HUMAN HEMOGLOBIN AS AN IRON SOURCE OF *STAPHYLOCOCCUS AUREUS*

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CFU	colony forming units
DIP	2,2-dipyridyl
EDDHA	ethylenediamine-di(o-hydroxyphenylacetic acid
FACS assay	fluorescently-activated cell sorting assay
Fur	ferric uptake regulator
Hb	hemoglobin
hHb	human hemoglobin
IP	immunoprecipitation
Isd	iron-regulated surface determinant
Mb	myoglobin
mHb	mouse hemoglobin
MRSA	methicillin-resistant *Staphylococcus aureus*
PBS	phosphate-buffered saline
PMSF	phenylmethylsulphonyl fluoride
rhHb	recombinant human hemoglobin
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TSB	tryptic soy broth

CHAPTER I

INTRODUCTION

"I think I'll take a bath in his blood"
Mike Tyson

A previously healthy 23 year old man arrived at an emergency room at Vanderbilt University Medical Center with a complaint of decreased movement and weakness in his arms that lasted for nearly two weeks. He did not have a fever or upper respiratory tract infection and his skin looked normal. The symptoms included anxiety, low blood pressure and decreased reflexes. Magnetic resonance imaging revealed a retropharyngeal/epidural abscess (Figure 1A). The patient recovered after six weeks of treatment with parenteral antibiotics. About the same time a previously healthy 16 year old boy developed vomiting and fever in the middle of the night. Upon arrival to the hospital he collapsed in the parking lot. Due to deteriorating respiratory status, mechanical ventilation and eventually extracorporeal life support were initiated. The patient died from severe pneumonia on day 6 after arriving in the hospital (Figure 1B). Both of these cases were caused by the same infectious agent that until recently was associated exclusively with the hospital setting. The superbug of the hospital rooms is now on the loose.

Figure 1. Common diseases caused by *S. aureus*[1]. A. Epidural abscess. **B.** Pneumonia. **C.** Purpura fulminans. **D.** Endocarditis. **E.** Osteomyelitis.

[1] Images kindly provided by Dr. C. Buddy Creech

Staphylococcus aureus is a non-motile catalase positive Gram-positive coccus, which occurs in grape-like clusters due to alternating division planes and incomplete cell wall separation between daughter cells. *S. aureus* is a commensal organism colonizing the anterior nares of approximately thirty percent of the human population (67). Upon breakage of host protective barriers, *S. aureus* is capable of establishing infection by avoiding the immune system, adhering to cellular surfaces, and acquiring nutrients from host tissues. *S. aureus* stands out due to its ability to produce an arsenal of virulence factors that enable it to invade and multiply within virtually any host tissue. Invasive infections caused by *S. aureus* include bacteremia, pneumonia, cellulitis, osteomyelitis, endocarditis and septic shock (Figure 1) (65, 66). The diverse ecological niches encountered by *S. aureus* necessitate a variety of systems devoted to the acquisition of nutrients during infection. Consistent with this, *S. aureus* succeeds in its ability to acquire iron within the host (9, 107, 120).

S. aureus is an important human pathogen responsible for almost 300,000 hospitalizations and 19,000 deaths each year in the USA alone. *S. aureus* infections are an increasing problem in developed countries, as the incidence of drug-resistant strains is steadily rising. In fact, invasive methicillin-resistant *S. aureus* (MRSA) infections are responsible for more deaths annually in the United States than AIDS (16, 65). Of invasive infections caused by methicillin resistant *S. aureus* (MRSA), 75.2 % result in bacteremia, 6.3% result in endocarditis, and 13.3% result in pneumonia (64-66). The dynamics and characteristics of staphylococcal infections are even more alarming than the incidence data. For instance, there has been (i) no decrease in hospital associated infections despite continuous efforts to improve measures to prevent the spread of nosocomial diseases; (ii)

a dramatic increase in infection occurrence and pathogenicity of community associated strains of *S. aureus* (84); and (iii) unremitting emergence of antibiotic resistance of staphylococci (64, 84). While 60% of current *S. aureus* isolates are antibiotic resistant, only four new classes of antibiotics were introduced in the past forty years (27). These points demonstrate the need for the identification of novel targets for therapeutic intervention against staphylococcal infection. Dissecting one such mechanism, hemoglobin-derived iron acquisition, is the goal of this thesis.

S. aureus typically shifts from a commensal colonizer to invading pathogen upon breakage of the skin or mucosal barrier whereupon *S. aureus* employs virulence factors that allow it to survive within the host and cause considerable damage (75). The majority of these virulence factors are either secreted from the bacterial cell or anchored to the cell wall through the action of transpeptidases known as sortases (75, 81). The functions of cell wall anchored proteins include adherence, immune evasion, nutrient acquisition and resistance to antimicrobials: all processes important for survival of *S. aureus* in the context of infection (23, 24, 39, 41, 46, 47, 80, 83, 85, 89, 102, 103, 118). The contribution of cell wall anchored proteins to the pathogenicity of *S. aureus* is evident through a decrease in the virulence of sortase mutants in animal models of infection (60, 61, 81, 83, 129). Among the functions carried out by sortase-anchored proteins is the acquisition of iron (80), which is a vital nutrient that is concealed from invading bacteria by host iron sequestering proteins (13, 29). To understand the mechanisms *S. aureus* employs to acquire iron during infection one must understand the basics of vertebrate iron regulation.

Host iron metabolism and infection

Iron is thought to be the most critical elemental nutrient in determining the outcome of host-pathogen interactions (96). This is because the lack of available iron within vertebrates provides a nutritional barrier to colonization by microbial invaders. Due to the requirement for iron in numerous physiological processes its acquisition is an important task for all living organisms. Mammals have adapted to exploit the necessity of iron for microbial replication by sequestering it away from microbes that have breached the skin or mucosal layers (32). Pathogens in turn have evolved mechanisms to remove iron from the host and internalize it for their own uses (29).

There are two primary reasons for the scarcity of free iron within vertebrate tissue. First, iron is insoluble at physiologic pH. Second, vertebrates take immense measures to carefully regulate iron homeostasis by sequestering it via host molecules with very high binding affinities. These measures both protect the host from iron toxicity and ensure that the concentration of free iron within tissues is orders of magnitude below that required to support bacterial growth and virulence. The active sequestration of nutrient metals in order to defend against microbial growth is a process termed nutritional immunity. It should be noted that the host is forced to maintain a certain level of bioavailable iron for cellular metabolic needs and thus employs intricate mechanisms to maintain iron balance. This is demonstrated by the observation that depriving cells of intracellular iron leads to rapid cell death (37). Conversely, if dietary or genetic factors shift the balance to a condition of iron overload, mammals are less able to resist infections (12). Among the most well documented pieces of evidence supporting the role of iron in infections is that tuberculosis patients supplemented with iron are much more

susceptible to the pathogenesis of *Mycobacterium tuberculosis*, and individuals with iron overload diseases such as hemochromatosis routinely suffer reinfections (14, 95). Furthermore, animal models have revealed that *S. aureus* and other pathogens inactivated for iron acquisition systems display significantly reduced virulence (1, 17, 29, 98, 113, 118). Taken together, these findings underscore the importance of nutritional immunity in protecting against bacterial infections.

In order to appreciate the sources of iron that are available to invading pathogens during infection, it is important to first understand the physiology of iron in a healthy vertebrate host. Under normal conditions 1-2 milligrams of iron enters the body of an adult human through absorption in the duodenum and small intestine. This process maintains the total amount of iron within a healthy adult at approximately 3-4 grams (44). Upon absorption, iron is bound by transferrin, which is secreted into the plasma and trafficked to tissues so that iron can be delivered throughout the body. In a healthy individual virtually all extracellular iron is bound by transferrin, accounting for about 0.1% of total iron within the organism (37). Due to the high affinity of transferrin for iron and the fact that only about 30% of transferrin is iron saturated at any given time, the extracellular concentration of free iron within vertebrates is extremely low (106). Upon cellular contact, transferrin-iron is taken up by cells via interaction with a transferrin receptor through clathrin-mediated endocytosis. Once endocytosed, iron is released from transferrin in a pH-dependent process mediated by the influx of protons into the endosomes. The vast majority of absorbed iron (~80%) is taken up by erythroid precursors in the bone marrow and incorporated into heme, which is bound by hemoglobin, the primary oxygen transport protein of vertebrates. In addition, a significant

amount of absorbed iron (~10%) is incorporated within heme into the oxygen storage protein myoglobin which is found within muscle cells known as myocytes (37). Excess intracellular iron is stored within the protein ferritin to protect against the reactivity of free iron. Ferritin can be found in the cytoplasm, nucleus, and mitochondria and its multimeric form can contain up to 4,500 atoms of iron (77). Finally, a small fraction of iron is bound by the antimicrobial protein lactoferrin found in secretions such as breast milk and tears. The remaining iron is used by different cell types and iron-binding proteins with roles in a variety of physiological processes. During infection, the primary sources of iron available to invading bacterial pathogens include transferrin-iron, heme-iron bound to hemoglobin or myoglobin, and iron complexed to ferritin.

Iron regulation in *Staphylococcus aureus*

Iron is an essential nutrient required by virtually all organisms. In the case of *S. aureus*, this requirement is demonstrated by failure to proliferate in an iron-free environment due to an inability to carry out such vital processes as respiration, nucleotide metabolism, and coping with oxidative stress (13, 29). The success of *S. aureus* as an infectious agent relies on a diverse panel of virulence factors. It has become increasingly evident that the iron status of *S. aureus* and the production and function of many of these virulence factors are interconnected. These emerging findings suggest a paradigm whereby *S. aureus* senses iron availability and modifies its physiology to effectively colonize its host. One method by which *S. aureus* adapts to iron-starved environments is through iron-dependent alterations in gene expression mediated by the ferric uptake regulator (Fur). Fur is a ubiquitous transcription factor in bacteria that regulates gene expression in response to alterations in cellular iron status. Iron-bound Fur binds consensus DNA sequences located upstream of the transcriptional start sites of iron-regulated genes and represses transcription. Repression is alleviated when iron is scarce as Fur releases from the DNA, allowing access to RNA polymerase (6). Over forty cytoplasmic proteins are negatively regulated by Fur in *S. aureus*, highlighting the complexity of the bacterial response to iron-deprivation (42). In addition to iron acquisition, many Fur-regulated proteins are involved in pathogenic functions such as biofilm formation, adhesion, evasion of the immune system and inhibition of wound healing, suggesting that iron-dependent regulation of these proteins has a considerable impact on the host-pathogen interaction (4, 7, 18, 19, 55, 58, 59, 90). The importance of Fur-mediated iron-dependent gene regulation during pathogenesis is demonstrated by the

significant attenuation of a *fur* mutant in a murine skin abscess model of *S. aureus* infection (54).

S. aureus is capable of acquiring iron from several different sources during infection. As described in detail in the next section, *S. aureus* expresses specific transport systems dedicated to heme-iron acquisition from host hemoglobin. Ferritin is an intracellular iron storage protein that is unlikely to be used as an iron source by extracellular bacteria such as *S. aureus*. Iron acquisition from transferrin is mediated primarily by secreted siderophores in *S. aureus*. Rather than promoting growth by providing *S. aureus* with iron, lactoferrin exhibits a bacteriostatic effect on *S. aureus in vitro*. This growth inhibition is attributed to both its iron-chelating properties and serine protease activity (2, 22). Although it is not yet clear how lactoferrin functions *in vivo* during staphylococcal infection, it is not expected to be a viable iron source to *S. aureus*.

A number of reports support the relevance of iron availability to the clinical disease course of *S. aureus* infections. For example, in patients with tunneled dialysis catheters, dose of supplemented iron is associated with increased risk of developing infections, including *S. aureus* bacteremia (56). Further, the administration of iron sucrose results in non-transferrin-bound iron in the serum of hemodialysis patients and this increased iron concentration enhances the growth of *S. aureus* and the risk of developing sepsis (8, 52). Finally, *S. aureus* has become a leading cause of bacterial infections in individuals with thalassemia, a congenital defect that results in reduced hemoglobin synthesis and anemia. The most common treatment for these patients is injection of the iron chelator desferrioxamine. Unfortunately, treatment with desferrioxamine further increases the rate of infections in these patients, presumably due

to the ability of *S. aureus* to utilize desferrioxamine as an iron source (11, 97). In light of the severity of *S. aureus* infections and the unremitting antibiotic resistance exhibited by this organism, continuous efforts to describe iron acquisition strategies and their role in the pathogenesis of *S. aureus* may be crucial to the development of future therapeutic agents.

Heme-iron acquisition in *S. aureus*

Most iron within the mammalian host is contained within the tetrapyrrole heme. Heme typically is not found in its free form but is instead bound to proteins that use it as a cofactor. The most abundant mammalian heme containing protein is hemoglobin (Hb) located within erythrocytes (28, 32, 122). Hemoglobin is a sufficient source of iron *in vitro* for many bacterial pathogens including *S. aureus* (29, 118). In order to capture iron, staphylococci need to employ methods to overcome at least three lines of defense: i) release Hb from the erythrocyte; ii) remove heme from Hb and import it into the cell; and iii) release iron from heme inside the cytoplasm.

To overcome the first line of defense and to access hemoglobin *S. aureus* lyses erythrocytes through the secretion of hemolytic toxins. Accordingly many of these toxins are upregulated in iron poor conditions (119). Upon erythrocyte lysis, to import heme *S. aureus* binds hemoglobin on the surface of the bacterial cell wall. The iron-containing heme co-factor is then extracted from hemoglobin and passed through the cell envelope into the cytoplasm. To release iron in the cytoplasm heme is degraded.

S. aureus acquires iron from hemoglobin through the cooperative action of the iron-regulated surface determinant (Isd) system, which is conserved in many Gram

positive pathogens (Figure 2) (80, 87, 93, 98-100, 113, 114, 118, 131). The proteins of the Isd family were named in alphabetical order from A to I (80, 112). IsdA, IsdB, and IsdH are covalently anchored to the cell wall exposed to the surrounding of the bacterium (33, 80, 86, 118). IsdC is also a cell wall anchored protein but is completely embedded in the cell wall and hence not surfaced-exposed (80). IsdDEF comprise an ABC-type transporter expressed in the cytoplasmic membrane of the bacterium (80), and IsdG and IsdI are cytoplasmically localized (112).

The expression of Isd proteins is tightly regulated by the cytoplasmic ferrous iron concentration through a canonical Fur box in their promoter region (33, 80, 86, 118). In high iron concentrations the ferric uptake regulator (Fur) binds to the Fur box repressing the transcription of downstream genes (29, 49). During infection, it is believed that the low level of available iron derepresses the expression of genes under Fur control, including *isd*. Cell wall anchored proteins contain an N-terminal signal peptide that is necessary for their translocation across the cytoplasmic membrane. Proximal to the C terminus of IsdA and IsdB is a LPXTG anchoring motif. LPXTG motifs are recognized by a transpeptidase Sortase A for covalent anchoring to the pentaglycine cross bridge of newly synthesized peptidoglycan (81, 82, 108, 118). IsdC is anchored by Sortase B, which recognizes a NPQTN motif of IsdC (80, 83).

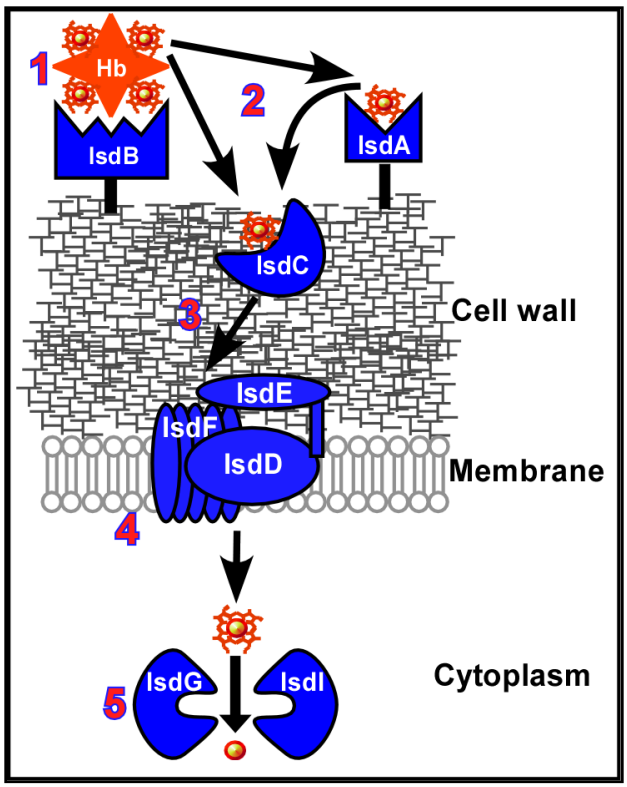

Figure 2. Heme-iron acquisition in *S. aureus*. In order to acquire iron from hemoglobin during infection, *S. aureus* utilizes a group of proteins collectively known as the Iron-regulated Surface Determinant (Isd) system. This process occurs in a step-wise fashion. (1) Cell wall anchored IsdB binds hemoglobin at the surface of the Gram-positive cell wall. (2) IsdB removes heme from the peptide portion of hemoglobin and passes it either directly to IsdC or to IsdA, which passes heme to IsdC. (3) IsdC then passes heme to the IsdDEF membrane transport system, (4) which pumps heme into the cytoplasm. (5) Once in the cytoplasm, heme is degraded by the heme oxygenases IsdG and IsdI to release iron.

The critical first step in this process of heme-iron acquisition is hemoglobin binding to its receptor IsdB on the surface of *S. aureus* and heme extraction from the protein portion of hemoglobin (33, 80, 92, 118). The staphylococcal cell wall is an extremely thick and rigid structure. It is improbable that any one protein transports a molecule through a 60 nm thick assembly or that Isd proteins covalently anchored to peptidoglycan migrate through the cell wall with their cargo. In this regard, IsdB must pass heme from Hb to another protein(s) in order to transport heme into the bacterium. It is predicted that IsdB removes heme from Hb and passes it to the surface exposed protein IsdA, or to IsdC which is embedded within the cell wall (87, 92, 131). Specifically it is predicted that IsdB physically interacts with IsdA and IsdC to pass heme through the cell wall. IsdC in its turn interacts with IsdDEF to pass heme for transport through the membrane into the cytoplasm. This model for heme-iron transport is supported by the observations that: (i) *isdABCDEF* are genomically associated, (ii) IsdA, IsdB, IsdC, IsdD, and IsdE bind heme, and (iii) Isd components are located in the cell wall, membrane and the cytoplasm according to their predicted function (46, 76, 80, 86, 109, 124). Further, IsdB is required for hemoglobin binding and utilization as an iron source, while IsdA, IsdE, IsdG and IsdI are necessary for heme-iron utilization (46, 47, 70, 98, 118). In support of this model, heme transfer from Hb to IsdB to IsdA to IsdC to IsdE has been reconstructed *in vitro* (72, 87, 131).

It should be noted that *S. aureus* genome encodes for another hemoglobin receptor called IsdH/HarA (33, 34, 91, 92). IsdH/HarA has been demonstrated to bind Hb with high affinity *in vitro*. However, deletion of IsdH does not affect *S. aureus* hemoglobin binding or iron acquisition from Hb. Further IsdH does not appear to

contribute to virulence in murine models of infection and is truncated in certain clinical isolates of *S. aureus* (118). A recent report implicated IsdH in immune evasion (127).

In the cytoplasm heme is degraded by heme oxygenases IsdG and IsdI that are unique in their structure and function (87, 98, 100, 112, 131). IsdG and IsdI are the first members of newly identified family of IsdG heme oxygenases conserved in Gram positive bacteria. IsdG and IsdI are 64% identical, and are both regulated by Fur. IsdG, however is rapidly degraded in the absence of heme, while IsdI is stable irrespective of heme availability. Both enzymes are required for full virulence of *S. aureus* demonstrating the importance of fine tuning heme oxygenase activity during infection (98). IsdG and IsdI degrade heme to a novel molecule named staphylobilin (100). All other heme oxygenases across all kingdoms of life produce biliverdin as a byproduct of heme degradation. The biological function of staphylobilin is currently unknown.

Despite this accumulating knowledge, numerous aspects of heme import including the mechanism and specificity of Hb recognition, the interactions between Isd proteins *in vivo*, and the role of Hb as an iron source during infections remain unexplored. This thesis primarily focuses on the role of IsdB in hemoglobin-derived iron acquisition of *S. aureus* in the context of infection. In Chapter II, we elucidate expression and surface localization of IsdB in iron-deplete and iron-replete conditions, establishing IsdB as a surface exposed iron-regulated protein. We provide evidence that IsdB co-localizes and interacts with IsdA in support of their role as conduits of heme through the cell wall. We establish differential localization patterns of the Isd machinery depending on its level of expression. This phenomenon to our knowledge has not been previously reported for bacterial surface proteins. We identify the location of Isd deposition to the

cell wall at the site of cell division. This result explains how *S. aureus* ensures co-localization of Isd proteins. Finally, we establish differential expression and contribution of Hb-derived iron acquisition depending on the site of infection.

Understanding heme transfer through the cell wall represents an important contribution to a broader understanding of staphylococcal and Gram-positive physiology. The mechanism of transport of molecules across the cell wall has long been a mystery and describing the transport of this vital nutrient source provides clues to the fundamental process of substrate trafficking within Gram-positive bacteria.

In Chapter III, I report on our discovery that *S. aureus* has adapted to bind human hemoglobin more efficiently than hemoglobin from other species. This is explained by the fact that hemoglobin sequence varies considerably across species. To focus our studies we investigated binding of human (hHb) versus mouse hemoglobin (mHb) in more detail. We report that enhanced binding of hHb allows for improved iron acquisition from hHb as compared to mHb. Enhanced iron utilization is explained by the fact that *S. aureus* IsdB binds hHb with higher affinity than mHb.

