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, 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 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.
INTRODUCTION

S. aureus cause a wide spectrum of human diseases, including skin and soft tissue infections, 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 resistance-genes to all antimicrobial therapies (5, 33). Thus, there is 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).

Due to the requirement for iron in multiple cellular processes, most bacterial pathogens have evolved strategies for the scavenging of iron from host proteins. The most well studied bacterial iron acquisition systems are siderophore-based. Siderophores are low molecular weight iron binding complexes that are secreted from the bacterial cell for the purposes of 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. Due to the extreme reactivity of heme, it is generally sequestered within human cells by hemoproteins such as hemoglobin and myoglobin. Keeping with this, many bacterial pathogens possess systems dedicated to the utilization of host hemoproteins as a nutrient source.

While the study 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* locus (iron-regulated surface determinants) and *hts* (heme transport system) membrane transport system provides for heme-iron transport into *S. aureus* (30, 37). The Isd system encodes cell wall anchored surface proteins (IsdA, IsdB, IsdC, IsdH), a membrane transporter (IsdD, IsdE, IsdF), a transpeptidase (SrtB), and cytoplasmic heme degrading monooxygenases (IsdG, 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 the C-terminal end of surface protein substrates and the crossbridge 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 their 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 the 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 functioning as receptors during infection (15, 30). This contention is supported by the observation that IsdB
binds hemoglobin \textit{in vitro} with characteristics consistent with a receptor ligand interaction (30). Although \textit{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 \textit{S. aureus} is capable of utilizing purified hemoglobin or intracellular erythrocyte hemoglobin as sole iron sources 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 \textit{isdB} are impaired in their ability to grow using hemoglobin as the sole iron source. Importantly, staphylococcal mutants lacking \textit{isdB} also display a 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- \textit{S. aureus} Newman, a human clinical isolate, was used in this study (16). Isogenic variants lacking \textit{fur}, \textit{srtA}, \textit{isdB}, \textit{isdA}, or \textit{isdC} have been previously described (30, 31). \textit{isdH} was inactivated following a protocol described by Bae and Schneewind (1). Briefly, sequences flanking \textit{isdH} were PCR-amplified using primers IsdH3-51-AttB1 (GGGGACAAGTTTGTACAAAAAAGCAGGCTCGTACGAATTGCCTTAACTG) and IsdH3-31-StuI (AGGCCTAGTGATACTGGTTGCGTAG) for the upstream fragment and primers IsdH3-52-StuI (AGGCCTTATTAGCTCCGTATCACAAAG)
and IsdH3-32-attB2 (GGGGACCACCTTTGTACAAGAAAGCTGGGTACTCGGC)

AGATGGTCA-GTTG) 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 pKOR1ΔisdH. Transduction of the
ΔisdB::ermC mutation into the chromosome of the ΔisdH 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 was
PCR-amplified from genomic DNA of the Newman strain. 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 of
chloramphenicol. As control, Newman (WT) and the isdB mutant strain were
transformed with pOS1 lacking insert.

Growth assays- S. aureus cultures were grown overnight under low-iron
conditions by inoculating strains in RPMI supplemented with 1% casamino acids +
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 ZnCl2, 25 µM MnCl2, 1 mM MgCl2, 100 µM CaCl2)
supplemented with 500 µM 2-2’ dipyridyl, and 0.5 µM of 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 increase of absorbance
(O.D.600) over time. For hemin and hemoglobin pre-exposure experiments, overnight
cultures were grown as described above. Bacteria were then incubated for 30
minutes with or without 0.5 µM hemoglobin or 2 µM hemin at 37°C with aeration. Staphylococci were washed, diluted one hundred-fold into fresh NRPMI+ containing 450 µM 2-2’ dipyridyl and grown at 37°C with aeration.

