review of Literature

The recent emergence of methicillin resistance and multidrug resistance in *Staphylococcus aureus* and *Staphylococcus pseudintermedius* has shifted the focus of research from conventional antibiotic-based therapies to the development of novel therapeutics or prophylactic means of dealing with these bacteria. This, in part, has been a challenge due to the heterogeneity of the species. There are two factors that play a crucial role in the development of a successful vaccine; (a) broad understanding of the nature of host immune response to staphylococcal infections and (b) the role of bacterial surface proteins and secreted virulence factors in pathogenesis.

Introduction to *Staphylococcus pseudintermedius*

S. pseudintermedius is a Gram-positive coccus. It is a commensal and an opportunistic pathogen in dogs, implicated as the most frequent cause of canine pyoderma. It causes other infections, including those of the urinary tract, wounds, and otitis externa. It has zoonotic potential and can cause sporadic infections in humans as well [1-5]. *S. pseudintermedius* was classified as a new species in 2005. Until then, it was classified as *S. intermedius*. Since its reclassification, *S. pseudintermedius* has been the most common staphylococcal species associated with infections in dogs. As with any 'new' species, there is a lot to discover in terms of genotype and phenotype and to understand any similarities or differences at the molecular level. Much of what is known today about this bacterium comes from comparative studies with *S. aureus*. Similar to *S.aureus*, the incidence of methicillin resistance and multidrug resistance in this species has been increasing in the past few years. This has made treatment of infection difficult especially in the veterinary setting due to the limited number of drug choices that

are available currently [6]. Therefore, there is an urgent need for the development of alternate treatment strategies.

History and Characterization

Staphylococcus intermedius was first described in 1976 and was thought to be the causative agent of skin infections in dogs. Advancements in molecular techniques led to the identification of a novel species, *S. pseudintermedius*, in 2005 as the causative agent of pyoderma and other skin infections in dogs [7]. Isolates that were previously classified as *S. intermedius* based on phenotypic methods were reclassified based on molecular typing methods. Thus, the *Staphylococcus intermedius* group (SIG) was created consisting of three species, namely, *S. intermedius* (type strain of pigeon origin), *S. pseudintermedius* (common causative agent of skin infections in dogs) and *S. delphini* (first isolated from purulent skin lesions of dolphins in 1988) [8].

Identification and Typing

Traditionally, isolates from canine skin infections had been identified by colony morphology and standard phenotypic tests, but in recent times, this has been complicated due to changes in the taxonomy of the species. Phenotypic methods are not reliable for distinguishing between members of the SIG. *S. intermedius* can be distinguished from *S. pseudintermedius* by a combination of biochemical tests that include arginine dihydrolase test, β-gentiobiose test and D-mannitol test. Currently, there are no known biochemical tests that can reliably distinguish between *S. pseudintermedius* and *S. delphini* [7].Clinical identification of *S. pseudintermedius* in a diagnostic setting relies on the fact that *S. intermedius* and *S. delphini* are not associated with infections in dogs. Molecular methods are the only way by which it is possible to correctly identify the species within the SIG.

Isolates previously identified as *S. intermedius* in dogs have now been classified as *S. pseudintermedius* based on molecular typing methods [7, 8].

Molecular (Genomic) Methods for Species Identification

The first molecular method that was discriminative for *S. intermedius* and *S. pseudintermedius* was phylogenetic analysis based on partial sequences of *sodA* gene and *hsp60* gene. Since then, various DNA-based methods have been developed primarily for typing and epidemiological surveillance. These include ribotyping, PFGE, *spa* typing, SCC-*mec* typing, and various PCR-based methods [7, 8]. *S. pseudintermedius* can be distinguished from *S. intermedius* and *S.delphini* by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). This method relies on the presence of a single MboI restriction site in the pta gene of *S. pseudintermedius*, which is absent in *S. intermedius* and *S.delphini*. The only disadvantage of this method is the heterogeneity associated with the MboI site in a small proportion of the S.pseudintermedius strains which either results in the absence of the restriction site or an additional band. A similar approach but with a different gene, the kat gene harboring the restriction site for TaqI restriction endonuclease, has also been evaluated for species identification of coagulase-positive staphylococci [8-10]. Another method for routine species identification of coagulase-positive staphylococci in veterinary medicine is based on a multiplex PCR developed by Sasaki et al. targeting the thermonuclease (nuc) gene [11].

Pulsed-field gel electrophoresis (PFGE) is one other method for bacterial typing in general. It is based on analysis of the DNA fingerprint of the bacteria generated upon treatment with selected restriction endonucleases. It has high discriminatory power compared to other typing methods. PFGE for

S. pseudintermedius is done using a protocol that was developed for *S. aureus* but with slight modifications. Although this method is useful in studies investigating the clonal relationships between MRSP isolates, it is time consuming and difficult to standardize for inter-laboratory comparison. There are several modifications of the protocol using the *Sma*I restriction endonuclease, making it difficult to share and communicate PFGE data [7, 8, 12-14].

Staphylococcal Protein A (*spa*) typing developed by Moodley *et al*. is based on the sequence variation of the polymorphic X-region of the *spa* gene. It is currently employed for easy and rapid typing of *S. pseudintermedius*. It has high discriminatory power and allows data sharing and harmonization between labs. There are about 53 *spa* types, although an online database for this is currently not available [5, 8, 15].

Multilocus Sequence Typing (MLST) is a valuable method for genotyping bacteria based on variation in the sequence of slowly evolving housekeeping genes. Data from MLST is portable, accurate and useful for global epidemiological studies involving population genetic of bacteria. The earlier version of MLST was based on four loci [16]. The current MLST scheme, which uses seven genes, developed by Solyman *et al*. [6] has greater genetic diversity and discriminatory power compared to other typing methods. The housekeeping genes used for this typing scheme are *tuf*, *cpn60*, *pta*, *purA*, *fdh*, *sar* and *ack*. Based on the MLST scheme, the predominant clonal population in the United States and Europe are designated ST68 and ST71, respectively [12].

Staphylococcal chromosome cassette *mec*A (*SCC mec*) typing is based on the sequence variation in the mobile genetic element (MGE), SCCmec. The SCCmec region comprises the *mecA* gene, the cassette chromosome recombinase (*ccr*) gene and three joining regions (J1-J3). The *mecA* gene is responsible for

conferring methicillin resistance. Therefore this typing method can be applied to only methicillin resistant isolates. Based on the allotype of the *ccr*, the class of *mecA* gene complex and sequence variation in the joining regions, there are at least ten SCCmec types that are known, designated I-X [17] .

Automated systems designed for high-throughput screening and identification of human pathogens such as microarrays may be used for *S. pseudintermedius* as well, but because they are not designed for veterinary pathogens, their accuracy in identification of veterinary pathogens is debatable. Most recently, a matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been developed for species differentiation within the SIG [18].

Pathogenesis of *S. pseudintermedius*

S. pseudintermedius is an opportunistic pathogen. It does not cause any disease unless the immune system of the host is compromised or there is a breach in the external skin barrier, such as wounds and surgical procedures or other factors predisposing the host to atopic dermatitis. The pathogenesis of *S. pseudintermedius* remains largely unknown. Some genetic lineages and sequence types are more virulent than the others and the reason behind this is not fully understood [7] . Host factors likely play a crucial role in the pathogenesis of *S. pseudintermedius* infection [8]. *S. pseudintermedius* adheres better to corneocytes of atopic dogs whether sampled from an inflamed area or not, than corneocytes of healthy dogs. There could be several reasons for this including a change in the immune status of the dog or altered expression of surface adhesins in atopic dogs compared to healthy dogs. More research in this area is needed to fully understand the complex nature of host-pathogen interaction [19-21].

Virulence Factors and Immune Evasion Factors Produced by *S. pseudintermedius*

S. pseudintermedius produces a number of virulence factors, including some functionally related to those produced by *S.aureus* [22]. Recent advancements in genome sequencing technology and bioinformatics have added a wealth of information to the current knowledge with respect to proteins and virulence factors produced by *S. pseudintermedius*.

S. pseudintermedius produces several enzymes that are involved in virulence such as coagulase, proteases, hyaluronidase, phospholipase, staphylokinase and thermonuclease. It produces toxins such as leukocidins, exotoxins, leukotoxins, exfoliative toxins, enterotoxins and hemolysins [8]. *S. pseudintermedius* also has the ability to bind to fibrinogen, fibronectin and cytokeratin using surface molecules that serve as microbial surface components recognizing adhesive matrix molecules (MSCRAMM) [8, 22]. *S. pseudintermedius* produces the immunoglobulin binding protein, Protein A, which is similar to that found in *S. aureus*. Predicted Protein A genes from *S. pseudintermedius* have been identified but their role in virulence and immune evasion are yet to be determined [5].

Cytotoxins

S. pseudintermedius produces α-hemolysin and β-hemolysin and causes hemolysis of rabbit erythrocytes and hot-cold hemolysis of sheep erythrocytes [23-25]. The β-hemolysin from *S. pseudintermedius* has high-affinity sphingomyelinase activity and is responsible for the characteristic partial zone of hemolysis observed around colonies on sheep blood agar [8, 22]. It also produces a bicomponent leukotoxins, Luk-I, encoded by two co-transcribed genes, *lukS* and

lukF, which has leucktoxic activity on polymorphonuclear cells. Luk-I is associated with the most predominant clonal population in Europe, ST71, particularly methicillin-resistant isolates [7, 8, 22].

Exfoliative toxin

S. pseudintermedius produces an exfoliative toxin called SIET. This toxin causes a rounding effect on cultured epithelial cells. In animal models, SIET was shown to cause an exfoliative effect in 1-day-old chickens, hamsters and dogs but not in rats and mice. Dogs that were injected with purified SIET developed clinical signs such as erythema, exfoliation and crusting similar to symptoms seen in canine pyoderma, human staphylococcal scalded skin syndrome and porcine exudative epidermitis (greasy pig disease). A second exfoliative toxin EXI (renamed ExpA) encoded by the *exi* gene has been identified in *S. pseudintermedius*. The predicted protein shows about 43-68% sequence identify with known staphylococcal exfoliative toxins. A recombinant version of this protein caused exfoliative skin lesions in neonatal mice. Apart from SIET and EXI, a third exfoliative toxin ExpB has been identified in *S. pseudintermedius*, which causes intraepidermal splitting and degradation of desmoglein-a (Dsg-1) [8, 22].

Enterotoxins and Superantigens

S. pseudintermedius produces proteins that cross-react with antibodies raised against staphylococcal enterotoxins (SEs) and superantigens (SAs); SEA, SEB, SEC, SED and toxic shock syndrome toxin-1 (TSST-1) [8, 22, 26]. A canine type C enterotoxin (SEC_{canine}) has been identified from canine pyoderma isolates. This is distinct from other staphylococcal enterotoxins but shares their ability to induce vomiting and T-cell proliferation [27]. A novel enterotoxin-related gene, *se-int* was detected by PCR but its function remains to be examined [24, 28].

Cell-Wall Associated Proteins

Cell wall-associated (CWA) proteins, such as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), play a crucial role in bacterial attachment to the host extracellular matrix. The repertoire of MSCRAMMs in each bacterial species contributes to its host adaptation and survival. CWA proteins are characterized by an N-terminal signal sequence, LPXTG motif, tandem repeats and immunoglobulin-like folds. Several CWA proteins have been identified in *S. pseudintermedius*, designated as 'Sps' (*Staphylococcus pseudintermedius* surface proteins). In particular, the isolate ED99 has at least 19 putative CWA proteins designated SpsA through SpsR [15, 29]. Many strains adhere to fibrinogen, fibronectin, cytokeratin 10, elastin, collagen type 1, vitronectin and laminin [29, 30]. Much remains to be understood about the interaction of *S. pseudintermedius* with the host extracellular matrix.

Protein A

Protein A in *S. aureus*

Protein A is one of the most potent immune evasion factors in *S. aureus* [31]. It is a cell wall-associated protein that binds to IgG from various mammals including goat, rabbit, mouse and humans specifically via its Fcγ region. This binding in the 'wrong' orientation prevents it from binding to Fc receptors on immune cells such as neutrophils, thereby preventing opsonization and phagocytosis [32-35] . Binding of Protein A to IgG also prevents it from activating the complement via C1q. Both the classical and alternate pathways of the complement can be inhibited by virtue of this binding [36-39]. Thus, *S. aureus* evades the immune system and causes persistent infections.

Protein A is also secreted into the extracellular milieu during staphylococcal growth and active infection [40-43]. The secreted form of Protein A functions as a B-cell super antigen (SAg). It binds to V_H3 B-cell receptors and limits their ability to produce antibodies. Also, marginal zone B cells and B-1 cells undergo, preferentially, induced cell death with supraclonal depletion and immune tolerance upon exposure to Protein A. There is a clonal expansion at first followed by rapid apoptosis of B-cells. Thus, Protein A may limit the efficacy of a vaccine that depends on opsonophagocytic antibody production. It can also interfere with vaccine efficacy by binding to B-cells and interfering with their function [44-48].

Structure and Function

Protein A is encoded by the *spa* (staphylococcal protein A) gene. The *spa* gene in *S. aureus* is variable in length with approximately 1500bp. It encodes a protein of about 480-500 amino acids with a molecular weight of ~40-60kDa [49, 50]. It is synthesized as a precursor with an N-terminal signal sequence and a C-terminal sorting peptide. The N-terminal part of SpA consists of four to five repeats of 56-61 amino acids that serve as immunoglobulin binding domains (IgBD), designated E,D,A,B and C, followed by a variable region, X [51]. The C-terminal region X is comprised of Xr, a highly repetitive yet variable octapeptide, and Xc, a domain with unique sequence in close proximity to the cell wall anchor structure of SpA [52]. The C-terminal also harbors an LPXTG motif which is recognized by the transpeptidase enzyme Sortase A, responsible for anchoring the protein to the pentaglycine residues of the peptidoglycan cell wall. Each IgBD folds into a triple-helical bundle, connected by short linkers with specific binding sites for Fc_γ and V_H3 Fab domains of human and animal immunoglobulin. Glutamine (Q) residues in position 9 and 10 in the α-helix 1 of each IgBD are critical for Fc_γ binding, thereby preventing antibody-mediated opsonization and phagocytosis. Aspartic acid (D) residues in position 36 and 37 in the linker region between α-helix 2 and 3 of each

IgBD are essential for Fab binding of V_H3-type IgG and IgM. This association triggers the B-cell superantigen activity of Protein A. *spa* mutants have been shown to be more susceptible to opsonophagocytic killing and are unable to block adaptive immune responses in mouse models of *S.aureus* infection. Kim *et al*. have demonstrated that a nontoxigenic Protein A variant, SpA_{KKAA}, with 20 amino acid substitutions replacing Q^9 and Q^{10} with lysine (K) as well as D^{36} and D^{37} with alanine (A) in each of the immunoglobulin binding domains, cannot bind Fc_γ or V_H3 Fab domains [53]. Immunization of mice with SpA_{KKAA} elicited antibodies that neutralized immunoglobulin-binding activities of Protein A, conferred some protection in a mouse model of *S. aureus* infection, and augmented immunogenicity of unrelated antigens [53, 54]. SpA_{KKAA} monoclonal antibodies promoted opsonophagocytic killing of *S.aureus* in mouse and human blood, provided protection from abscess formation, and stimulated pathogen-specific immune responses in a mouse model of staphylococcal disease [54, 55]. A monoclonal antibody that specifically recognizes the E domain of SpA has also been developed which neutralizes only the E domain of SpA but does not provide disease protection in mice [44, 55].

Protein A in *S. pseudintermedius*

A putative staphylococcal protein A (*spa*) gene was initially described by Moodley *et al*. in 2008 in the *S. pseudintermedius* isolate ED99 [5]. This gene was used to develop a species-specific *spa* typing protocol. The *spa* gene in ED99 is 1389bp in length and has 68% nucleotide and 55% predicted amino acid identity to the homologous gene in the *S. aureus* reference strain 8325-4 where Protein A was originally described. The predicted protein contained functional regions and conserved domains similar to those described in *S.aureus*, including four IgG-binding domains and a polymorphic X-region [5, 15]. The sequence variation in the variable region X in *S. pseudintermedius* had duplications or deletions of whole

repeats or point mutations within the repeats. Lack of IgG-binding domain region B (58 amino acids) partly accounted for the shorter protein in *S. pseudintermedius* ED99 [5]. Bannoehr *et al*. in 2011 suggested that there were two predicted orthologues of *S. aureus* Protein A in ED99, SpsP (designated as Spa2) and SpsQ (which is a full-length Protein A designated as Spa1). The genes are located along the oriC part of the genome and are adjacent to each other [15].

spsP

The *spsP* gene encodes a protein that consists of 377 amino acids in ED99. This protein is 73% identical to SpsQ at the amino acid level. It has a predicted N-terminal sequence of 33 amino acids, followed by a repeat region consisting of three predicted IgG-binding domains. Each IgG-binding domain consists of 55 amino acids with 78%-85% identity between domains. The C-terminal region has a predicted X-region which shares 63% overall sequence similarity to the X-region of *S.aureus* [15].

spsQ

The *spsQ* gene encodes a protein that is 462 amino acids in ED99. It has a predicted N-terminal sequence of 33 amino acids, followed by a repeat region consisting of four IgG-binding domains. Domains 1 and 2 have 59 amino acids; domain 3 has 55 amino acids while domain 4 has 52 amino acids. The domains have between 59%-86% protein sequence identity. The repeated IgG-binding domains are conserved between SpsP and SpsQ, with 67% to 90% sequence identity in pairwise alignments. The C-terminal region consist of a predicted X-region which consists of a 77 amino acid-long repeat sequence (Xr) and a constant region (Xc) with 70% similarity to the X-region of *S. aureus*. The Xr repeat region of SpsQ was used by Moodley *et al*. to develop the spa typing method for *S. pseudintermedius* [5, 15]. Whole genome sequencing of several *S. pseudintermedius* isolates in this lab has shown that most isolates have a full-

length *spsQ* gene while they may or may not harbor the full length *spsP* gene. Some have a truncated *spsP* gene or the gene may be split into two separate fragments.

Sortase A

Role of Sortase A in Protein Anchoring

Sortase A is a cysteine transpeptidase enzyme that is produced by most Gram-positive bacteria. Surface proteins in Gram-positive bacteria play a crucial role during pathogenesis. The main function of Sortase A is to anchor surface proteins with an N-terminal LPXTG motif on to the peptidoglycan cell wall. Sortase A substrates function as adhesins, internalins, blood clotting and immune evasion factors, and transporters for nutrients across the microbial cell wall envelope [56]. Sortase A-deficient mutants of *S. aureus* fail to assemble LPXTG proteins on their surface and are unable to form abscess lesions in organs or cause lethal bacteremia in animal models of infection [57].

Sortase A is the prototypical enzyme for members of the class A family (house-keeping sortase) and has been widely studied. Sortase A is present in Firmicutes, and is responsible for anchoring of proteins that mediate bacterial adhesion. Other sortase genes and their corresponding enzymes have been identified. Class B (sortase B) enzymes are responsible for attaching hemoglobin receptors to the peptidoglycan cell wall and in the assembly of pili. These enzymes are also present in Firmicutes. Class C sortases function as pilin polymerases and anchor pili on bacterial surfaces. Class D sortases are found in bacilli and streptomyces and are expressed during sporulation. Other less studied sortases include Class E and F enzymes in actinobacteria whose functions are unknown [56, 58-60].

Sortase A in *S. aureus*

Structure and Function

Sortase A in *S. aureus* has been well studied and characterized. The *srtA* gene in *S. aureus* is 618bp in length. It encodes a 206-amino acid protein with an N-terminal hydrophobic segment that functions as a signal peptide for secretion and as a stop transfer signal for membrane anchoring. The C-terminal cell wall sorting signal typically consists of a 35-reside peptide with an LPXTG motif, followed by a hydrophobic domain and a positively charged tail [61]. The enzyme adopts a type II membrane topology, with the N-terminus inside the cytoplasm and the C-terminal enzymatic portion located across the plasma membrane [62]. NMR spectroscopy and X-ray crystallography of Sortase A revealed that the enzyme assumes a unique fold, consisting of an eight-stranded β-barrel that includes one or two helices and several loops. Strands β7 and β8 form the floor of the hydrophobic depression where the active site is located. Within the active site are the highly conserved Cys^{184} and His^{120}. The Cys^{184} is anchored in β7, while the His^{120} is located within a helical region that connects β3 and β3, with its imidazole group in the vicinity of the sulfhydryl side chain of Cys^{184}. Arg^{197} is another amino acid anchored in β8 that is located in close proximity and parallel to the active-site cysteine [63]. Amino acid replacement of either Cys^{184} or His^{120} completely abolished the activity of Sortase A both *in vivo* and *in vitro* and replacement of Arg^{197} greatly reduced its enzymatic activity. Thr^{180}, Ile^{182}, and Ala^{118} are involved in recognition and stabilization of bound substrate. Amino acid substitutions in each of these residues can have profound effect on enzyme activity. It has been observed that the presence of Ca^{2+} ions greatly enhances the activity of the enzyme [56, 63-68].

Surface proteins are synthesized in the bacterial cytoplasm as precursors harboring an N-terminal signal peptide and a C-terminal sorting signal comprising the LPXTG motif (P1 precursor) [64]. The surface proteins are then initiated into the Sec secretion pathway. Signal peptidase cleaves the N-terminal signal peptide to generate the P2 precursor. The hydrophobic domain of the sorting signal is thought to retain the polypeptide in the membrane, thereby allowing the LPXTG motif to be recognized by Sortase A. The sulfhydryl group of the active site Cys^{184} (possibly activated for deprotonation by Agr^{197}) performs a nucleophilic attack at the peptide bond between the threonine and glycine residue of the conserved LPXTG. This generates an acyl-enzyme intermediate that undergoes a nucleophilic attack by the amino group of the pentaglycine (Gly_5) cross-bridge within the peptidoglycan precursor lipid II and forms the P3 intermediate. P3 intermediates are subsequently covalently incorporated into the peptidoglycan cell wall via the transpeptidation and transglycosylation reactions of the cell wall biosynthesis, ultimately giving rise to the mature product (M). Thus, the surface proteins are covalently linked at the C-terminal end to the cell wall envelope [56, 57, 63, 69].

Sortase A in *S. pseudintermedius*

Our lab is currently studying Sortase A and trying to find inhibitors that can effectively block Sortase A activity. A putative *srtA* gene was identified from whole genome sequences of various isolates representing the major clonal populations in the United States and Europe. Based on the predicted protein sequence, functional domains were identified. Sortase A in *S. pseudintermedius* is 57% identical to *S. aureus* Sortase A at the protein level. They share the same sortase family domains. Multiple sequence alignment of *srtA* gene from several *S. pseudintermedius* isolates revealed that the gene is 597bp in length, 21bp shorter than that found in *S. aureus*. It encodes a protein with 198 amino acids with a

theoretical molecular weight of 22.3kDa. As with *S. aureus* Sortase A, it contains highly conserved amino acids. Based on homology modeling and active site prediction done as part of this project, Cys^{177}, His^{114}, and Arg^{190} are predicted to be present in the active site and critical for enzyme activity. The crystal structure and NMR spectra of *S. pseudintermedius* Sortase A are yet to be resolved. However, based on the conservation of amino acids and predicted structural similarity, it is hypothesized that Sortase A in *S. pseudintermedius* performs a similar function as Sortase A in *S. aureus*. The predicted amino acid sequence of Sortase A in *S. pseudintermedius* with the amino acids involved in catalytic activity (highlighted) is as follows:

MQKLTRGLVPLIGIALILAGVYLIFKPKIDAYLTNKENEQKIEVYEESQQNAPSKVPEI
PKDPSKVVGVLEVPSVGIKEAVYPGPATPEQLERGVSLAEKDESLKDQNIAIAGHTNYS
LNYQFTELHKAKKGAEVIFKLGKETRKYQITSIKDVDPYQVEVLEEMKKDKDQLTLITC
DDYDEKTGQWLTRKIYVAERV

Inhibition of Sortase A and its Biological Effects

The recent emergence of methicillin resistance and multidrug resistance is a major concern because conventional antibiotic treatments do not work against such 'superbugs' [56, 70]. Sortase A plays a crucial role in the pathogenesis of Gram-positive bacteria by modulating the ability of the bacterium to adhere to host tissues via the covalent anchoring of adhesins and other virulence factors onto the peptidoglycan cell wall. *S.aureus* mutants lacking Sortase A fail to display surface proteins and are defective in the establishment of infections in mouse models. Therefore, inhibition of Sortase A is a promising strategy for treatment and prevention of Gram-positive bacterial infections [56, 57, 71].

