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# *Staphylococcus aureus* IsdB Is a Hemoglobin Receptor Required for Heme Iron Utilization▽

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**The pathogenesis of human infections caused by the gram-positive microbe *Staphylococcus aureus* has been previously shown to be reliant on the acquisition of iron from host hemoproteins. The iron-regulated surface determinant system (Isd) encodes a heme transport apparatus containing three cell wall-anchored proteins (IsdA, IsdB, and IsdH) that are exposed on the staphylococcal surface and hence have the potential to interact with human hemoproteins. Here we report that *S. aureus* can utilize the host hemoproteins hemoglobin and myoglobin, but not hemopexin, as iron sources for bacterial growth. We demonstrate that staphylococci capture hemoglobin on the bacterial surface via IsdB and that inactivation of *isdB*, but not *isdA* or *isdH*, significantly decreases hemoglobin binding to the staphylococcal cell wall and impairs the ability of *S. aureus* to utilize hemoglobin as an iron source. Stable-isotope-tracking experiments revealed removal of heme iron from hemoglobin and transport of this compound into staphylococci. Importantly, mutants lacking *isdB*, but not *isdH*, display a reduction in virulence in a murine model of abscess formation. Thus, IsdB-mediated scavenging of iron from hemoglobin represents an important virulence strategy for *S. aureus* replication in host tissues and for the establishment of persistent staphylococcal infections.**

*Staphylococcus aureus* causes a wide spectrum of human diseases, including skin and soft-tissue infections and infectious endocarditis (17), as well as septicemia with abscess formation in diverse organ tissues (6). Human morbidity and mortality caused by this pathogen are compounded by the propensity of staphylococci to acquire genes conferring resistance to all antimicrobial therapies (5, 33). Thus, there is a pressing need for the development of novel therapeutic strategies against this important human pathogen. Promising targets for the development of novel antimicrobials are bacterial iron acquisition systems, as many bacterial pathogens, including staphylococci, require iron as an essential nutrient to successfully mount human infections (7).

Because of the requirement for iron in multiple cellular processes, most bacterial pathogens have evolved strategies for the scavenging of iron from host proteins. The best-studied bacterial iron acquisition systems are siderophore based. Siderophores are low-molecular-weight iron-binding complexes that are secreted from the bacterial cell for iron retrieval. Many bacterial pathogens employ siderophore-mediated iron acquisition strategies during infection, and *S. aureus* is no exception, as it has been shown to elaborate at least four separate siderophores (10, 11, 14, 22). The contribution of siderophores to *S. aureus* pathogenesis is underscored by the demonstration that a siderophore synthesis mutant exhibits a defect in virulence in a mouse model of abscess formation at late times during infection (11). Although siderophore-based acquisition systems contribute to infection in certain cases, the host iron sources that are potential siderophore targets represent a small percentage of the total iron content of the vertebrate host. In fact, iron in the form of heme is the most abundant source of iron in mammalian tissues (12), and this iron source is not accessible to siderophore-based systems. Because of the extreme reactivity of heme, it is generally sequestered within human cells by hemoproteins such as hemoglobin and myoglobin. In keeping with this, many bacterial pathogens possess systems dedicated to the utilization of host hemoproteins as a nutrient iron source.

While studies of bacterial heme acquisition systems have focused mostly on gram-negative microbes (19, 26, 32, 39, 41), comparatively less is known about how gram-positive pathogens utilize host hemoproteins as an iron source. A transport system responsible for the utilization of heme or hemoglobin has been described for *Corynebacterium diphtheriae*, the causative agent of diphtheria (13). Other work identified surface proteins of *Streptococcus pyogenes* that capture heme or hemoglobin (2, 24, 25). In addition, we have shown that the *isd* (iron-regulated surface determinant) locus and *hts* (heme transport system) membrane transport system provide for heme iron transport into *S. aureus* (30, 37). The Isd system encodes cell wall-anchored surface proteins (IsdA, IsdB, IsdC, and IsdH), a membrane transporter (IsdD, IsdE, and IsdF), a transpeptidase (SrtB), and cytoplasmic heme-degrading monooxygenases (IsdG and IsdI) (30, 31, 36, 38, 42). In gram-positive bacteria, surface proteins are covalently anchored to peptidoglycan by sortases (29), membrane-anchored transpeptidases that catalyze the formation of an amide bond between

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the C-terminal end of surface protein substrates and the cross-bridge of wall peptides (27, 40). *S. aureus* sortase A (*srtA*) catalyzes the cell wall anchoring of about 20 proteins, including IsdA, IsdB, and IsdH (29, 31). The contribution of SrtA-anchored surface proteins to virulence is documented by the fact that *srtA* mutant staphylococci are severely impaired in the ability to cause infections in animal disease models (4, 21, 28).

*S. aureus* can grow on heme or hemoglobin as a sole iron source in vitro (30, 37), and heme acquisition is vital to staphylococcal pathogenesis (37). The fact that free heme is virtually undetectable in the vertebrate host suggests that heme acquisition is initiated upon the bacterial surface recognition of heme bound to hemoproteins. In this regard, staphylococci are thought to lyse host erythrocytes, capture host hemoproteins, remove the heme cofactor, transport heme into the cytoplasm, and finally release iron from the tetrapyrrole through the action of heme-degrading monooxygenases (19, 38). This model is supported by several published findings, including the observation that staphylococcal *srtA* mutants, which exhibit a general block in surface protein anchoring, are also defective in utilizing heme as a sole iron source for growth (30). In addition, the sortase-anchored protein IsdH, which is also known as HarA, has been shown to bind haptoglobin, hemoglobin, and haptoglobin-hemoglobin complexes (15). All proteins of the Isd system, excluding SrtB, are each individually capable of binding hemin in vitro (15, 30), and inactivation of *isdA* or *isdF* decreases heme transport into the cytoplasm of staphylococci (30). Finally, once inside the cytoplasm, free iron is released from heme through the action of IsdG and IsdI acting as heme monooxygenases (36, 42). The surface proteins IsdB and IsdH are 85% identical; thus, they have been suggested to be similarly involved in the binding of hemoproteins and to function as receptors during infection (15, 30). This contention is supported by the observation that IsdB binds hemoglobin in vitro with characteristics consistent with a receptor-ligand interaction (30). Although in vitro analysis supports a role for IsdB in the recognition of host hemoproteins, the pathophysiological ramifications of the IsdB-hemoprotein interaction have not been evaluated.