For the growth assay using the erythrocyte precursor line K-562, we induced hemoglobin expression over 5 days following addition of 15 µM hemin to the media. Cells were then washed twice with TBS and resuspended in NRPMI+ supplemented with 500 µM 2,2’-dipyridyl. 1x10^4 K-562 cells per ml (induced and uninduced) were mixed with a 1:500 dilution of a S. aureus culture that had been grown for 15 hrs 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 were grown for 12 hours in RPMI + 200 µM 2,2’-dipyridyl. Following incubation, bacteria were mixed with top agar containing 1 mM 2,2’-dipyridyl and poured onto TSB plates containing 4 mM 2,2’-dipyridyl. Discs (8.5 mm in diameter) were impregnated with 10 µL of human hemoglobin at 25 µM concentrations, placed onto plates, and incubated for 24 hours at 37°C. Following incubation, growth surrounding the discs was photographed, and the diameter of the growth zones was determined using 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 densities of O.D. 600 0.40-0.55, cultures were treated with 1 mM 2-2’ dipyridyl for 1 hour. Staphylococci were collected by centrifugation and suspended in TSM buffer (100 mM Tris-HCl pH 7.0, 500 mM sucrose, 10 mM MgCl2). Following addition of
[\textsuperscript{14}C]hemoglobin, suspensions were incubated at room temperature for 5 minutes and ice cold ethanol:acetone [1:1 (vol:vol)] was added to quench iron uptake. Mixtures were incubated on ice for 10 minutes, and subsequently centrifuged at 10,000 \textit{g} for 10 minutes at 4°C. Supernatant was aspirated, and bacterial sediment was suspended in 100 \textmu L TSM and subjected to scintillation counting to determine the total amount of [\textsuperscript{14}C]hemoglobin associated with bacterial cells. To determine the percent amount of [\textsuperscript{14}C]hemoglobin in protoplast preparations, staphylococci were suspended in 0.1 M Tris-\textit{HCl} (pH 7.0) and incubated with 100 \textmu g/mL lysostaphin for 10 minutes at 37°C. After digestion, protoplasts were sedimented at 10,000 \textit{g} for 5 minutes, suspended in 0.1 M Tris-\textit{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 using antisera against a cytoplasmic protein (IsdI) (36). Following ethanol:acetone treatment, bacteria were sedimented by centrifugation at 6,000 \textit{g} for 10 minutes, 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 solublized with lysostaphin for 30 minutes at 37°C. Following cell wall digestion, the protoplasts were separated from the cell wall fraction by centrifugation at 13,000 \textit{g} for 2 minutes. All fractions (cell wall, protoplasts, ethanol:acetone supernatant) were subjected to immunoblot using antisera specific to the cytoplasmic protein IsdI (data not shown).

