

IsdG and IsdI, Heme-degrading Enzymes in the Cytoplasm of *Staphylococcus aureus**

Received for publication, July 22, 2003, and in revised form, October 9, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M307952200

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***Staphylococcus aureus* requires iron for growth and utilizes heme as a source of iron during infection. Staphylococcal surface proteins capture hemoglobin, release heme from hemoglobin and transport this compound across the cell wall envelope and plasma membrane into the bacterial cytoplasm. Here we show that *Staphylococcus aureus* *isdG* and *isdI* encode cytoplasmic proteins with heme binding properties. IsdG and IsdI cleave the tetrapyrrol ring structure of heme in the presence of NADPH cytochrome P450 reductase, thereby releasing iron. Further, IsdI complements the heme utilization deficiency of a *Corynebacterium ulcerans* heme oxygenase mutant, demonstrating *in vivo* activity of this enzyme. Although *Staphylococcus epidermidis*, *Listeria monocytogenes*, and *Bacillus anthracis* encode homologues of IsdG and IsdI, these proteins are not found in other bacteria or mammals. Thus, it appears that bacterial pathogens evolved different strategies to retrieve iron from scavenged heme molecules and that staphylococcal IsdG and IsdI represent examples of bacterial heme-oxygenases.**

In order to successfully colonize the mammalian host, bacterial cells must overcome a number of host defense systems both innate and acquired. A primary obstacle to colonization is the lack of available iron. Most living microorganisms require iron in the range of 0.4–4.0 μM (1); however in mammals, the concentration of free ionic iron is maintained at a level of about 10^{-9} M (2, 3). Iron sequestration in host tissues is caused by a combination of factors including the low solubility of iron at physiological pH (4), the intracellular location of iron (99.9% of total body iron is found within mammalian cells) (4), and the sequestration of this ion within the iron-binding glycoproteins transferrin and lactoferrin or heme-containing proteins such as hemoglobin. Utilization of hemoglobin as an iron source presumably requires bacterial binding of the hemoglobin polypeptide, removal, and transport of the heme molecule, and opening of the heme porphyrin ring to remove the single iron atom. All identified enzymes capable of heme degradation are monooxygenases known as heme oxygenases. Heme oxygenases are ubiquitous in nature, and are responsible for the oxidative degradation of heme to biliverdin, CO, and free iron (5–7). In mammals, this process is believed to be important in protecting the organism against oxidative damage (8–11), however, in

bacteria heme degradation is essential in order to access the iron atom for use as a nutrient source. Bacterial pathogens representing more than fifteen genera are capable of utilizing heme as a sole iron source, however, a comparatively smaller number of bacterial heme-degrading enzymes have been identified (12–15). Examining the currently available complete or incomplete bacterial genome sequences with BLAST homology searches using known bacterial heme oxygenase enzymes as queries does not reveal additional candidates for enzymes capable of oxidatively degrading heme (data not shown). This paucity of identifiable heme-degrading enzymes in bacteria that utilize heme as an iron source allows for speculation as to how these bacteria are accessing the iron atom of the heme porphyrin ring.

We have previously described a heme-uptake system in the pathogenic bacterium *Staphylococcus aureus* (16), which is encoded by a cluster of genes known as (*isd*)¹ iron-regulated surface determinants. This cluster encompasses three transcriptional units, *isdA*, *isdB*, and *isdCDEFsrtBisdG*, specifying three cell-wall anchored proteins capable of binding hemin: IsdA, IsdB, and IsdC. In addition, IsdB binds hemoglobin with characteristics resembling receptor-ligand interactions. IsdD (a membrane protein), IsdE (a lipoprotein ATPase), and IsdF (a polytopic transmembrane protein) each display homology to Gram-positive and Gram-negative heme-iron transporters, whereas SrtB functions as a sortase and is responsible for anchoring IsdC to the cell wall (17). The product of the last gene within the multiple cistronic *isdCDEFsrtBisdG* transcript, IsdG, is hypothesized to reside in the cytoplasm. In this report we describe purification of IsdG, and its intrachromosomal paralogue IsdI. We show that sequence signatures place these proteins in a family of known monooxygenases, and we demonstrate that IsdG and IsdI bind hemin with characteristics consistent with known heme-degrading enzymes. In addition, we show that both IsdG and IsdI are capable of degrading the heme macrocyclic porphyrin ring, with subsequent release of free iron for use by the pathogen as a nutrient source. Analysis of the reaction products of IsdG- and IsdI-mediated heme degradation reveal compounds that are chromatographically similar to those produced by mammalian HO-1 heme oxygenase. Finally, IsdI is shown to complement the heme utilization defect of a *C. ulcerans* heme oxygenase mutant (18).