Most *S. aureus* animal experiments are performed in mice. The importance of the Isd system to iron acquisition and staphylococcal pathogenicity has been demonstrated using murine models of infection (20, 81, 93, 118). Our finding that mHb is a poorer iron source than hHb for *S. aureus*, suggests that murine models may underestimate the contribution of Hb utilization to human infections. To circumvent this limitation we have acquired hHb transgenic mouse strain ($\alpha^{H}\beta^{H}$) that expresses hHb. Increased susceptibility of $\alpha^{H}\beta^{H}$ to *S. aureus* infection demonstrates translation of increased binding of hHb into enhanced infection, and establishes $\alpha^{H}\beta^{H}$ mice as an improved model for *S. aureus*

infections. Finally, we demonstrate that certain polymorphisms within hHb found in the population affect hHb binding to *S. aureus*. These findings suggest that hHb polymorphisms may affect individual susceptibility to *S. aureus* infection. Notably, the Isd system is conserved throughout Gram-positive pathogens and it is conceivable that hHb polymorphisms affect the severity of numerous bacterial infections in humans (80, 86, 91). Findings and methods described in this thesis lay the foundation for studies into a genetic component of an interaction between a vital host molecule and a deadly pathogen.

CHAPTER II

SUBCELLULAR LOCALIZATION OF THE *Staphylococcus aureus* HEMOGLOBIN RECEPTOR IsdB

"Location, location, location"

Introduction

The proposed model for heme-iron transport through the Isd system predicts that the protein constituents of the Isd system physically interact with each other to form a molecular conduit for heme transport through the cell wall. However, the subcellular localization pattern of the Isd proteins has not been reported (93). In addition, it is not known if proteins of the Isd system physically engage with one another within the bacterium. Finally, the contribution of hemoglobin capture to staphylococcal virulence is incompletely defined.

Upon extraction of heme from Hb *in vitro*, IsdB passes heme to IsdA (87, 131). In this chapter we demonstrate that IsdB co-localizes with IsdA to discrete sites within the staphylococcal cell wall, and these sites correspond to regions of hemoglobin capture. IsdAB localization and subsequent hemoglobin binding is regulated by iron availability, and appears to occur at the site of new cell wall formation. In support of this localization pattern, we demonstrate that IsdA and IsdB physically interact within the staphylococcal cell wall providing direct evidence that proteins of the Isd system act as a coordinated unit to mediate hemoglobin recognition and heme-iron acquisition. Finally, we report that IsdB exhibits an organ-specific regulation pattern which corresponds to an organ-specific requirement for IsdB during the pathogenesis of *S. aureus*. Taken together, these

results expand our understanding of the mechanism and function of hemoglobin capture by Gram positive pathogens.

Methods

Bacterial strains and growth conditions. All experiments were carried out with *S. aureus* strain Newman, or with mutants generated in its background. The protein A knock-out mutant (Δ*spa*) was generated through allelic replacement as previously described (5). Briefly, *spa* and approximately 1 kb of flanking DNA were cloned into pCR2.1. The primers used were: spa-51-AttB1: GGGGACAAGTTTGTACAAAAAAGCAGGCT-TCGAAGTAAAATTGATGAGCG and spa-32-AttB2: GGGGACCACTTTGTACAAGAAAGCTGGGT-CAACCTGGAGGTGCACTTG. The plasmid was then digested with NruI and Bpu10I, which excised *spa*, treated with T4 DNA polymerase to generate blunt ends and religated. The resulting construct was recombined into pKOR1 and used for allelic replacement in Newman (5). Strains inactivated for *spaisdB*, *spaisdA* and *spaisdAB* were generated by transducing the Δ*isdB::ermC*, Δ*isdA::ermC* or Δ*isdAB::ermC* alleles into the Δ*spa* background using bacteriophage ϕ-85 (80) to create Δ*spa*Δ*isdB*, Δ*spa*Δ*isdA* and Δ*spa*Δ*isdAB*. Strain inactivated for *spaisdBisdH* was generated by transducing Δ*spa*Δ*isdB* with the Δ*isdH::tetR* allele (kindly provided by Dr**.** Eszter Nagy) to create Δ*spa*Δ*isdB*Δ*isdH* using similar techniques (33). All transductions were confirmed by PCR and/or immunoblotting. All *S. aureus* cultures were inoculated from a single colony and grown overnight (~15 hours) in 5 ml tryptic soy broth (TSB) in 15 ml conical tubes at 37°C with shaking at 180 rpm unless noted otherwise.

Immunofluorescence. Bacteria were grown overnight in 5 ml TSB or TSB supplemented with 2,2-dipyridyl (DIP) in 15 ml conical tubes at 37°C with shaking at 180 rpm. Bacteria were sedimented at 3,000 *g* for 5 minutes and washed 3 times in 1 volume of ice cold phosphate-buffered saline (PBS) (pH 7.4). The following procedures were carried out at room temperature. One hundred and fifty μl of bacteria were applied to poly-L-lysine coated cover slip for 5 minutes. The cover slips were then floated on 2% formaldehyde in PBS for 20 minutes to fix the bacteria. Cover slips were then washed once with PBS and blocked in PBS + 3% (w/v) bovine serum albumin (BSA) for 1 hour. Cover slips were then incubated with BSA and primary antibody, washed 3 times with PBS, incubated in BSA with secondary antibody, washed 3 times, sealed to a slide by nail polish and visualized on an Olympus BX60 microscope. Pictures were taken with an Olympus DP71 camera using DP Controller and analyzed using DP Manager software. For simultaneous labeling of IsdB and hemoglobin the primary antibodies were added simultaneously followed by the secondary antibodies. For simultaneous labeling of IsdA and IsdB, IsdA antibody was biotinylated by EZ-Link NHS-PEO Solid Phase Biotinylation Kit. The labeling included 4 successive incubations: 1. Rabbit α-IsdB. 2. α-rabbit-488. 3. Rabbit biotinylated α-IsdA. 4. Streptavidin-555. The antibodies and concentrations used for immunofluorescence were: Rabbit α-IsdB 1:5,000; Rabbit α-IsdA 1:15,000 (80); Mouse α-human hemoglobin (Santa Cruz) 1:250; Rabbit biotinylated α-IsdA 1:50; Alexa Fluor 488 goat α–rabbit IgG (H+L) (green) 1:250; Alexa Fluor 488 goat α–mouse IgG (H+L) 1:250; Alexa Fluor 555 goat α–rabbit IgG (H+L) (red) 1:250; streptavidin, Alexa Fluor 555 conjugate 1:250.

Fluorescently-activated cell sorting (FACS) assay. For FACS assays the cells were labeled similar to the method used for immunofluorescence, except bacteria were fixed and incubated with antibodies in solution as opposed to on cover slips. The antibodies and concentrations were similar to immunofluorescence with an exception of Hb labeling, where all antibodies were used at 1:1,000 dilution. The cells were fixed with 2% formaldehyde for the second time after labeling. FACS analysis was performed with FACSCalibur (BD) using Cell Quest Pro software. Mean fluorescence intensity was quantified with Flow Explorer 4.2.

Hemoglobin binding. For hemoglobin binding assays, overnight cultures were spun down for 5 minutes at 3,000 *g* and resuspended in 1 volume PBS (pH 7.4) containing 0.5 μM hemoglobin (Sigma) for immunofluorescence and 1 μM hemoglobin for FACS analysis. They were then incubated at room temperature for 0.5 hour on a rotisserie, washed in PBS and labeled for hemoglobin as described in the immunofluorescence section.

Fluorescent hemoglobin binding assay. Human hemoglobin was conjugated to a fluorescent molecule with DyLight 549 Microscale Antibody Labeling Kit (Pierce) in borate buffer (pH 8.5) according to the manufacturer's recommendations. Forty μl of the overnight cultures grown in TSB + 1mM DIP were resuspended in 40 μl borate buffer containing 4 μM labeled hemoglobin, and incubated at room temperature for 30 minutes. The samples were then washed 3 times in borate buffer and fixed with 2% formaldehyde in PBS in a 100 μl volume. The cells were attached to cover slips and visualized. In competition assays unlabeled hemoglobin was added at 12 μM, while BSA was added at 50 μM.

Trypsin treatment. S. aureus overnight cultures were resuspended in 1 volume of PBS + 250 μg/ml trypsin (Sigma). The suspension was incubated at 37°C with shaking for 1 hour. Cells were then washed 3 times with PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF) followed by incubation at 37°C with shaking in TSB + 1 mM DIP + 1 mM PMSF. Aliquots were taken after 0, 5, 15, 30 and 60 minutes of incubation, immediately placed on ice and washed with ice cold PBS 3 times. Once all the samples were collected they were processed for immunofluorescence.

Immunogold labeling and electron microscopy. For immunogold labeling the primary antibody concentrations and incubation times were the same as for immunofluorescence. Bacterial cells were attached to poly-L-lysine coated Nickel Formvar grids. The samples were fixed with 2% formaldehyde in sodium cacodylate buffer. The secondary antibody, 6 nm Colloidal Gold-Affinipure Goat α-rabbit IgG (H+L), was used at 1:50 dilution. Blocking and antibody labeling were carried out in TBS (pH 7.1) + 3% BSA. After all labeling and washing steps, the grids were briefly washed 3 times with double deionized water. Samples were viewed using an FEI CM12 transmission electron microscope (FEI, Hillsboro OR).

Immunoblotting. Immunoblotting was performed using nitrocellulose membranes. Membranes were blocked in 5% milk made in TBS + 0.1% Tween 20 (TBST) from 1 hour to overnight. They were then incubated in milk plus primary antibody, washed 3 times with TBST, incubated in milk plus secondary antibody, and washed 3 times in TBST. Membranes were visualized using an Odyssey infrared imaging system (LI-COR), which was also used to quantify intensities of the blots. The antibodies used for

immunoblotting were rabbit α-IsdB (1:10,000), rabbit α-IsdA (1:25,000), and Alexa Fluor 680 goat α-rabbit IgG (H+L) (1:25,000).

Immunoprecipitation. Immunoprecipitation was carried out using the Protein A Seize X kit (Pierce). Agarose beads were crosslinked to α-IsdB according to the manufacturer's recommendations. Briefly, 35 μl of α-IsdB antiserum was bound to 200 μl of the beads and crosslinked by 25 μl DSS. Overnight *S. aureus* cultures were resuspended in 1ml TSM (100 mM Tris pH 7.0, 500 mM sucrose, 10 mM $MgCl_2$) containing 20 μg lysostaphin and incubated at 37°C for 1 hour. PMSF was added to 100 μM upon completion of incubation. The protoplasts were pelleted at 16,000 g for 2 minutes. 200 μl of the supernatant was mixed with 200 μl PBS (400 μl total) and loaded onto a column containing 50 μl agarose protein A beads crosslinked to α-IsdB. The samples were incubated at 4°C overnight on a rotisserie. The beads were then washed 5 times with PBS and bound proteins were eluted 3 times with low pH in 150 μl volumes for each elution. The elutions were pooled together (Elution 1). The beads were then transferred to a microfuge tube and boiled for 5 minutes in 100 μl 4% (w/v) sodium dodecyl sulfate (SDS), 0.5M Tris pH 8.0. This fraction was added to the pooled fractions eluted with low pH (Elution 2). The samples were normalized before loading onto 12% SDS-PAGE. The immunoprecipitation of rIsdA (15 μM) with rIsdB (7.5 μM) (80), was carried out in a similar manner, with omission of elution 2. The proteins were mixed and incubated at 37°C for 0.5 hour prior to immunoprecipitation.

Systemic mouse infections. Six to eight week old C57BL/6J mice were infected retroorbitaly with ~10^7 colony forming units (CFU) grown to mid-log phase and resuspended in sterile PBS. Ninety six hours post infection the mice were euthanized with

forced inhalation of CO_2. The hearts and livers were removed post-mortem and homogenized in 1 ml PBS for further processing. For immunofluorescence and quantification of IsdA and IsdB, the homogenized organs of 4 infected mice were transferred to microfuge tubes and centrifuged at 1,000 g for 1 minute. The supernatant was transferred to another tube and centrifuged for 3 minutes at 16,000 g. The supernatant was decanted and the pellet was resuspended in 1 ml PBS and spun again at 16,000 g for 3 minutes. The supernatant was removed and the pellet was resupended in 200 μl TSM. Twenty μls were removed to determine the CFU/ml of the samples. Twenty μg lysostaphin was added to the remaining suspension (100 μg/ml lysostaphin final concentration) and incubated at 37°C for 1 hour. PMSF was added to 1 mM and the samples were frozen at -20°C. The following morning the CFUs/ml were quantified, and normalized samples were assessed for relative amount of IsdB and IsdA in the infected organs by quantitative immunoblotting. For immunofluorescence, the samples were not treated with lysostaphin, but rather prepared to visualize using a fluorescent microscope. CFUs/ml were quantified by serial dilutions in PBS and plating on TSA. Each group included at least 9 mice.

Results

IsdB surface expression is regulated by iron availability. Proteins of the Isd system are likely to be targeted to the same subcellular locale in order to ensure efficient acquisition of heme-iron. To test the hypothesis that the cell wall anchored Isd proteins are proximal to each other, we chose to use immunofluorescence. Proteins of the Isd system are up-regulated in low iron conditions, therefore we grew *S. aureus* in tryptic soy broth (TSB) supplemented with the iron chelator 2,2-dipyridyl (DIP). IsdB expression on the staphylococcal surface was evaluated using IsdB-specific rabbit antiserum (α-IsdB) and a secondary antibody conjugated to a fluorophore. As expected for these conditions the primary antibody bound to the surface of *S. aureus* (Figure 3A). To control for non-specific binding of α-IsdB, we tested antibody binding to Δ*isdB* grown in TSB + DIP, and wild type *S. aureus* grown in iron sufficient TSB, a growth condition that is not permissive to IsdB expression (80, 118). α-IsdB bound to *S. aureus* in all of these conditions indicating significant non-specific binding in these experiments (Figure 3A-C).

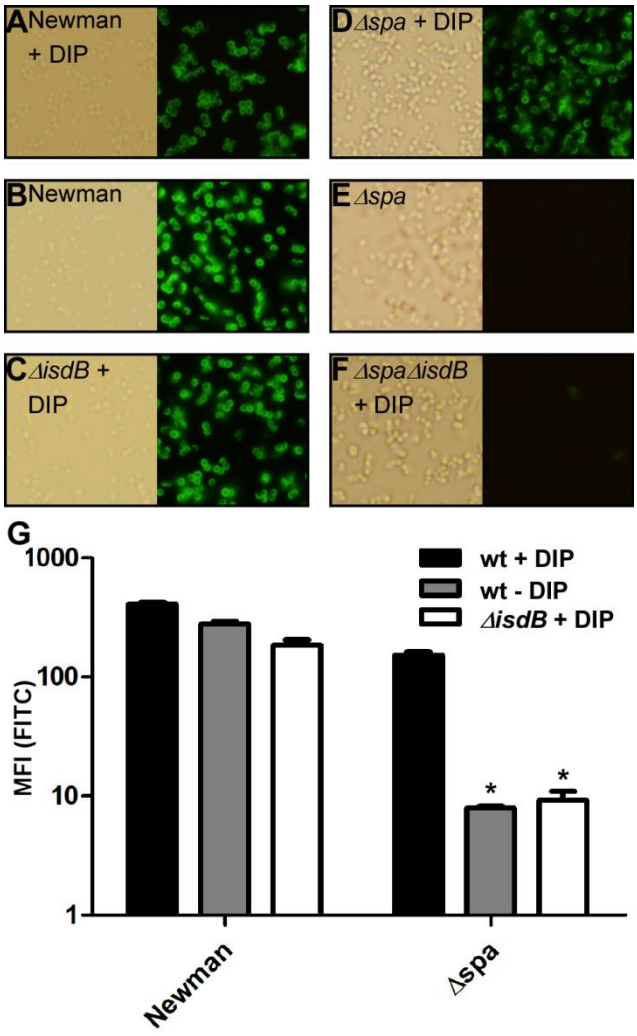

Figure 3. Creation of a Protein A-deficient strain for specific labeling of *S. aureus* surface exposed proteins. A-F. *S. aureus* strains were grown overnight in TSB with 1 mM of iron chelating agent 2,2-dipyridyl (DIP) where indicated. Bacteria were subsequently labeled with rabbit α–IsdB antibody and Alexa Fluor 488 goat α–rabbit IgG (H+L). **G.** Bacteria grown under the same conditions and labeled similarly to *A-F* were subjected to FACS analysis to determine mean fluorescence intensity (MFI). Error bars represent standard error. Asterisks denote statistically significant differences in relation to Δ*spa* + DIP as determined by Student's *t* test ($P < 0.05$).

S. aureus protein A is a cell wall factor that non-specifically binds the constant regions of immunoglobulin G (41). We reasoned that Protein A might be responsible for the non-specific labeling observed upon staining with α-IsdB (40). To circumvent this issue, we created a *S. aureus* strain inactivated for the gene encoding protein A (Δ*spa*) and analyzed IsdB expression. Immunoblotting demonstrated that inactivation of *spa* does not affect the expression of IsdB (data not shown). Δ*spa* expressed IsdB on the surface when grown in iron deplete conditions, whereas IsdB was not detectable when Δ*spa* was grown in iron-sufficient conditions (Figure 3D-E). In addition, Δ*spa*Δ*isdB* did not elaborate an IsdB signal regardless of the iron status of the bacterium (Figure 3F). To quantify the effects of *spa* deletion on IsdB detection, we performed a fluorescently-activated cell sorting (FACS) assay on *S. aureus* grown in both iron-sufficient and iron-deplete conditions (Figure 3G). These experiments confirmed that IsdB is expressed on the surface of *S. aureus* in an iron-dependent manner, and alterations in iron status lead to an approximately 19-fold change in IsdB surface expression as measured by FACS. Taken together, these results establish that iron restriction increases the expression and surface anchoring of IsdB.

The subcellular distribution of IsdA and IsdB is regulated by iron availability. In order to determine how iron availability affects localization of the Isd system on the surface of *S. aureus*, we grew Δ*spa* overnight in TSB supplemented with varying concentrations of DIP. As expected, we were unable to detect IsdB on the surface of *S. aureus* grown in iron-sufficient conditions (Figure 4A). Bacteria grown in increasing concentrations of iron chelator exhibited a commensurate increase in IsdB expression and surface anchoring (Figure 4C). Further, addition of excess iron to medium containing DIP suppressed IsdB expression, eliminating the possibility that IsdB up-regulation was induced by DIP independently of its iron chelating activity (Figure 4A and C). During the course of these experiments, it was noted that the distribution pattern of IsdB on the surface of staphylococci is affected by the iron status of the organism. More specifically, at 250 μM DIP IsdB localizes to discrete puncta throughout the cell surface, whereas *S. aureus* grown at 1 mM DIP distributes IsdB in a uniform circumferential pattern around the cell wall (Figure 4A). A similar pattern of IsdB expression and surface localization was observed upon iron starvation induced by a different iron chelator (ethylenediamine-di(o-hydroxyphenylacetic acid) (data not shown).

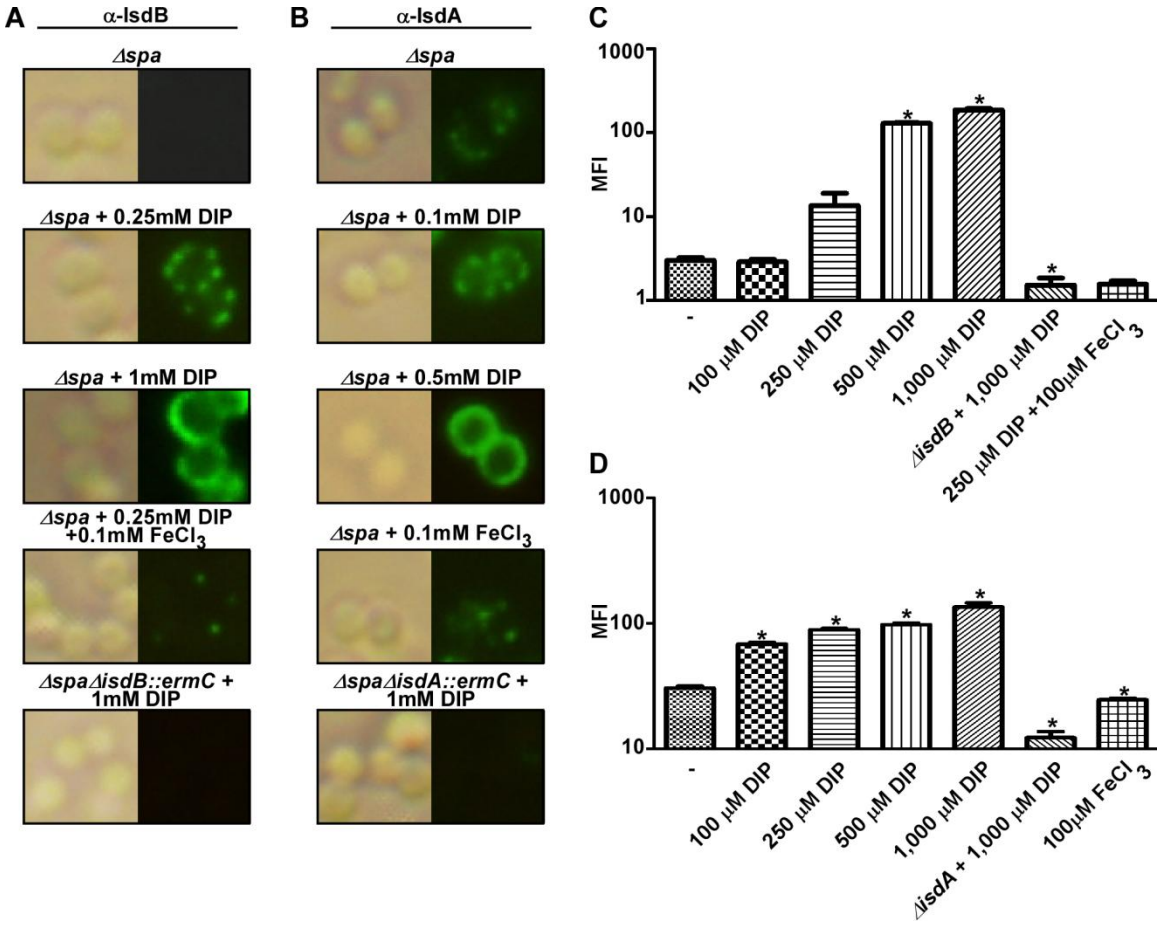

Figure 4. Iron availability influences the expression and localization of IsdB and IsdA on the staphylococcal surface. Δ*spa* was grown overnight in medium supplemented with the indicated concentrations of DIP. Bacteria were subsequently labeled with rabbit α–IsdB (**A**) or rabbit α–IsdA (**B**) antibodies followed by Alexa Fluor 488 Goat α–rabbit IgG (H+L). Quantification of relative amounts of IsdB (**C**) and IsdA (**D**) expressed on the surface of Δ*spa* was determined by FACS. Asterisks denote statistically significant difference in relation to Δ*spa* grown in plain TSB (-) as determined by Student's *t* test ($P < 0.05$). In fluorescent images green color was increased by 100% in *A* and by 25% in *B*.