\textit{Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)-} Staphylococci were incubated in the presence of equal amounts of \textsuperscript{54}Fe\textsuperscript{2+}hemoglobin (Scipac) and...
[\textsuperscript{57}Fe]\textsuperscript{} transferrin (Scipac) as previously described (37). Normalizations were performed for the predicted number of iron atoms to account for differences in iron binding capacity of hemoglobin (4 atoms) vs. transferrin (2 atoms). Samples of bacterial cultures (1 ml aliquots) were removed at 6 and 9 hours and sedimented by centrifugation. Supernatants were mixed with high purity [\textsuperscript{15}N]nitric acid (Seastar\textsuperscript{TM}), and bacterial sediments were washed 3 times with NRPMI prior to suspension in [\textsuperscript{15}N]nitric acid. Samples were processed as described previously (37) with exceptions as noted below. Iron isotopic composition and abundance (\textsuperscript{54}Fe/\textsuperscript{56}Fe and \textsuperscript{57}Fe/\textsuperscript{56}Fe) were determined using a Finnigan Element 12 mass spectrometer (37). Samples were introduced into the ICP-MS with an all-Teflon sample introduction system consisting of an ESI\textsuperscript{TM} PFA 100 µl/min nebulizer, an ASX-100 autosampler with 2 ml PFA cups, and an ESI\textsuperscript{TM} PFA spray chamber. A 2-minute wash-time and 1 minute take-up time was used between samples. Briefly, 50 scans of the entire peak of each of \textsuperscript{53}Cr, \textsuperscript{54}Fe, \textsuperscript{56}Fe and \textsuperscript{57}Fe were collected at a mass-resolving power of M/ΔM=4300, sufficient to completely separate the atomic isobars from interfering molecular isobars (\textsuperscript{40}Ar\textsuperscript{13}C\textsuperscript{+} from \textsuperscript{53}Cr\textsuperscript{+}, \textsuperscript{40}Ar\textsuperscript{14}N\textsuperscript{+} from \textsuperscript{54}Fe\textsuperscript{+}, \textsuperscript{40}Ar\textsuperscript{16}O\textsuperscript{+} from \textsuperscript{56}Fe\textsuperscript{+}, etc.). The isobaric interference of \textsuperscript{54}Cr on \textsuperscript{54}Fe was corrected by monitoring \textsuperscript{53}Cr, and assuming a constant \textsuperscript{54}Cr/\textsuperscript{53}Cr=0.2489 ratio. Data is represented as isotope ratios with each reported data point representing an isotope ratio of an average of 50 experimentally determined ratios. In all experiments, isotopically labeled Fe was introduced as [\textsuperscript{54}Fe]\textsuperscript{} hemoglobin (90.65% \textsuperscript{54}Fe) and [\textsuperscript{57}Fe]\textsuperscript{} transferrin (94.4% \textsuperscript{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 FACS-based assay was performed using purified
hemoglobin, myoglobin, or hemopexin labeled with biotin (EZ-Link Sulfo-NHS-LC-
Biotin, Pierce) at a 12-molar excess of biotin to hemoproteins for 30 min at RT. *S.
aureus* cells were grown in RPMI containing 200 µM 2-2' dipyridyl until mid-log
phase (O.D.₆₀₀~1.0) and cells were diluted to $5 \times 10^6$ colony forming units in
phosphate buffered saline. Biotinylated hemoproteins (20 µg) were added to
staphylococci and incubated for 30 min at room temperature. Staphylococci were
then washed in Tris-buffered saline (TBS, 50 mM Tris-HCl (pH 7.5), 150mM NaCl)
and streptavidin-FITC was added at a 1:100 dilution. Bacterial complexes with
biotinylated hemoproteins and streptavidin were fixed with 3.5 % formaldehyde and
fluorescent intensity quantified by FACSCan (Becton Dickinson)

**Cosedimentation assays** - *S. aureus* cultures were grown overnight in RPMI
supplemented with 1% casamino acids and 200 µM 2-2’ dipyridyl (iron deplete) or in
RPMI supplemented with 10 µM FeSO₄ (iron replete). Staphylococci were washed
with TBS and diluted to the same optical density (O.D.₆₀₀ of ~1.0). Bacteria were
incubated with hemoglobin (0, 1, 5, and 10 µg/ml) in TBS for 30 minutes at room
temperature, washed with TBS and hemoglobin was eluted from the staphylococcal
surfaces by boiling for 15 minutes in 0.5M Tris-HCl buffer (pH 8.0), 4% SDS.
Following sedimentation of staphylococci, solubilized hemoglobin was
electrophoresed on 15% SDS-PAGE and electrotransferred to nitrocellulose
membrane (Amersham Biosciences). Hemoglobin was detected by immunoblotting
with rabbit anti-hemoglobin (Sigma) and anti-rabbit AlexaFluor 680-conjugate
(Molecular Probes) using an Odyssey Infrared Imaging system (LI-COR). As a
loading control, we took advantage of the fact that staphylococcal protein A non-
specifically 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-S. aureus wild-type, Δfur, and ΔisdB::ermC were
grown in either iron replete (RPMI + 10 µM FeSO₄), or iron deplete (RPMI + 300
µM 2,2’-dipyridyl) conditions. Following 18 hour incubations, the bacteria were
pelleted by centrifugation at 6,000 g for 10 minutes. The cell wall and protoplasts
were separated and subjected to immunoblot as described above

Mouse model of infection- Six to eight week old BALB/c mice (Jackson
Laboratories) were infected with 1 × 10⁶ colony forming units of S. aureus wild-type
(Newman) and the ΔisdB::ermC, ΔisdH, and ΔisdB::ermCΔisdH mutant strains
suspended in phosphate buffered saline (PBS) by injection into the retro-orbital vein
complex. Four days after infection, mice were euthanized with CO₂. Livers and
kidneys were removed, analyzed for abscess formation, and homogenized in PBS.
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 using the Student’s t test.
RESULTS

*S. aureus* utilize hemoglobin or myoglobin as nutrient iron sources.