The idea of Sortase A inhibition was explored as early as in 1999 when Ton-That and Schneewind demonstrated that methanethiosulphonstes and

organomercurials displayed inhibitory effect on Sortase A. It was also observed that vancomycin and moenomycin, two antibiotics that are inhibitors of cell wall polymerization into peptidoglycan strands, inhibit Sortase A [64, 72]. Several natural or chemical compounds ranging from plant extracts, alkaloids, and flavonoids have been tested for their abilities to inhibit Sortase A *in vitro* with limited success. Most of these compounds have been evaluated for the ability to inhibit Sortase A *in vitro* by determining the IC_{50}. There are several concerns with *in vitro* inhibition based solely on IC_{50} values; (a) the mechanism of inhibition needs to be completely understood i.e., whether the reaction is reversible or irreversible (b) the enzyme kinetics assay parameters have to be evaluated (c) target site validation studies have to be performed to determine if the inhibitor binds to the active site of the enzyme or elsewhere in the polypeptide (d) the effect of the substrate on the inhibition reaction i.e., whether or not there is competitive inhibition (e) the nature of enzyme inhibition i.e., the effect of redox action, protein aggregation, pH changes on inhibition and (f) the cytotoxic effects (if any) of the compounds. All these factors need to be evaluated thoroughly before the inhibitor can be cleared for use in humans or animals.

Recent advancements in proteomics and bioinformatics have made it possible to identify novel inhibitors of Sortase A. Molecular Dynamic (MD) simulations (modeling studies) can predict the binding/active site of the enzyme and also facilitate rational design of inhibitors based on their affinities [73, 74]. There are a large number of small molecules, drug-like libraries, and robotic automations that allow for high-throughput screening [75]. Using synthetic chemistry and medicinal chemistry, it is also possible to introduce side chain modifications or remove reactive functional groups from the inhibitors such that the affinity of the inhibitor to the enzyme to facilitate greater inhibition [76]. Several thousands of compounds can be screened virtually and those compounds that display inhibitory potential can then be tested in *in vitro* and *in vivo* models.

Emergence of Methicillin Resistance and Multidrug Resistance

The emergence of methicillin resistance and multidrug resistance in staphylococci has been an important problem both in human and veterinary medicine [77, 78]. Virulence genes and antibiotic resistance genes can be transferred between isolates of the same species or between different species. This has the potential to make a 'drug-susceptible, avirulent' strain resistant and highly virulent. There are several ways by which this horizontal gene transfer (HGT) is mediated among staphylococcal species. (a) Conjugation, mediated by plasmids, is one of the most common and frequent methods of HGT. Plasmids carry several antibiotic resistance genes and virulence genes that can be transferred to susceptible strains upon successful conjugation. (b) Transduction, mediated by bacteriophages, is another method by which susceptible strains acquire resistance and virulence genes. (c) Transformation, which occurs via uptake of naked (exogenous) DNA from the environment [79].

Resistance to methicillin is conferred by the *mecA* gene. This gene encodes penicillin-binding protein 2A (PBP-2A) with reduced affinity for methicillin. The *mecA* gene is contained within a mobile genetic element known as staphylococcal cassette chromosome (SCCmec) [80]. Since its first report in 1999, the incidence of methicillin resistance in staphylococcal isolates of canine origin has been significantly increasing. Methicillin resistance in *S.pseudintermedius* is of major concern because many of the methicillin resistant isolates are also multidrug resistant [1, 78]. Treatment options are complicated due to the limited number of drug choices to treat infections and the often extremely long and repeated treatments needed to clear infections. The problem associated with prolonged use of antibiotics, especially in terms of the prudent use of antimicrobials, is the emergence of resistance [81].

Towards Vaccine Development – Efforts and Reasons for Failure

As previously described, the increasing prevalence of antibiotic resistance among staphylococci is a threat in both human and veterinary medicine [80, 82]. Non-antibiotic based alternate treatment strategies to efficiently combat staphylococcal infections are urgently needed. Immunological strategies based on vaccine development or therapeutic antibodies may have significant roles in the control of staphylococcal infections. Vaccines are considered one of the most promising approaches especially with the breakthrough of next generation sequencing applications which provide novel methods for selecting antigens [22]. Most vaccine targets are surface components of staphylococci or soluble virulence determinants such as alpha-toxin, Panton-Valentine leukocidins, or superantigenic enterotoxins [83]. Much effort has gone into vaccine development for *S. aureus*, yet none of these have been translated into a commercially available protective vaccine. There have been several reasons attributed to the failure of an *S. aureus* vaccine. First of all, successful preclinical results obtained with antigens tested in clinical trials were likely overestimated by vaccine manufacturers because antigens tested in clinical trials provided only partial protection in mice. Also, the protective immunity in mice cannot be directly translated into protective immunity in humans. Further, vaccines tested in humans to date, since they all targeted single antigens, were probably disproportionate to the complex pathogenic mechanisms of the bacterium.

S. aureus produces a wide variety of immune evasion factors that complicate vaccine development. In addition, the nature of protective immunity has not been clearly understood which in turn has severely limited the ability to interpret both preclinical and clinical data. Finally, the vaccines did not contain new generation adjuvants, which may be critical in augmenting antibody production and steering the T-cell response toward the proper profile of cytokine production [44, 84-86].

Recent developments in whole genome sequencing and bioinformatics have made it possible to identify novel vaccine candidates in a short span of time by replacing time- consuming experimental tasks using *in silico* prediction steps. This way of finding novel antigens has been called reverse vaccinology [87-89]. A successful vaccine against staphylococcal infections should be able to elicit three major immune responses:

(i) Humoral immune response i.e., antibodies to directly inhibit bacterial viability and/or toxicity

(ii) Antibodies to facilitate opsonophagocytosis

(iii) Cell-mediated T-helper 1 (Th1)/Th17 response [84].

However, there are a number of unresolved issues in vaccine development that are yet to be elucidated; optimal antigenic target identification, criteria for acceptable efficacy, identification of target population, optimal timing of immunization strategy and storage to name a few [90].

Concluding Statement

The objective of this dissertation was to characterize the roles of Protein A and Sortase A as potential targets for novel therapeutic approaches against *S. pseudintermedius* infection, along with the analysis of the secretome and surface-associated proteins in *S. pseudintermedius* by mass spectrometry to identify novel antigenic and vaccine targets.

It is hypothesized that Protein A is a potent immune evasion factor in *S. pseudintermedius*. Understanding the nature of Protein A and its role in pathogenesis and immune evasion could lead to the development of a non-toxigenic Protein A vaccine that could be useful to treat *S. pseudintermedius*

infection. The increase in methicillin resistance and multidrug resistance among staphylococci has sparked interest in the development of novel non-antibiotic based treatment strategies to combat this problem. One such attractive target in *S. pseudintermedius* is Sortase A, the transpeptidase enzyme responsible for anchoring proteins with an LPXTG motif onto the peptidoglycan cell wall. Many of the LPXTG proteins function as potent virulence factors. Inhibition of Sortase A could potentially lead to decreased surface expression of all those proteins harboring the LPXTG motif, thus rendering the organism less virulent.

The development of a successful vaccine against *S. pseudintermedius* may require a multicomponent vaccine, for which it is essential to identify novel antigen and vaccine targets. Also, a good vaccine should include B-cell and T-cell epitopes to facilitate the generation of both humoral and cell-mediated immune response, thus resulting in protective immunity. This can be achieved by analyzing the secretome and surface proteins using mass spectrometry and vaccine prediction software.

The immune response to *S. pseudintermedius* has not been widely studied. A deeper knowledge of the virulence factors and immune evasion factors will help understand the pathogenesis of *S. pseudintermedius*. This will aid in the rational design and development of vaccines and novel therapeutics, and will ultimately pave the way towards treatment and prevention of *S. pseudintermedius* infections. Therefore, I propose using Sortase A inhibition along with a multicomponent vaccine containing non-toxigenic Protein A and other potent antigens as a potential strategy against antibiotic-resistant *S. pseudintermedius* infection.

This dissertation is organized in a manuscript format. Each chapter is a manuscript. Chapter 1 describes Protein A in *S. pseudintermedius*. In Chapter 2,

the molecular basis for Protein A deficiency in the *S. aureus* strain Wood 46 has been explained. As an important addition to this manuscript, the whole genome sequence of Wood 46 was published and this is presented in Chapter 3. The impact of Sortase A inhibition has been detailed in Chapter 4 followed by the mass spectrometric analysis of the secretome and surface associated proteins in *S. pseudintermedius* in Chapter 5.

CHAPTER 1.

Protein A in *Staphylococcus pseudintermedius*

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This chapter is a manuscript that will be submitted to an appropriate journal in the Spring of 2017. My contributions to this paper include : (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) all of the writing.

Abstract

Staphylococcus pseudintermedius is an opportunistic pathogen in dogs and the most frequent cause of canine pyoderma. Much of what is known today about *S. pseudintermedius* comes from comparative research with *Staphylococcus aureus*. Protein A, a potent virulence factor in *S. aureus* is encoded by the *spa* gene. *S. pseudintermedius* possesses genes seemingly analogous to *spa*, but the expression and the characteristics of their products have not been directly determined, and the proteins they encode only share about a fifty percent identify with *S. aureus* Protein A. The purpose of this study was to test isolates from major clonal groups for the presence of *spa* gene orthologs, quantitate their expression levels, and to characterize Protein A in *S. pseudintermedius*.

Results from conventional and quantitative real-time PCR suggest that *S. pseudintermedius* isolates contain genes analogous to *spa* in *S. aureus* and the mRNA could be quantified. Isolates representing the major clonal populations in the United States and Europe, ST68 and ST71 respectively, bound significant amounts of canine IgG and more than isolates with other genetic backgrounds. Canine IgG bound to Protein A on *S. pseudintermedius* via its Fc region similar to Protein A from *S. aureus*. The expression profile of *spa* differed based on the sequence types and was correlated to the density of Protein A on the bacterial surface. It was also observed that Protein A was secreted during the exponential growth phase. Phagocytosis experiments with *S. pseudintermedius* show that blocking Protein A enhanced phagocytosis in whole blood, neutrophils and DH82 canine macrophage-like cell line. Taken together, the results demonstrate that *S. pseudintermedius* produces Protein A that shares *S. aureus'* ability to bind the Fc region of immunoglobulins and may serve as a potential virulence factor by evading the host immune system.

Introduction

S. pseudintermedius is the most common species of bacteria isolated from canine pyoderma. Recently, this organism has developed widespread resistance to methicillin [91-97] . Many methicillin-resistant *S. pseudintermedius* (MRSP) strains are also multidrug resistant. In one study by Sasaki *et al*., all 18 of the MRSP strains detected in their patients were resistant to erythromycin, clindamycin, trimethoprim-sulfamethoxazole, and levofloxacin [98]. The emergence of antimicrobial resistance has gradually shifted the focus of research in this field to searching for prophylactic methods of control for these pathogens. Vaccine development, however, requires a deeper understanding of the surface properties and virulence mechanisms of the various strains of *S. pseudintermedius*

Several sequence types (STs) of *S. pseudintermedius* have been described, but two in particular have been reported in significantly higher numbers than the rest: ST68 in the United States and ST71 in European countries [6, 12, 99]. These two sequence types are considered the most dominant and successful clonal populations in their respective geographic locations, and the reason for their success over other strains has yet to be elucidated.

Staphylococci have numerous defenses that allow evasion of the host immune system including immunoglobulin binding proteins such as staphylococcal Protein A. Protein A is produced by many species of staphylococci [31]. Within *S. aureus* it is a 40–60 kDa cell-wall protein encoded by the *spa* gene [49, 50] and secreted during exponential growth phase [41-43]. It is synthesized by nearly all *S. aureus* isolates and binds to the Fc and Fab regions of immunoglobulins, [33, 35] inhibits opsonophagocytic killing [31, 32, 100] and serves as a B cell superantigen [47, 48, 101]. Furthermore, marginal zone B cells and B-1 cells undergo, preferentially, induced cell death with supraclonal depletion and immune tolerance upon

exposure to Protein A [45]. Thus, Protein A may interfere with the efficacy of vaccines that depend on opsonophagocytic antibody production by binding to B-cells and preventing their function [44]. On the other hand, antibodies directed against Protein A have protected mice in experimental models of *S. aureus* infection [53-55].

Putative Protein A activity has been correlated with virulence in *S. pseudintermedius*; however, this conclusion was based on a semi-quantitative dot blot IgG binding assay, did not take other immunoglobulin-binding proteins into consideration or demonstrate the presence of *spa* genes [102]. Genome sequencing studies have shown that *S. pseudintermedius* contains genes analogous to those encoding *S. aureus* Protein A as well as genes encoding other IgG binding proteins [15]. A putative staphylococcal protein A (*spa*) gene was initially described by Moodley *et al*. in 2008 in the isolate ED99 [5]. This gene was used to develop a species-specific *spa* typing protocol. The *spa* gene ortholog in ED99 is 1389bp in length and has 68% nucleotide and 55% predicted amino acid identity to the homologous gene in the *S. aureus* reference strain 8325-4 in which Protein A was originally described. The predicted protein contained a number of functional regions and conserved domains that were previously described in *S. aureus*, including four IgG-binding domains and a polymorphic X-region [5, 15]. Lack of IgG-binding domain region B (58 amino acids) partly accounted for the shorter protein in *S. pseudintermedius* ED99 compared to Protein A in *S. aureus*. [5] .

Bannoehr *et al*. in 2011 suggested that there were two predicted orthologues of *S. aureus* protein A in ED99, SpsP (designated as Spa2) and SpsQ (which encodes a full length protein A designated as Spa1) [15]. The *spsP* gene encodes a protein that consists of 377 amino acids in ED99. This protein is 73% identical to SpsQ at the amino acid level. It has a predicted N-terminal sequence of 33 amino acids,

followed by a repeat region consisting of three predicted IgG-binding domains. Each IgG-binding domain consists of 55 amino acids with 78%-85% identity between domains. The C-terminal region has a predicted X-region which shares 63% overall sequence similarity to the X-region of *S. aureus*.

The *spsQ* gene encodes a protein that is 462 amino acids in ED99. It also has a predicted N-terminal sequence of 33 amino acids, followed by a repeat region consisting of four IgG-binding domains. The four IgG-binding domains vary from 59 amino acids for the first and second, to 55 amino acids for the third, and 52 amino acids for the fourth domain. They have between 59%-86% identity at the protein level. The repeated IgG-binding domains are well conserved between SpsP and SpsQ, with 67% to 90% sequence identity in pairwise alignments. The C-terminal region consists of a predicted X-region which includes a 77 amino acid-long repeat sequence (Xr) and a constant region (Xc) with 70% similarity to the X-region of *S.aureus* [5].

Whole genome sequencing of several *S. pseudintermedius* isolates [103] has shown that most isolates have a full-length *spsQ* gene while they may or may not harbor the full length *spsP* gene. It is not known, however, whether or not these genes are expressed and produce functional protein in *S. pseudintermedius* or if there are differences in the level of Protein A expression in different strains. The purpose of this study was to characterize Protein A in *S. pseudintermedius*. In order to determine this, the ability of *S. pseudintermedius* Protein A to bind canine IgG and its digestion fragments, Fab and Fc, was determined. Gene expression profiles were correlated with surface protein expression. In addition to surface-associated Protein A, secreted Protein A in *S. pseudintermedius* was also investigated, because the secreted form might function as a potent superantigen.

Materials and Methods

Bacterial Strains and Growth Conditions

S. pseudintermedius clinical isolates (Table 1.1) were used for all experiments. *S. aureus* strain Cowan 1 and the *S. pseudintermedius spa* negative isolate 57395 (a kind gift from Dr. Vincent Perreten, Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland) were used as the positive control and negative control strains for *spa* respectively wherever applicable. The strains were selected based on their sequence types. Representatives from the most prevalent sequence types in the US and Europe were used in this study. The genetic background of the isolates was determined by multilocus sequence typing (MLST) [6]. Bacteria from blood agar plates were grown overnight in 5ml of Trypticase Soy Broth (TSB, Becton Dickinson, Franklin Lakes, NJ) at 37°C in a shaker incubator at 225rpm. Fifty microliters of overnight culture was inoculated into 5ml of fresh, sterile TSB and incubated for 3hr at 37°C with shaking to obtain log phase cultures (OD_{600} = 0.4-0.6) which were used for all the experiments unless mentioned otherwise.

DNA Extraction and Conventional PCR

Bacterial DNA was extracted from overnight cultures using a commercial kit (MoBio Ultra Clean DNA Isolation Kit, MoBio, Carlsbad, CA) according to the manufacturer's protocol. PCR for detection of *spsQ* gene in clinical isolates was performed using the following primers: spa forward 5'-ATCTCAACCTGCTCCTGATTAC-3' and spa reverse 5'-GCATCTTTCGCTTTGTCCATAC-3'. The following cycling conditions were performed: initial denaturation at 95°C for 90s, 30 cycles of annealing at 55°C for

Table 1.1 Bacterial isolates used in this study along with their sequence type and methicillin resistance profile

Strain	Sequence Type	Methicillin Resistance
ED99	ST25	S
07 676c	ST29	R
08 547a	ST30	R
08 1781	ST43	S
57395 (*spsQ* negative)	ST45	R
08 1791	ST46	S
07 1447	ST54	R
NA47	ST56	S
NA12	ST64	R
06 3228	ST68	R
08 1294	ST68	R
08 521a	ST68	R
08 1661	ST71	R
E140	ST71	R
NA16	ST71	R
07 5066	ST83	R
NA45	ST84	R
KM241	ST93	R

30s and extension at 72°C for 1min followed by a final extension at 72°C for 5min. PCR products were sequenced (The University of Tennessee Genomics Core Facility), aligned and compared using Geneious 9.1.0 (Biomatters, Auckland, New Zealand).

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from log-phase bacterial cultures using a commercial kit (MoBio Ultra Clean RNA Isolation kit, MoBio, Carlsbad, CA) according to the manufacturer's protocol. Quantitative reverse-transcriptase PCR (RTqPCR) for *spsQ* was performed using the TaqMan RNA-to-C_T 1-Step kit on an ABI StepOne PCR System (Applied Biosystems, Foster City, CA) using the primer-probe set : 5'-/56-FAM/CGCCAAGTT/ZEN/TCGATGAAGCGCAA/3IABkFQ/-3', 5'CCGTTACGTTGCTCTTCAGTA-3', 5'-TAACCAAACACCTACACAACCT-3'. The following cycling conditions were performed: 48°C for 30min, 95°C for 10min and 40 cycles of 95°C for 15s and 60°C for 1min. qPCR data were analyzed by the $\Delta\Delta C_T$ method and normalized against 16S rRNA as the endogenous control as previously described [104].

Preparation of Canine Fab and Fc Fractions from Canine IgG and Biotin Conjugation

Lyophilized canine IgG (IR-DG2-GF, Innovative Research, Novi, MI) was dissolved in sterile PBS (pH 7.2). Canine Fab and Fc fractions were prepared using a kit according to the manufacturer's instructions (Pierce Fab preparation kit, Thermo Scientific, Waltham, MA). Briefly, 1mg/ml of canine IgG equilibrated in digestion buffer was added to papain (immobilized on beads) and incubated for six hours at

37°C with shaking. Enzyme treated-IgG was then added to a Protein A column to bind Fc fragments. Unbound Fab fragments were collected and Fc fragments were eluted and immediately adjusted to neutral pH. All fragments were equilibrated in PBS (pH 7.2).

Fc and Fab fragments prepared above and whole canine IgG were conjugated to biotin (EZ-link NHS-PRG4-Biotin, No-Weigh Format, ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol. Two milligrams each of whole IgG, Fab and Fc were biotinylated. Unbound biotin was removed and the fractions were equilibrated in PBS by ultrafiltration, and stored at 4°C until used.

Canine IgG Binding Assay

One milliliter of log phase bacterial culture was centrifuged at 10,000xg for 1min. The bacteria were washed and suspended in 1ml of PBS and incubated with 100µl of whole canine IgG (described above) at concentrations of 10µg/ml, 100µg/ml or 1000µg/ml for 30min at room temperature. The cells were washed twice with PBS and incubated with 100µl of 1:100 dilution of goat anti-canine IgG (H+L)-FITC conjugate (Southern Biotech, Birmingham, AL) for 30min at room temperature in the dark. For the binding assay with canine Fab and Fc fractions, log phase bacteria were incubated with 2µg/ml biotin-conjugated whole IgG, Fab or Fc prepared previously at room temperature for 30min. Bacteria were washed as before and incubated with 1:500 dilution of avidin-FITC conjugate (Sigma-Aldrich, St. Louis, MO) at room temperature for 30min in the dark. The cells were washed and the amount of binding was determined by flow cytometry.

Protein A Binding Assay

One milliliter of log phase bacterial culture was centrifuged at 10,000xg for 1min. The pellets were washed twice with PBS and resuspended in 1ml PBS and incubated with 100µl of 1:100 dilution of chicken anti-Protein A-FITC conjugate (Gallus Immunotech, Cary, NC) at room temperature for 30min in the dark. Bacteria were washed again and the amount of binding was determined by flow cytometry.

Competitive Binding Assay

One milliliter of log phase bacterial culture was centrifuged at 10,000xg for 1min. The pellet was washed and suspended in 1ml PBS and incubated with 100µl of 1:100 dilution of chicken anti-Protein A antibody (Gallus Immunotech, Cary, NC) for 30min at room temperature. The bacteria were washed as previously described. One hundred microliter of 2µg/ml of biotin-conjugated canine Fc was added and incubated at room temperature for 30min. After incubation, the bacteria were washed and a 1:500 dilution of avidin-FITC conjugate (Sigma-Aldrich, St. Louis, MO) was added and incubated for an additional 30min in the dark. Bacteria were washed again and the amount of binding was determined by flow cytometry.

Enzyme Linked Immuno Sorbent Assay

Secreted Protein A in log-phase culture supernatants was measured using an antigen capture ELISA. Briefly, 96-well flat bottom, costar plates (Corning Inc., Corning, NY) were coated overnight at 4°C with 1µg/ml of mouse monoclonal anti-protein A antibody clone SPA-27 (Mouse IgG1 isotype, Sigma-Aldrich, St. Louis, MO,) in PBS. Bacterial supernatant containing secreted Protein A was added and incubated at 37°C for 1 hour. Bound antigen was detected using a 1:500 dilution

of HRP-conjugated chicken anti-protein A antibody (Gallus Immunotech, Cary, NC) followed by addition of 100µl/well of tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO). Plates were washed in between each step with PBS containing 0.05% polysorbate-20 using an EL_X405 auto plate washer (BioTek Instruments Inc., Winooski, VT). The reaction was stopped by adding 50µl/well of 0.18M sulfuric acid. The optical density was measured at 450nm using an EL_X800 Universal Microplate Reader (BioTech Instruments Inc. Winooski, VT).

Labeling of Bacteria with pHrodo™ Red Amine-Reactive Label

Bacterial isolate 08-1661 (ST71) was labeled with pHrodo Red, succinimidyl ester (pHrodo™ Red, SE, ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions with minor variations. Briefly, 1mg of the pHrodo™ Red SE was dissolved in 150µl of dimethyl sulfoxide (DMSO) to make a stock solution of 10.2mM. 5ml of overnight bacterial culture was centrifuged 10,000g for 5 min and the pellet was washed twice with phosphate buffered saline (PBS, pH 7.2). One milligram of the pellet was resuspended in 750µl of fresh sodium bicarbonate buffer, pH 8.5. The prepared dye was diluted into the bacterial suspension at a final concentration of 1mM. The tubes were incubated at room temperature for 60 min in the dark with gentle rocking. After incubation, the cells were washed with 750µl of Hank's balanced salt solution (HBSS, ThermoFisher Scientific, Waltham, MA) by centrifugation at 15,000g for 1min. The bacterial pellet was resuspended in 1ml of 100% methanol (Sigma-Aldrich, St. Louis, MO). Aggregates, if any, were removed by gently vortexing. The bacteria were centrifuged again at 15,000xg for 1min to remove the methanol. Finally, the labeled bacteria were resuspended in 500µl of HBSS and stored in aliquots at -80°C until use.

Whole Blood Phagocytosis Assay

A pH-sensitive dye, pHrodo Red, which fluoresces only in the acidic environment of the phagolysosome was used to label bacteria in order to effectively visualize phagocytosis and distinguish phagocytosed bacteria from those that are merely adhered to the cell surface.

Fresh blood was collected in heparinized tubes from healthy dogs following an approved IACUC protocol. Briefly, 100µl of blood was mixed with an equal volume of pHrodo^{TM} Red-labeled bacteria (containing 5X10^5 bacteria) and incubated at 37°C for 30min to facilitate phagocytosis. Control tubes containing all components were incubated on ice for the same duration. After 30 min, phagocytosis was stopped by addition of ice-cold PBS containing 5mM EDTA. The cells were washed by centrifugation at 5000xg for 10min, suspended in 2ml of RBC lysing buffer (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 20min. The lysed RBCs were removed by centrifugation. The pellet was washed twice with PBS and the amount of phagocytosis was determined by flow cytometry. The same assay was performed after isolating neutrophils from whole blood using a method described previously [105] and the results were compared. .