To determine if this iron-dependent localization pattern was specific to IsdB, we analyzed IsdA expression and distribution using antisera specific for IsdA (Figure 4B and D). We found that IsdA was expressed at low levels when grown in iron-sufficient conditions and its expression increased upon iron starvation. Furthermore, we found that the distribution pattern of IsdA mirrored that of IsdB. When exposed to intermediate iron stress (100 μM DIP), IsdA localized to distinct puncta around the cells. In contrast, IsdA was evenly distributed around the cell wall in maximally iron starved *S. aureus* (Figure 4B). These results indicate that both the expression and subcellular distribution of IsdA and IsdB are regulated by iron availability, and that the Isd system localizes to discrete regions within the cell in conditions of moderate iron stress.

Hemoglobin capture by *S. aureus* is iron-regulated and IsdA/IsdB/IsdH-dependent.

The expression and surface distribution of IsdB is regulated by iron (Figure 3), and IsdB has been shown to bind hemoglobin (34, 80, 118). In order to determine the effect that iron availability and IsdB expression have on hemoglobin binding to the surface of *S. aureus* we analyzed hemoglobin binding to Δ*spa* grown in iron-replete or iron-deplete conditions. These experiments revealed that Δ*spa* grown in iron-replete conditions did not bind detectable levels of hemoglobin (Figure 5A). However, when grown in the presence of the iron chelating agent DIP, *S. aureus* exhibited a dose-dependent increase in hemoglobin binding (Figure 5B, C). Hemoglobin binding to the staphylococcal surface was localized to discrete puncta, reminiscent of the iron-dependent distribution pattern observed for IsdB and IsdA (Figure 4A, B). To assess whether the observed iron-dependent hemoglobin binding was mediated by IsdB, we measured hemoglobin binding to Δ*spa*Δ*isdB* (Figure 5E-G). These experiments revealed that iron-starved Δ*spa*Δ*isdB* is deficient in binding hemoglobin as compared to Δ*spa* (Figure 5G). To confirm that the punctate binding distribution of hemoglobin was not an artifact of antibody-based detection, we incubated bacteria with fluorescently labeled hemoglobin (Figure 6). This assay confirmed that hemoglobin binding to *S. aureus* is IsdB-dependent and specific as fluorescently labeled hemoglobin could be outcompeted by excess non-labeled hemoglobin.

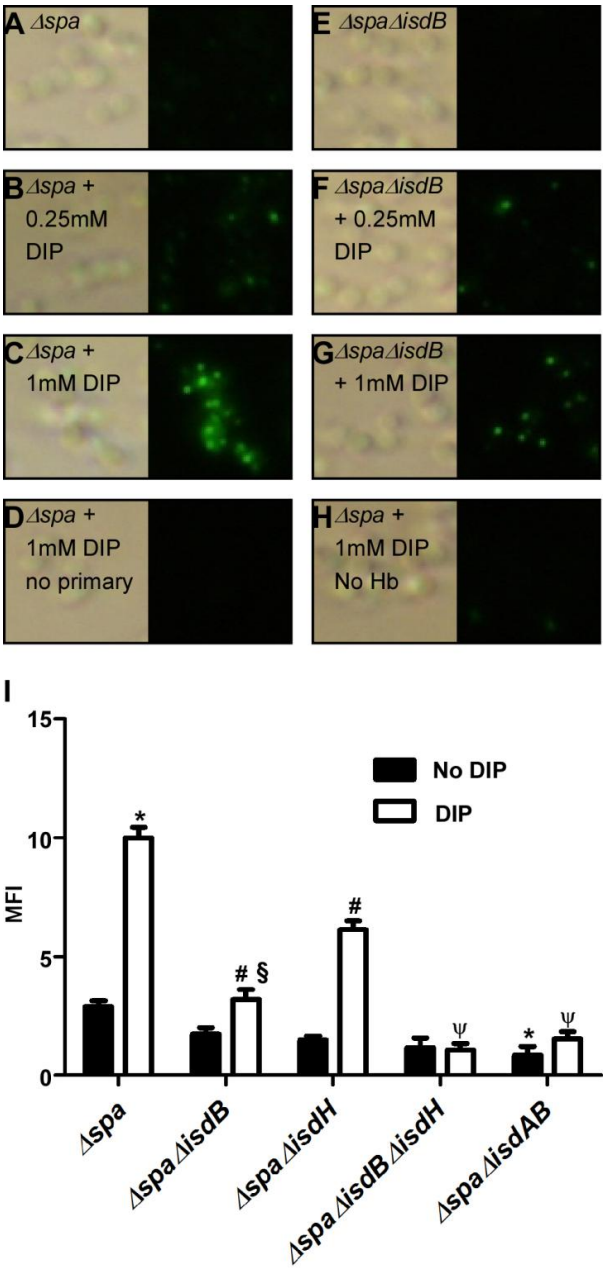

Figure 5. Iron- and Isd-dependent hemoglobin binding to the surface of *S. aureus*. Δ*spa* (**A-D**) or Δ*spa*Δ*isdB* (**E-G**) were grown overnight in TSB supplemented with the indicated concentrations of DIP. Bacteria were then incubated in PBS with human hemoglobin and washed with PBS. Cells were subsequently labeled with mouse α–hemoglobin IgG followed by Alexa Fluor 488 goat α–mouse IgG (H+L). **I.** Quantification of the relative amount of human hemoglobin bound to the surface of indicated strains, grown in presence of 1 mM DIP where indicated, determined by FACS. Error bars represent standard error. The symbols denote statistically significant differences as determined by Student's *t* test ($P < 0.05$) in relation to: * *Δspa* (no DIP), # *Δspa* (DIP), § *ΔspaΔisdB* (no DIP), ψ *ΔspaΔisdB* (DIP).

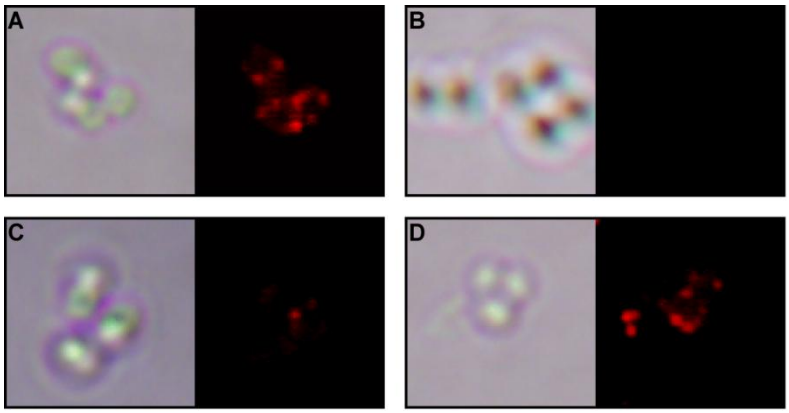

Figure 6. Hemoglobin-549 binding to the surface of *Staphylococcus aureus*. Δ*spa* (**A**) or Δ*spa*Δ*isdB* (**B**) was grown overnight in TSB supplemented with 1 μM 2,2-dipyridyl. Bacteria were then incubated in borate buffer with 4 μM human hemoglobin conjugated to DyLight 549 fluorophore and washed with borate buffer. **C, D.** Δ*spa* were grown and processed as in *A*. In addition to fluorescent hemoglobin, samples were incubated with 12 μM unlabeled hemoglobin (**C**) or 50 mM bovine serum albumin (**D**). In fluorescent images brightness and contrast was increased by 75% and red color was increased by 25%.

Despite the reduction in hemoglobin binding, Δ*spa*Δ*isdB* was capable of binding detectable levels of hemoglobin in an iron-dependent manner (Figure 5E, G). To confirm this observation we measured relative hemoglobin binding to Δ*spa* and Δ*spa*Δ*isdB* grown in high or low iron with FACS (Figure 5I). We observed that iron-starved Δ*spa*Δ*isdB* displayed a significant decrease in hemoglobin binding when compared to Δ*spa*. However, iron-starved Δ*spa*Δ*isdB* bound hemoglobin at levels higher than iron-replete Δ*spa*Δ*isdB*, suggesting the presence of an iron-regulated IsdB-independent hemoglobin binding activity (Figure 5G, I). To date, IsdH and IsdA are the only staphylococcal proteins other than IsdB that have been shown to bind hemoglobin (24, 33, 91). To investigate whether IsdH was responsible for the residual levels of hemoglobin binding detected in Δ*spa*Δ*isdB*, we inactivated *isdH* in both the Δ*spa* and Δ*spa*Δ*isdB* backgrounds (creating Δ*spa*Δ*isdH* and Δ*spa*Δ*isdB*Δ*isdH* respectively) and measured the ability of these strains to bind hemoglobin. When grown in iron-deplete conditions, Δ*spa*Δ*isdH* bound hemoglobin at reduced levels compared to Δ*spa*. Accordingly, Δ*spa*Δ*isdB*Δ*isdH* bound hemoglobin at lower levels than Δ*spa*Δ*isdB*, suggesting that IsdH is at least partially responsible for the observed secondary binding activity. To assess whether IsdA contributes to capturing hemoglobin at the staphylococcal surface we created a strain lacking *isdA* and *isdB* in the Δ*spa* background (Δ*spa*Δ*isdAB*) and tested the ability of Δ*spa*Δ*isdAB* to bind hemoglobin. These experiments revealed that iron-starved Δ*spa*Δ*isdAB* exhibited decreased hemoglobin binding when compared to Δ*spa* and Δ*spa*Δ*isdB*. Further, *Δspa*Δ*isdAB* displayed decreased levels of hemoglobin binding as compared to *S. aureus* grown in iron-replete conditions, suggesting that IsdA contributes to hemoglobin binding in both iron-replete and iron-deplete conditions (Figure 5I). As a

control for the specificity of the α-hemoglobin antisera, we found that no strains stained positive for antibody binding in the absence of hemoglobin addition (data not shown). These results indicate that IsdB is the primary hemoglobin receptor on the surface of *S. aureus* in iron-starved environments, however IsdH and IsdA also contribute to hemoglobin capture.

We next sought to determine whether hemoglobin bound to the surface of staphylococci co-localizes with IsdB. To determine the relative localization of IsdB and hemoglobin, we grew Δ*spa* overnight in iron-deplete conditions and labeled the two molecules simultaneously on the surface of staphylococci with fluorophores emitting at distinct wavelengths. Both IsdB and hemoglobin displayed iron-dependent patterns of localization similar to that seen when labeled individually (Figures 7A, 4A, and 5C). At 1 mM DIP, IsdB is diffusely distributed on the surface, whereas hemoglobin binding is punctate. Nevertheless, hemoglobin and IsdB co-localize on the surface, and hemoglobin binding is maximal in regions of the cell surfaces where IsdB is more abundant (Figure 7A, B). Both IsdB and hemoglobin fluorescent labeling are specific as indicated by the absence of the appropriate fluorescence when one of the primary antibodies is omitted (Figure 7C, D). Cumulatively, these results suggest that hemoglobin binding to the surface of *S. aureus* is mediated by IsdB and occurs at distinct foci throughout the cell wall.

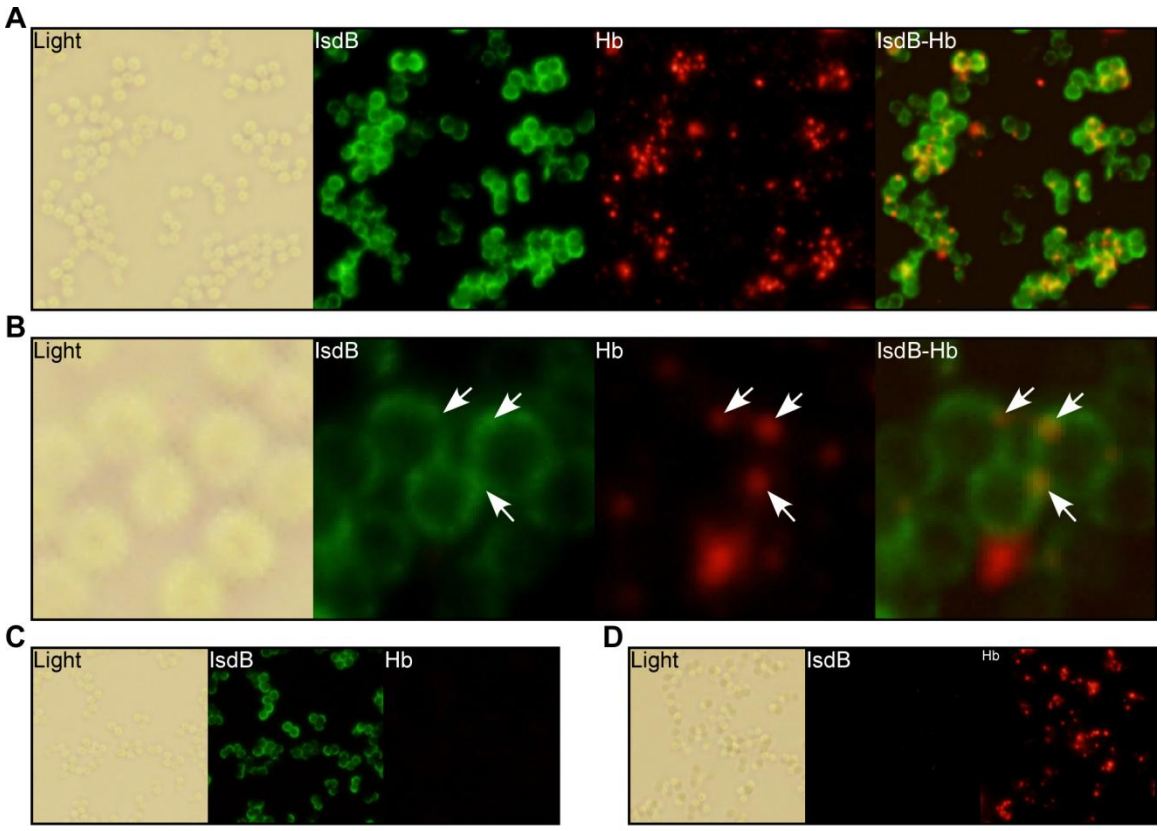

Figure 7. Hemoglobin co-localizes with IsdB on the surface of *S. aureus*. A. Δ*spa* was grown overnight in TSB supplemented with 1 mM DIP. Bacteria were then incubated with human hemoglobin and washed with PBS. Cells were then simultaneously labeled with rabbit α–IsdB and mouse α–hemoglobin antibodies followed by Alexa Fluor 488 goat α–rabbit and 555 goat α–mouse IgG (H+L). **B.** Close-up of *A*, the arrows point to locations on the cell surface where hemoglobin is bound. To control for antibody cross-reactivity either mouse α–hemoglobin (**C**), or rabbit α–IsdB (**D**) antibodies were omitted. In fluorescent images green color was increased by 25% and red by 75%.

IsdA and IsdB form a complex at discrete regions within the cell wall. IsdB is capable of removing heme from hemoglobin and transferring it to IsdA *in vitro* (87, 131). In order for this process to occur *in vivo*, IsdA and IsdB, which are restrained to their anchoring site, are likely to be in direct proximity to each other. To test whether IsdA and IsdB co-localize on the surface of *S. aureus*, we labeled the proteins simultaneously and viewed their localization using immunoflourescence. IsdB was detected using α-IsdB counterstained with secondary-488 antibody, while IsdA was detected using biotinylated α-IsdA counterstained with streptavidin-555. These experiments revealed that IsdB and IsdA labeling co-localize on the surface of *S. aureus* (Figure 8A), suggesting that IsdA and IsdB are deposited proximally to each other within the cell wall. To improve the resolution of this assay we sought to determine the initial anchoring sites of IsdA and IsdB within the cell wall. To achieve this, we first treated staphylococci with trypsin, which effectively digested both IsdA and IsdB on the surface of staphylococci as indicated by the absence of their labeling following treatment (Figure 8B). The cells were then washed, resuspended in iron-deficient medium and allowed to recover for 15 minutes. This assay enables the visualization of proteins that are newly deposited to the cell wall, thus identifying the location of their anchoring. These experiments revealed that IsdA and IsdB are both deposited to the same location on the surface of staphylococci (Figure 8C). In addition, IsdA exhibits a slightly more diffuse localization pattern possibly due to its less stringent regulation (Figure 4). Among a total of 97 cells observed in this analysis, 81 cells (84%) deposited IsdA and IsdB at the same location. To confirm that IsdA and IsdB engage one another at these discrete anchoring sites, we performed immunoprecipitation (IP) of IsdB from cell wall lysates. Both IsdA and IsdB

were precipitated by α-IsdB consistent with IsdAB complex formation occurring in the cell (Figure 8D). IP of lysates from Δ*spa*Δ*isdB* did not result in the precipitation of IsdA confirming that IsdA pull-down requires IsdB. In further support of a physical interaction between IsdA and IsdB, recombinant IsdA was immunoprecipitated with recombinant IsdB by an α-IsdB antibody following incubation of these two proteins (Figure 8D). These results indicate that IsdA and IsdB co-localize on the surface of staphylococci and that their co-localization is achieved through anchoring to the same site within the cell wall. Further, these data suggest that IsdA and IsdB physically interact to promote hemoglobin capture during infection.

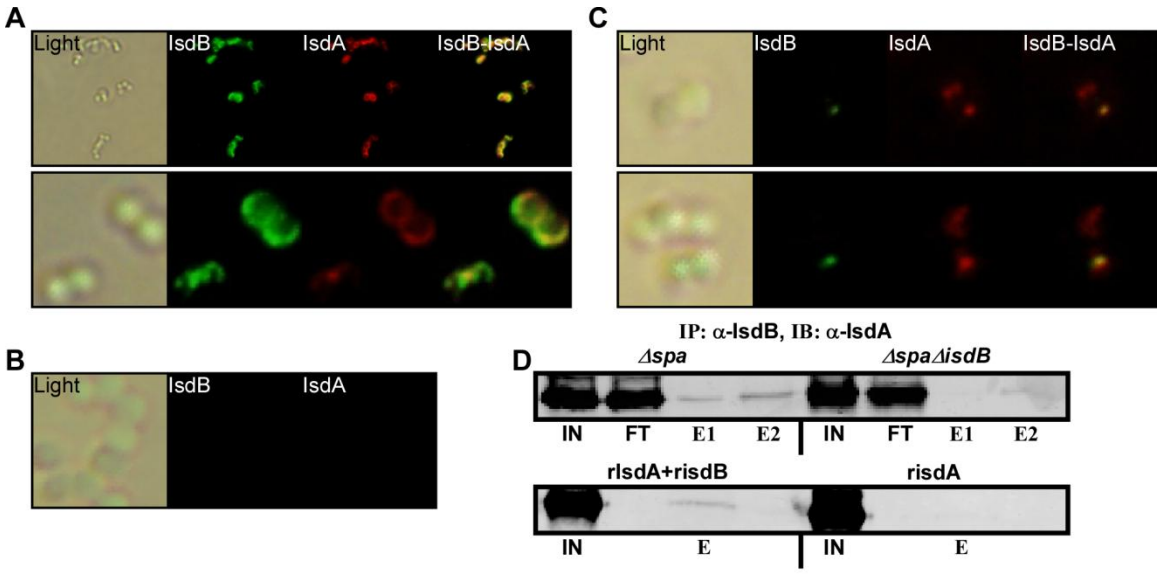

Figure 8. IsdA and IsdB co-localize and interact on the cell wall of *S. aureus*. A. Δ*spa* was grown overnight in TSB supplemented with 1 mM DIP. Bacteria were then sequentially labeled with rabbit α–IsdB, Alexa Fluor 488 goat α–rabbit IgG (H+L), biotinylated rabbit α–IsdA, and streptavidin Alexa Fluor 555 conjugate. **B, C.** Cells from the same culture were subjected to trypsin digestion, washed, resuspended in TSB + 1 mM DIP, and incubated. Aliqouts were taken after 0 (**B**) or 15 (**C**) minutes and labeled as in *A.* **D.** Top: Δ*spa* or Δ*spa*Δ*isdB* were grown overnight in TSB + 1 mM DIP. The cell wall proteins were solubilized by lysostaphin. IsdB was pulled down with Seize X Protein A Immunoprecipitation Kit and rabbit α–IsdB antibody. Input (IN), non-precipitated flow through (FT), and eluted proteins (E1, E2) were subjected to SDS-PAGE, transferred onto nitrocellulose and immunoblotted with α–IsdA antibody. Bottom: Recombinant rIsdA and rIsdB were combined and incubated at 37°C for 30 minutes. Immunoprecipitation was performed as in top panel and input (IN) and elution (E) are shown.

