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School of Medicine
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This is to certify that the dissertation prepared by Nicole Marie Lawrence entitled CHARACTERIZATION OF THE STAPHYLOCOCCUS AUREUS IMMUNODOMINANT SURFACE ANTIGEN B, ISAB has been approved by his or her committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy

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CHARACTERIZATION OF THE STAPHYLOCOCCUS AUREUS IMMUNODOMINANT SURFACE ANTIGEN B, ISAB

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Dedication

I would like to dedicate my Dissertation to my Family, especially my parents Robin and Stephen Mackey, my husband John, and my siblings Lantz, Jessica and Rachel. Their love, support, understanding and encouragement were essential to my successes both academically and personally and I will always be thankful for them.

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List of Abbreviations

°C	degree centigrade
A	adenine
a.a.	amino acid
AMP	Antimicrobial Peptides
B. cereus	*Bacillus cereus*
B. *subtilus*	*Bacillus subtilis*
BHI	brain heart infusion
BHIG	BHI + 1% glucose
BLAST	basic local alignment search tool
bp	base pair
C	cytosine
C. elegans	*Caenorhabditis elegans*
CBD	chitin binding domain
CcpA	Carbon catabolite control protein A
CCR	Carbon Catabolite Repression
cDNA	complementary DNA
CFU	colony forming unit
CREs	Catabolite-responsive elements
C-terminal	carboxy-terminal
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
eDNA	Extracellular DNA
EDTA	ethylenediamine tetraacetic acid
EMSA	electro-mobility shift assay
EPS	exopolymeric Matrix
g	gram
G	guanine
gDNA	genomic DNA
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IsaB	Immunodominant surface antigen B
Ka	association constant
kb	kilobase pairs
Kd	dissociation constant

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L	liter
L. lactis	*Lactococcus lactis*
LB	Luria-Bertani
LBF	LB + 1% fructose
LBG	LB + 1% glucose
LBS	LB + 1% sucrose
LTA	lipoteichoic acid
M	molar
M. tuberculosis	*Mycobacterium tuberculosis*
M.O.I.	multiplicity of infection
ml	milliliter
mM	millimolar
mmol	millimole
mol	mole
mRNA	messenger RNA
MRSA	Methicillin resistant *S. aureus*
MSCRAMMS	microbial surface components recognizing adhesive matrix molecules
MWCO	molecular weight cut-off
n	nano
N. meningitidis	*Neisseria meningitidis*
NETs	Neutrophil Extracellular Traps
nM	nano molar
nm	nanometer
NMR	Nuclear Magnetic Resonance
N-terminal	amino-terminal
OD	optical density
oligo	oligomer
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pM	pico molar
PNAG	poly-N-acetylglucosamine
RT-PCR	real-time quantitative PCR
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. mutans	*Streptococcus mutans*
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes*

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S. xylosus	*Staphylococcus xylosus*
SarA	Staphylococcal accessory regulator A
SDS	sodium dodecyl sulfate
SERAMs	secreatable expanded repertoire of adhesive molecules
SPR	Surface Plasmon Resonance
ssDNA	single Stranded DNA
SSS	sonicated Salmon Sperm DNA
TAE	Tris acetate EDTA
TLR	toll-like receptor
tRNA	transfer RNA
TSB	Tryptic soy broth
TSBG	TSB + 1% glucose
U	uracil
UV	ultraviolet
WT	wild-type
WTA	wall teichoic acid
µ	micro
µg	micro gram

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Abstract

CHARACTERIZATION OF THE S. AUREUS IMMUNODOMINANT SURFACE ANTIGEN B, ISAB

By Nicole Marie Lawrence, PhD

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

Major Director: Dr. Kimberly K. Jefferson
Assistant Professor of Microbiology and Immunology

Staphylococcus aureus is a significant cause of morbidity and mortality world-wide. This opportunistic pathogen is capable of causing several severe diseases that are exacerbated by its diverse and widespread antibiotic resistance profile. Therefore it is necessary to identify novel therapeutic targets to effectively treat *S. aureus* disease. Lorenz et al first described the Immunodominant Surface Antigen B, IsaB, because it was 1 of 4 unique proteins immunogenic during septicemia and not colonization, suggesting that IsaB may be a virulence factor and a possible novel therapeutic target. Interestingly, IsaB has no homology to proteins of known function and appears to be found only in Staphylococci. We sought to characterize the function of IsaB in *S. aureus*. We began our studies by

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determining how *isaB* was regulated by known *S. aureus* regulators and environmental stimuli. It was observed that the transcriptional regulator SarA represses expression of *isaB*, while serum and acidic pH induce expression. We found that IsaB is an extracellular nucleic acid binding protein, able to bind to dsDNA, ssDNA, and RNA and leads significant accumulation of eDNA on the cell surface. We employed multiple virulence models to ascertain the role of IsaB in virulence. Excitingly, we found that IsaB significantly protects *S. aureus* from antimicrobial peptides and Neutrophil Extracellular Traps, both components of the innate immune system. Another virulence mechanism of *S. aureus* is the ability to form biofilms. While recent studies show a significant role for eDNA in *S. aureus* biofilms, we found that IsaB actually had a negative affect on biofilms under certain growth conditions. Finally, to group IsaB into a known functional class, we successfully expressed and purified mature IsaB for structural determination by Nuclear Magnetic Resonance, which is currently underway. Our studies show that IsaB is a novel virulence factor of *S. aureus,* able to bind eDNA and significantly protect from AMPs and NETs, and could therefore play a key role in immune evasion.

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CHAPTER 1

Introduction

Clinical relevance of *S. aureus*

Staphylococcus aureus is a potent opportunistic pathogen, causing significant morbidity and mortality on a global scale and is the leading cause of nosocomial infections in the United States. The genus *Staphylococcus* is made up of over 40 species, most of which are not significant contributors to human disease [1]. *S. aureus* is the headlining member of the genus due to its imposing ability to cause a variety of potentially life-threatening human diseases. The staphylococci are non-motile, facultative anaerobic Gram-positive cocci. All staphylococci possess catalase, however only *S. aureus* produces coagulase, which is the standard marker used to differentiate *S. aureus* from other *Staphylococcus* species. The genus name comes from the Greek word staphyle, for the grape-like cluster morphology observed upon Gram-staining the organism. The species name comes from the Latin word aurum, gold, for its golden colony pigmentation which is due to the presence of staphyloxanthin [2].

While *S. aureus* is an important human pathogen, it is also capable of colonizing 30-50% of the population either permanently or transiently [3, 4]. An important distinction

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between colonization and infection is that during colonization, unlike infection, the host is not adversely affected, and oftentimes there is only a very acute local immune response if any is observed at all [5, 6]. *S. aureus* can be found on moist areas of the skin (interior of elbows and armpits), however, colonization most often occurs in either the anterior nares or the back of the throat [3, 4]. While colonization is not likely to harm a healthy individual, it is a risk factor for disease once normal protective barriers or defenses are weakened. Carriers who become infected with *S. aureus* are usually infected with their own resident strain.

Once *S. aureus* crosses the epithelial boundary, it can cause a large number of disease manifestations with varying degrees of severity. *S. aureus* is the leading cause of purulent soft tissue infections, causing folliculitis, impetigo and carbuncles (boils). These disease manifestations are generally self-limiting and not severe [7, 8]. However, *S. aureus* is also a leading cause of a wide variety of life-threatening infections such as osteomyelitis, and infective endocarditis, which are chronic infections of bone tissue and heart valves, respectively [7, 8]. *S. aureus* is also capable of causing both necrotizing pneumonia and fasciitis, as well as toxinoses such as toxic shock syndrome, all of which are often fatal.

Due to severity of disease caused by *S. aureus*, it is essential to start effective antibiotic intervention immediately. Antibiotic choice is complicated by diverse mechanisms of resistance and the widespread presence of methicillin resistant *S. aureus* strains (MRSA) [9]. Until recently MRSA was thought to be a significant threat only in the hospital setting, as *S. aureus* is the leading cause of nosocomial infections [9, 10].

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However, it has been shown that normal healthy individuals are becoming infected with community acquired MRSA strains, CA-MRSA, which are thought to have evolved separately from HA-MRSA, and are frequently hypervirulent [9, 11, 12].

While oxacillin (clinically-approved form of methicillin) is the drug of choice against *S. aureus*, it is ineffective against MRSA, therefore alternate antibiotic interventions must be employed immediately upon confirmation of MRSA in order to stop disease progression before *S. aureus* overwhelms the host defenses and causes death. It is important to note that *S. aureus* is inherently susceptible to a number of other drugs such as erythromycin and tetracycline, however the chromosomal cassette carrying the *mecA* methicillin resistance gene also contains genes that confer resistance to many of these other antibiotics [13]. Two antibiotics that are used clinically to treat MRSA are vancomycin and daptomycin [13-15]. These second line antibiotics are used sparingly so as not to promote the spread of resistant strains, which have been clinically documented, especially for vancomycin intermediate resistant (VISA) and vancomycin resistant *S. aureus* (VRSA) [13, 14, 16]. Due to the continuously rising prevalence of strains resistant to all relevant antibiotics, there has been a strong push from clinicians and scientists to find other effective treatment modalities. In order to find therapeutic targets, it is essential to understand the pathogenesis and virulence factors of this organism.

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Virulence factors

Immune Evasion and Virulence

Neutrophils. While *S. aureus* is, on occasion, able to subvert the host barriers and the immune system in order to cause disease, host defenses are usually capable of eradicating *S. aureus* before establishment of infection begins. Neutrophils are the body's primary defense against *S. aureus* [2, 17, 18]. Thus, neutrophils are extremely well adapted and equipped to thwart these invaders. Neutrophils can kill by a number of different mechanisms: releasing granules full of peroxide as well as antimicrobial proteins and peptides, by production of ROSs through an oxidative burst, and their calling-card method, phagocytosis/opsonophagocytosis [2, 17-19]. Not surprisingly, *S. aureus* is similarly well equipped to take on neutrophils and knock them out. Some examples of proteins that *S. aureus* use to avoid killing by neutrophils are: chemotaxis inhibitory proteins of Staph (CHIPs), which prevents neutrophil recruitment, and Panton Valentine Leukocidin (PVL), which is a pore forming leukotoxin [17, 18, 20-23].

Opsonophagocytosis. While neutrophils are potent killers, their innate activity is greatly enhanced in a process known as opsonization. Opsonization is the process in which complement, a major antimicrobial component of serum, and pathogen specific antibodies, are deposited on the bacterial cell surface. Opsonization kick-starts a dramatic antibacterial sequence of events in a number of ways. When complement binds to the bacterial cell surface it becomes activated through proteolysis that releases a number of factors, which actively recruit chemotactic cells, including neutrophils, to the site of intrusion [17, 18, 24].

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Once the neutrophils arrive on scene they recognize and bind to either the Fc portion of specific antibodies bound to the bacterial cell with Fc receptors, or they can bind to the deposited complement (c3b) via CR1 [17, 18, 24, 25]. Binding of either ligand to its receptor induces phagocytic pathways ending with the bacteria being internalized within a phagolysosome that efficiently destroys the bacteria [17, 18]. However, *S. aureus* has ways to inhibit this process at a number of points, including the inactivation of complement by Staphylococcal Complement Inhibitor (SCIN), evasion of opsonization by binding to host fibrinogen via CflA, and also by binding the Fc regions of antibodies which is one function of Protein A [2, 17-19, 25].

*Antimicrobial peptides***.** Antimicrobial peptides, AMPs, are another important arm of innate immunity that target and kill *S. aureus*. These small cationic peptides, which are found in virtually all higher organisms, have recently become attractive drug candidates, due their natural production in and protection of many host tissues and cell types [26-28]. AMPs are highly effective against most bacteria, regardless of Gram-type, because the overall external charge on the bacterial envelope is negative, which differentiates them from self, as eukaryotic cells are typically "seen" as neutral [27, 28]. Interestingly, production of AMPs is not exclusive to the typical immune cells (i.e. neutrophils), they are produced by numerous cell/tissues types, most notably epithelial cells in the skin and lungs, and also by the cells of the gastrointestinal tract [27, 29, 30].

While *S. aureus* is susceptible to a number of AMPs, it has evolved mechanisms of defense, proteins and systems associated with protection from these bactericidal AMPs [26, 29-34]. AMPs act by binding to the bacterial membrane via charge interactions and

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can form pores in the membrane, they can activate hydrolases or autolysins, and can even be internalized and disrupt cellular processes [27, 32, 35, 36]. Therefore, most *S. aureus* resistance strategies involve alteration of the overall charge of the cell wall, proteolysis, as well as active transporter systems [26, 31, 32, 34]. A few of the genes that have been associated with AMP resistance are: *mprF*, which encodes a membrane protein involved in membrane modification, *dlt* operon whose gene products are required for efficient D-alanylation of teichoic acid which inhibits autolysin activity, and finally *graRS*, a two-component system [26, 31, 32, 35].

Adhesion and Invasion

Along with the virulence factors involved in immune evasion, *S. aureus* also has a diverse array of other factors that allow for adherence to host tissues. MSCRAMMs, microbial surface components recognizing adhesive matrix molecules, and SERAMS, secretable expanded repertoire of adhesive molecules, are critical for the initiation of disease as these proteins are responsible for *S. aureus* binding intimately to host tissues [37]. MSCRAMMs and SERAMs are protein families with multiple members that allow for binding to host fibrinogen, fibronectin, elastin, collagen, vitronectin, β-neurexin, and cytokeratin 10 [37-43]. Furthermore, *S. aureus* deficient in many of these proteins, including SdrC, FnBPA, FnBPB, and Eap have been shown to significantly decreased adherence to various human cell lines [37, 38, 41].

In concert with adherence, recent studies have shown that *S. aureus* can invade and survive intracellularly in both phagocytic and non-professional phagocytic cells [42, 44-

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49]. One process by which *S. aureus* can be actively internalized is through fibronectin binding proteins interacting with receptors on the host cell surface, initiating uptake of the bound bacteria [37, 44]. The invasive strategy is likely a process to avoid innate and cellular host defenses, as once inside cells they are protected from professional phagocytes and also from AMPs, antibodies and complement. Recently a study by Zautner et al, have demonstrated that intracellular *S. aureus* is responsible for a large proportion of recurring tonsillitis infections [50]. Due to the amount of resources and proteins committed to promoting adherence and even invasion in to host-tissues, it easy to see how important these strategies are to the pathogenesis of *S. aureus* within the host.

Biofilm formation

Biofilms are sessile multi-cellular communities of bacteria that are encased in an exopolymeric substance [51, 52]. Many environmental and pathogenic bacteria have been found to form biofilms, either in the environment or within a host during infection [52]. The biofilm growth phenotype is now thought to be the default mode of growth for bacteria, insomuch as approximately 75% of infections are biofilm related [51]. Biofilms are likely preferred because this growth phenotype confers increased survival of the bacteria under many stressful conditions such as low nutrient availability, presence of antibiotics, phagocytes, and both oxidative and shear stresses [53].

S. aureus biofilms are clinically relevant as they have been found to be essential in the pathogenesis of a number of chronic infections such as catheter infections, infections of indwelling medical devices, chronic rhinosinusitis [54-58]. While *S. aureus* infections can

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be difficult to treat as a result of antibiotic resistance determinants, the chronic biofilm diseases are even more so, and often require the removal of the infected device or surgical debridement of infected tissues. Part of the difficulty of treating biofilms is due to the fact that biofilms are intrinsically up to a 1,000X more resistant to antibiotics. Also compounding treatment and causing recurring infections is the presence of persister cells which can re-colonize a bacterial biofilm after antibiotics are removed, thus re-initiating infection [51, 59].

Biofilm formation is a multi step process relying first on attachment to a surface, followed by secretion of an exopolymeric substance (EPS) which leads to irreversible attachment, and finally outgrowth and maturation of the biofilm structure [51, 60, 61]. An important EPS of the *S. aureus* biofilm matrix component is the exopolysaccharide poly-N-acetylglucosamine, PNAG, which is encoded by the intracellular adhesin locus, *ica* [61-63]. However several recent studies have found that both extracelluar DNA (eDNA) and several surface proteins, such as the SERAM Eap, can play a more significant role in biofilm formation than PNAG under certain conditions [61, 62, 64-67]. Furthermore, a number of transcriptional regulators have been found to be important in biofilm formation, for example SarA, which is considered a major regulator, is capable of regulating biofilm formation in both *ica* dependent and independent mechanisms [63]. While much is now known about *S. aureus* biofilm formation, the production and regulation of biofilms is a very active topic of study and it is likely that many novel biofilm associated factors and mechanisms will be described in the near future.

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Regulation of virulence

S. aureus not only needs to control its normal cellular functions involved in metabolism and growth, it also must have efficient ways to respond quickly to an infinite number of environmental stimuli. This network is further expanded once you take into account the myriad of environmental information it must respond to, such as antibiotics, phagocytes, antimicrobial peptides, complement, etc. In response to these signals *S. aureus* is able to regulate its plethora of virulence factors in a manner that is specific and appropriate for the current stage of infection. To accomplish this large feat, *S. aureus* has multiple regulatory mechanisms involving 3 sigma factors, 16 two-component systems, a quorum sensing system through the Agr/RNAIII pathway, and a large repository of transcriptional regulators [68-70]. While hundreds of reviews on *S. aureus* regulation have been published, for the purposes of this report we will focus on two "global" regulators, SarA and CcpA, both of which have been implicated in virulence, metabolism, and biofilm formation.

Staphylococcal accessory regulator A. The Staphylococcal accessory regulator A, SarA is the prototype for the 11-member Sar family of DNA binding regulators that *S. aureus* uses to respond to changing local environments [71]. SarA is a DNA binding protein which is known to regulate the expression of many virulence factors, especially extracellular proteases, cell wall proteins and biofilm formation [72-75]. Active SarA forms a homo-dimer which results in a winged helix motif, allowing for DNA binding to occur by both the winged and helix-turn-helix motifs, which interact with the minor and major grooves, respectively [71, 76]. It is also been shown that SarA binds to

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the promoter regions of its targets by recognizing a specific consensus sequence. A number of studies have been performed by various labs to characterize and predict the SarA consensus sequence, which can be a helpful tool for determining a direct or indirect SarA regulatory effects [75, 77]. Importantly, SarA has been found to both positively and negatively regulate factors depending on how it interacts with the targeted promoter; it can bend DNA in such a way that it is either optimal or inhibitory for transcription to occur [71].

Expression of SarA is dependent upon growth phase, with its greatest expression observed during the late exponential growth phase [15]. Thus, it can regulate virulence determinants in a temporal manner. Interestingly, SarA has been found to be both a positive and negative regulator of different virulence factors and its regulatory effects can be predicted by protein class. SarA has been consistently found to repress expression of extracellular proteases while positively regulating expression of proteins involved in biofilm formation and cell wall-associated proteins [15-18]. One can surmise that SarA is turned on when cells are at a high enough density where adherence to tissues is desired, and where extracellular proteases can be detrimental, possibly by eliciting an immune response. Furthermore, SarA has been found to play a significant role in the regulation of biofilm formation and also of both the *agr* locus and RNAIII, which constitute the main components of the quorum sensing system [63, 71].

Carbon Catabolite Protein A. *S. aureus* metabolism is a highly regulated and tightly orchestrated activity that allows *S. aureus* to efficiently use every energy source it can. During aerobic growth and in the presence of simple sugars (glucose, fructose,

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sucrose, etc) *S. aureus* will begin metabolism through Embden-Meyerof-Parna pathway, commonly referred to a glycolysis [69]. Glycolysis is only the first step in aerobic metabolism in *S. aureus*, which can also utilize the TCA and pentose phosphate pathways. The TCA cycle typically takes over during the transition to exponential phase once the available supply of glucose has been extinguished. Importantly, during glycolysis there is a build-up of by-products, one of which is acetic acid. Aside from causing a drastic decrease in pH, acetic acid can be used as an acetate source which is an important metabolic substrate of the TCA cycle [69].

So in rich media, where many potential carbon sources available, how does *S. aureus* choose what it would like to "eat"? Metabolism is like a popularity contest where available carbon sources (i.e. sugars) compete for favor, with the prom-queen, glucose, ultimately beating out other contestants. Not only does glucose trump the other contestants/sugars, it starts a regulatory system that will shut-down genes useful for metabolism of other carbon sources, while turning on genes for its metabolism as well as other factors [78-81]. Glucose's handy-man is the Carbon Catabolite Protein A, CcpA, which is a global regulator in *S. aureus* and has homologues in most other bacteria. CcpA, along with its partner HPr, is responsible for carbon catabolite repression (CCR) in a number of low GC content Gram-positive bacteria, such as *B. subtilis* (in which CCR has been extensively studied), *B. cereus, L. lactis*, *S. mutans*, *S. pyogenes*, *S. epidermidis* and *S. xylosus* [69, 78, 80, 82, 83].

CcpA, binds to DNA promoter regions called CREs (catabolite-responsive elements), and becomes "activated" when its co-factor, HPr is phosphorylated, which

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occurs through the sensing of glucose intermediates [69, 84]. Interestingly, the level of CcpA stays at a constant level in the cell and is not expressed in response to glucose [69]. While CcpA is typically thought of as a repressor, it can act as both a repressor and an activator depending on where/how the active CcpA/P-ser-HPr complex binds to promoters [69, 78, 84-87]. CcpA has also been found to have significant roles in regulating virulence factors such as *hla*, and *spa* [86]. Furthermore, a role for CcpA has been implicated in biofilm formation as a CcpA deficient strain exhibited decreased ability to form biofilms [86]. While CcpA has been studied exhaustively in *B. subtilis*, only a few published reports have looked into the CcpA regulon and activity in *S. aureus*, and it will be interesting to see the extent of its role in virulence.

The Immunodominant surface antigen B (IsaB)

A study by Lorenz et al, investigated the antibody response of patients who recovered from MRSA septicemia in an effort to identify possible targets for antibody therapy [88]. In their study, they compared the antibody response at the onset of MRSA septicemia to the one observed during convalescence. Screening pre- and post-infection serum against nine clinical MRSA strains they found that upon patient recovery, at least four distinct proteins had induced an immune response; and the authors hypothesized that these antibodies would be protective. They designated one of the four proteins as Immunodominant surface antigen B (IsaB), a 17kDa *S. aureus* protein with no significant homology to other proteins with known function. While all septic patient sera tested

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contained antibodies towards IsaB, sera collected from healthy or healthy-colonized individuals had little or no antibody reactivity against IsaB.

The findings that IsaB antibodies are absent in individuals who have not experienced a *S. aureus* infection but present in individuals who have recovered from septicemia suggest that IsaB is immunogenic during acute infection. This also suggests that IsaB is expressed during infection and may indicate that it is a virulence factor of *S. aureus*. The identification of novel virulence factors, in particular those that elicit a robust immune response, could lead to new therapeutic agents to combat this important pathogen.

Our goal therefore was to characterize the regulation, structure, and function of IsaB, as well as its role in virulence. From these studies we have identified a number of external and internal factors involved in the regulation of *isaB* expression. We found that IsaB is a nucleic acid-binding protein and used Electro-mobility shift assays (EMSAs) and surface plasmon resonance (SPR) to elucidate and characterize its affinity for RNA and DNA. We investigated role of IsaB in virulence, immune evasion, and biofilm formation, and found that IsaB is protective against both AMPs and NETs. Finally, we characterized the structure of IsaB by nuclear magnetic resonance.

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CHAPTER 2

Methods and Materials

Strains and growth conditions

Bacterial strains used in these studies are listed in **Table 1**. *S. aureus* strains were grown on Tryptic Soy Agar plates (TSA) (BD, Sparks, Md), or TSA plates supplemented with (10µg/ml) erythromycin (erm) as required for selection at 37℃ overnight, or as indicated. Liquid cultures of *S. aureus* were grown in either Tryptic Soy Broth (TSB)(EMD Chemicals Inc, Gibbstown, NJ) or Luria Bertani Broth (LB)(EMD Chemicals Inc), with or without supplementation of 1% D-Glucose (TSBG or LBG) (Fisher Scientific, Fair Lawns, NJ) at 37℃ overnight with shaking at 200 rpm. *E. coli* strains were grown in LB, either broth or plates, supplemented with (100µg/ml) ampicillin, (35µg/ml) chloramphenicol, or (50µg/ml) kanamycin as required for antibiotic selection, at 37℃ overnight. Primers used throughout these studies are listed in **Table 2**.

RNA Extraction

Samples were separated by centrifugation at 25℃, the spent media was discarded and pellets were frozen immediately at -80℃ until use. The Qiagen RNeasy® mini kit

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Table 1. Bacterial Strains Used.

Strain	Species	Resistance
10833	*S. aureus*	
10833Δ*isaB*	*S. aureus*	Erm
10833Δ*nPase*	*S. aureus*	Erm
10833Δ*tagO*	*S. aureus*	Erm
Sa113	*S. aureus*	
Sa113Δ*isaB*	*S. aureus*	Erm
Sa113Δ*sarA*	*S. aureus*	Erm
Sa113Δ*ccpA*	*S. aureus*	Erm
RN450	*S. aureus*	
502	*S. aureus*	
502Δ*srtB*	*S. aureus*	Erm
Top10	*E. coli*	
BL21-pLYS(DE3)-pRIL	*E. coli*	Cm
Op50	*E. coli*	
BL21-pLysS(DE3)-pRIL + *isaB*	*E. coli*	Cm, Kan
BL21-pLysS(DE3)-pRIL + m-*isaB*	*E. coli*	Cm, Amp

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Table 2. Primers and Oligos.

Name	Sequence
WTUTR-c	5'-BIOTIN-TGCaauuacaaauauuuccguuuaauuauaacaacaaucuauuGCA-3'
IsaBIntein	5'-GGGCATATGAATAAAACCAGTAAAGTTTGTGTAGC-3'
IsaBInteinREV	5'-GGTTGCTCTTCCGCAACCTTTACTTGTTTTGTATGGTGTATGTCC-3'
*isaB*XhoFWD	5'- GGGCATATGGTTTGTGTAGCAGCAACATTAGC-3'
*isaB*XhoREV	5'-GGGCTCGAGCGAAGTAACAGTTGGACATACACC-3'
icaUTR6	5'-GUUUAAUUAUAACAACAAUCUAUUGCA-3'
BioticaPRO	5'-BIOTIN-ATTGVGTTATCAATAATCTTA-3'
IcaRcloneFWD	5'-GGTGGGATCCTTGAAGGATAAGATTA-3'
WTUTR(RNA)	5'-Biot-tegugcaauuacaaauauuuccguuuaauuauaacaacaaucuauuGCA-3'
*isaB*RT-PCRFwd	5'-GCAAAAGGAAACGAAGCAAG-3'
*isaB*RT-PCRRev	5'-AACGACAAACCAGATGCCTAA-3'
Trunc-*isaB*Bam5'	5'-GGATCCGCAATAACCCCATATTATAC-3'
Trunc-*isaB*Xho3'	5'-CTCGAGTTATTTACTTGTTTTGTATGG-3'
*isaB*Northern	5'CGTTTTAGCTAATTTAATACCATTGAATTTCACATTATCATACTTAATCGCG-3'
16sRT-PCRFwd	5'-GAACCGCATGGTTCAAAAGT-3'
16sRT-PCRRev	5'-TATGCATCGTTGCCTTGGTA-3'
IsaBprobe	5'GTATCTGCATCCAAGCACATTACATAATCATATGAAGCCTGTTTAATGCC-3'

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(Qiagen, Valencia, CA) was used with some modification. Samples were thawed on ice and immediately resuspended in 500 µl RLT® lysis buffer (Qiagen), mixed, and added to Lysing Matrix B® tubes (MP Biomedicals, Solon, OH). 500 µl Acid Phenol:Choloroform (Ambion, Austin, TX) was added to the sample, which was mixed by inverting and immediately processed in a FastPrep-FP120 (MP Biomedicals) at a speed setting of 6.0 for 45s. The tubes were cooled 5min on ice and the bead beating was repeated. Samples were centrifuged at 14,000 rpm for 7 min at 4℃. The aqueous phase was collected and 500µl 100% ETOH (Invitrogen, Carlsbad, CA) was added and mixed. Samples were then loaded into the RNAeasy column (Qiagen). Following the manufacturers' instructions, columns were washed with 700µl RW1 (Qiagen) followed by 2X washes of RPE (Qiagen). The RNA was eluted twice with 50µl nuclease-free H_20 (American Bioanalytical). Eluted RNA was immediately treated with TurboDNAse (Ambion) according to the manufacturer's instructions, for 30-40 min. TurboDNAse was inactivated and removed using DNAse Inactivation Reagent (Ambion) according to manufacturers instructions. A NanoDrop1000 spectrophotometer (Thermo Scientific, Milford, MA) was used to obtain the readings at 230ηm, 260ηm and 280ηm in order to determine the RNA concentration and the amount of protein/ethanol contamination. Absorbancy at 280ηm was used to determine the amount of protein contamination with a 260/280 ratio of 2.0 being ideal. The 230ηm absorbency was used to determine the relative amount of ethanol contamination from the extraction procedure. RNA concentration was determined from the 260ηm absorbency using the following equation: 44ug/ml x OD260nm.

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Northern Blot Analysis

Northern analysis was performed using the NorthernMax®–Gly Kit (Ambion, Austin, Texas) according to the manufacturer's instructions. 20µg of RNA was used per sample. ULTRAhyb®–Oligo Hybridization Buffer (Ambion) was used instead of the hybridization buffer contained in the kit. The ULTRAhyb®–Oligo Hybridization Buffer protocol was followed per the manufacturer's instructions from pre-hybridization until the final washes. The biotinylated nucleotide "IsabNorthern" was used as the probe. BrightStar®–BioDetect Kit (Ambion) was used following the manufacturer's instructions for detection and the blots were exposed to autoradiographic film.

Cellular localization of IsaB

Sa113 and Sa113Δ*isaB* were grown in 1L TSB for 6-10 hours. Cultures were centrifuged and both the cell pellet and spent medium were collected. Protein from 400 ml spent medium was precipitated by 70% saturation $(NH_4)_2SO_4$, while stirring at 4°C for 1 hour. Precipitated proteins were collected by centrifugation, the resulting pellet was resuspended in 1 ml of PBS with complete protease inhibitor cocktail tablets (Roche Diagnostics). The samples were dialyzed against 3 L of 0.1X PBS overnight at 4°C before gel electrophoresis. The cell pellet was washed with PBS and resuspended in 20 ml of Buffer A (40mM Tris-Cl, 100 mM NaCl, 27% Sucrose, 20 mM $MgCl_2$, and protease inhibitor cocktail 1/50 ml). 500 µg lysostaphin was added and the cells were incubated for 4 hours at 37°C. The pellet (protoplasts) and supernatant (peptidoglycan) were separated by centrifugation. The cell pellet was resuspended in 10 ml of water, 1% Triton X was added

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and mixture was rocked for 10 min at RT. Samples were centrifuged 10,000Xg for 20 min to remove intact cells and membranes were collected by centrifugation at 100,000Xg for 1 hr. Following centrifugation the supernatant (cytoplasm) was collected and the pellet (membrane) was resuspended in water. Equal amounts of protein from the four cellular fractions were analyzed by denaturing PAGE using NuPAGE® 4-12% Bis-Tris gels (Invitrogen) according to manufacturer's instructions. The proteins were transferred onto a PVDF membrane which was then blocked 1 hr in PBS containing 5% skim milk. The blot was probed with a 1:5,000 dilution of IsaB-specific rabbit antisera in PBS containing 0.05% tween (PBST) and 0.5% skim milk followed by a 1:10,000 fold dilution of goat anti-rabbit horseradish peroxidase conjugated IgG in PBST. Proteins were detected using the ECL Plus detection system (Amersham) and analyzed with a CCD camera (Kodak).

Quantitative RT-PCR

1µg of RNA from each sample was converted into cDNA by a two-step reverse transcriptase reaction using Superscript II Reverse Transcriptase kit (Invitrogen, Carlsbad, Ca) following the manufacturer's instructions. The RNA, and 1:10 dilution of (10pM) of primers *isaB*RT-PCRRev and 16sRT-PCRRev were combined and heated @ 70°C for 10 minutes. Samples were snap cooled on ice for at least 30 seconds. Following incubation on ice, the reverse transcriptase mixture was added each to sample and incubated at 42°C for 2 hours, followed by 15 minutes at 70°C. The cDNA was diluted 1:10 for *isaB* samples and 1:1000 for the *16s* samples. These samples were subjected to RT-PCR using the SensiMix *Plus*, SYBR® + FLUORESEIN (Bioline, Randolph, Ma) following the

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manufacturer's instructions. Real-Time RT PCR was performed using the following cycle conditions: 1 cycle 95°C for 3 min.; 40 cycles of 10 sec 95°C, 30 sec 58°C and 15 sec 72°C (realtime data collection) followed by a melt-curve using 1:10 dilutions of *isaB*RT-PCRRev+*isaB*RT-PCRFwd or 16sRT-PCRRev+16sRT-PCRFwd. Cycle thresholds from the *isaB* samples and 16S rRNA samples were used to determine the normalized expression (*E*) of *isaB*, using the equation *E*=2^(16s ct – *isaB* ct). Samples lacking the reverse transcriptase enzyme were assembled as controls for contamination of the RNA samples with genomic DNA, and each PCR run included a no template control.