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 a source of iron by staphylococci, we measured the ability of *S. aureus* to grow on hemoglobin, myoglobin, or hemopexin in media 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-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* utilize 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 developed bright red color and expressed large amounts of hemoglobin, whereas uninduced K-562 cells maintained a pale white color 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 media did not support bacterial growth (data not shown). *S. aureus* incubated with uninduced K-562 cells in the same media, replicated 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-iron (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 as 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 isotopic concentrations in staphylococci are determined using inductively coupled plasma mass spectrometry (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 efficiency (data not shown). To determine the iron source preference, iron starved bacteria were subcultured into chemically defined medium and supplemented with equimolar amounts of $[^{54}\text{Fe}]{\text{hemoglobin}}$ and $[^{57}\text{Fe}]\text{transferrin}$. 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 7-fold 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 $[^{54}\text{Fe}]{\text{hemoglobin}}$ from conditioned media 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 hours) (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 using a 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 a *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 *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*, *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. Co-sedimentation assays with hemoglobin and
staphylococci grown under iron replete or deplete 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 similar decrease in hemoglobin binding
as observed for 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 in iron-deficient conditions, at levels
similar to wild-type (Fig. 4B). These results conclusively demonstrate that the
impairment in 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, in iron replete, or in iron deplete
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 co-sedimentation 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 [14C] 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}\text{C}]\text{hemoglobin} bound to the surface of \textit{S. aureus} and this interaction was significantly reduced upon inactivation of \textit{isdB} (Fig. 3-4 & Table 2). To measure globin (polypeptide)-derived internalization of \[^{14}\text{C}]\text{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, which had captured \[^{14}\text{C}]\text{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.

\textit{isdB} is required for \textit{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 wild-type, \textit{isdB}, \textit{isdH}, and \textit{isdBH} mutant strains were compared in media where hemoglobin served as the sole source of iron. Initial observations suggested that inactivation of \textit{isdB} does not abrogate the ability of \textit{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 non-specific 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 non-specifically bound hemoglobin can be removed from staphylococci by washing in iron-free media, staphylococci were incubated with hemoglobin for thirty minutes, 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 staphylococcal strains examined were unable to grow in iron free medium unless the bacteria were pre-exposed 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, *isdB* or *isdBH* double mutant strains were impaired in their 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 media 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, based on 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* possess additional mechanisms of access 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 systemic mouse model of 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. 3-4**) (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 1×10^6 CFUs of *S. aureus* Newman (wild-type) and *isdB, isdH, or isdBH* mutant strains. Enumeration of bacteria from abscesses in various organ tissues removed from mice 4 days after infection revealed a 1-log reduction in bacterial load for *isdB* mutant staphylococci in both spleen and kidneys when compared to wild-type (*p* < 0.03) (**Fig. 6**). Inactivation of *isdH* resulted in a small reduction in bacterial load in kidney tissue, however the observed difference was not statistically significant (*p* < 0.07). Moreover, *isdBH* double mutant strains exhibited a similar decrease in bacterial load as observed for *isdB* mutant staphylococci (*p* <
Together, these results suggest that IsdB is required for \textit{S. aureus} virulence \textit{in vivo} and that IsdB is responsible for a significant portion of the virulence defect observed upon inactivation of \textit{srtA}.

\section*{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 inherited diseases or tissue injury, predisposes 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 \textit{S. aureus} bind and use hemoglobin as a sole iron source through the 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 \textit{S. aureus} can use hemoglobin or myoglobin as a sole iron source (Fig. 1-2). Based on our inability to detect myoglobin binding to \textit{S. aureus}, it appears as if staphylococci have evolved an alternate mechanism to access myoglobin-Fe. In contrast, hemoglobin readily binds to the staphylococcal surface (Fig. 3-4 & Table 2). The ability of \textit{S. aureus} to use hemoglobin as an iron source is not limited to \textit{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 system (Isd) 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. 3-4), 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. 3-4 & 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 is 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 recognition 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 a *srtA* 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 of how these bacteria are able to bind and transport heme-iron through their membranes is not well understood. Based on 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 blood borne infection, *S. aureus* encounter red blood cells that are lysed via the secretion of potent hemolysins (3). Erythrocyte lysis liberates large quantities of intra-cellular 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 yet understood and then translocated across the cell wall envelope by interacting with IsdC and/or IsdA. Heme then enters the cytoplasm via the 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 the iron for use as a
staphylococcal nutrient.