Newly formed IsdA and IsdB are anchored to the cell wall at the site of cell division.

It has been shown that cell wall proteins in Gram positive bacteria are destined for two primary anchoring locations (15, 31). One destination is the site of cell division where new cell wall is rapidly synthesized, while the other is distant from the cell division site. In order to precisely determine the cell wall destinations of IsdA and IsdB, we utilized electron microscopy to localize immunogold labeled IsdA and IsdB. Bacteria were grown in iron-deplete medium, treated with trypsin, and recovered in iron-deplete medium. Aliquots were taken before or immediately following trypsinization, and at different time points after recovery. Bacteria were attached to nickel formvar grids and labeled with α-IsdB or α-IsdA antibodies followed by α-rabbit antibodies conjugated to 6 nm gold beads. Consistent with data acquired using immunofluorescence (Figure 4A and B), both IsdA and IsdB were distributed throughout the cell surface when *S. aureus* was grown in maximally iron-starved conditions (Figs. 9A, B, and 10A, B). Gold beads were not detectable on the surface of Δ*spa*Δ*isdB* and Δ*spa*Δ*isdA* indicating the specificity of the labeling procedure (Figs. 9C and 10C). Further, gold beads were not detected following trypsin treatment of wild type *S. aureus* grown under iron-deficient conditions, indicating that IsdA and IsdB are removed from the cell wall by trypsin digestion (Figures 9D and 10D). Cells which were allowed to recover for 5 minutes following trypsinization displayed IsdB and IsdA deposition on the cell walls (Figures 9E-L). Specifically, we observed localization of newly synthesized IsdA and IsdB to the site of cell division (Figure 9I, J and K). In addition to localization at the site of new cell wall formation, IsdA was found more diffusely throughout the staphylococcal cell (Figure 9I, J, and L). Bacteria which were allowed to recover for longer periods of time displayed

progressively more diffuse localization patterns of IsdA and IsdB, with uniform circumferential distribution following 60 minutes of recovery in iron-deficient medium (Figure 10E-H). These data confirm that IsdA and IsdB are co-localized within the cell wall and establish the site of cell division as their common deposition location. However, it should be noted that the site of cell wall division does not appear to be the exclusive site of deposition for IsdA.

IsdA and IsdB exhibit organ-specific patterns of expression. Strains lacking IsdB exhibit decreased colonization of murine kidneys and spleen in systemic models of infection, and mice immunized with IsdB are protected against staphylococcal infection (68, 115, 118). These observations imply that IsdB is expressed within the vertebrate host. To test this directly, we monitored IsdB expression in a murine model of infection. C57BL/6J mice were infected with Δ*spa*. After 96 hours the mice were sacrificed, and hearts were removed and homogenized with PBS. The homogenates were subjected to a series of centrifugations to remove larger mammalian cells and cellular debris. The resulting suspension was labeled with α-IsdB antibody. Using this protocol we recovered staphylococcal cells which bound α-IsdB antibody indicating expression of IsdB on their surface. Heart tissue from an uninfected mouse processed in the same way did not stain positive for IsdB (Figure 11A). These experiments demonstrate that IsdB is expressed during staphylococcal infection.

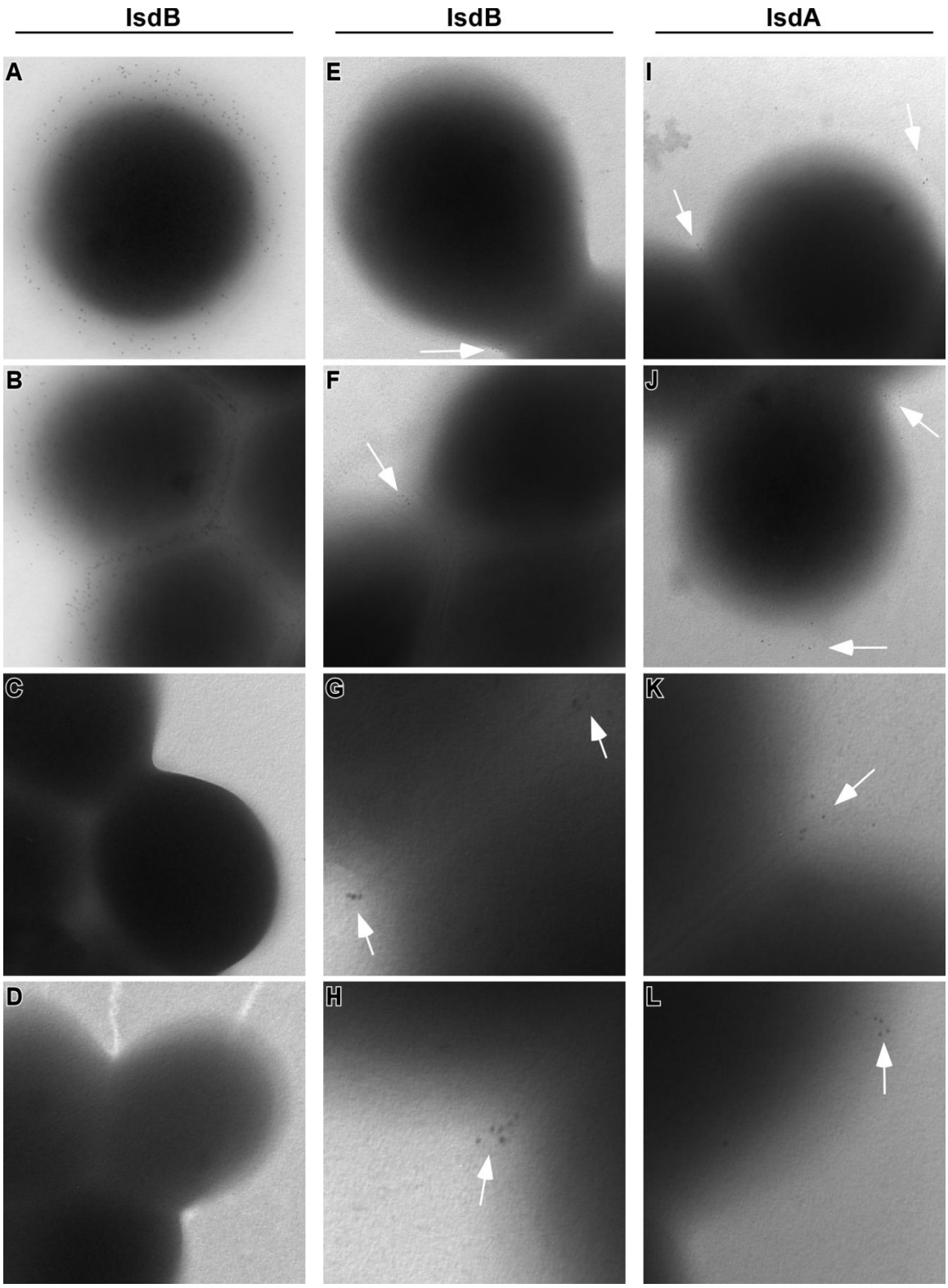
IsdB
IsdB
IsdA
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Figure 9. IsdA and IsdB are anchored at sites of nascent cell wall formation. Δ*spa* was grown overnight in TSB supplemented with 1 mM DIP. Cells were treated with trypsin, washed, resuspended in TSB + 1mM DIP and incubated. Aliquots were taken at different time points, washed, attached to nickel formvar grids and sequentially labeled with indicated primary antibodies and secondary 6 nm Colloidal Gold-Affinipure Goat α–rabbit IgG (H+L). **A**, **B.** Non-trypsinized Δ*spa* labeled for IsdB. **C.** Non-trypsinized Δ*spa*Δ*isdB* labeled for IsdB. **D.** Trypsinized Δ*spa* labeled for IsdB. **E-L.** Δ*sp*a upon trypsin treatment and 5 minute recovery in TSB, labeled for IsdB (**E-H**) or IsdA (**I-L**)**.**

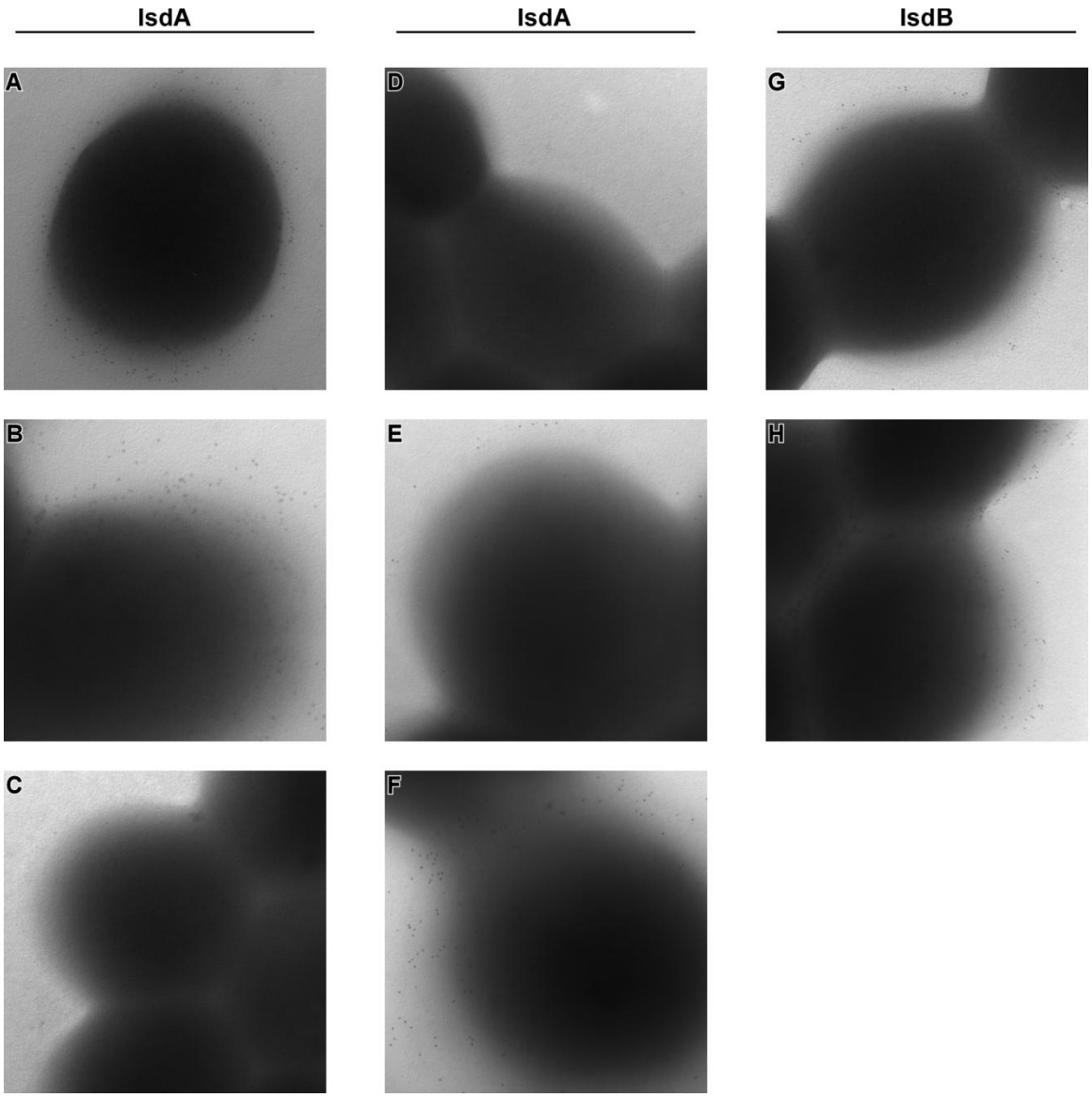

Figure 10. IsdA and IsdB distribution on the cell wall of *Staphylococcus aureus*. Δ*spa* was grown overnight in TSB supplemented with 1 μM 2,2-dipyridyl. Cells were treated with trypsin, washed, resuspended in TSB + 1 μM 2,2-dipyridyl and incubated at 37°C. Aliquots were taken at different time points, washed, attached to nickel formvar grids and sequentially labeled with indicated primary antibodies and secondary 6 nm Colloidal Gold-Affinipure Goat α-rabbit IgG (H+L). **A**, **B.** Non-trypsinized Δ*spa* labeled for IsdA. **C**. Non-trypsinized Δ*spa*Δ*isdA* labeled for IsdA. **D**. Trypsinized Δ*spa* labeled for IsdA. **E**-**H**. Δ*spa* upon trypsin treatment and 15 (**E, G**) and 60 (**F, H**) minute recovery in TSB + 1 μM DIP.

To quantify the relative expression levels of IsdB and IsdA during infection, we infected mice with wild type *S. aureus*. After harvesting and processing the organs as described above, we normalized the samples to colony forming units (CFU) of infecting *S. aureus* (see methods). Following normalization, cell wall lysates were immunoblotted for the presence of IsdA and IsdB. As depicted in Figure 11B, we detected IsdA both in livers and hearts of infected mice, but not in the organs of uninfected animals. In contrast, IsdB was detected in the hearts, but not the livers of infected mice. Quantification of the band intensities from the samples of individual mice indicated lower levels of IsdA and IsdB expression in the livers of infected mice when compared to the hearts of the same animals (Figure 11C). These experiments demonstrate that IsdB and IsdA are differentially expressed across organs.

To establish the contribution of IsdB to *S. aureus* virulence in hearts and livers, we infected mice with either wild type or Δ*isdB*. We observed a significant decrease in virulence of Δ*isdB* in the hearts demonstrated by a reduction in CFU of invading bacteria by almost two orders of magnitude (Figure 11D). Consistent with the undetectable level of IsdB expression in murine livers, strains lacking IsdB colonized this organ as efficiently as wild type (Figure 11D). Together, these results demonstrate that *S. aureus* requires IsdB for growth in the murine heart and accordingly induces its expression when colonizing this organ. However, IsdB is not expressed during liver colonization and hence is dispensable for colonization in this host environment. This discrepancy in IsdA and IsdB expression and contribution to infection is potentially due to differences in iron availability within the heart and livers of mice.

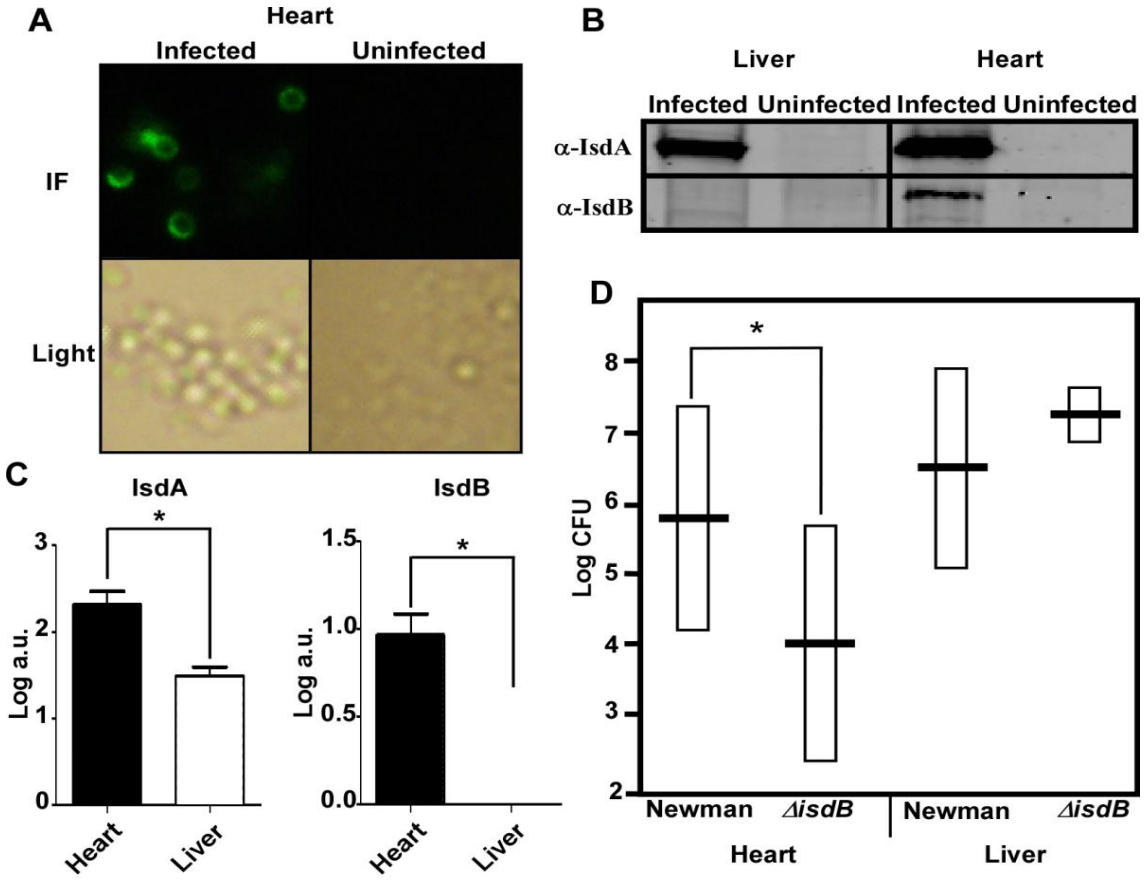

Figure 11. IsdB is expressed within the hearts of infected animals and contributes to cardiac colonization. **A.** C57BL/6J mice were retroorbitaly infected with 10^7 CFU of Δ*spa* in 100 µl PBS, and sacrificed 96 hours post-infection. Hearts and livers were removed and homogenized in 1 ml sterile PBS. Bacteria were then partially separated from the mammalian cells by centrifugation, washed and immunofluorescently labeled with α–IsdB. **B.** Wild type *S. aureus* recovered from the organs of C57BL/6J mice was separated from the mammalian cells as in *A*, normalized to $1x10^5$ CFUs, lysed with lysostaphin to release cell-wall proteins which were separated on SDS-PAGE gel, and transfered onto nitrocellulose membrane. The membrane was immunoblotted with α–IsdA and α–IsdB antibodies. **C.** Relative amount of IsdA and IsdB in the infected organs was quantified based on immunoblot intensity. Error bars represent standard error. Asterisks denote statistically significant differences as determined by Student's *t* test ($P < 0.05$). **D.** Organ colonization was estimated based on CFU quantification by serial dilution and plating on TSA. The horizontal bars represent the mean, boxes represent standard deviation. Asterisks denote statistically significant differences as determined by Student's *t* test ($P < 0.05$). Each group included at least 9 mice.

Discussion

Here we demonstrate subcellular co-localization and interaction of IsdA and IsdB, which function together to provide nutrient heme-iron to *S. aureus* during infection. In Gram positive bacteria, the thick cell wall poses an additional obstacle to the transport of iron (99, 114). To acquire iron during infection *S. aureus* utilizes the Isd system, which allows it to bind hemoglobin, remove and transport heme across the cell wall and membrane into the cytoplasm, where heme is degraded to release iron (114). The properties of individual factors constituting the Isd system have been investigated; however the mechanics of their cooperation remain to be established. Recent studies have shown that recombinant IsdB (rIsdB) is capable of removing heme from hemoglobin and passing it to rIsdA in solution (87, 131). *In vivo*, IsdA and IsdB are predicted to be located in direct proximity to each other in order to remove and pass heme iron. Testing this model has proven difficult due to the inherent complexities associated with performing protein localization studies in *S. aureus*. Few reports on the localization of *S. aureus* proteins have been published to date due to the small size of staphylococcal cells and the non-specific binding of IgG by Protein A.

Here we demonstrate that IsdA and IsdB expression and localization is regulated by iron availability. In conditions under which their expression is limited, IsdA and IsdB are concentrated to distinct puncta located throughout the cell surface. One possible explanation for this organized localization pattern is that under these conditions the Isd proteins are produced in successive waves of expression and are therefore anchored to the cell wall periodically. Another mechanism through which punctate localization could be achieved is suggested by the location of IsdA and IsdB deposition at the sites of cell

division, possibly through a single secretion portal. Because cell division is periodical, it is possible that when the cell starts dividing this portal "opens", and all synthesized IsdB swiftly gets incorporated into the cell wall. A "pause" follows, during which the IsdB levels are exhausted and it is not incorporated into the cell wall, accounting for the interruptions in its distribution. Although these models are speculative at this point, the proteins of the Isd system provide excellent tools to test these models, because their expression levels are easily manipulated by iron availability. Notably, another report has demonstrated punctuate distribution of a hemoglobin receptor on the surface of *Bacillus cereus* (30).