Phagocytosis in DH82 Cells

The canine macrophage-like cell line, DH82, was obtained from the American Type Culture Collection (ATCC, CLR-10389), propagated and maintained in Eagle's Minimum Essential Medium (EMEM, Quality Biological Inc., Gaithersburg, MD) containing 15% heat-inactivated fetal bovine serum (Sigma Aldrich, St. Louis, MO) and 1% penicillin/streptomycin/amphotericin-B mixture (Lonza, Walkersville, MD). Cells were grown to confluency, trypsinized and seeded into 24-well costar tissue culture plates (Corning Inc., Corning, NY) at a density of 1x10^6 cells per well and allowed to adhere. pHrodo^{TM} Red-labeled bacteria were added at an MOI of 100

and incubated for 30min at 37°C to facilitate phagocytosis. A control plate with all the components was incubated on ice for the same duration. After incubation, phagocytosis was stopped by the addition of ice cold PBS containing 5mM EDTA. The plate was allowed to sit for 5 min at room temperature. The PBS-EDTA was gently aspirated and the adherent cells were harvested by standard trypsin treatment for 5 min at 37°C. The contents from each well were transferred into a vial and centrifuged at 5000g for 10 min. Cells were washed twice and suspended in 1ml of PBS and analyzed by flow cytometry. Each sample was run in triplicate and analyzed individually. Non-infected cells served as negative control to set the cut-off for the discrimination of pHrodo^{TM} Red-negative and -positive cells, and the cells incubated on ice served as control to determine the percentage of phagocytosis.

Flow Cytometry

Binding experiments with canine IgG, anti-Protein A antibody and phagocytosis assay using labeled bacteria were performed by flow cytometry. The amount of binding or phagocytosis was determined by measuring the fluorescence using an Attune acoustic focusing cytometer (Applied Biosystems, Foster City, CA) at excitation/emission wavelengths of 488/519 nm for green fluorescence and 560/585 nm for red fluorescence. Data were acquired in linear mode for forward and side scatter and logarithmic mode for fluorescence. For each sample, 10,000 events were measured.

Statistical Analysis

A one-way ANOVA and multiple comparisons using Tukey's honest significant difference were performed to test if there was a significant difference in the

expression of *spsQ* among bacterial isolates, and also to determine if there was a significant difference in the amount of surface-expressed Protein A, secreted Protein A and percentage of phagocytosed bacteria. Each experiment was repeated at least three times and a *p-value* of <0.05 was considered significant. All analyses were conducted using the GraphPad Prism software (Version 7, GraphPad Software Inc, La Jolla, CA).

Results

S. pseudintermedius Contains Genes Analogous to *spa* in *S. aureus*

Whole genome sequences of isolates representing MLST sequence types ST68 (06-3228), ST71 (08-1661) and ST84 (NA45) were examined for the *spa* gene ortholog [103]. Gene-specific primers were designed from a consensus sequence after performing multiple sequence alignment, to amplify both the partial and full-length *spsQ* gene. The PCR results showed that all but one isolate (the *spsQ* negative isolate 57395) examined in this study contained the *spsQ* gene. Further, sequence analysis of the *spsQ* gene from isolates representing ST68 and ST71 showed that they share 96% identity with that of the previously published *spa* gene from ED99, while the *spsQ* gene from NA45 shared about 53% identity with that of ED99. The *spsQ* gene sequences from 06-3228, 08-1661 and NA45 are available in GenBank [103].

Expression of *spsQ* Gene in *S. pseudintermedius*

Reverse transcriptase qPCR was used to analyze the expression of *spa* in various *S. pseudintermedius* isolates. The results showed that expression profiles of *spsQ*

varied among sequence types. Isolates representing ST68 had lower expression (approximately 1.5 log reduction) compared to isolates from ST71, but in general, ST68 and ST71 isolates had higher *spsQ* expression than isolates from other sequence types. The *spsQ* expression levels in isolates NA12, NA45 and KM241 could not be quantified because they were below the detection limits of the assay. Isolate 57395, which was negative for *spsQ*, did not produce detectable amounts of *spsQ* mRNA. *spsQ* expression levels were normalized against 16S rRNA as the endogenous control and the results were expressed as fold change in mRNA levels using the $\Delta\Delta C_T$ method. To ensure equal amplification efficiencies of the target and control genes, four 10-fold serial dilutions of the target RNA were tested. The efficiencies of both the target gene and the endogenous control were comparable (Figure 1.1).

Cell Wall-Associated Protein A in *S. pseudintermedius*

The density of surface-anchored Protein A in *S. pseudintermedius* was determined by flow cytometry. Since there was no commercially available antibody against Protein A from *S. pseudintermedius*, FITC conjugated anti-Protein A antibody raised against *S. aureus* Protein A was used for this experiment. The amount of binding was normalized against *S. aureus* Cowan 1 and expressed as percentage bound relative to positive control. The results showed that isolates representing ST68 and ST71 bound significantly more antibody compared to isolates representing other sequence types suggesting that both ST68s and ST71s express a higher density of Protein A on their

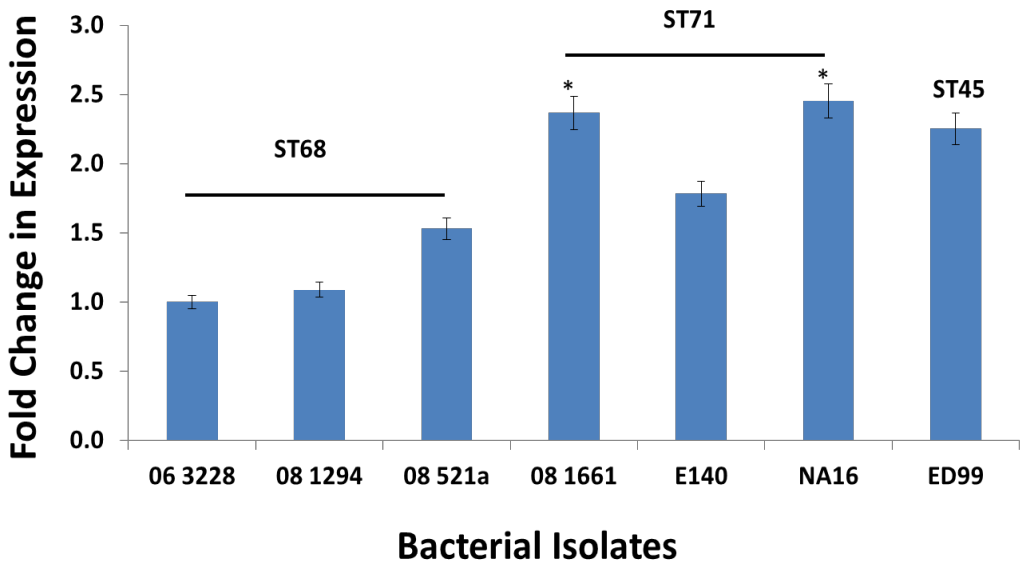

Figure 1.1 Expression levels of *spsQ*.

qPCR was performed to measure the amount of *spsQ* mRNA in log phase bacterial cells. Relative gene expression was calculated using the $\Delta\Delta C_T$ method and expressed as fold change. 16S rRNA was used as the endogenous control. The values represent the average from three independent experiments. (*P<0.05 was considered significant).

[#]*The spsQ mRNA levels in isolates NA12, NA45 and KM241were below the detection limits of the assay*.

surface. Isolates 06-3228, 08-1294 and 08-521a (representing ST68) bound 34.47%, 20.94% and 27.54% of the labeled antibody respectively; while isolates 08-1661, E140 and NA16 (representing ST71) bound 40.77%, 29.92% and 38.26% of the labeled antibody respectively, relative to Cowan 1 (Figure 1.2).
Between isolates from ST68 and ST71, it was found that ST71 isolates bound a higher percentage of the antibody, suggesting that these isolates may have a higher density of Protein A on the surface. Since the antibody raised against Protein A in *S. aureus* recognizes and binds to Protein A on *S. pseudintermedius*, Protein A in these two species apparently share epitopes, however, differences between the two species may affect relative antibody reactivity.

Canine IgG Reacts with Protein A on the Surface of *S. pseudintermedius* and Binds to Protein A via its Fc Region

To determine if canine IgG binds to Protein A on the surface of *S. pseudintermedius*, a binding experiment with canine IgG was performed. Canine IgG was diluted 10-fold from 10µg/ml to 1mg/ml and incubated with log phase cultures of various strains of *S. pseudintermedius*. A dose response was observed for binding with increasing amounts of canine IgG (Figure 1.3), suggesting that canine IgG recognizes and binds to Protein A on *S. pseudintermedius*.

Isolates representing various sequence types (listed in Table 1.1) were reacted with 100µg/ml of canine IgG. It was observed that isolates representing ST68 and ST71 bound significantly more canine IgG than isolates from other sequence types suggesting that they may have a higher density of cell-wall associated Protein A on their surface (Figure 1.4).

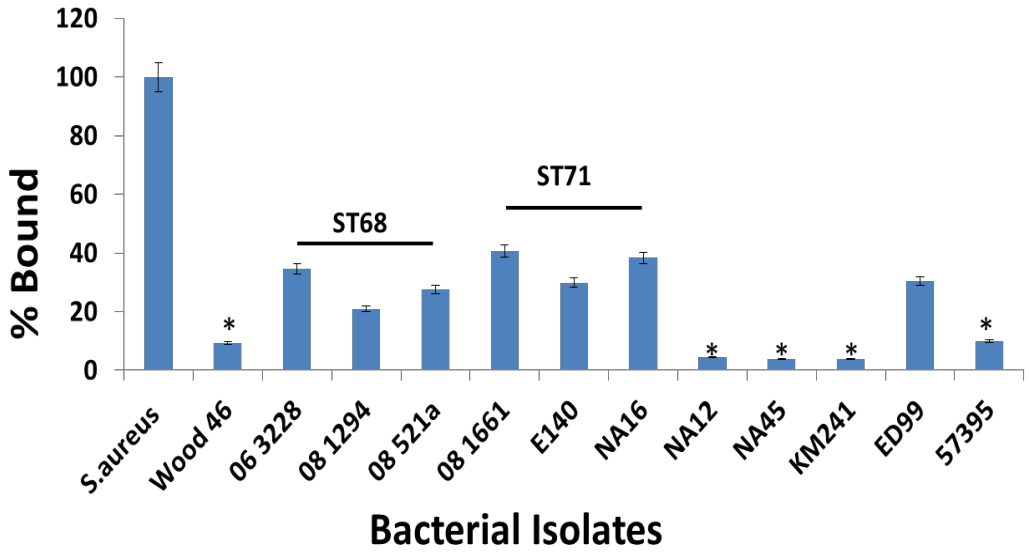

Figure 1.2 Protein A on the surface of *S. pseudintermedius*.

The density of cell wall-associated Protein A in various strains of *S. pseudintermedius* was measured using FITC-conjugated anti-Protein A antibody by flow cytometry. The values represent the average from three independent experiments. (*P<0.05 was considered significant).

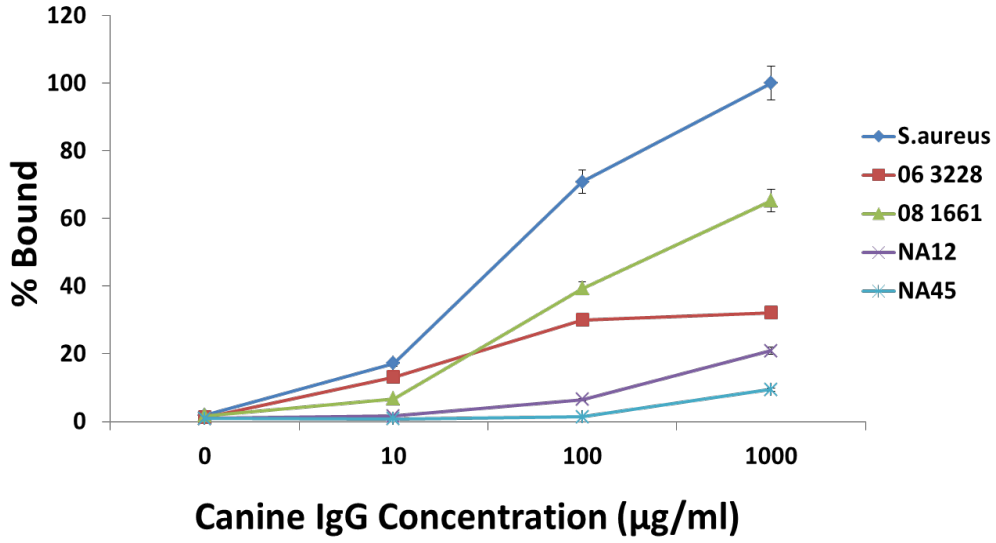

Figure 1.3 Binding of canine IgG to Protein A on *S. pseudintermedius* (dose-response).

A dose-response effect observed when varying concentrations of canine IgG ranging from 10µg/ml-1000µg/ml was treated with *S. pseudintermedius*. The values represent average from three independent experiments. ($P<0.05$ was considered significant).

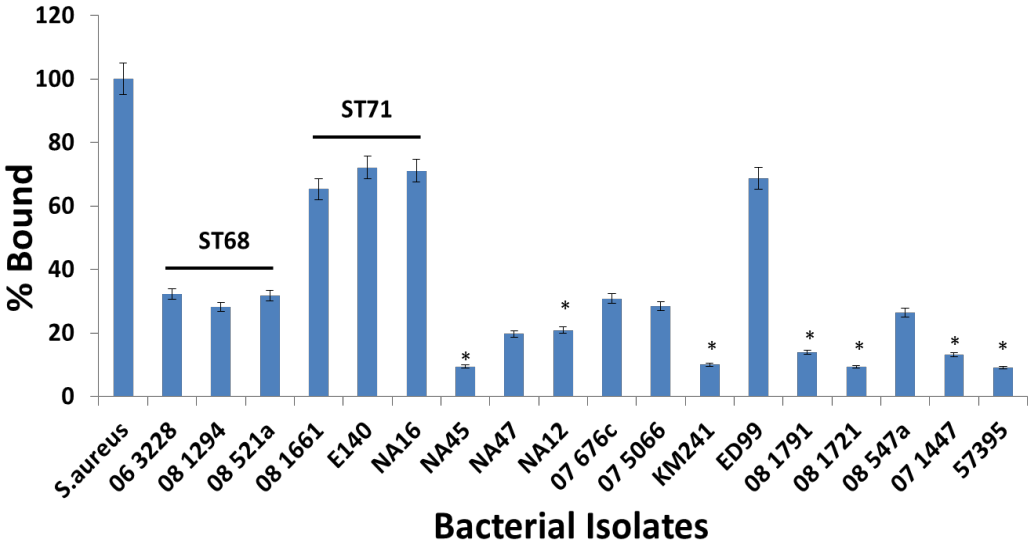

Figure 1.4 Canine IgG recognizes and binds to Protein A on *S. pseudintermedius*.

Isolates representing various sequence types of *S. pseudintermedius* were reacted with 100µg/ml of canine IgG. The amount of binding was determined by flow cytometry and expressed as percentage bound relative to the positive control *S. aureus* Cowan 1 strain. The values represent average from three independent experiments. (*P<0.05 was considered significant).

After determining that canine IgG recognizes and binds to Protein A on *S. pseudintermedius*, the next step was to examine which fraction of canine IgG specifically binds to Protein A. Protein A in *S. aureus* binds to IgG primarily via its Fc region [54]. It was hypothesized that Protein A in *S. pseudintermedius* also binds IgG via its Fc region. In order to test this, canine IgG was digested with papain to generate Fab and Fc fragments and reacted with bacteria. Two microgram of each of the fragments, Fab and Fc, along with whole IgG were reacted with various strains of *S. pseudintermedius*. The results showed that Protein A in *S. pseudintermedius* binds to canine IgG via its Fc region (Figure 1.5). Data for isolates representing the predominant sequence types 06-3228 (ST68), 08-1661 (ST71), NA12 (ST64) and NA45 (ST84) have been presented.

Anti-Protein A Antibody Inhibits the Binding of Canine Fc Fragment to *S. pseudintermedius*

In order to determine whether anti-Protein A antibody (raised in chickens against *S. aureus* Protein A) binds to Protein A on the surface of *S. pseudintermedius*, a competitive inhibition assay was performed. Bacterial cells were incubated with excess of unlabeled chicken anti-Protein A antibody prior to adding biotin-conjugated canine Fc. Anti-Protein A antibody competitively inhibited the binding of canine Fc as determined by a decrease in fluorescence when detected using flow cytometry (Figure 1.6). A titration experiment was performed to determine the optimal dilution of both the anti-Protein A antibody and the canine Fc fraction that results in at least 50% reduction in binding of canine Fc. Data for isolates from strains 06-3228 (ST68), 08-1661 (ST71), NA12 (ST64) and NA45 (ST84) have been presented in (Fig 1.6). Since isolates NA12 and NA45 showed very little binding to canine IgG and anti-Protein A antibody (Figure 1.2 and Figure 1.4), these isolates were not expected to show any inhibition (Figure 1.6).

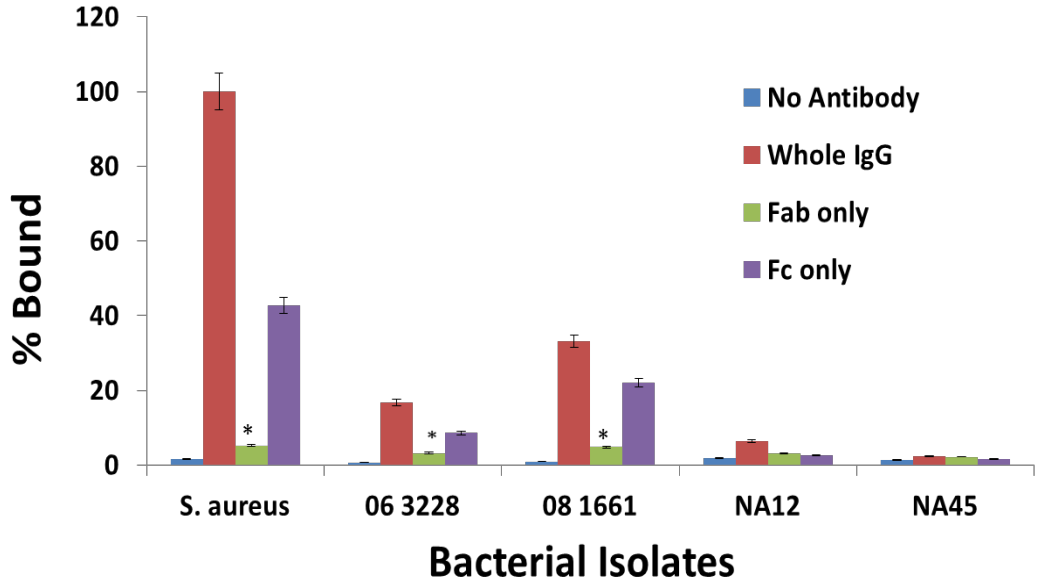

Figure 1.5 Protein A binds to canine IgG primarily via its Fc region.

S. pseudintermedius isolates were treated with IgG and its papain digestion fragments, Fab and Fc. The amount of binding was determined by flow cytometry. The values represent average from three independent experiments. (*P<0.05 was considered significant).

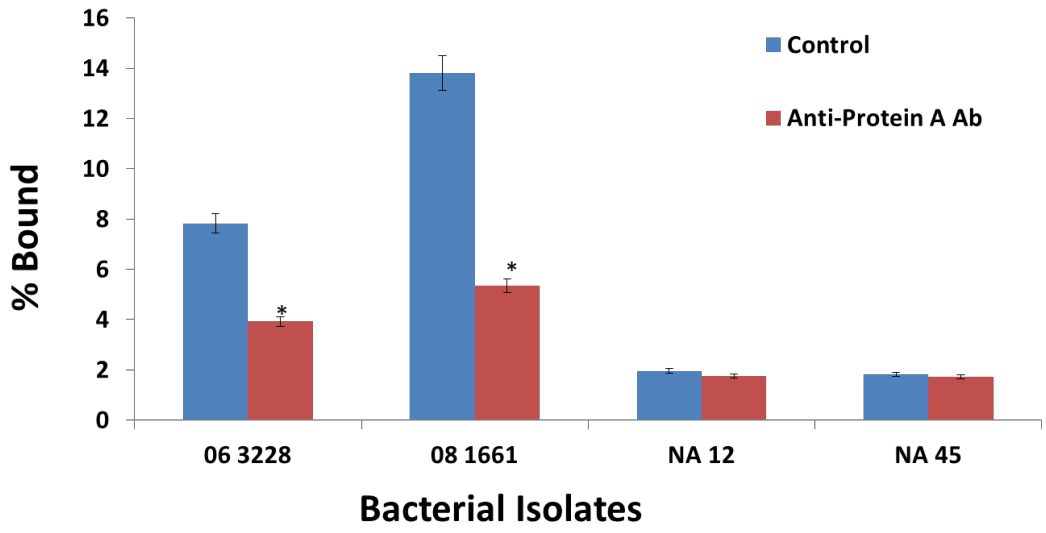

Figure 1.6 Binding of anti-Protein A antibody prevents the binding of canine Fc to Protein A on *S. pseudintermedius*.

Bacterial isolates were incubated with an excess of chicken anti-Protein A antibody prior to the addition of canine Fc. There was at least 50% reduction in the binding of canine Fc because of anti-Protein A antibody binding. The values represent average from three independent experiments. (*P<0.05 was considered significant).

This further strengthened the fact that *S. pseudintermedius* expresses Protein A on its surface and is recognized by the commercially available anti-Protein A antibody.

Protein A in Bacterial Culture Supernatant

S. aureus isolates secrete Protein A into the culture supernatant during log phase [41, 42]. In order to determine if *S. pseudintermedius* isolates also secrete Protein A during log phase, a capture ELISA was used to measure the amount of Protein A in bacterial culture supernatants. Log phase culture supernatants from various bacterial isolates were tested. The results showed that isolates representing ST68 and ST71 secreted higher amounts of Protein A into the culture supernatant compared to isolates from other sequence types. *S.aureus* Cowan 1, used as the positive control, secreted 23.1ng/ml of Protein A. Isolates 06-3228, 08-1294 and 08-521 secreted between 0.98ng/ml-2.19ng/ml of Protein A. Isolates 06-1661, E140 and NA16 secreted between 3.45ng/ml-9.94ng/ml of Protein A (Figure 1.7). Among isolates from ST68 and ST71, a significant difference was observed in the amount of secreted Protein A, with ST71s secreting higher amounts than ST68s. As expected, isolate 57395, which was negative for the *spsQ* gene by both conventional and quantitative real-time PCR did not produce detectable amounts of Protein A. The lower limits of detection and quantification of the assay were 0.125ng/ml and 0.3ng/ml respectively. Taken together, these results suggest that *S. pseudintermedius* not only expresses Protein A on its surface but also secrets it into the culture supernatant during log phase.

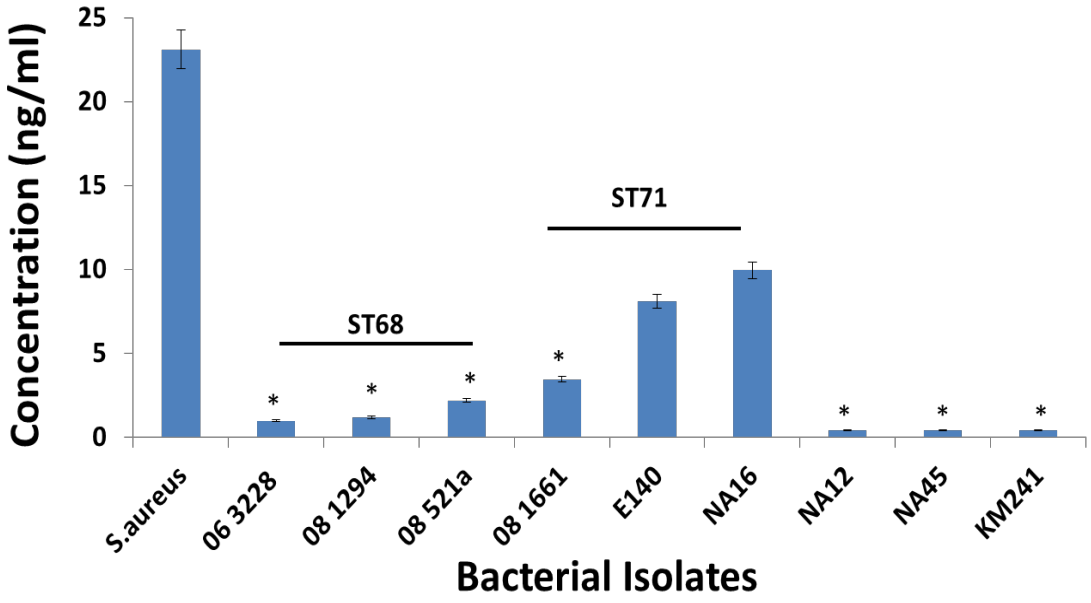

Figure 1.7 Production of extracellular Protein A in *S.pseudintermedius*.

The amount of extracellular Protein A was measured using an antigen-capture ELISA. Commercially available Protein A from *S.aureus* was used to generate the standard curve. The values represent the average from three independent experiments. (*P<0.05 was considered significant).

Binding of Anti-Protein A Antibody makes *S. pseudintermedius* more Susceptible to Phagocytosis in Whole Blood, Canine Neutrophils and DH82 Cells

In order to determine if Protein A in *S. pseudintermedius* can function as an immune evasion factor, phagocytosis was performed using canine whole blood, neutrophils and DH82 canine macrophage-like cells. *S. pseudintermedius* isolate 08-1661 was labeled with a pH-sensitive dye, pHrodo^{TM} Red which enables easy visualization of phagocytosis. The labeled bacteria fluoresce in the acidic environment of phagosomes, allowing the differentiation and visualization of phagocytosed bacteria from those that are merely adhered to the cell surface.