We report here that *S. aureus* is capable of utilizing purified hemoglobin or intracellular erythrocyte hemoglobin as a sole iron source for growth. Our data demonstrate that hemoglobin is captured by IsdB on the staphylococcal surface and that heme iron, but not the polypeptide of hemoglobin, is transported into the bacterial cytoplasm. Staphylococcal mutants lacking *isdB* are impaired in the ability to grow by using hemoglobin as the sole iron source. Importantly, staphylococcal mutants lacking *isdB* also display reduced virulence in a mouse model of abscess formation, suggesting that staphylococcal heme iron scavenging from hemoglobin is an important pathogenic strategy.

## MATERIALS AND METHODS

**Bacterial strains.** *S. aureus* Newman, a human clinical isolate, was used in this study (16). Isogenic variants lacking *fur*, *srtA*, *isdB*, *isdA*, or *isdC* have been previously described (30, 31). *isdH* was inactivated by following a protocol described by Bae and Schneewind (1). Briefly, sequences flanking *isdH* were PCR amplified with primers IsdH3-51-AttB1 (GGGGACAAGTTTGTACAAAAAAGCAGGCTCGTACGAATTGCCTTAACTG) and IsdH3-31-StuI (AGGCCTAGTGATTACTGGTTGCGTAG) for the upstream fragment and primers IsdH3-52-StuI (AGGCCTTATTAGCTCCGTATCACAAAG) and IsdH3-32-attB2 (GGGGACCACTTTGTACAAGAAAGCTGGGTACTCGGCAGATGTTCAGTTG) for a downstream fragment. The PCR fragments were then assembled into pCR2.1 (Invitrogen) and recombined into pKOR1 (1). Inactivation of *isdH* was achieved by allelic replacement with pKOR1Δ*isdH*. Transduction of the Δ*isdB*::*ermC* mutation into the chromosome of the Δ*isdH* mutant was carried out with phage Φ85 (30) to generate the double-mutant strain.

**Complementation of the *isdB* mutant strain.** To complement the hemoglobin-binding defect of the *isdB* mutant strain, the *isdB* gene and its promoter sequence were PCR amplified from genomic DNA of the Newman strain. A complementing plasmid was generated by ligating the *isdB* amplicon into the shuttle vector pOS1 (34). *S. aureus* strains containing pOS1 were grown in the presence of 10 μg/ml chloramphenicol. As a control, strain Newman (wild type) and the *isdB* mutant strain were transformed with pOS1 lacking an insert.

**Growth assays.** *S. aureus* cultures were grown overnight under low-iron conditions by inoculating strains in RPMI supplemented with 1% Casamino Acids and 200 μM 2,2′-dipyridyl. Overnight cultures were washed twice in NRPMI (Chelex-treated RPMI) containing 500 μM 2,2′-dipyridyl and inoculated into NRPMI+ (NRPMI containing 25 μM, $ZnCl_2$, 25 μM $MnCl_2$, 1 mM $MgCl_2$, and 100 μM $CaCl_2$) supplemented with 500 μM 2,2′-dipyridyl and 0.5 μM human hemoglobin (Sigma), 2 μM myoglobin, or 2 μM hemopexin, as indicated. Cultures were grown at 37°C with aeration, and bacterial growth was monitored by measuring the increase in absorbance (optical density at 600 nm [$OD_{600}$]) over time. For hemin and hemoglobin preexposure experiments, overnight cultures were grown as described above. Bacteria were then incubated for 30 min with or without 0.5 μM hemoglobin or 2 μM hemin at 37°C with aeration. Staphylococci were washed, diluted 100-fold into fresh NRPMI+ containing 450 μM 2,2′-dipyridyl, and grown at 37°C with aeration.

For the growth assay with the erythrocyte precursor line K-562, we induced hemoglobin expression over 5 days following addition of 15 μM hemin to the medium. Cells were then washed twice with Tris-buffered saline (TBS; 50 mM Tris-HCl [pH 7.5], 150 mM NaCl) and resuspended in NRPMI+ supplemented with 500 μM 2,2′-dipyridyl. K-562 cells ($1 \times 10^4$/ml; induced and uninduced) were mixed with a 1:500 dilution of an *S. aureus* culture that had been grown for 15 h in NRPMI+ supplemented with 500 μM 2,2′-dipyridyl. Cultures were grown at 37°C with aeration, and staphylococcal growth was determined by colony formation on tryptic soy agar.

**Hemoglobin nutrition plate assay.** *S. aureus* was grown for 12 h in RPMI plus 200 μM 2,2′-dipyridyl. Following incubation, bacteria were mixed with top agar containing 1 mM 2,2′-dipyridyl and poured onto tryptic soy agar (TSA) plates containing 4 mM 2,2′-dipyridyl. Discs (8.5 mm in diameter) were impregnated with 10 μl of human hemoglobin at a 25 μM concentration, placed onto plates, and incubated for 24 h at 37°C. Following incubation, growth surrounding the discs was photographed and the diameter of the growth zones was determined with an AlphaImager.

**[$^{14}$C]hemoglobin binding to staphylococci.** *S. aureus* strains were grown in NRPMI+ containing 500 μM 2,2′-dipyridyl at 37°C with aeration. At bacterial $OD_{600}$s of 0.40 to 0.55, cultures were treated with 1 mM 2,2′-dipyridyl for 1 h. Staphylococci were collected by centrifugation and suspended in TSM buffer (100 mM Tris-HCl [pH 7.0], 500 mM sucrose, 10 mM $MgCl_2$). Following addition of [$^{14}$C]hemoglobin, suspensions were incubated at room temperature for 5 min and ice-cold ethanol-acetone (1:1, vol/vol) was added to quench iron uptake.

Mixtures were incubated on ice for 10 min and subsequently centrifuged at $10,000 \times g$ for 10 min at 4°C. Supernatant was aspirated, and bacterial sediment was suspended in 100 μl TSM and subjected to scintillation counting to determine the total amount of [$^{14}$C]hemoglobin associated with bacterial cells. To determine the percentages of [$^{14}$C]hemoglobin in protoplast preparations, staphylococci were suspended in 0.1 M Tris-HCl (pH 7.0) and incubated with 100 μg/ml lysostaphin for 10 min at 37°C. After digestion, protoplasts were sedimented at $10,000 \times g$ for 5 min, suspended in 0.1 M Tris-HCl (pH 7.0), and subjected to scintillation counting.