RNA Stability Assay

Performed essentially as described in Roberts et al, with the following modifications [89]. Overnight cultures of Sa113 grown in LB were diluted 1:100 in fresh LBG or LBG supplemented with 50mM HEPES Buffer (USB Corporation, Cleveland, OH) at a flask to volume ratio of 1:2.5 and grown at 37℃ with shaking at 200rpm for 6 hours. 200µg/ml rifampin was added to cultures and time-points collected at 0, 5, 30 and 60 min. Each sample was immediately treated with ice-cold RNAlater® (Ambion) and centrifuged at 4℃ for 10min and pellets were immediately frozen at -80℃ until RNA extraction was performed followed with RT-RT-PCR.

Expression of IsaB in *E. coli*

BL21-pLysS(DE3)-pRIL + *isaB* strain of *E. coli* (Stratagene, La Jolla, CA) previously constructed was used. 1L of LB containing 50 µg/ml kanamycin and 35 µg/ml

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chloramphenicol was inoculated with 50 mL of overnight culture and incubated at 37ºC for 3 hours. The culture was induced with 1 mM IPTG and incubated 3 hours at 37ºC. The bacteria were collected by centrifugation, and resuspended in 25 ml of CBD buffer (20mM Tris-CL pH 7.0 containing 0.5M NaCl) with 0.1% Triton X-100 and protease inhibitors (Roche, Indianapolis, IN). The bacteria were lysed using a French pressure cell followed by 6 X 20 sec, 9 Watt pulses with a probe-type sonicator. Intact cells and debris were removed by centrifugation, and the supernatant was filtered through a 0.45 µm filter. 8 mL chitin resin (New England Biolabs, Ipswitch, MA) was poured into a column, washed once with 10 mL H_2O and twice with CBD buffer. The lysate was applied to the column, and the column was rinsed 3X with 15 ml of CBD buffer, once with 15 mL CBD buffer containing 1% TritonX-100, 3X with 15 mL CBD buffer, and finally with 15 mL CBD buffer containing 50 mM dithiothreitol (DTT), and the column was incubated 16 hours at 4 ºC. The column was eluted with 50 mL of CBD buffer and the eluate was concentrated and desalted using 5,000 MW Amicon Ultra® concentrators (Millipore Corporation, Billerica, MA).

Electrophoretic mobility shift analysis

Probes for EMSAs were fluorescently labeled with the ULYSIS™ Alexa Fluor® 594 Nucleic Acid labeling kit (Invitrogen) according to manufacturer's instructions. Mobility shift reaction mixtures containing 20 µL binding buffer (BB1: 20mM HEPES, 1mM DTT, 20 mM KCl, 200 µg BSA/ml, 10% glycerol), 480 pmol purified, recombinant IsaB (optimal concentration determined from Fig. 3A, which had either 3.84 nmol, 1.92 nmol,

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960 pmol, 480 pmol, or 240 pmol of purified, recombinant IsaB), and 270 pmol RNA probe icaUTR6 were incubated for 10 minutes at room temperature. The reactions were loaded onto a 2% acrylamide gel, bromophenol blue was added to one lane as a marker, and the gel was electrophoresed at 100V for 30 min. Bands were visualized using a CCD camera. Salmon sperm DNA (SSS) was serially diluted 10-fold and added to designated reactions at final concentrations ranging from 1.35 nmol-1.35 pmol. For inhibition analysis, 2.7 nmol of either salmon sperm DNA (Invitrogen), nucleotides, or yeast tRNA (Sigma, St. Louis, MO) were added in addition to the standard mobility shift reaction mixtures.

Surface Plasmon Resonance

IsaB interactions with RNA, DNA, and dsDNA were analyzed using a BIAcore Model T100 (GE Healthcare, Piscataway, NJ) following manufacturer's instructions. Biotinylated oligos, DNA and RNA, were immobilized on a Streptavidin chip (SA sensor chip, GE Healthcare) in 0.33X HBS-EP buffer, supplemented with 1x of non-specific binding inhibitor (GE Healthcare). Double-stranded DNA was created by loading the SA DNA coated chip with the complementary strand, icaRcloneFWD. The first flow chamber was left blank to allow for normalization and subtraction of non-specific binding. Resonance units were determined using decreasing concentrations of IsaB that were loaded onto the chip at a flow rate of 30 µl/min. The kD and kA were determined with the BIA Evaluation Software version 3.0.

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S. aureus binding to fluorescently labeled oligonucleotide

Overnight cultures of *S. aureus* strains 10833 and 10833Δ*isaB* were diluted 1:20 in fresh media (TSB+1% glucose) and incubated at 37°C with shaking. After 4 hours of incubation, approximately 10^8 bacteria were collected by centrifugation and resuspended in binding buffer (20mM HEPES, 1mM DTT, 20 mM KCl, 200 µg BSA/ml). 40ng ULYSIS™ Alexa Fluor® 488-labeled sonicated salmon sperm (SSS) was added and the reactions were incubated for 15 minutes at room temperature. Control reactions lacked the fluorescent oligonucleotide. Following incubation, the cells were washed once in binding buffer, and resuspended in 200 ml of water. Fluorescent counts were determined using an Flx800 (BioTek, Winooski, VT). Experiments were performed in triplicate and statistical significance was determined using an unpaired Student's t-test.

Growth Curves

Overnight cultures of 10833 or 10833Δ*isaB* were grown in LB, were diluted 1:100 in fresh LB or LBG with a media to flask ratio of 1:2.5. Cultures were grown at 37℃ with shaking at 200 rpm. At the indicated time-points 1 ml of culture was removed and analyzed with a BioMate3 spetrophotometer at (Thermo Scientific) at OD_{600}, to determine culture density, and plotted from time 0.

C. elegans Killing Assay

In these experiments we used a protocol adapted from Begun et al [90]. Briefly, *C. elegans* were allowed to feed on a lawn of *S. aureus* on LB agar containing 1% glucose and 5mg

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nalidixic acid/mL. *S. aureus* was grown for 4-6hrs at 37ºC on the plate prior to addition of *C. elegans*. 10 nematodes were added per plate (each strain was tested in duplicate) and placed at room temperature. *C. elegans* that failed to respond to touch were considered dead and were counted over a five-day period. We tested *S. aureus* strains 10833, its isogenic *isaB* deletion mutant (10833Δ*isaB*), and a strain complemented for the deletion in trans by a multi-copy plasmid containing the *isaB* gene under control of an inducible promoter (10833Δ*isaB*+*isaB*). OP50 is an avirulent strain of *E. coli* that was used as a negative control.

Survival in Whole Blood

Whole human blood was purchased from Biochemed (Winchester, VA) for use in survival assays. Overnight cultures of *S. aureus* strains Sa113 and Sa113Δ*isaB* were grown overnight in TSBG and used the following day. Cultures were diluted 1:50 in whole blood and samples were collected at 30 minutes, 2 and 4 hours. Red blood cells were lysed by sonication, samples were collected by centrifugation, resuspended in water and sonicated again. Cells were collected by centrifugation and resuspended in LB prior to plating for CFUs. Percent survival was determined from CFUs of blood treated samples to the CFUs of the input culture: (CFU blood/ CFU input)*100.

Invasion Assays

In order to determine intracellular survival in epithelial cells we used a protocol adapted from Hess et al [30]. Briefly, lung epithelial A549 cells were grown to confluence in

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standard tissue culture six well plates with 3mls RPM1 (Gibco, Grand Island, NY). 100ml of bacteria at OD_{600} 0.5 were added to the epithelial cells and cocultured for one hour at 37ºC. Following incubation, 100 μg/ml of gentamicin was added to the co-culture to kill extracellular bacteria. Following gentamicin treatment, the epithelial cells were lysed with EDTA+ 0.1% saponin, and the bacteria serially diluted and plated. Enumeration of CFUs was done the following day, and "percent invasive" was calculated by taking the number of (CFUs of invasive/CFUs input)*100 per strain. Thus wild-type and Δ*isaB* were compared by "percent invasive".

Adherence Assays

This assay was performed essentially as described for the invasion assay, however cells were lysed following the first 1 hour co-culture and CFUs were plated. Comparisons were made using "percent adherent" again using input culture as a means to normalize cultures.

Neutrophil Bactericidal Assay

Neutrophil mediated killing via phagocytosis was performed using a protocol adapted from Corbin et al, to assess the ability of IsaB to protect *S. aureus* from neutrophil mediated killing [91]. Briefly, cultures of *S. aureus* strains 10833 and 10833Δ*isaB* were grown overnight in TSBG. The bacterial cells were pre-opsonized with 50% human serum/50% PBS at 37℃ for 30 minutes, which allowed for complement and antibody deposition. Meanwhile, human neutrophils were isolated using a Ficol sucrcose gradient and red blood cells were lysed using a water wash. $2x10^7$ neutrophils were co-cultured with the pre-

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opsonized bacteria at an MOI of 1 for 2 hours at 37℃ with rotation. Neutrophils were then lysed with saponin, samples were sonicated and then plated for CFUs. Percent survival from neutrophil mediated killing was determined using the following equation: (CFUs neutrophil/ CFUs pre-opsonized input)*100.

NETs Killing Assay

Performed using a protocol adapted from Jann et al [92]. Neutrophils were isolated from whole blood separated with a Ficol sucrose gradient. 500,000 cells were added to wells in a 96 well plate and treated with 50nM PMA in RPMI for 4 hours at 37℃. Neutrophil media was replaced with fresh RPMI containing 10nM cytochalsin D. For bacteria, overnight cultures of Sa113 and Sa113Δ*isaB* grown in LBG. Bacteria were washed in RPMI and $1x10^3$ bacteria were co-cultured with the NETs for 30 minutes. Samples were sonicated briefly with a probe-type dismembranator, and CFUs were used to enumerate killing by NETs. Percent survival was determined by the following equation: (CFUs treated/CFUs input)*100. Statistical analysis was done using the Student's t-test with significance cutoff of $p \leq 0.05$.

AMP Survival Assays

S. aureus strain 10833 and 10833Δ*isaB* were grown overnight in LBG. Bacteria from 100µl of overnight cultures were collected by centrifugation and resuspended in 1 ml of PBS. 10µl of the bacteria, containing approximately $1X10^6$ bacteria was added to 100µl of PBS. Experimental samples were treated with 2.4µg/ml of each HNP1 (PeproTech Inc,

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Rocky Hill, NJ), HBD2 (PeproTech Inc), Buforin (AnaSpec Inc, San Jose, CA), and HBD3 (PeproTech Inc) and controls were left untreated. AMPs and bacteria were incubated together for 2 hours at 37℃ with rotation. Samples were sonicated briefly and then plated for CFU enumeration of percent survival. Percent survival was calculated by the following equation: (CFUs treated/CFUs control)*100. Statistical analysis was done using the Student's t-test with significance cutoff of $p \leq 0.05$.

Confocal Microscopy

Overnight cultures of either 10833, or 10833Δ*isaB* grown in TSB were diluted 1:20 and added to wells of a 6-well tissue culture plate. After overnight growth the media was removed and biofilms were resuspended in PBS and stained with Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes) following manufacturer's instructions. Stained biofilms were visualized with a Zeiss confocal scanning laser microscope.

96 Well Biofilm Micro-titer Plate Assay

Biofilm assays were performed essentially as described by Christensen [93]. Overnight cultures of *S. aureus* strains 10833, 10833Δ*isaB*, Sa113, and Sa113Δ*isaB* were diluted 1:20 in fresh media (TSB, TSBG, TSBG +3.5% NaCl, BHI, BHIG, LB, or LBG) in a microtiter plate. Cultures were incubated overnight at 37°C. The following day, the media was removed, plates were washed with 1X PBS, dried and stained with safranin. For visualization of biofilms, images were obtained by the use of an image scanner. Stained

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biofilms were resuspended in 100µl in 33% acetic acid and absorbency at 564nm was determined using an ELISA plate reader.

For DNAse treated biofilms the same protocol was followed with the addition of treatment with experimental biofilms with 2.5U TurboDNase per sample. DNase was incubated with the biofilms for 4 hours before processing as normal. The control biofilms were processed prior to the 4 hour incubation. Percent degradation was calculated by comparing the mean biofilm absorbency on the control plate to the mean biofilm absorbency of the biofilms treated with TurboDNase; (treated biofilm/control biofilm)*100.

Serum biofilm formation was assessed with the standard amount of cells being inoculated directly into 100%, 50%, 25%, 12.5%, or 6.25% human serum. Serum dilutions (when required) were made with TSBG.

Western Analysis

Performed essentially as described above for "Cellular Location of IsaB". Samples were collected by centrifugation at 4,000 rpm for 15 min and resuspended in 250µl of 5%SDS, and incubated at room temperature with agitation for 30 min. Following SDS treatment, the bacteria were centrifuged at 13,300 rpm for 5 minutes, and the cleared supernatant, containing the soluble protein, was collected. 200 µg of protein/sample was used. Then treated as described above.

For western time course, the cells were diluted 1:100 from overnight LB cultures into LB, LBG, or LBG + 50mM HEPES. 10 ml samples were taken at time-points 2, 4, 6,

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8, and 24 hours. Cells were collected and processed with SDS as described above. Supernatant from these cultures was concentrated 10X using micron-concentrators (Millipore) with LDS buffer being added directly to samples and processed normally.

For surface-association assays, 5 mls of overnight culture of indicated strains were grown in TSBG. Cells and supernatant were separated by centrifugation. Cells were treated as described above and supernatants were concentrated as described previously.

Expression and Purfication of m-IsaB.

The mature *isaB* (m-*isaB*) gene excluding the signal sequence was amplified from Sa113 total DNA by PCR using primers truncIsaB-Bam and truncIsaB-Xho. The PCR product was digested with BamI and XhoI (New England BioLabs)and cloned into the pET32-XT vector (kindly provided by Dr. David Willaims) using Ready-to-go ligase (Amersham, Piscataway, NJ). The pET32-XT vector fuses an amino terminal thioredoxin-6His onto the protein. The ligation reaction was used to transform TOP10 *E. coli* (Invitrogen) and successful transformants were selected by resistance to 100 μg/ml ampicillin. The plasmid was sequenced to confirm the lack of mutations within m-*isaB* and was used to transform the BL21-(DE3)-pRIL strain of *E. coli* (Stratagene, La Jolla, CA). 1L of LB containing 100 μg/ml ampicillin and 35 μg/ml chloramphenicol was inoculated with 50 mL of overnight culture and incubated at 37ºC for 3 hours. The culture was induced with 1 mM IPTG and incubated 3 hours at 37ºC. The bacteria were collected by centrifugation, washed in PBS and the pellet frozen at -80℃. Cells were lysed with BPER lysing reagent (Invitrogen) supplemented with 150mM NaCl and 2mM β-Merceptoethanol. Lysates were

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cleared via centrifugation and immediately loaded into Akta HPLC and separated over a nickel column. Protein peaks were collected and then dialyzed against 3L of 20mM Tris-HCL pH 8.0 with 2mM β-Me and 150mM NaCl using a 10,000 mw Slide-A-Lyzer® Dialysis Cassette from (Thermo Scientific). 150U Thrombin was added to the dialysis reaction which was incubated at RT overnight. Dialyzed samples were again loaded into the Akta and m-IsaB was separated over a mono-S ion exchange column. Protein peaks were collected and then purified once more over a large gel filtration column. Protein concentration was determined using the 280ηm on a spectrophotometer using the equation: (absorbance at 280nm*dilution factor)/extinction coefficient. The extiniction coefficient used was 1.292 as was determined for m-IsaB using the ExPasy ProtParam Tool, available at http://expasy.org/cgi-bin/protparam[94]. To ensure purity ~5 µg of protein was used and NuPAGE® LDS Sample Buffer (4X) was added to samples and then heated at 75°C for 10 minutes. Following the denaturation step, the samples were loaded onto NuPAGE® 4-12% Bis-Tris Gel 1.5mm x 10 well and electrophoresed for 30 minutes at 200V. After gel electrolysis, the gel was washed once in 50mls of dH_2O followed by staining with Imperial™ Protein Stain (Thermo) according to the manufacturer's instructions. The stained gel was then decolorized with 3X 50mls washes with dH_2O until no background staining was observed.

Labeling procedure was adapted from Cai et al [95]. Briefly, ^{13}C-glucose and $^{15}NH_4Cl$ (Cambridge Isotope Laboratories Inc., Andover, MA) were provided as the sole carbon and nitrogen source to ensure isotope incorporation into the protein. For expression of labeled m-IsaB, BL21+m-*isaB* was grown overnight in 1LB at 37℃, 500 µl were used

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to inoculate 10mls of M9 buffer (containing both ^{13}C-glucose and $^{15}NH_4Cl$) which was grown for approximately 8 hours at 37℃. 10ml was then used to inoculate 100mls of M9 buffer that was then grown overnight at 37℃. The overnight culture was inoculated into 1L of M9 and grown to an OD_{600} of 0.8, where it was then induced with the addition of 1mM IPTG and incubated for 4 hours before cells were collected by centrifugation and storage at -80℃. Samples were then subjected to the purification process previously described for m-IsaB.

m-IsaB EMSA

Mobility shift reaction mixtures containing 20 mL binding buffer (BB1: 20mM HEPES, 1mM DTT, 20 mM KCl, 200 µg BSA/ml, 10% glycerol), with either 1.6 nmol, 160pmol, 16pmol, or 0.16pmol purified, recombinant m-IsaB, and 1 fmol biotinylated probe IsaBprobe were incubated for 20 minutes at room temperature. The reactions were loaded onto a 2% acrylamide gel, bromophenol blue was added to one lane as a marker, and the gel was electrophoresed at 100V for 30 min. The gel was then transferred to a BrightStar®-Plus Positively charged nylon membrane (Ambion) by wet-transfer at 30V for 1 hour. The membrane was then blocked, probed, washed, and developed using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific) according to the manufacturer instructions.

For teichoic acid competition assay lipoteichoic acid (LTA) (Sigma Aldrich, St. Louis, MO) was used. The above EMSA reaction containing 160nmol of purified

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recombinant IsaB (optimal shift) was used with the addition of LTA at a concentration of 1µg, 100ηg or 10ηg. Reactions were then completed as described above.

Assay for Degradation of DNA by m-IsaB

EMSA reactions setup as described above using either 1.6pmol or 160nmol IsaB in either BB1 or in BB1 where 20mM HEPES buffer was replaced with 20mM phosphate buffer pH 5.8. Samples were incubated at RT for 30min and then treated with 2X Novex®® TBE-Urea Sample Buffer (Invitrogen) and heated for 3min at 75℃. Samples were then loaded onto a 15% TBE-Urea gel (Invitrogen) and electrophoresed at 100V for 2 hours. Samples were then transferred and reacted as described previously using the Chemiluminescent Nucleic Acid Detection Module.

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CHAPTER 3

Regulation of IsaB

Introduction:

The observation by Lorenz et al. that IsaB elicits an antibody response during acute infection but not during colonization led us to hypothesize that expression of IsaB is induced during active infection, and that this expression has a role in the modulation of *S. aureus* virulence [87]. The expression of IsaB during sepsis may contribute to disease progression and the characteristic robust immune response seen in these patients, whereas suppression of IsaB during colonization may contribute to the lack of a specific immune response to this protein in carriers. Further suggestive of its relationship to infection, other labs have reported microarray data showing *isaB* expression to be increased; 1.) in response to neutrophil exposure, 2.) in biofilms, 3.) under anaerobic conditions and 4.) following internalization in human epithelial cells [2, 49, 96, 97]. All of these phenomena support the hypothesis that IsaB is a virulence factor of *S. aureus*.

Because the function of IsaB in vivo was unknown, we first wanted to determine the regulation of *isaB*. A number of global regulators in *S. aureus*, such as SarA, control the expression of genes within a specific regulon. Characterization of the regulatory

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network, and inducing stimuli, of *isaB* would serve as a foundation for a hypothesis regarding the role of IsaB in the virulence of *S. aureus*. Therefore we began our studies by determining the stimuli responsible for inducing *isaB* expression and how global regulators affected expression.

Results:

isaB is Transcribed as a Single Transcript

To begin our studies we first examined the location of *isaB* on the chromosome to determine the likelihood of it existing within an operon, which could lead to clues to its function or regulation. **Figure 1** shows the surrounding genes of *isaB*, and as the schematic shows, *isaB* does not appear to be located within an operon. However, we were intrigued by the possibility that *isaB* and *aur*, the gene encoding for aureolysin, were cotranscribed, as *aur* is immediately downstream of *isaB*. Aureolysin is a previously described extracellular zinc-metalloprotease that contributes to the virulence of *S. aureus*. To address whether *isaB* and *aur* were cotranscribed, Northern blot analysis was used to determine the size of message recognized by the biotinylated *isaB* specific probe. As shown in **Figure 2** we detected a message of approximately 500 bases, which is consistent with predicted size of the *isaB* transcript alone. If the two genes were co-transcribed, then a transcript of approximately 2,000 bases would have been observed. Therefore, *isaB* is not located within an operon and is it not co-transcribed with the immediately downstream *aur* gene.

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Figure 1. Chromosomal Location of *isaB*. *isaB* and immediately surrounding genes on the chromosome of *S. aureus* strain Col. Arrows indicate the direction of transcription.

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Figure 1. Chromosomal Location of *isaB*.

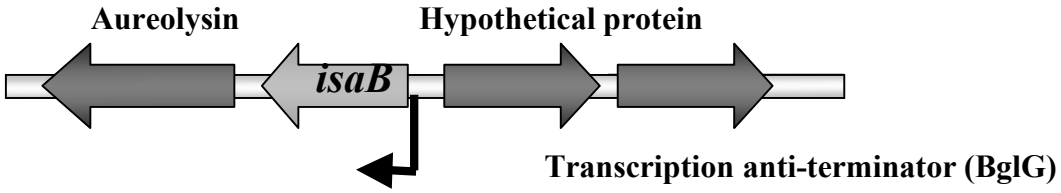

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Figure 2. *isaB* is a Monocystronic Transcript. If *isaB* was co-transcribed with *aur* a band with the predicted size of ~2000 bp would be observed by Northern blot analysis. Using a biotinylated *isaB* specific probe, a band corresponding to the predicted 566 bp size of the *isaB* transcript was observed.

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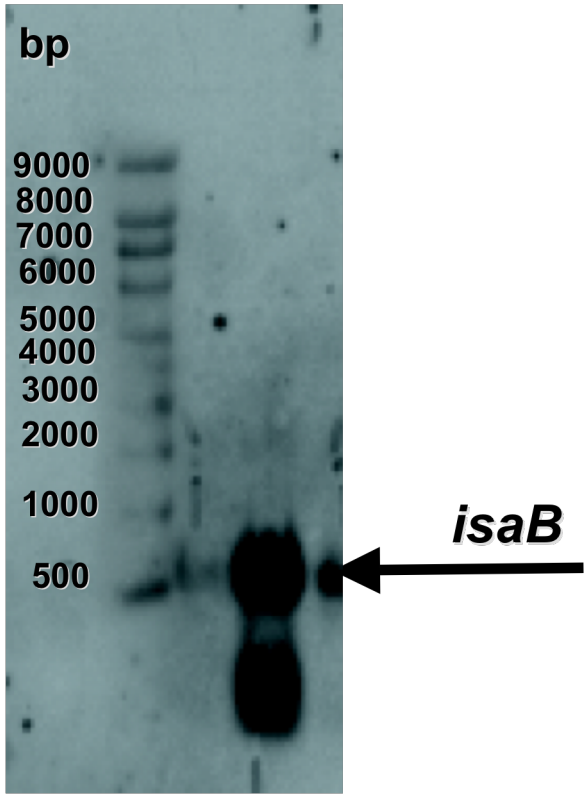
Figure 2. *isaB* is a Monocystronic Transcript.



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IsaB is Secreted

In Gram-positive bacteria most virulence factors are exposed on the cell surface or secreted into the extracellular milieu in order to function against the adverse conditions found in vivo [7]. *S. aureus* generally uses its sec-pathway for translocation of proteins across the cell membrane, which are targeted for export by a N-terminal signal peptide sequences. Following translocation of the protein, the signal peptide sequence is cleaved by the signal peptidase SpsB releasing the mature protein [7, 98]. Using the prediction server PSORTb we found that, like other virulence factors, IsaB was predicted to have an N-terminal signal peptide sequence for secretion [99]. Therefore Western blot analysis was used to confirm the predicted extracellular location of IsaB. Stationary phase cultures of *S. aureus* were fractionated into cell surface associated, supernatant, membrane associated and cytoplasmic fractions. The presence of IsaB in these fractions was detected using anti-IsaB Rabbit anti-serum. As shown in **Figure 3**, IsaB was detected exclusively on the cell surface and in the spent medium. Therefore IsaB is secreted from *S. aureus*, likely through the sec-pathway due to the presence of an N-terminal signal peptide sequence.

isaB is Not Regulated Temporally Under Normal Culture Conditions

While IsaB has been shown to be immunogenic, which lead to our hypothesis that it is expressed in vivo, no information was known about its expression under normal laboratory conditions. *S. aureus* encodes numerous "accessory" genes that are directly related to pathogenesis, which are often described as the "virulon" [100].

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Figure 3. IsaB is Secreted. Sa113 and Sa113Δ*isaB* cultures were fractionated into: supernatant (lanes 1 and 2), cell wall associated (lanes 3 and 4), cell membrane (lanes 5 and 6) and cytoplasmic (lanes 7 and 8) fractions. IsaB bands were observed in both the supernatant and cell wall associated fractions in wild-type Sa113 (lanes 1 and 3, arrows) but not in Sa113Δ*isaB* (lanes 2 and 4 respectively). Proteins that reacted non-specifically with IsaB antiserum were observed in all lanes, but were present in the *isaB* mutant as well as wild-type.

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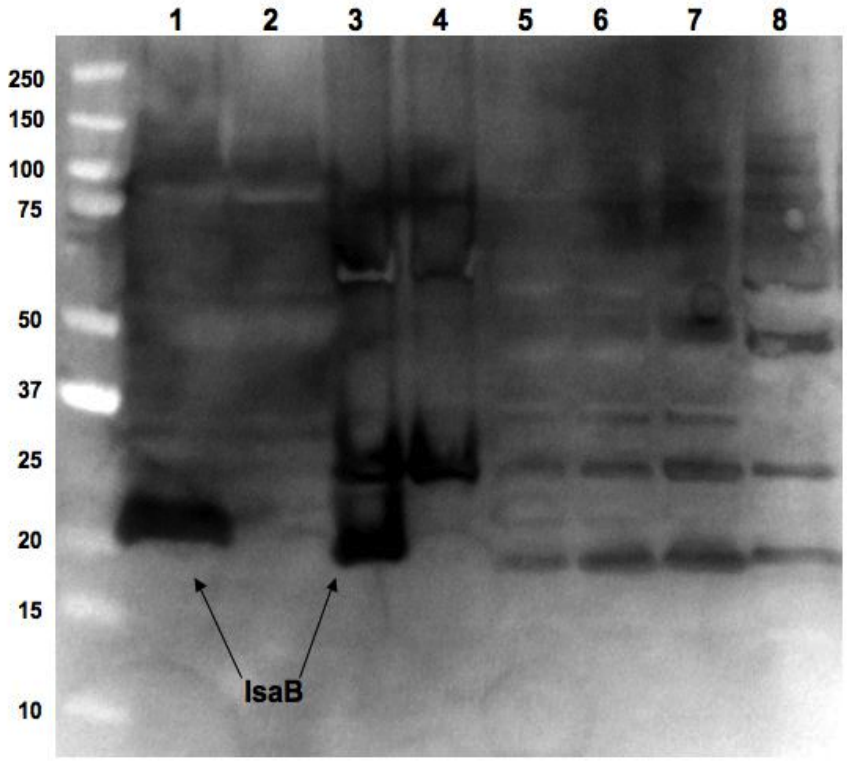
Figure 3. IsaB is Secreted.



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The expression of many of these virulence factors and other genes in *S. aureus* can be dependent upon external signals; however many are regulated temporally dependent upon growth stage and culture density. This temporal regulation is carried about by numerous transcription factors and/or two-component systems such as the transcriptional regulator SarA, or the Agr two-component system [100-102]. Therefore we sought to determine if *isaB* is temporally regulated during in vitro culture conditions, including growth in complex rich media such as Luria Bertani Broth (LB). Overnight cultures of wild-type *S. aureus* strain(s) were diluted 1:100 in fresh LB and samples collected at time-points 2, 4, 6, 8, 24, 48, and 72 hours. RNA was extracted from the samples and analyzed by quantitative reverse transcriptase PCR, RT-PCR. Following normalization with the internal control, 16s rRNA, it was found that *isaB* is expressed at a fairly consistent level throughout the time course varying only slightly (< 2 fold), until 72 hours post-dilution, where we observed an approximately 4.5 fold reduction in *isaB* expression, as shown in **Figure 4**. This data suggests that *isaB* under normal conditions is not significantly temporally regulated.

Glucose and Other Simple Sugars Induce *isaB* Expression.

IsaB stimulates an antibody response during septicemia. Therefore potential "in vivo" cues were assessed for their ability to induce *isaB* expression. The first cue tested was glucose, which would be available to the organism in blood during septicemia. Previously, glucose has been shown to both activate and repress transcription of factors associated with virulence; this discrepancy is likely due to environmental and metabolic

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Figure 4. *isaB* Does Not Exhibit Significant Temporal Regulation. To determine if *isaB* is temporally regulated throughout the growth cycle of *S. aureus*, RT-PCR of *S. aureus* strain Sa113 grown in LB was performed. Time-points were taken post-dilution into fresh media and normalized to the *16s rRNA* ct. No significant amount of temporal regulation was observed.

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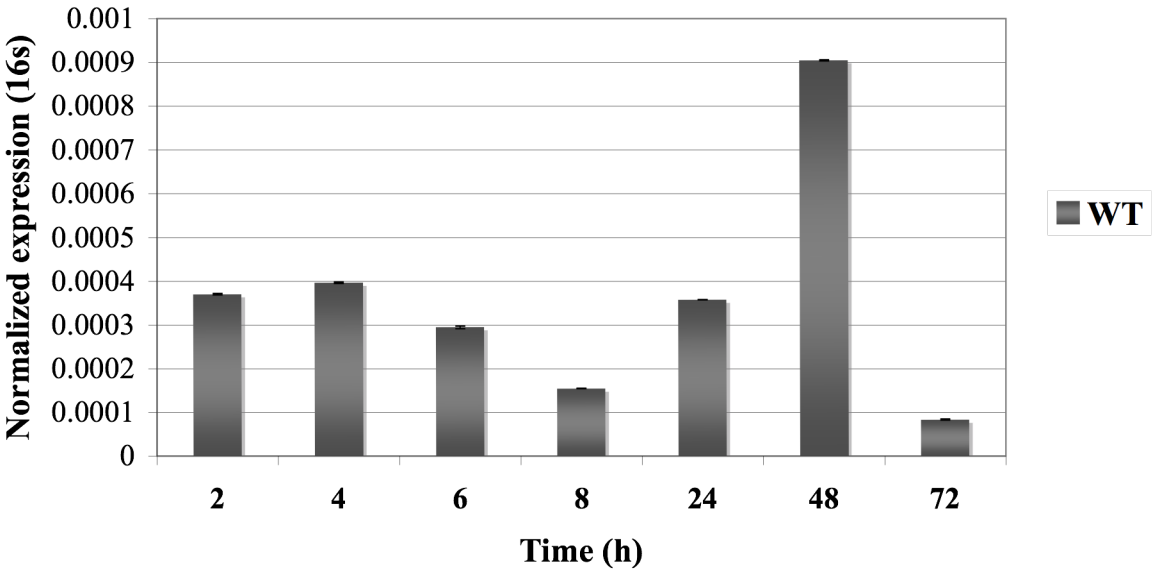
Figure 4. *isaB* Does Not Exhibit Significant Temporal Regulation.