The percentage of phagocytosis after 30min at 37°C in whole blood, neutrophils and DH82 macrophage-like cells was 57.8%, 54.6% and 44.3% respectively compared to the control which was incubated on ice for the same duration. To test the involvement of Protein A in preventing phagocytosis, labeled bacteria were pre-incubated with 100µg/ml of either chicken anti-Protein A antibody or canine IgG before the start of phagocytosis. With both canine blood and neutrophils, pre-incubation of the bacteria with anti-Protein A antibody enhanced phagocytosis by at least 10%, although this difference was not significant (Figure 1.8 and Figure 1.9). In DH82 cells however, phagocytosis was significantly enhanced by 17.8% in the presence of anti-Protein A antibody (Figure 1.10) compared to the control without anti-Protein A antibody. Canine IgG was also used in the experiment, but it did not have any effect on phagocytosis. The duration for phagocytosis was determined by a time course experiment and 30min was found to be optimal as with previous studies involving *S. aureus* (Figure 1.11) [106, 107].

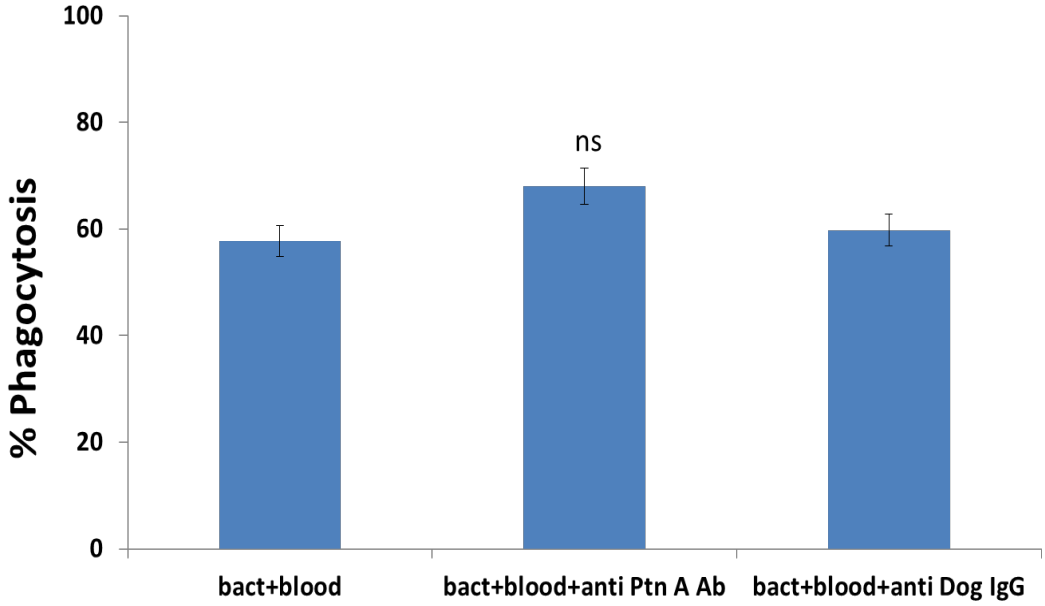

Figure 1.8 Phagocytosis in whole blood.

The involvement of Protein A in immune evasion was demonstrated by phagocytosis using pHrodo™ Red-labeled 08-1661 in whole blood. Incubation of bacteria with anti-Protein A antibody enhanced phagocytosis. The values represent average from three independent experiments. (*P<0.05 was considered significant).

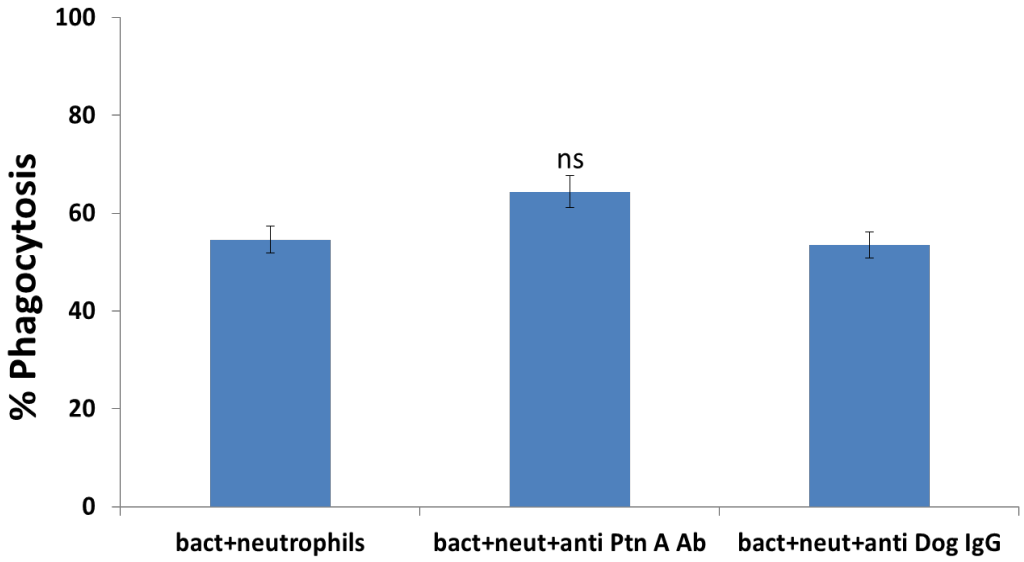

Figure 1.9 Phagocytosis in neutrophils.

The involvement of Protein A in immune evasion was demonstrated by phagocytosis using pHrodo^{TM} Red-labeled 08-1661 in neutrophils. Incubation of bacteria with anti-Protein A antibody enhanced phagocytosis. The values represent average from three independent experiments. (*P<0.05 was considered significant).

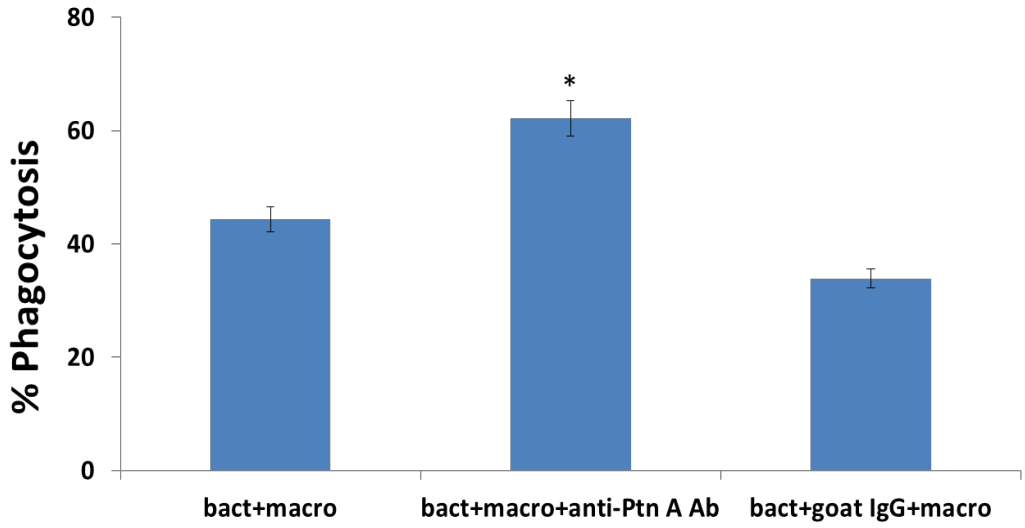

Figure 1.10 Phagocytosis in DH82 canine macrophage-like cell line.

The involvement of Protein A in immune evasion was demonstrated by phagocytosis using pHrodo^{TM} Red-labeled 08-1661 in DH82 canine macrophage-like cell line. Incubation of bacteria with anti-Protein A antibody significantly enhanced phagocytosis. The values represent average from three independent experiments. (*P<0.05 was considered significant).

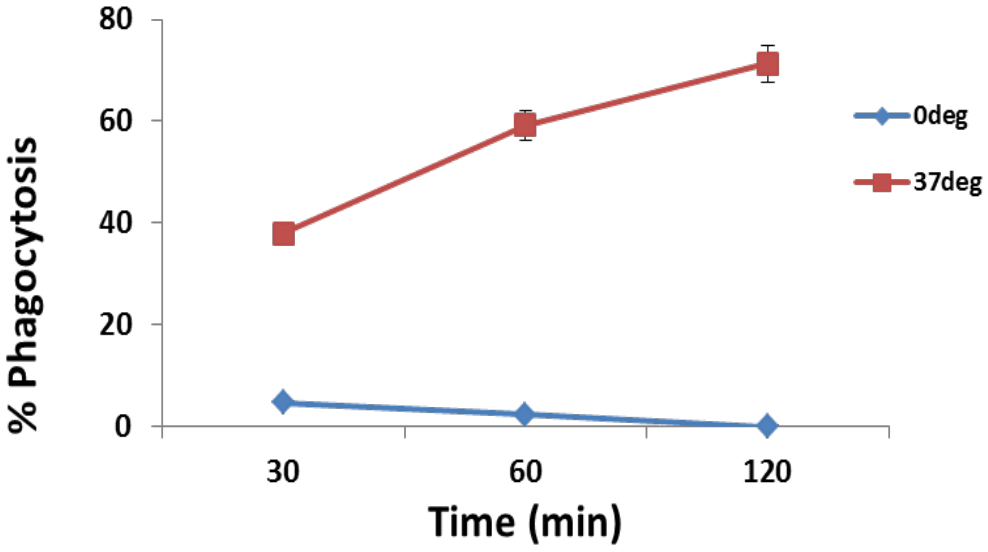

Figure 1.11 Phagocytosis of pHrodo™ Red-labeled 08-1661.

Time-course experiment to demonstrate phagocytosis at 37°C and a control experiment at 0°C.

Taken together, these results suggest that Protein A in *S. pseudintermedius* could function as an immune evasion factor similar to Protein A in *S. aureus*, and prevent bacteria from being opsonized and phagocytosed.

Discussion

Protein A is a widely studied virulence and immune evasion factor. Nearly all *S. aureus* isolates are known to produce Protein A [31]. It is expressed on the peptidoglycan cell wall and is also secreted into the bacterial culture supernatant during exponential growth phase [41-43, 49, 108]. One of the most important features of Protein A is its ability to bind IgG via its Fc region, thereby preventing opsonization and phagocytosis [33-35]. This helps the bacteria evade the host immune response, leading to persistent infections [32, 36, 37, 100]. *S. pseudintermedius*, the canine pathogen implicated as the causative agent of pyoderma also contains genes predicted to encode Protein A [7, 15]. Protein A in *S. pseudintermedius* has not been previously studied or characterized.

In this study, the presence of cell-wall anchored Protein A was measured by flow cytometry. It was found that Protein A is a component of the cell wall of most of the isolates tested in this study (Fig 1.2). It was also found that canine IgG was able to react with Protein A on the surface of *S. pseudintermedius*. Protein A on *S. pseudintermedius* bound to canine IgG via its Fc fragment (Fig 1.4 and Fig 1.5) suggesting that it may escape phagocytosis and serve as an immune evasion factor. It should be noted that the anti-Protein A antibody used in this study is raised against *S. aureus* Protein A. Also, canine IgG used in this study could be binding the bacterial surface via its Fab region. In fact, isolate 57395 which was negative for the *spa* gene by conventional PCR showed some binding to anti-Protein A antibody. Since this antibody is raised against *S. aureus*

Protein A, there is a possibility that it reacts with other surface proteins, particularly immunoglobulin binding proteins like Sbi, besides Protein A on the surface of *S. pseudintermedius*. This is true in the case of *S. aureus*, where *spa* deletion mutants have been shown to bind with anti-Protein A antibody [109-111]. Isolates from the dominant clonal populations in the United States and Europe, ST68 and ST71, bind more IgG and have a higher density of Protein A on their surface than isolates of other STs. This property could explain why isolates from ST68 and ST71 are so successful in the population compared to other sequence types. The presence of extracellular Protein A in S*. pseudintermedius* suggests that it could also function as a superantigen. Phagocytosis experiments with whole blood, neutrophils and DH82 macrophage-like cells indicate that blocking bacterial surface Protein A with anti-Protein A antibody makes it more susceptible to phagocytosis. Taken together, the results suggest that Protein A in *S. pseudintermedius* may share common features with its *S. aureus* counterpart, and serve as a potent virulence factor.

Vaccine studies with *S. aureus* have indicated that a multivalent vaccine with different epitopes would probably work best [44, 84, 112]. Keeping the same factors in mind, the successful development of a vaccine against *S. pseudintermedius* would involve identifying antigenic targets representative of a wide variety of strains and sequence types. Understanding the nature of surface proteins and their role in virulence and pathogenesis is critical for vaccine development against *S. pseudintermedius* infection.

CHAPTER 2.

Molecular Basis of Surface Anchored Protein A Deficiency in the *Staphylococcus aureus* Strain Wood 46

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This chapter is a manuscript that has been submitted to PLoS One in March 2017. My contributions to this paper include : (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) all of the writing.

Abstract

Protein A in *Staphylococcus aureus* is encoded by the *spa* (*s*taphylococcal *p*rotein *A*) gene and binds to immunoglobulin (Ig). The *S. aureus* strain Wood 46 has been variously reported as Protein A-deficient and/or *spa* negative and used as a control in animal models of staphylococcal infections. The results of this study indicate that Wood 46 has normal *spa* expression but transcribes very low levels of the *srtA* gene which encodes the Sortase A (SrtA) enzyme. This is consistent with unique mutations in the *srtA* promoter. In this study, a low level of Sortase A explains deficient anchoring of proteins with an LPXTG motif, such as Protein A, fibrinogen-binding protein and fibronectin-binding proteins A and B on to the peptidoglycan cell wall. The activity of secreted Protein A is an important consideration for use of Wood 46 in functional experiments and animal models.

Introduction

Protein A produced by *S. aureus* is a potent virulence factor and immune-modulator [31, 113-116]. It is a 40–60 kDa cell-wall protein encoded by the *spa* gene [49, 50]. It is synthesized by nearly all *S. aureus* isolates and binds to the Fc and $F(ab)_2$ regions of immunoglobulins [33, 35], prevents opsonophagocytic killing [31, 32, 100] and serves as a B-cell superantigen [47, 48, 101]. Furthermore, marginal zone B cells and B-1 cells undergo, preferentially, induced cell death with supraclonal depletion and immune tolerance upon exposure to Protein A [45]. Thus, Protein A may interfere with vaccine efficacy that depends on opsonophagocytic antibody production by binding to B-cells and preventing their function. The *S. aureus* strain Wood 46 had been considered *spa* negative and Protein A-deficient. For this reason, it was used as a negative control in studies involving *S. aureus* virulence [31, 117-123]. There is evidence that Wood 46 produces extracellular Protein A during exponential growth phase and steady state [119]. This has been demonstrated indirectly by immunoglobulin binding

experiments [119]. Extracellular Protein A has also been isolated from Wood 46 using IgG Sepharose chromatography, although the amount of putative Protein A recovered from Wood 46 is lower compared to *S. aureus* strain Cowan 1, a high producer of Protein A [118, 119]. *S.aureus* produces a second immunoglobulin-binding protein (Sbi), which is also capable of binding to the Fc portion of IgG similar to Protein A and protects the bacteria against innate immune responses [109].

Sortase A (SrtA) is a transpeptidase commonly produced by Gram-positive bacteria. It has specificity for proteins with an LPXTG motif and cleaves between the threonine (T) and glycine (G) residues to form an acyl enzyme intermediate that is relieved by the nucleophilic attack of the amino group in the pentaglycine cross bridge of the peptidoglycan precursor[62]. Modified proteins are subsequently incorporated into the peptidoglycan cell wall and displayed on the surface [56, 62-64, 66, 108, 124]. *S. aureus* isolates encode approximately 17-21 surface proteins with an LPXTG motif [70, 115, 125]. These include Protein A, fibrinogen-binding proteins - clumping factors A and B (Clf A/B) and fibronectin binding proteins (FnBP A and FnBP B) that function as potent virulence factors [126]. *S. aureus* mutants lacking SrtA fail to covalently attach proteins with an LPXTG motif onto their cell wall, and are thus less virulent than their wild-type counterparts. They do not form abscess lesions in organs or cause bacteremia in mouse models [70, 74]. In this study, the reason for Protein A deficiency on the surface of Wood 46 was investigated and the defective expression of *spa* and *srtA* genes encoding functional proteins was examined as a possible cause.

Materials and Methods

Bacterial Strains and Growth Conditions

S. aureus strain Wood 46 (ATCC 10832), Seattle 1945 (ATCC 25923) and Cowan 1 (ATCC 12598) were obtained from the American Type Culture Collection. The *S. aureus* Newman strain and its corresponding *spa* and *sbi* mutants (Newman WT, Newman sbi::Em^r, Newman spa::Ka^r and Newman sbi::Em^r spa::Ka^r) were a kind gift from Drs. Tim Foster and Joan Geoghegan (Department of Microbiology, Trinity College Dublin) [109]. Bacteria were grown overnight in Trypticase Soy Broth (TSB, Becton Dickinson, Franklin Lakes, New Jersey) at 37°C in a shaker incubator and diluted 1:50 in fresh TSB to initiate log-phase cultures.

DNA Extraction and Conventional PCR

Bacterial DNA was extracted from overnight cultures using a commercial kit (MoBio Ultra Clean DNA Isolation Kit, MoBio, Carlsbad, CA) according to the manufacturer's protocol. PCR for *spa, srt A* and the -35 regulatory region was performed using primers listed in Table 2.1. The following cycling conditions were performed: initial denaturation at 95°C for 90s, annealing at 55°C for 30s and extension at 72°C for 1min (30 cycles), and a final extension at 72°C for 5min. PCR products were sequenced (The University of Tennessee, Genomics Core Facility) and the sequences of *spa, srtA* and the regulatory region in all isolates were aligned and compared using Geneious (version 9.1.6) (Biomatters, Auckland, New Zealand).

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from log phase bacterial cultures using a commercial kit (MoBio Ultra Clean RNA Isolation kit, MoBio, Carlsbad, CA) according to the manufacturer's protocol. Quantitative Real-Time PCR (qPCR) for *spa* and *srt A* were performed using the TaqMan RNA-to-C_T 1-Step kit on an ABI StepOne PCR System (Applied Biosystems, Foster City, CA). The primers and probe used for the qPCR are listed in Table 2.1. The following cycling conditions were performed: 48°C for 30min, 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min. qPCR data were analyzed by the $\Delta\Delta C_T$ method and normalized against 16S rRNA as the endogenous control as previously described [104].

Protein A ELISA

Secreted Protein A in culture supernatants was measured using an antigen capture ELISA. Briefly, 96-well Costar plates (Corning Inc, Corning, NY) were coated with 1µg/ml of mouse monoclonal anti-protein A antibody clone SPA-27 (Sigma-Aldrich, St. Louis, MO) in PBS pH 7.2. Bacterial supernatant was added and incubated at 37°C for 1 hour. Bound antigen was detected using a 1:500 dilution of HRP-conjugated chicken anti-protein A antibody (Gallus Immunotech, Cary, NC) followed by addition of 100µl/well of tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO). Plates were washed in between each step with PBS containing 0.05% polysorbate-20 using an EL_X405 auto plate washer (BioTek Instruments Inc., Winooski, VT). The reaction was stopped by adding 50µl/well of 0.18M sulfuric acid. The optical density was measured at 450nm using an EL_X800 Universal Microplate Reader (BioTech Instruments Inc.

Table 2.1 List of primers used in this study

Gene	Primers	Reference
spa (conventional PCR)	F 5'-ATCTGGTGGCGTAACACCTG-3' R 5'-CGCTGCACCTAACGCTAATG-3'	This study
srtA (conventional PCR)	F 5'-AAGCGTTTCGTTATTTGAATGC-3' R 5'-TCGTCATTGCTACCTCATACC-3'	This study
spa (qPCR)	5'-/56-FAM/TTTGTCAGC/ZEN/AGTAGTCGCGTTTGC/3IABkFQ/-3' 5'-ATGTCGTTAAACCTGGTGATACA-3' 5'-GGTTTGCTGGTTGCTTCTTATC-3'	This study
srtA (qPCR)	5'-/56-FAM/AGCAAGCTA/ZEN/AACCTCAGATTCCGAAAGA/3IABkFQ/-3' 5'-GAACAGGCGAGTAAAGACAATAAG-3' 5'-GGTCCTGGATATACTGGTTCTTT-3'	This study
16S (qPCR)	5'-/56-FAM/CCGGCAGTC/ZEN/AACTTA/3IABkFQ/-3' 5'-CACCTTCCTCCGGTTTGTCA-3' 5'-CCCTTGAACTTAGTTGCCATCATTC-3'	[104]

Winooski, VT). Commercially available *S. aureus* Protein A (Thermo Scientific, Rockford, IL) was used to generate the standard curve.

Flow Cytometry to Measure Surface-Expressed Protein A, Fibrinogen-Binding Protein and Fibronectin-Binding Protein

One milliliter of log phase bacterial cells were washed and incubated with either no conjugate (NC, negative control), 2µg/ml of chicken anti-protein A FITC conjugate (Gallus Immunotech, Cary, NC), 1µg/ml human fibrinogen FITC conjugate (Zedira GmbH, Darmstadt, Germany) or 1µg/ml bovine fibronectin HiLyte Fluor ^{TM} 488 conjugate (Cytoskeleton Inc., Denver, CO) for 30 min at room temperature in the dark. Excess conjugate was removed by washing and the amount of binding determined by measuring fluorescence using an Attune acoustic focusing cytometer (Applied Biosystems, Foster City, CA) at excitation/emission wavelengths of 488/519 nm. Data were acquired in linear mode for forward and side scatter and logarithmic mode for fluorescence. For each sample, 10,000 events were measured.

Statistical Analysis

A one-way ANOVA and multiple comparisons using Tukey's honest significant difference were performed to test if there was a significant difference in the expression of *spa* and *srt* among bacterial isolates, and also to determine if there was a significant difference in the amount of surface-expressed Protein A, fibrinogen-binding protein and fibronectin-binding protein. Each experiment was repeated at least three times and a *p-value* of <0.05 was considered significant. All analyses were conducted using the IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY).

Results

Wood 46 Expresses Lower Amounts of Cell Wall-Associated Protein A

Flow cytometry using chicken anti-Protein A antibody was performed to determine the amount of cell wall-associated Protein A. The amount of binding was normalized against Seattle 1945 which showed maximum binding in this assay (assigned 100% binding). Wood 46 bound lower amounts of the antibody (about 19%) relative to Seattle 1945. The *Δ*sbi, *Δ*spa and *Δ*sbi-*Δspa* mutants bound 76%, 27% and 9% of the antibody respectively. In contrast, Cowan 1 and the Newman WT isolate, both reported to be high producers of Protein A bound 69% and 83% respectively (Figure 2.1), indicating that Wood 46 may have lower amounts of cell wall-associated Protein A compared to the positive control strains used in this study.

Secreted Protein A in Culture Supernatant of Wood 46

S. aureus releases Protein A during the exponential growth phase [41, 42]. To confirm this in Wood 46, a Protein A-capture ELISA was used to measure the amount of Protein A secreted into culture supernatant. Both overnight (data not shown) and log phase culture supernatants were tested. Wood 46 secreted 12.42 ng/ml of Protein A into the culture supernatant compared to Seattle 1945 which secreted the highest amount of Protein A (22.56ng/ml) during log phase (Figure 2.2). These data indicate that Wood 46 produces and secretes Protein A, although the amount is lower, although not statistically significant, compared to Seattle 1945 or wild-type Newman isolates.

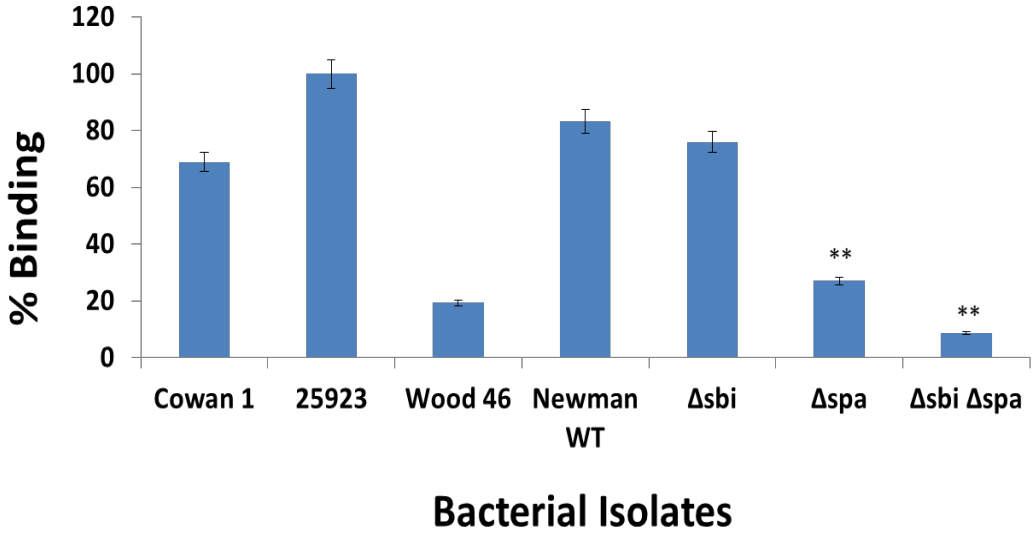

Figure 2.1 Cell wall-associated Protein A in *S. aureus*.