We have shown that IsdA and IsdB are deposited at the same location on the cell wall of *S. aureus*. This might ensure that IsdA and IsdB, which function together in heme import, are proximal to each other. Notably, some IsdA is deposited on the cell wall at locations distant from IsdB, which might be due to the fact that IsdA has functions distinct from heme-iron acquisition such as resistance to antimicrobials and adherence to the host epithelium (22-24). Studies have indicated that not all cell wall proteins of Gram positive bacteria are destined for the same site, suggesting differential localization of surface proteins depending on their function (15, 31, 105). In keeping with this, the specific deposition of IsdB at the cell division site can be attributed to the YSIRK/GS sequence found within its signal peptide domain, which primes cell wall anchored proteins to the sites of cell division (15, 31). In contrast, IsdA does not encode for YSIRK/GS within its signal peptide and is anchored to sites both within and outside of the sites of cell division. These facts suggest that additional factors which contribute to the localization of cell wall anchoring are yet to be determined. It is possible that IsdA

anchoring is not restricted to the site of cell division due to its higher basal level of expression. In addition to spatial proximity, the possibility that IsdA/IsdB interact *in vivo* is supported by the observation that IsdA co-immunoprecipitates with IsdB.

A primary function of the Isd system is to capture hemoglobin for use as a nutrient iron source. We have found that hemoglobin binding to the surface of *S. aureus* depends on iron availability and the presence of IsdB. Surprisingly, staphylococci which are maximally iron starved and therefore display IsdB uniformly throughout the cell surface, bind hemoglobin at discrete locales. It is possible that in order to effectively bind hemoglobin, multiple tightly packed molecules of IsdB are employed or that IsdB requires another factor to bind Hb, and this factor is only present at these locales. Additionally, we observed an iron-dependent IsdB-independent hemoglobin binding activity conferred by IsdA and IsdH, consistent with their *in vitro* hemoglobin binding activity (24, 33, 91). Interestingly, Δ*spa*Δ*isdAB* was impaired in its ability to bind hemoglobin when grown in iron rich medium compared to Δ*spa*. This indicates that IsdA binds minimal levels of hemoglobin in iron-sufficient conditions, potentially providing *S. aureus* with a mechanism to acquire low levels of heme when the organism is not in an iron-starved state.

Levels of IsdA and IsdB expressed by *S. aureus* isolated from the hearts of infected mice are lower than those of "maximally starved" bacteria grown in TSB + 1 mM DIP (data not shown). By comparing the expression pattern of IsdB from *S. aureus* grown in iron-deplete media to that of IsdB from *S. aureus* removed from infected animals, we can estimate that *S. aureus* experience a level of iron starvation within the heart similar to that seen upon growth in TSB + 250 μM DIP. Furthermore, IsdB is not

uniformly localized on the surface of bacterial cells removed directly from infected organs. These results suggest that the punctate localization of IsdB observed in our *in vitro* experiments is recapitulated during staphylococcal infection.

Consistent with the observation that IsdB is detected by *S. aureus* in the hearts but not the livers of infected mice, Δ*isdB* is impaired in colonizing the heart while this strain colonizes livers at levels equivalent to wild type. Expression of IsdA is also lower in murine livers as compared to hearts. Taking into consideration the varying degrees by which iron impacts the regulation of IsdB and IsdA, we suggest that the heart provides an environment with less available iron than that found in the liver. This idea is supported by the fact that the liver is the major storage site for iron in vertebrates (43, 45). Further, liver is a common site of *S. aureus*–induced abscess formation and iron overload is associated with increased susceptibility to staphylococcal liver infection (21, 111). It has recently been shown that *S. aureus* recovered from murine airways bind considerable hemoglobin, suggesting that the respiratory tract is an iron-poor environment similarly to the heart (123). In addition, we have found that *S. aureus* is iron deficient when inside murine kidney abscesses (98). Thus, monitoring expression of Isd proteins and hemoglobin binding across murine organs can be used to predict the level of iron restriction experienced by *S. aureus* during the course of an infection. Heme-iron acquisition systems have been considered viable targets for novel antimicrobials and anti-staphylococcal vaccines (68, 115). The differential expression of these systems across organs should be taken into consideration when designing future therapeutic and preventive regimens.

CHAPTER III

SPECIFICITY FOR HUMAN HEMOGLOBIN ENHANCES *Staphylococcus aureus* INFECTION

I was a vampire, and she had the sweetest
blood I'd smelled in eighty years.
Edward Cullen, *Midnight Sun*

Introduction

Capture of hemoglobin by the hemoglobin receptor IsdB is a critical step in heme-iron acquisition (80, 93, 118). This process is required for pathogenesis as demonstrated by the decreased proliferation of *S. aureus* strains inactivated for *isdB* in murine models. Notably, the primary amino acid sequence of hemoglobin differs across species and variation within hemoglobin primarily localizes to surface exposed residues that are likely recognized by IsdB (Figure 12A). Further, nearly a thousand polymorphisms within the human hemoglobin (hHb) sequence have been identified to date (50). This suggests that interspecies variation and variation within hHb may impact iron capture, the host range, and severity of infection caused by *S. aureus*. Here we report that *S. aureus* IsdB preferentially recognizes human hemoglobin as compared to hemoglobin derived from other animal species. This preferential recognition results in enhanced bacterial proliferation and increased susceptibility of mice carrying hHb to staphylococcal infection. We show that preference for hHb is not confined to human isolates of *S. aureus* and is characteristic for animal isolates as well. Further, we demonstrate that certain hHb variants are differentially recognized by *S. aureus*. Finally, we identify tyrosine 165 within IsdB as an essential residue for both hHb and mHb binding and heme as a regulator of Hb binding.

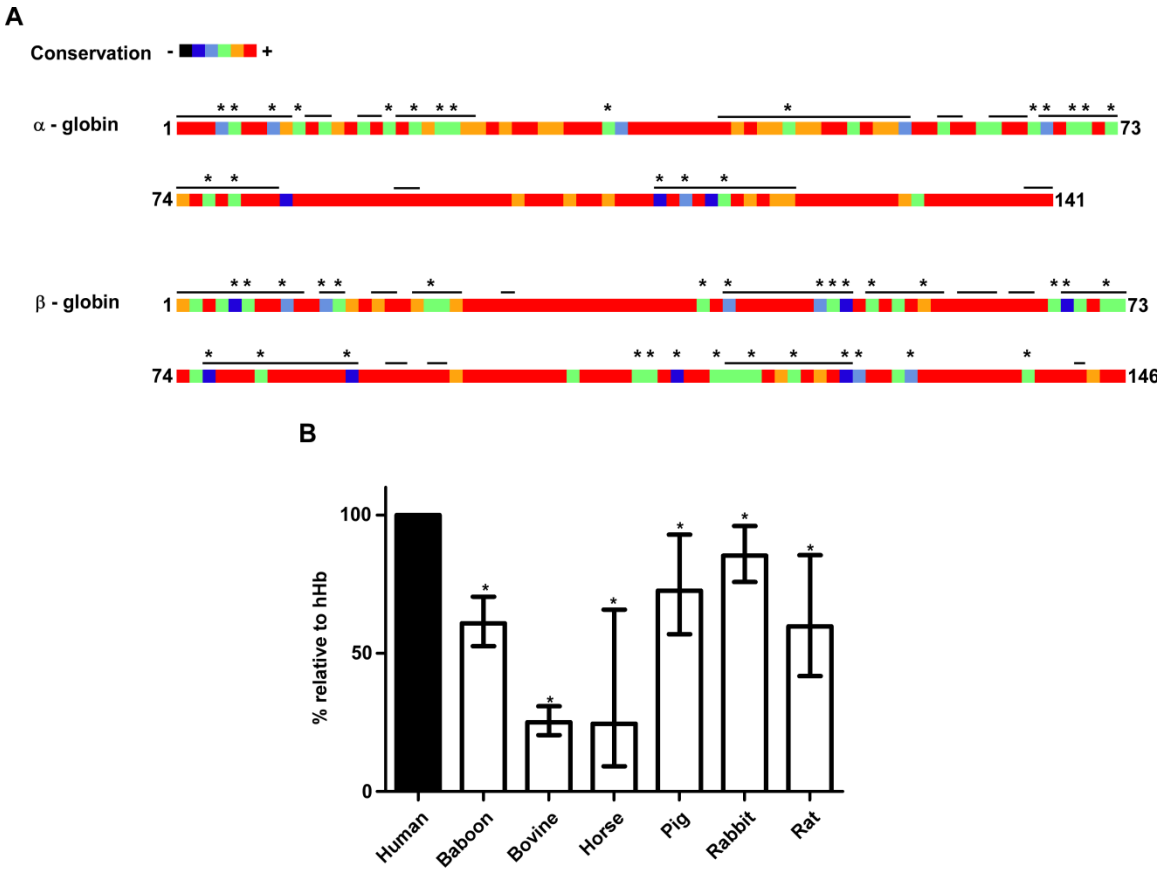

Figure 12. Sequence conservation and binding by *S. aureus* of hemoglobin derived from different species. A. Hemoglobin amino acid conservation based on alignment of human, mouse, baboon, bovine, horse, pig, rabbit and rat sequences prepared with Lasergene 6 software. Blue indicates little conservation, while red represents absolute conservation. Surface-exposed residues are marked with black horizontal bars. Residues that are divergent between mouse and human hemoglobin are marked with asterisks. **B.** Binding of animal hemoglobin (supplemented at 50 μg/ml) to the cell wall of *S. aureus* expressed in percent of bound human hemoglobin. Means and statistical significance were calculated based on logarithmically transformed fractions. Error bars represent confidence intervals (α=0.05); asterisks denote quantities of bound Hb statistically different from bound hHb (Student's two-tailed *t*-test, P<0.05). The graphs resulted from at least three independent experiments.

Methods

Bacterial strains and growth conditions. All experiments were carried out with *S. aureus* strain Newman (35), or with mutants generated in its background, unless indicated otherwise. The following strains of other bacteria were used for growth assays: *Acinetobacter baumannii* 17978, *Pseudomonas aeruginosa* PAO1, *E. coli* DH5α, *Staphylococcus lugdunensis* HKU09-01, *Staphylococcus simulans* TNK3, *Staphylococcus epidermidis* NRS6, *Bacillus cereus* 569, *Bacillus anthracis* Sterne, *Staphylococcus haemolyticus* NRS9, *Corynebacterium diphtheriae* C7(-) and *Shigella flexneri* SC560 (an M90T derivative with a Δ*icsA*::ΩSp^r mutation). All cultures were inoculated from a single colony and grown overnight (~20 hours) in 5 ml RPMI (Thermo) medium supplemented with 1% casamino acids (RPMI + CA) in 15 ml conical tubes at 37°C with shaking at 180 rotations per minute (rpm) unless noted otherwise. The isogenic variant lacking *isdB* (Δ*isdB)* has been described previously (80). A complementing plasmid containing Newman *isdB* (pOS1-isdB) has also been previously described (118). Alanine substitution mutations within *isdB* at positions Y165 and Y440 were generated using Pfu mutagenesis and confirmed by sequencing. In order to maintain the plasmids, the complemented strains were grown in the presence of chloramphenicol (10 μg/ml). RN6390Δ*isdB* has been described previously (117). Strains inactivated for *isdB* in RN4220 and USA300 were generated by transducing the Δ*isdB*::*ermC* allele from Newman Δ*isdB* using bacteriophage ϕ-85 (80). To generate a complementing plasmid containing *isdB* from RF122, *isdB* and its promoter were PCR-amplified from the chromosome of RF122 with IsdB-5_RF122-PstI (GGGGCTGCAG-ATAAATCATAATCACACTCATAAC) and IsdB-3_RF122-BamHI (CCCCGGATCC-

ACAGGCTACCTCATCCACC) primers. To generate a complementing plasmid containing *isdB* from ST398, *isdB* and its promoter were PCR-amplified from the chromosome of ST398 with IsdB-5_ST398-NsiI (GGGGATGCAT-TAAATAAATCTTCGTCACACTC) and IsdB-3_ST398-BamHI CCCCGGATCC-ATCGGAATACCTCATCTGCC) primers. The PCR reactions were digested with PstI (NsiI for ST398 *isdB*) and BamHI and cloned into pOS1 digested with PstI and BamHI. Cloning was confirmed with sequencing of the inserts. *isdB* Y165A mutation within the chromosome of *S. aureus* Newman was generated by PCR amplifying *isdB* from pOS1-*isdB* Y165A using IsdB-5'-AttB1 (GGGG ACAAGTTTGTACAAAAAAGCAGGCT - ATGAACAAACAGCAAAAAGAATT) and IsdB-3'-AttB2 (GGGG ACCACTTTGTACAAGAAAGCTGGGT – TTATTAGTTTTTACGTTTTCTAGG) primers. PCR was recombined into pKOR1 vector and mutagenesis was carried out as described previously (5). Mutation was confirmed by PCR.

Purification of human and mouse hemoglobin from fresh blood. Erythrocytes were sedimented by centrifugation (1,500 x *g*, 20 minutes, 4°C) from fresh human or mouse blood supplemented with anticoagulant. Erythrocytes were then washed 3 times with 3 volumes of ice cold saline (0.9% NaCl). Hemoglobin was released from erythrocytes by gently resuspending red blood cells in 1.5 volumes of 10 mM Tris-HCl (pH8.0) and 20% toluene (v/v) overnight on a rotisserie at 4°C. Hemolysate was separated from insoluble cellular debris (pellet) and membranes (toluene, upper layer) by a single centrifugation (20,000 x *g*, 1 hour, 4 C°). Hemolysate was then passed through a 0.4 μm filter. Hemoglobin was purified using an HPLC anion exchange column (Varian, PL-SAX 1000Å 8μm, 150mm x 4.6mm). The mobile phase A was 10 mM Tris-HCl (pH 8.0) and

mobile phase B was 10 mM Tris-HCl (pH 8.0) + 0.5 M NaCl. A 0-100% gradient of solvent B was run over 2 minutes at 2.0 ml/min flow rate. The eluant was monitored based on absorption (λ: 410 nm and 280 nm). Purified hemoglobin was dialyzed twice at 4°C against phosphate buffered saline (PBS). Final hemoglobin concentrations were measured by Drabkin's reagent (Sigma). Purified hemoglobin was stored in liquid nitrogen.

S. aureus hemoglobin binding assay. Hemoglobin was either purified from blood (Figure 13) or purchased (Sigma) (Figure 12). *S. aureus* was grown overnight in RPMI + CA supplemented with 0.5 mM of the iron chelator 2,2-dipyridyl (iron deplete) or 100 μM $FeCl_3$ (iron replete). Bacterial numbers were normalized to an optical density at 600 nm (OD_{600}) of 2.0. One ml of each culture was sedimented by centrifugation (3,000 x *g*, 10 minutes), resuspended in 1 ml of PBS containing the indicated concentrations of hemoglobin and incubated at 37°C for 0.5 hour with shaking at 180 rpm. Heme, where added was purchased from Sigma (51280). Upon completion of incubation, bacteria were washed 3 times with 1 ml of ice cold PBS, resuspended in 30 μl of 4% sodium dodecyl sulfate (SDS) 0.5M Tris-HCl (pH 8.0) and boiled for 5 minutes to release bound hemoglobin. *S. aureus* was then sedimented by centrifugation (16,000 x *g*, 5 minutes) and the supernatant containing hemoglobin was collected. Solubilized hemoglobin was subjected to 15% SDS-PAGE. Gels were silver stained (GE Healthcare Kit) and the relative abundance of bound hemoglobin was estimated based on the density of hemoglobin bands quantified by the Odyssey infrared imaging system (LI-COR) at 800 nm.

Immunoblotting of cell wall IsdB. Cell walls were solubilized by incubation of *S. aureus* in 20 μg/ml lysostaphin for 0.5 hour at 37°C. Cell wall proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk made in TBS with 0.1% Tween 20 (TBST) from 1 h to overnight. The membranes were then incubated in milk plus primary rabbit anti-IsdB (1:10,000), washed three times with TBST, incubated in milk plus 0.1% sodium dodecyl sulfate plus secondary Alexa Fluor 680 goat anti-rabbit IgG (H+L) (1:25,000), and washed three times in TBST. Membranes were visualized using an Odyssey infrared imaging system (Li-Cor).

IsdB–hemoglobin affinity measurement. Purification of recombinant IsdB has been previously described (80). Hemoglobin was biotinylated using EZ-Link NHS-LC-LC-Biotin (Pierce) at 1:2 protein:biotin ratio according to manufacturer's recommendations. Unbound biotin was removed with Zeba™ Desalt Spin Columns (Pierce 89889). Binding kinetics were measured with an Octet QK (ForteBio, Inc., Menlo Park, CA) apparatus. Briefly, streptavidin high binding capacity FA biosensors (ForteBio 18-5019) were loaded with biotinylated hemoglobin at 25 μg/ml. Upon washing in PBS the sensors were transferred to rIsdB solution (5 – 10,000 nM) to allow association between rIsdB and hemoglobin. The sensors were then transferred to PBS to measure dissociation. Dissociation constants were calculated using Origin 7.5 SR6 software (OriginLab Corp., Northampton, MA) based on data acquired from three experiments using an automated curve fitting prompted by the Octet 4 software (ForteBio).

Growth in liquid medium. Single colonies of *S. aureus* were inoculated into RPMI + CA supplemented with 0.5 mM of the iron chelator ethylenediamine-di(o-

hydroxyphenylacetic acid) (EDDHA, LGC Standards GmbH) and grown overnight. EDDHA was used in place of 2,2-dipyridyl due to the fact that EDDHA is less toxic to *S. aureus* in growth assays. One ml of overnight cultures was normalized to OD_{600} of 3.0, bacteria were sedimented (3,000 x *g*, 10 minutes), and resuspended in 1 ml NRPMI + 0.5 mM EDDHA. NRPMI was prepared in advance by treating RPMI + CA with Chelex 100 (Sigma) according to the manufacturer's recommendations and supplementing the resulting ion-deficient medium with 25 μM $ZnCl_2$, 25 μM $MnCl_2$, 100 μM $CaCl_2$ and 1 mM $MgCl_2$. The resulting suspension of *S. aureus* was subcultured (1:100) into 1 ml of NRPMI + 0.5 mM EDDHA + hemoglobin at indicated concentrations. One ml cultures were incubated at 37°C in 15 ml conical tubes on a rotating wheel. OD_{600} measurements were taken at indicated time-points by mixing 10 μl aliquots of the culture with 90 μl PBS in 96 well plates. The number of colony forming units per milliliter of culture were quantified by serial dilution and plating on tryptic soy agar.

Growth on solid medium. Single colonies of bacteria were inoculated into RPMI + CA + EDDHA (500 μM for *S. aureus* strains and *P. aeruginosa*, 250 μM for *B. anthracis*, and *S. lugdunensis*, 100 μM for *A. baumannii*, *S. haemolyticus*, *S. simulans*, *E. coli*, and *S. flexneri*, 25 μM for *S. epidermidis*, 10 μM for *B. cereus* and none for *C. diphtheriae*) and grown overnight. Overnight cultures were spread with cotton swabs on NRPMI agar (NRPMI + 1.2% Bacto Agar) supplemented with EDDHA (500 μM for *S. aureus* strains, *P. aeruginosa*, *B. anthracis*, *S. lugdunensis*, and *B. cereus*, 100 μM for *A. baumannii*, *S. haemolyticus*, *S. simulans*, *E. coli*, *S. flexneri*, and *S. epidermidis*). *C. diphtheriae* were grown on RPMI + CA agar supplemented with 1 μM EDDHA. Sterile Whatman (d = 7 mm) disks were impregnated with 10 μl PBS-hemoglobin (1 mg/ml), placed onto agar

and incubated at 37°C. Pictures were taken at a 72 hour time point except: USA500 at 96 hours, *B. cereus* at 14 hours, *B. anthracis* at 20 hours. Growth was measured by quantifying the distance between the edge of the disk and the edge of the zone of growth.

Systemic mouse infections. Seven week old C57BL/6J or human hemoglobin transgenic $\alpha^{H}\beta^{A}$ mice that were hemizygous for the transgene (104), but had no knock-outs or deletions, were infected retroorbitaly with ~10^7 CFU grown to mid-log phase in tryptic soy broth and resuspended in sterile PBS. Ninety-six hours post infection the mice were euthanized with forced inhalation of CO_2. The hearts and livers were removed *post mortem* and homogenized in 1 ml sterile PBS. Organ suspensions were serially diluted, plated on tryptic soy agar and incubated overnight at 37°C. The following morning the numbers of CFU/organ were quantified. Animal experiments were approved by the institutional animal care and use committee of Vanderbilt University.

Expression of recombinant human hemoglobin. pHb0.0 plasmid (a gift of Dr. John Olson) encoding hHb genes was transformed into *E. coli* strain BL21(DE3) and maintained by supplementation of tetracycline to Luria Bertani (LB) growth medium at 5 μg/ml (126). pHUG21 (a gift of Dr. Doug Henderson) harboring *Plesiomonas shigelloides* heme transport system was co-transformed in order to enhance rhHb expression and maintained by supplementation of ampicillin at 25 μg/ml. Five milliliter overnights were sub-cultured into 1L LB containing antibiotics and iron chelator ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) to 6.25 μg/ml. Cultures were incubated with shaking at 225 rotations per minute at 37°C until OD_{600} reached ~0.4. At that point the temperature was switched to 16°C and cultures were cooled for 1 hour. rhHb gene expression was then induced by the addition of isopropyl β-D-1-thiogalactopyranoside

(IPTG) to 40 μg/ml. Heme was supplemented to 15.6 μM. rhHb expression was allowed to continue at 16°C overnight. In the morning the bacteria were sedimented by centrifugation at 8,000 *g* for 10 minutes and frozen at -80°C. The pellet was thawed on ice and resuspended in 20 ml ice cold PBS containing 10 mM imidazole, 2 Mini Complete Protease Inhibitor tablets (Roche), 5 mM $MgCl_2$ and 1 mg/ml lysozyme. The pellets were homogenized with a glass homogenizer and incubated at room temperature for 15 minutes with gentle rocking. Bacteria were lysed by passage through a French press twice at 1,200 psi. Soluble fraction was separated by centrifugation at 100,000 *g* for 45 minutes. The supernatant was passed twice through 0.45 μm filter, loaded onto 1.5 ml of Ni-NTA beads (Qiagen) and incubated at room temperature for 15 minutes to allow Hb binding to the beads. Unbound protein was run off and the beads were washed with PBS containing 10 mM imidazole. Hb was eluted with 500 mM imidazole and dialized twice against PBS. Hb concentration was measured using Drabkin's reagent. Substitution mutations within hemoglobin genes were generated using Pfu mutagenesis and confirmed by sequencing.