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cues that regulate disease progression by *S. aureus* [84, 85, 87]. Therefore, overnight cultures of wild-type *S. aureus* were diluted 1:100 in fresh media, containing 1% glucose, LBG, which is approximately 10X greater than concentrations likely experienced in vivo [103]. Following six hours of induction samples were collected and RNA extracted for analysis by Northern Blots.

As shown in **Figure 5a**, after six hours of growth in glucose a dramatic increase in the amount of *isaB* message was detected by Northern blot. In order to quantify the magnitude of the change in expression due to glucose, RT-PCR was employed. As shown in **Figure 5b**, upon induction with 1% glucose *isaB* transcript increases approximately 14-fold relative to media without glucose. Carbon uptake and metabolism in *S. aureus* is controlled by a complex regulatory network, known as the phosphotransferase system (PTS), and is largely dependent on the type of carbon source available, i.e. preferred versus non-preferred [81, 84, 104, 105]. Fructose and sucrose, both simples sugars that can be used in glycolysis were next tested for their ability to induce *isaB*, again utilizing RT-PCR [79]. As shown in **Figure 5b**, both fructose and sucrose were capable of increasing detectable *isaB* transcript by approximately 13 and 8 fold, respectively.

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Figure 5. *isaB* Expression is Induced by Sugar(s). To determine if *isaB* is induced by glucose or other sugars *S. aureus* strains were grown in LB, LBG, LB+Sucrose (LBS) and LB+Fructose (LBF) for 4 hours before *isaB* transcript analysis. a) Northern blot analysis of *isaB* levels in *S. aureus* strain 10833 grown in either LB or LBG shows a dramatic increase in the level of transcript detected. b) RT-PCR of 10833 grown in LB, LBG, LBF, or LBS. The greatest increase of *isaB* was observed in samples grown in LBG followed by LBF, and LBS with fold increases of 13.5, 12.5, and 7.8 respectively. All samples were normalized to *16s rRNA* ct values.

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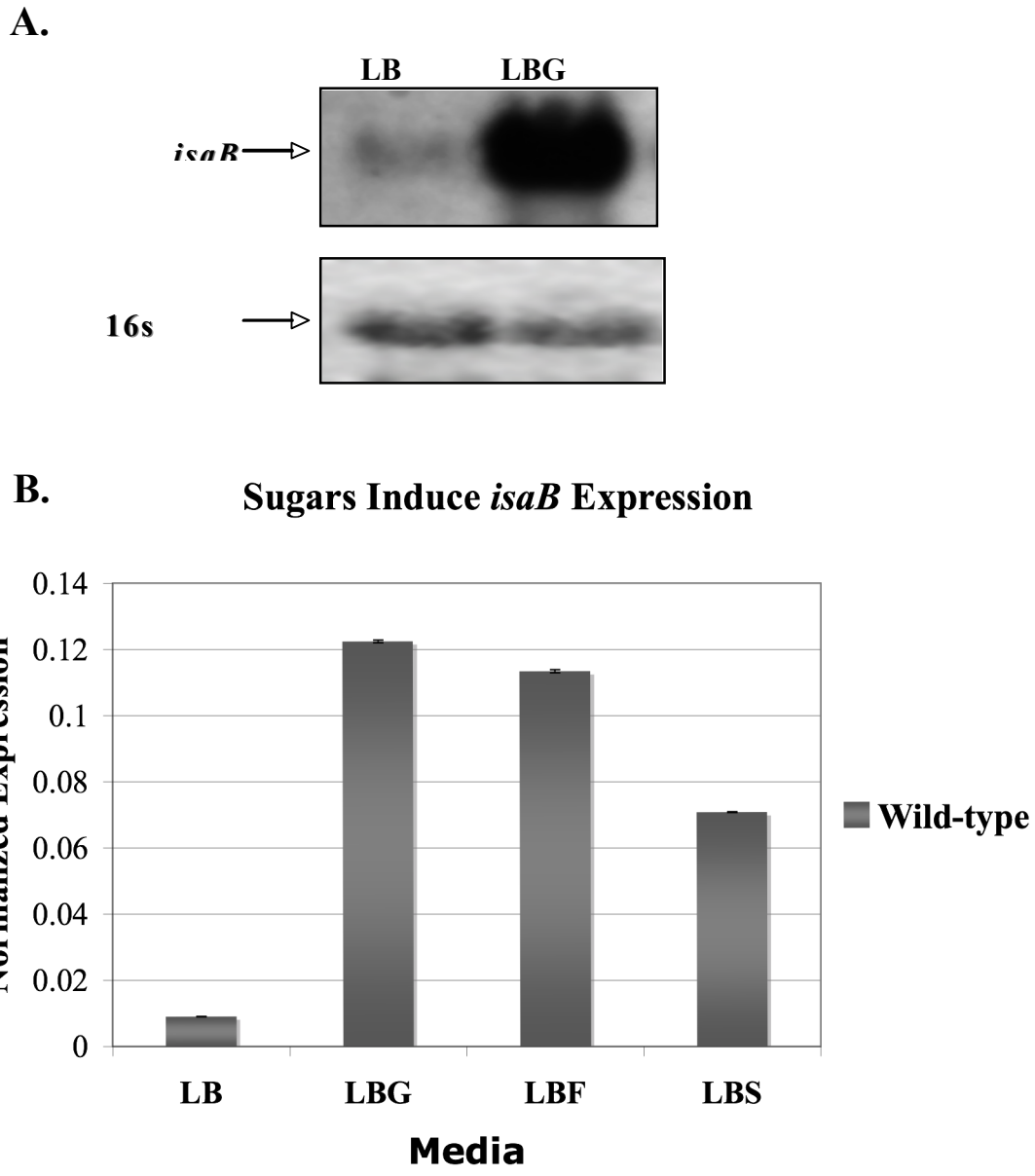
Figure 5. *isaB* expression is Induced by Sugar(s).



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Maximum *isaB* Expression Occurs Between 8-24 hours.

Finally, a long-term time course of glucose induction was performed to determine when the maximal induction of *isaB* occurred, and how long the induction lasts. Overnight cultures were diluted as described above, and samples for RNA extraction were collected at 2, 4, 6, 8, 24 post-dilution into LBG. As shown in **Figure 6**, *isaB* expression increases over time, with maximal expression observed at approximately 24 hours post dilution into media supplemented with 1% glucose. Similar experiments were performed on two other *S. aureus* strains: Sa113 and 10833, and the same trend was observed with *isaB* expression peaking between 8-24 hours, followed by decreasing levels out to 72 hours.

m-IsaB Expression Coordinates with *isaB* Expression.

To ensure that IsaB was being translated at greater rates in concert with gene transcription, western blots were performed to determine relative expression of IsaB. For these experiments, overnight cultures of wild-type and Δ*isaB* strains were diluted 1:100 in LBG, and 5 mls of culture were collected at time-points 2, 4, 6, 8, and 24 hours. Both cell surface associated protein, and concentrated supernatant was taken at each time-point to assay any variation in the cell associated and secreted proportions. Δ*isaB* samples taken at 6 and 24 hours were used as a negative control because of the presence of a consistently observed cross-reactive band that runs just slightly smaller than IsaB.

The blots in **Figure 7** show that very little IsaB is observed for cell-surface associated samples at 2 hours post-dilution. However by 4 hours IsaB expression was

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Figure 6. Time-course of *isaB* Expression in Glucose. RT-PCR analysis of *isaB* expression in *S. aureus* strain RN496 following growth in glucose. Dark bars indicate cultures grown in LB while light bars represent samples grown in LBG. Time points indicate time after dilution into fresh media. Maximal expression of *isaB* is observed at 8 hours post-dilution. All samples were normalized to their *16s rRNA* ct values.

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Figure 6. Time-course of *isaB* Expression in Glucose.

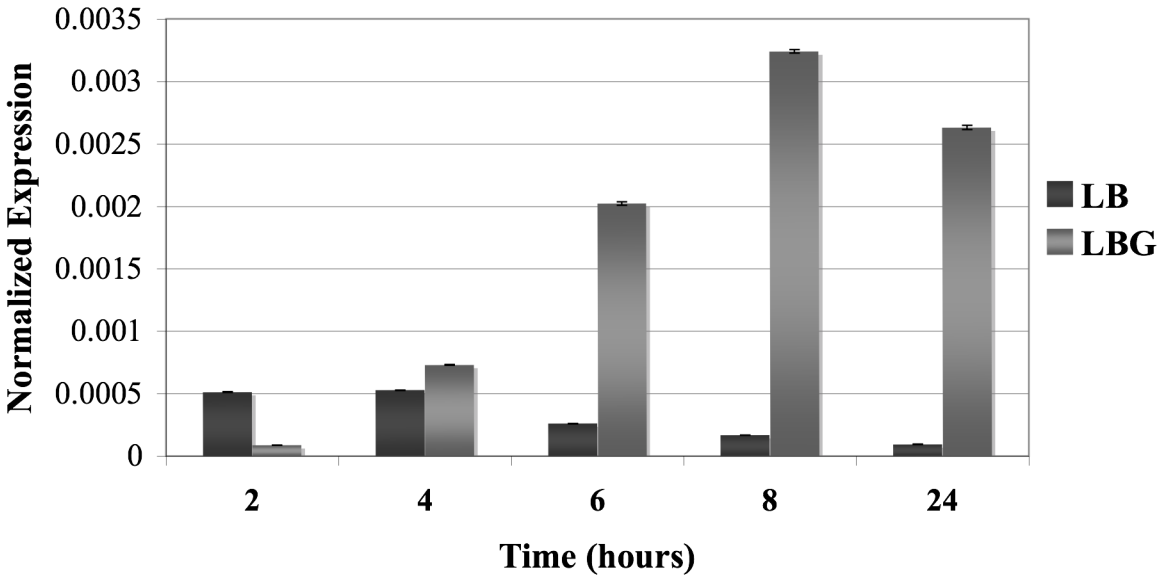

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Figure 7. IsaB Expression is Coordinately Regulated With the Gene. To ensure m-IsaB was being regulated in the same fashion as its gene western blot analysis was used. Cultures of *S. aureus* strains 10833 and 10833 Δ*isaB* were diluted 1:100 in LBG. Samples of both cells and supernatants were collected at the time points indicated. IsaB was detected using our anti-IsaB polyclonal antibody. Very little of IsaB was detected at 2 hours post-dilution (left arrow), however like transcript maximal expression was observed between 8 and 24 hours (right arrow). The non-specific band under the IsaB band is never observed in the culture supernatants.

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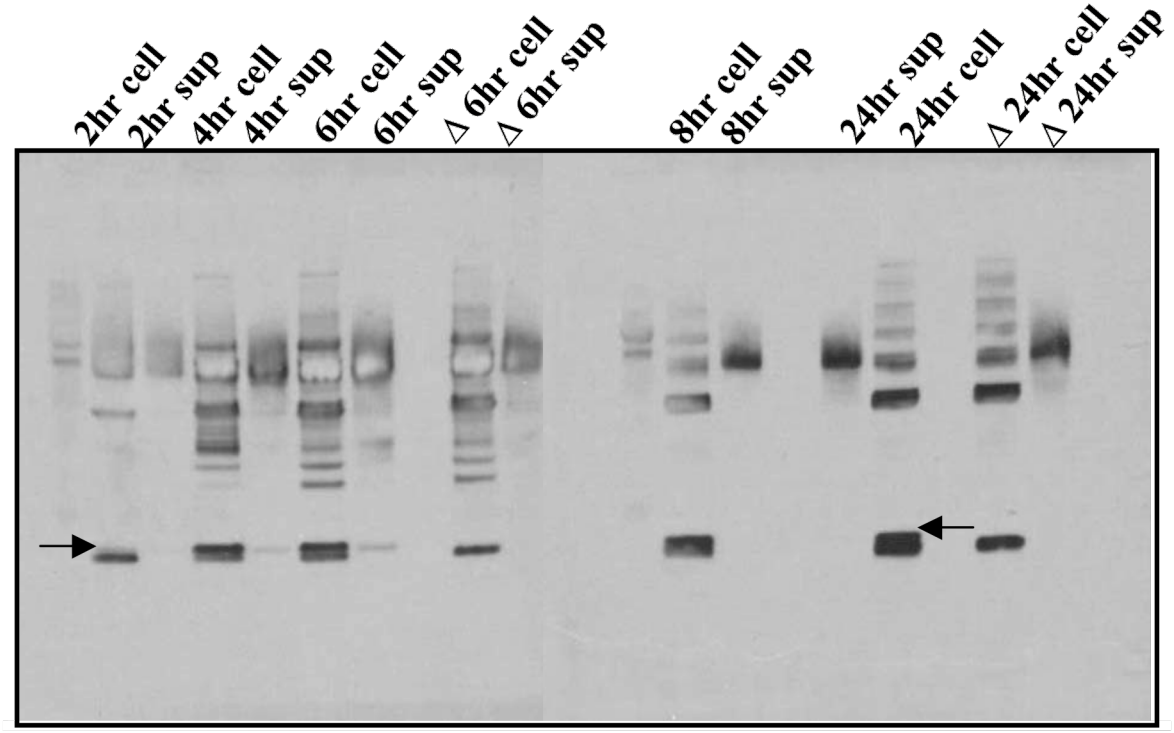
Figure 7. Time-course of IsaB Expression in Glucose.



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noticeably increased and continued to increase over the next 20 hours. Interestingly, IsaB was most highly detected in the supernatant fractions between 4-6 hours, with very little being observed in the 8 and 24 hour fractions. These results show that expression of IsaB protein undergoes a pattern that is similar to expression of the *isaB* gene in response to the addition of glucose.

Exposure to Serum and/or Plasma Upregulates *isaB* levels.

After discovering that simple sugars induce *isaB* expression, two complex in vivo cues, plasma and serum, were tested for their ability to induce *isaB* transcript levels, again using RT-PCR. Overnight cultures of *S. aureus* were grown in LB and subsequently diluted into either whole human serum or human plasma and grown for 2 hours, before collecting samples for RNA extraction using a modified protocol incorporating trizol. The results obtained from these experiments are found in **Figure 8**, which shows that after two hours of incubation in plasma or serum (compared to growth in LB) *isaB* levels were increased by approximately five-fold in each condition, further supporting evidence that IsaB is expressed in vivo.

SarA Represses Expression of *isaB*

The staphylococcal accessory regulator A, SarA, is a well characterized transcription factor considered to be a global regulator of many virulence genes, and was recently suggested, from microarray data by Dunman et al; to repress *isaB* [106][15]. Furthermore, when examining the predicted promoter region of *isaB*, we found a putative

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Figure 8. Plasma and Serum Induce *isaB* Expression. To determine how *isaB* was expressed in blood components, RT-PCR of *isaB* expression following incubation in either LB (blue), plasma (purple) or serum (yellow) were performed. Graph shows that incubation in either serum or plasma increases *isaB* expression approximately 5 fold. All values were normalized to their *16s rRNA* ct values.

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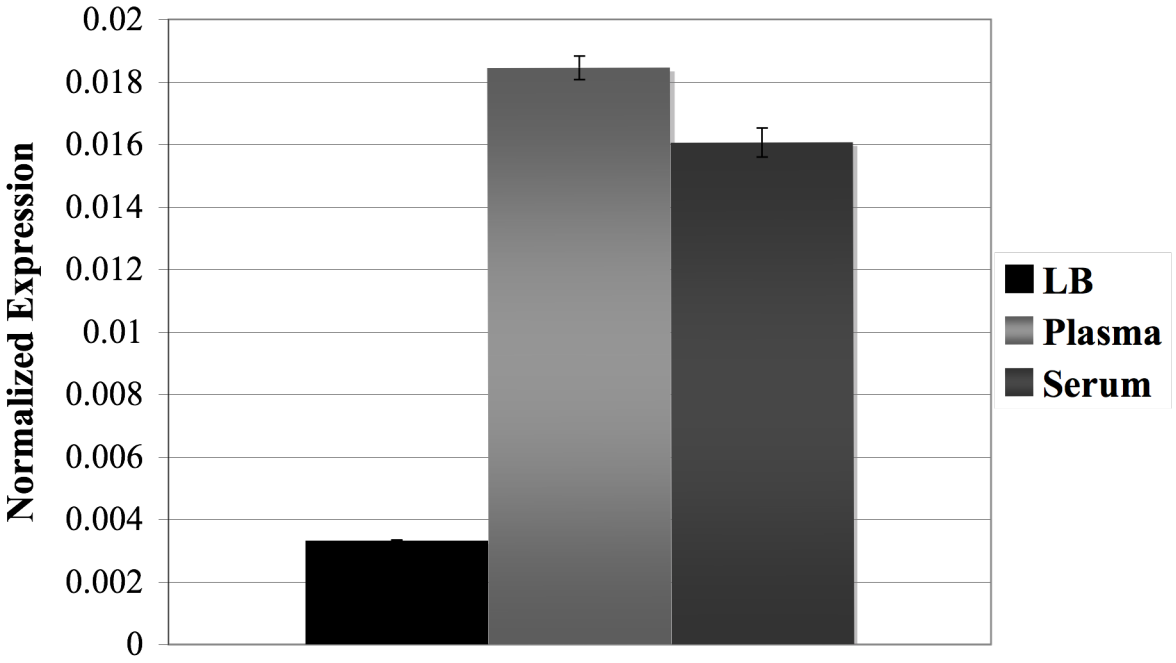
Figure 8. Plasma and Serum Induce *isaB* Expression.



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SarA binding site, as shown in **Figure 9**, consisting of TTTTTAT [75, 77]. Utilizing *sarA* isogenic deletion mutants available, the effect of *sarA* on *isaB* expression levels was determined. First, overnight cultures of the wild-type Sa113 and Sa113Δ*sarA* strains were diluted as described above, and samples were collected 4 hours post-dilution. RNA was extracted from the collected samples, and using an *isaB* specific probe, transcript levels were observed by Northern blot analysis. As shown in **Figure 10a**, upon deletion of *sarA* a marked increase in *isaB* transcript is observed, suggesting that *sarA* is a negative regulator of *isaB*.

In order to further quantify the level of SarA-mediated repression a long-term time-course was performed with the wild-type and *ΔsarA* strains as previously described in LB, with samples collected at 2, 4, 6, 8, and 24 hours. The results in **Figure 10b** show that at all time-points tested *ΔsarA* had more *isaB* transcript present, with fold differences ranging from approximately 2 fold to 88 fold over the time-course, with the greatest increase of *isaB* over wild-type observed at 24 hours (88 fold). These data support the conclusion that SarA is a negative regulator of *isaB* expression. SarA is a known negative regulator of extracellular proteases, enzymes, and other secreted proteins; therefore, regulation of *isaB* expression by SarA is not unexpected [73-75, 106].

Acidification of Media Following Glycolysis Induces *isaB*.

Since *isaB* was dramatically increased following induction in glucose, we hypothesized that the Carbon catabolite protein A, CcpA, of *S. aureus* could play a role in expression of *isaB*. We constructed a *ccpA* isogenic deletion mutant, which would allow

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Figure 9. Structure of Predicted IsaB Promoter. The predicted organization of the *isaB* promoter region, that also contains a putative SarA consensus binding sequence. Bprom promoter finding tool was used to predict promoter structure.

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Figure 9. Structure of Predicted IsaB Promoter.

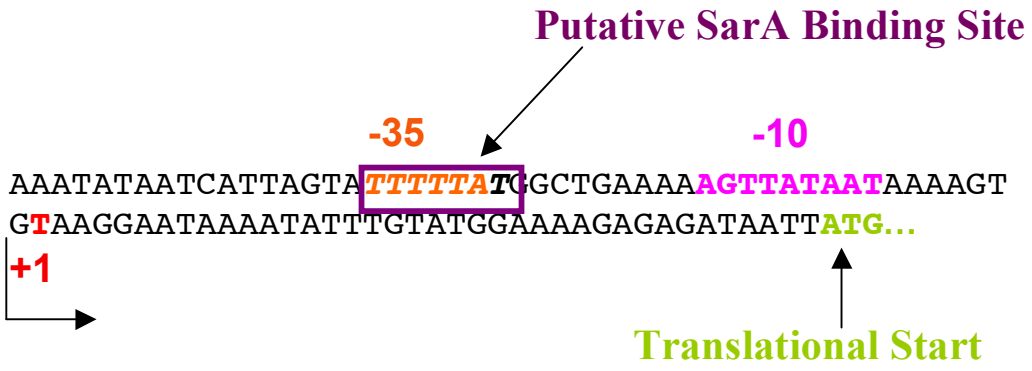

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Figure 10. SarA Negatively Regulates *isaB* Expression. To determine the ability of SarA to regulate *isaB* transcription, *ΔsarA* mutants were assessed for differences in *isaB* levels compared to Wild-type. **a)** Northern blot analysis of the levels of *isaB* transcript in *S. aureus* 10833 and 10833 Δ *sarA*. Increased expression is observed in 10833 Δ *sarA*. **b)** RT-PCR of *isaB* expression in *S. aureus* Sa113 (dark) and Sa113 Δ *sarA* (light). Time-points indicate time post dilution into fresh LB. Deletion of SarA leads increased *isaB* detected at all time-points with the greatest difference of 88 fold observed at 24 hours post-dilution into fresh LB. All samples were normalized to the *16s rRNA* ct values.

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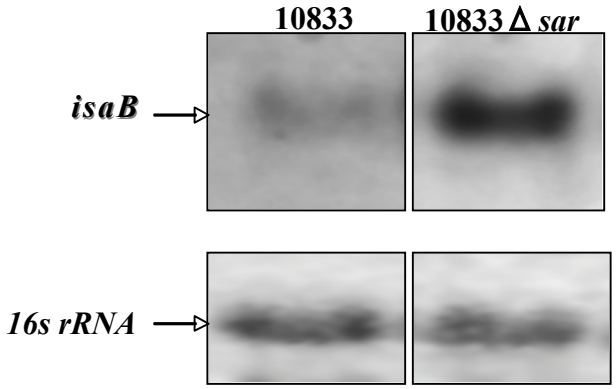
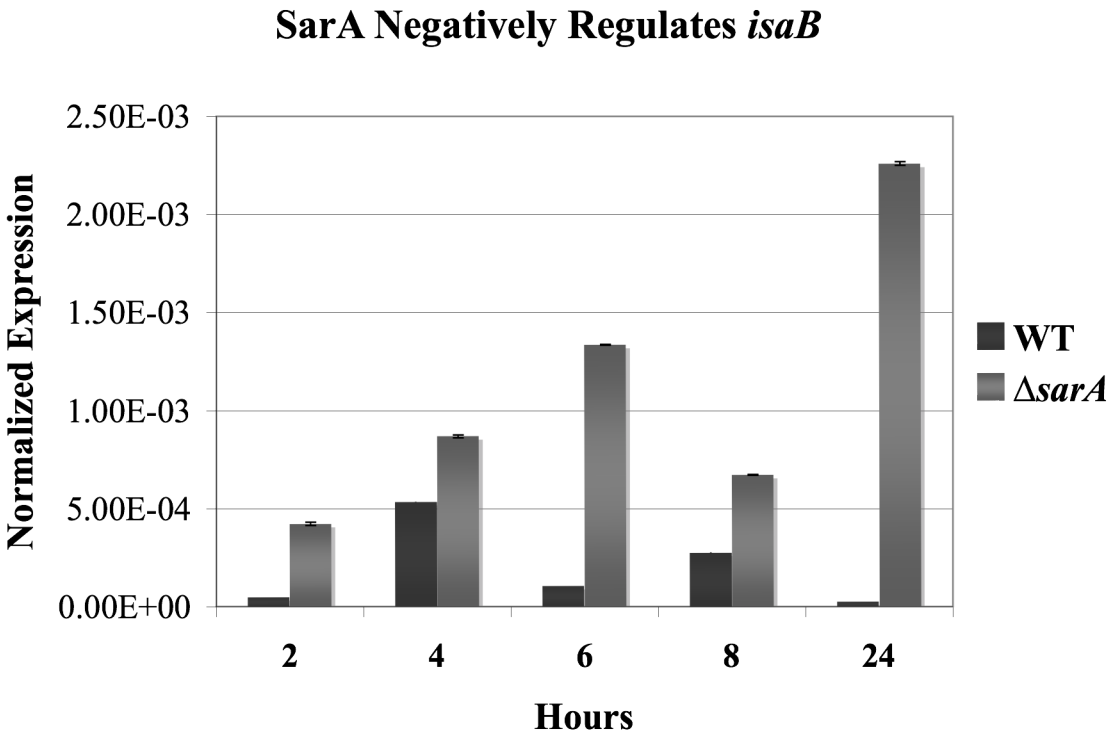
Figure 10. SarA Negatively Regulates *isaB*.

A.



B.



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for studies to determine the affect *ccpA* has on *isaB* expression. To that end, RT-PCR was used to analyze *isaB* transcript levels between wild-type and *ΔccpA* strains, using the protocol described above with time-points taken at 2, 4, 6, 8, and 24 hours post-dilution. As **Figure 11** shows, deletion of *ccpA* led to a dramatic decrease in the amount of *isaB* transcript induced by the presence of glucose. These findings suggested that *isaB* expression is activated through the carbon catabolite control system, mediated by CcpA.

We also investigated the possibility that the effect of the glucose was mediated through an effect on pH rather than through a direct effect of CcpA. Through personal observation and previously published literature, it is well known that following addition of glucose to a growing culture of *S. aureus* there is a subsequent decrease in the pH of the media [87, 107]. To address if pH and not glucose was inducing *isaB* expression, a time-course was performed, however in addition to the normal LBG culture, 50mM HEPES was added to LBG to effectively buffer the media to prevent or delay the acidification of the media without affecting growth [84]. Surprisingly, as shown in **Figure 12**, when the pH of the media was buffered to maintain a neutral pH longer (than unbuffered with glucose) we did not observe the typical levels of *isaB* induction by glucose. Therefore, it is likely that the falling pH, a consequence of carbon metabolism, is in fact turning on *isaB* expression. However it is yet unknown through which pathway the change in pH is acting to cause induction of *isaB*.

Upon further analysis of *isaB* levels and fold increases during the pH buffering and *ΔccpA* experiments, it became obvious that the fold decrease from these two experimental

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Figure 11. CcpA Activates *isaB* Expression. RT-PCR analysis of *S. aureus* Sa113 (dark) and Sa113 Δ *ccpA* (light) grown in LBG were used to test the CcpA deletion mutants ability to express *isaB* following glucose induction. Time-points indicate time post-dilution into fresh media. Decreased *isaB* levels were detected in the CcpA deletion mutant from 4 hours post-dilution onward. All samples were normalized to their *16s rRNA*.

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Figure 11. CcpA Activates *isaB* Expression.

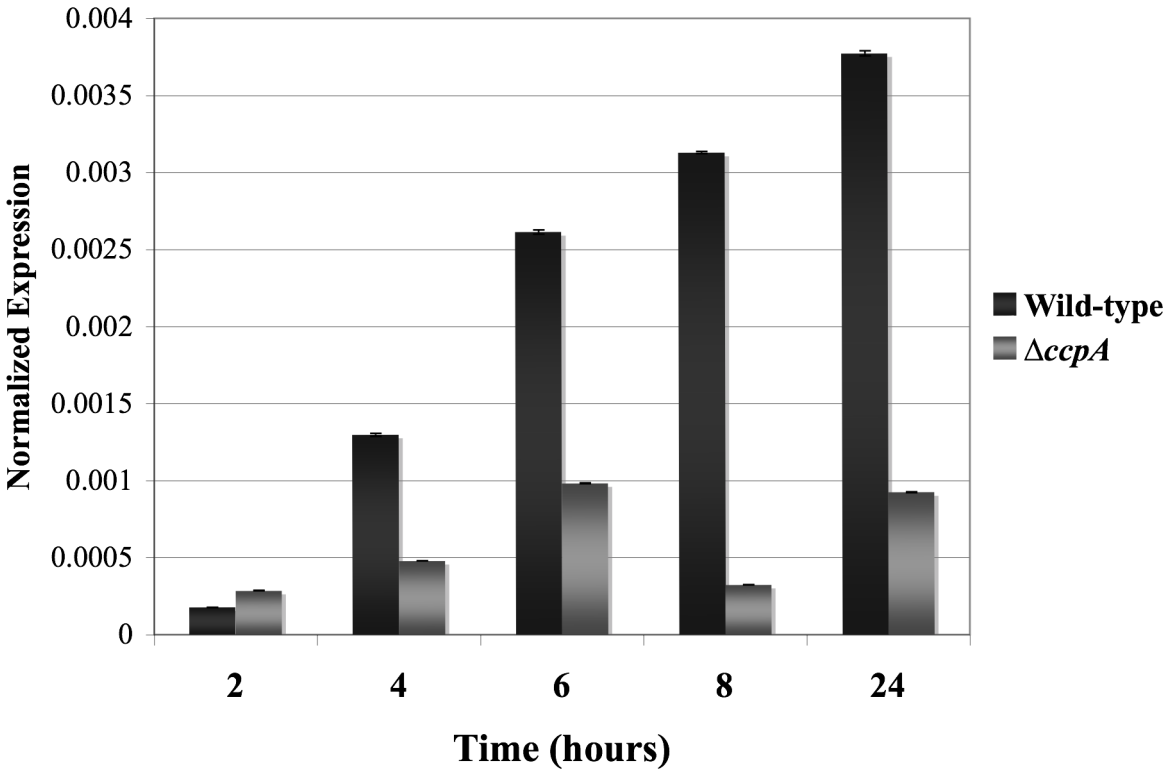

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Figure 12. Acidic pH Following Glycolysis Induces *isaB*. To determine the affect that the decreasing pH following glucose metabolism has on expression of *isaB* in *S. aureus,* LBG was buffered with 50mM HEPES and transcript levels were analyzed using RT-PCR. Wild-type Sa113 was grown in LB, LBG, LBG Buffered or LB Buffered. Results demonstrate show a significant reduction in *isaB* expression in the presence of glucose when media is buffered. All samples were normalized to their *16s rRNA* ct values.

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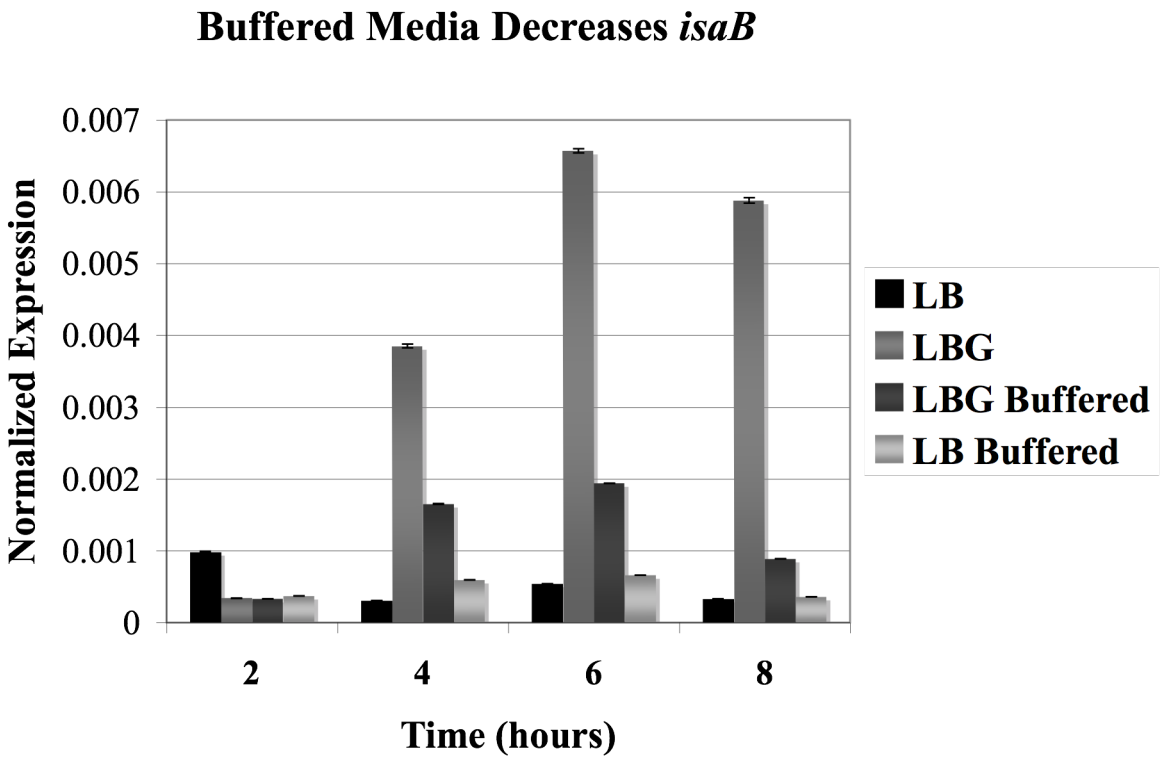
Figure 12. Acidic pH Following Glycolysis Induces *isaB*.