Protein A expressed on the surface of *S. aureus* strains Cowan1, Seattle 1945 (25923), Wood 46, Newman WT and its corresponding deletion mutants, *Δ*sbi, *Δ*spa and *Δ*sbi-*Δ*spa, as measured by flow cytometry with chicken anti-Protein A antibody. The values represent the average from three independent experiments. (*P<0.05 and **P<0.02 were considered significant).

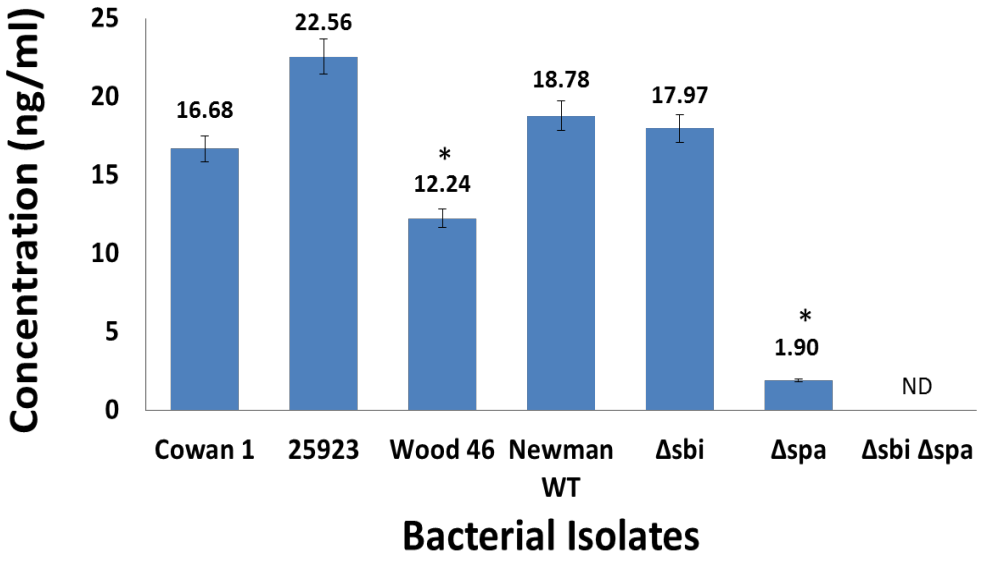

Figure 2.2 Production of extracellular Protein A in *S. aureus*.

The amount of secreted Protein A was measured using an antigen-capture ELISA. Commercially available Protein A from *S.aureus* was used to generate the standard curve. The values represent the average from three independent experiments. (*P<0.05 was considered significant).

Gene Sequence Analysis of *spa, srtA* and Regulatory Region of *srtA*

It was hypothesized that if the *spa* gene in Wood 46 harbors a mutation, it may lead to a truncated or non-functional protein. In order to test this hypothesis, the *spa* gene in Wood 46 and other *S. aureus* isolates was amplified using PCR and the products were sequenced and analyzed. Results from the PCR and DNA sequence analysis of the *spa* gene showed that Wood 46 not only possesses the full-length *spa* gene, but also the sequence of the *spa* gene is in complete agreement with those from Cowan 1, Seattle 1945 and wild-type Newman (data not shown). Therefore, mutation in the *spa* gene was ruled out as the possible cause for lower expression of Protein A in Wood 46.

Sequence analysis of the *srtA* gene in Wood 46, however, showed that it has a 12bp deletion at the 3' end of the gene that results in a four amino acid difference in the protein compared to *S. aureus* Cowan 1, Seattle 1945 and wild-type Newman (Figure 2.3). Also, analysis of the upstream promoter and regulatory region showed that Wood 46 contains mutations in both the -10 and -35 regions (Figure 2.4). These mutations likely adversely affect RNA polymerase binding and transcription as mutations in the upstream promoter and regulatory regions have been associated with reduced gene expression [127-129].

mRNA Levels of *spa* and *srtA* in Wood 46

If the transcription of *spa* is adversely affected, it could lead to lower expression of Protein A on the bacterial surface. Reverse transcriptase qPCR was used to analyze the expression of *spa* in *S. aureus* isolates. The results showed that Wood 46 transcribes *spa* mRNA although the levels were lower compared to

```
Consensus sequence from Cowan 1, Seattle 1945 and Newman
                155 MTSIRDVKPTDVEVLDEQKGKDKQLTLITCDDYNEKTGVWEKRKIFVATEVK 206
Wood 46         155 MTSIRDVKPTDVEVLDEQKGKDKQLTLITCDDYNEKTGVWEKRKI....EVK 202
```

Figure 2.3 Amino acid composition of Sortase A.

The amino acid sequence of Sortase A in Cowan 1, Seattle 1945, Newman and Wood 46 were aligned. Amino acids from position 155 to the end of the protein are shown. Wood 46 contains a 12bp deletion at the 3' end of the *srtA* gene that leads to a corresponding loss of four amino acids at the C-terminus of the protein.

```
S.aureus_Consensus   ATGCAAATATGTGTAAATGAAATCTAACGTGACAATATATGTGC
          Wood 46    ATCCCTCTATGTATCKACGAAA----------AGTATACATGT

                                                          +1
AAATTTAATATTATAAAATGAATTGCTATGAGTCATTTTATAATTAATGGTATACTATATGAA
AAATTTTATAGTATAAAATGAATTGCTATGAGTCATTTTGAAATTAATGGTATACTATATGAA
```

Figure 2.4 Upstream promoter and regulatory region of *srtA*

Consensus promoter and upstream regulatory sequence from Cowan 1, Seattle 1945 and Newman was aligned with that of Wood 46. The boxes highlight regions with mutations. The transcription start site ATG is indicated as +1.

Cowan 1 (assigned as the calibrator for this experiment), Seattle 1945 (0.74 fold) and wild-type Newman isolate (Figure 2.5). As expected, the *Δ*spa and *Δ*sbi, *Δspa* mutants did not produce detectable *spa* mRNA. This suggests that inefficient *spa* transcription was not the reason for low surface expression of Protein A in Wood 46. The *spa* expression level was normalized against 16S rRNA as the endogenous control and the results were expressed as fold change in mRNA levels using the $\Delta\Delta C_T$ method. To ensure equal amplification efficiencies of the target and control genes, four 10-fold serial dilutions of the target RNA were tested. The efficiencies of both the target gene and the endogenous control were comparable.

Conversely, the expression level of *srtA* mRNA in Wood 46 was below the limit of detection of the assay. Cowan 1 was assigned as the calibrator strain for this experiment. The expression levels of Seattle 1945 and Newman WT were 0.26 fold and 0.28 fold relative to Cowan 1, while that of the deletion mutants *Δ*sbi, *Δ*spa and *Δ*sbi,*Δ*spa were 0.14 fold, 1.03 fold and 0.51 fold respectively (Figure 2.6). Taken together, the results suggest the lack of Sortase A could be responsible for the deficient anchoring of Protein A in Wood 46.

Wood 46 Expresses Lower Amounts of the LPXTG Motif-Harboring Fibrinogen-Binding Protein and Fibronectin-Binding Protein

Sortase A is responsible for anchoring proteins with an LPXTG motif on to the peptidoglycan cell well [62, 64, 66, 124, 130]. If lack of this enzyme is indeed the reason for low surface density of Protein A in Wood 46, there should be a global decrease in the expression of all surface proteins that bear the LPXTG motif. In order to test this hypothesis, two well-studied proteins with LPXTG motifs were chosen. Fibrinogen-binding protein (Clf A and B) and fibronectin-binding protein

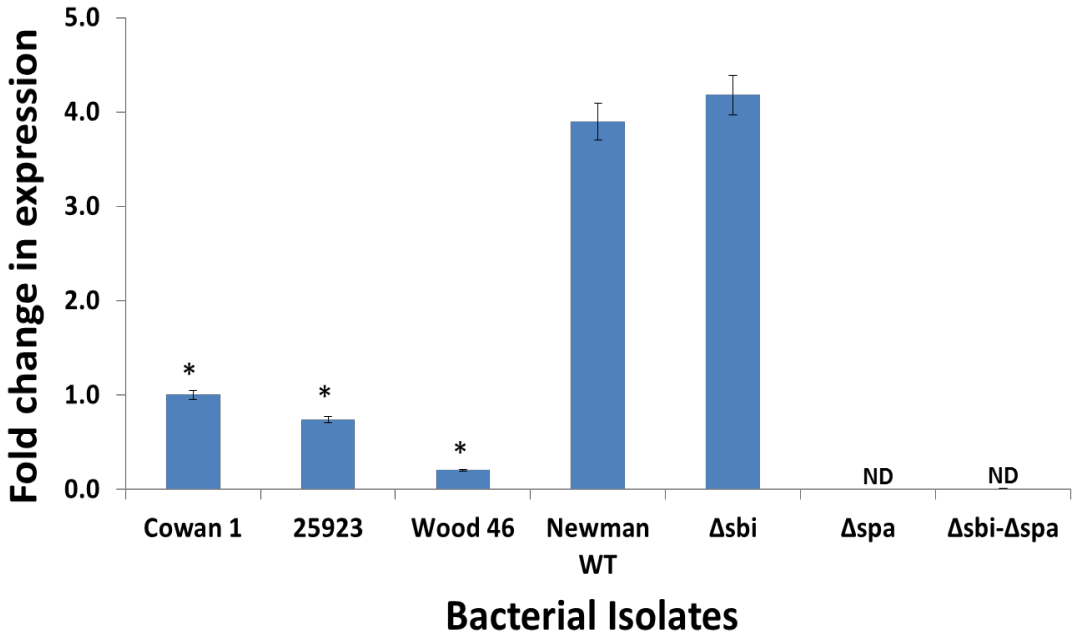

Figure 2.5 Expression levels of *spa* in *S. aureus*

Quantitative real-time PCR was used to measure the amount of *spa* mRNA in log phase bacterial cells. Relative gene expression was calculated using the $\Delta\Delta C_T$ method and expressed as fold change. 16S rRNA was used as the endogenous control. The values represent the average from three independent experiments. (*P<0.05 was considered significant). ND- not detected.

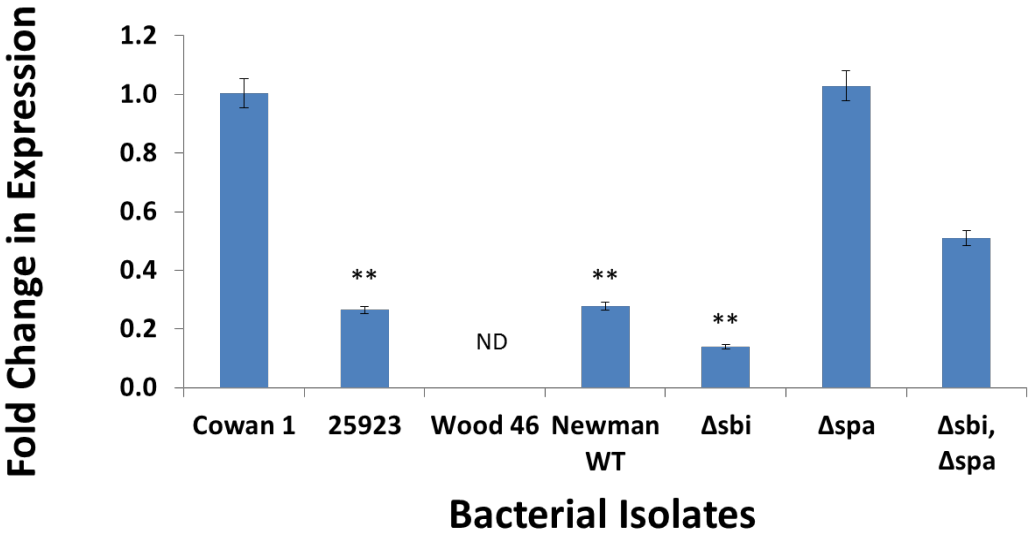

Figure 2.6 Expression levels of *srtA* in *S. aureus*.

Quantitative real-time PCR was used to measure the amount of *srtA* mRNA in log phase bacterial cells. Relative gene expression was calculated using the $\Delta\Delta C_T$ method and expressed as fold change. 16S rRNA was used as the endogenous control. The values represent the average from three independent experiments. (*P<0.05 and **P<0.02 were considered significant). ND- not detected.

(FnBP A and B) are anchored on the bacterial surface and known immune evasion factors in *S. aureus*. A binding assay was performed with FITC-labeled human fibrinogen to detect surface-expressed fibrinogen-binding protein, and HiLyte Fluor™ 488 conjugated bovine fibronectin to detect surface-expressed fibronectin-binding protein. Flow cytometry results showed that Wood 46 bound only 0.49% of labeled fibrinogen on its surface compared to the *Δ*spa isolate which bound the highest amount of fibrinogen in this study (Figure 2.7). Also, Wood 46 bound only 11.4% of the labeled fibronectin relative to Cowan 1 (considered as 100%) and Seattle 1945 (31.2%) (Figure 2.8). The Newman isolate and its corresponding deletion mutants were not tested in the fibronectin-binding assay because they have truncations in both FnBP A and FnBP B [131]. These data suggest the lack of Sortase A in Wood 46 likely results in lower amounts of not just Protein A, but other proteins that bear the LPXTG motif.

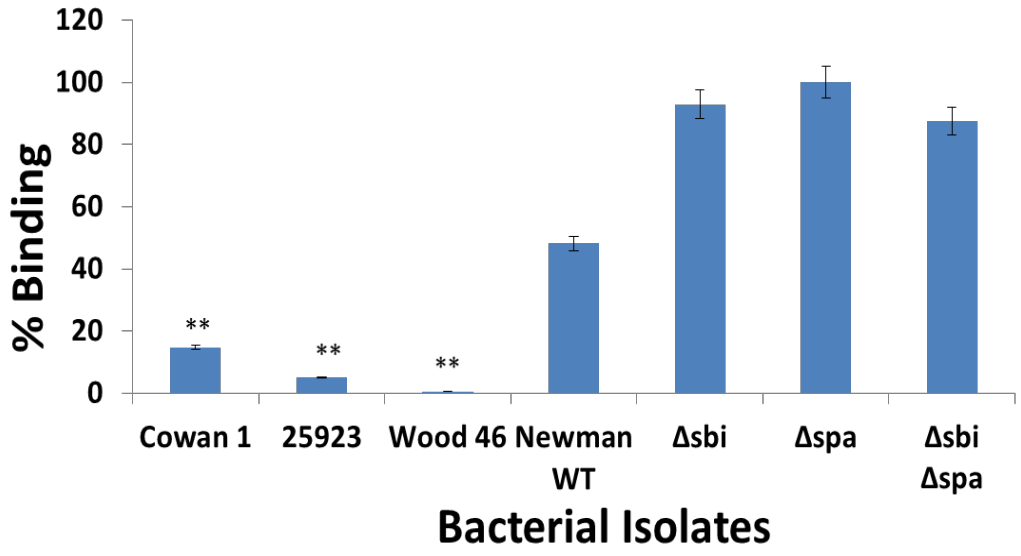

Figure 2.7 Fibrinogen-binding assay.

The amount of Sortase A-anchored, LPXTG-motif containing fibrinogen-binding protein was measured by flow cytometry using FITC-conjugated fibrinogen. The percentage binding was determined relative to highest value obtained for that experiment. The values represent the average from three independent experiments. (*P<0.05 and **P<0.02 were considered significant).

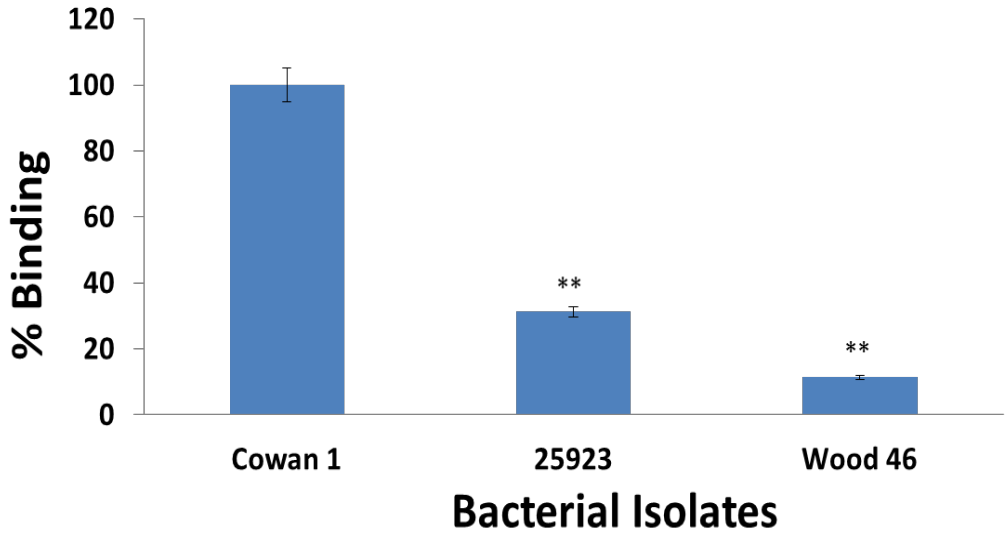

Figure 2.8 Fibronectin-binding assay.

The amount of Sortase A-anchored, LPXTG-motif containing fibronectin-binding protein was measured by flow cytometry using HiLyte Fluor ™ 488-conjugated fibronectin. The percentage binding was determined relative to highest value obtained for that experiment. The values represent the average from three independent experiments. (*P<0.05 and **P<0.02 were considered significant). *(Since the Newman isolate has truncations in both FnBP-A and FnBP-B, it was not tested in the fibronectin binding assay).*

Discussion

S. aureus produces a number of virulence factors including secreted toxins, enzymes and cell wall-associated proteins that bind to and interact with host extracellular matrix [132]. In an *in vitro* environment, the production of virulence factors is growth-phase dependent [133]. The secreted proteins are produced during post-exponential (stationary) phase wheras the cell wall-associated proteins are predominantly produced during the logarithmic growth phase [134, 135]. Many of the known virulence factors such as Protein A, fibrinogen-binding proteins like clumping Factors A and B, fibronectin-binding proteins A and B are synthesized and anchored onto the peptidoglycan cell wall by the transpeptidase enzyme, Sortase A [66, 124, 130, 136].

The *S. aureus* isolate Wood 46 has played an important role in studies designed to examine the biological function of Protein A and its role in pathogenesis and virulence. There have been contradictory reports on the production, display and secretion of Protein A [119, 137]. The results have either been ambiguous or lacked definitive experimental data. Wood 46 has been described as a *spa*-negative and Protein A-deficient strain although there is evidence that Wood 46 expresses low amounts of Protein A on its surface as evidenced by its ability to bind low amounts of IgG [119, 138]. It is also reported that Wood 46 secretes small amounts Protein A into the culture supernatant during exponential growth phase [118, 119]. Recently, several mutant strains have replaced Wood 46 as the preferred choice for negative control in both *in vitro* and *in vivo* studies. However, the mechanism for low expression of Protein A on the surface of Wood 46 had not been previously investigated.

Cell wall-associated Protein A binds to IgG via its Fc region, thereby preventing it from opsonization and phagocytosis [36]. In this study, it was observed that Wood

46 had a lower density of Protein A on its surface and secreted less Protein A compared to the Cowan 1, Seattle 1945 or Newman isolates. It was observed that even the *Δ*spa isolate bound some anti-Protein A antibody. This could be due to the ability of the antibody to react with other immunoglobulin-binding proteins on the surface of the bacterium. Secreted Protein A functions as a superantigen (SAg) [48]. It binds to VH_3-clan B-cell receptors and limits their abilities to produce antibody. Also, marginal zone B-cells and B1-cells undergo apoptosis upon exposure to Protein A [44-48, 108]. There have been reports of secreted Protein A in Wood 46 activating human B-cells in a mechanism that is different from that seen in Cowan 1 [139]. Therefore, it is important to consider the toxic effects of secreted Protein A, if Wood 46 is used as a control in animal models to test vaccine efficacy.

Conventional PCR and sequencing showed that Wood 46 possesses the *spa* gene with a complete open reading frame. The gene sequence is identical to that of Seattle 1945, Cowan 1 and Newman. qPCR for *spa* expression showed that Wood 46 does transcribe *spa* mRNA but at lower amounts compared to that of Cowan 1, Seattle 1945 and Newman isolates. Because this did not explain the low surface levels of Protein A, the transpeptidase enzyme Sortase A responsible for anchoring surface proteins on the peptidoglycan cell wall, was examined. Conventional PCR with *srtA*-specific primers showed that Wood 46 possesses the *srtA* gene with a complete open reading frame. However, it harbors a 12bp deletion at the 3'-end of the gene resulting in a corresponding loss of four amino acids at the N-terminus of the protein sequence. Further analysis of the upstream region of the *srt A* gene shows mutations in the -10 and -35 regions of the promoter and regulatory that would be expected to adversely affect binding of RNA polymerase and transcription. The functional effects of these mutations are yet to be evaluated and may be pursued at a later stage. Promoter site mutations have been associated with impaired transcription and in turn affect protein expression [127-

129, 140-143]. These mutations could affect the expression of *srtA* which in turn may be responsible for the low surface density of Protein A. If this is true, there should be a decrease in the expression of other surface proteins that harbor the LPXTG motif. This was confirmed by examining the surface expression of two known LPXTG proteins, fibrinogen-binding protein (Clf A and Clf B) and fibronectin-binding protein (FnBP A and FnBP B). Both these proteins in *S. aureus* are virulence factors similar to Protein A [62, 66, 108, 124, 125, 144]. Wood 46 expressed very low amounts of both fibrinogen-binding protein and fibronectin-binding protein on its surface suggesting that it may express lower amounts of all surface proteins that have an LPXTG motif. The complete coding sequence of the Wood 46 *srtA* gene along with the upstream promoter and regulatory region has been deposited in GenBank (accession number: KY514391). The whole genome sequence of Wood 46 has been determined and deposited in GenBank (accession number: MTFQ00000000, version MTFQ00000000.1). This could provide a wealth of information about other immunoglobulin- binding proteins with or without an LPXTG motif. It will also help identify novel genes whose products may be involved in immune evasion. Sequence information about promoters and regulatory elements of virulence genes could help explain why some strains of *S. aureus* are innately less virulent compared to other highly virulent strains.

In conclusion, the results from this study suggest that defective expression of *srtA* in Wood 46 accounts for the failure of Protein A anchoring to its surface with the majority of the Protein A being secreted. This may be accompanied by deficient anchoring of all LPXTG bearing surface proteins. The Wood 46 promoter at both the -10 and -35 regions contained mutations compared to the control strains used in this study. Promotors that vary from consensus sequences generally are associated with decreased gene expression [140, 141, 143]. Understanding the nature of surface protein expression and its role in virulence is crucial towards successful vaccine development against *S. aureus*

CHAPTER 3.

Complete Genome Sequence of *the Staphylococcus aureus* Strain Wood 46

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This chapter is a manuscript that has been accepted for publication in Genome Announcements in February 2017. My contributions to this paper include : (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) all of the writing.

Here, we report the first complete genome sequence of the *Staphylococcus aureus* strain, Wood 46. Wood 46 has played an important role in understanding the virulence and pathogenesis of *S. aureus* infections. This report will assist efforts in vaccine development against MRSA infections.

Staphylococcus aureus is a Gram-positive opportunistic pathogen that infects both humans and animals. It causes skin and soft tissue infections, and is also associated with septic arthritis, pneumonia, post-surgical and implant infections, osteomyelitis, septicemia and toxic shock syndrome [145, 146]. Methicillin-resistant *S. aureus* (MRSA) is an important concern in hospitals (HA-MRSA), communities (CA-MRSA) and among livestock (LA-MRSA) [82, 146]. New vaccine discovery efforts rely on understanding the role of surface and/or secreted proteins in pathogenesis and infection [44, 147]. The *S. aureus* Wood 46 strain was reported to be Protein A-deficient and assumed to be *spa* negative [148]. It has also been shown to have reduced virulence in animal models of *S. aureus* infection [31, 107, 117-122, 149]. To date, the reason for reduced virulence and Protein A expression in the Wood 46 strain remains unknown. Whole genome sequence of this strain could provide insights into the genes, promoters and regulatory regions involved in pathogenesis and virulence. This in turn could help understand the role of specific virulence factors during infection and identify novel vaccine targets and/or develop novel strategies to combat MRSA infections.

The *S. aureus* Wood 46 strain was sourced from the American Type Culture Collection (ATCC 10832). DNA was sequenced using Illumina MiSeq (Illumina, Inc., USA). *De novo* assemblies were individually produced and merged using Geneious (version 9.1.6) and CLC Genomics Workbench (version 9.0) (http://www.qiagenbioinformatics.com). Automated annotation was performed

using the NCBI Prokaryotic Genome Annotation Pipeline (http://ncbi.nlm.nih.gov/genome/annotation_prok).