Results

***S. aureus* preferentially recognizes human hemoglobin.** Many bacterial pathogens acquire nutrient iron from hemoglobin during infection. Interspecies variation in the primary amino acid sequence of hemoglobin suggests that bacterial pathogens may differentially recognize hemoglobin from distinct animals. Due to the extensive use of mice as animal models of *S. aureus* infections, we sought to compare the efficiencies with which *S. aureus* recognizes human (hHb) and mouse (mHb) hemoglobin. Hemoglobin was purified from fresh human or mouse blood and incubated with iron-starved *S. aureus* expressing the Isd system. Bound hemoglobin was then eluted and the relative amounts of hHb and mHb associated with the surface of *S. aureus* were compared. These experiments revealed that *S. aureus* binds hHb more effectively than mHb across a range of concentrations (Figures 13A). To test whether this preferential binding is dependent on IsdB we measured relative quantities of hHb and mHb bound by an isogenic *isdB* mutant (Δ*isdB*). *S. aureus* Δ*isdB* fails to bind increased quantities of hHb compared to mHb and this phenotype is fully complemented by providing a full length copy of *isdB in trans* (Figures 13B, C). These results demonstrate that *S. aureus* has evolved to bind hHb through IsdB with increased efficiency compared to mHb. The hHb preference of *S. aureus* is evident amongst hemoglobins from a variety of animal species suggesting that *S. aureus* has evolved to most efficiently recognize hemoglobin from its human host (Figure 12B).

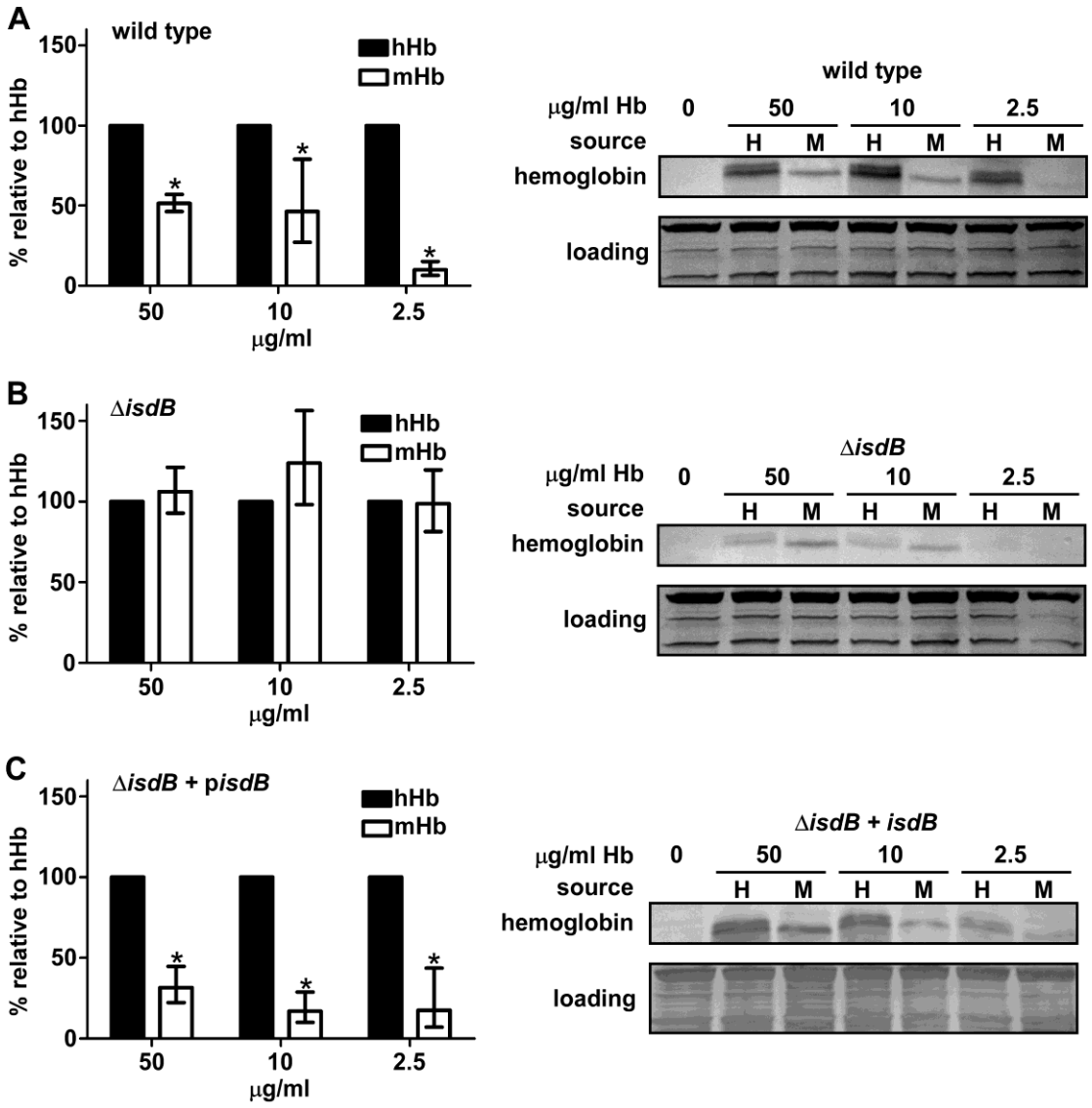

Figure 13. *S. aureus* displays increased binding of hHb as compared to mHb. Iron-starved *S. aureus* strain Newman were incubated with hemoglobin at the indicated concentrations and washed. Captured hemoglobin was eluted, subjected to SDS-PAGE, and silver stained. Bound hemoglobin was quantified based on the relative intensity of Hb bands. Relative quantities of cell-wall bound hemoglobin are expressed as percent of hHb bound by wild type (**A**), Δ*isdB* (**B**), and Δ*isdB* + p*isdB* (**C**). Representative silver stained gels of solubilized human (H) and mouse (M) hemoglobin eluted from the cell wall of *S. aureus* are on the right. "Loading" refers to non-hemoglobin banding patterns which were used as a loading control to confirm equal loading. Means and statistical significance were calculated based on logarithmically transformed fractions. Error bars represent confidence intervals (α=0.05); asterisks denote quantities of bound mHb statistically different from hHb supplemented at the same conditions (Student's two-tailed *t*-test, $P<0.05$). Each graph is a result of three independent experiments.

The IsdB-dependent requirement for the preferential binding of hHb to the surface of *S. aureus* suggests that IsdB binds hHb with an increased affinity as compared to mHb. To test this hypothesis, we measured the affinity of recombinant IsdB (rIsdB) for hHb and mHb by biolayer interferometry (Figure 14). In support of our *in vivo* findings, the K_D of the rIsdB-hHb interaction (5.5×10^{-8} M) is significantly stronger than the K_D of the rIsdB-mHb interaction (9.8×10^{-7} M). Notably, this calculated affinity for the interaction of IsdB and hHb is consistent with previously published findings (34). This result indicates that the preferential binding of hHb to the cell wall of *S. aureus* is achieved through a stronger interaction with the Hb receptor IsdB.

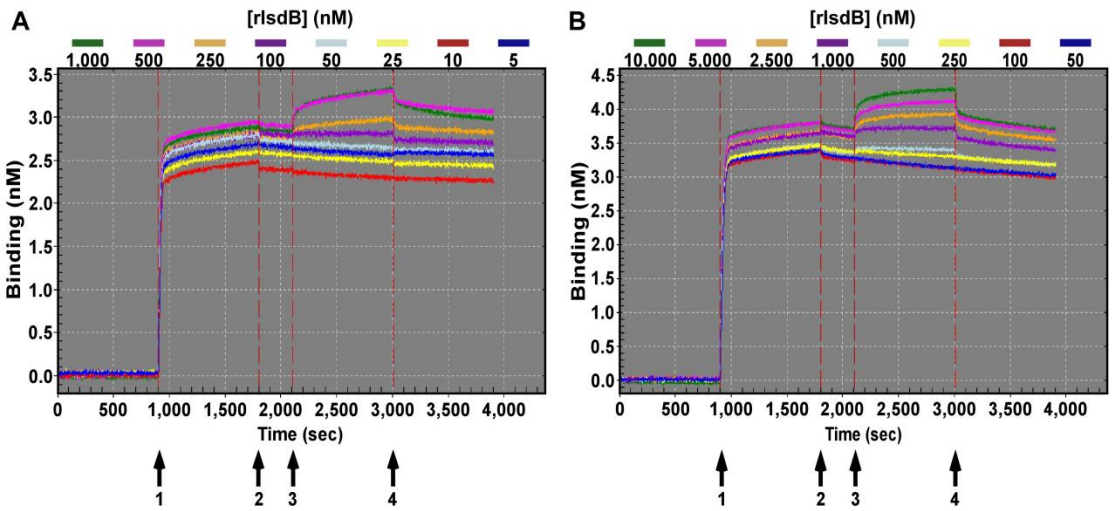

Figure 14. Real-time, label-free protein association or dissociation as detected by biolayer interferometry signal. Signal of rIsdB-hHb (**A**) and rIsdB-mHb (**B**) interactions. Numbers below the graphs represent critical steps in the experiments: 1. Streptavidin sensors are placed into wells containing biotinylated hemoglobin for 900 seconds. Increase in signal indicates loading of hemoglobin. 2. Sensors are transferred into buffer to wash off unbound hemoglobin and establish a baseline (300 seconds). 3. Sensors are transferred to wells containing indicated concentrations of rIsdB (900 seconds). Increase in signal indicates association of rIsdB with hemoglobin. 4. Sensors are transferred into buffer, decrease in signal indicates dissociation of rIsdB from hemoglobin (900 seconds).

***S. aureus* has evolved to acquire nutrient iron from hHb more efficiently than from mHb.** In iron-limiting conditions such as those encountered during infection (93, 98), hemoglobin is a preferred source of iron that is sufficient to provide *S. aureus* with iron necessary for growth (113, 118). Therefore, we sought to determine whether preferential binding of hHb correlates with an improved ability to utilize hHb as an iron source. To test this hypothesis we measured the capacity of hHb and mHb to support *S. aureus* proliferation in an otherwise iron-deficient medium. Iron-starved *S. aureus* were inoculated into medium containing either hHb or mHb as a sole source of iron and bacterial replication was monitored over time as a function of either optical density or enumeration of colony forming units. *S. aureus* supplemented with mHb displayed a significant delay in growth as compared to hHb supplementation (Figure 15A, B). These results reveal that *S. aureus* more efficiently utilizes hHb as an iron source as compared to mHb. The enhanced growth of *S. aureus* in the presence of hHb is dependent on IsdB, as indicated by Δ*isdB* exhibiting similar growth rates on either hHb or mHb (Figure 15C, D). Notably, Δ*isdB* does not display an altered growth pattern when non-hemoglobin sources of iron are available (118).

To further evaluate the efficiency of hHb-iron utilization, we monitored the ability of *S. aureus* to grow on solid medium where either hHb or mHb was the sole iron source. *S. aureus* was spread on iron-deficient medium containing discs impregnated with either mHb or hHb. The zone of growth around the disks was recorded as a measure of the ability of *S. aureus* to utilize Hb as an iron source. Growth around disks containing hHb was observed by 24 hours and continued to expand over the course of the experiment. In

contrast, growth was not detectable around the mHb containing disc until approximately 72 hours after inoculation (Figure 15E). These findings demonstrate that *S. aureus* has evolved to acquire nutrient iron from hHb more efficiently than from mHb, and the enhanced recognition of hHb is mediated by the hemoglobin receptor IsdB.

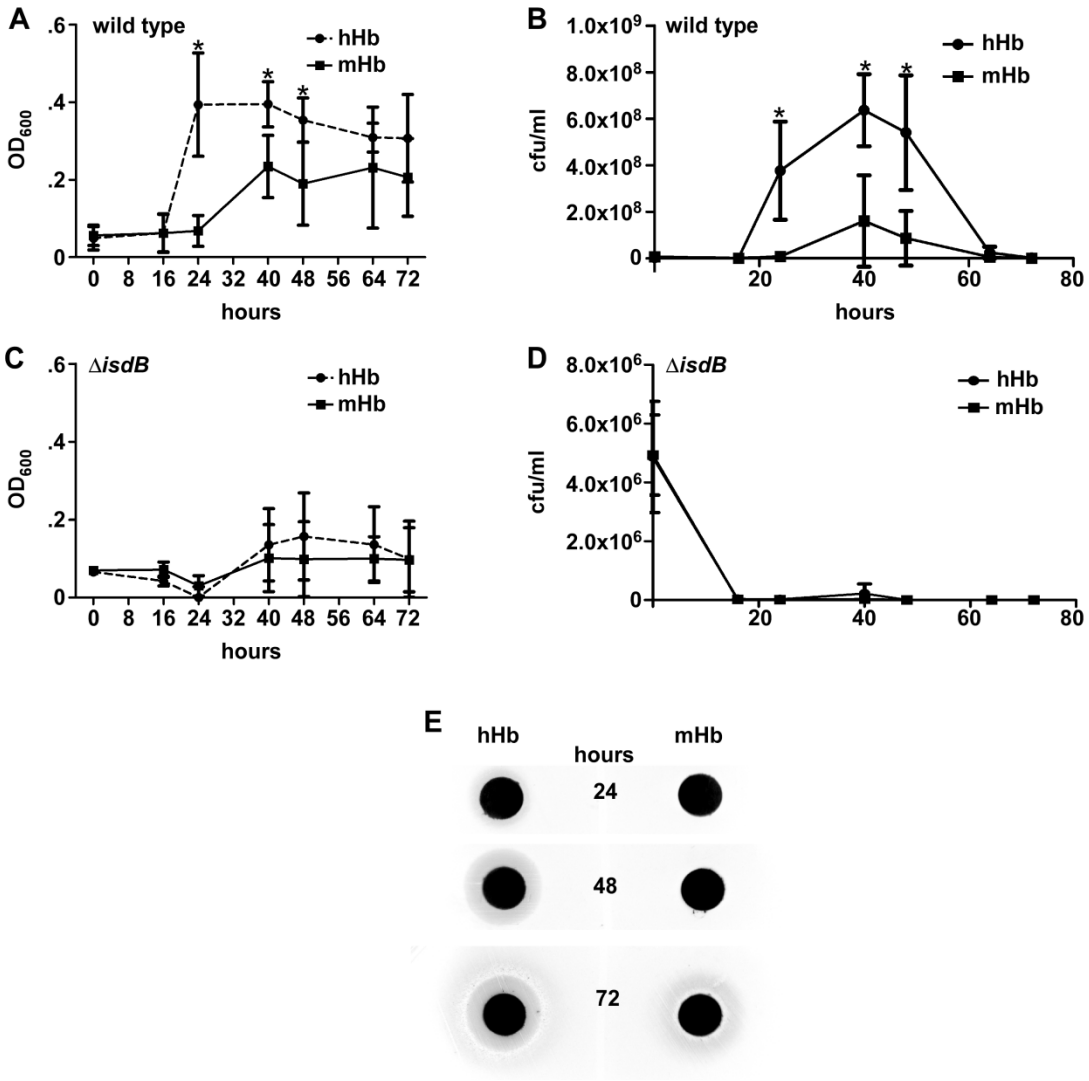

Figure 15. hHb promotes *S. aureus* replication in iron-limiting conditions. Growth of *S. aureus* Newman wild type (**A, B**) or Δ*isdB* (**C, D**) in liquid medium supplemented with 5 μg/ml hemoglobin as a sole source of iron was measured based optical density at 600 nm (OD_{600}) (**A, C**) or colony forming units per milliliter of liquid (**B, D**) over 72 hours (cfu/ml). The graphs represent a mean of three independent experiments. Error bars represent standard deviation; asterisks denote values upon hHb supplementation significantly different from values upon mHb supplementation at the same time point (Student's two-tailed *t*-test, $P<0.05$). **E.** Petri dishes containing iron-restrictive agar were streaked with bacterial cultures. Disks impregnated with 10 μg of hemoglobin were placed on top of the agar and *S. aureus* growth surrounding the disks was monitored over 72 hours. Opaque gray zones around disks indicate zone of growth. The images are a representative of five independent experiments.

Human hemoglobin exacerbates *S. aureus* infection in a murine model. Hemoglobin binding is a prerequisite for heme-iron acquisition during infection and as such, plays a critical role during *S. aureus* infection (93, 118). In order to test whether the increased specificity for hHb benefits *S. aureus* during infection, we examined the susceptibility of transgenic $\alpha^{H}\beta^{A}$ mice that express normal adult human hemoglobin to systemic staphylococcal infection (104). *S. aureus* were inoculated intravenously into wild type or $\alpha^{H}\beta^{A}$ mice and the infection was allowed to proceed for 96 hours. Following this time course, mice were sacrificed, organ tissues were removed and homogenized, and bacterial counts were enumerated. In accordance with an increased ability of *S. aureus* to utilize hHb as an iron source, $\alpha^{H}\beta^{A}$ mice were more efficiently colonized as compared to wildtype animals (Figure 16, top). The presence of human hemoglobin does not affect infection by Δ*isdB* as demonstrated by the similar susceptibility of wildtype and $\alpha^{H}\beta^{A}$ mice to Δ*isdB* (Figure 16, bottom). Thus, the increased susceptibility of $\alpha^{H}\beta^{A}$ mice to systemic *S. aureus* infection is fully dependent on hemoglobin binding by IsdB. These results demonstrate that the enhanced specificity of *S. aureus* for hHb translates into increased colonization and establishes $\alpha^{H}\beta^{A}$ as a humanized mouse that exhibits increased susceptibility to *S. aureus* infections. Importantly, $\alpha^{H}\beta^{A}$ mice express approximately equal levels of both hHb and mHb suggesting that the effects observed here may underestimate the contribution of hHb to staphylococcal infection in humans. In addition, total Hb concentration does not differ between $\alpha^{H}\beta^{A}$ and wild type animals therefore variations in susceptibility are not due to hemoglobin abundance (data not shown). Knock-in mice that express exclusively hHb have been previously generated. However, hHb knock-in mice are notoriously difficult to breed and are therefore

unsuitable for infection models, which require high numbers of subjects in order to evaluate statistical significance (130).

Preference for human hemoglobin varies across pathogens. The experiments described above were performed using *S. aureus* Newman, a commonly studied laboratory strain. To assess the ability of other laboratory and clinically relevant *S. aureus* isolates to acquire iron from mHb and hHb we tested the Hb preference of a panel of staphylococcal strains. As demonstrated in Figure 17A, all tested *S. aureus* isolates display increased binding of hHb as compared to mHb. Isogenic Δ*isdB* mutants of clinically relevant USA300 and the common laboratory strains RN4220 and RN6390 lost the ability to bind increased quantities of hHb as observed with strain Newman (Figures 17B and 13B). In support of the role of IsdB in increased hHb binding, *S. aureus* do not differentiate between hHb and mHb when grown under iron-replete conditions that prohibit *isdB* expression (Figure 17C). Further, disk diffusion assays demonstrated increased proliferation using hHb as a sole iron source as compared to mHb for all tested *S. aureus* strains (Figure 17D). These results demonstrate that the preferential utilization of hHb as an iron source by IsdB is conserved among tested *S. aureus* isolates.

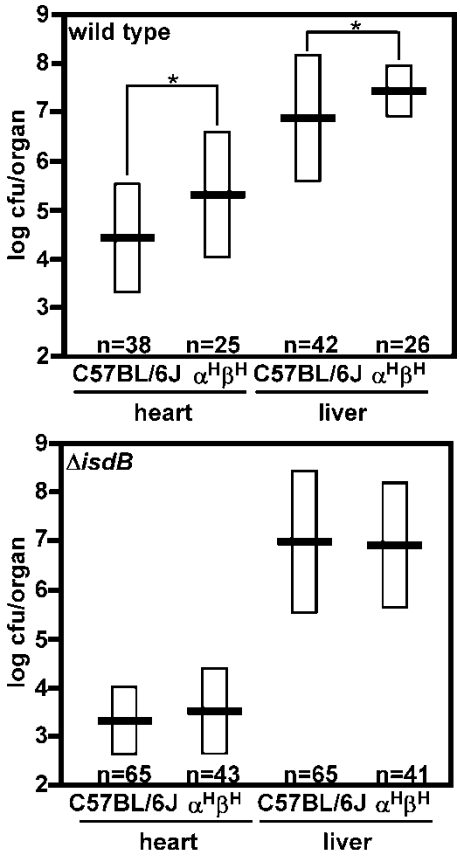

Figure 16. Mice expressing human hemoglobin exhibit increased susceptibility to *S. aureus*. Number of colony forming units (CFU) of *S. aureus* Newman isolated from organs of systemically infected C57BL/6J and $\alpha^{H}\beta^{A}$ mice 96 hours post-inoculation as determined by serial dilution. Data were logarithmically transformed prior to statistical analyses. Horizontal bars represent the average values of CFU/organ, boxes represent standard deviation. Asterisks denote significantly different values (Student's two-tailed *t*-test, $P<0.05$). The graphs represent combined data acquired from multiple independent experiments.