The integrity of the bacteria following ethanol-acetone treatment was confirmed by immunoblotting the ethanol-acetone suspension with antiserum against a cytoplasmic protein (IsdI) (36). Following ethanol-acetone treatment, bacteria were sedimented by centrifugation at $6,000 \times g$ for 10 min and the supernatant was removed and concentrated by centrifugation under vacuum at room temperature. The bacterial pellet was resuspended in 1 ml TSM, and the cell wall was solubilized with lysostaphin for 30 min at 37°C. Following cell wall digestion, the protoplasts were separated from the cell wall fraction by centrifugation at $13,000 \times g$ for 2 min. All fractions (cell wall, protoplasts, and ethanol-acetone supernatant) were subjected to immunoblotting with antiserum specific to the cytoplasmic protein IsdI (data not shown).

**Inductively coupled plasma mass spectrometry (ICP-MS).** Staphylococci were incubated in the presence of equal amounts of [$^{54}$Fe]hemoglobin (Scipac) and

[$^{57}$Fe]transferrin (Scipac) as previously described (37). Normalizations were performed for the predicted number of iron atoms to account for differences in iron-binding capacity between hemoglobin (four atoms) and transferrin (two atoms). Samples of bacterial cultures (1 ml aliquots) were removed at 6 and 9 h and sedimented by centrifugation. Supernatants were mixed with high-purity [$^{15}$N]nitric acid (Seastar), and bacterial sediments were washed three times with NRPMI prior to suspension in [$^{15}$N]nitric acid. Samples were processed as described previously (37), with exceptions as noted below. Iron isotopic composition and abundance ($^{54}$Fe/$^{56}$Fe and $^{57}$Fe/$^{56}$Fe) were determined with a Finnigan Element mass spectrometer (37). Samples were introduced into the ICP-MS with an all-Teflon sample introduction system consisting of an ESI PFA 100-μl/min nebulizer, an ASX-100 autosampler with 2-ml PFA cups, and an ESI PFA spray chamber. A 2-min wash time and a 1-min take-up time were used between samples. Briefly, 50 scans of each entire peak of $^{53}$Cr, $^{54}$Fe, $^{56}$Fe, and $^{57}$Fe were collected at a mass-resolving power of M/ΔM = 4,300, sufficient to completely separate the atomic isobars from interfering molecular isobars ($^{40}$Ar$^{13}$C$^+$ from $^{53}$Cr$^+$, $^{40}$Ar$^{14}$N$^+$ from $^{54}$Fe$^+$, $^{40}$Ar$^{16}$O$^+$ from $^{56}$Fe$^+$, etc.). The isobaric interference of $^{54}$Cr on $^{54}$Fe was corrected by monitoring $^{53}$Cr and assuming a constant $^{54}$Cr/$^{53}$Cr ratio of 0.2489. Data are presented as isotope ratios, with each reported data point representing an average of 50 experimentally determined isotope ratios. In all experiments, isotopically labeled Fe was introduced as [$^{54}$Fe]hemoglobin (90.65% $^{54}$Fe) and [$^{57}$Fe]transferrin (94.4% $^{57}$Fe). Natural Fe was present as a ubiquitous contaminant in all experiments, being introduced by contaminants during handling or present in reagents, and from the natural Fe stores of bacteria. Thus, all measured ratios reflect a combination of natural Fe (dominated by $^{56}$Fe, 91.7%) and isotopically labeled Fe.

**Binding assays.** A fluorescence-activated cell sorter (FACS)-based assay was performed with purified hemoglobin, myoglobin, or hemopexin labeled with biotin (EZ-Link Sulfo-NHS-LC-Biotin; Pierce) at a 12 M excess of biotin to hemoproteins for 30 min at room temperature. *S. aureus* cells were grown in RPMI containing 200 μM 2,2′-dipyridyl until mid-log phase ($OD_{600}$ of ~1.0), and cells were diluted to $5 \times 10^6$ CFU in phosphate-buffered saline (PBS). Biotinylated hemoproteins (20 μg) were added to staphylococci, and the mixture was incubated for 30 min at room temperature. Staphylococci were then washed in TBS, and streptavidin-fluorescein isothiocyanate was added at a 1:100 dilution. Bacterial complexes with biotinylated hemoproteins and streptavidin were fixed with 3.5% formaldehyde, and fluorescence intensity was quantified by FACScan (Becton Dickinson) analysis.

**Cosedimentation assays.** *S. aureus* cultures were grown overnight in RPMI supplemented with 1% Casamino Acids and 200 μM 2,2′-dipyridyl (iron depleted) or in RPMI supplemented with 10 μM $FeSO_4$ (iron replete). Staphylococci were washed with TBS and diluted to the same $OD_{600}$ of ~1.0. Bacteria were incubated with hemoglobin (0, 1, 5, and 10 μg/ml) in TBS for 30 min at room temperature and washed with TBS, and hemoglobin was eluted from the staphylococcal surfaces by boiling for 15 min in 0.5 M Tris-HCl buffer (pH 8.0)–4% sodium dodecyl sulfate. Following sedimentation of staphylococci, solubilized hemoglobin was subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membrane (Amersham Biosciences). Hemoglobin was detected by immunoblotting with rabbit anti-hemoglobin (Sigma) and anti-rabbit Alexa Fluor 680 conjugate (Molecular Probes) with an Odyssey infrared imaging system (LI-COR). As a loading control, we took advantage of the fact that staphylococcal protein A nonspecifically binds to the antisera used in this analysis, leading to the appearance of a cross-reactive band whose intensity can be used to compare loadings across samples.

**Fur regulation of IsdB.** Wild-type, Δ*fur*, and Δ*isdB*::*ermC S. aureus* strains were grown under either iron-replete (RPMI plus 10 μM $FeSO_4$) or iron-depleted (RPMI plus 300 μM 2,2′-dipyridyl) conditions. Following 18-h incubations, the bacteria were pelleted by centrifugation at $6,000 \times g$ for 10 min. The cell wall and protoplasts were separated and subjected to immunoblotting as described above.

**Mouse model of infection.** Six- to eight-week-old BALB/c mice (Jackson Laboratories) were infected with $1 \times 10^6$ CFU of wild-type *S. aureus* Newman and the Δ*isdB*::*ermC*, Δ*isdH*, and Δ*isdB*::*ermC*Δ*isdH* mutant strains suspended in PBS by injection into the retroorbital vein complex. Four days after infection, mice were euthanized with $CO_2$. Livers and kidneys were removed, analyzed for abscess formation, and homogenized in PBS. The staphylococcal load was determined by colony formation on tryptic soy agar. Ten or more mice were infected with each strain of *S. aureus*. Statistical analyses were performed with the Student *t* test.