A total of 3,985,114 high-quality reads resulted in >180-fold overall coverage. The genome is 2,824,597 bp with a 32.8% GC content. The number of predicted coding sequences (CDS) and genes (RNAs) were 2806 and 73 respectively. The Wood 46 genome shared 98%-99% identity with other published *S. aureus* genomes. The bacterium is susceptible to amoxicillin/clavulanic acid, cefoxitin, cephalothin, chloramphenicol, clindamycin, erythromycin, gentamicin, marbofloxacin, oxacillin, tetracycline and trimethroprim/sulfa, and resistant to ampicillin and cefpodoxime [150].

Nucleotide sequence accession number: The Whole Genome Shotgun project of the *S. aureus* strain Wood 46 has been deposited at DDBJ/ENA/GenBank GenBank under the accession number MTFQ00000000. The version described in this paper is MTFQ00000000.1.

CHAPTER 4.

Mass Spectrometric Analysis of the Secretome and Surface Associated Proteins in Staphylococcus *pseudintermedius*

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This chapter is a manuscript that will be submitted for publication to an appropriate journal in the Spring of 2017. My contributions to this paper include : (i) selection of isolates (ii) experimental design and set up (iii) part of the experimental testing (iv) part of the data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) all of the writing except the LC-MS/MS section.

Abstract

Staphylococcus pseudintermedius is a Gram-positive coccus and a common opportunistic pathogen in dogs. It causes skin and soft tissue infections, and is the most frequent cause of canine pyoderma. In the past few years, this organism has developed widespread resistance to methicillin. Many of the methicillin resistant *S. pseudintermedius* (MRSP) isolates are also multidrug resistant. The emergence of antimicrobial resistance has spurred the search for prophylactic methods for the control of these bacteria. Vaccine development, however, requires a deeper look into the surface properties of varying strains of *S. pseudintermedius*. *S. pseudintermedius* is known to produce a wide variety of virulence factors. Some of these are surface-associated while others are secreted.

The purpose of this study was to analyze the whole secretome (secreted proteins) and surface-associated proteins in *S. pseudintermedius* by Mass Spectrometry (MS) in an effort to identify potential vaccine candidates. Three isolates representing the major clonal populations of *S. pseudintermedius* in the United States and Europe- 06-3228 (ST68), 08-1661 (ST71) and NA45 (ST84) - were selected for this study. The specific objectives of this study were: (a) to analyze the entire bacterial secretome (b) to identify secreted immunoglobulin-binding (Ig-binding) proteins and (c) to identify proteins accessible on the surface of the bacteria.

The secretome, Ig-binding proteins and trypsin-treated proteins were compared among the three isolates using their respective genomes as reference databases. Orthologous proteins among the three isolates were identified and from this group, proteins common to all three isolates were further analyzed to determine if they were good vaccine candidates.

Introduction

S. pseudintermedius is an opportunistic pathogen residing on the skin and mucosa of dogs and is frequently isolated from the nares, mouth, pharynx, forehead, groin and anus of healthy dogs and cats [7]. When the host immune system is suppressed or weakened, or if there is a breach in the external skin barrier, it can cause severe skin and soft tissue infections. It is the leading cause of clinical infections in dogs suffering from atopic dermatitis and is implicated in the pathogenesis of canine pyoderma [7, 8, 29]. Recently, the emergence of methicillin resistance and multidrug resistance in *S. pseudintermedius* has complicated treatment. This is due to limited treatment options and the often long duration and repeated treatments that are required to clear infection, sometimes extending over 3-4 weeks along with a topical treatment [7, 8, 29, 81]. Such prolonged therapies may result in emergence of resistant bacteria. Once selected, this resistance may be horizontally transferred to other bacteria which may include human pathogens. Also, *S. pseudintermedius* has zoonotic potential and in rare cases has been reported to cause severe infections in humans, the most common route being dog bite wounds [7]. Therefore, there is an urgent need for effective methods to prevent and control *S. pseudintermedius* infections in dogs.

The development of a successful vaccine against *S. pseudintermedius* requires a thorough understanding of the ecology and epidemiology of the bacteria, knowledge of bacterial and host factors involved in pathogenesis, and the nature of immune response. In the past, crude preparations based on *S. aureus* phage lysate or *S. pseudintermedius* autogenous bacterin had shown promise as adjunctive therapies to treat pyoderma [8, 15]. An efficacious vaccine administered either prophylactically or after the onset of an infection may be the most effective way of combating canine pyoderma.

Cell-wall associated (CWA) proteins produced by Gram-positive pathogens play a crucial rule in the pathogenesis of infection [15]. Surface-exposed and secreted proteins are more likely to interact with the host immune system. Several studies with *S. aureus* have indicated that CWA proteins represent promising targets for immunotherapeutic and vaccine design [151] . Little is known about the pathogenesis of *S. pseudintermedius*. However, *S. pseudintermedius* produces a number of virulence factors, including some which are functionally similar to those produced by *S. aureus* [22]. These include enzymes such as coagulase, proteases, thermonuclease, and toxins including hemolysins, exfoliative toxins and enterotoxins. *S. pseudintermedius* produces several CWA proteins that closely resemble those of *S.aureus*, including extracellular matrix (ECM) proteins fibrinogen-binding proteins, fibronectin-binding proteins, and cytokeratin [15, 22, 29, 151]. Protein A encoded by the *spa* gene in *S. aureus*, one of the most potent immune evasion factors, also has a homologue in *S. pseudintermedius* designated as SpsQ (spa1) [15]. Along with Protein A, another immunoglobulin-binding protein, Sbi, in *S. aureus*, designated SpsK in *S. pseudintermedius* also functions as an immune evasion factor. Both these proteins are found abundantly on the bacterial surface but are not good vaccine candidates because of their toxicity and B-cell super-antigen activity [151].

Vaccine studies with *S. aureus* suggest that multicomponent vaccine would be advantageous over killed or attenuated whole bacterial vaccine [152, 153]. Recent advancements in genome sequencing have made it possible to identify novel vaccine targets using reverse vaccinology. This technology has successfully been employed in *S. aureus* and vaccine development based on surface-exposed and secreted proteins have seen success in mouse models [154, 155]. The other important consideration during vaccine development is identification of targets conserved among a variety of strains. The identification of orthologous groups in prokaryotic genomes allows cross-referencing of genes from multiple

species/strains and facilitates genome annotation. Further, it makes possible the classification of protein families, bacterial evolution and identification of novel targets for antimicrobial drug development [156-159]. Mass spectrometry has also emerged as a powerful and efficient tool for analyzing proteins to identify biomarkers and vaccine targets [160].

In the present study, mass spectrometry was used to identify the surface proteins and secreted proteins from three strains of *S. pseudintermedius*; 06-3228 (ST68), 08-1661 (ST71) and NA45 (ST 84) which are representatives of the major clonal populations in the United States and Europe. Comparative analysis of the surface proteins and secreted proteins among these isolates could provide a wealth of information about strain similarities and differences, and identify novel proteins that could serve as vaccine targets.

Materials and Methods

Bacterial Strains and Growth Conditions

S. pseudintermedius clinical isolates 06-3228 (ST68), 08-1661 (ST71) and NA 45 (ST 84) were used for this study. The isolates were obtained from the Clinical Bacteriology Lab at the Veterinary Medical Center, University of Tennessee, Knoxville. Bacterial colonies from blood agar plates were inoculated into 5ml of sterile Trypticase Soy Broth (TSB) (BD Biosciences, San Jose, CA) and incubated overnight at 37°C with shaking at 225rpm (Excella E24 Incubator Shaker, New Brunswick Scientific). Fifty microliters of overnight culture were inoculated into 5ml of fresh, sterile TSB to initiate log-phase bacterial cultures. The bacteria were grown at 37°C with shaking at 225rpm until an optical density of OD_{600} = 0.4-0.6 was reached.

Preparation of Whole Secretome

Log phase bacterial cultures were centrifuged at 10,000xg for 30 minutes at 4°C (Sorvall RC-5C Plus Superspeed Centrifuge). Without disturbing the bacterial pellet, the supernatant was collected and passed through a 0.45µm filter (Whatman, GE Healthcare Lifesciences, Pittsburgh, PA). The filtrate was concentrated using an Amicon® Ultra-4 centrifugal filter (EMD Millipore Corp., Billerica, MA) and stored at -20°C until further analysis.

Purification of Immunoglobulin-Binding Proteins from Bacterial Culture Supernatants

Log phase bacterial cultures were centrifuged at 10,000xg for 30 minutes at 4°C. The supernatant was passed through an IgG-sepharose column (GE Healthcare Lifesciences, Pittsburgh, PA). Briefly, the column was equilibrated with five bed volumes of binding buffer containing 50 mM Tris, 2.7 mM potassium chloride and 0.137 M NaCl pH 8.0. The bacterial supernatant was added to the column to allow attachment of IgG binding proteins. Unbound proteins were washed off with 15 bed volumes of binding buffer. The bound proteins were eluted in 0.1-0.2 M Glycine/HCl pH 2.5-3.0. The pH of the elution fraction was adjusted to 7.0 using 0.5M Tris and stored at -20°C until further analysis.

Purification of Trypsin-Treated Surface Proteins

Log phase bacterial cultures were centrifuged at 4,200xg for 3min and washed three times with PBS. The pellet was suspended in 1ml digestion buffer (0.6M sucrose buffered with 50mM Tris, pH 7.5). Two hundred microgram of porcine trypsin (Sigma-Aldrich, St.Louis, MO) was added and the cells were digested for 1

hour at 37°C. Protease inhibitor cocktail set IV (Calbiochem, EMD Millipore Corp, Temecula, CA) was then added to the mixture at a final concentration of 0.1µg/ml. The supernatant containing tryptic peptides was stored at -20°C until further analysis.

Biotin Labeling of Surface Proteins

Log phase bacterial cells were harvested by centrifugation at 10,000xg for 1min and washed twice with ice-cold phosphate buffered saline (PBS) pH 8.0. One hundred microliters of 10mM EZ-Link Sulfo-NHS-LC-Biotin reagent (Thermo Scientific, Waltham, MA) per 1ml of cell suspension was added and incubated for 30min at room temperature. The cells were washed three times with PBS and 100mM glycine was added to quench and remove excess biotin. The biotin-labeled bacteria were stored in aliquots at -20°C until further use. The biotin-labeled proteins were digested with trypsin as described previously and were stored at -20°C until further use.

Purification of Biotin-Labeled Surface Proteins Using Monomeric Avidin

Biotin-labeled surface proteins were purified using a monomeric avidin column (Pierce Monomeric Avidin Kit, Thermo Scientific, Waltham, MA) as per manufacturer's protocol. Briefly, the monomeric avidin column was washed with 8ml of PBS followed by the addition of 6ml of biotin blocking buffer to block any non-reversible biotin binding sites. The excess biotin was removed from the reversible binding sites by addition of 12ml of regeneration buffer. The biotin-labeled, trypsin-cleaved proteins were loaded onto the column. Unbound proteins were washed off with PBS. The bound proteins were eluted using elution buffer in 6 X 2ml fractions. The eluates were stored at -20°C until further use.

LC-MS/MS Analysis

Samples interrogating *S. pseudintermedius* supernatant and surface-associated proteins were prepared for shotgun liquid crystallography-mass spectrometry (LC-MS/MS) analysis. Trypticase soy broth (media alone) and bacterial culture supernatant passed through control Sepharose beads (without IgG) served as controls. Prior to protein denaturation with sodium dodecyl sulfate (SDS), samples were concentrated / solvent exchanged on molecular weight cut-off (MWCO) spin filters (Vivaspin, GE Healthcare Lifesciences, Pittsburgh, PA) that retain proteins/polypeptides > 5kDa in size (~ 50 amino acids or larger). Retained proteins were then re-suspended in 4% SDS, 100mM Tris-HCl, 5mM dithiothreitol (DTT), transferred to microcentrifuge tube, and incubated at 95°C for 5min. Iodoacetamide was then added to a final concentration of 15mM and samples incubated for 20 min in the dark at room temperature. Denatured and reduced proteins were then digested to peptides with 2µg of proteomics-grade porcine trypsin (Sigma-Aldrich, St.Louis, MO) for 4 hours. Following digestion, peptide samples were adjusted with 200mM NaCl-0.1% formic acid and passed through a 10kDa MWCO Vivaspin2 spin filter to collect appropriately sized tryptic peptides for LC-MS/MS analysis.

With the aid of a pressure cell, peptides were loaded onto a biphasic MudPIT back column containing both strong-cation exchange and reversed-phase (RP) resins-5u Luna or 5u Kinetex, respectively (Phenomenex, Torrance, CA) and separated as previously detailed [161]. Briefly, loaded samples were washed offline then placed in line with an in-house pulled nanospray emitter packed with 15cm of RP resin. Peptides were then separated and analyzed with either a 2-step MudPIT LC-MS/MS protocol (salt cuts of 50mM and 50mM ammonium acetate) over a 4-hr period (supernatant samples) or a 1-step protocol (500mM ammonium acetate) over a 2-hr period (surface proteins and pulldown samples). All samples were measured with a hybrid LTQ XL-Orbitrap (Thermo Scientific, Waltham, MA) mass

spectrometer (MS) as follows: data-dependent acquisition, 1 full scan followed by 10 MS/MS scans. Orbitrap mass analyzer was set to 15K resolution, LTQ isolation window = 2.2 m/z, monoisotopic precursor selection, dynamic exclusion window, duration, and max = 3 m/z, 60s (30s for supernatant samples), and 500 respectively. The immunogenicity of peptides was predicted using the POPI v.2.0 web server.

Results

LC MS/MS Data Analysis

LC-MS/MS spectra were searched against sample-specific proteome databases [103] concatenated with common protein contaminants using MyriMatch v.2.2 [162]. Peptide spectrum matches (PSM) were filtered and assembled to proteins using IDPicker v.3.0 [163] with false-discovery rates, as assessed by evaluating MS/MS spectra that matched to reversed decoy sequences, controlled at < 1% at the peptide-level. Protein calls required two distinct peptides at a minimum. Peptide intensities were assessed by chromatographic area-under-the-curve (AUC) using IDPicker's embedded spectra/label-free quantification option and protein abundances derived via summation of constituent peptides intensities. To qualitatively compare the proteins identified across each strain of *S. pseudintermedius*, OrthoMCL (OrthoMCL Database, Version 5.0) [164-166] was employed to identify orthologous protein sequences. Identified protein data for each specific *S. pseudintermedius* sequence type were then re-cast into their orthologous groups and compared across all sample types to identify proteins common to all three pathogenic strains – with special emphasis on those that are surface-associated and/or secreted. To help winnow down the list of potential secreted and surface-bound antigen targets, PSORTb v.3.0 [167] was used to assign subcellular location to each identified protein.

Identification of Surface Proteins, IgG-Binding Proteins and Extracellular (Secreted) Proteins by Mass Spectrometry

Using the analysis method described above, a total of 580 proteins were identified in the isolate 06-3228 (ST68), 596 in 08-1661 (ST71) and 542 in NA45 (ST84). The number of proteins in each fraction is listed in Table 4.1 and the percentage of proteins in each fraction for individual isolates, 06-3228 (Figure 4.1), 08-1661 (Figure 4.2) and NA45 (Figure 4.3) is depicted.

Orthologous Proteins in 06-3228, 08-1661 and NA45

In order to narrow down the spectrum of proteins for analysis across all three sequence types, orthologous protein prediction was performed using the web-based tool OrthoMCL. A total of 364 predicted orthologous proteins were identified across all three sequence types. Excluding proteins that were classified as cytoplasmic in origin, there were 72 proteins predicted to be either secreted (17/72), cell wall-associated (10/72) or of unknown cellular location (45/72) across all three sequence types. The percentage of orthologous proteins based on their location is shown in Figure 4.4. Isolate 06-3228 had at least 10 proteins that were predicted to be cell wall-associated and 16 extracellular proteins. Isolate 08-1661 had 10 proteins predicted to be cell wall-associated and 17 extracellular proteins while isolate NA45 had 9 predicted cell wall-associated proteins and 14 extracellular proteins. From this list, eight proteins common to all three isolates were identified that could potentially function as antigens. This includes: predicted cell wall-anchored protein SasF (LPXAG motif), beta-hemolysin, gamma-hemolysin component B, secretory antigen precursor, SsaA, fibronectin-binding protein FnbB, oligopeptide ABC transporter, virulence-associated cell wall-anchored protein SasH (LPXTG motif) and exotoxin 15.

Table 4.1 Total number of predicted proteins in each of the fractions analyzed by mass spectrometry for isolates 06-3228, 08-1661 and NA45

Isolate	Sequence Type	Secreted Proteins	IgG-Binding Proteins	Surface Proteins	Biotinylated Surface Proteins	Total
06-3228	ST68	202	38	234	106	**580**
08-1661	ST71	237	26	274	59	**596**
NA45	ST84	162	66	260	54	**542**

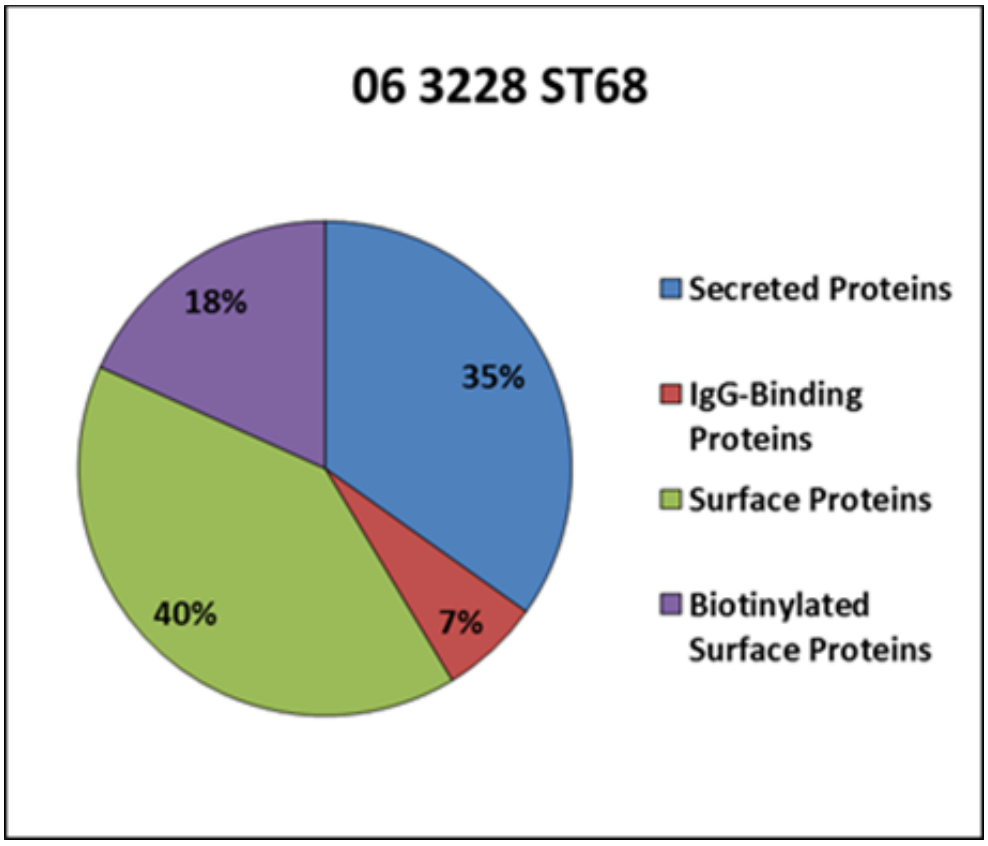

Figure 4.1 Percentage of proteins predicted in each fraction for isolate 06-3228

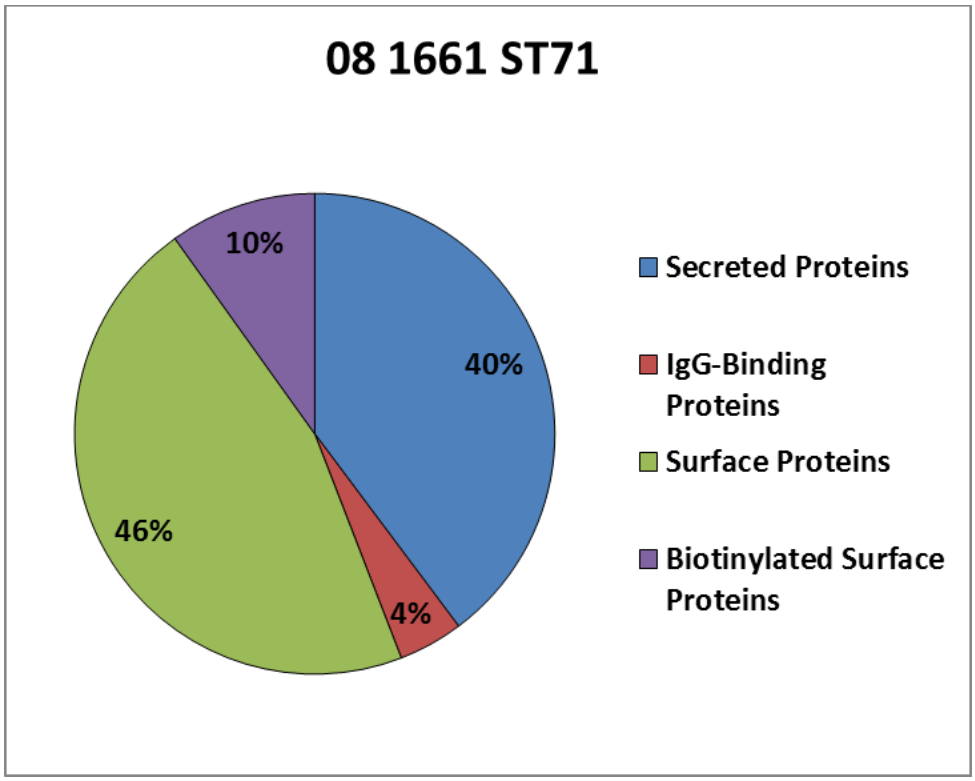

Figure 4.2 Percentage of proteins predicted in each fraction for isolate 08-1661

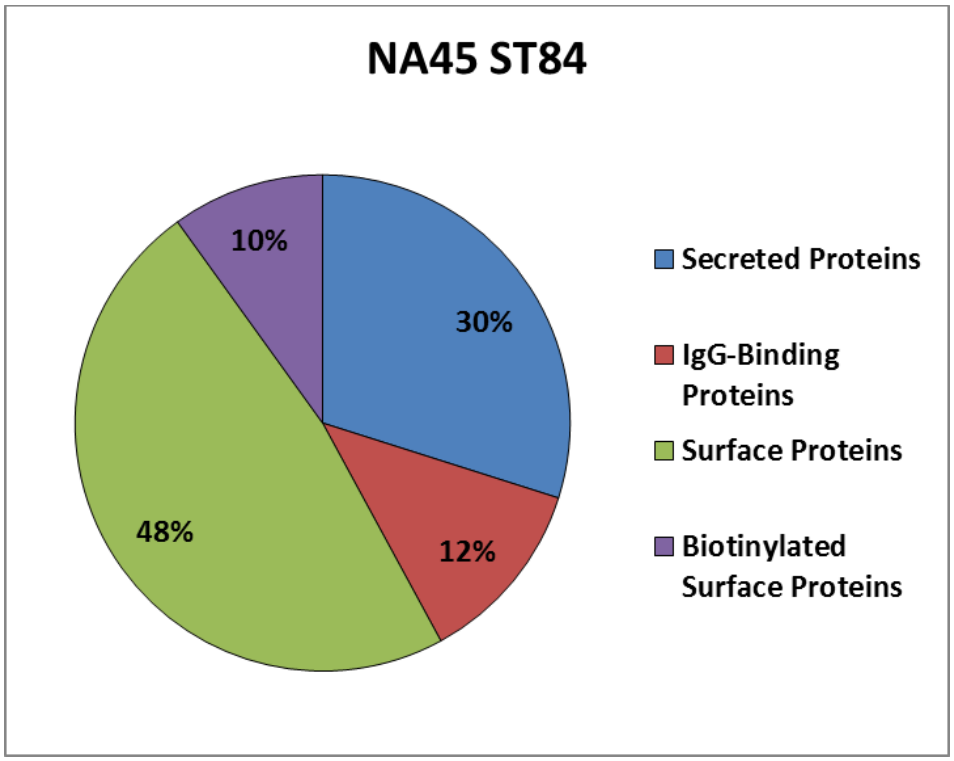

Figure 4.3 Percentage of proteins predicted in each fraction for isolate NA45

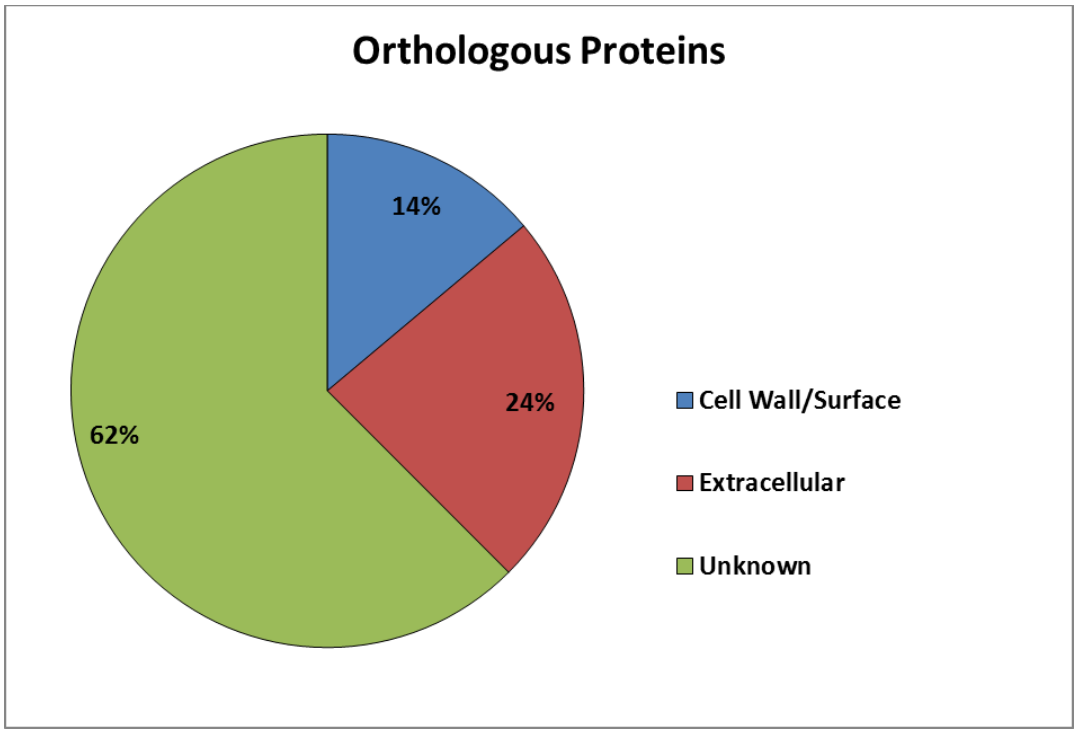

Figure 4.4 Percentage of predicted orthologous proteins across all three isolates.