Further identification and detailed description of the mechanism by which
pathogens like S. aureus acquire 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 homologous systems to
the Isd heme transport apparatus exist in multiple Gram-positive pathogens including
Bacillus anthracis (35), Clostridium tetani (38), and Listeria monocytogenes (38).
ACKNOWLEDGEMENTS

We would like to thank Dr. Ann Smith for her generous gift of purified hemopexin. This work was enabled by United States Public Health Service Grants AI38897 (O.S.), AI52474 (O.S.) and AI69233 (E.P.S.) from the National Institute of Allergy and Infectious Diseases and the Division of Microbiology and Infectious Diseases. V. J. T. was supported by a Ruth L. Kirschstein NRSA AI071487.
REFERENCES


**FIGURE LEGENDS**

**Figure 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 optical density (O.D. 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 ± S.D. of triplicate experiments.

**Figure 2.** Growth of *S. aureus* using intracellular hemoglobin as a sole iron source. A) Immunoblot of extracts from K-562, an erythrocytes precursor cell line that was induced (I) or left uninduced (U) using an anti-hemoglobin antibody as a measure for hemoglobin expression. B) *S. aureus* strain Newman was cultured in iron-free medium in the presence of K-562 cells induced (*Gray line*) or uninduced (*Black line*) for the expression of hemoglobin (Hb). Data represent the mean ± S.D. of triplicate bacterial enumeration on agar plates (CFU; colony forming units). Asterisks denote statistical significance differences from wild-type as determined by a Student’s *t* test (*p* < 0.05).

**Figure 3.** Hemoprotein binding to *S. aureus* surface. FACS-based assay measuring the binding of hemoglobin (Hb; A), myoglobin (Mb; B), and hemopexin (Hp; C) to the surface of different strains of *S. aureus*. MFI stands for Mean Fluorescence Intensity. Results represent the mean ± S.D. from triplicate determinations. Asterisks denote statistical significance differences from wild-type as determined by a Student’s *t* test (*p* < 0.05).
**Figure 4.** Hemoglobin binding to whole cell *S. aureus*. A) Whole cells of the indicated *S. aureus* strains were incubated with various concentrations of hemoglobin (Hb; µg/ml) followed by immunoblot with an antiserum specific for Hb. Left panel represents Hb and right panel represents the loading control. B) Whole cells of the complemented *isdB* mutant strain (Δ*isdB/pOS1IsdB*) and controls strains containing the pOS1 plasmid without the *isdB* gene (*i.e.*, WT/pOS1 and Δ*isdB/pOS1) were grown in iron deplete (-Fe) or iron replete (+Fe) medium. Whole cells were then incubated with various concentrations of Hb (µg/ml) followed by immunoblot with an antiserum specific for Hb. Left panel represents Hb and right panel represents the loading control. C) *S. aureus* Newman (WT) and the isogenic Δ*isdB*, and Δ*fur* mutant strains were grown in iron deplete (-Fe) or iron replete (+Fe) medium. Whole cells were then analyzed by immunoblot with an antiserum specific for IsdB.

**Figure 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 pre-incubated with hemin (2 µM) or hemoglobin (0.5 µM) for 30 minutes or not. Cells were then washed and cultured in iron-free NRPMI+. Bacterial growth was determined by measuring the optical density (O.D. _600_) of cultures at 6, 9, and 12 hours. Results represent the mean ± S.D. from triplicate determinations. Asterisks denote statistically significance differences from wild-type as determined by a Student’s _t_ test (_p_ < 0.007).
Figure 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. Horizontal gray line represents the mean of log colony forming units on the Y-axis. The horizontal black line represents the limit of detection. Each data point represents the number of bacteria (colony forming units; CFU) per milliliter of tissue homogenate in a single animal. Asterisks denote statistically significant differences between wild-type and mutant strains as determined with the Student’s *t* test (*p* < 0.03).
### Table 1. Isotopic tracking of Fe-hemoglobin and Fe-transferrin uptake