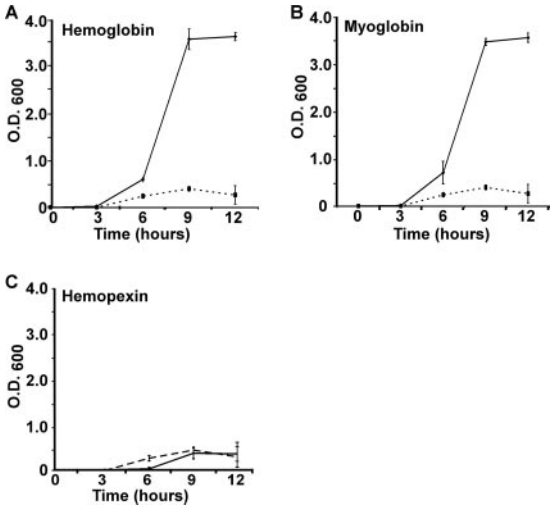


FIG. 1. Growth of *S. aureus* using hemoproteins as a sole iron source. *S. aureus* strains were grown in iron-free NRPMI+ supplemented with 0.5 μM hemoglobin (A), 2 μM myoglobin (B), or 2 μM hemopexin (C). Bacterial growth was determined by measuring the $OD_{600}$ of cultures. Solid black lines represent *S. aureus* wild-type strain Newman, whereas dashed black lines indicate growth in the absence of hemoproteins. Data represent the mean ± the standard deviation of triplicate experiments.

## RESULTS

***S. aureus* utilizes hemoglobin or myoglobin as a nutrient iron source.** Staphylococci are capable of using hemoglobin as a sole iron source for growth (30); however, the ability of *S. aureus* to utilize additional host hemoproteins has not been evaluated. To determine which hemoproteins can be utilized as sources of iron by staphylococci, we measured the ability of *S. aureus* to grow on hemoglobin, myoglobin, or hemopexin in medium lacking all other sources of iron. Staphylococci were able to utilize hemoglobin and myoglobin for growth, whereas hemopexin did not serve as a nutrient iron source (Fig. 1A to C). This result suggests that staphylococci are capable of acquiring iron from the two most abundant heme sources in the host, hemoglobin and myoglobin.

***S. aureus* utilizes intracellular hemoglobin as nutrient iron.** We considered the possibility that hemoglobin can be accessed directly from red blood cells during staphylococcal infection. To test this hypothesis, a growth assay was developed which utilizes an erythrocyte precursor line (K-562) as a sole iron source for staphylococcal growth (18). The erythroleukemia cell line K-562 can be induced to express large amounts of intracellular hemoglobin upon the addition of exogenous hemin (18). K-562 cells were therefore incubated with 15 μM hemin for 5 days to induce hemoglobin production. Hemin-induced cells became bright red and expressed large amounts of hemoglobin, whereas uninduced K-562 cells stayed pale white and expressed only very low levels of hemoglobin (Fig. 2A and data not shown). Cells from both uninduced and induced cultures were washed extensively and then suspended in iron-free growth medium as a sole iron source. As expected, incubation of *S. aureus* in iron-free growth medium did not support bacterial growth (data not shown). *S. aureus* incubated with uninduced K-562 cells in the same medium replicated

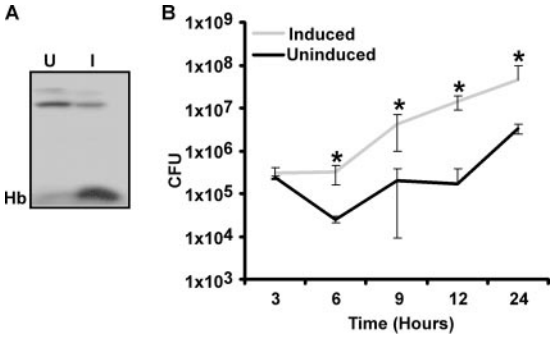


FIG. 2. Growth of *S. aureus* using intracellular hemoglobin as a sole iron source. (A) Immunoblot assay of extracts from K-562, an erythrocyte precursor cell line that was left uninduced (U) or induced (I) by using an anti-hemoglobin antibody as a measurement of hemoglobin expression. (B) *S. aureus* strain Newman was cultured in iron-free medium in the presence of K-562 cells left uninduced (black line) or induced (gray line) for the expression of hemoglobin (Hb). Data represent the mean ± the standard deviation of triplicate bacterial enumerations on agar plates. Asterisks denote statistically significant differences from the wild type as determined by Student's *t* test ($P < 0.05$).

approximately 1 log after a prolonged lag period (Fig. 2B). This residual growth is presumably due to the staphylococci gaining access to non-hemoglobin iron stores of K-562 cells undergoing spontaneous lysis. In contrast, incubation of *S. aureus* with hemin-induced K-562 cells resulted in significant growth with cell densities approximately 2 logs greater than the starting inoculum. As hemoglobin production is the only described difference between hemin-induced and uninduced K-562 cells (18), it appears that *S. aureus* can indeed access and utilize intracellular erythrocyte hemoglobin as an iron source for growth.

***S. aureus* preferentially acquires hemoglobin-derived iron.** *S. aureus* is able to utilize multiple iron sources for growth, including heme, hemoproteins, and transferrin-Fe (15, 30, 37). The mechanisms for metabolizing these iron sources are distinct, with transferrin-Fe acquisition likely being mediated by siderophore uptake systems and hemoglobin-Fe acquisition systems being mediated by hemoglobin-specific receptors. Therefore, we compared the ability of *S. aureus* to acquire iron from hemoglobin-Fe compared to transferrin-Fe by using a stable-isotope-tracking assay (37). This assay exposes *S. aureus* to equal molar amounts of individual iron sources labeled with distinct minor stable isotopes of iron. After growth in isotopically labeled medium, bacteria are removed and the iron isotope concentrations in staphylococci are determined by ICP-MS (37). To measure the iron source preference of *S. aureus*, we obtained Fe-hemoglobin and Fe-transferrin samples consisting almost exclusively of $^{54}$Fe and $^{57}$Fe, respectively. Isotopic labeling did not affect the ability of *S. aureus* to use these compounds as iron sources for growth, and bacteria growing in the presence of individual labeled-Fe sources replicated with equal efficiencies (data not shown). To determine the iron source preference, iron-starved bacteria were subcultured into chemically defined medium and supplemented with equimolar amounts of [$^{54}$Fe]hemoglobin and [$^{57}$Fe]transferrin. Natural Fe was present as a ubiquitous contaminant in all experiments; therefore, all measured ratios reflect a combination of natural Fe (dominated by $^{56}$Fe, 91.75%) and isotopically labeled Fe. Analyses of bacteria collected during the growth of the culture revealed a sevenfold enrichment in the ratio of hemoglobin-derived iron to transferrin-derived iron (Table 1). This Fe source preference was further documented by the depletion of [$^{54}$Fe]hemoglobin from conditioned medium of growth cultures (Table 1). This preferred acquisition of hemoglobin-derived iron was partially affected by the growth phase of cultures, as evidenced by a shift to increased transferrin-Fe uptake during late-log phase (9 h) (Table 1). Together, these results suggest that *S. aureus* preferentially uses hemoglobin over transferrin as an iron source.