Additionally, three proteins, putative peptidoglycan bound protein (LPXTG) motif Lmo1799 homolog, immunodominant staphylococcal antigen A precursor and zinc metalloproteinase precursor/aureolysin) that were identified only in isolates 08-3228 and 08-1661 could also be potential antigenic targets (Table 4.2).

Identification of Peptides with High Immunogenic Potential

Peptide sequences from the eight proteins were tested for their immunogenicity using the online immunogenicity prediction tool POPI v.2.0. Sequences corresponding to SasF, SsaA, SasH, FnBP and exotoxin 15 are predicted to be good vaccine candidates. Taken together, the results suggest that Mass Spectrometry, along with orthologous protein prediction could be a good strategy to scan for potential vaccine targets in *S. pseudintermedius*.

Table 4.2 List of orthologous proteins identified across all three isolates with their predicted cellular location

	Predicted Protein	**Cellular Location**
All 3 sequence types	Predicted cell-wall-anchored protein SasF (LPXAG motif)	Cell wall
	Virulence-associated cell-wall-anchored protein SasH (LPXTG motif)	Cell wall
	Fibronectin binding protein FnbB	Cell wall
	Oligopeptide ABC transporter	Cell wall
	Beta-hemolysin	Extracellular
	Gamma-hemolysin component B	Extracellular
	Secretory antigen precursor SsaA	Extracellular
	Exotoxin 15	Extracellular
ST68 and ST 71	Putative peptidoglycan bound protein (LPXTG motif) Lmo1799 homolog	Cell wall
	Immunodominant staphylococcal antigen A precursor	Extracellular
	Zinc metalloproteinase precursor/aureolysin	Extracellular

Discussion

The increasing prevalence of methicillin resistance and multidrug resistance among staphylococci has given rise to 'superbugs' that can no longer be treated with conventional antibiotics. This is especially difficult in veterinary medicine where the number of antibiotic choices available to treat *S. pseudintermedius* infections is limited. Therefore, it has become important to seek alternative treatment strategies, such as preventative or therapeutic vaccines. In the past, attempts to generate *S. aureus* vaccines based on whole-cell determinants and/or virulence factors have failed to induce protective immunity in humans, although they have demonstrated promising results in animal models [44, 84-86, 168, 169]. A multivalent vaccine is considered the best strategy for vaccine development against *S. aureus* [112, 153, 169-171]. A successful vaccine against staphylococcal infections should be able to elicit three major immune responses (a) humoral immune response i.e., antibodies to directly inhibit bacterial viability and/or toxicity (b) antibodies to mediate opsonophagocytosis and (c) a cell-mediated T-helper 1 (Th1)/Th17 response. Identifying novel vaccine candidates requires extensive analysis of the proteome of *S. pseudintermedius*. Surface proteins, particularly cell wall-associated proteins and secreted proteins are most likely to come in contact with the host, and therefore contribute to the virulence and pathogenesis. Keeping these factors in mind, the present study analyzed the secretome and surface proteins in three distinct isolates, 06-3228 (ST68), 08-1661 (ST71) and NA45 (ST84) of *S. pseudintermedius* by mass spectrometry to identify novel vaccine targets. Bannoehr et al., had employed a combination of genomic and proteomic approach to analyze the surface proteins in the *S. pseudintermedius* strain ED99 [15]. They were successful in identifying 60 surface proteins, including those involved in adhesion.

In the present study, the secretome and surface-associated proteins in three different *S. pseudintermedius* isolates was analyzed by mass spectrometry. LC-

MS/MS analysis of surface-associated/exposed supernatant and proteins expressed by *S. pseudintermedius* sequence types identified a total of 364 orthologous proteins across all sample types. As revealed by PSORT, a large majority of these proteins were cytoplasmic in origin and were largely identified in the supernatant proteomes of each subspecies – a finding that often results from natural and unavoidable cell lysis over the course of organism growth. These cytoplasmic "contaminants" are known to be abundant and are generally involved in central carbon metabolism and organism biogenesis. As these proteins are irrelevant to this current study, only those proteins classified a non-cytoplasmic in origin, i.e. those that are cell wall-associated, membrane-associated, extracellular or unknown/unclassified were the focus of this study. When confining our view to only these non-cytoplasmic proteins, only 72 proteins remained. As the goal of this study was to identify secreted or surface-bound protein antigen targets for vaccine development against pathogenic (MRSP) strains of *S. pseudintermedius*, these 72 proteins were analyzed in the context of the various enrichment strategies discussed above with particular attention paid to those that were surface-exposed and/or secreted, found across all three sequence-types. Eight candidate proteins were identified that could serve as viable antigen targets. These peptides are as yet to be tested for their immunogenic potential. Proteins that were annotated as hypothetical or those predicted with an unknown cellular location were excluded from this study. Further characterization of these hypothetical proteins is required to determine if any of them are good vaccine candidates.

Using a combination of mass spectrometry and orthologous protein prediction, it was possible to identify novel antigenic targets in three *S. pseudintermedius* isolates representing three distinct sequence types. Using the same strategy, it is possible to determine if all clinically relevant *S. pseudintermedius* strains express these proteins in high frequency which will further aid in the development of a multicomponent vaccine against *S. pseudintermedius* infections.

CHAPTER 5.

Identification of *Staphylococcus pseudintermedius* Sortase A Inhibitors

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This chapter is a manuscript that will be submitted for publication to an appropriate journal in the Spring of 2017. My contributions to this paper include : (i) selection of isolates (ii) experimental design and set up (iii) experimental testing (iv) data analysis (v) gathering and review of literature (vi) formulation of discussion topics (vi) all of the writing except the section on homology modeling.

Abstract

Conventional antibiotic-based therapies against *S. pseudintermedius* infections have failed in recent years, due to the increased prevalence of methicillin resistance and multidrug resistance. Therefore, alternate strategies for treatment have to be explored. Anti-infective therapy based on sortase inhibition holds potential to address this shortcoming. Sortase A (SrtA) is a transpeptidase, commonly produced by Gram-positive bacteria, that recognizes proteins with a C-terminal LPXTG motif and anchors them on the peptidoglycan cell wall. SrtA substrates include numerous virulence factors and proteins that may overcome the host immune response including Protein A, fibrinogen binging proteins and fibronectin binding proteins.

In the present study, a *srtA* gene from *S. pseudintermedius* was synthesized, inserted into an expression vector and expressed in *E.coli*. A commercially available synthetic substrate for SrtA, Abz-LPETG-K(Dnp)-NH_2, was used to assess the function of the recombinant enzyme. Using molecular dynamic simulations, a 3-D homology model of SrtA was created to screen a library of compounds from drug databases for potential inhibitors. From the predicted top twenty most active inhibitors, ten were selected and tested for their ability to inhibit sortase *in-vitro*. Four out of ten compounds showed ~50% inhibition of recombinant SrtA activity. These compounds were further evaluated for their ability to inhibit SrtA in bacterial culture. The results indicate the possibility of SrtA inhibition as a potential therapeutic strategy in the treatment of staphylococcal infection in both humans and animals.

Introduction

S. pseudintermedius is a skin and mucous membrane commensal of dogs and is the organism most frequently associated with atopic dermatitis and wound infections in dogs. It is also implicated in the pathogenesis of canine pyoderma [7, 8]. In the past, antimicrobial agents have been used to successfully control *S. pseudintermedius* infections. However, in recent years, there has been an increased prevalence of methicillin resistance and multidrug resistance among *S. pseudintermedius* isolates [5, 7, 80, 95, 98, 172]. This has made treatment of infection difficult due to the limited number of antimicrobial agents used in veterinary medicine [173]. Therefore, there is an urgent need for the development of non-antibiotic based treatment strategies to combat *S. pseudintermedius* infections.

Surface proteins in *S. aureus* harboring a C-terminal sorting signal with an LPXTG motif are covalently attached onto the peptidoglycan cell wall by the enzyme SrtA which is encoded by the *srtA* gene [62, 69]. This 618bp gene encodes a protein of 206 amino acids with a molecular weight of 23.59kDa. The enzyme is a cysteine transpeptidase that recognizes the conserved LPXTG motif in surface proteins and specifically cleaves between the threonine (T) and glycine (G) residues. The active site cysteine of SrtA forms an acyl enzyme intermediate that is relieved by the nucleophilic attack of the amino group of the pentaglycine cross bridge in peptidoglycan synthesis precursors. The N-terminus of the enzyme is located in the cytoplasm while the C-terminal enzymatic region is located across the plasma membrane (type II membrane topology) [62]. His^{120}, Cys^{184} and Arg^{197} form the catalytic triad of the enzyme and are found to be conserved among sortases in *S. aureus* [62, 68]. Surface proteins attached to peptidoglycan precursors are then incorporated into the bacterial cell wall and displayed on the surface [58, 62, 63, 65, 66, 68, 70, 124]. There are at least 17-21 surface proteins in *S. aureus* that harbor the LPXTG motif. Most of these proteins are involved in virulence and

pathogenesis [62, 124]. SrtA is essential for the functional assembly of all surface proteins harboring an LPXTG motif, and therefore plays a crucial role in the pathogenesis of *S. aureus* infections. *S. aureus* mutants that do not have a functional *srtA* gene fail to assemble surface proteins on to the peptidoglycan cell wall. Also, they are unable to form abscess lesions in organ tissues or cause lethal bacteremia in mice models of *S. aureus* infections [57]. Therefore, inhibition of SrtA could result in defective display of all the proteins harboring the LPXTG motif and render the organism less virulent. SrtA inhibitors, therefore, may be useful as anti-infective agents particularly in cases where there is methicillin resistance and multidrug resistance, and disrupt the pathogenesis of bacterial infections without affecting microbial viability [57, 70, 174]. The earliest described inhibitors of SrtA include methane-thiosulfonates such as MTSET, and *p*-hydroxymercuribenzoic acid. All these compounds interact with Cys^{184} and render the enzyme inactive [62]. Other natural or chemical compounds have also been examined for their abilities to inhibit SrtA [62, 175]. Although identification of inhibitors for SrtA has been ongoing for several years, it has gained momentum in the last decade due to the advancements in genomics and proteomics [56].

SrtA in *S. pseudintermedius* has not been previously examined. In this study, the *srtA* gene in *S. pseudintermedius* was identified, cloned and expressed in *E. coli*. Molecular dynamic (MD) simulation using the bioinformatics tool MOE (Mathematical Operating Environment) was used to generate a 3-D homology model of *S. pseudintermedius* SrtA using the existing 3-D model of *S. aureus* SrtA. After identifying the active site of *S. pseudintermedius* SrtA, scaffold hopping and molecular docking were combined to virtually screen the drug databases NCI (National Cancer Institute) and Zinc to identify potential inhibitors of *S. pseudintermedius* SrtA. Ten potential inhibitors were identified and purchased from NCI. Of these ten compounds, four inhibited recombinant SrtA in an *in vitro*

functional assay using a synthetic SrtA substrate Abz/DNP (Abz-LPETG-K(Dnp)-NH_2 by at least 50%. These inhibitors were further tested for their ability to inhibit SrtA in actively growing bacterial cultures. One of the compounds, 1847, showed at least 50% inhibition of SrtA in actively growing bacterial cultures, suggesting the possibility of SrtA inhibition as an anti-infective therapy against *S. pseudintermedius* infection.

Materials and Methods

Identification of the *srtA* gene in *S. pseudintermedius*

Whole genome sequences of several *S. pseudintermedius* isolates representing the major clonal populations in the United States and Europe were available for analysis [103]. The *srtA* gene from these isolates was identified by a combination of BLAST (using the *S. aureus srtA* gene as reference) and conserved domain search. Multiple sequence alignment (MSA) of the *srtA* gene from various isolates was performed to generate a consensus sequence. This sequence was used to design primers for conventional and quantitative real-time PCR (qPCR) assays.

Bacterial Strains and Growth Conditions

S. pseudintermedius clinical isolates (Table 5.1) were selected based on their multilocus sequence types [6]. Bacteria from blood agar plates were used to inoculate trypticase soy broth (TSB, Becton Dickinson, Franklin Lakes, NJ) and were grown at 37°C overnight with shaking at 225rpm. After an overnight incubation, 50μl of broth culture were inoculated into 5ml of sterile TSB and incubated for 3hr at 37°C with shaking to obtain log phase cultures (OD_{600} = 0.4-0.6) which were used for all experiments.

Table 5.1 Bacterial isolates used in this study along with their sequence type and methicillin resistance profile

Strain	Sequence Type	Methicillin Resistance
06 3228	ST68	R
08 1294	ST68	R
08 521a	ST68	R
E140	ST71	R
08 1661	ST71	R
NA16	ST71	R
NA12	ST64	R
NA45	ST118	R
KM 241	ST73	S
ED99	ST25	S

DNA and RNA Extraction for Conventional and Real-Time PCR

Bacterial DNA was extracted from overnight cultures using a commercial kit (MoBio Ultra Clean DNA Isolation Kit, MoBio, Carlsbad, CA) according to the manufacturer's protocol. PCR for *srtA* gene was performed using the following primers. SP sortase fwd: 5'-ACTGCGATAAACAGTAACCACTA-3' and SP sortase rev: 5'-CGCTCTCCAACACCATTAAC-3'. The following cycling conditions were performed: initial denaturation at 95°C for 90s followed by 30 cycles of annealing at 55°C for 30s and extension at 72°C for 1min, and a final extension at 72°C for 5min. PCR products were sequenced (The University of Tennessee, Genomics Core Facility), aligned, and compared using Geneious 7.1.7.0 (Biomatters, Auckland, New Zealand).

Total RNA was extracted from log phase bacterial cultures using a commercial kit (MoBio Ultra Clean RNA Isolation kit, MoBio, Carlsbad, CA) according to the manufacturer's protocol. Quantitative Real-Time PCR (qPCR) for *srtA* was performed using the TaqMan RNA-to-C_T 1-Step kit on an ABI StepOne PCR System (Applied Biosystems, Foster City, CA) using the following primer-probe set. Probe: 5'-/56-FAM/TCGCTATTG/ZEN/CGGGACATACGAATT/3IABkFQ/-3', Primer 1: 5'-GGATGAATCACTCAAAGACC-3' and Primer 2: 5'-CGCTTTGCTTAACTCTGTAA-3'. The following cycling conditions were performed: 48°C for 30min, 95°C for 10min, 95°C for 15s, 60°C for 1min (40 cycles). qPCR data were analyzed by the $\Delta\Delta C_T$ method and normalized against 16S rRNA as the endogenous control as previously described [104].

SrtA Expression and Purification

A synthetic *srtA* gene for *S. pseudintermedius* Sortase A with a 6X-His tag at the 3' end was designed, codon optimized for expression in *E. coli*, synthesized and inserted into the expression vector pET11a (GenScript Inc., Piscataway, NJ). Briefly, 5ng of the pET11a plasmid harboring the *srtA* gene was transformed into competent *E. coli* BL21 (DE3) (New England BioLabs, Ipswich, MA) by heat shock for exactly 10s at 42°C. The mixture was immediately placed on ice for 5min and 950µl of SOC outgrowth medium (New England BioLabs, Ipswich, MA) was added, and allowed to incubate at 37°C with shaking for 1hr. After 1hr, 25µl and 75µl of the transformed *E.coli* was plated on pre-warmed LB-amp (Luria-Bertani with 100µg/ml ampicillin) plates and incubated overnight at 37°C.

Recombinant colonies were picked and inoculated into LB-amp broth and grown overnight at 37°C on a shaker incubator at 225rpm. One milliliter of overnight culture was inoculated into 100ml of LB-amp broth and allowed to grow till an OD_{600}=0.4-0.6 was reached. Protein expression was induced by adding 1mM isopropyl 1-thio-β-d-galactopyranoside (IPTG, Sigma-Aldrich, St.Louis, MO) at 30°C for ~4hr. Cells were harvested by centrifugation at 10,000xg for 30min. The bacterial pellet was resuspended in 5ml of Bug Buster solution (Millipore-Novagen, Temecula, CA) along with protease inhibitor cocktail IV (Millipore-Calbiochem, San Diego, CA) and incubated at room temperature for 20-30min with shaking to facilitate cell lysis. The lysate was clarified by centrifugation at 10,000g for 30min at 4°C and recombinant protein was enriched on an Ni-TED column (PrepEase His-tagged protein purification kit, Affymetrix Inc, Santa Clara, CA) and His-tagged SrtA was eluted from the column as per the manufacturer's protocol. The purity of the protein was determined by SDS-PAGE and western blot using monoclonal anti-His antibody-HRP conjugate (Thermo Scientific, Rockford, IL).

Homology Modeling of Sortase A and Virtual Screening for Sortase A Inhibitors

The predicted *S. pseudintermedius* SrtA protein sequence was compared to that of *S. aureus* SrtA using the BLAST algorithm and conserved domain search feature in NCBI. The proteins share 57% identity at the amino acid level. His^{114}, Cys^{177} and Arg^{190} are predicted to be a part of the catalytic triad site involved in attacking the LPXTG motif in *S. pseudintermedius* [68]. Molecular Dynamic (MD) simulation was performed using the bioinformatics tool MOE (Molecular Operating Environment) to generate a 3-D homology model of *S. pseudintermedius* SrtA. Enzyme coordinates were mapped and the catalytic and active sites including the amino acids involved in substrate binding were predicted. The enzyme structure at one of many coordinates was fixed and this structure was initially used to screen the drug database NCI (National Cancer Institute). Compounds predicted to bind to the active site were identified. Initially, from the 200 highest ranked compounds, twenty were selected. Of the 20, the 10 most active compounds were purchased and tested in the *in vitro* assay.

For the second iteration, compounds that showed >50% inhibition in the *in vitro* assay were selected. The common structural features of these compounds were identified in an attempt to find inhibitors with higher binding affinities for SrtA. With the refined search parameters, the drug database Zinc was screened. Ten compounds were evaluated for their inhibitory potential in the *in vitro* assay.

Enzymatic Activity of Sortase A

The functional activity of recombinant SrtA was performed in 96-well black microtiter plates with clear bottom (Corning, Fisher Scientific, Hampton, NH) in a final reaction volume of 200µl. Fluorescent- quenched bacterial sortase substrate

III, Abz/DNP (Abz-LPETG-K(Dnp)-NH_2 (GenScript Inc., Piscataway, NJ) dissolved in isopropanol was used for K_m determination. Varying concentrations of the substrate (2.5μM - 200μM) were incubated at 25°C with 1μM of SrtA diluted in assay buffer (50mM Tris-HCl, 150mM NaCl, 5mM $CaCl_2$, and 0.1% Triton X-100, pH 7.5). The increase in fluorescence was measured in a kinetic assay for 20min at 2min intervals using a Synergy HTX multimode reader (BioTek, Winooski, VT) at wavelengths 320 excitation/420 emission. The initial velocity V_0 was determined from the linear portion of the curve and the K_m was determined using the Michaelis-Menten equation where $V=V_{max}[S]/([S]+K_m)$.

In vitro Sortase Inhibition

The assay developed by Kruger et al. was modified and used to determine the activity of recombinant SrtA [176]. Bacterial sortase substrate III, Abz/DNP (Abz-LPETG-K(Dnp)-NH_2 (GenScript Inc., Piscataway, NJ) was dissolved in isopropanol to a final concentration of 1mM. Recombinant SrtA was pre-incubated with varying amounts of the inhibitor at 37°C for 30min. One hundred micromolar of the FRET substrate was added to the wells of a 96-well black microtiter plate with clear bottom (Corning, Fisher Scientific, Hampton, NH). One hundred microliters of the SrtA-inhibitor mixture was added and the incubated at room temperature for 20min. The amount of fluorescence was measured using a Synergy HTX multimode reader (BioTek, Winooski, VT) at wavelengths 320 excitation/420 emission. A titration experiment was performed to determine the optimal amount of the enzyme and inhibitor to be used for each assay.

Sortase Inhibition in Bacterial Cells

Bacterial colonies from blood agar plates were inoculated into 5ml of TSB and grown overnight at 37°C with shaking. Fifty microliters of overnight culture were inoculated into 5ml of fresh TSB along with 100µg/ml of the inhibitor and allowed to grow until log phase (OD_{600} = 0.4-0.6) was reached. One milliliter of culture was aliquoted into a micro centrifuge tube and centrifuged at 10,000xg for 1min. The pellet was washed twice and suspended in 1ml of PBS. One hundred microliters of chicken anti-protein A antibody conjugated to FITC (Gallus Immunotech, Cary, NC) was added and incubated at room temperature for 30min in the dark. Unbound antibody was washed off and the pellet was suspended in a final volume of 1ml PBS. The amount of bound fluorescent antibody was measured by flow cytometry using an attune acoustic focusing cytometer (Applied Biosystems, Foster City, CA) at excitation/emission wavelengths of 488/519 nm. Data were acquired in linear mode for forward and side scatter and logarithmic mode for fluorescence. For each sample, 10,000 events were measured. Also, for each isolate, cells incubated with PBS instead of the inhibitor served as the negative control to distinguish between inhibition and non-inhibition.

Statistical Analysis

A one-way ANOVA and multiple comparisons using Tukey's honest significant difference were performed to test if there was a significant difference in the expression of *srtA* among bacterial isolates, and also to determine if there was a significant difference in the inhibition assay. Each experiment was repeated at least three times and a *p-value* of <0.05 was considered significant. All analyses were conducted using the GraphPad Prism software (Version 7, GraphPad Software Inc, La Jolla, CA).

Results

S. pseudintermedius contains an *srtA* Gene Homologous to *srtA* Found in *S. aureus*

Whole genome sequence analysis of several *S. pseudintermedius* isolates revealed that *S. pseudintermedius* contained a *srtA* gene homologous to the one found in *S.aureus*. The *srtA* gene in *S.pseudintermedius* is 597bp in length and codes for a 198 amino acid protein (Figure 5.1) with a predicted molecular weight of 22.36kDa.

Conventional PCR with *S. pseudintermedius srtA*-specific primers showed that isolates representing the major clonal populations in the United States and Europe (Table 5.1) contained the *srtA* gene. qPCR data suggested that the fold change in expression for isolates 08-1294 and 08-521a were 0.76 fold and 0.4 fold respectively. Fold change in expression for isolates form sequence type ST71 were 0.75 fold (08-1661), 4.12 fold (E140) and 3.47 fold (NA16). Isolate 06- 3228 was assigned as the calibrator for this experiment. The amount of *srtA* mRNA was normalized against 16S rRNA as the endogenous control (Figure 5.2). These results indicate that the *srtA* mRNA in *S. pseudintermedius* could be detected and quantified.

Recombinant *S. pseudintermedius* SrtA

The full-length recombinant, his-tagged SrtA protein was purified from *E. coli* BL21 (DE3). The molecular weight of the recombinant protein was ~23kDa and was confirmed by SDS-PAGE and western blot (Figure 5.3). The concentration of the protein was estimated by Bradford method and found to be 0.5-1mg/ml per 100ml batch of *E. coli* culture.