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conditions were suspiciously similar. This coupled with the fact that glucose is depleted long before maximal *isaB* induction occurs suggested that pH could be responsible for the observed CcpA effect [84, 87]. Therefore culture pH was monitored during a subsequent biological repeat of the *ΔccpA* time-course, and as suspected it was found that the *ΔccpA* mutant grown in LBG exhibited delayed media acidification compared to the wild-type strain. This effect is illustrated in **Figure 13**. The delayed drop in pH is likely responsible for the observed effect that deletion of *ccpA* had on *isaB* levels, and therefore from these studies it appears that CcpA does not have a direct regulatory role on *isaB*.

Transcriptional activity rather than mRNA Stability Accounts for the Increase in *isaB* Message.

To help further elucidate the apparent regulation of *isaB* expression by a pH dependent mechanism, RNA stability experiments were performed to determine if de novo transcription or increased stability was responsible for the rise in detectable *isaB* transcript levels. For this experiment, overnight cultures of wild-type *S. aureus* were grown, diluted 1:100 in either LBG or LBG+50mM HEPES. Cultures were grown for 6 hours post-dilution before 200µg/ml rifampin was added and samples taken at 0, 5, 30, and 60 minutes to assess the rate of message degradation between "induced" and "buffered" conditions. The results of these experiments shown in **Figure 14** demonstrate that there does not appear to be a significant difference in the rate of degradation, indicating that *isaB* transcript levels are increasing by de novo transcription.

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Figure 13. Deletion of CcpA Delays Media Acidification. pH values of *S. aureus* grown in LB: Sa113 (black dashed line with filled box), Sa113 Δ*ccpA* (light dashed line with open triangle) or LBG: Sa113 (black line with open box), Sa113 Δ*ccpA* (light line with an x) were collected. Time-points indicate time post-dilution into fresh LBG. Data shows that deletion of CcpA delays the pH decrease following glycolysis.

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Figure 13. Deletion of CcpA Delays Media Acidification.

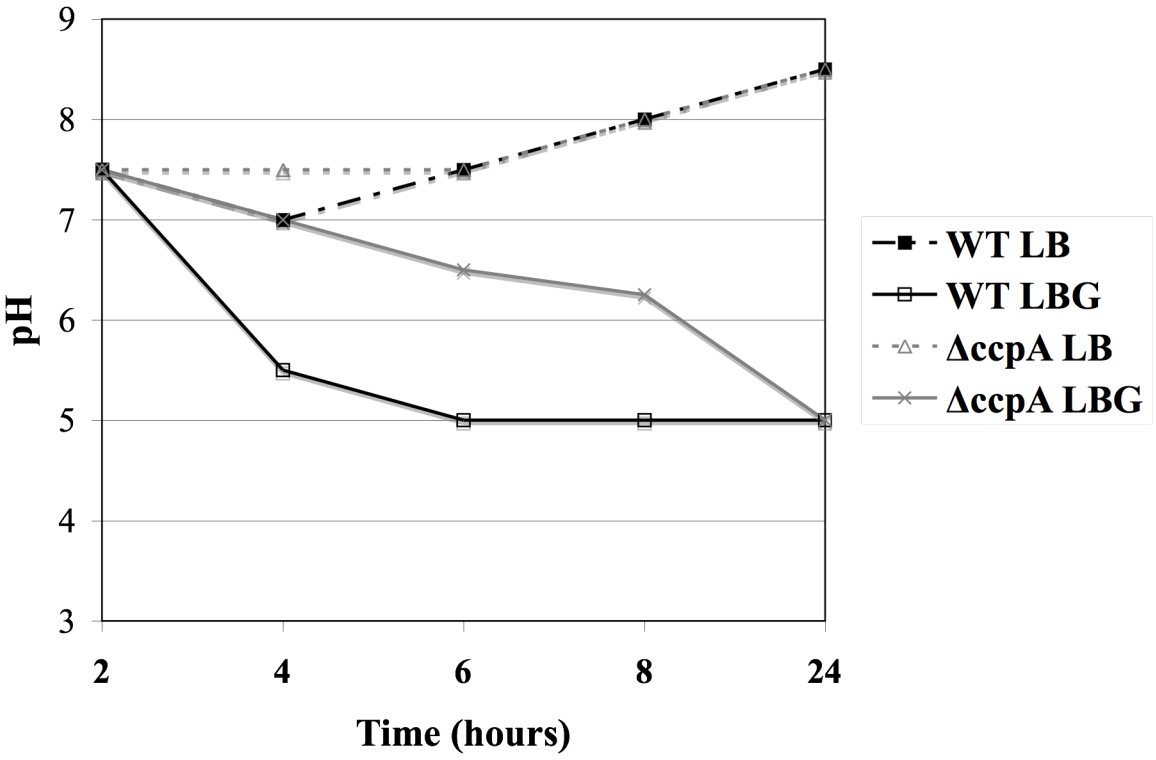

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Figure 14. *isaB* Transcript Stability is Not Affected by pH.
Transcript levels in the absence of de novo transcription (rifampicin treatment) were measured to determine if the increase in *isaB* is due to increased stability or de novo transcription. RNA stability assay using *S. aureus* Sa113 grown for 6 hours in either LBG (dark circles) or LBG+50mM HEPES (light triangles) prior to rifampin treatment. Time-points indicate time after addition of 200µg/ml rifampin. Due to the similar slopes of transcript decay observed it does not appear that the stability of the *isaB* transcript is altered.

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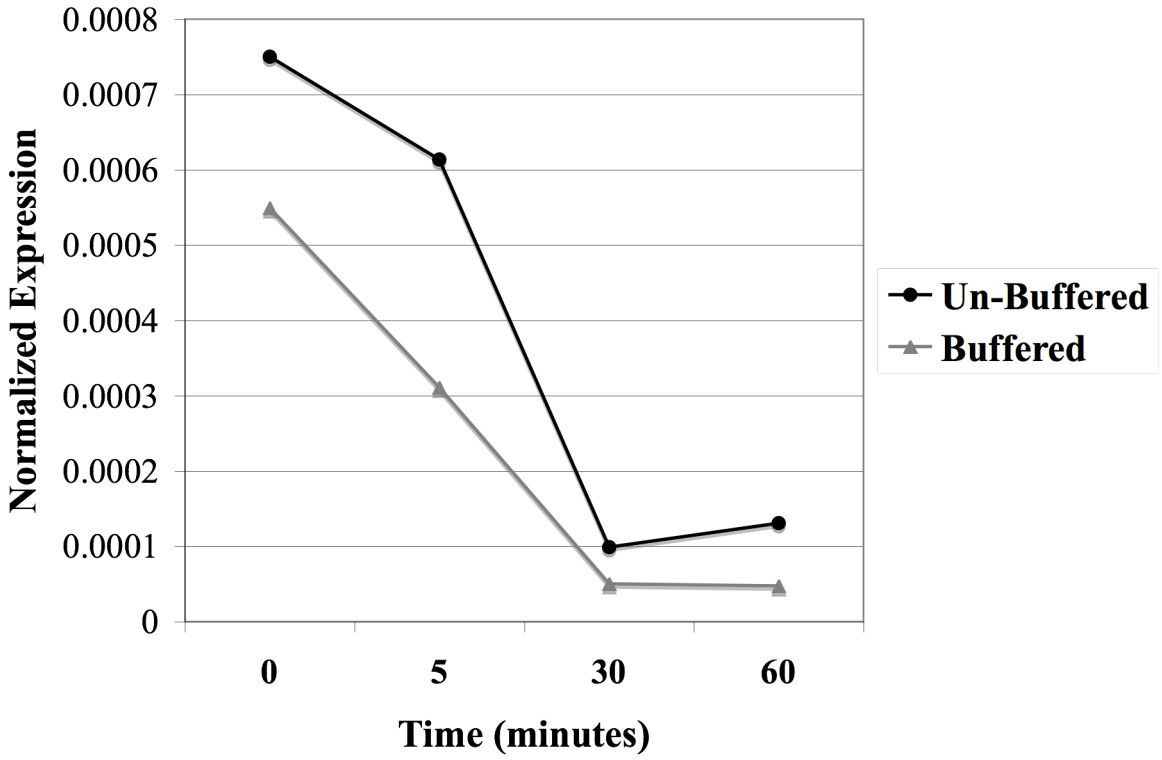
Figure 14. *isaB* Transcript Stability is Not Affected by pH.



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Discussion:

IsaB was first identified due to its strong immunogenicity during MRSA septicemia. In support of our hypothesis that IsaB is a virulence factor produced during infection we found that IsaB is secreted by *S. aureus*. This finding suggests that IsaB is immunogenic due to its active secretion by *S. aureus* and not a side-effect of bacterial cell lysis, further arguing that IsaB is an important virulence factor. The above results illustrate that regulation of *isaB* is likely due to a complex regulatory network, with expression being orchestrated by external/environmental signals, as well as internal factors, such as the SarA transcription factor. These studies show that the main (as characterized thus far) environmental factor that leads to de novo transcription of *isaB* is not likely mediated through glucose or carbon catabolite regulation, but in fact through a drop in pH. Importantly, *isaB* expression does not reach peak levels until long after all glucose in the culture is depleted, and the pH has dropped to approximately 5. Furthermore we found that IsaB is translated at rates that coordinate with the observed increase in transcription following exposure to glucose and media acidification.

It important to note that under normal/non-inducing growth conditions *isaB* does not exhibit significant amounts of temporal regulation. While there was an increase in *isaB* levels at 48 hours it is unknown which stimuli are inducing expression and how expression of *isaB* is important at this late stage of growth. However an interesting observation is that while acidification is delayed, the pH in both the *ΔccpA* and buffered cultures did eventually reach normal/control levels when supplemented with glucose, and yet *isaB* was not highly induced. Therefore, even though the pH ultimately decreased,

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isaB expression never reached (in our time-course) levels observed normally. This could indicate that *isaB* is being regulated in a temporal or growth stage specific manner under acidic conditions, which could be a consequence of its regulation by SarA, which regulates genes temporally, and has recently been shown to be affected by pH [106]. Also it is prudent to point out that while most of the upregulation of *isaB* is ablated by buffering conditions, the addition of glucose does appear to have some role in increasing expression as the levels observed in the buffered LBG are consistently greater than *S. aureus* grown in buffered LB only.

Of clinical relevance, these studies have also found that *isaB* expression increases significantly following culture in either serum or plasma. Again showing that IsaB is likely expressed in vivo during septicemia, a systemic infection of the blood, and is likely important for *S. aureus* survival. However as these fluids are composed of a great number of factors, it is unknown which components in are responsible for stimulating increased *isaB* transcription. These results further increase the complexity of the regulation of *isaB* as the same signals or proteins that sense acidic conditions are not likely to be stimulated in serum/plasma as these biological fluids are inherently buffered to maintain a tight homeostasis at neutral pH. However, *isaB* is induced following phagocytosis, where *S. aureus* would be exposed to low pH inside the phagolysosome of neutrophils. Important future experiments would be to elucidate through which mechanisms, such as one of the 16 two-component systems in *S. aureus* is responsible for each stimulus [69].

Finally these studies have demonstrated that SarA is a negative regulator of *isaB*, although it is currently unknown if this is due to direct or indirect regulation. Two

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independent labs have proposed consensus sequences for SarA binding sites, one of which is 26bps, the other only 7 (with one allowed mismatch) [75, 77]. As **Figure 9** highlights, the predicted promoter of *isaB* contains a 6 base pair match to the predicted 7 base pair SarA binding consensus sequence, suggesting that *isaB* could be directly regulated by SarA. However, even the authors caution that *S. aureus* is very AT rich and the proposed consensus sequence of ATTTTAT without direct observation of SarA binding, through EMSAs, may not be stringent enough to determine direct binding or regulation by SarA, this caveat is especially true as the *isaB* gene itself contains two of these "consensus" sites [77].

While the studies presented above, give us a great deal of information about the regulation of *isaB*, there may be other regulators as well as other stimuli that regulate expression of *isaB*. Importantly, the increased expression due to serum/plasma gives weight and credibility to the hypothesis that IsaB is expressed during septicemia and is therefore likely to play a role in pathogenesis of *S. aureus*.

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CHAPTER 4

Nucleic Acid Binding by IsaB

Introduction:

IsaB was previously isolated in our laboratory in an affinity chromatography screen for *S. aureus* proteins that bound to 5-untranslated region (5'-UTR) of the intercellular adhesin (*ica*) transcript. The primary research focus of the laboratory is on the regulation and production of biofilms in *S. aureus* and other Gram-positive pathogens. The prototypic *S. aureus* biofilm matrix is primarily composed of the extracellular polysaccharide, PNAG, which is encoded by the intracellular adhesin locus, *ica*. The purpose of the affinity chromatography was to determine if there was an RNA binding protein involved in the post-transcriptional regulation of the *icaADBC* transcript. The 5' UTR of the *icaADBC* transcript was used as "bait" to pull down proteins from whole cell lysates of *S. aureus*. Only one protein was isolated from this screen, the corresponding protein band was excised from an SDS-Page gel and used for Mass-spectral analysis to determine the peptide composition. This singular protein was determined to be IsaB (Unpublished Data).

The lab moved forward to validate the regulatory roles for IsaB on *icaADBC* translation and production of PNAG. Following production of *isaB* isogenic deletion

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mutants, changes in *icaADBC* transcript levels were assessed using RT-PCR and changes in the production of PNAG were assessed by immunoblot. Through these experiments, we determined that IsaB had no observable role in post-transcriptional regulation of *icaADBC* nor did it affect PNAG production. The argument for the lack of a role in PNAG production was further strengthened by the fact that our experiments demonstrated IsaB secretion and cell wall association [99]. Therefore, IsaB does not have a regulatory role in PNAG production, however these studies suggested that IsaB was a novel extracellular RNA binding protein. In this study we sought to further characterize the nucleic acid-binding activity of IsaB.

Results:

Purification of IsaB

IsaB was previously cloned into and expressed in *E. coli*, using the pKYB1 vector, which contains an IPTG-inducible promoter. Recombinant protein, which included a C-terminal chitin-binding domain, was purified over a chitin column, and IsaB was released from the column by activation of the self-cleaving intein tag by reducing conditions. A schematic showing the procedure and a representative SDS-PAGE protein gel are shown in **Figure 15.**

IsaB binds to RNA.

The first experiment performed was designed to validate the RNA-binding ability of IsaB. Using the same RNA probe initially used to purify endogenous IsaB, Electro-

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Figure 15. Schematic of IsaB Purification. a) Diagram of purification scheme using a chitin-binding domain with an intein tag. **b)** Typical SDS-Page gel following purification of IsaB at the predicted size of approximately 17 kDa.

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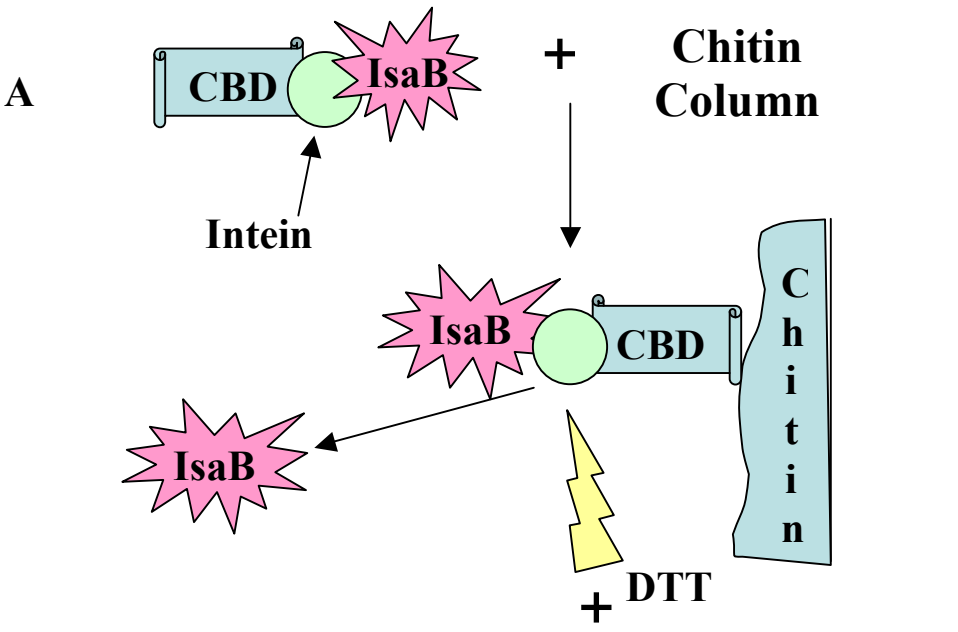
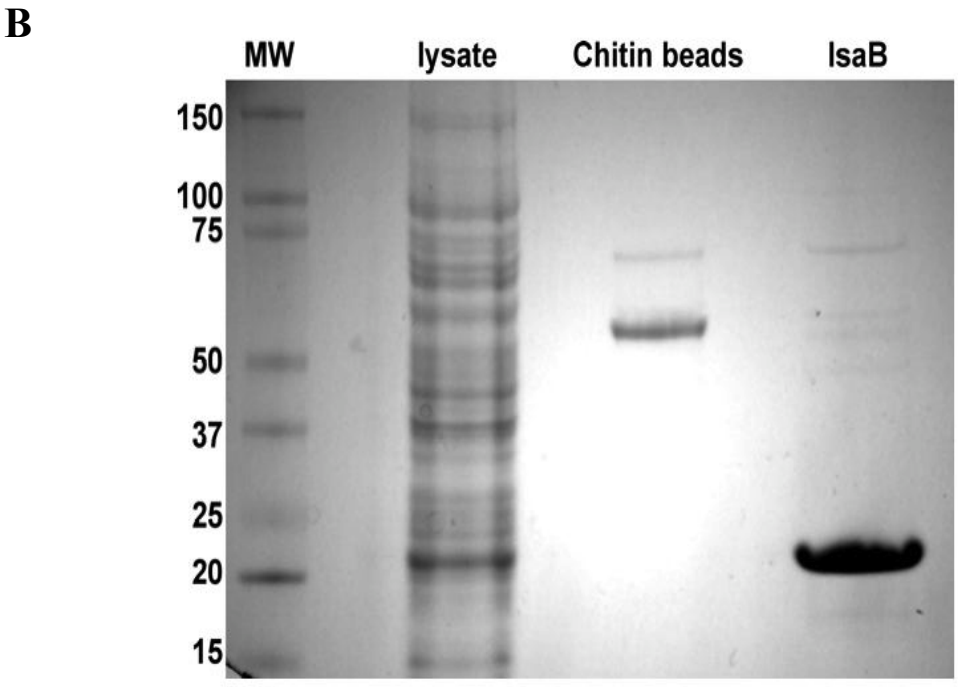
Figure 15. Schematic of IsaB Purification.





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Mobility Shift Assays (EMSAs) were performed in order to directly observe IsaB-RNA interactions. EMSA reactions were performed as previously described. Briefly, the fluorescently labeled probe was mixed with varying concentrations of IsaB and allowed to incubate at RT for 15 minutes in freshly prepared binding buffer. These reactions were separated electrophoretically over a 5% acraylamide gel, and the probe signal was detected by excitation with UV light. As shown in **Figure 16**, IsaB was able to bind to the probe and retard its movement through the gel (when compared to the probe only control), demonstrating that IsaB is readily able to bind to RNA and is a novel extracellular binding protein of *S. aureus*.

IsaB binds to Nucleic Acids.

To proceed with EMSA analysis, the concentration of IsaB that produced the most significant shift was used for all further studies. However, as shown in **Figure 16** there is a large amount of RNA/IsaB that was unable to migrate into the gel, likely due to large complex formation. The addition of non-specific carrier DNA is generally added to EMSA reactions to inhibit non-specific binding and typically breaks up such large complexes ensuring that all probe is able to be visualized within the gel. Therefore, sonicated salmon sperm DNA, SSS, was added to the EMSA reactions starting at a 2:1 ratio of SSS to labeled probe. Surprisingly, as **Figure 17** shows, the SSS was able to out-compete the labeled probe as seen by the ablation of the RNA shift at SSS ratios 2:1 and 0.5:1. This EMSA demonstrates that not only can IsaB interact with RNA, it can also interact with

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Figure 16. IsaB Binds RNA. EMSA reaction showing ability of IsaB to shift a fluorescently labeled RNA oligo. All lanes contained 270 pmol labeled RNA: Lane 1, no IsaB control, Lane 2, 3.84 nmol IsaB, Lane 3, 1.92 nmol IsaB, Lane 4, 960 pmol IsaB, Lane 5, 480 pmol IsaB, Lane 6, IsaB.

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Figure 16. IsaB Binds RNA.

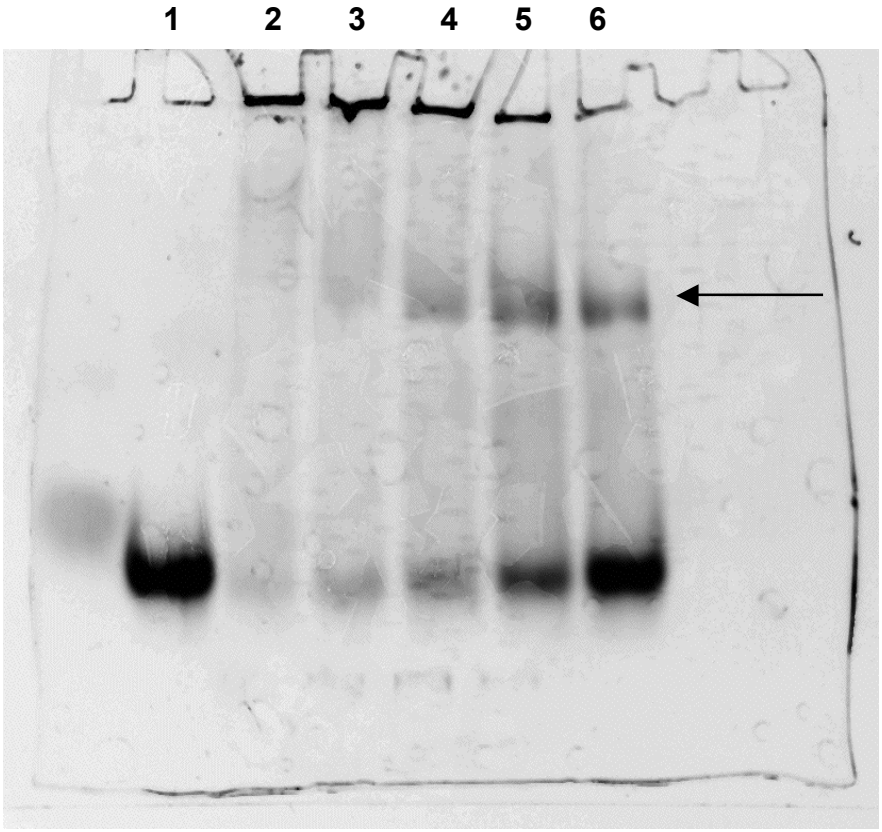

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Figure 17. IsaB Binds Nucleic Acids. In order to block non-specific binding, SSS DNA was added to EMSA reactions. EMSA reactions contained 480 pmol purified IsaB and 270 pmol labeled RNA. Lane 1, RNA only, Lane 2 EMSA, Lane 3, EMSA + 1.35 nmolSSS, Lane 4, EMSA + 135 pmol SSS, Lane 5, EMSA + 13.5pmol SSS, Lane 6, EMSA + 1.35 pmol SSS. At the two highest concentrations of DNA the shift was ablated indicating that IsaB binds DNA as well.

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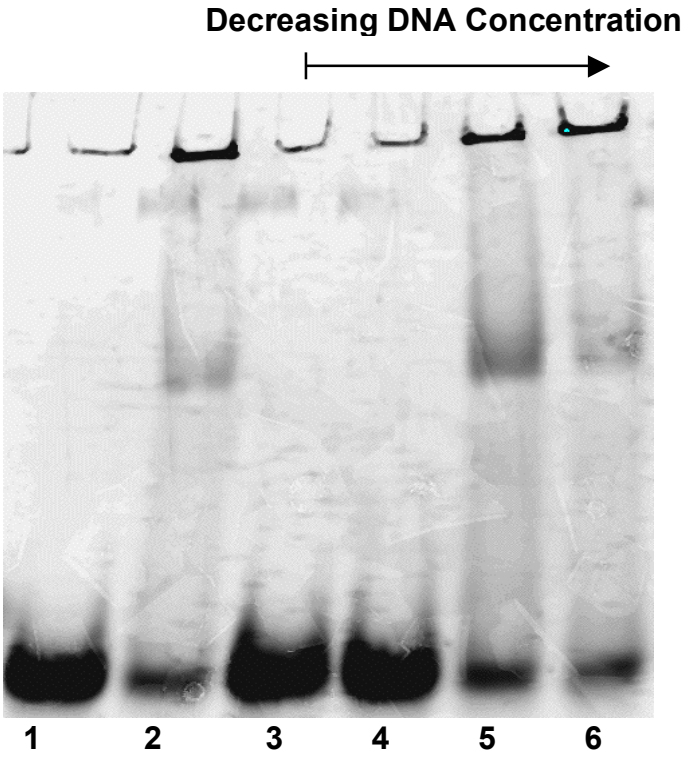
Figure 17. IsaB Binds Nucleic Acids.



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DNA, which IsaB appeared to have a slight preference for. Secondly, this experiment strongly suggests that the binding activity observed is non-sequence specific, as SSS, which is made up of oligonucleotides of random sequence and size, is able to effectively compete with the RNA probe for binding to the protein. Therefore IsaB is binding to nucleic acids, both ds-DNA and ss-RNA, in a non-sequence specific manner.

IsaB Binds Nucleic Acids, Not Nucleotides.

Due to the ability of IsaB to bind all nucleic acids tested, its lack of a conserved/predicted binding domain, and apparent lack of sequence specificity, we considered the possibility that IsaB could have a nucleotide-binding domain. To investigate its nucleotide binding activity, a competitive EMSA was performed. This experiment was performed as described above, however, to each reaction, a 10-fold greater concentration of unlabeled competitor to probe was added, either SSS, yeast tRNA, or dNTPs. If IsaB bound to any of these substrates, even if the affinity was less than its affinity for the labeled probe, a decrease in the shift should be observed. **Figure 18** indicates, as expected, that when competitor SSS or tRNA was added to EMSA reactions there was no shift observed as IsaB bound to the unlabeled probe added in excess. Importantly, in the EMSA reaction supplemented with 10X dNTPs, the labeled RNA probe is still shifted, indicating that IsaB binds to nucleic acids and not to monomeric nucleotides.

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Figure 18. IsaB Does Not Bind Nucleotides. Unlabeled competitors were added to EMSA reactions to determine their effect on the shift. EMSA reactions contain 270 pmol RNA and 480 pmol IsaB. 2.7 nmol of unlabeled competitors were added to reactions. Lane 3, SSS, Lane 4, nuleotides, and Lane 5, yeast tRNA.

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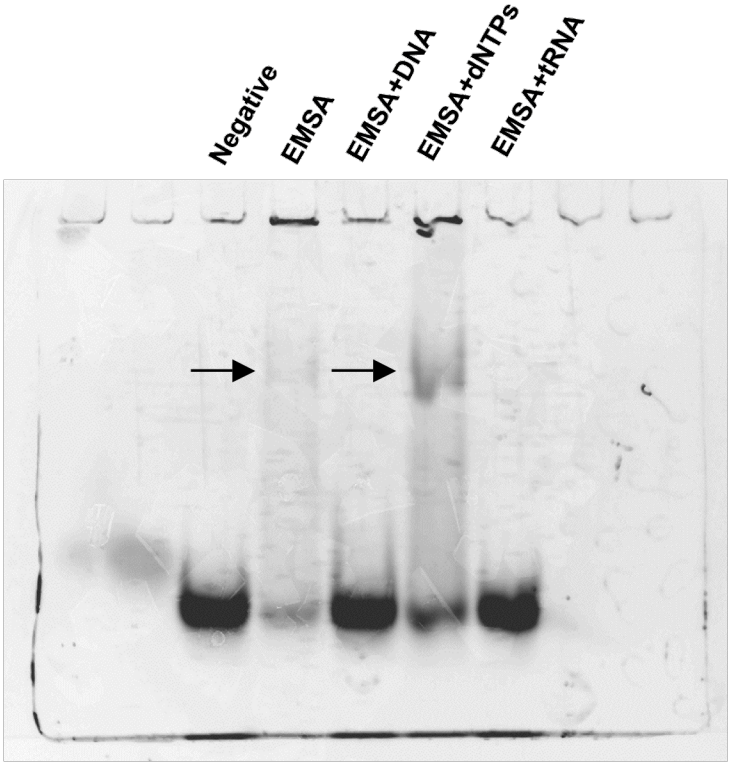
Figure 18. IsaB Does Not Bind Nucleotides.



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IsaB Binds to Nucleic Acids in the Low ηM Range.

In order to determine the affinity of IsaB for dsDNA, ssDNA, and ssRNA, Surface Plasmon Resonance, SPR, was employed. In this automated system, "bait" is immobilized onto a thin metal chip, and as the sample with the "target" is passed above the chip, the amount of target binding to bait can be measured. For our purposes, the bait used was dsDNA, ssDNA, and ssRNA, each of which was biotinylated to allow for immobilization onto a strepavidin-coated chip. By flowing decreasing concentrations of IsaB over the chip and monitoring the strength of the interactions, the resonance units were collected and BiaCore Evaluation software was able to determine the dissociation (Kd) constant for the IsaB binding reactions. The sensogram shown in **Figure 19a** demonstrates that IsaB binds to the respective targets with significantly greater affinity then to the chip alone. Furthermore any observed non-specific binding was compensated for during analysis. However, due to the nature of the curve, no accurate stoichiometric analysis was able to be performed to due poor fit with modeling curves. **Figure 19b** shows that IsaB binds slightly more avidly to dsDNA with binding occurring at $8.1x10^{-9}$ (8.1nM). Therefore we can conclude that IsaB does bind all nucleic acids tested with similar affinity in the low nM range.

Endogenous IsaB Contributes to Accumulation of eDNA on the Bacterial Surface.

We wanted to determine if deletion of *isaB* could have any significant effect on the accumulation of eDNA within *S. aureus* cultures. In these assays, wild-type and Δ*isaB* strains were diluted from overnight cultures in TSB and 1% glucose was added to induce

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Figure 19. Affinity of IsaB for Nucleic Acids. From SPR experiments, the affinity for dsDNA, ssDNA, and RNA was determined. **a)** Representative sensogram, with non-specific binding subtracted, demonstrating the ability of decreasing concentrations of IsaB to bind to target. **b)** Kd = dissociation constant, Ka = association constant. Binding of IsaB to the bait is significantly above background binding, and exhibits a slightly higher affinity for ds DNA followed by ssDNA and RNA.

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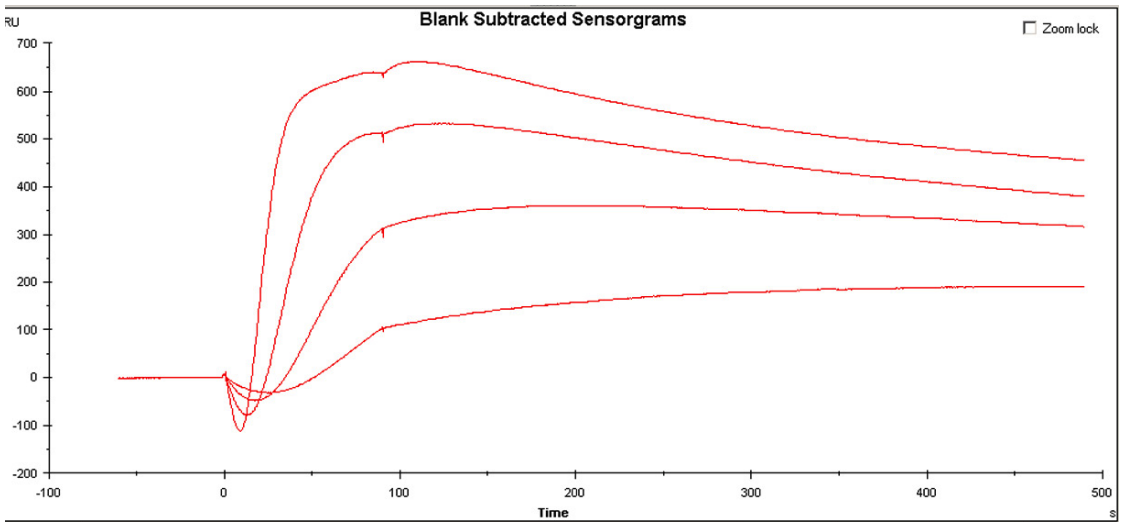
Figure 19. Affinity of IsaB for Nucleic Acids.