***S. aureus* binds hemoglobin.** The ability of *S. aureus* to utilize hemoproteins as a nutrient iron source might require recognition of these host proteins on the bacterial cell surface. To evaluate this, hemoglobin, hemopexin, and myoglobin were biotinylated and incubated with staphylococci. Bacteria were washed, and bound hemoprotein was detected with fluorescein-labeled streptavidin and fluorescence-based detection. These experiments demonstrated significant hemoglobin binding to *S. aureus*; however, we were unable to detect binding of myoglobin or hemopexin in these assays (Fig. 3).

In *S. aureus*, up to 20 proteins are anchored to the cell wall by sortase A (SrtA) (28, 29). To determine if SrtA cell wall-anchored proteins are involved in staphylococcal hemoglobin recognition, we analyzed the ability of an *S. aureus srtA* mutant strain to bind hemoglobin. Inactivation of *srtA* virtually eliminated our ability to detect hemoglobin binding in this assay (Fig. 3A). As SrtA is responsible for anchoring IsdA, IsdB, and IsdH to the cell wall envelope (30, 31), we compared the ability of *S. aureus* strains inactivated for *isdA*, *isdB*, or *isdH* to bind hemoglobin. Inactivation of *isdB* almost eliminated the ability of mutant staphylococci to bind hemoglobin (Fig. 3A), suggesting that IsdB functions as a primary receptor for hemoglobin in

TABLE 1. Isotopic tracking of Fe-labeled hemoglobin and Fe-labeled transferrin uptake

| Sample or condition | % Hb[a] | % Tf[b] | $^{54}$Fe/$^{56}$Fe ratio | $^{57}$Fe/$^{56}$Fe ratio |
|---|---|---|---|---|
| [$^{54}$Fe]Hb/[$^{57}$Fe]Tf | | | | |
| Whole cells, 6 h | 88 | 12 | 5.35 | 0.74 |
| Supernatant, 6 h | 35 | 65 | 0.45 | 0.84 |
| Whole cells, 9 h | 71 | 29 | 1.05 | 0.39 |
| Supernatant, 9 h | 53 | 47 | 2.78 | 2.48 |
| [$^{54}$Fe]Hb | | | | |
| Whole cells, 6 h | 100 | 0 | 0.55 | 0.03 |
| Whole cells, 9 h | 100 | 0 | 0.71 | 0.03 |
| [$^{57}$Fe]Tf | | | | |
| Whole cells, 6 h | 2 | 98 | 0.07 | 0.26 |
| Whole cells, 9 h | 1 | 99 | 0.08 | 1.06 |
| Growth in: | | | | |
| Normal growth medium[c] | NA[e] | NA | 0.06 | 0.02 |
| Labeled growth medium[d] | 60 | 40 | 1.78 | 1.19 |

[a] Hb, hemoglobin.
[b] Tf, transferrin.
[c] The normal growth medium used was TSB.
[d] The labeled growth medium used was TSB supplemented with $^{54}$Fe-labeled hemoglobin and/or $^{57}$Fe-labeled transferrin.
[e] NA, not applicable.

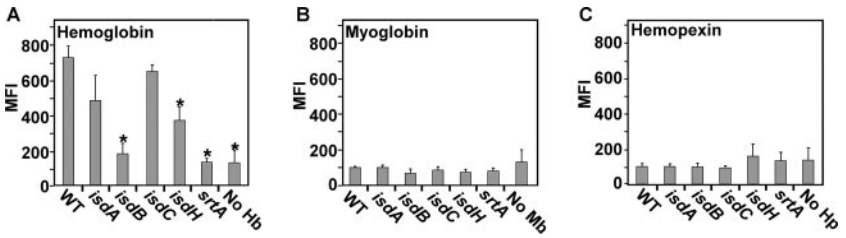


FIG. 3. Hemoprotein binding to *S. aureus* surface. Shown are results of FACS-based assays measuring the binding of hemoglobin (Hb; A), myoglobin (Mb; B), and hemopexin (Hp; C) to the surface of different strains of *S. aureus*. MFI, mean fluorescence intensity. Results represent the mean ± the standard deviation from triplicate determinations. Asterisks denote statistically significant differences from the wild type (WT) as determined by Student's *t* test ($P < 0.05$).

*S. aureus*. In contrast, inactivation of *isdA* or *isdC*, specifying a sortase B-anchored heme-binding protein (31), did not affect hemoglobin binding of *S. aureus* (Fig. 3A). In agreement with a previous report (15), inactivation of *isdH* caused a significant reduction in hemoglobin binding, albeit this decrease was much less severe than that observed for the *isdB* mutant strain (Fig. 3A).