Numerous bacterial pathogens express hemoglobin receptors and utilize hemoglobin as an iron source during infection (29). In keeping with this, we evaluated the ability of a number of bacterial species to grow in the presence of hHb and mHb. Many organisms that do not express hemoglobin receptors were unable to proliferate in the presence of either hHb or mHb, including *Escherichia coli* DH5α, *Staphylococcus haemolyticus, Staphylococcus epidermidis* and *Shigella flexneri* (data not shown). In contrast, *Staphylococcus lugdunensis, Staphylococcus simulans* and *Corynebacterium diphtheriae* displayed a preference for hHb similar to *S. aureus* (Figure 17E). Finally, *Acinetobacter baumannii, Pseudomonas aeruginosa, Bacillus anthracis* and *Bacillus cereus* utilized mHb and hHb with equal efficiency. These results demonstrate that the preferential utilization of hHb as an iron source is conserved across some bacterial pathogens while others do not discriminate between hHb and mHb.

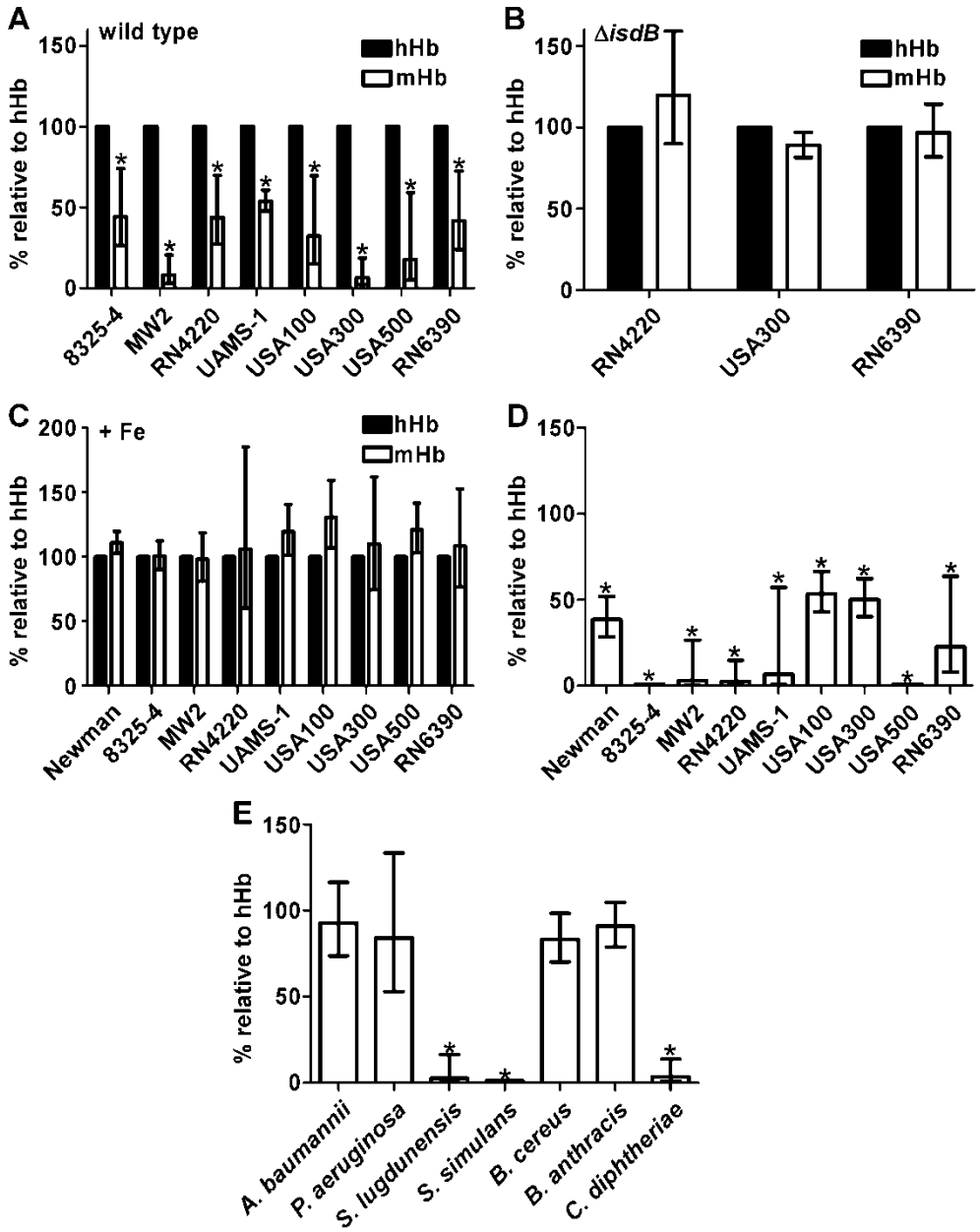

Figure 17. Bacterial pathogens vary in preference of hHb over mHb. A., **B.** and **C.** Binding of hHb and mHb by *S. aureus* strains was assessed as in Figure 1. Petri dishes containing iron-restrictive agar were streaked with strains of *S. aureus* (**D**), and other bacterial pathogens (**E**). Disks impregnated with 10 μg of hHb or mHb were placed on top of the agar and bacterial growth surrounding the disks was measured. The graphs depict growth on mHb as a percentage of growth on hHb in the same conditions (growth on hHb = 100%). The graphs represent a mean of three to four independent experiments. Means and statistical significance were calculated based on logarithmically transformed fractions. Error bars represent confidence intervals (α=0.05); asterisks denote growth on mHb that is statistically different from growth on hHb supplemented at the same conditions (Student's two-tailed *t*-test, P<0.05).

Animal isolates of *S. aureus* display hHb preference. *S. aureus* causes infections in numerous species. Because human isolates of *S. aureus* uniformly display preference for hHb, we investigated whether isolates from other species preferentially bind Hb derived from their host. To this end we have acquired isolates from bovine (RF122), pig (ST398 and t337), mouse (DAK), rat (ALR), and primate (NPRC3, NPRC6, and NPRC53) hosts. All of the above isolates, as well as all sequenced strains of *S. aureus*, carry full-length *isdB*. Surprisingly, all tested strains displayed preferential cell wall binding of hHb over Hb derived from the host they were isolated from (Figure 18A, B and data not shown). To confirm that hHb preference was due to IsdB-mediated binding, we have cloned *isdB* from RF122 and ST398 into the pOS1 expression vector, which was then transformed into Newman Δ*isdB*. RF122 and ST398 *isdB* expressed in Newman Δ*isdB* background conferred increased binding of hHb to the cell wall of the bacterium (Figures 18C and 13C). These results indicate that animal isolates of *S. aureus* display increased binding of hHb through interaction with IsdB.

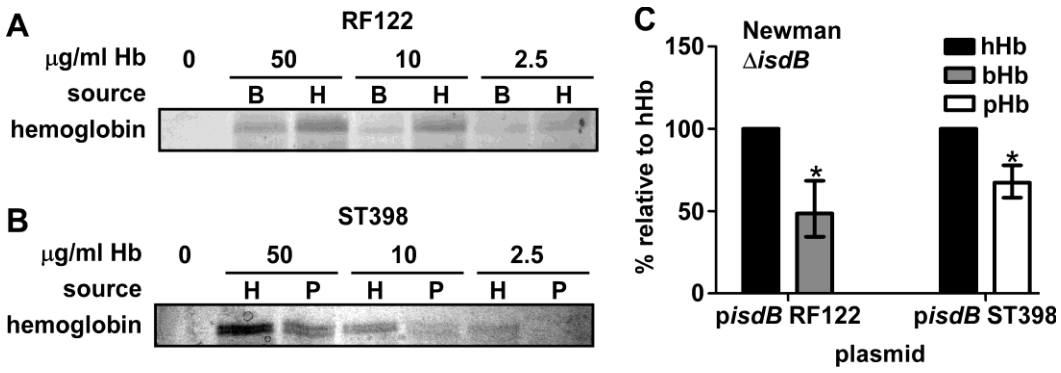

Figure 18. Animal isolates of *S. aureus* display increased binding of hHb as compared to Hb of their host. Iron-starved *S. aureus* strain Newman were incubated with hemoglobin at the indicated concentrations and washed. Captured hemoglobin was eluted, subjected to SDS-PAGE, and silver stained. **A.** and **B.** Silver stained gels of solubilized human (H), bovine (B) and pig (P) hemoglobin eluted from the cell wall of indicated strains of *S. aureus*. **C.** Relative quantities of human (hHb), bovine (bHb) and pig (pHb) hemoglobin bound by Newman Δ*isdB* carrying a plasmid with *isdB* from an animal isolate. Hb was supplemented at 10 μg/ml. Bound hemoglobin was quantified based on the relative intensity of Hb bands. Means and statistical significance were calculated based on logarithmically transformed fractions. Error bars represent confidence intervals (α=0.05); asterisks denote quantities of bound Hb statistically different from hHb supplemented at the same conditions (Student's two-tailed *t*-test, $P<0.05$). Each graph is a result of three independent experiments.

Human hemoglobin variants are differentially recognized by *S. aureus*. We have demonstrated that *S. aureus* has adapted to bind hHb with a higher affinity than Hb derived from other mammals. Importantly, hHb is highly diverse across the human population with numerous polymorphisms identified to date. Certain substitutions found within hHb result in debilitating diseases such as sickle cell anemia, while others are not associated with disorders. To generate a subset of hHb variants, we have acquired a plasmid that allows expression and purification of recombinant hHb (rhHb) from *E. coli*. Due to the fact that mHb is bound by *S. aureus* with a decreased affinity compared to hHb, we have chosen to test hHb variants with substitutions in the residues that are: a) different between hHb and mHb and are b) on the outer, external face of the molecule (Figure 19 A,B). With an exception of the β-globin V20E substitution that is associated with mild erythrocytosis (increase in RBC count), none of the selected variants are known to cause abnormalities. A number of substitutions found within the human population resulted in decreased binding of rhHb to the cell wall of *S. aureus* (Figure 19C). In particular, we observed a pronounced reduction in binding of α-globin A5D variant to the surface of *S. aureus*. Notably, mHb also contains an aspartate at the position 5. This indicates that aspartate instead of alanine at position 5 of mouse α-globin is at least in part responsible for the decreased affinity of IsdB to mHb as compared to hHb. These results indicate that certain hHb variants found in the human population are bound by *S. aureus* with a decreased efficiency compared to the most prevalent allele.

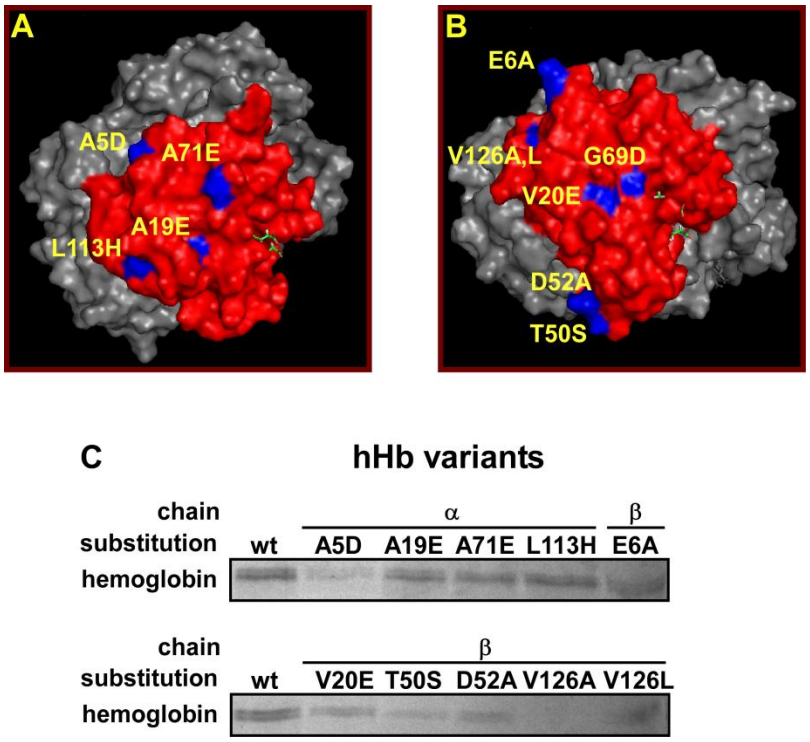

Figure 19. *S. aureus* differentially binds hHb variants. A. and **B.** Residues within recombinant hHb (rhHb) that were selected for mutagenesis are depicted in blue. One of the two α (**A**) or β (**B**) chains of the Hb tetramer is depicted in red. wt refers to the most prevalent variant of Hb. **C.** Iron-starved *S. aureus* strain Newman were incubated with Hb at 2.5 μg/ml and washed. Captured hemoglobin was eluted, subjected to SDS-PAGE, and silver stained.

Tyrosine Y165A is required for Hb binding by IsdB. We have established that IsdB is a cell wall anchored, surface exposed receptor that is adapted to bind hHb with increased efficiency compared to Hb derived from other species. To test whether IsdB interacts with hemoglobin from different species through the same mechanism, we have generated a mutant IsdB with a conserved tyrosine 165 residue substituted for alanine (*isdB* Y165A). This residue has been shown to contribute to Hb binding by IsdH (92). Cell wall expression of a *isdB* Y165A eliminates hemoglobin binding, establishing this residue as critical for both hHb and mHb recognition by IsdB (Figure 20A). IsdB Y165A is expressed at the same amount as wt IsdB from the pOS1 vector (Figure 20A, bottom insert). To confirm the role of Y165 in Hb binding, we generated a chromosomal Y165A mutation in Newman and assessed the ability of this mutant to bind Hb. IsdB expressed from the chromosome carrying Y165A mutation was deficient in binding Hb, although its expression level was not altered (Figure 20B,C). These results demonstrate the absolute requirement of the conserved tyrosine 165 within IsdB for Hb binding to the cell wall of *S. aureus.*

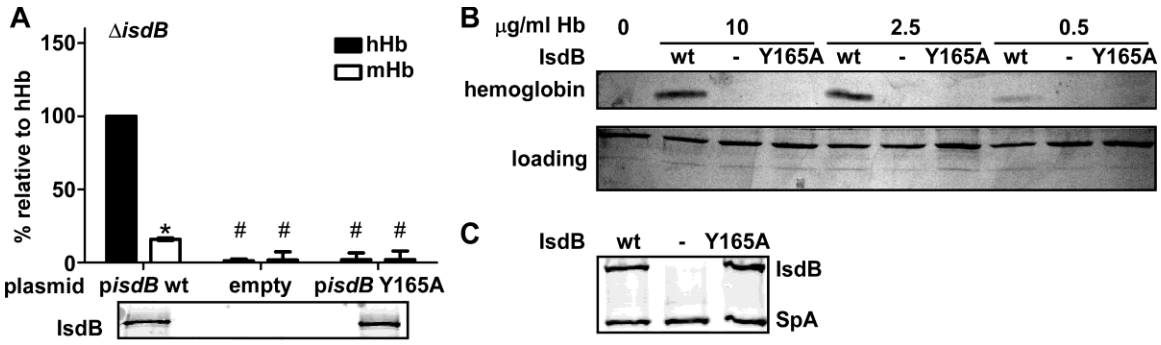

Figure 20. Tyrosine-165 is required for Hb binding activity of IsdB. A. Iron-starved *S. aureus* strain Newman Δ*isdB* harboring the indicated plasmids were incubated with hemoglobin at 10 μg/ml. Captured hemoglobin was eluted, subjected to SDS-PAGE, and silver stained. Bound hemoglobin was quantified based on the relative intensity of Hb bands. Relative quantities of cell-wall bound hemoglobin are expressed as percent of hHb bound. Insert below panel is an image of an anti-IsdB immunoblot, demonstrating cell wall IsdB expression. Means and statistical significance were calculated based on logarithmically transformed fractions. Error bars represent confidence intervals (α=0.05). * denotes quantities of bound mHb statistically different from hHb, # denotes quantities that are significantly different from hHb and mHb bound by Δ*isdB* + p*isdB* (wt) supplemented at the same conditions (Student's two-tailed *t*-test, $P<0.05$). Each graph is a result of three independent experiments. **B.** Silver stain of hHb bound by Newman wt, Δ*isdB*, and Newman carrying Y165A mutation within chromosomal *isdB*. Hemoglobin was supplemented at indicated concentrations. **C.** Anti-IsdB immunoblot, demonstrating cell wall IsdB expression by Newman wt, Δ*isdB*, and Newman carrying Y165A mutation within chromosomal *isdB*. SpA is protein A, used here as a loading control.

Heme regulates Hb binding by *S. aureus.* IsdB contains distinct putative hemoglobin and heme-binding domains (92). Upon binding of Hb, IsdB extracts heme and subsequently passes it to IsdA or IsdC (87, 131). The mechanism of hemoglobin release upon heme extraction, however, has not been elucidated. To test whether heme binding regulates Hb binding in IsdB, we cultured *S. aureus* in iron poor conditions in the presence of heme. *S. aureus* grown in the presence of as little as 2 μM heme was unable to bind hemoglobin (Figure 21A). Notably the expression levels of IsdB on the cell wall were not affected by the presence of heme in the growth medium (Figure 21B). Other changes in the expression profile of *S. aureus* induced by heme might have affected its affinity for Hb. To eliminate this possibility we have cultured *S. aureus* in the absence of heme and supplemented heme together with Hb. The concentrations of heme above 0.5 μM were inhibitory to Hb binding to the cell wall of *S. aureus* (Figure 21C). To test whether heme inhibition occurred through specific binding to IsdB, we have expressed an IsdB mutant predicted to be deficient in heme binding (Y440A). Heme-binding-deficient IsdB is not sensitive to heme inhibition of Hb binding (Figure 21C). These results suggest that IsdB-Hb interaction is regulated by the heme status of IsdB.

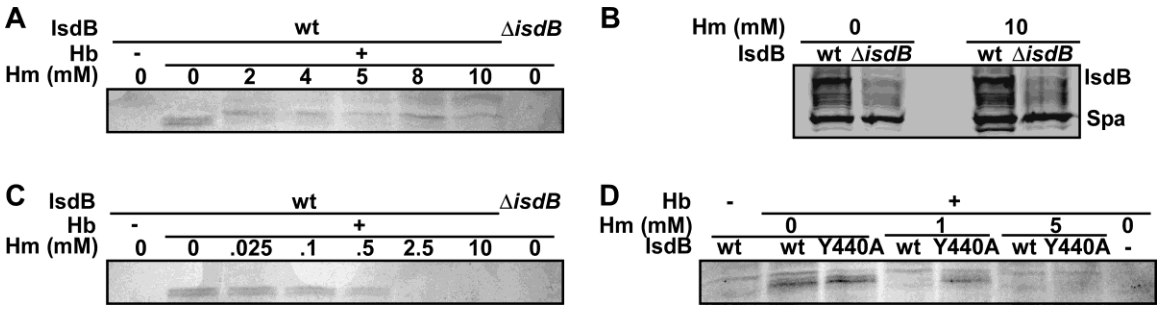

Figure 21. Heme regulates Hb binding to the cell wall of *S. aureus.* A. *S. aureus* were grown overnight in the presence of indicated concentration of heme. *S. aureus* were then incubated with hemoglobin at 2.5 μg/ml. Captured hemoglobin was eluted, subjected to SDS-PAGE, and silver stained. Note: the band in heme-grown cultures is likely a *S. aureus* protein up-regulated by heme. **B.** Anti-IsdB immunoblot, demonstrating cell wall IsdB expression by *S. aureus* grown in the presence or absence of heme. **C.** Iron-starved *S. aureus* strain Newman were incubated with hemoglobin at 2.5 μg/ml and heme at indicated concentrations. Captured hemoglobin was eluted, subjected to SDS-PAGE, and silver stained. **D.** Iron-starved *S. aureus* strain Newman Δ*isdB* harboring plasmids with wt or Y440A *isdB* were incubated with hemoglobin at 10 μg/ml. Captured hemoglobin was eluted, subjected to SDS-PAGE, and silver stained.

Discussion

Herein, we demonstrate that human hemoglobin is a factor that impacts the host susceptibility to *S. aureus* through its interaction with the hemoglobin receptor IsdB. The significant affinity of IsdB for human hemoglobin permits the efficient utilization of hHb as an iron source leading to increased colonization and disease. Importantly, all tested clinically relevant strains display increased iron acquisition from hHb. By exploiting these observations, we have established $\alpha^{H}\beta^{A}$ mice as an improved murine model for studies into the pathogenesis of staphylococcal infections.

Our results reveal that a variety of distinct pathogens display an enhanced ability to utilize hHb as an iron source, while others do not exhibit hemoglobin preference (Figure 4D). Notably, bacteria that primarily associate with humans (*S. aureus, S. lugdunensis, S. simulans, C. diphtheria*) display preference for hHb over mHb, whereas environmental bacteria that infect numerous hosts (*A. baumannii, P. aeruginosa, B. anthracis* and *B. cereus*) grow at comparable levels on hHb and mHb. In this regard, *S. aureus* IsdB binds hHb with a much stronger K_D value (5.5×10^{-8} M) than the *B. anthracis* hemoglobin binding protein IsdX1 (7.3×10^{-6} M) (78). This supports the hypothesis that *S. aureus* IsdB is optimized to bind hHb in order to acquire iron and colonize humans.