A.



B.

Ligand	Kd	Ka	(M)
Double-stranded DNA	8.10×10^{-9}	1.23×10^{8}	8.1 nM
Single-stranded DNA	1.08×10^{-8}	9.28×10^{7}	10.8 nM
RNA	1.65×10^{-8}	6.07×10^{7}	16.5 nM

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Figure 20. IsaB Aids in Accumulation of eDNA. *S. aureus* strains Sa113, Sa113 Δ *isaB*, 10833 and 10833 Δ *isaB* were tested for their ability to bind exogenously added fluorescent eDNA. Measurements were made using a fluorimeter and displayed as relative light units. Wildtype fluorescence levels were significantly higher with a probability value of $p=0.006$ for 10833 versus 10833 Δ *isaB* and Sa113 versus Sa113 Δ *isaB* (Student's unpaired T test).

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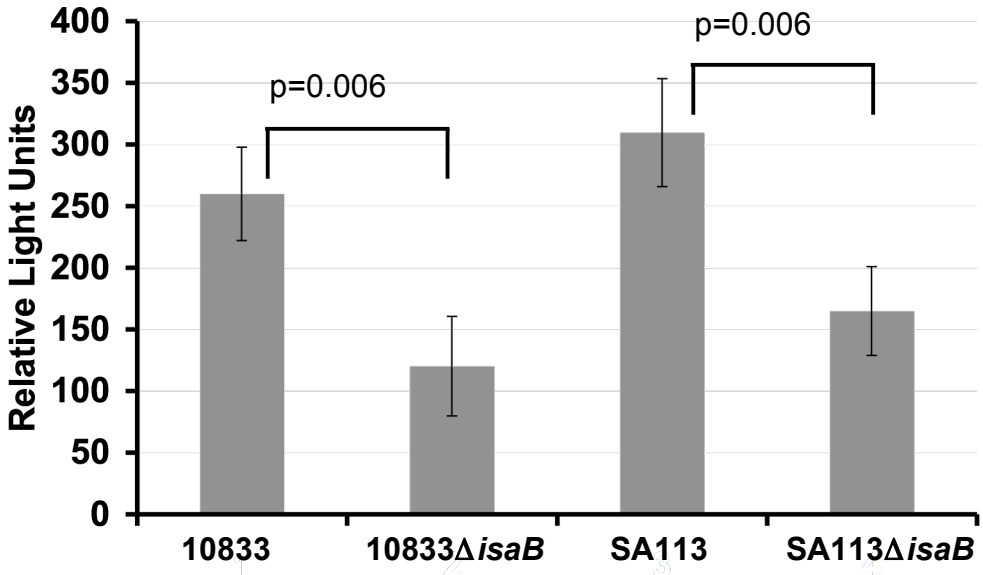
Figure 20. IsaB Aids in Accumulation of eDNA.



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isaB expression for four hours. After incubation in the presence of glucose to induce expression of *isaB*, fluorescently labeled SSS was added to cultures and incubated at 37℃ for 15 minutes before washing 3X to remove any unbound SSS DNA. Surface-bound fluorescent DNA was quantified using a fluorimeter to determine relative light units. As displayed in **Figure 20**, eDNA accumulated on the bacterial surface even in the absence of IsaB, but endogenously expressed IsaB lead to significantly greater accumulation of eDNA on the cell surface of *S. aureus* in both wild-type strains tested. This finding demonstrates that while there may be other factors on the cell surface that interact with eDNA, IsaB does play a role. This suggests that binding of eDNA by IsaB is physiologically relevant.

Discussion:

The findings presented above show that IsaB is a novel extracellular nucleic acid binding protein. While its nucleic acid binding ability did not exhibit sequence specificity, the interaction appeared to be dependent upon oligomeric nucleic acids, as monomeric dNTPs were unable to compete with nucleic acid binding. Furthermore, we found that the binding affinity of IsaB for its nucleic acid targets was in the low ηM range, which is indicative of strong binding. The lack of sequence specificity and the fact that IsaB binds strongly to any nucleic acid tested, suggests that IsaB may be interacting with the phosphate backbone of nucleic acids.

To put the strength of the DNA binding activity into perspective, we compared the binding affinity of IsaB to affinities of previously characterized DNA binding proteins in *S. aureus*. The affinity for the transcriptional regulators SarA and SarZ have both been

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described for promoter sequences they are known to regulate, and were found to be in the low pM range which is far stronger binding than that observed by IsaB [108, 109]. However these reports also found that each regulator exhibited binding in the low ηM range when binding to promoters they were not specific for (heterologous DNA) [108, 109]. These studies suggest that the affinity of IsaB is in line with affinities of other DNA binding proteins interacting with DNA in a non-sequence-specific manner. It is important to note that while it is unlikely that an extracellular nucleic-acid binding protein has sequence specificity, we cannot rule out the potential of an IsaB specific binding sequence, and if one were to exist, we would predict binding in the low pM range based upon the SarA and SarZ reports.

The only published protein(s) known to be secreted by *S. aureus* that bind nucleic acids are the Thermostable nuclease(s) that were initially identified in the mid 1900's [110-113]. While it appears that many bacterial species actively secrete nucleases, there are only a few published reports of extracellular DNA-binding proteins. Two extracellular DNA binding proteins from the literature are the Mycobacterium DNA-binding Protein 1 (MDP1), and the Histone-like Protein A (HlpA), from *M. tuberculosis* and *Streptococcal* species, respectively. It has been subsequently demonstrated that these proteins, while they bind DNA, are involved in binding to Glycosaminoglycans (GAGs), which aids in adhesion of bacterial cells to host tissues [114-116]. Furthermore, HlpA is capable of inducing a potent pro-inflammatory immune response [116]. This raises the possibility that, similar to other extracellular proteins with DNA-binding activity, IsaB may have

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some other function. Highly intriguing future studies would be to determine the ability of IsaB to promote inflammatory cytokine production and adherence to GAGs.

It is also interesting to speculate how IsaB binding to DNA would affect Toll-like receptor 9 (TLR-9) activation which recognizes unmethylated CpG motifs found with high frequency in bacterial DNA [117-119]. Activation of signaling pathways through TLR-9 leads to a significant cytokine production prompting a Th1 mechanism, leading to cellular based immune responses [117, 119]. Since *S. aureus* and other *Staphylococci* have been intimately associated with higher vertebrates throughout their evolution, perhaps IsaB could be playing a role in inhibiting TLR-9 activation, thus dampening the host immune response. A mechanism can be envisioned wherein IsaB binds to bacterial CpG DNA, inhibiting TLR-9 recognition and the subsequent immune response. Further studies of the DNA binding activity of IsaB and its involvement in pathogenesis could demonstrate previously unknown immune evasion mechanisms.

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CHAPTER 5

Role of IsaB in *S. aureus* Virulence

Introduction:

IsaB, one of four "immunodominant" proteins identified during sepsis, is described in the literature as a putative virulence factor [96,120]. Virulence factors are proteins or molecules secreted by pathogens to promote disease establishment and/or propagation in vivo but are not required for growth in vitro. *S. aureus* has an immense arsenal of virulence factors that it skillfully regulates to cause significant morbidity and mortality worldwide. Some of these virulence factors protect *S. aureus* from the host immune system by attacking cellular mediators (Panton-Valentine Leukocidin) or by hiding *S. aureus* from immune factors (Protein A). Still others help *S. aureus* adhere to (MSCRAMMS and SERAMS) and invade (multiple Fibronectin binding proteins) host cells to help establish infection and promote dissemination through tissues (numerous super-antigens and toxins). Finally a number of internal factors such as transcriptional regulators can be described as virulence factors due to their necessity for expression of external factors involved in pathogenesis (Sar family of transcriptional regulators).

The immense number and diversity of virulence factors, and great amount of energy spent to make and regulate them, demonstrates how important they are for *S. aureus* to survive in vivo and to cause disease. Due to their clinical importance, hundreds of studies have been performed in hopes of characterizing known or novel virulence

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factors. From these extensive studies there have been many simplified tools and techniques developed to demonstrate a role for a protein in the virulence of *S. aureus*. These techniques allow researchers to simulate in vivo conditions by exploiting model systems, such as the *C. elegans* killing model, or to directly determine the effect a protein has on protection from single factors such as AMPs or even ex vivo neutrophils. We therefore sought to use a number of these previously described models to determine if IsaB does actually play a significant role in *S. aureus* virulence.

Results:

IsaB is not Required for Growth.

In order to determine if deletion of *isaB* has deleterious affects on the growth of *S. aureus*, which would thus lead to bias in subsequent assays, growth curve experiments of wild-type and Δ*isaB* isogenic strains grown in normal culture conditions were performed. For these experiments *S. aureus* strain 10833 and 10833Δ*isaB* were used. Overnight cultures of these were diluted 1:100 in fresh LB or LBG and incubated with vigorous shaking at 37℃. OD_{600} readings were taken at time-points 0, 2, 4, 6, 8, and 24 hours post-dilution. As **Figure 21** depicts there was no decrease in growth rate or culture density observed in the Δ*isaB* strain. Similar experiments were performed in which media was supplemented with potentially harmful factors such as hydrogen peroxide, low pH, and sub-inhibitory concentrations of antibiotics. Even in the presence of these stressors there was no significant difference in bacterial growth observed. These data show that any

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Figure 21. Deletion of IsaB Does Not Affect Growth. Growth curve was performed to determine IsaBs affect on overall growth. *S. aureus* strain Sa113 and Sa113 Δ*isaB* were grown in LB or LBG and monitored at indicated time-points using OD_{600}. No obvious growth defect was observed in the deletion mutant.

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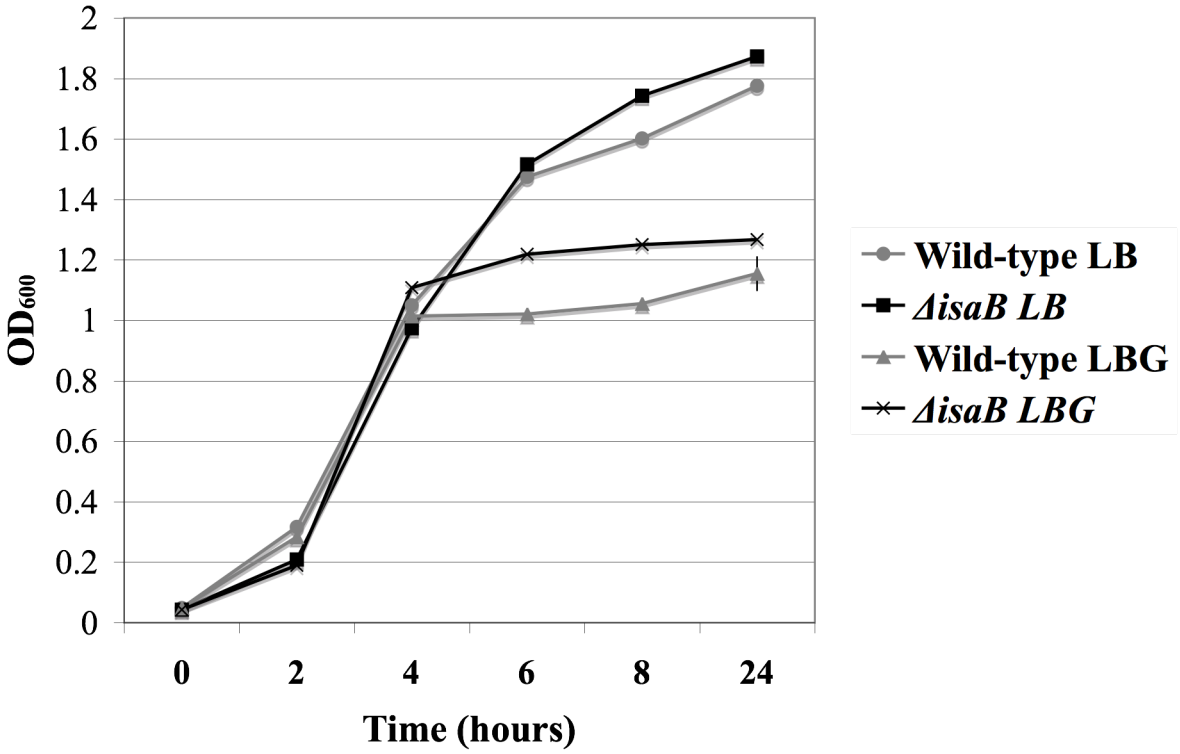
Figure 21. Deletion of IsaB Does Not Affect Growth.



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difference in virulence observed is due solely to the presence or absence of IsaB, and not a difference in growth rate or fitness between the strains tested.

IsaB Does Not Contribute to Virulence in *C. elegans*.

Invertebrates can serve as rapid, tractable, and inexpensive models for the initial characterization of virulence factors [121]. One such model, *C. elegans*, has been developed and used to study the pathogenesis of *S. aureus* for a number of years [121-123]. *C. elegans* has many properties inherent to vertebrate innate immune systems such as Toll-like receptors (TLRs), highly conserved signaling cascades, the production of reactive oxygen species, and also produce anti-microbial peptides or defensins [90, 121, 122, 124-128] . Due the significant overlap in innate and fundamental cellular targets, *C. elegans* killing assays have successfully been used to find or confirm virulence factors in *S. aureus*, *E. faecalis,* and *S. pneumoniae*, which are all important human pathogens [121, 124].

Upon ingestion of bacterial pathogens, which the worms attempt to avoid, the bacteria infect the intestinal epithelium of *C. elegans* and cause death by a variety of mechanisms, including blockage of the gastrointestinal tract by the formation of a bacterial biofilm, and by secretion of toxins [90, 122]. Proteomic studies of *C. elegans* during infection with pathogens have found that these nematodes sense infections and "mount" a protective response against pathogens, showing the ability to raise defenses similar to those found in humans [124]. Due to large amount of overlap with human innate immunity, previous proof of principle for identification of virulence factors, and the elegance and ease

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of working with *C. elegans*, we sought to determine if IsaB, a putative virulence factor, could play a role in virulence in this model organism.

In these experiments a protocol adapted from Begun et al, was employed [90]. Lawns of each *S. aureus* strain: wild-type, Δ*isaB*, or Δ*isaB* +*isaB*, were grown on LBG agar containing 5mg nalidixic acid/mL for 4-6 hours at 37ºC on plates. Following initial growth of *S. aureus*, 10 nematodes were added per plate and placed at room temperature. The *C. elegans* fed upon the *S. aureus*, and this served as the mode of infection/inoculation. We hypothesized that the wild-type 10833 and complemented *S. aureus* strains would be significantly more virulent than the Δ*isaB* mutant, and would lead to an increased rate of *C. elegans* killing. *C. elegans* that failed to respond to touch were considered dead, and were tallied in order to calculate killing by each strain over a five-day period. OP50, an avirulent strain of *E. coli* that the nematodes are fed in lab, was used as a negative control. The results shown in **Figure 22** suggest that while we did see increased death in nematodes grown on *S. aureus* strains, there was no significant difference in the rate of death between the three strains tested regardless of the presence or absence of IsaB. Therefore it is unlikely that IsaB contributes significantly to virulence in this model. *C. elegans* lacks an adaptive humoral immune response and most importantly lacks phagocytic cell lines, both of which are vital human host defenses against *S. aureus* so these results do not preclude a role for IsaB in virulence in higher organisms.

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Figure 22. *C. elegans* Killing Assay. The nematode *C. elegans* was used a virulence model to determine if Δ*isaB S. aureus* was less virulent then wild-type. Worms were grown on LBG plates feeding upon either 10833, 18033 Δ*isaB* or 10833 Δ*isaB*+*isaB*. Dead worms were counted everyday over five days. No significant difference in virulence of the S. aureus strains were observed.

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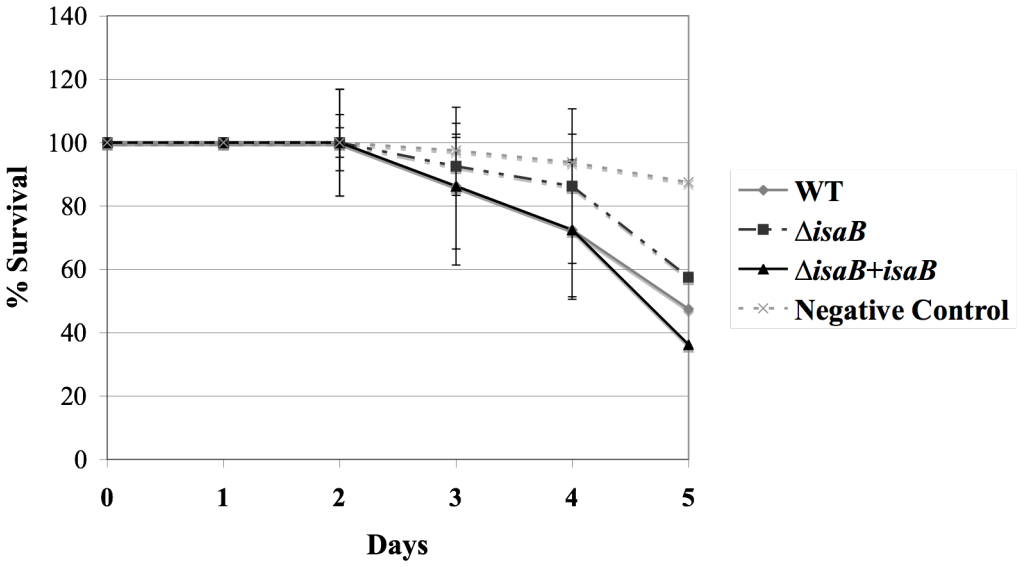
Figure 22. *C. elegans* Killing Assay.



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IsaB Does Not Increase Adherence or Invasion to Epithelial Cells.

S. aureus has a large number of proteins involved in the adherence to and invasion of host cells and we wanted to determine if IsaB could be playing a role in either of these two mechanisms. One piece of information that supported our hypothesis was a study done by Garzoni et al, which suggested through microarray analysis that *isaB* was upregulated approximately 5-fold 2 hours following internalization of *S. aureus* into human epithelial cells [49]. Using the human lung epithelial cell line A549 cells and both wild-type 10833 and 10833Δ*isaB* strains, we performed experiments to determine whether IsaB has a role in either adherence or in antibiotic stimulated invasion.

Briefly, bacterial cells were washed in PBS and added to confluent wells of A549 cells. For adherence assays, the bacteria and A549 cells were co-cultured for 1 hour before nonadherent bacteria were removed by washing with PBS. Adherent bacteria were collected by lysing the A549s with PBS containing saponin, and were plated for the enumeration of CFUs. For invasion assays, a similar procedure was performed as for adherence, except after the initial 1 hour co-culture, media was replaced with media containing gentamicin and incubated for 1 hour to kill all extracellular cells. The gentamicin was removed by washing with PBS, and invasive cell counts were enumerated as CFUs. As shown in **Figure 23** there was no difference observed in the amount of adherence or invasion between the two strains, suggesting that IsaB does not likely play a significant role in either of these two pathways.

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Figure 23. IsaB Does Not Increase Adherence or Invasion. IsaB was analyzed for its ability to aid in *S. aureus* adherence or invasion into A549 lung epithelial cells. *S. aureus* strains Sa113 and Sa113 Δ *isaB* were induced in TSBG prior to co-culture with A549 cells. Adherence was assayed after 1 hour co-culture. For invasion, non-invasive cells were killed with 1hr gentamycin treatment. Adhesion and invasion were determined by lysins of A549 cells and plating for CFUs. Percentages were determined by comparing inoculum to adherent or invasive.

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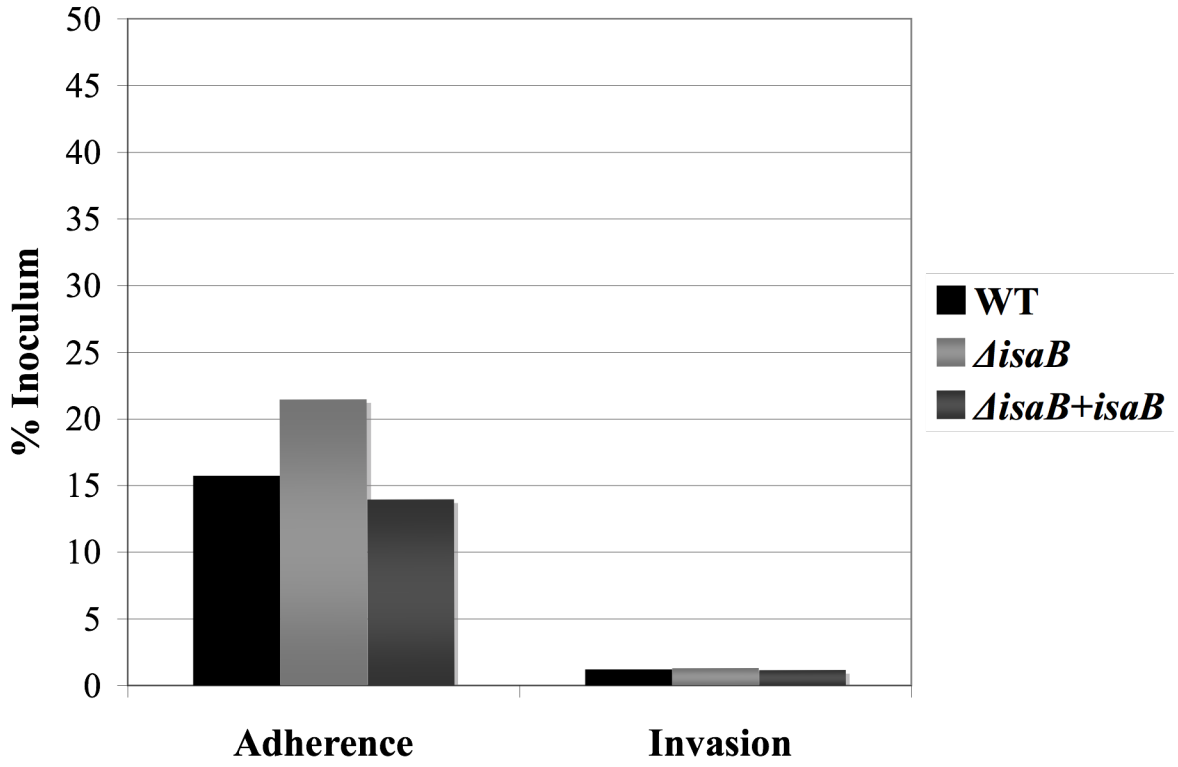
Figure 23. IsaB Does Not Increase Adherence or Invasion.



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IsaB Does Not Increase *S. aureus* Survival in Whole Blood.

As discussed above, IsaB was first characterized by its observed immunogenicity following septicemia, suggesting that it is actively expressed during these systemic blood infections. Furthermore, as shown in Chapter 3, *isaB* expression is induced approximately 5 fold upon exposure to either plasma or serum. This information led us to hypothesize that IsaB could play an important role in *S. aureus* survival in whole blood, which inherently consists of numerous adverse or bactericidal components, such as complement and antibodies.

To address this, overnight cultures of *S. aureus* 10833 and 10833Δ*isaB* were grown in TSBG. Cultures were diluted 1:10 in pooled human blood and incubated up to 4 hours with samples taken at 30 minutes, 2 and 4 hours. Samples were sonicated 2X separated by a wash with sterile water between, which was done in order to break up *S. aureus* aggregates and to lyse red blood cells to allow for accurate CFU counts. CFUs obtained were then used to determine the amount of survival observed. As shown in **Figure 24** there was no significant decrease in percent survival in the Δ*isaB* mutant compared to wild-type demonstrating that IsaB does not affect survival in whole blood culture.

IsaB Does Not Protect Against Neutrophil Mediated Killing.

Neutrophils are considered to be the body's primary defense against *S. aureus*. Therefore, *S. aureus* has a number of virulence factors it employs to evade neutrophil-mediated killing. There are a number of assays used to assess the protective role virulence factors have in circumventing neutrophil mediated killing through phagocytosis.

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Figure 24. IsaB Does Not Protect *S. aureus* in Whole Blood. To determine if IsaB could increase *S. aureus* survival in whole blood strains 10833, 10833 Δ *isaB*, Sa113, and Sa113 Δ *isaB* were incubated in freshly collected whole human blood. Percent survival was determined by CFUs comparing samples treated with blood to untreated. Δ*isaB* had no affect on *S. aureus* ability to adhere to or invade A549 epithelial cells.

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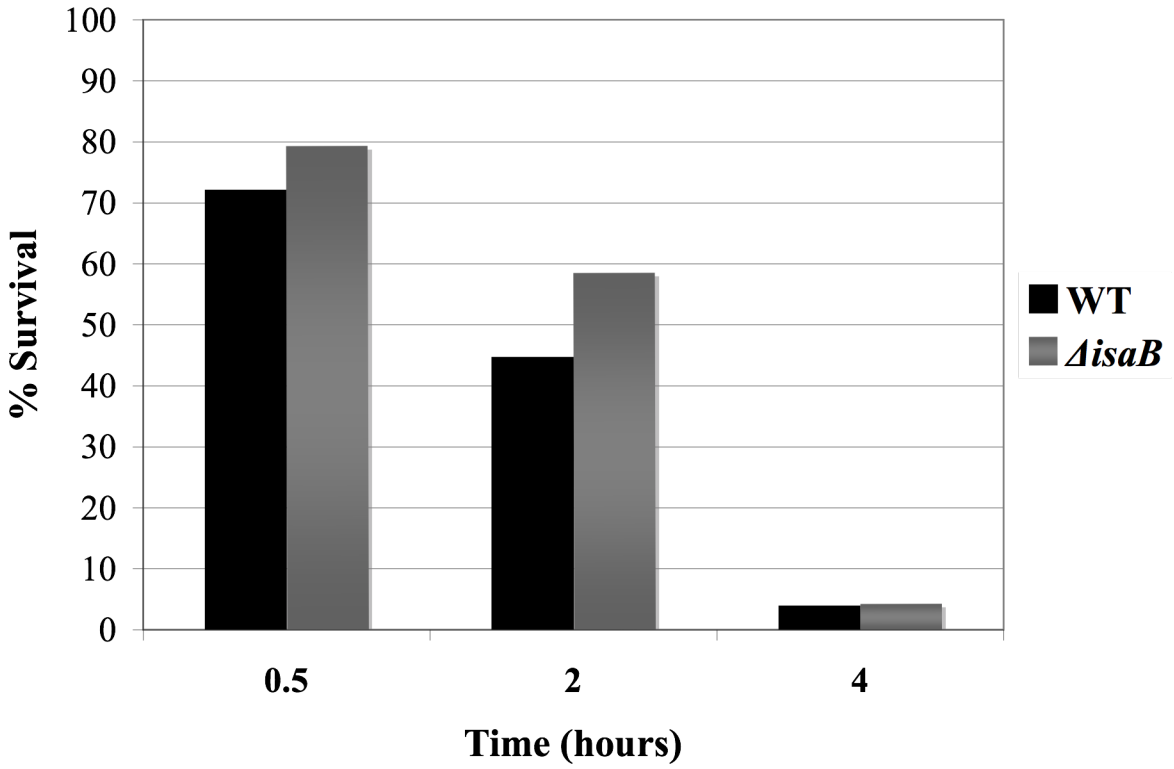
Figure 24. IsaB Does Not Protect *S. aureus* in Whole Blood.



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One of the most commonly used are opsonophagocytic assays that can be easily modified to provide different information, i.e. evasion of phagocytosis or survival after phagocytosis [91, 129, 130]. Because *S. aureus* causing septicemia had to continuously subvert these killing mechanisms to establish systemic infection, and because *isaB* was induced in response to serum, and putatively in response to neutrophils and AMPs, we hypothesized that IsaB could play a pivotal role in *S. aureus* evasion of neutrophil mediated killing.

We therefore used a protocol adapted from Corbin et al to assess the ability of IsaB to protect *S. aureus* from neutrophil mediated killing [91]. Briefly, cultures of *S. aureus* strains 10833 and 10833Δ*isaB* were grown overnight in TSBG. The bacterial cells were then pre-opsonized with 50% human serum, which allowed for complement and antibody deposition that greatly increases the efficiency of phagocytosis. $2x10^7$ human neutrophils were co-cultured with the pre-opsonized bacteria at an MOI of 1 for 2 hours. Neutrophils were then lysed with saponin, samples were sonicated and plated for CFUs. **Figure 25** shows the results from this assay. There was a slight decrease in survival in the absence of *isaB*, but this did not reach statistical significance. These data suggest that IsaB does not significantly protect from phagocytosis by human neutrophils.

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Figure 25. Evasion of Neutrophil Mediated Killing. To determine IsaBs role in protection of *S. aureus* from opsonophagocytosis, pre opsonized 10833 and 10833Δ*isaB* were co-cultured with human neutrophils at an MOI of 1. CFUs were used to compare percent survival from CFUs of pre-opsonized input. No significant difference in survival from phagocytosis was observed after 2 hours of co-culture.

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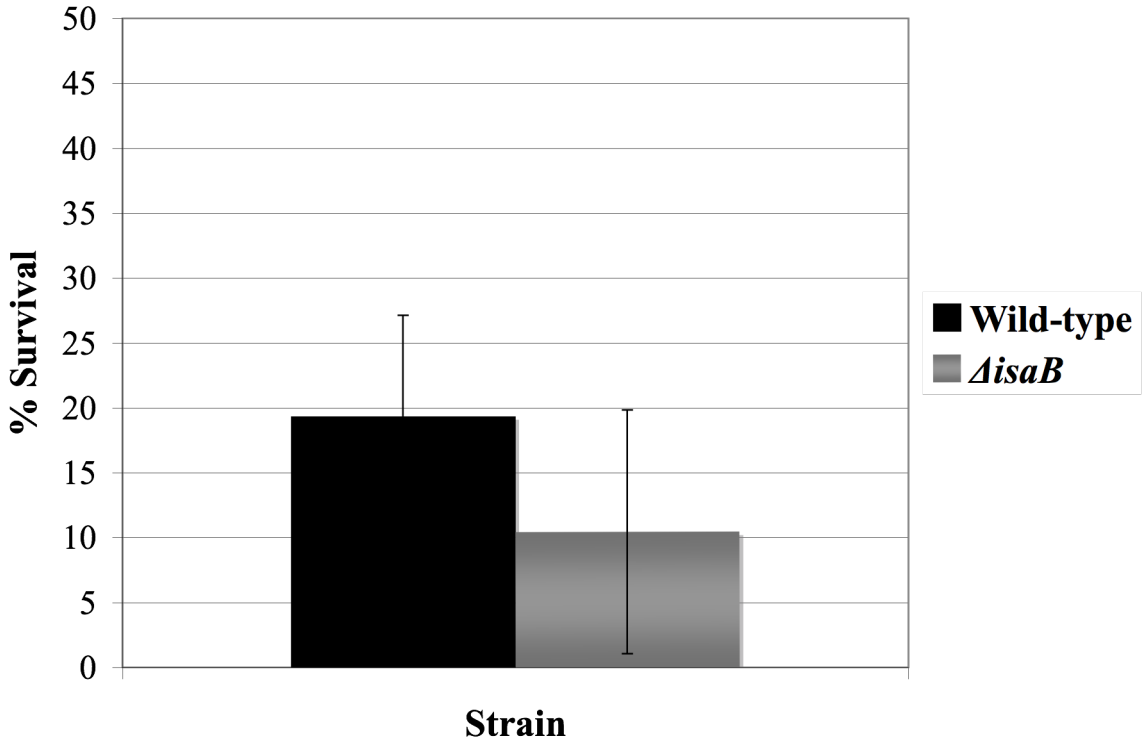
Figure 25. Evasion of Neutrophil Mediated Killing.



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IsaB Protects *S. aureus* Against NETs

While neutrophils are excellent phagocytes, they are also capable of secreting neutrophil extracellular traps, NETs, which occurs through "neutrophil suicide" [17, 19, 92, 131, 132]. Formation of NETs is induced in response to a number of threatening stimuli, including bacteria, and are made up of secreted neutrophil DNA along with its bound histone proteins, and a number of Antimicrobial Peptides and Proteins [92, 131, 132]. In vivo NETs can either kill *S. aureus*, which is likely due to the increased local concentrations of antimicrobial agents, or they can trap the bacteria and prevent their spread further into host tissues, also allowing other effectors and cells to be recruited [92, 131, 132]. Due to ability of IsaB to bind eDNA, which is the main component of NETs, it was intriguing to observe whether deletion of *isaB* had an affect on *S. aureus* survival following incubation with NETs. Neutrophils were isolated, and secretion of NETs was induced using PMA following the protocol from Jann et al [92]. Stationary phase 10833 and 10833Δ*isaB* strains were then co-cultured with the NETs for 30 minutes to allow for killing. **Figure 26** shows the percent survival of wild-type and Δ*isaB* strain. Excitingly we found that the wild-type strain survived NET killing 2.5 fold better than deletion mutant strain, which was statistically significant with a p-value of 0.02. These findings indicate that IsaB plays a significant role in protecting *S. aureus* from NETs.