Bacterial iron uptake systems are often under the control of metal-dependent transcriptional regulators such as the ferric uptake repressor Fur, which inhibits transcription of Fur-regulated genes under iron-replete conditions (15, 20, 31, 43). The genes specifying the cell wall-anchored proteins of the Isd system (*isdA*, *isdB*, *isdC*, and *isdH*) are also regulated in an iron-dependent manner (15, 30, 31), prompting us to investigate if hemoglobin binding to staphylococci is influenced by the iron and Fur status of the bacterium. Cosedimentation assays with hemoglobin and staphylococci grown under iron-replete or iron-depleted conditions demonstrated that the capacity of *S. aureus* to bind hemoglobin increased upon iron starvation (Fig. 4). Consistent with results obtained in the FACS-based binding assay, inactivation of *srtA* or *isdB* decreased the binding of hemoglobin to mutant staphylococci under iron-starved conditions. In contrast, inactivation of *isdH* did not result in a reduction in hemoglobin binding. To investigate whether IsdB and IsdH cooperate in capturing hemoglobin on the bacterial surface, a double-mutant strain was generated (*isdBH*). The *isdBH* double-mutant strain displayed a decrease in hemoglobin binding similar to that observed in the *isdB* and *srtA* mutant strains (Fig. 4A). We were able to complement the hemoglobin-binding defect of the *isdB* mutant strain by providing *isdB* in *trans*. The complemented strain binds hemoglobin under iron-deficient conditions at a level similar to that of the wild type (Fig. 4B). These results conclusively demonstrate that the impairment of hemoglobin binding exhibited by the *isdB* mutant strain is dependent on *isdB*. To confirm a role for Fur in the iron-dependent regulation of *isdB*, we compared IsdB expression in the absence of Fur under iron-replete and iron-depleted conditions (Fig. 4C). These experiments demonstrated that *isdB* is under Fur-mediated iron-dependent repression and provide a mechanistic explanation for the increase in hemoglobin cosedimentation seen upon iron starvation. Together, these results suggest that IsdB functions as the staphylococcal hemoglobin receptor elaborated under iron-starved conditions.

**Removal of heme from hemoglobin at the staphylococcal surface.** To determine whether or not staphylococci remove heme from hemoglobin, bacteria were incubated with hemoglobin that was labeled by incorporation of [$^{14}$C] into the polypeptide. The carbons contained within the tetrapyrrole of hemin are not isotopically labeled in this experiment. As expected from the studies reported above, [$^{14}$C]hemoglobin bound to the surface of *S. aureus* and this interaction was significantly reduced upon inactivation of *isdB* (Fig. 3 and 4 and Table 2). To measure globin (polypeptide)-derived inter-

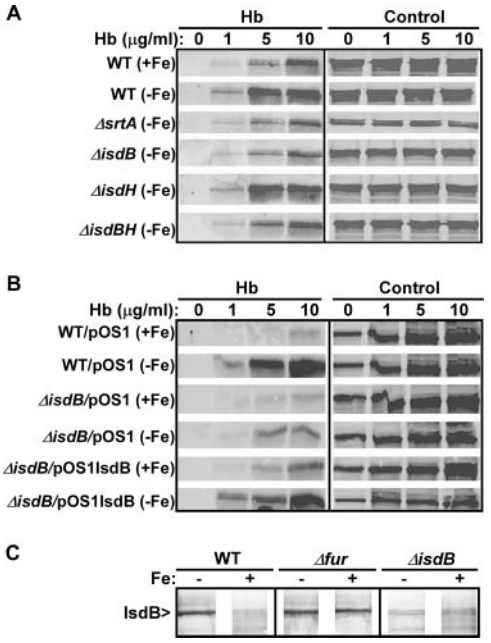


FIG. 4. Hemoglobin binding to whole *S. aureus* cells. (A) Whole cells of the indicated *S. aureus* strains were incubated with various concentrations of hemoglobin (Hb; micrograms per milliliter), followed by immunoblotting with an antiserum specific for Hb. The left side represents hemoglobin, and the right side represents the loading control. (B) Whole cells of the complemented *isdB* mutant strain (Δ*isdB*/pOS1IsdB) and control strains containing the pOS1 plasmid without the *isdB* gene (i.e., wild type [WT]/pOS1 and Δ*isdB*/pOS1) were grown in iron-depleted (−Fe) or iron-replete (+Fe) medium. Whole cells were then incubated with various concentrations of hemoglobin (micrograms per milliliter), followed by immunoblotting with an antiserum specific for hemoglobin. On the left is hemoglobin, and on the right is the loading control. (C) *S. aureus* Newman (wild type) and the isogenic Δ*isdB* and Δ*fur* mutant strains were grown in iron-depleted (−Fe) or iron-replete (+Fe) medium. Whole cells were then analyzed by immunoblotting with an antiserum specific for IsdB.

TABLE 2. [$^{14}$C]hemoglobin binding to *S. aureus*

| Strain | Avg counts per minute (SD) | Avg %[a] of whole strain Newman cells (SD) |
|---|---|---|
| Whole WT[b] cells | $3.31 \times 10^3$ (243.8) | 100.000 (00.000) |
| WT protoplasts | $5.54 \times 10^1$ (51.1) | 0.017 (00.014) |
| Whole Δ*isdB* mutant cells | $8.37 \times 10^2$ (204.3) | 10.742 (18.240) |
| No label | $8.00 \times 10^0$ (1.4) | 0.004 (00.001) |

[a] Percentage calculated from the counts per minute.
[b] WT, wild type.

TABLE 3. Hemoglobin nutrition plate-based assay

| Strain | Avg diam ± SD (mm) | *P* value[a] |
|---|---|---|
| Wild type | 17.06 ± 0.53 | |
| Δ*isdB* mutant | 15.10 ± 0.39[b] | $4.08 \times 10^{-8}$ |
| Δ*isdH* mutant | 17.18 ± 0.51 | $5.9 \times 10^{-2}$ |
| Δ*isdB* Δ*isdH* mutant | 16.15 ± 0.37[b] | $3.5 \times 10^{-4}$ |

[a] *P* values were determined by Student's *t* test.
[b] Statistically significant differences ($P < 0.05$).

nalization of [$^{14}$C]hemoglobin captured on the bacterial surface, the cell wall of staphylococci was removed with lysostaphin and protoplasts were sedimented. Scintillation counting of protoplasts revealed that staphylococci that had captured [$^{14}$C]hemoglobin via IsdB on their surface did not import the polypeptide into the bacterial cytoplasm (Table 2). This result must be viewed in light of the fact that similar preparations of staphylococci are indeed capable of utilizing hemin as a nutrient iron source through the import of isotopically labeled heme from hemoglobin into the bacterial cytoplasm (30). Thus, it appears that IsdB-mediated capture of hemoglobin on staphylococcal surfaces is accompanied by a release of heme from globin and subsequent transport of heme into the bacterial cytoplasm.