ATGCAAAAATTAACACGTGGGTTGGTTCCACTTATTGGTATTGCATTGATTTTAGCAGG
CGTATATTTAATCTTTAAGCCGAAAATTGATGCATATTTGACCAATAAAGAAAATGAAC
AAAAAATTGAAGTATATGAAGAAAGTCAACAAAATGCACCATCAAAAGTGCCTGAAATT
CCGAAAGACCCGTCGAAAGTCGTTGGTGTATTAGAAGTGCCGTCAGTCGGTATAAAAGA
AGCTGTGTATCCTGGTCCTGCGACACCTGAACAATTGGAGCGCGGTGTCAGTTTGGCGG
AAAAGGATGAATCACTCAAAGACCAAAATATCGCTATTGCGGGACATACGAATTATAGT
TTGAACTATCAATTTACAGAGTTACACAAAGCGAAAAAGGGTGCAGAAGTAATTTTTAA
ACTGGGTAAAGAAACGCGCAAATATCAAATTACGTCGATTAAAGATGTCGATCCGTATC
AAGTTGAAGTGTTGGAAGAGATGAAGAAAGATAAAGACCAACTGACACTCATTACTTGT
GATGATTACGATGAGAAAACAGGTCAGTGGCTGACACGCAAAATTTATGTAGCTGAACG
TGTGTAG

MQKLTRGLVPLIGIALILAGVYLIFKPKIDAYLTNKENEQKIEVYEESQQNAPSKVPEI
PKDPSKVVGVLEVPSVGIKEAVYPGPATPEQLERGVSLAEKDESLKDQNIAIAGHTNYS
LNYQFTELHKAKKGAEVIFKLGKETRKYQITSIKDVDPYQVEVLEEMKKDKDQLTLITC
DDYDEKTGQWLTRKIYVAERV

Figure 5.1 Consensus gene sequence and predicted amino acid sequence of SrtA in *S. pseudintermedius*.

The *srtA* gene in *S. pseudintermedius* is 597bp in length and codes for a protein with 198 amino acids. Based on amino acid conservation with *S. aureus* SrtA, the amino acids predicted to be critical for enzymatic activity are highlighted.

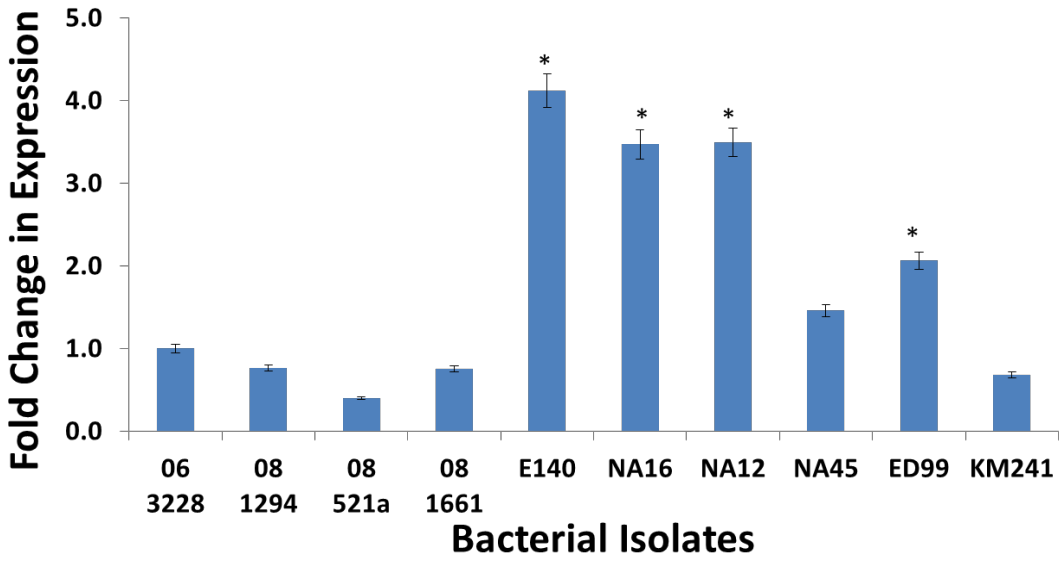

Figure 5.2 Expression levels of *srtA* in *S. pseudintermedius*.

Quantitative real-time PCR was used to measure the amount of *srtA* mRNA in log phase bacterial cells. Relative gene expression was calculated using the $\Delta\Delta C_T$ method and expressed as fold change. 16S rRNA was used as the endogenous control. The values represent the average from three independent experiments. (*P<0.05 was considered significant).

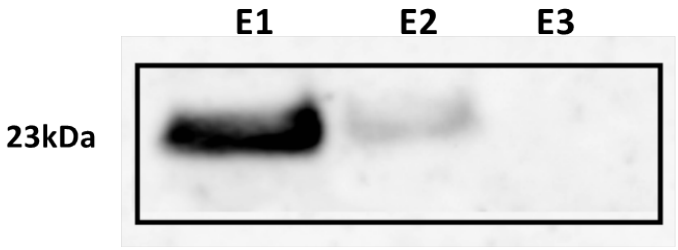

Figure 5.3 SDS-PAGE and Western Blot analysis of recombinant SrtA.

Western blot analysis of recombinant SrtA in one of the elution fractions using anti-His antibody. The molecular weight of the protein corresponded to ~23kDa. E1, E2 and E3 are three separate elution fractions.

3-D homology model of SrtA and Identifying Sortase Inhibitors

The *S. pseudintermedius* SrtA protein sequence was compared to that of *S.aureus* SrtA. Molecular Dynamic (MD) simulation was performed using the bioinformatics tool MOE (Molecular Operating Environment) to generate a 3-D homology model of *S. pseudintermedius* SrtA. A potential structure of SrtA was built and atomic coordinates for the protein, including for the active site's amino acids involved in substrate binding, were generated. The predicted 3-D homology model of SrtA with a potential inhibitor bound to its active site is shown in Figure 5.4.

Inhibition of SrtA activity *in vitro*

To determine the activity of recombinant SrtA, an *in vitro* functional assay using the synthetic SrtA substrate, bacterial sortase substrate III, Abz/DNP (Abz-LPETG-K(Dnp)-NH_2 was performed. Functional SrtA cleaves the substrate between threonine (T) and glycine (G) and this is measured by an increase in fluorescence. A dose dependent increase in the cleavage of the substrate was observed when 1μM of recombinant SrtA was used in the FRET assay (Figure 5.5).

Next, to determine the inhibitory potential of the compounds from NCI, recombinant SrtA was pre-incubated with the inhibitors at 37°C for 30min and the FRET assay was performed. From the first round of screening, four out of the ten compounds from NCI (117466, 117922, 64876 and 1847) showed between 40-60% inhibition of SrtA. Of these, Compound 1847 consistently showed 50-80% inhibition of recombinant SrtA depicted in (Figure 5.6). MTSET which is a known inhibitor of SrtA [70] was used as the control and showed 57.5% inhibition in this assay.

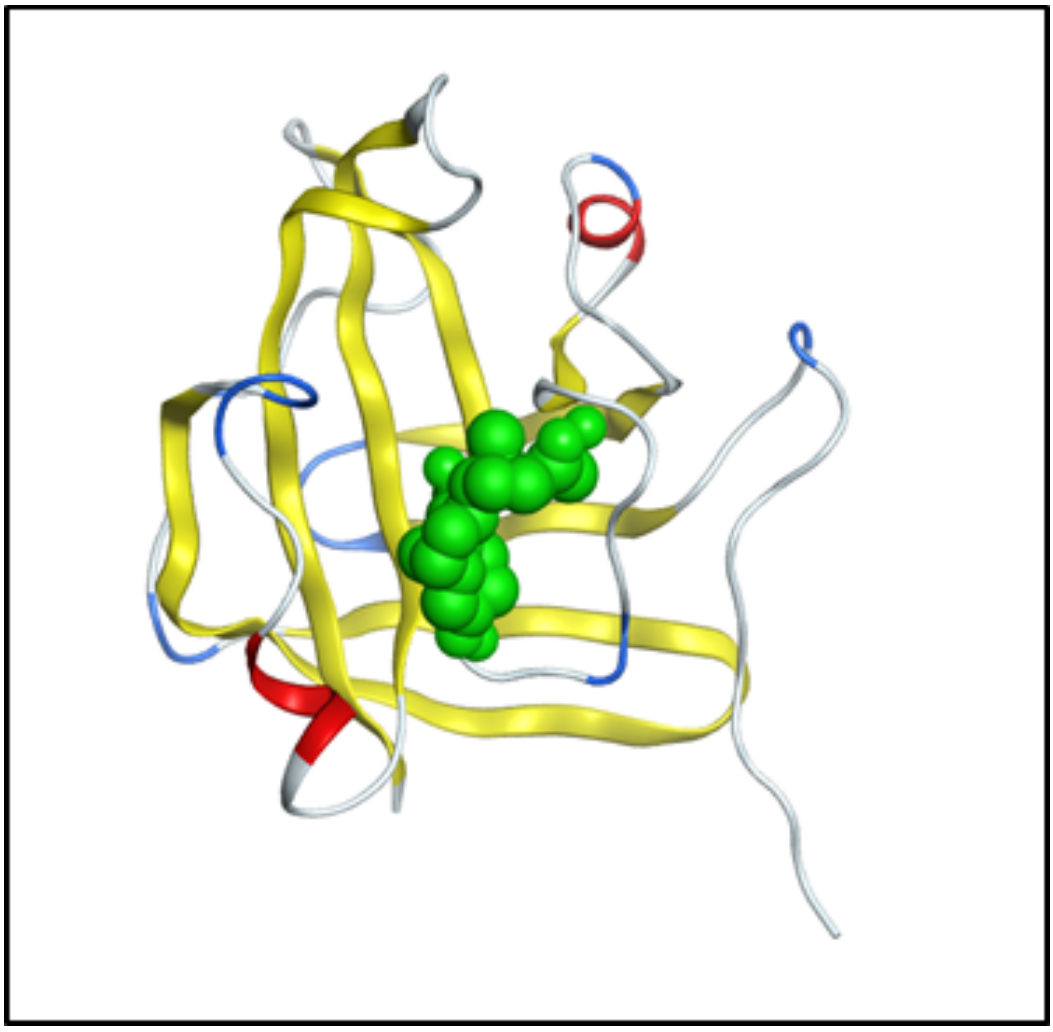

Figure 5.4 3-D homology model of SrtA.

3-D homology model of *S. pseudintermedius* SrtA with a representative inhibitor (shown in green) bound to the active site. The β-sheets, α-helices and loop regions are indicated in yellow, red and blue respectively.

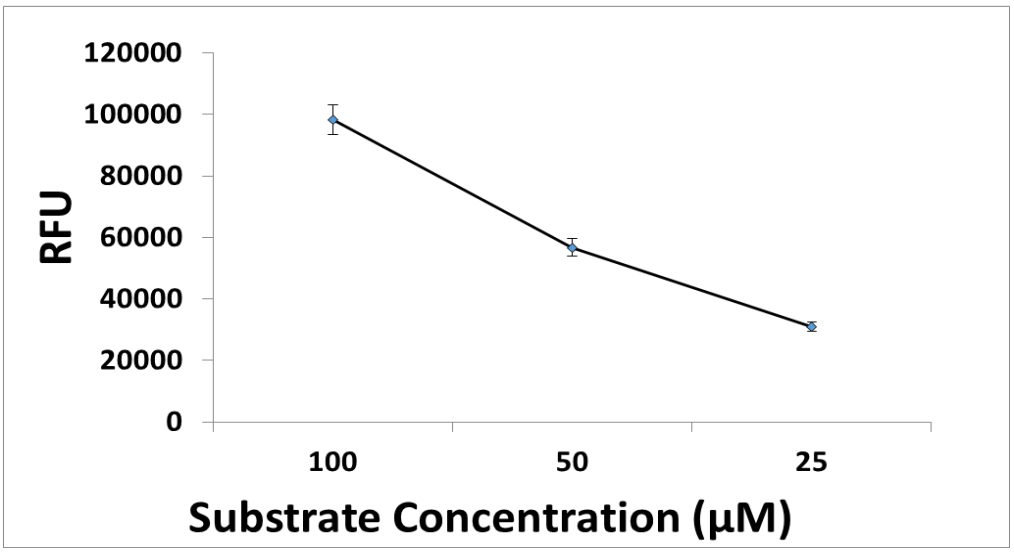

Figure 5.5 SrtA functional assay.

The activity of recombinant of SrtA was measured using a FRET assay. Varying concentrations of the FRET substrate were incubated with 1µM of the enzyme. The cleavage of the substrate was monitored by measuring an increase in fluorescence.

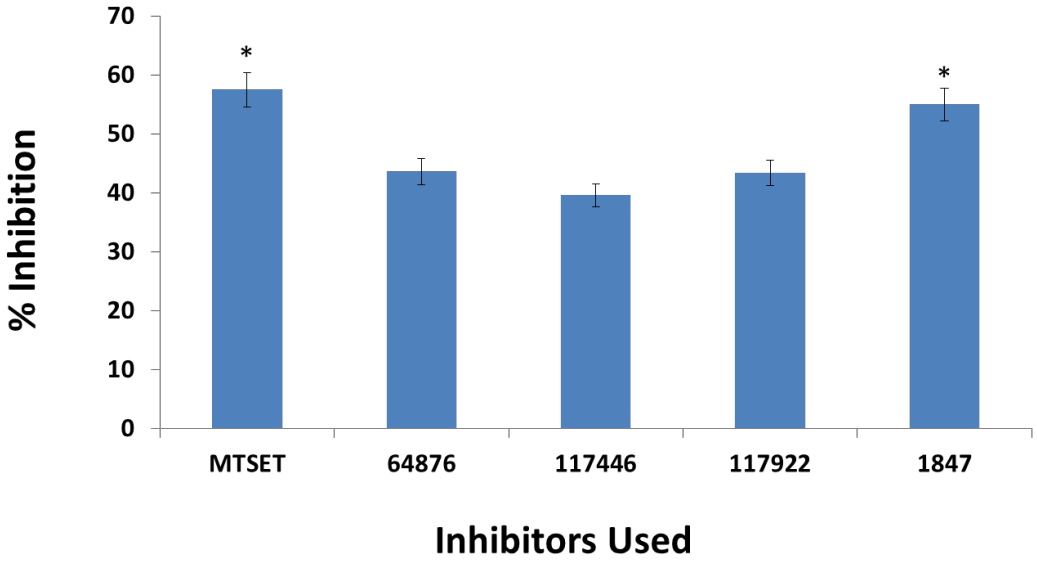

Figure 5.6 First round of screening for inhibitors.

Compounds from NCI tested for their ability to inhibit SrtA in the *in vitro* FRET-based functional assay. Four of the ten compounds showed ~40%-60% inhibition of Srt A. The values represent average from three independent experiments. (*P<0.05 was considered significant).

Since compound 1847 showed promising results, the second search iteration was based on the structure of 1847 to identify similar compounds but with higher inhibitory potential. Four compounds were identified from the Zinc database. Of the four compounds, only one compound, 5930504, showed 51.76% inhibition. 5510335 and STL223442 showed 9.88% and 13.75% inhibition respectively. SKT022091 did not have inhibitory effect on SrtA. However, none of the compounds from Zinc were better than the parent compound, 1847 (Figure 5.7).

Inhibition of SrtA Activity during Bacterial Growth

SrtA catalyzes the anchoring of surface proteins with a specific LPXTG motif on to the peptidoglycan cell wall during active bacterial growth (log phase). Inhibition of SrtA in bacterial cells should result in decreased amounts of cell wall-associated proteins harboring the LPXTG motif. This can be measured indirectly by measuring the decrease in the density of cell-wall associated LPXTG proteins. In *S. pseudintermedius*, there are several proteins with a predicted LPXTG motif, including but not limited to protein A, clumping factors A and B, fibrinogen binding protein and fibronectin binding protein. In this assay, the inhibition of SrtA was measured indirectly by measuring the decrease in the amount of Protein A on the bacterial surface using flow cytometry. Five representative *S. pseudintermedius* isolates were tested. Isolates 06-3228, 08-1661, NA12 and NA16 showed 38.7%, 74.3%, 42.8% and 89.4% reduction in the density cell wall-associated Protein A compared to the respective controls without the inhibitor for each isolate, as a result of SrtA inhibition (Figure 5.8). Isolate NA45 was not affected by compound 1847. These results suggest that compared to the controls (without inhibitor), the bacterial cells exposed to the inhibitor 1847 showed a decrease in the density of cell wall-associated Protein A due to inhibition of SrtA.

Figure 5.7 Second round of screening for inhibitors.

Inhibitory effect of compounds from ZINC. Of the four compounds tested, only 5930504 showed ~50% inhibition of Srt A in the in-vitro FRET-based functional assay. 5510335 and STL223422 showed 10-20% inhibition while STK022091 did not have any inhibitory effect on SrtA. The values represent average from three independent experiments. (*P<0.05 was considered significant).

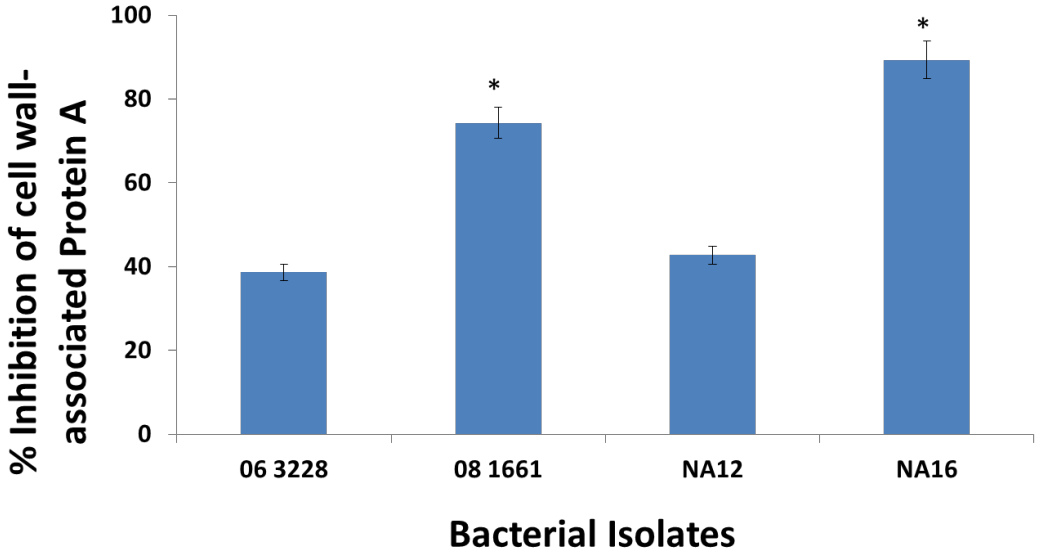

Figure 5.8 Inhibition of SrtA in whole bacterial cells.

Four bacterial isolates 08-3228, 08-1661, NA12 and NA16 were grown in presence of inhibitor 1847 till exponential log phase. SrtA inhibition was measured indirectly by measuring the decrease in the density of cell wall associated Protein A (one of the LPXTG proteins targeted by SrtA) using flow cytometry. The values represent average from three independent experiments. (*P<0.05 was considered significant). Isolate NA45 which does not express functional Protein A on its surface was not affected by inhibitor 1847.

To determine the effect of inhibitor on bacterial viability, bacterial growth was monitored in the presence and absence of inhibitors at varying concentrations. Bacterial growth was determined by measuring the turbidity of the culture medium at A_{600}. It was found that the bacterial growth remained unaffected in the presence of the inhibitor suggesting that the inhibitor affected the function of SrtA but had no direct effect on growth of the bacteria.

Discussion

In the last decade, there has been a steady increase in the incidence of microorganisms with resistance to methicillin both in human and veterinary medicine [80, 92]. Most of the organisms that display methicillin resistance are also multidrug resistant and cannot be treated with conventional antibiotics. This is particularly difficult in the veterinary setting due to the limited number of antibiotic choices that are approved for treatment [81]. Hence, there is an urgent need for the development of novel therapeutics that can be administered either prophylactically or after the onset of infection. Ideally, any novel therapeutic should not have a high level of selective pressure that is observed with current antibiotics. This will help reduce the risk of developing new forms of resistance [177].

Cell wall-associated (CWA) proteins are responsible for virulence and immune evasion in staphylococci [126]. SrtA is a cysteine transpeptidase produced by most Gram positive bacteria. It catalyzes the anchoring of proteins harboring the LPXTG motif on to the peptidoglycan cell wall [58, 62]. Inhibition of SrtA will prevent proteins harboring the LPXTG motif from anchoring on to the cell wall, thereby preventing the interaction between the CWA proteins and the host. Since this is a crucial step in the establishment of infection, inhibiting SrtA would make the bacteria less virulent [56, 57, 70, 124]. Inhibition of SrtA has several advantages. (a) Firstly, SrtA is expressed on the outside of the bacterial cell membrane which

makes it readily accessible to inhibitors without the need for penetrating the cell membrane, (b) SrtA inhibition will not affect the viability of the bacteria directly. Therefore, the risk of bacteria developing resistance against the inhibitor is greatly reduced and (c) there is no known homologue for SrtA in eukaryotes, thus it is possible to achieve selectivity more easily. SrtA inhibition has been extensively studied in *S. aureus* [71, 175]. Virtual screening of drug databases to identify inhibitors for *S. aureus* SrtA has also been explored [70, 74].

In this study, recombinant SrtA from *S. pseudintermedius* was expressed and purified from *E. coli*. A homology model of the enzyme was generated. Using this model, virtual screening of NCI database for compounds that bind to the catalytic site of SrtA was performed. Ten compounds were selected and tested for their abilities to inhibit recombinant SrtA in an *in vitro* FRET-based assay. Compound 1847 consistently showed >50% inhibition in this assay. The structure of 1847 was used as the template to screen a second database, Zinc, in an attempt to find compounds with higher inhibitory potential. Four compounds were identified and tested again using the in vitro FRET-based assay, although none of the new compounds were better than the parent compound, 1847. Since 1847 was the most promising candidate, it was tested in whole bacterial cells during active growth phase to determine its ability to inhibit SrtA. The results showed that in all of the bacterial isolates tested, except NA45, there was between 35%-80% reduction in the amount of CWA Protein A, suggesting that SrtA inhibition could work as an anti-infective therapy against staphylococcal infections. Future experiments in this study include analysis of enzyme kinetic parameters, target validation using surface plasmon resonance and/or isothermal colorimetry, side chain modification to enhance the inhibitory potential of compound 1847 and finally, testing the inhibitor in an animal model of *S. pseudintermedius* infection.
The role of SrtA in anchoring CWA proteins and its contribution to virulence is not just limited to staphylococcal species. SrtA and surface proteins with LPXTG

sorting signals have also been studied in streptococcal species and other nosocomial pathogens like *Enterococcus faecalis, Enterococcus faecium, and Clostridium difficile* [58]. Therefore, small molecule inhibitors that can specifically block sortase-catalyzed transpeptidation reaction could work against a broad spectrum of pathogenic, antibiotic-resistant Gram-positive bacteria.

CONCLUSION

Conclusions and Recommendations

The canine pathogen *S. pseudintermedius* has been associated with an increased prevalence of methicillin resistance and multidrug resistance. Due to the limited number of antibiotics available for treatment in the veterinary setting, it is important to develop novel strategies to treat or prevent infection. Understanding the nature of immune response to *S. pseudintermedius* infection is crucial for vaccine development.

Characterization of the virulence factor Protein A in *S. pseudintermedius* has provided information about the nature of canine IgG binding to this protein. It is now evident that the most predominant clonal populations in the United States and Europe, ST68 and ST71 respectively, bind higher amounts of canine IgG and anti-Protein A antibody, suggesting that these sequence types may express a higher amount of Protein A on their surface. Protein A binds to canine IgG via its Fc region. This binding in the 'wrong' orientation facilitates Protein A to function as an immune evasion factor by preventing opsonization and phagocytosis. This has also been demonstrated in canine blood, neutrophils and DH82 macrophage-like cell line wherein pre-incubation of bacteria with anti-Protein A antibody enhances phagocytosis. Once the IgG-binding sites on Protein A are identified, it will be possible to generate a non-toxigenic form of the protein that can be tested both *in vitro* and *in vivo* as a potential vaccine candidate.

S. pseudintermedius employs several immune evasion strategies to persist in the host and establish infection. Inhibiting one virulence/immune evasion factor may not be the best strategy for treatment of infection. Instead, targeting a global protein or enzyme that is central to the regulation of multiple virulence factors could be a potential strategy to combat infection. Sortase A is a transpeptidase enzyme produced by all Gram-positive bacteria that is responsible for anchoring proteins

with an LPXTG domain onto the peptidoglycan cell well. Most proteins with an LPXTG domain are potent virulence factors. In this study, the possibility of inhibiting Sortase A as an anti-infective therapy was explored. 3-D homology modeling and molecular dynamic simulation helped map the active site of Sortase A. Virtual screening of drug databases provided a list of potential inhibitors that were tested both in an *in vitro* assay and in bacterial cells. Compounds that were able to inhibit the activity of Sortase A were identified. Further optimization and testing of the compounds in an animal model could prove to be useful in determining if co-administration of a Sortase A inhibitor along with antibiotic treatment will be a more effective treatment strategy than what is currently being followed.

Several studies with *S. aureus* have indicated that a successful vaccine could be one with several different antigenic determinants (multivalent vaccine) that can stimulate both B-cell and T-cell responses. Keeping these factors in mind, the secretome, surface-associated proteins and IgG-binding proteins from three representative sequence types were analyzed by mass spectrometry. From the data, eight target proteins were identified across all three sequence types. Further testing and characterization of these proteins need to be performed.

This work has laid the foundation for identifying, characterizing and testing proteins that could serve as potential vaccine candidates, and explore non-antibiotic strategies such as Sortase A inhibition as alternate anti-infective therapy against methicillin-resistant *S. pseudintermedius* infection.

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