<table>
<thead>
<tr>
<th>Samples</th>
<th>% Hb&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Tf&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$^{54}$Fe/$^{56}$Fe</th>
<th>$^{57}$Fe/$^{56}$Fe</th>
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<tbody>
<tr>
<td>$[^{54}\text{Fe}]\text{Hb} / [^{57}\text{Fe}]\text{Tf}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Whole cells 6 hrs</td>
<td>88%</td>
<td>12%</td>
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<td>0.74</td>
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<td>Supernatant 6 hrs</td>
<td>35%</td>
<td>65%</td>
<td>0.45</td>
<td>0.84</td>
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<tr>
<td>Whole cells 9 hrs</td>
<td>71%</td>
<td>29%</td>
<td>1.05</td>
<td>0.39</td>
</tr>
<tr>
<td>Supernatant 9 hrs</td>
<td>53%</td>
<td>47%</td>
<td>2.78</td>
<td>2.48</td>
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<tr>
<td>$[^{54}\text{Fe}]\text{Hb}$</td>
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<td></td>
<td></td>
<td></td>
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<td>Whole cells 6 hrs</td>
<td>100%</td>
<td>0%</td>
<td>0.55</td>
<td>0.03</td>
</tr>
<tr>
<td>Whole cells 9 hrs</td>
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<td>0%</td>
<td>0.71</td>
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<td>$[^{57}\text{Fe}]\text{Tf}$</td>
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<td></td>
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<tr>
<td>Whole cells 6 hrs</td>
<td>2%</td>
<td>98%</td>
<td>0.07</td>
<td>0.26</td>
</tr>
<tr>
<td>Whole cells 9 hrs</td>
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<tr>
<td><strong>Growth Condition</strong></td>
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<tr>
<td>Normal growth medium&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.06</td>
<td>0.02</td>
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<tr>
<td>Labeled growth medium&lt;sup&gt;d&lt;/sup&gt;</td>
<td>60%</td>
<td>40%</td>
<td>1.78</td>
<td>1.19</td>
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<sup>a</sup> Hb; hemoglobin.  
<sup>b</sup> Tf; transferrin.  
<sup>c</sup> TSB.  
<sup>d</sup> TSB supplemented with $[^{54}\text{Fe}]\text{Hb}$ and/or $[^{57}\text{Fe}]\text{Tf}$.  
<sup>e</sup> NA; not applicable.

### Table 2. $[^{14}\text{C}]$hemoglobin binding to S. aureus

<table>
<thead>
<tr>
<th>Strain</th>
<th>CPM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% of whole cell Newman&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT whole cell</td>
<td>$3.31 \times 10^4$ (243.8)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100.000% (00.000%)</td>
</tr>
<tr>
<td>WT protoplast</td>
<td>$5.54 \times 10^4$ (51.1)</td>
<td>0.017% (00.014%)</td>
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<tr>
<td>ΔisdB whole cell</td>
<td>$8.37 \times 10^4$ (204.3)</td>
<td>10.742% (18.240%)</td>
</tr>
<tr>
<td>No label</td>
<td>$8.00 \times 10^4$ (1.4)</td>
<td>0.004% (00.001%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> CPM; counts per minute  
<sup>b</sup> Percent calculated from the CPM.  
<sup>c</sup> Standard error.
Table 3. Hemoglobin nutrition plate-based assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diameter (mm)</th>
<th>Standard Deviation (mm)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>17.06</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>ΔisdB</td>
<td>15.10*</td>
<td>0.39</td>
<td>4.08x10⁻⁸</td>
</tr>
<tr>
<td>ΔisdH</td>
<td>17.18</td>
<td>0.51</td>
<td>5.9x10⁻²</td>
</tr>
<tr>
<td>ΔisdBΔisdH</td>
<td>16.15*</td>
<td>0.37</td>
<td>3.5x10⁻⁴</td>
</tr>
</tbody>
</table>

* P-value determined by Student’s t-test, asterisks denote statistical significance (P<0.05)
Torres et al. Figure 1
Torres et al. Figure 2
Torres et al. Figure 3.
Torres et al. Figure 4.
Torres et al. Figure 5
Torres et al. Figure 6.