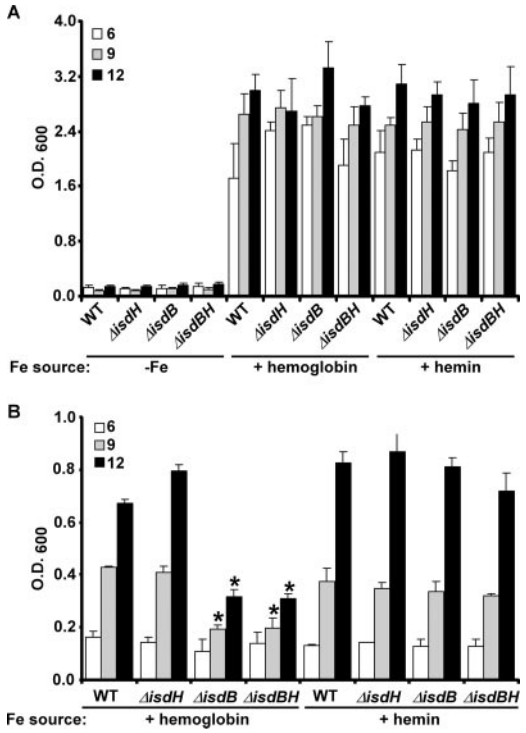


FIG. 5. Growth of *isdB* mutants using hemin or hemoglobin as the sole iron source. (A) *S. aureus* strains were grown in iron-free medium continuously supplemented with hemin (2 μM) or hemoglobin (0.5 μM) as an iron source or without iron (−Fe). (B) *S. aureus* strains were grown in iron-free medium preincubated with hemin (2 μM) or hemoglobin (0.5 μM) for 30 min or not. Cells were then washed and cultured in iron-free NRPMI+. Bacterial growth was determined by measuring the $OD_{600}$ of cultures at 6, 9, and 12 h. Results represent the mean ± the standard deviation from triplicate determinations. Asterisks denote statistically significant differences from the wild type (WT) as determined by Student's *t* test ($P < 0.007$).

***isdB* is required for *S. aureus* utilization of hemoglobin as an iron source.** To test the hypothesis that IsdB-mediated capture of hemoglobin on the staphylococcal surface is important for heme iron scavenging, the growth rates of the wild-type and *isdB*, *isdH*, and *isdBH* mutant strains were compared in medium where hemoglobin served as the sole source of iron. Initial observations suggested that inactivation of *isdB* does not abrogate the ability of *S. aureus* to utilize hemoglobin as an iron source in this assay (Fig. 5A). A trivial explanation for this result is that extended exposure to soluble hemoglobin facilitates nonspecific interactions between *S. aureus* and hemoglobin, thereby masking a possible deleterious effect of inactivation of the structural gene for IsdB, the staphylococcal hemoglobin receptor. In support of this hypothesis is the observation that when *S. aureus* is exposed to excess hemoglobin, appreciable amounts of hemoglobin are bound to *S. aureus* inactivated for *isdB* (Fig. 4A and B). As the majority of nonspecifically bound hemoglobin can be removed from staphylococci by washing in iron-free medium, staphylococci were incubated with hemoglobin for 30 min, followed by extensive washing and transfer into iron-free medium. Under these conditions, hemoglobin bound to bacterial surfaces represents the predominant source of iron. All of the staphylococcal strains examined were unable to grow in iron-free medium unless the bacteria were preexposed to hemin or hemoglobin (Fig. 5). Following capture of hemoglobin on staphylococcal surfaces, both wild-type and *isdH* mutant strains were able to grow, demonstrating utilization of surface-bound hemoglobin as an iron source (Fig. 5B). In contrast, the *isdB* mutant strain and the *isdBH* double-mutant strain were impaired in the ability to grow after the bacteria were exposed to hemoglobin (Fig. 5B). Importantly, inactivation of *isdB* or *isdH* did not negatively affect the ability of *S. aureus* to use hemin as a sole iron source in these assays (Fig. 5B).

To confirm the role of *isdB* in the utilization of hemoglobin as an iron source, we developed a plate-based nutrition assay which measures the ability of *S. aureus* to grow on solid medium lacking available iron supplemented with hemoglobin through disc diffusion. These experiments revealed a decreased ability of *S. aureus* strains to utilize hemoglobin as an iron source when inactivated for *isdB* alone or in combination with *isdH* (Table 3). Consistent with the growth assays described above, inactivation of *isdH* alone did not adversely affect growth on hemoglobin as a sole iron source in these plate-based assays (Table 3). Together, these data confirm that the interaction between IsdB and hemoglobin contributes to staphylococcal heme iron scavenging. However, on the basis of

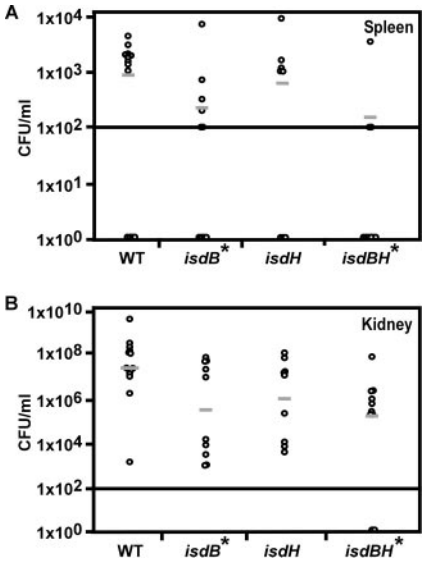


FIG. 6. Contribution of IsdB-mediated hemoglobin binding to staphylococcal pathogenesis. *S. aureus* colonization of murine spleen (A) or kidney (B) tissue was measured by tissue homogenization, dilution, and colony formation on agar medium. The horizontal gray line represents the mean log CFU on the *y* axis. The horizontal black line represents the limit of detection. Each data point represents the number of bacteria (CFU) per milliliter of tissue homogenate in a single animal. Asterisks denote statistically significant differences between the wild-type (WT) and mutant strains as determined by Student's *t* test ($P < 0.03$).

the measurable binding of hemoglobin to *isdB* mutants (Fig. 4) and detectable growth of *isdB* mutants using hemoglobin as a sole iron source, it is likely that *S. aureus* possesses additional mechanisms of access to hemoglobin-derived iron during infection.