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Figure 26. IsaB Significantly Protects *S. aureus* From NETs. Neutrophil Extracellular Traps, NETs, are an alternative form of killing by neutrophils. To determine if IsaB played a significant role in S. aureus evasion of these NETs *S. aureus* strain 10833 and 10833Δ*isaB* were co-cultured with NETs secreted by freshly isolated human neutrophils. *S. aureus* strains were grown overnight and then used at an MOI of 0.01 to infect Neutrophils induced to secret NETs. Bacteria were centrifuged onto the NETs and co-cultured for 30 min. Samples were then sonicated and used for CFU enumeration. Percent survival was determined by comparing CFUs from NETs to CFUs of input. The data show that deletion of *isaB* had a significant and negative impact on S. aureus ability to survive NETs exposure, $p = 0.02$. Student's t-test was used for statistical analysis.

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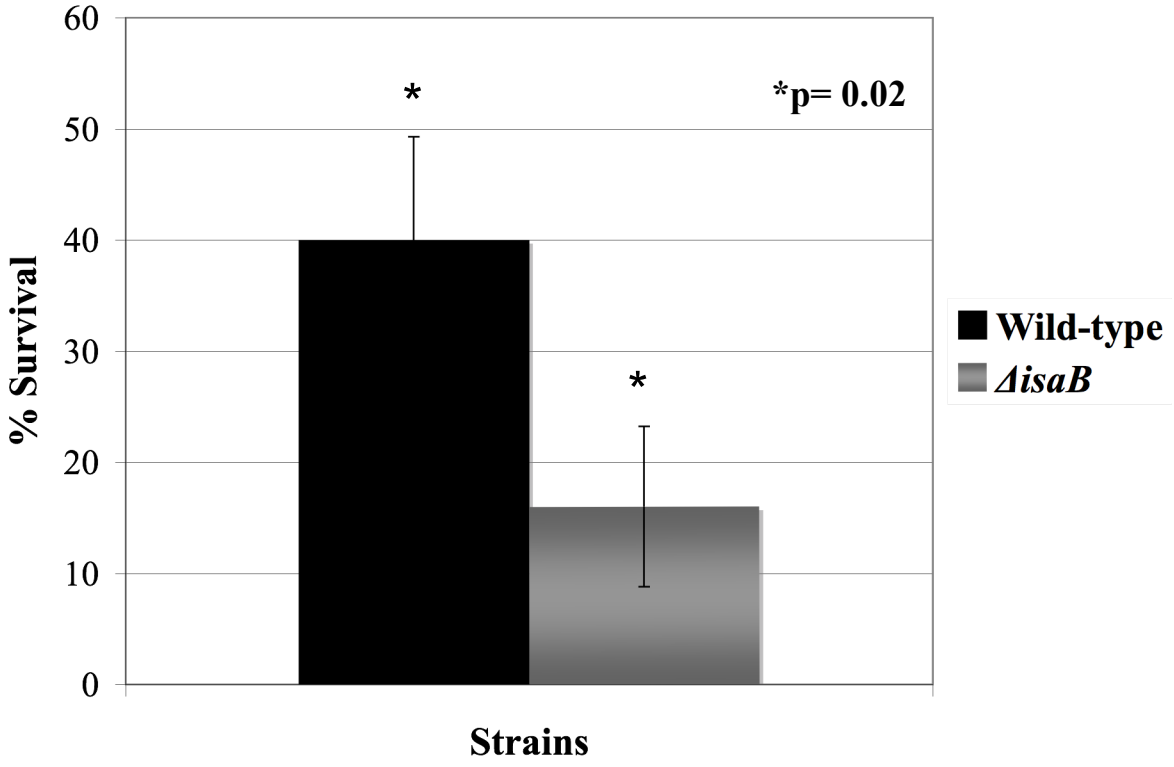
Figure 26. IsaB Prevents Killing By NETs.



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IsaB Significantly Increases Survival Following Treatment With Antimicrobial Peptides.

Antimicrobial peptides or AMPs, are highly cationic small peptides that have been found to be potent bactericidal innate immune components. Interestingly microarray data from Herbert et al, revealed that *isaB* is upregulated by the two-component system GraRS, which plays a major role in *S. aureus* resistance to AMPs [32]. Furthermore, one of the hypothesized mechanisms of NET mediated killing is the NETs ability to sequester AMPs and locally increase their concentration, allowing the AMPs to interact more intimately with the bacteria. Therefore, we hypothesized that IsaB, which is highly cationic, could aid in the repulsion of the cationic AMPs from the cell surface of *S. aureus*.

Because it was unknown how IsaB could affect individual AMPs four were chosen for study based on their previous validated potency against *S. aureus;* HNP1, HBD3, HBD2, and Buforin [29, 30, 133]. The chosen AMPs were mixed together and used to treat either wild-type or Δ*isaB* strains. Briefly, overnight cultures of 10833 and 10833Δ*isaB* were grown in LBG. These stationary phase cultures were then diluted 1:100 in PBS containing 2.4μg/ml of each AMP and incubated together for 2 hours before plating for CFUs. As shown in **Figure 27**, deletion of IsaB significantly decreased *S. aureus* survival compared to wild-type in response to AMP treatment. The wild-type survived approximately 4 fold better than the *isaB* deletion mutant strain with $p = 0.001$. Therefore IsaB significantly protects *S. aureus* from AMPs.

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Figure 27. IsaB Protects *S. aureus* From AMPs. To determine IsaBs ability to protect *S. aureus* from AMPs, *S. aureus* strains 10883 and 10833Δ*isaB* were treated with a combined mixture of AMPs: HNP1, HBD2, and HBD3. After 2 hour treatment, samples were plated for CFUs. Percent survival was determined by comparing untreated to AMP treated samples. IsaB was found to significantly increase *S. aureus* ability to survive AMP exposure, $p = 0.001$. Student's t-test was used for statistical analysis.

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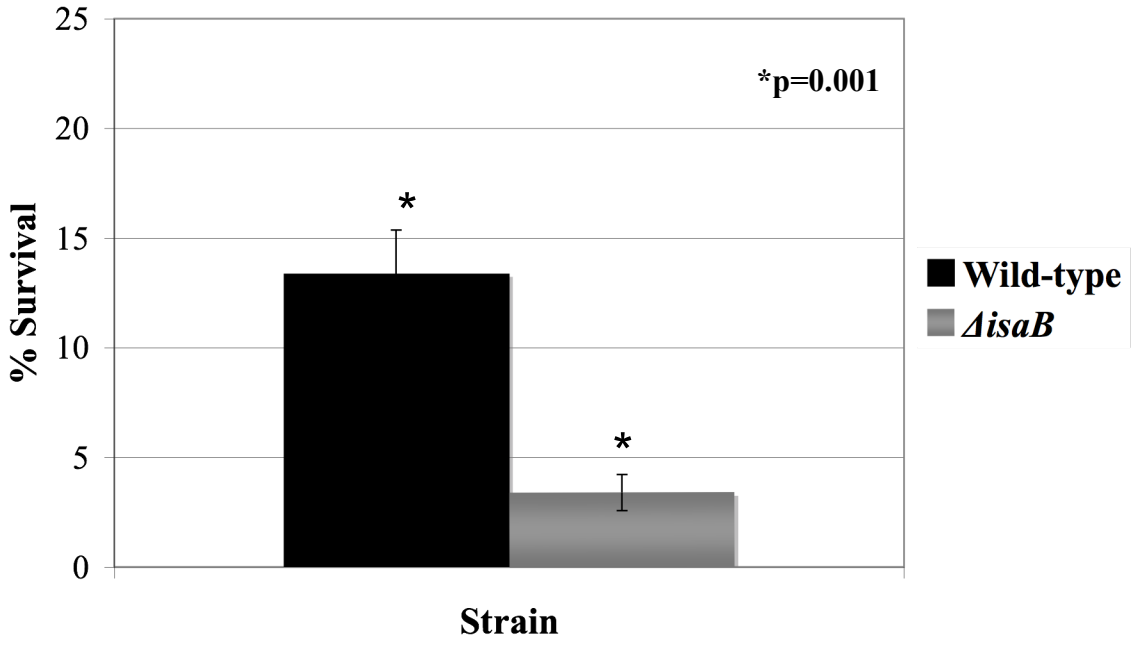
Figure 27. IsaB Protects *S. aureus* From AMPs.



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Discussion:

From the above studies we found that IsaB does not appear to have a significant role in growth, adherence, invasion or survival in whole blood. However we have shown that IsaB is in fact a virulence factor, one that significantly protects *S. aureus* from Antimicrobial Peptides and Neutrophil Extacellular Traps. These findings are extremely exciting as they lend significant clinical relevance to our studies and show that as hypothesized, IsaB is an important and novel virulence factor of *S. aureus*. It is important to note that the significant role IsaB has in evasion of AMPs and NETs is likely intertwined and due to the same phenomenon.

As described above it is hypothesized that NETs have a high concentration of AMPs associated with them and that it is the AMPs in the NETs that are responsible for bactericidal activity. Furthermore, the neutrophils in our assays were inactivated during co-culture with *S. aureus* by the addition of cytochalsin D to the media, thus showing that it is NET killing and not a combination of NET and phagocytosis-mediated killing. The fact that IsaB had a significant role in evasion of NET killing strongly suggests that IsaB acts in a physiologically relevant manner, supporting the data from the more extrapolated AMP killing assays.

While IsaB played a significant role in these two assays, we found no significance in its ability to protect against phagocytosis or promote killing in *C. elegans*. In the case of the opsonophagocytic assay, many AMPs are likely kept sequestered within the neutrophil where they have an active role in the phagolysosome. Additionally if AMPs were

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secreted they may not have reached a great enough local concentration to significantly inhibit/kill the bacteria. Also, the opsonophagocytic assay is a much more complex assay in which *S. aureus* has many previously described virulence factors which could mask the significance of IsaB. As to why no significant role for IsaB was observed in the *C. elegans*, it is likely due to many of the same reasons given for evasion of neutrophil mediated killing. However, it could also be argued that IsaB is a protective mechanism and while it helps the *S. aureus* survive in vivo or within the *C. elegans*, it does not appear to directly target or affect the host on its own, and thus it does not significantly affect killing in this assay. However it is interesting that while there was no significant difference between the wild-type, complemented and Δ*isaB* strains, the overall trend suggested reduced killing of the nematodes by the Δ*isaB* mutant; that the wild-type killed more, and that the complemented strain exhibited the highest killing rate, however there was great variability within each experiment and between repeats. Therefore IsaB could play a role in this model that might be observed if the variability in the assay was somehow decreased.

As discussed above, one of the common ways that pathogens including *S. aureus* subvert AMPs is through extensive modification of their cell surfaces to help neutralize the overall negative charge of their surface. It is therefore intuitive to postulate that IsaB, which is highly cationic and associated with the cell surface, would have a significant effect on repelling AMPs, thus not allowing them to bind and perform their bactericidal activities. A second mode of action could relate to the ability of IsaB to bind to eDNA. AMPs have been found to interact with NETs that are primarily composed of DNA. It

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could therefore be possible that IsaB binding to eDNA acts as a decoy causing the AMPs to bind to it instead of the bacterial surface. We strongly believe that IsaB is likely acting in one of these two functions and that IsaB is not playing a role in degradation of the AMPs as we have not observed any proteolytic or enzymatic activity with purified protein.

From these studies we now know that IsaB is a novel AMP evasion virulence factor, which can account for its immunogenicity/expression in vivo. Due to its ability to protect *S. aureus* from AMPs, it strongly suggests that IsaB is an important factor not only in septicemia but potentially all disease manifestations of *S. aureus* as AMPs are found throughout the body. Furthermore, if IsaB is expressed in response to serum, AMPs and neutrophils, it would be expressed during all acute *S. aureus* disease manifestations, making IsaB an extremely attractive antibody therapeutic target or potential vaccine candidate.

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CHAPTER 6

Role of IsaB in *S. aureus* Biofilms

Introduction:

There is a well-established precedent for the role of extracellular DNA within bacterial biofilms. eDNA has been found to be a major biofilm matrix component in many Gram-positive and Gram-negative pathogens such as *S. epidermidis, N. meningitidis*, *L. monocytogenes*, *S. mutans*, *E. faecalis*, and *P. aeruginosa* [15, 134-138]. However, until recently, it was believed that the *S. aureus* biofilm was made up predominantly of PNAG. Several studies performed by Dr. Kenneth Bayles (University of Nebraska, Omaha, NE) have implicated eDNA in the formation and maintenance of the *S. aureus* biofilms [66, 67, 139]. These studies have shown that under certain conditions, *S. aureus* undergoes autolysis/lysis, releasing genomic DNA, which is then used as a matrix component for the developing biofilm, a system similar to those in other bacterial species that utilize eDNA in their biofilm matrices [15, 66, 67, 136-139].

Because it is unknown how the eDNA is incorporated into the matrix, we hypothesized that IsaB could be playing an important role in biofilm formation. One could rationalize that IsaB, as an extracellular nucleic acid binding protein, could play a significant role in either the incorporation of eDNA into the biofilm, or in promoting adherence of the bacterial cells to the biofilm matrix. Therefore the studies that follow

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were performed with the intent of determining the role of IsaB in biofilm formation of *S. aureus*.

Results:

isaB Is Not Differentially Expressed Between Biofilms and Planktonic Cultures.

We hypothesized that if IsaB played a role in biofilm formation then transcription of the *isaB* gene would be elevated in biofilms with respect to planktonic cultures. A study by Resch et al, suggested that *isaB* was upregulated approximately 3-fold in 24, and 48 hour biofilms compared to planktonic [96]. Briefly, overnight wild-type cultures were diluted 1:10 in TSBG and added to either 96-well plate, or into 10mls TSBG. These cultures were grown overnight, and the following day the media was removed and replaced with fresh TSBG and incubated for 2 hours prior to collection for RNA extraction. Using RT-PCR, no difference was observed in the expression of *isaB* between the two modes of growth as shown in **Figure 28.** Therefore, under our standard laboratory conditions there was not a differential regulation in *isaB* expression between biofilms and planktonic.

IsaB Negatively Impacts Biofilm Formation.

The use of confocal microscopy is advantageous for the direct visualization of *S. aureus* biofilms because it allows for estimation of biofilm depth and architecture. Therefore, biofilms of either wild-type or Δ*isaB* were grown under static conditions in 6-well plates overnight at 37℃. The biofilms were washed in PBS and stained with BacLight Live/Dead, which imparts green fluorescence to viable bacteria and red to dead

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Figure 28. *isaB* is Not Differentially Expressed. To determine if *isaB* is expressed more or less in the biofilm mode of growth RT-PCR was done.
S. aureus 10833 was grown as either a biofilm or planktonic culture in TSBG overnight. The cultures were then "fed" with fresh TSBG for 2 hours to stimulate metabolic activity. RT-PCR for *isaB* expression was performed. No difference was observed in *isaB* expression between the two modes of growth. All samples were normalized to their *16s rRNA* ct values.

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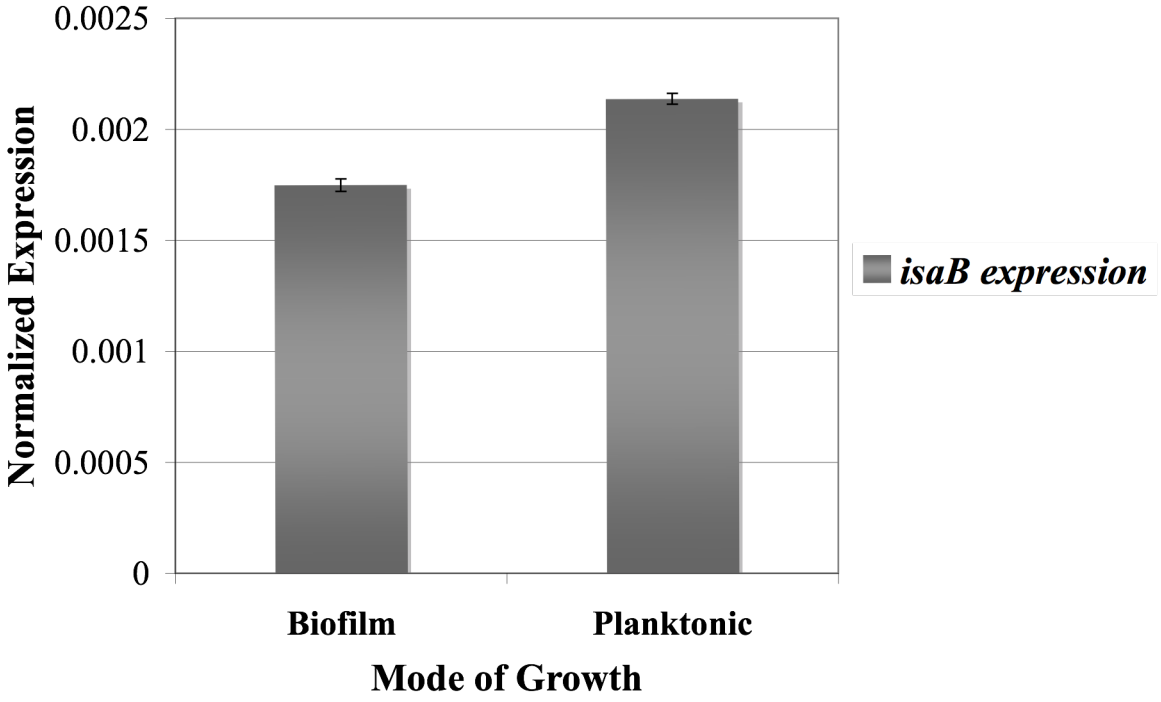
Figure 28. *isaB* is Not Differentially Expressed in Biofilms.



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bacteria. The biofilms were visualized directly by confocal microscopy. We had hypothesized that IsaB would likely have a positive affect on biofilm formation and therefore when *isaB* was deleted a decrease in biofilm formation would be observed. However, as shown in **Figure 29a**, deletion of *isaB* appeared to have a positive affect on *S. aureus* biofilm, countering the hypothesis.

To confirm the visualized increase of biofilm in the deletion mutant, and to get quantitative data, the 96-well microtiter biofilm plate assay was utilized. In these experiments, overnight cultures of wild-type or Δ*isaB* were diluted 1:10 in TSBG, added to each well and grown overnight at 37℃. The following day the biofilms were washed in PBS to remove any non-adherent cells, stained with safranin, and dried. Safranin was solubilized using 33% acetic acid and read on a plate reader at OD_{564}. As shown in **Figure 29b**, these experiments confirmed the previously seen increase, which was equivalent to an approximately 12% increase in biofilm compared to the wild-type strain. These experiments suggest that under the conditions tested and for the strain tested, not only does IsaB not augment biofilm formation, it may actually hinder it in some way.

The Negative Effect of IsaB on Biofilm Formation Depends upon Culture Conditions.

To determine if IsaB negatively affected biofilms in all growth conditions and in multiple wild-type strains, biofilms were grown in a variety of medias. The biofilms were grown as described above, however they were grown in LB, LBG, BHI, BHIG, TSB or TSBG+NaCl. TSBG was supplemented with 3.25% NaCl because a recent publication

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Figure 29. IsaB Decreases Biofilm Formation. To determine if IsaB was significant in eDNA biofilm incorporation biofilm assays were performed. *S. aureus* strain 10833 and 10833 Δ *isaB* were grown in TSBG. **a)** For confocal microscopy, biofilms were grown in a 6-well plate and stained with Live/Dead stain prior to microscopy. **b)** 96 well biofilm microtiter plate assay was used to get quantitative data about the observed increase in biofilm formation. Biofilms were stained with safranin, solubilized with acetic acid and read at OD_{564}. IsaB produced significantly more biofilm than wildtype p= 0.004. Student's t-test was used for statistical analysis.

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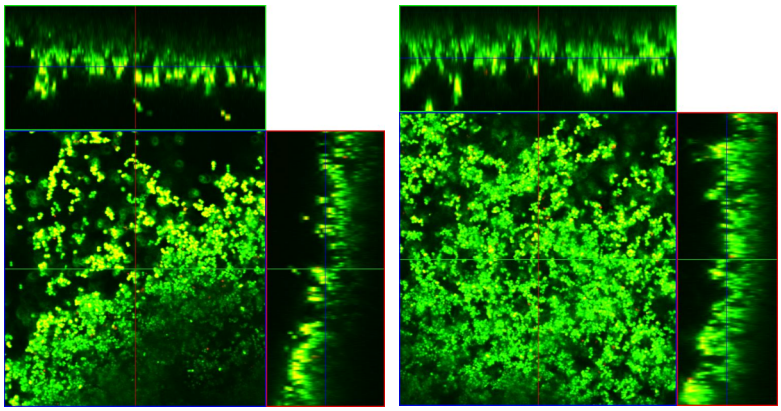
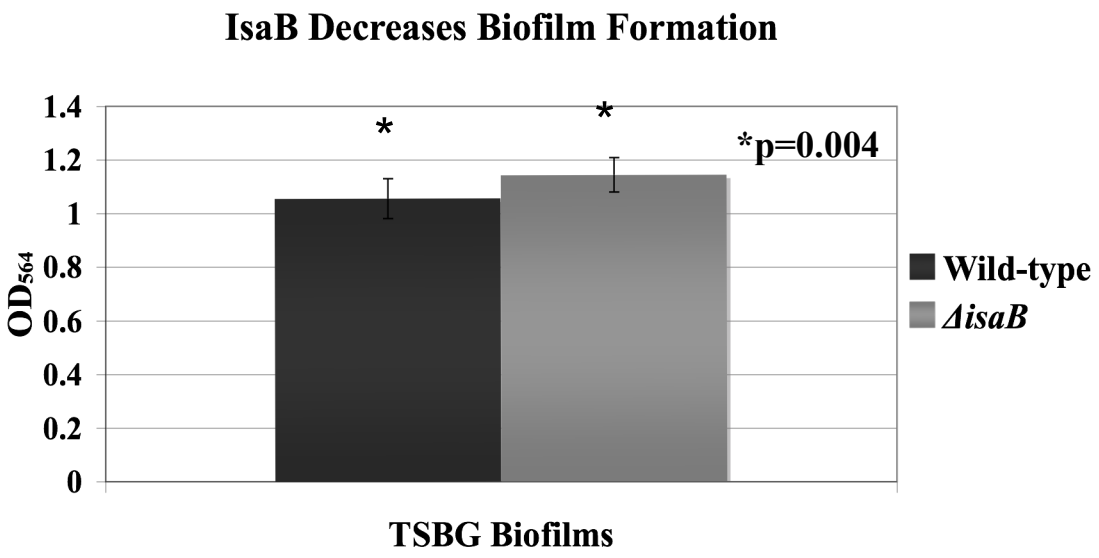
Figure 29. IsaB Decreases Biofilm Formation.

A.



10833 **10833Δ*isaB***

B.



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suggested that growth in either glucose or glucose + NaCl could induce biofilm formation [61]. A representative photo of the biofilm plate before solubilization with acetic acid is shown in **Figure 30a**. The graph in **Figure 30b**, shows the quantitative data obtained from the plate reader. As the graph shows, under most conditions tested there is no observed effect of deletion of *isaB*. However, when both wild-type strains were grown in LBG the same significant increase of biofilm formation in the Δ*isaB* strains was observed. These findings suggest that IsaB interferes with biofilm formation only under certain inducing conditions.

IsaB Does Not Affect the Role of eDNA in Biofilms.

DNAse has been used as a tool to assess the role of eDNA in biofilms [66, 86]. We therefore used a similar assay to determine the extent of eDNA biofilm incorporation between the wild-type or Δ*isaB* strain. For these experiments, biofilms were grown in TSBG as previously described. Following overnight growth, the cultures were either left untreated, or treated with 2.5 Units of DNAse for 4 hours prior to washing, staining, and reading with the plate reader. **Figure 31a** is a representative microtiter plate demonstrating the extent of visual DNAse degradation. **Figure 31b** shows the data from the plate reader expressed as % intact biofilm following DNAse treatment. As this graph shows, there was not a significant difference observed in the amount of DNAse degradation between the wild-type or Δ*isaB* strains tested. Suggesting that IsaB does not significantly modulate the amount of eDNA within the biofilm matrix.

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Figure 30. IsaB Only Affects Biofilms Under Specific Growth Conditions. To observe how consistent the increase in biofilm formation occurred when *isaB* was deleted biofilms were grown in a wide variety of media with different supplementation. **a)** Representative image of stained biofilms prior to solubilizing. **b)** quantittative results from biofilms using the plate reader. Δ*isaB* biofilms grown in LBG for both displayed significantly greater biofilm formation p=0.005 (10833) and p=0.0003 (Sa113) than parental strains. Student's t-test was used for statistical analysis.

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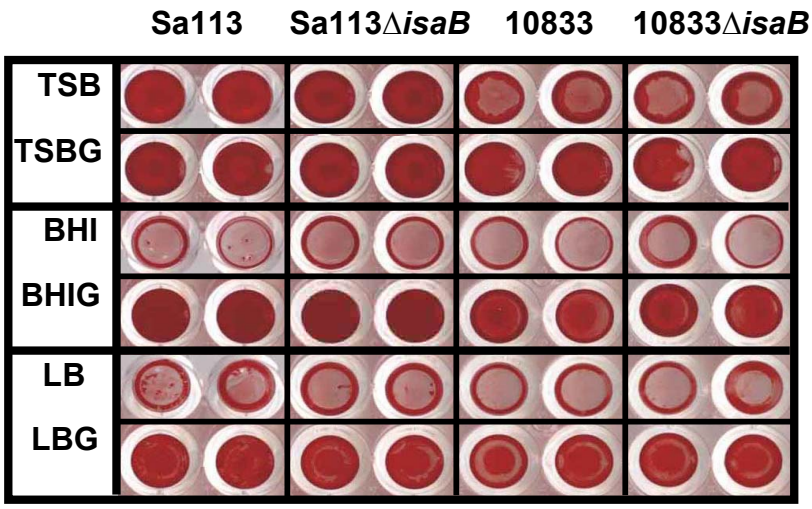
Figure 30. IsaB Only Affects Biofilms Under Specific Conditions.

A.



B.

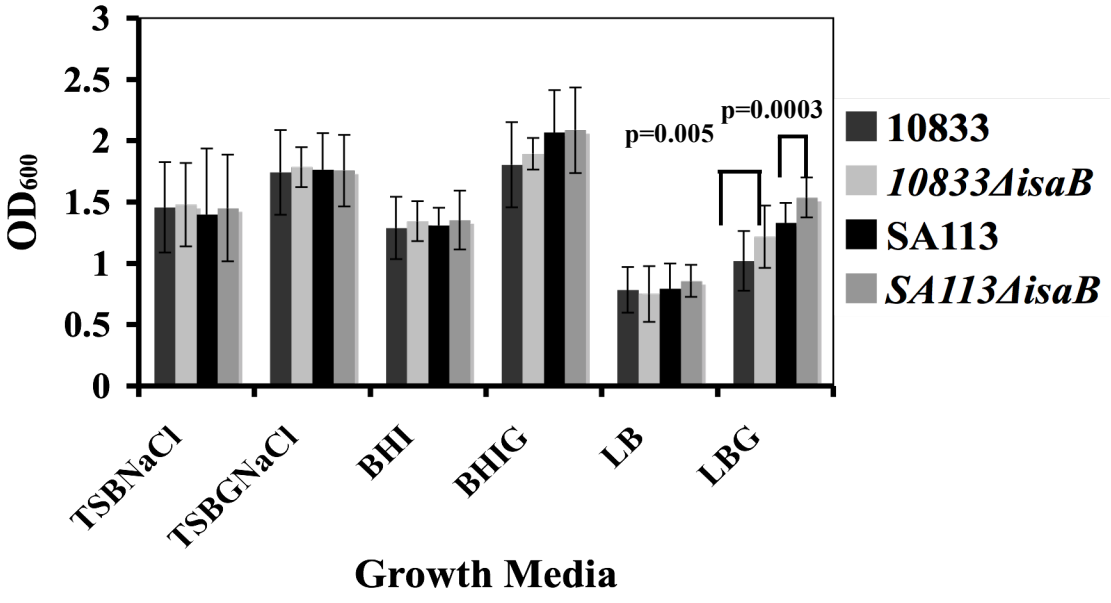


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Figure 31. IsaB Does Not Play a Role in eDNA Incorporation. To determine if IsaB was able to change the amount of eDNA in the biofilm matrix DNAse sensitivity assays were performed. *S. aureus* strains 10833, 10833 Δ *isaB*, Sa113, and Sa113 Δ *isaB* were grown as biofilms in TSBG in a 96 well microtiter plate. Biofilms were then DNAse treated with 2.5U DNAse for four hours. **a)** Representative image of stained biofilms following DNAse treatment. **b)** Graph of quantitative data from OD_{564}. Statistical analysis was performed using the Student's t-test. No Statistically significant difference was observed as % degradation by DNAse.

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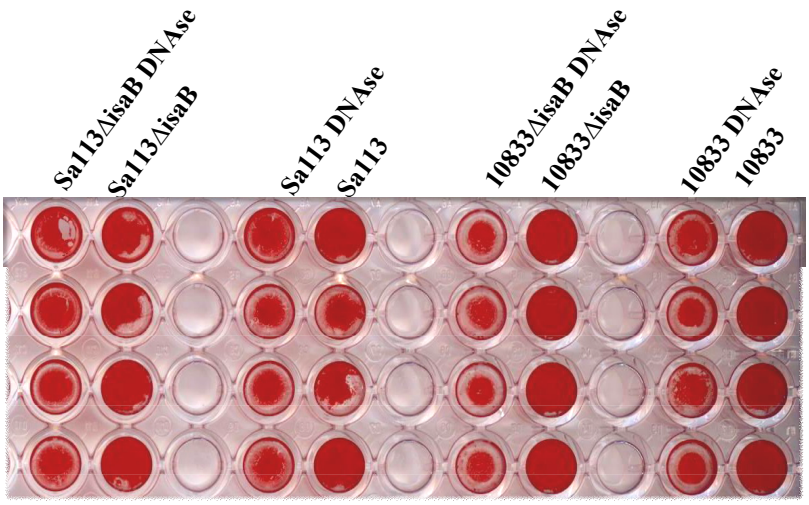
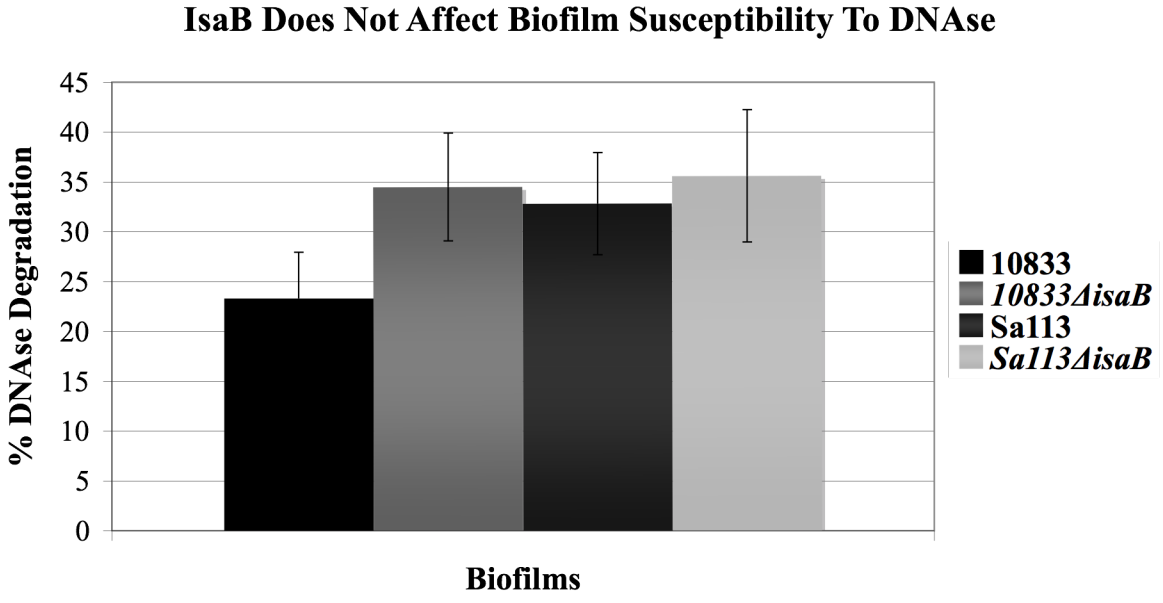
Figure 31. IsaB Does Not Play a Role in eDNA Incorporation.