Much of what has been learned regarding the pathogenesis of *S. aureus* infection has been obtained from murine models of infection. However, due to inherent differences between mice and humans, murine infection models do not perfectly recapitulate human disease. To improve on this shortcoming, significant effort has been devoted to the development of humanized mouse models that more accurately reflect human disease

(71, 110). To date, few humanized mouse models have been established that exploit non-immune host factors (57, 69, 116). Our findings add the human hemoglobin expressing mouse to the list of humanized animals that are valuable tools for modeling infection. Moreover, these findings demonstrate that humanized mouse models can be created that exploit the nutrient requirements of bacterial pathogens. Importantly, many bacterial pathogens utilize hemoglobin as an iron source; therefore human hemoglobin expressing mice may be valuable for studies into a variety of infectious diseases (29).

We have found that animal isolates of *S. aureus* preferentially bind human hemoglobin through the interaction with IsdB similarly to human isolates. This could be explained by the fact that strains of *S. aureus* that associate with animals have their origins in humans and have switched their host in a single jump (48, 52, 74). In support of this notion animal isolates can readily infect humans as demonstrated by transmission of ST398 from pigs to humans. Human ST398 infections lead to a significant increase in community acquired infections in Europe and spread of the isolate to the United States (38). It is therefore possible that all *S. aureus* strains preferentially recognize human hemoglobin due to the fact that all strains of *S. aureus* were at one point associated with humans. Interestingly, rabbit hemoglobin is recognized by *S. aureus* nearly as well as human hemoglobin. In parallel are observations that other *S. aureus* virulence determinants interact with human and rabbit factors with an increased specificity as compared to other animals. Examples include increased binding of rabbit and human IgGs by *S. aureus* protein A, enhanced cell lysis by α-toxin and Panton-Valentine leukocidin, and fibrinogen coagulation by coagulase (26, 53, 73, 101, 125).

Numerous polymorphisms are identified within hHb. Many of these polymorphisms are located on the outer face of the Hb tetramer within residues that are potentially recognized by IsdB and may therefore affect IsdB-Hb interaction. We have chosen to initially focus on polymorphisms in amino acids that are different between hHb and mHb, due to the difference in the affinity of IsdB for these two hemoglobins. Here we demonstrate that single amino acid changes corresponding to hHb polymorphisms may affect the ability of *S. aureus* to bind hHb. The change in the affinity to hHb may potentially translate into altered ability of *S. aureus* to acquire iron and infect the carrier of the hHb polymorphism. Encouraged by these results we will extend our studies into other variants of hHb. As discussed in the future directions, we will utilize a bacterial two-hybrid system to screen other hHb variants to identify those that are differentially recognized by IsdB. Notably, at least one hHb polymorphism is known to affect resistance to infectious disease. Valine substituted for glutamate in the β-chain of hHb renders hHb unavailable to *Plasmodium* species, conferring its carrier resistance to malaria (3). This is a classic example of how a substitution within a host molecule affects the pathogen-host interaction. Together these findings raise the exciting possibility that hHb polymorphisms may have implications regarding individual susceptibility to bacterial infections (50).

Until this work residues involved in the IsdB-Hb interaction have not been identified. Here we demonstrate the requirement of tyrosine 165 to hemoglobin binding by *isdB*. Introduction of Y165 alanine substitution leads to an elimination of hHb and mHb binding by *S. aureus* similar to knocking out IsdB. We have confirmed the critical role of Y165 in Hb binding when IsdB is expressed from either plasmid or chromosome.

To our knowledge this is the first report of a successful single amino acid substitution within a chromosomal gene of *S. aureus.* These results lay the groundwork for further investigation of the mechanism of IsdB-Hb interaction.

Upon binding of holo-Hb (heme-bound), IsdB extracts heme and passes it to IsdA and IsdC (87, 131). The mechanism of Hb release, however, remains to be elucidated. The Hb receptor in *B. anthracis* was found to bind apo-Hb (heme-free) with a lower affinity than holo-Hb (78). This allows *B. anthracis* to release apo-Hb upon heme extraction. In contrast, apo-Hb binds to the cell wall of *S. aureus,* as efficiently as holo-Hb (preliminary data not shown). Here we report that heme inhibits Hb binding to *S. aureus.* We propose that upon heme extraction from hemoglobin, heme-bound IsdB lowers its affinity to Hb. In other words, the heme status of IsdB and not Hb determines the affinity of interaction. In support of this hypothesis, IsdB that is predicted to be deficient in heme binding, is not inhibited in its ability to bind Hb by the addition of heme. Further experiments are warranted to test the hypothesis and are described in the future directions.

CHAPTER IV

CONCLUSIONS

"Blood is a very special juice"
Johann Wolfgang von Goethe, *Faust*

Summary

S. aureus is the deadliest infectious agent in the United States (65). In addition to the toll on human life, *S. aureus*, is responsible for an estimated 14 billion dollars in health-care costs due to health-care associated infections alone (88). The data in the developing world are not readily available, but the burden of *S. aureus* infections in third world countries is likely as substantial as in the U.S. More alarming is the increase in incidence of *S. aureus* infections in recent three years (62). A number of characteristics allow *S. aureus* to stand out as a successful pathogen. First, *S. aureus* is endowed with an impressive arsenal of virulence factors that damage host tissues and manipulate the immune system. Second, *S. aureus* has the capacity to invade and proliferate within virtually any tissue of the host. Third, *S. aureus* is rapidly acquiring antibiotic resistance to all current antimicrobials, frequently without fitness costs that are typically associated with resistance. Fourth, newly emerging strains are more virulent and antibiotic resistant than the preceding isolates. These strains are not confined to the hospital setting and frequently infect otherwise healthy individuals. These points emphasize the importance of identifying novel targets for future antimicrobial development.

One such target is iron acquisition which is absolutely required for the survival and replication of bacteria. Therefore, antimicrobial therapies which inhibit iron acquisition would target a variety of essential cellular processes of the bacterium. The

most abundant and bioavailable source of iron during infection is Hb-bound heme. In order to acquire iron from Hb, *S. aureus* lyses red blood cells releasing Hb. Cell wall-expressed IsdB binds Hb and extracts heme from the globin portion of the molecule. IsdB subsequently passes heme to IsdA and other cell envelope Isd proteins, which transport heme into the cytoplasm. Finally, heme is degraded to release elemental iron. Isd-mediated iron acquisition from the host Hb is critical for *S. aureus* infection as indicated by a significant decrease in virulence of strains inactivated for components of the Isd system.

Since its recent discovery the Isd has become one of the most studied iron acquisition systems in bacteria. Interest in the Isd system is fueled by the fact that its different components are conserved among numerous Gram positive pathogens. Further, Isd is the only identified Gram positive cell wall transport system and delineation of its mechanism uncovers aspects of basic bacterial physiology. Studies outlined in this thesis are primarily focused on the function and interactions of the Hb receptor IsdB in the context of infection. Our findings contribute to the model of iron acquisition and nutrient transport in Gram positive bacteria.

In Chapter II, I describe the elucidation of the regulation and localization of IsdB at the cell wall and its expression during *S. aureus* infection. Cell wall Isd proteins are covalently anchored to murein and are therefore immobile. To bind Hb, IsdB has to be exposed on the surface. Further, to pass heme to the Isd system, IsdB has to be in direct proximity to other Isd proteins. Because of the sub-micrometer size of a *S. aureus* bacterium and the presence of non-specific antibody-binding Protein A (SpA), studies of protein localization within *S. aureus* are few. In order to circumvent the antibody-binding

activity of SpA, we have generated a knock out strain (Δ*spa*) deficient in SpA. This strain was used for all immunofluorescence and immunogold studies we have carried out. Importantly, expression levels of Isd proteins are unaffected in Δ*spa*. Using immunofluorescence we have demonstrated iron regulation and surface localization of IsdB. We have also observed a similar pattern of IsdA expression and localization. Double staining revealed that IsdA and IsdB co-localize on the cell surface. Additionally IsdA and IsdB form an interaction within staphylococcal cell wall and *in vitro*. Co-localization and interactions of IsdB with IsdA are consistent with their function as a conduit of heme through the cell wall.

To get insight into the mechanism which allows for IsdA and IsdB co-localization, we carried out experiments which pinpoint the site of their deposition to the cell wall. To increase resolution we utilized electron microscopy coupled with immunogold labeling. We have found that IsdB is exclusively anchored at the site of cell division. IsdA is also deposited at the same site, although some of it is deposited at a distant locale. This indicates that *S. aureus* ensures co-localization of the Isd components by anchoring them to the cell wall at the same site.

While investigating cell wall expression of the Isd machinery, we have discovered that the pattern of its localization is dependent on the quantity of expressed protein. In low-iron conditions, when expression is maximal, Isd is uniformly distributed throughout the cell surface. In contrast, in the conditions of lower expression levels under moderate iron stress, Isd is confined to distinct puncta within the cell wall. Discrete localization increases the local concentration of the Isd components, which may be necessary for their function as a heme import machinery. Importantly, we have found that staphylococci

isolated from the hearts of infected mice display punctuate localization of IsdB. This indicates that targeting of Isd to discrete locales on the surface of *S. aureus* is relevant in the context of infection.

Systemic murine infections revealed differential expression of IsdB dependent on the site of invasion. *S. aureus* infecting mouse hearts express both IsdA and IsdB at significant levels. In contrast, *S. aureus* isolated from the livers express lower amounts of IsdA and undetectable levels of IsdB. In support of organ specific expression profiles, IsdB is required for full virulence of *S. aureus* within the hearts, but not the livers of infected mice. We propose that differential expression of IsdA and IsdB are indicative of different levels of available iron within these two organs. This is supported by the fact that liver is a major site of iron storage in the body. Therefore it is likely that bacteria invading the liver experience a relatively iron-rich environment and down-regulate iron acquisition systems. Vaccines against IsdB have been shown to elicit a response against *S. aureus* infections in mice, non-human primates, and humans, supporting its role in *S. aureus* pathogenesis (36, 51, 63, 68, 115). Antibodies directed against IsdB appear to function through inhibition of iron acquisition rather than through opsonization of *S. aureus* (63). We propose that by monitoring the expression of Isd proteins during infection, one can predict the iron availability within the infection site. Considering the impact of iron concentration on staphylococcal virulence, organ specific variations in iron availability may contribute significantly to the types of *S. aureus* infections that present clinically.

In chapter III, I describe investigations into the specificity of IsdB-Hb interaction. The amino acid sequence of Hb significantly varies across animal species. Most variation

occurs within the external residues that are more likely to interact with IsdB than residues embedded within Hb. We demonstrate that these differences result in differential recognition of Hb from distinct species by *S. aureus*. Further, *S. aureus* preferentially binds human Hb (hHb) over Hb from other animals. Because mouse models of infection are commonly used to study bacterial pathogenicity, we focused on differences between the interaction of hHb and mouse Hb (mHb) with IsdB. As with Hb derived from other animals, *S. aureus* demonstrates a significant preference for hHb over mHb. This preference is entirely dependent on IsdB, as confirmed by the lack of discrimination between mHb and hHb by Δ*isdB*. Further, IsdB binds hHb *in vitro* with a much stronger affinity than mHb. We have found that the increased specificity of IsdB for hHb results in enhanced iron acquisition from hHb as compared to mHb (94).

To assess the contribution of enhanced iron acquisition from hHb to infection, we acquired hHb-expressing mice ($\alpha^{H}\beta^{A}$). In a systemic model of infection these mice display increased number of colony forming units of *S. aureus* in their hearts and livers as compared to mice expressing mHb (C57BL/6J). hHb expression does not boost the number of infecting Δ*isdB*, indicating that the increase in susceptibility of $\alpha^{H}\beta^{A}$ is due to enhanced hHb binding and not potential secondary effects. Infection of the $\alpha^{H}\beta^{A}$ mice revealed the contribution of IsdB to liver infection, which was not obvious in C57BL/6J (Chapter II). Whereas infection with either wild type or Δ*isdB* resulted in similar number of CFUs in the liver of C57BL/6J mice, IsdB allowed for increased colonization of the livers of $\alpha^{H}\beta^{A}$ animals. We propose the following explanation of liver infection data. Non-hemoglobin iron stored in the liver allows for *S. aureus* proliferation independent of IsdB-Hb interaction as indicated by high CFU numbers in the livers infected by Δ*isdB*

mutant (Figures 11 and 16). C57BL/6J mice infected with wild type *S. aureus* do not show increase in virulence as compared to Δ*isdB* due a local concentration of mHb that is too low to be utilized by IsdB. Wild type *S. aureus* infecting $\alpha^{H}\beta^{A}$ mice proliferate to higher numbers than Δ*isdB* because they have access to hHb, which is bound with a higher affinity than mHb. The magnitude of the effect we report is comparable to other humanized mouse models of bacterial infection which have profoundly impacted studies into the host-pathogen interaction (57, 69, 94, 116).

We demonstrate that all tested *S. aureus* isolates preferentially bind hHb. This is true for strains isolated from human and animal hosts. The explanation for hHb preference of animal isolates of *S. aureus* likely lies in their human origin. These isolates are thought to have been transmitted from humans to animal hosts recently in a single jump (48, 52, 74). We show that certain bacterial pathogens display preference for hHb, while others utilize mHb and hHb with similar efficiency. hHb preference appears to correlate with the host specialization of the pathogen. Human pathogens utilize hHb better than mHb, while pathogens with a wider host range do not differentiate between the two sources of Hb. Our experiments establish $\alpha^{H}\beta^{A}$ as an improved model for studies into iron acquisition. Humanized mouse models are a valuable tool in biomedical research, underscoring the impact that our findings may have on studies into bacterial virulence (25, 71).

Numerous polymorphisms are found with the Hb sequence in the human population. Changes in the amino acid sequence of Hb can potentially affect acquisition of iron and severity of infections caused by *S. aureus.* To lay the groundwork for investigations into the contribution of hHb polymorphisms to *S. aureus* infections, we

have expressed recombinant versions of select hHb variants. As a proof of principle, we demonstrate that single amino acid substitutions within hHb found in the population affect hHb binding to *S. aureus.* These results encourage analysis of other variants and progression to clinical studies. Based on the ability of most vertebrate pathogens to use heme as a nutrient iron source, and the conservation of the Isd system across numerous Gram-positive pathogens, these studies may be applicable to a wide variety of disease-causing microbes (29). Understanding the effect of hHb variations on *S. aureus* and other bacterial infections will provide insight into the role of host genetics in infectious diseases.

Future directions

Identify the structural determinants of IsdB required for Hb binding We have determined that tyrosine Y165 is required for IsdB-Hb interaction. However, other IsdB residues involved in Hb recognition have not yet been identified. Residues necessary for Hb binding by IsdH are conserved within IsdB and will therefore be targeted for mutagenesis (92). The generation of point mutant forms of IsdB will be done by a Pfu mutagenesis technique. We will purify the mutants using our established purification protocol for IsdB (93). To ensure that the proteins are properly expressed and folded we will assess expression using SDS-PAGE and folding using circular dichroism spectroscopy at the Structural Biology Core Facility at Vanderbilt University. Purified forms of IsdB will be tested for binding to hHb using Octet technology (94). To extend the biochemical characterization of mutant IsdB we will test the ability of mutant IsdB proteins to complement the Hb utilization defect of Δ*isdB*. For these studies, we will clone deletion constructs into the staphylococcal expression vector pOS-1, and transform these complementation constructs into Δ*isdB*. We will confirm the expression and surface localization of truncated forms of IsdB by FACS and immunoblot. We will then assess the ability of the complemented strains to bind Hb by pull downs followed by silver stains as described in Chapter III. We will infect human hemoglobin expressing mice ($\alpha^{H}\beta^{A}$) with *isdB* Y165A intrachromosomal mutant of *S. aureus*. The decrease in virulence of *isdB* Y165A mutant similar to that of Δ*isdB* would establish Hb binding as the primary function of IsdB during infection. These experiments will allow us to pinpoint the domains and residues within IsdB necessary for Hb recognition.

Elucidate the role of heme in regulation of Hb binding to IsdB Incubation of *S. aureus* with heme inhibits IsdB-dependent binding of Hb. Expression of IsdB that is deficient in heme binding abolishes heme-induced inhibition of Hb binding. These results suggest a mechanism whereby binding of heme to IsdB upon its extraction from Hb weakens the interaction between IsdB and Hb. This allows the release of apo-Hb from IsdB. To test this hypothesis we will express rIsdB from *E. coli* using techniques described in Chapter II. rIsdB purifies as a mixture of apo- and holo-protein. We will generate exlusively holo-rIsdB by incubating rIsdB with heme. Heme binding will be assessed spectrophotometrically. Apo-IsdB will be generated by extraction of heme with concentrated urea and refolding of the protein in buffer. We will then measure the affinity of the interaction between apo-IsdB or holo-IsdB with Hb using Octet. Upon extraction of heme from Hb, IsdB passes heme to IsdA. We will also determine the strength of the interaction between apo-IsdB or holo-IsdB with apo-IsdA or holo-IsdA. In addition to heme, IsdB can bind metalloporphyrin analogues (heme backbone with non-iron metals substituted). We will test if IsdB binding to non-heme metalloporphyrins inhibits Hb binding similarly to heme. If this is the case we will perform growth curve experiments where metalloporphyrins will be added together with Hb as a sole iron source. If metalloporphyrins can inhibit Hb binding to IsdB, they will shut down heme-iron acquisition and will slow growth. Data obtained from these experiments will allow us to get insight into the mechanism of apo-Hb release from IsdB upon heme extraction.

Identify human hemoglobin polymorphisms that affect heme-iron acquisition by *S. aureus* We have found that certain polymorphisms within hHb decrease the efficiency of hHb binding by *S. aureus*. Numerous other polymorphisms remain to be tested. In order to bypass the necessity to purify rhHb variants we will utilize the BACTH two-hybrid interaction system (Euromedex). This system allows detection of an interaction between two protein partners by cloning them into vectors containing two separate domains of an adenylate cyclase within an *E. coli* reporter strain. The activity of adenylate cyclase resulting from the interaction of the two binding partners is spectrophotmetrically measured. To this end we have fused wild type IsdB and Hb into these vectors and have detected the interaction occurring between the two proteins. We will focus on polymorphisms within the amino acid residues that are exposed on the surface and are likely recognized by IsdB. Because many of these polymorphisms are within the same residues, we will test the least conservative substitutions within each residue (~200 total). Upon identification of such residues, we will express the Hb with identified polymorphisms and test their binding to the cell wall of *S. aureus.* Hb variants that pass the above two screens will be supplied to *S. aureus* in iron-limiting growth assays and will be tested for Hb-IsdB interaction strength *in vitro*.

In addition to variants with polymorphisms within the external amino acids we will test variants with polymorphisms within the heme binding pocket. These polymorphisms likely affect the affinity with which Hb binds heme. It is possible that heme is differentially extracted from these variants, facilitating or slowing heme import into *S. aureus*. We will express hemoglobin variants with polymorphisms within the heme binding pocket and test their ability to supply iron to *S. aureus.* Finally, upon

identification of hemoglobin variants with altered affinity and/or ability to supply iron for *S. aureus* growth we will attempt to acquire blood samples from carriers of identified polymorphisms. We will purify native hemoglobin from blood samples and confirm the phenotypes identified with the recombinant Hb. Because we have experience with all the proposed techniques, we do not anticipate technical difficulties with their execution. These experiments will allow us to identify hHb variants that provide iron to *S. aureus* with altered efficiency. Data acquired from these experiments will be used to identify sequence polymorphisms within hHb that are associated with severe staphylococcal disease through the use of BioVU DNA repository.

Elucidate the role of myoglobin and albumin as potential sources of heme-iron to *S. aureus* Hb is the most abundant source of heme-iron within vertebrates. Other heme-binding proteins are potentially available to *S. aureus* during infection. Myoglobin (Mb) is a heme-binding protein that stores oxygen in the heart and muscle tissue. *S. aureus* causes heart and muscle infections and Mb is structurally related to Hb, therefore upon cell damage induced by *S. aureus* toxins, myoglobin can potentially become available to *S. aureus*. Albumin is an extracellular protein that binds heme and is present in the serum at concentration of 50 mg/ml (128). We will test myoglobin and albumin binding and utilization as an iron source by *S. aureus* using the techniques as we used to define Hb utilization. Notably, Mb-deficient mice are available and will provide with an animal model to discern the potential role of myoglobin as an iron source during infection. These experiments will define the role of myoglobin and albumin as potential sources of nutrient iron to the invading *S. aureus.*

Define the role of IsdB-Hb interaction in bacterial pathogenesis We have demonstrated an increase in susceptibility of mice to *S. aureus* systemic infection due to expression of hHb. We will expand our studies with $\alpha^{H}\beta^{A}$ mice to additional infection models to determine the benefit that enhanced iron acquisition has on bacterial pathogenesis within distinct organs. The contribution of the IsdB-Hb interaction to *S. aureus* lung infections will be tested in a murine model of pneumonia (79). The contribution of IsdB-Hb interactions to *S. aureus* bone infections will be tested using a murine model of osteomyelitis that has been developed in the laboratory of Dr. Mark Smeltzer (10). The contribution of IsdB-Hb interactions to heart colonization will be tested in a murine model of infectious endocarditis which has been developed by Dr. Arnold Bayer. *S. lugdunensis* is an emerging pathogen responsible for numerous invasive infections. Recently sequenced genome of *S. lugdunensis* has revealed the presence of a gene encoding a putative Hb receptor (121). In chapter III we have demonstrated preference for hHb displayed by *S. lugdunensis.* Mouse infections delineating the contribution of *S. lugdanensis* heme acquisition system will be carried out in $\alpha^{H}\beta^{A}$ mice. The results of these experiments will reveal the contribution of the IsdB-Hb interaction during a variety of clinically relevant staphylococcal infections.

LIST OF PUBLICATIONS

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