**IsdB-mediated capture of hemoglobin contributes to the pathogenesis of staphylococcal infections.** SrtA is one of the most significant virulence determinants of staphylococcal pathogenesis, exemplified by a 3-log decrease in virulence in a mouse model of systemic infection (21, 28). A significant portion of the virulence defect of *srtA* mutants may be attributable to defects in hemoglobin binding and heme iron transport (Fig. 3 and 4) (30). To determine the contribution of IsdB- and IsdH-mediated hemoglobin and haptoglobin binding to staphylococcal virulence, a mouse model of systemic abscess formation was employed. Mice were infected intravenously with $1 \times 10^6$ CFU of *S. aureus* Newman (wild type) or the *isdB*, *isdH*, or *isdBH* mutant strain. Enumeration of bacteria from abscesses in various organ tissues removed from mice 4 days after infection revealed a 1-log reduction in the load of *isdB* mutant staphylococci in both the spleen and kidneys compared to that in the wild type ($P < 0.03$) (Fig. 6). Inactivation of *isdH* resulted in a small reduction in the bacterial load in kidney tissue; however, the observed difference was not statistically significant ($P < 0.07$). Moreover, the *isdBH* double-mutant strain exhibited a decrease in the bacterial load similar to that observed in *isdB* mutant staphylococci ($P < 0.007$). Together, these results suggest that IsdB is required for *S. aureus* virulence in vivo and that IsdB is responsible for a significant portion of the virulence defect observed upon inactivation of *srtA*.

## DISCUSSION

Most bacteria require iron, an atom that is an essential cofactor for many biochemical processes. The ability to sequester free iron has long been recognized as a natural resistance mechanism of humans to fight infection, and alterations of available-iron levels, brought about by inherited diseases or tissue injury, predispose humans to infection with a variety of pathogens (8). In mammals, most of the host iron is sequestered intracellularly in the form of the tetrapyrrole heme, with the large majority being associated with the oxygen-carrying molecule of erythrocytes, hemoglobin (12). Thus, heme and hemoglobin are rich potential iron sources for invading pathogens. In this study, we show that *S. aureus* bind and use hemoglobin as a sole iron source through elaboration of the hemoglobin receptor IsdB and that this interaction is required for full virulence in a mouse model of infection.

Our data indicate that *S. aureus* can use hemoglobin or myoglobin as a sole iron source (Fig. 1 and 2). On the basis of our inability to detect myoglobin binding to *S. aureus*, it appears that staphylococci have evolved an alternate mechanism to access myoglobin Fe. In contrast, hemoglobin readily binds to the staphylococcal surface (Fig. 3 and 4 and Table 2). The ability of *S. aureus* to use hemoglobin as an iron source is not limited to in vitro growth in the presence of purified hemoglobin (Fig. 1), as intracellular hemoglobin, produced by erythrocyte precursor cells, also provides rich hemoglobin iron resources for bacterial growth (Fig. 2). *S. aureus* prefers hemoglobin over transferrin as an iron source under the in vitro conditions tested (Table 1), underscoring the importance of heme to staphylococcal pathogenesis. These results are in agreement with previous work demonstrating that free heme iron is a preferred source of iron over transferrin iron (37).

We have previously described the iron-regulated surface determinant (Isd) system as the first heme uptake system identified in *S. aureus* (30, 31). The staphylococcal Isd system encompasses three surface-exposed, SrtA-anchored proteins (IsdA, IsdB, and IsdH/HarA) involved in binding and transport of heme and/or hemoproteins (15, 30). In this study, we determined the contribution of several SrtA-anchored Isd proteins to *S. aureus* hemoglobin recognition. Our data demonstrate that inactivation of *srtA* results in decreased hemoglobin binding (Fig. 3 and 4), suggesting that SrtA cell wall-anchored proteins play an important role in hemoprotein recognition. Furthermore, inactivation of *isdB* inhibited the ability of *S. aureus* to bind hemoglobin (Fig. 3 and 4 and Table 2). These data support our previous observation that purified recombinant IsdB binds hemoglobin in vitro (30). Together, these data demonstrate that *S. aureus* binds hemoglobin via its IsdB receptor.

The differential roles in hemoprotein binding and heme uptake by IsdB and IsdH are evidenced by the fact that IsdB, but not IsdH, is required for *S. aureus* growth using hemoglobin as the sole iron source (Fig. 5B). We have shown that purified recombinant IsdB binds hemoglobin in vitro with characteristics consistent with a receptor-ligand interaction (30). Furthermore, IsdH has been implicated in the surface recog-

nition of hemoglobin-haptoglobin complexes (15). We demonstrate that in the animal model used here, IsdB, but not IsdH, contributes to the pathogenesis of *S. aureus* infections (Fig. 6). Strains inactivated for *isdB* or *isdBH* did not reach the approximately 3-log virulence defect of an *srtA* mutant strain in a similar animal model (28), implying that a combinatorial effect on other cell wall-anchored proteins is responsible for the significant virulence defect of *srtA* mutant strains. Nevertheless, IsdB is the first gram-positive hemoprotein receptor shown to contribute to in vitro growth on hemoglobin as an iron source, as well as virulence in vivo.

Although systems involved in hemoprotein usage and heme uptake in gram-positive bacteria are beginning to emerge, the precise mechanism by which these bacteria are able to bind and transport heme iron through their membranes is not well understood. On the basis of the results presented here, we are able to add a mechanism for hemoglobin recognition to the proposed model of *S. aureus* heme iron acquisition (38). Our model proposes that during a blood-borne infection, *S. aureus* encounters red blood cells that are lysed via the secretion of potent hemolysins (3). Erythrocyte lysis liberates large quantities of intracellular hemoglobin or hemoglobin/haptoglobin complexes that can be captured on staphylococcal surfaces by binding to IsdB or IsdH, respectively. Heme is then removed for transport from hemoglobin in a manner that is not understood, and it is then translocated across the cell wall envelope by interacting with IsdC and/or IsdA. Heme then enters the cytoplasm via two heme-specific membrane transport systems, IsdDEF and HtsABC (30, 37). In the cytoplasm, the tetrapyrrole of heme is cleaved by the staphylococcal heme oxygenases IsdG and IsdI (36), releasing iron for use as a staphylococcal nutrient.

Further identification and detailed description of the mechanism by which pathogens like *S. aureus* acquires iron from hemoproteins, a process required for pathogenesis (Fig. 6) (37), may lead to the identification of novel targets for the development of molecules that inhibit staphylococcal infection. In fact, cell wall-anchored proteins of the Isd system have recently been highlighted as potential vaccine candidates against staphylococcal infection (9, 23). A functional understanding of the contribution of the Isd system to pathogenesis may facilitate the successful implementation of Isd system-based vaccine strategies. This strategy for vaccine development is made more important by the fact that systems homologous to the Isd heme transport apparatus exist in multiple gram-positive pathogens, including *Bacillus anthracis* (35), *Clostridium tetani* (38), and *Listeria monocytogenes* (38).

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