A.



B.



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IsaB Does Not Affect Biofilm Formation in Serum.

Evidence suggests that IsaB is expressed during septicemia. Therefore, we hypothesized that IsaB may play a more important role in biofilm formation under physiologically relevant conditions. Because biofilms have been found to be important contributing factors in a number of diseases, we wanted to determine if IsaB could promote biofilm formation in various concentrations of serum. Aside from being more relevant to human disease, **Figure 8** shows that *isaB* is induced by exposure to serum. Therefore 96-well biofilm microtiter assays were performed as described above, however instead of bacterial growth media, 1:2 dilutions of serum diluted in TSBG were used. **Figure 32** shows the results from reading at OD_{564nm}, and as can be seen, there is no difference observed between wild-type or Δ*isaB* strains at any concentrations of serum tested.

Discussion:

We hypothesized, due to the ability of IsaB to bind DNA and to increase the amount of eDNA accumulated on the cell surface, that IsaB would have a significant role in biofilm formation. Through our studies, it was determined that IsaB, surprisingly, had a negative impact on biofilm formation (under certain conditions) in contrast to our expected result. Biofilms were also grown in various concentrations of human serum in the hopes that *isaB* could potentially augment biofilm formation under conditions that most closely mimic human disease. However, we again observed that IsaB had no significant affect on biofilm formation in human serum.

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Figure 32. IsaB Does Not Affect Biofilm Formation in Serum. To look at IsaBs role in biofilm formation under more physiologically relevant conditions, biofilms were grown in human serum. *S. aureus* strains 10833 and 10833 Δ*isaB* were grown in 100%, 50%, 25%, 12.5% and 6.25% human serum diluted in TSBG. Biofilms were stained with safranin and quantitative data was obtained by OD564. No significant difference in Biofilm formation was observed in either wild-type or Δ*isaB* strain.

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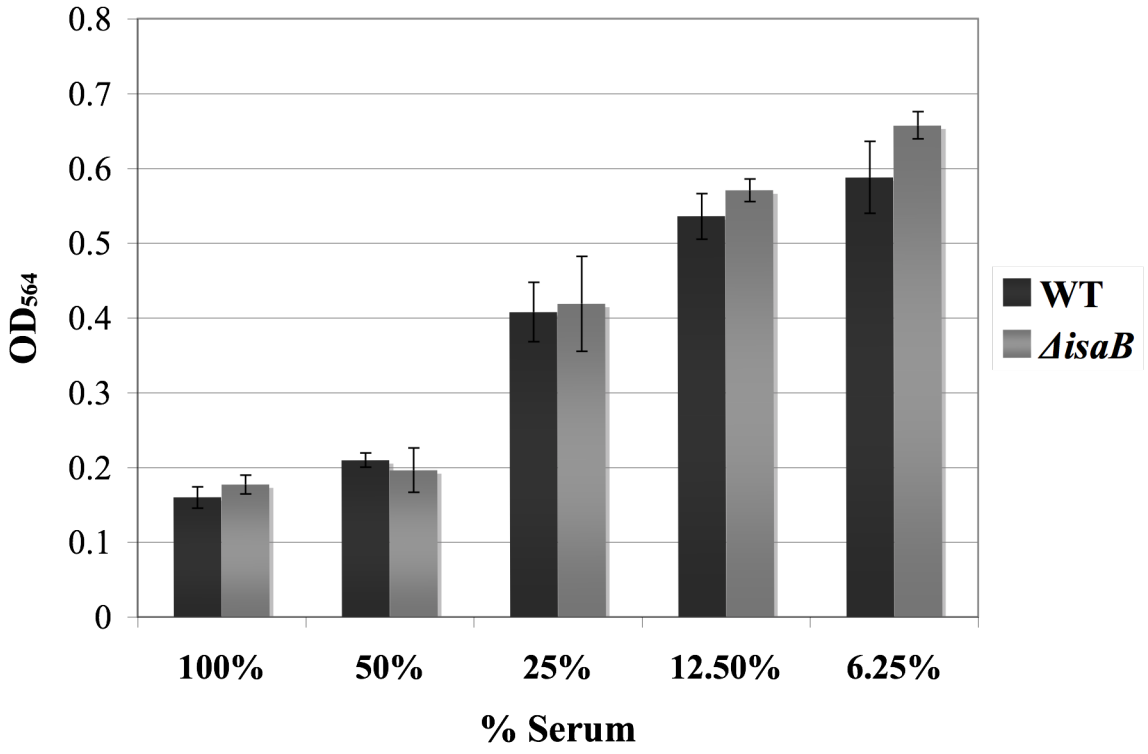
Figure 32. IsaB Does Not Impact Biofilm Formation in Serum.



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It is important to note, that while the increase in biofilm observed with the Δ*isaB* was statistically significant (possibly due to a large n), it is not clear that this slight increase is physiologically relevant. However, the increase could be due to binding of eDNA by IsaB, which could block binding sites used in biofilm formation and attachment. Why would IsaB not be involved in augmenting biofilm formation by aiding in the incorporation of eDNA? Perhaps IsaB could be involved in biofilm formation under growth conditions that were untested, or another un-characterized protein with redundant function could be masking the deletion. Either way, we have shown that IsaB, while expressed in biofilms (at levels similar to planktonic), does not augment biofilm formation in the *S. aureus* strains tested.

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CHAPTER 7

Structural Analysis of IsaB

Introduction:

We have found that IsaB binds to nucleic acids in a non-specific manner and protects the bacteria from antimicrobial peptides and NET-mediated killing. However, IsaB does not have significant similarity, at least at the amino acid sequence level, to other proteins of known function (or even to hypothetical proteins from other Genera). Therefore, we do not know whether it functions in a manner similar to other nucleic acid-binding proteins or other proteins involved in AMP-evasion. Furthermore, we do not know whether IsaB has an additional primary metabolic function. We attempted to use protein structure/function prediction programs, such as I-TASSER Online (available at http://zhanglab.ccmb.med.umich.edu/I-TASSER/) to predict IsaB function and structure. However in silico analyses were unable to confidently predict a likely structure or function, therefore experimental methods were employed [140].

To date there are a number of techniques used to determine the 3-dimensitional structure of proteins, two of the most commonly used are X-Ray Crystallography and Nuclear Magnetic Resonance [141-143]. X-Ray Crstallography is considered to be the gold-standard used for protein structure determination. This technique requires successful formation of a protein crystal, which is then subjected to a X-Ray beam [142]. The protein structure is then determined by the amount and angle of X-ray beam diffraction. Unlike X-

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ray Crystallography, NMR does not require crystal formation for successful structure determination allowing for more rapid analysis. In NMR the protein is analyzed in solution during NMR, and is subjected to a large magnetic field. The magnetic resonance of individual atoms in each aa are assigned and used to determine the proximity to other amino acids which subsequently reveals the folding structure [142-144]. While NMR can produce accurate structural information more rapidly then X-Ray Crystallography, its use is often hindered by strict size constraints as it has only been used to identify structure in molecules under 30 kDa, and often requires labeling with isotopes [141-143].

The objective of this study was to determine the structure and localization of IsaB in an attempt to better understand its function. We found that IsaB is an extracellular protein that is largely associated with the bacterial cell surface. We purified protein for use in x-ray crystallographic analysis by our collaborators Drs. Martin Safo and Faik Musayev, but sufficient crystals for analysis could not be produced. We then purified protein for Nuclear Magnetic Resonance analysis in collaboration with Dr. David Williams to determine the definitive structure of IsaB.

Results:

IsaB in Staphylococci

isaB has been annotated in multiple *S. aureus* genomes (http://www.ncbi.nlm.nih.gov/nuccore), allowing us to use BLAST to determine the distribution of IsaB throughout the *S. aureus* genomes [145]. As shown in **Figure 33**, IsaB was highly conserved between all annotated *S. aureus* genomes. Interestingly, we found

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that IsaB homologues were in all sequenced *Staphylococcal* species with varying degrees of identity **Figure 33**. However, beyond the staphylococci, IsaB does not appear to have any significant similarity to any known proteins. Therefore, IsaB appears to be specific to this genus.

m-IsaB Predictions.

Prediction software was used to determine where the signal peptide sequence would be cleaved, and what the mature protein would "look" like. The SignalP 3.0 software (available at http://www.cbs.dtu.dk/services/SignalP/), was used to determine where a Gram-positive signal peptidase would cleave IsaB, the results are shown in **Figure 34** [146]. As the data and graph show, signal cleavage is predicted, with 97% probability, to occur between position 36-37, cutting at the sequence 5' AQA-AI 3'. Thus the most N-terminal aa of m-IsaB is an alanine. The predicted m-IsaB was then analyzed by the Expasy online tool (available at http://www.expasy.ch/cgi-bin/protparam), and was predicted to be 139 aa in length, with a molecular weight of ~15.8 kDa, and a theoretical pI of 9.66 [147].

Next Emboss (available at http://www.ebi.ac.uk/Tools/emboss/pepinfo/) was used to visualize the types of aa throughout the protein, as shown in **Figure 35**, which was useful to determine where pockets of positive and negative charges, along with polar vs

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Figure 33. Homology of *isaB* in Staphylococci. Using BLAST search IsaB has been found in all currently sequenced *S. aureus* strains. This chart depicts the ranges of homology between IsaB homologs. Putative IsaB genes have been found in all genomes of sequenced Staphylococci with varying degrees of similarity.

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Figure 33. Sequenced *Staphylococcus* Species Containing Putative IsaB

Species	Identity	Similarity
S. aureus	82-100%	93-100%
S. epidermidis	42-55%	63-76%
S. haemolyticus	48%	65%
S. capitis	39-47%	63-68%
S. hominis	46%	61%
S. warneri	37%	59%
S. saprophyticus	35%	56%
S. carnosus	31%	50%

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Figure 34. Predicted Singal Peptide Cleavage Site. Using SignalP 3.0 prediction server we determined that IsaBs signal peptide sequence is most likely cleaved between aa 36-37, with a probability of 0.97. Bolt indicated signal peptidase cleavage on the peptide backbone of IsaB. The mature 5' end is an Alanine.

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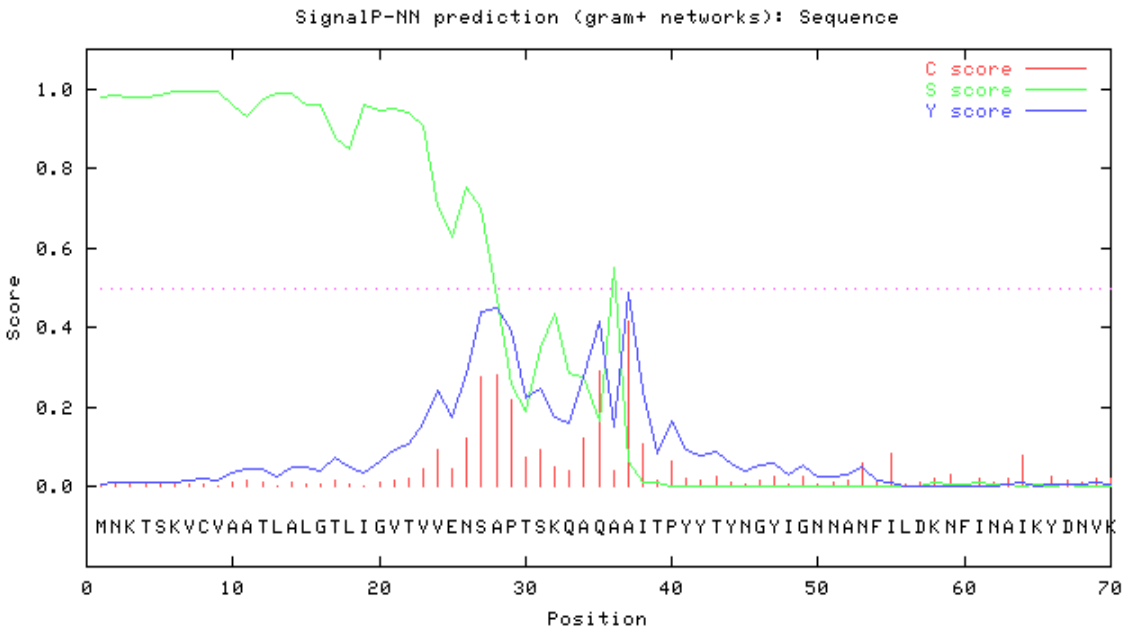
Figure 34. Predicted Signal Peptide Cleavage

 # Most likely cleavage site between pos. 36 and 37: AQA-AI
SequencePrediction: Signal peptideSignal peptide probability: 1.000Max cleavage site probability: 0.973 between pos. 36 and 37



p = 0.973

N-Terminal Signal Peptide

MNKTSKVCVAATLALGTLIGVTVVENSAPTSKQAQA-AITPYYTYNGYIGNN

5' m-IsaB

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Figure 35. Distribution of Amino Acids in IsaB. Using the Emboss prediction server we were able to analyze the aa characteristics throughout IsaB. There do not appear to be any significant patches of aa of a particular type or charge throughout the backbone.

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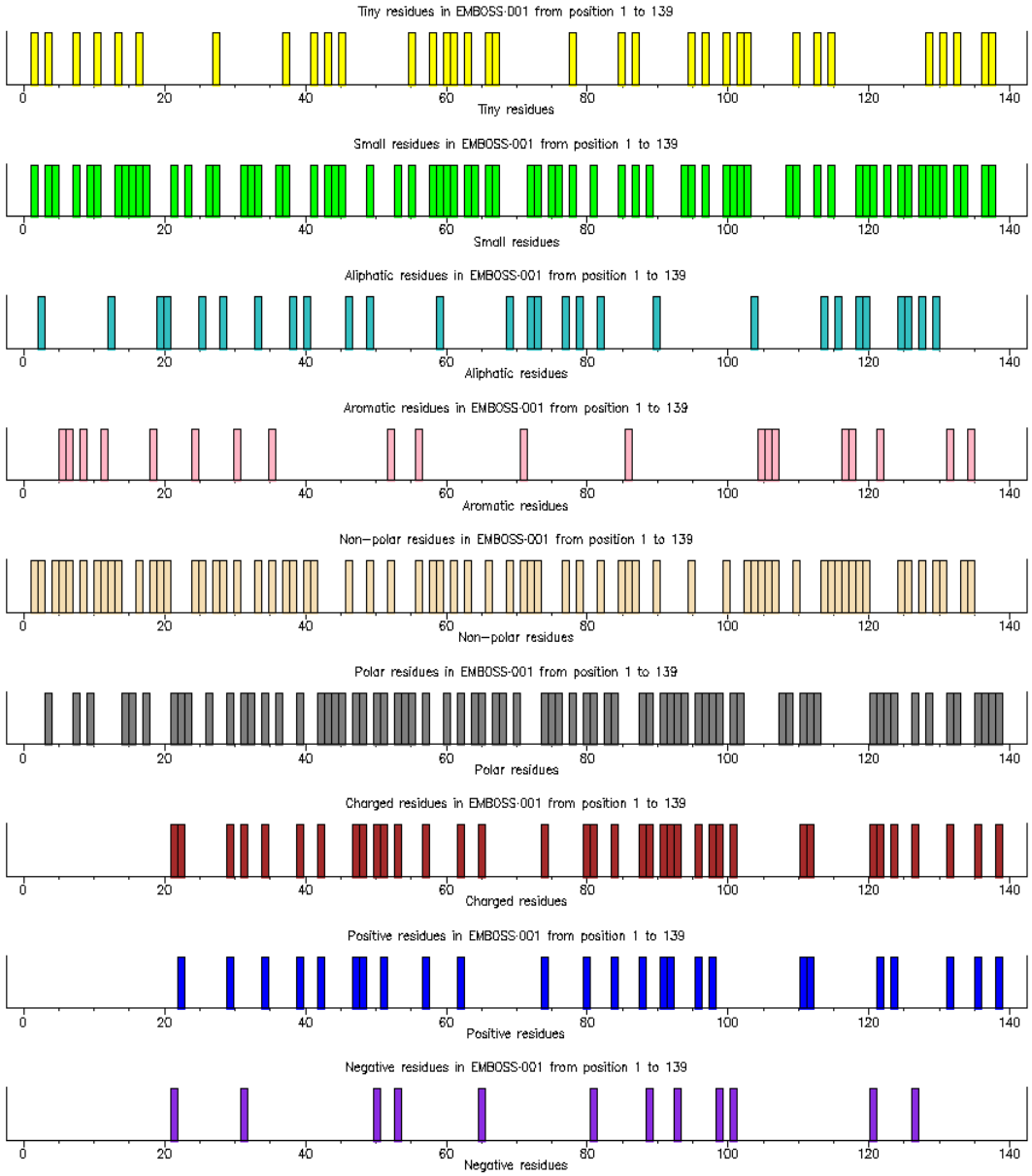
Figure 35. Distribution of Amino Acids in IsaB



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nonpolar aa [148]. The predicted hydrophathy chart was also obtained from Emboss and as shown in **Figure 36**, m-IsaB is predicted to be mainly hydrophilic (hydropathy < 0) aside from a hydrophobic patch at the C-terminus. It is therefore likely that the hydrophobic patch is sequestered within the folded protein, while strongly hydrophilic areas are predicted to be exposed on the surface of the folded protein. From these predictions, we now know the size and aa sequence of m-IsaB, and many of the characteristics governed by its aa sequence.

IsaB is an Anchorless Protein.

As mentioned previously, BLAST analysis of IsaB showed that IsaB had a Gram-positive signal peptide sequence, interestingly it did not predict the presence of a conserved Gram-positive cell-wall anchoring motif, commonly called a LPXTG motif. This motif allows for bacterial sortase genes to covalently anchor secreted proteins to the peptidoglycan layer, thus keeping them attached to the cell surface and preventing release beyond the cell [7].

Therefore, we wanted to ensure that the cell-surface association of IsaB was in fact independent of sortase. To answer this question, a *srtA* deletion mutant was used for western blot analysis. Overnight cultures of *S. aureus* strains 502, 502ΔsrtA, and 10833Δ*isaB* were grown in TSBG, and cell surface associated proteins were extracted. As shown in **Figure 37**, deletion of *S. aureus srtA* gene had no observed effect on IsaB association with the cell surface. This experiment confirms that association of IsaB with the surface of *S. aureus* is independent of Sortase A.

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Figure 36. Hydropathy Chart of IsaB (Kyte-Dolittle). Using Emboss prediction server, we obtained m-IsaBs proposed hydopathy chart. Most of the protein appears to be hydrophilic (< 0) however there is a hydrophobic patch on the carboxy terminus which would likely be folded within the mature secreted protein.

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Figure 36. Hydropathy Chart of IsaB (Kyte-Dolittle).

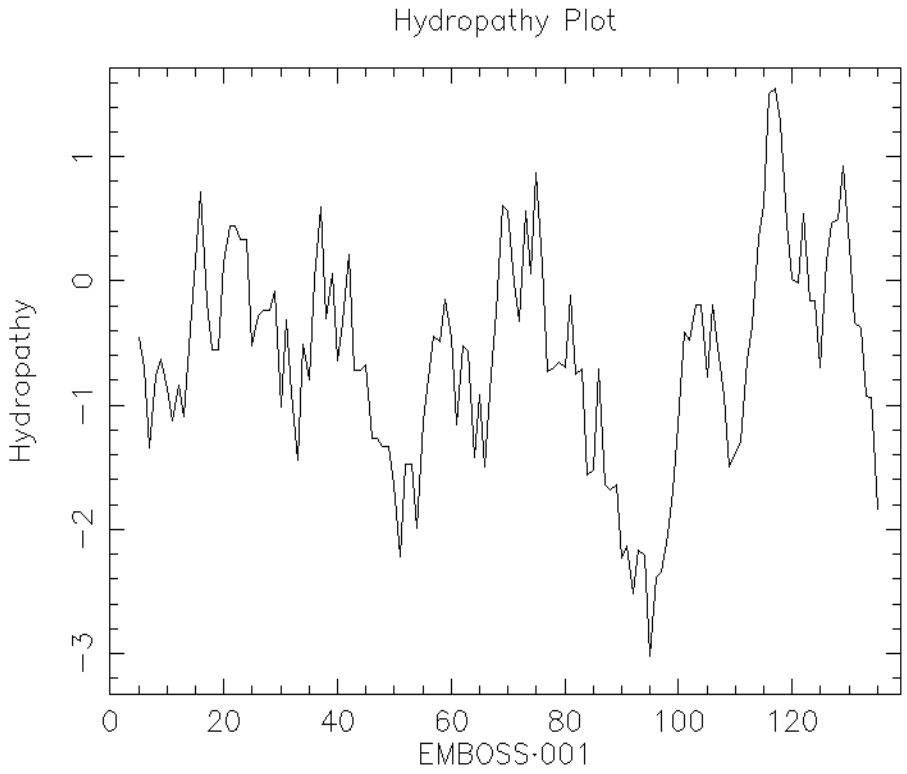

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Figure 37. IsaB Surface Association is Independent of Sortase A. To validate the prediction that IsaB is anchorless and to determine if IsaB could be binding to an anchored protein, a Sortase A deletion mutant was used. *S. aureus* strains RN450 and RN450 Δ *srtA* were grown overnight in TSBG. Cell samples were collected and screened with anti IsaB antibody. No difference in the amount of IsaB was detected between the two strains.

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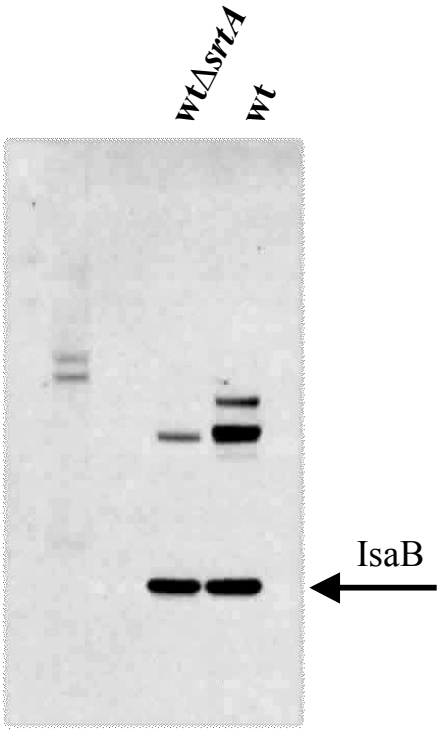
Figure 37. IsaB Surface Association is Independent of Sortase A.



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Lipoteichoic Acid Does Not Tether IsaB to the Cell Surface.

The histone-like protein of *Streptococcus* species was mentioned earlier because it is one of the few published extracellular DNA-binding proteins. Another similarity that HlpA shares with IsaB is that it also an anchorless cell surface associated protein. A study by Boleij et al, showed that HlpA associates with the cell surface via binding to lipoteichoic acid (LTA), which is found in many Gram-positvie bacteria, including *S. aureus* [115, 149]. Furthermore, teichoic acids have been shown to be significant defenses against AMPs and therefore IsaB binding to teichoic acids could have synergistic activity. Therefore we wanted to determine if IsaB was interacting with surface exposed teichoic acids.

We obtained a Δ*tagO* strain that is not able to secrete wall teichoic acids (WTA), however this mutant will still secrete LTA. To address the ability of IsaB to bind to WTA thus allowing it to associate with the cell surface, western blot analysis was used to compare IsaB cell surface association in the wild-type and Δ*tagO*. Overnight cultures of *S. aureus* strains 10833, 10833Δ*tagO* and 10833Δ*isaB* were grown in TSBG. Cells were collected by centrifugation and cell surface proteins were extracted with SDS. As the blot in **Figure 38** shows IsaB was detected in the wild-type and Δ*tagO* strains in similar amounts. These findings show that IsaB cell surface association was independent of WTA expression. Therefore, wall-teichoic acids do not contribute to IsaB binding to the cell surface.

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Figure 38. Teichoic Acid Does Not Tether IsaB to Surface. There is precedence for anchorless proteins to bind to LTA or WTA in order to associate with the cell surface. We obtained a WTA (*tagO*) deletion mutant and compared IsaB cell surface association with its parental strain. *S. aureus* strains 10833, 10833 Δ *tagO*, and 10833 Δ *isaB* were grown overnight in TSBG and cells were collected for western analysis using our anti-IsaB antibody. No difference in IsaB amount was observed between the wild-type and WTA deletion mutant.

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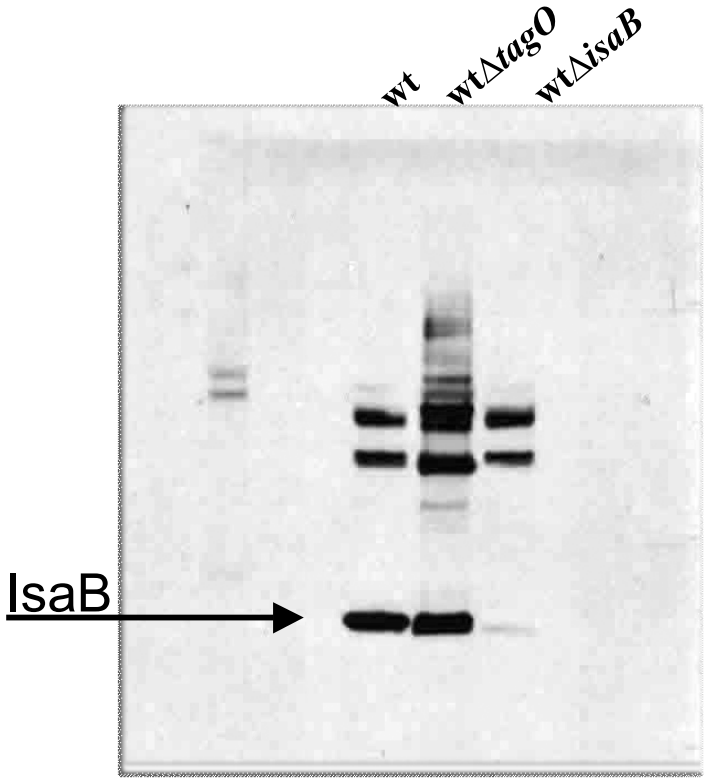
Figure 38. Teichoic Acid Does Not Tether IsaB to Surface.



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IsaB Cell Surface Association is Independent of Neutral Phosphatase.

There have been some reports in the literature describing anchorless protein association with the cell surface. One such report by Flock and Flock showed that Eap, an important MSCRAMM is a secreted anchorless protein which re-binds to the cell surface by binding to Neutral Phosphatase [150]. Interestingly, NPase is also an anchorless cell surface associated protein [150, 151]. Intrigued by this study, we looked at the dependence of IsaB surface association in wild-type, deletion mutant (Δ*nPase*), and complemented strain (Δ*nPase*+*nPase*). Using overnight cultures grown in TSBG, western blot analysis was performed as previously described. As **Figure 39** shows, levels of IsaB are constant in both the supernatant and cell surface extracts in samples irrespective of NPase status. Therefore, NPase does not aid in the ability of IsaB to re-associate with the cell surface.

X-ray crystallography

In order to get real answers about the structure of IsaB, we initially collaborated with Dr. Martin Safo and Dr. Faik Musayev at the VCU Center for Biologic Complexity to perform crystallography analysis. However, this approach proved to be technically challenging, and while some crystals were made, they were not of sufficient quality for resolution of the structure.

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Figure 39. IsaB Surface Association is Neutral Phosphatase Independent. To determine if IsaB like Eap could bind to NPase an nPase deletion mutant was used. *S. aureus* strains 10833, 10833 Δ *isaB*, 10833 Δ *nPase*, and 10833 Δ *nPase*+*nPase* were grown overnight in TSBG and supernatant and cell fractions were collected for western blot analysis. No difference in IsaB cell surface association was observed between the wild-type and Npase mutant.

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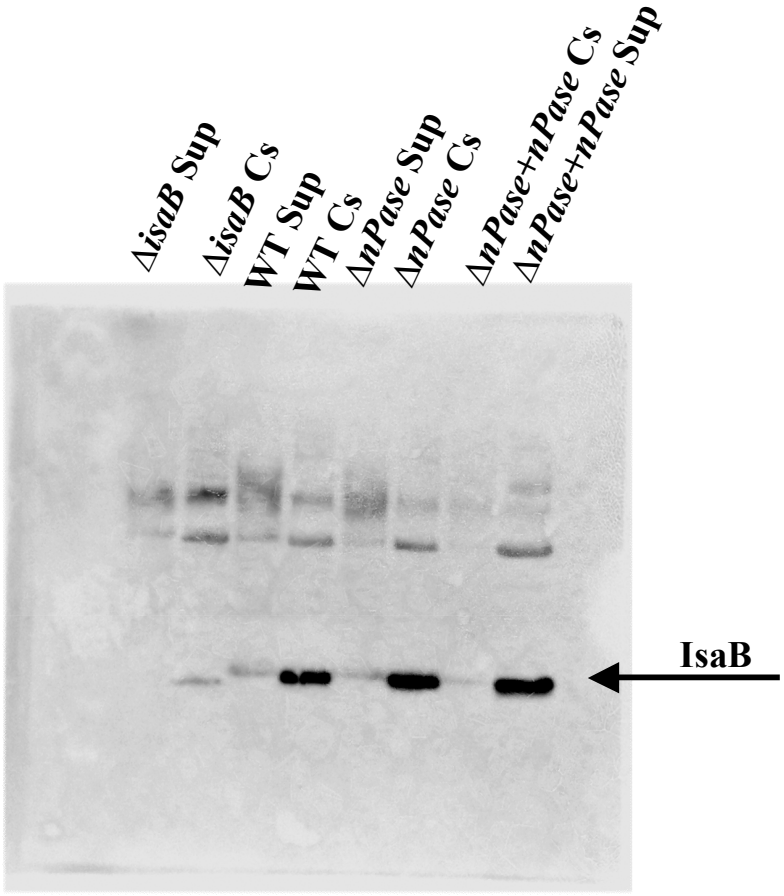
Figure 39. IsaB Surface Association is Neutral Phosphatase Independent.



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Modified Method for Expression and Purification of IsaB.

As discussed earlier, we had previous success with cloning and purifying recombinant pre-IsaB using a C-terminal CBD tag. However we required increased yield, purity, and production of mature IsaB lacking the signal sequence (m-IsaB). We cloned m-*isaB* into a modified pET vector (pET32XT) that contained an N-terminal thioredoxin tag immediately followed with a his-tag and thrombin cleavage site. A detailed schematic of the expression and purification steps is outlined in **Figure 40**. (m-IsaB for these studies was expressed in LB; m-IsaB for NMR was grown in M9 minimal media with N and C isotopes). As shown on the SDS Page in **Figure 40**, highly purified IsaB was obtained.

m-IsaB Binds DNA.

In order to confirm that removal of the signal peptide did not interfere with the nucleic acid-binding activity, we repeated the EMSA assay with m-IsaB. The DNA probe was labeled with biotin, and detected with strepavidin-HRP. As shown in **Figure 41**, m-IsaB is still capable of binding and shifting DNA in an m-IsaB concentration dependent manner.

We also wanted to determine whether or not IsaB might have nuclease activity or modify the DNA probe in any way. Reactions were setup as for a normal EMSA, however following 30 minutes incubation at room-temperature the reactions were electrophoresed under denaturing conditions on a TBE-Urea gel. Because m-IsaB is known to be expressed in acidic conditions and could be active at lower pH, one set of reactions were performed in binding-buffer at pH 5.8. The results of this experiment are shown in **Figure 42**.

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Figure 40. Overview of Expression and Purification. Schematic illustrating the methods used to purify recombinant m-IsaB. For activity and binding assays, m-IsaB was expressed in LB while protein purified for NMR purposes was grown in M9 supplemented with the indicated isotopes for incorporation into the protein.

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Figure 40. Overview of Expression and Purification.

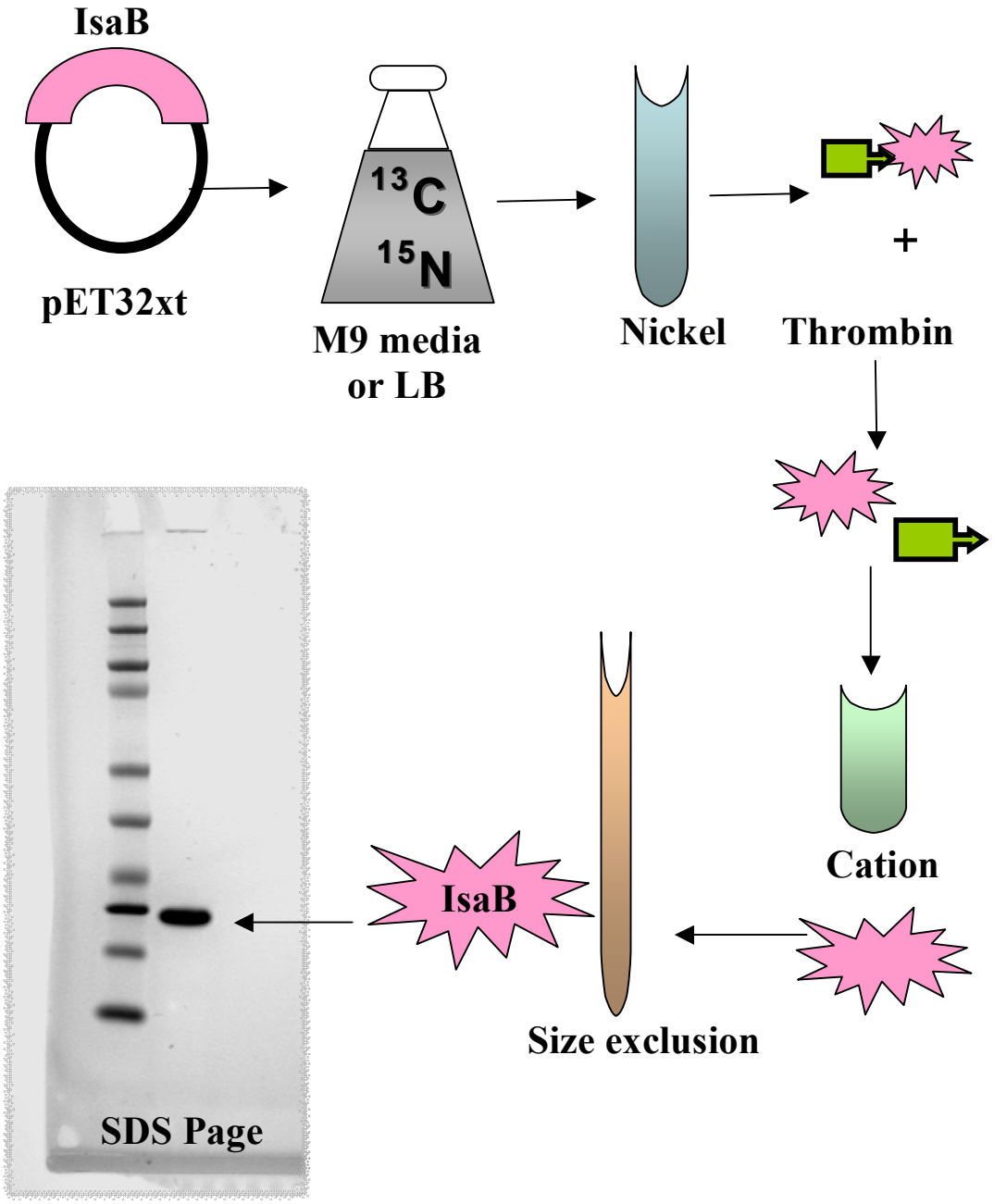

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Figure 41. m-IsaB Still Binds DNA. To ensure that purified recombinant m-IsaB still retained its Nucleic acid binding activity EMSAs were performed. All reactions contained 1fmol biotinylated DNA probe. Lane 1, no IsaB control, Lane 2, 1.6 nmol IsaB, Lane 3, 160 pmol IsaB, Lane 4, 16 pmol IsaB, Lane 5, 160 fmol IsaB. m-IsaB was able to shift the ssDNA probe in a concentration dependent manner.

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Figure 41. m-IsaB Still Binds DNA.

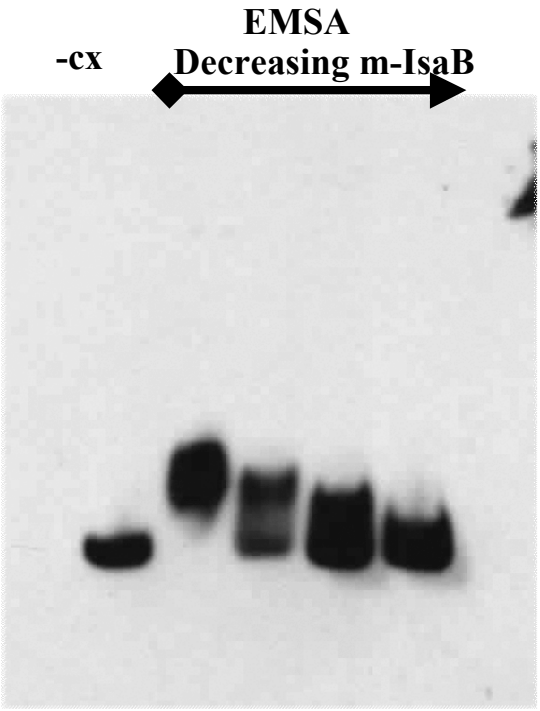

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Figure 42. IsaB Does Not Modify DNA. To observe if m-IsaB has any activity on bound DNA EMSA reactions were set up with normal or decreased pH and ran under denaturing conditions to break up the probe/m-IsaB complex. EMSA reactions were used at "normal" 7.6pH or were buffered with pH 5.8 phosphate buffer. Each pH had a complete set of reactions. Lane 1, no m-IsaB control, Lane 2, 1.6 nmol m-IsaB, Lane 3, 160 pmol IsaB. Reactions were denatured with heat and electrophoresed through a denaturing TBE-Urea gel. No modification of the probe nor decrease in signal intensity was observed.

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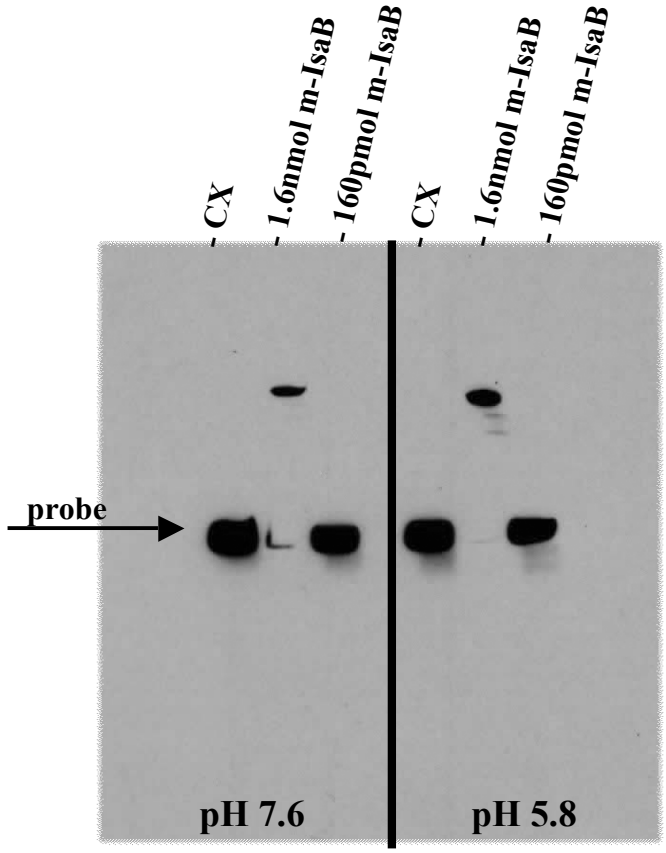
Figure 42. IsaB Does Not Modify DNA.



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From this blot it does not appear that m-IsaB is degrading or modifying the ssDNA in either of the reactions at pH 7.6 or 5.8. The signals at the top of the blot in the lanes with the greatest amount of m-IsaB are presumably aggregates of protein/DNA that were unable to migrate through the gel due to the high protein concentration.

LTA Does Not Compete with m-IsaB Binding DNA.

As mentioned previously, teichoic acids come in two varieties, LTA and WTA, both of which are very negatively charged. As discussed above, we found that WTA is not responsible for IsaBs binding to the cell surface. However, it had previously been observed that IsaBs nucleic acid binding could be successfully out competed with *S. aureus* cell membrane extracts (personal communication Dr. Kimberly Jefferson). To determine if the cell extract competition was due to LTA, competitive EMSAs were performed using LTA in great excess over the labeled probe. As shown in **Figure 43**, LTA was unable to successfully out compete binding of IsaB to the labeled probe. Again suggesting that IsaB is not significantly interacting with teichoic acids.

Drum-roll Please….and the Structure is…

In order to use NMR for structural determination of m-IsaB we needed to purify a large amount of labeled m-IsaB in solution that would not aggregate, and contained only a single species. For expression, cultures were serially grown in minimal M9 media in which the only sources of nitrogen and carbon were the $^{15}NH_4Cl$ and ^{13}C-glucose isotopes.

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Figure 43. LTA Does Not Compete With DNA for Binding. To visualize if lipoteichoic acid could ablate the DNA shift a competitive EMSA was performed where unlabeled teichoic acid at varying concentrations was added to the reactions. Each reaction contained 1 fmol probe, 160 pmol m-IsaB. Lane 1, no m-IsaB control, Lane 2, EMSA control, Lane 3, EMSA + 1 ug LTA, Lane 4, EMSA + 100 ng LTA, and Lane 5, EMSA + 10 ng LTA. No significant competition of IsaB binding was observed at any concentrations of LTA.

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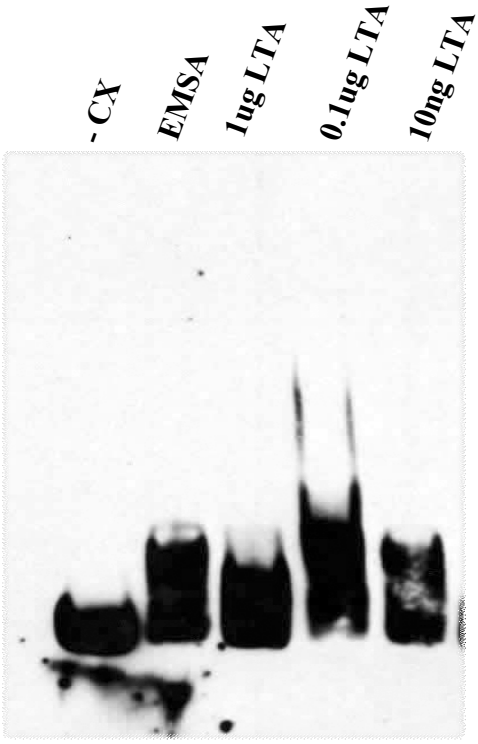
Figure 43. LTA Does Not Compete With DNA for Binding.



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Growth in this media was necessary to ensure that these atoms were used in the building of amino acids and subsequently in protein production. Using the purification process shown in **Figure 40**, we were able to successfully purify m-IsaB which appeared to be single species, to a concentration of ~1M without any aggregation being observed, which evidently is much more then needed for NMR. At this point the protein sample was turned over to Dr. David Williams for determination of structure. **Figure 44** illustrates the progress that has been made in determining the structure of IsaB. **Figure 44a/b** includes TALOS software predictions based on the assigned residues from the ^{15}N-HSQC plot that is shown in **Figure 44c [152].** From these data it appears that IsaB contains multiple β-sheets and at least two extended α-helices (**Figure 44b**). Furthermore the predictions suggest that IsaB is highly structured with only a small region predicted to be un-ordered (**Figure 44a**). However, these studies are currently in progress and determination of the structure of IsaB will by completed by the end of April 2010.

Discussion:

The studies presented in this chapter were aimed at characterizing the structure of mature IsaB. To begin we were intrigued by the lack of a predicted LPXTG motif and the ability of IsaB to associate with the bacterial cell surface. Using a Sortase A deficient mutant, we found that IsaB is a true anchorless protein, as its association with the cell surface is not dependent upon the Sortase A activity of covalently linking proteins to the cell wall. While these results are interesting, they are not entirely novel as other proteins in

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Figure 44. Nuclear Magnetic Resonance. Currently determinantion of the structure of IsaB is currently underway. However a large amount of data has been collected thus far allowing for highly accurate predictions to be formed with the TALOs software (95% accuracy). a) Readout from TALOs demonstrating the predicted ordered regions of protein structure. Only one area from ~95 to 100 aa appear to be unordered as shown by the dip in the chart. b) Secondary structure prediction from TALOs, lines above the axis indicate β-sheets while lines below are α-helices. IsaB appears to have two extended α-helices and several regions of β-sheets. c) ^{15}N-HSQC, shows the resonance of all labeled N throughout the protein in 2D.

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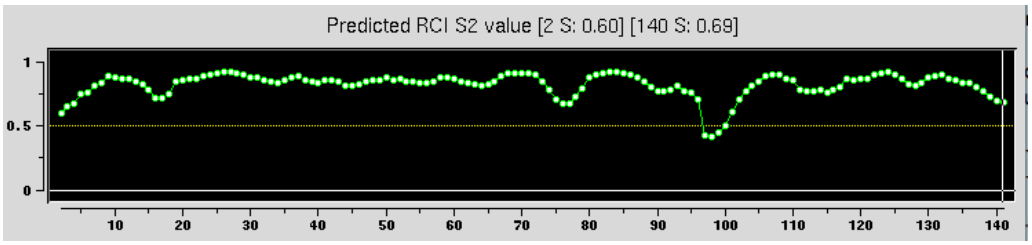
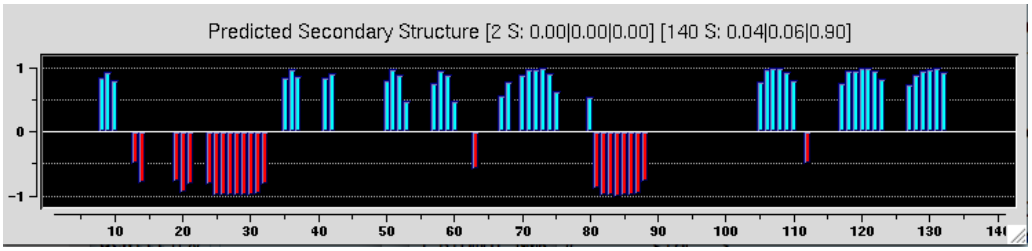
Figure 44. Nuclear Magnetic Resonance.

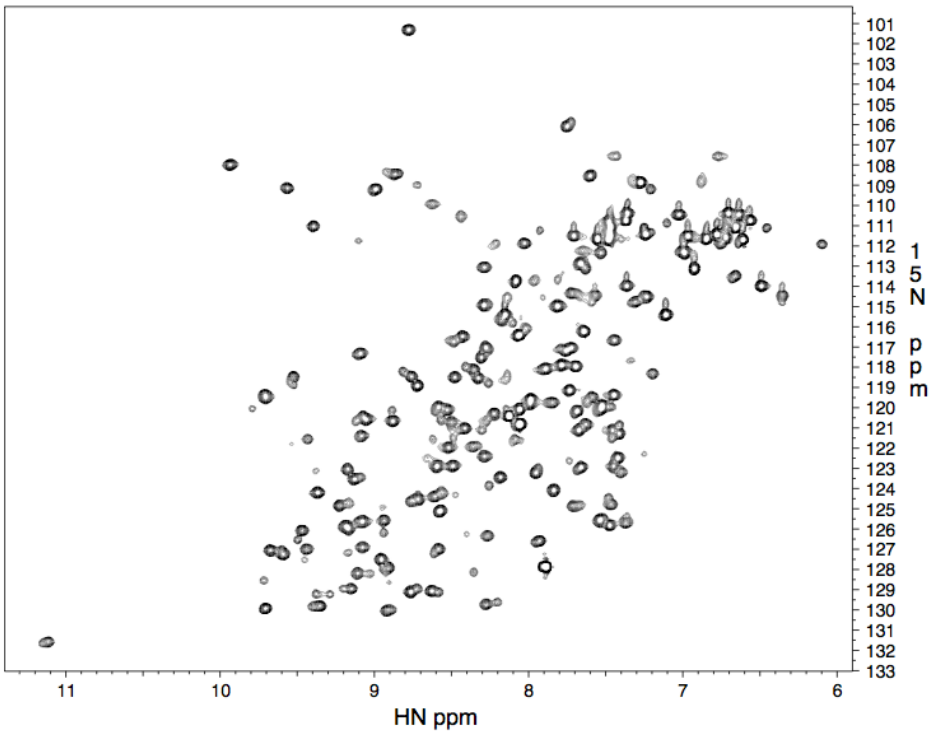
A.



B.



C.



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S. aureus and other Gram-positives exhibit similar non-covalent cell wall binding characteristics. Motivated by reports of how two anchorless proteins associate with the cell surface, we determined that neither WTA nor NPase were responsible for the rebinding of IsaB. The western blot on the WTA deficient strain could not rule out the potential that the cytoplasmic membrane tethered LTA was responsible for IsaB cell surface association and this scenario cannot be completely ruled out without testing a LTA deficient strain.

Another interesting observation made with the Sortase A deficient mutant was that no visible decrease was seen in the strength of the IsaB signal. This finding is significant because without Sortase A, cell-wall anchored proteins are released into the extracellular milieu and are not associated with the cell wall. This indicates that IsaB is interacting with a protein(s) or other substance that is not covalently linked by an LPXTG motif, which drastically decreases the number of binding suspects. It is also entirely possible that IsaB is interacting with the surface simply through charge attractions as the overall cell surface of *S. aureus* is negatively charged and IsaB is highly cationic. While these studies were interesting, they reveal little about the activity or structure of IsaB, therefore we focused on obtaining structural analysis of IsaB.

We first attempted to obtain crystal structure of IsaB through a collaboration with Drs. Martin Safo and Faik Musayev but this was not successful. One of the technical problems was related to degradation products that prevented crystal formation. As discussed in Chapter 3, IsaB was predicted to have an N-terminal signal peptide sequence

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for secretion, and in support of this, we found IsaB to be secreted and surface-associated. Importantly, the recombinant IsaB we were using for crystallography still contained the majority of the signal peptide sequence, which led us to hypothesize that the degradation product interfering with crystal formation had lost its signal peptide sequence. We needed to remove the signal peptide sequence to obtain the structure of the protein, and also to determine if removal of signal peptide sequence was required for activation of and potential enzymatic function of IsaB.

Protein activities are typically determined using recombinant purified protein of interest so that the function, which may not have a large phenotype under most conditions, can be elucidated. Using a number of purification strategies and a large amount of trial and error we finally found a system that expressed large amounts of m-IsaB. Through our numerous trials we believe that a large N-terminal tag was required for successful expression of IsaB, which we hypothesize was able to block m-IsaBs toxic activity inside of *E. coli*. Upon successful purification of m-IsaB we found that it, like pre-IsaB binds to ssDNA in a concentration dependent manner. Using denaturing Urea gels, we found that also like pre-IsaB, m-IsaB had no apparent role in modifying the DNA in the reactions.

While these experiments are positive reinforcement of what we previously found with pre-IsaB, the main goal of expressing and purifying recombinant m-IsaB was to use it for NMR to determine its protein structure. While we have shown that IsaB protects *S. aureus* against AMPs and can bind to eDNA, it was still unknown what its structure was. Currently Dr. David Williams is employing his NMR expertise to determine the structure of IsaB, and it is estimated that these studies will be successfully completed by the end of

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April 2010. Once the structure is obtained, it will be used to search for homology to other solved protein structures, which will help us to assign IsaB to a functional class.

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CHAPTER 8

Conclusions

When our studies first began, the only known property of IsaB was its immunogenicity. It was postulated to be involved in virulence but as this protein bears no homology to other proteins of known function and had never been studied, this claim was purely speculative. Our lab first became interested in IsaB following a screen performed in search of RNA binding proteins able to post-transcriptionally regulate the intercellular adhesin (*ica*) locus, a locus that is responsible for production of PNAG. While multiple experiments determined that IsaB does not regulate *ica* expression or PNAG production, we were still intrigued by this uncharacterized protein. As discussed earlier, IsaB does not have any significant similarity to proteins of known function and therefore we could gain no insight into its function.

Adding to the level of mystery about IsaB is the fact that it is found in all *S. aureus* strains sequenced, and has homologs in all *Staphylococcal* species currently annotated. However, IsaB homologs have not been found in other annotated genomes outside this genus. These results taken together suggest an important role for IsaB due to its widespread occurrence throughout a genus with great genetic diversity. One could argue that this prevalence among staphylococci makes IsaB an unlikely virulence factor, as widespread genes are usually involved in housekeeping. While this is a strong argument, it is prudent to note that staphylococci are typically found associated with higher hosts,

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which could be indicative of a role in virulence as all *Staphylococcal* species are challenged by host immune defenses. Therefore it is highly possible that IsaB, which is known to be immunogenic during human septicemia caused by *S. aureus*, could be not only an important virulence factor in *S. aureus* but also in other staphylococcal species which cause disease in a wide range of vertebrate hosts. Therefore characterization of this protein could lead to therapeutic interventions in both human and veterinary settings alike.

Regulation of IsaB

As nothing was known about IsaB, we first began our characterization of the *isaB* gene by characterizing its regulatory network, in the hope that this would shed light on possible roles in virulence and function. Perhaps the most potent regulator of *isaB* expression we observed was acidic pH, however it took numerous different experiments to determine this. In the beginning we found that *isaB* expression was dramatically induced by easily metabolized sugars such as glucose, and therefore hypothesized that *isaB* was one of many genes being regulated by glucose. These studies prompted us to examine the role that CcpA had on *isaB* expression, and as we hoped we found that CcpA was a positive regulator of *isaB*. While this made for a nice story, there was a red flag. Maximum *isaB* transcript levels were observed in post-exponential phase, when all easily metabolized glucose should be gone. We therefore needed to look into other possible factors that would be occurring in a time-dependent manner starting after glycolysis had initiated.

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As aerobic bacteria in culture grow, CO_2 and acetic acid accumulate in the media, which leads to a decrease in the pH from ~7.5 to ~4.5 over only a few hours. We speculated that acidic conditions were actually behind the induction of *isaB*. Because *S. aureus* grown in acidic media exhibit a significant delay in growth, to address this question we used HEPES buffer to significantly delay acidification of the media. In support of our hypothesis, we found that once the media was buffered and the drop in pH delayed, much of the induction by glucose was ablated. Another interesting finding was that the pattern of *isaB* induction of cultures grown in the buffered media was strikingly similar to what was observed for the CcpA mutant. By repeating the CcpA time-course and monitoring the change in pH we found that the CcpA mutation was associated with a significant delay in the acidification of the media. Therefore, we were able to conclude that pH, not glucose or CcpA was responsible for the majority of the *isaB* induction.

As IsaB has been found to be immunogenic during MRSA septicemia we wanted to determine the effect that culture in whole serum or plasma had on *isaB* expression levels. As we hypothesized, *isaB* expression was increased approximately 5-fold following two hours of culture in either serum or plasma. While there are many components in either fluid which could be stimulating *isaB* expression (antibodies, complement, hormones, etc) it is unlikely that clotting factors such as platelets, which are present in plasma and not serum, are responsible for the increase. These data support our overall hypothesis that IsaB is expressed during septicemia and is important in the pathogenesis of *S. aureus*.

Another significant piece of information obtained about *isaB* regulation was the finding that SarA negatively regulates *isaB* expression. SarA is a known regulator of

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secreted proteins and regulates genes in a temporal fashion, so this finding was not unexpected. SarA is also an important regulator of virulence determinants, so its role in *isaB* expression supported our hypothesis that IsaB is involved in virulence.

IsaB Binds Nucleic Acids

Prior to beginning this project, we had strong reason to believe that IsaB was able to bind to RNA, as it was the only protein which was previously isolated in an RNA affinity chromatography assay. Due to IsaB being fully secreted and not covalently anchored to the cell wall, the idea of an extracellular RNA binding protein was exciting and novel as the only other secreted protein known to bind nucleic acids in *S. aureus* is the Thermostable nuclease. Using recombinant purified full-length IsaB from a previously constructed *E. coli* strain, we first sought to visualize IsaB binding through EMSAs. Through a number of these experiments we determined that not only did IsaB bind RNA, it also bound to DNA. Furthermore, it appeared as though IsaB binding was occurring in a non-sequence specific manner because non-specific carrier DNA used in the gel shifts was able to out-compete the labeled probe for IsaB binding.

While the EMSAs allowed for direct observation of IsaB binding to nucleic acids, they did not tell us much about the affinity IsaB had for these ligands. In order to determine dissociation/association constants we used Surface Plasmon Resonance (SPR). Using biotinylated RNA, ss-DNA, or ds-DNA oligos as bait, and varying concentrations of IsaB we were able to determine that IsaB binds to the tested oligos in the low nM range. Interestingly, this binding affinity is very similar to the affinities of transcriptional

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regulators when they are bound to heterologous DNA. While unlikely, due to its extracellular nature, it is possible that IsaB may recognize a specific sequence that we have not yet identified. To address this in the future, assays such as selex could be used to determine if there is a sequence IsaB preferentially binds to. Furthermore, we found that endogenously expressed IsaB can aid in the accumulation of eDNA on the cell surface, supporting and lending relevance to our in vitro studies.

An extracellular nucleic acid binding protein could have many functions in *S. aureus*. IsaB binding nucleic acid could potentially block activation of TLR-9 which recognizes bacterial unmethylated CpG DNA leading to NfKb signaling and amplification of the immune response. Along these lines of thought, it would be interesting to determine affinity of IsaB for bacterial DNA or Human DNA, to see if IsaB could bind with higher affinity to unmethylated vs. methylated CpG DNA.

IsaB as a Virulence Factor

IsaB is immunogenic and expressed in vivo where it is actively targeted by the adaptive immune response. Why would *S. aureus* produce a protein in vivo that would serve as a target for host immune defenses? We hypothesized that IsaB was expressed in vivo, despite its immunogenicity, because it plays a critical role in the virulence of *S. aureus*. To test this we used a number of different assays to observe IsaBs role in virulence. We used assays ranging in complexity from single molecule killing assays (AMPs), neutrophil mediated killing (phagocytosis and NETs), adherence and intracellular survival, survival in whole blood, to the *C. elegans* nematode killing model.

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Through these assays we found that IsaB has a significant role in protecting *S. aureus* from Antimicrobial Peptides and Neutrophil Extracellular Traps. We hypothesize that IsaB protects against AMPs, which subsequently gives *S. aureus* increased resistance to NETs, whose killing mechanism relies heavily on AMPs. These findings suggest that IsaB is a novel virulence factor that increases *S. aureus* survival in vivo and in turn can lead to more severe disease. Furthermore, the ability of IsaB to protect against AMPs makes it relevant to all *S. aureus* diseases, as AMPs are part of the innate immune system secreted throughout the body. While at this time we do not know conclusively how IsaB protects against AMPs, it is highly likely that it is through repulsive forces. *S. aureus* goes through great lengths to modify its cell surface to change its overall charge from negative to neutral to prevent the cationic AMPs binding. Therefore we hypothesize that IsaB helps modify the surface charge, preventing AMPs from binding to the cell wall. These findings warrant further study to elucidate this mechanism.

As discussed above, AMPs are highly conserved throughout all animals, from nematodes to humans, and IsaB is conserved throughout the staphylococci. These two pieces taken together suggest that IsaB evolved early in the *Staphylococcal* genus and could have significant roles in AMP evasion in all staphylococci that are associated with higher organisms. Therefore this is an exciting finding as IsaB has implications in human disease but could also be relevant to veterinary diseases caused by staphylococci, making IsaB an exceptionally attractive therapeutic target.

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Biofilms

Recent studies by Dr. Bayles laboratory (University of Nebraska), have demonstrated that *S. aureus* biofilms can have an abundance of eDNA within their matrix. While this phenomenon has been established in many other bacterial biofilms, it was only recently confirmed for *S. aureus* biofilms, which were previously thought to primarily consist of PNAG. Given the novelty of eDNA in biofilms, and our possession/knowledge of a novel extracellular nucleic acid binding protein we were excited to determine how IsaB affected biofilm formation. We hypothesized that deletion of IsaB would decrease the overall biofilm formed due to a significant decrease in the amount of eDNA that was incorporated into the biofilm matrix.

Using the well-established 96 well biofilm microtiter plate assay we screened wild-type and *isaB* deletion mutants in a variety of growth media with different supplements. Under most conditions we found no difference in the amount of biofilm formed in the presence or absence of IsaB. However, in the conditions where a significant change was observed between the deletion mutant and wild-type, it was the deletion mutant which had slightly greater levels of biofilm formation. Therefore, under certain inducing conditions, IsaB seemed to negatively affect biofilm formation, although this effect was minor. However this negative role does not seem to be due the amount of eDNA in the biofilm as DNAse susceptibility assays suggest that similar amounts of eDNA were in the biofilms of two strains. We also hypothesized that we were perhaps not stimulating IsaB expression appropriately, and since *isaB* is expressed in serum, we performed the biofilm assays in the

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presence of serum, and again found no significant difference between the wild-type and deletion.

These findings suggest that IsaB plays little, if any role in biofilm formation. The increase observed in the mutant was only 12%, and while it was statistically significant, we are unable to comment on whether this is a physiologically relevant increase. One way to explain the observed increase in biofilm formation is that perhaps IsaB binding to eDNA blocks it from being properly incorporated into the biofilm and is acting as a mechanism to fine tune biofilm formation. However it is still be possible that IsaB plays a role in augmenting biofilm formation under conditions not addressed, or in *S. aureus* strains not used in these studies.

Structure of IsaB

As indicated by results in Chapter 3, IsaB was only found in extracellular fractions of *S. aureus* cultures wherein a minor fraction was present in the spent medium and the majority was associated with the cell surface. This was intriguing because IsaB is an "anchorless" protein, having no predicted cell-wall anchoring motif. Furthermore, using a Sortase A deletion mutant we confirmed that IsaB cell surface association is independent of Sortase A, and thus is not covalently linked to the cell-wall. While this is an interesting finding, it is not an entirely rare occurrence as other *S. aureus* proteins that are fully secreted such as Eap and NPase are able to "re-bind" the cell surface. This re-binding can occur through binding to other cell-surface associated proteins, and also by binding to LTA or WTA. Using deletion mutants of both NPase and WTA, we concluded that IsaBs cell-

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surface association is independent of these two factors. Therefore it is still unknown how IsaB is interacting with the cell surface.

Our initial studies used recombinant IsaB purified from *E. coli* containing an expression vector with the entire *isaB* open reading frame. IsaB is an extracellular protein, and upon examination of its amino acid sequence with SignalP 3.0 we were able to predict where its signal peptide would be cleaved once secreted. An important paradigm found for many secreted proteins is that the protein must be processed after secretion to become active. This mechanism is likely used to protect the intracellular components of an active enzyme that could potentially harm the bacteria producing it. Because of this phenomenon we predicted that we had not detected enzymatic activity of our previously produced recombinant protein because this construct included the Gram-positive signal peptide sequence, which would not be appropriately processed in the Gram-negative *E. coli*. Furthermore, in order to determine the structure of mature IsaB, the signal peptide sequence would need to be removed, and therefore we began experiments to express and purify large amounts of m-IsaB.

After a myriad of different techniques and cloning strategies we were finally able to express the mature, secreted form of IsaB (m-IsaB) in *E. coli*. In our successful approach a large N-terminal tag encoding thioredoxin, 6-his, and a thrombin cleavage site, was fused immediately prior to the start of the 5' end of m-IsaB, therefore purified protein would not have a signal peptide sequence on it. Once large amounts of highly pure m-IsaB were obtained we repeated a number of our EMSAs. These EMSAs showed that m-IsaB is still

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capable of binding DNA in an IsaB concentration dependent manner, however no enzymatic activity on DNA was observed.

Our main motivation behind producing m-IsaB was to use it for structural analysis through NMR, which was performed by our collaborators with significant experience. These studies are currently in progress and should be concluded shortly. By obtaining the structure of IsaB we hope to find clues as to how IsaB is interacting with either eDNA or AMPs. Furthermore, we can then search for structural homologs to determine if IsaB could in fact have another function in *S. aureus*.

In summary, these studies have determined that IsaB is an extracellular DNA binding protein that significantly protects the bacteria from AMPs and NETs. These findings demonstrate that IsaB is an important virulence factor in *S. aureus* and possibly in other staphylococci. Furthermore, our experiments have shown that *isaB* expression is regulated by a complex network including the global regulator SarA and a number of environmental stimuli including serum/plasma components and pH. These studies illustrate that IsaB is a clinically relevant virulence factor, with potential therapeutic implications. It will be exciting to observe how the story of the Immunodominant Surface Antigen B progresses and develops from these initial studies.

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Presentations

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