EXPERIMENTAL PROCEDURES

General Methods—Western blotting, transformations, plasmid purification, and subcloning were carried out as described previously (19). Deionized double distilled water was used for all experiments. Oligonucleotides were synthesized by Integrated DNA Technologies. Antibodies were created by injection of purified protein emulsified in com-

* This work was supported by United States Public Health Service Grants AI38897 and AI52474. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: *isd*, iron-regulated surface determinants; HO-1, heme oxygenase 1; HPLC, high performance liquid chromatography; *srtB*, sortase B; Fur, ferric uptake regulator, EDDA, ethylenediamine-*N,N'*-diacetic acid.

plete Freund's adjuvant (day 7 injection) or incomplete Freund's adjuvant (two subsequent injections) into female New Zealand rabbits.

Bacterial Strains and Growth Conditions—*Escherichia coli* strain DH5 α [F⁻ *ara* D(*lac-proAB*) *rpsL* Φ 80(*dlacZ*M15 *hsd* R17)] was used for DNA manipulation, and *E. coli* strain BL21 (DE3) [F⁻ *ompT* *hsdS*_B(*r*_B⁻ *m*_B⁻) *gal* *dcm* (DE3)] was used for the expression of *isdG* and *isdI*. *isdG* and *isdI* were amplified with PCR using *S. aureus* strain Newman chromosomal DNA as a template. *S. aureus* strains RN6390 and Newman were used as parent strains for the inactivation of *isdG*. *C. ulcerans* strain CU712 (18) and CU29 were obtained from the strain collection of Michael P. Schmitt. *C. ulcerans* strains were grown in heart infusion broth (Difco, Detroit, MI) with 0.2% Tween 80 (HIBTW). Ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), and chloramphenicol (30 μ g/ml for *E. coli* and 2 μ g/ml for *C. ulcerans*) were added to the media as required. All strains were incubated at 37 °C.

Construction of Vectors—To create vectors to be used in expression of *isdG* and *isdI*, the complete *isdG* and *isdI* coding sequences were PCR amplified. The amplified DNA fragments were cloned into pCR2.1 (Invitrogen) and successful transformants were sequenced for accuracy. Inserts containing the correct sequence were subcloned into pET15B (Novagen). To create vectors for use in the heme utilization assay, *orfXisdI* was amplified by PCR using *IsdI*5BglIIIB (AAAGATCTCGTAAATGCGTTAATGGGACAAG) and *IsdI*3BamHI (AAGATCCTCAGACAAGCCGGATGAATTC), and cloned into pCR2.1 (Invitrogen). Inserts were excised from pCR2.1 with BglII and BamHI for directional cloning into pCGL0243 (20) creating pEPS10. The corynebacterial expression vector pCM2.6, and pCD293 (pCM2.6 containing the cloned *C. diphtheria* heme oxygenase gene *hmuO*) were generous gifts of Michael P. Schmitt.

Mutagenesis of *IsdG*—Sequence flanking *isdG* was amplified in PCR using primers *IsdG*-51-EcoRI (AAAAGAATTCCTCCTTTAGAAATTC-CAG) and *IsdG*-31-KpnI (AAAAGGTACCCGTCAGCCTATTTTCTGC) for the upstream fragment and *IsdG*-52-KpnI (AAAAGGTACCATTAT-AAATAACAAGTAATTACA) and *IsdG*-32-BamHI (AAAAGGATCCGCATCCGCAGTTCTAATTA) for the downstream fragment. These fragments were first assembled in pCR2.1, and subsequently cloned into pTS1 (21). Cloning products were linearized with KpnI, and an *ermC* cassette was inserted. Inactivation of *isdG* was carried out using allelic exchange with pTS1*IsdG::erm* into the laboratory strain RN4220 (restriction-deficient). Subsequent phage transduction with the bacteriophage Φ -85 into strain Newman was used to transfer the *isdG::ermC* mutation.

Expression and Purification of *IsdG* and *IsdI*—*E. coli* BL21(DE3) strains carrying either pET15B*IsdG* or pET15B*IsdI* were grown overnight at 37 °C in Luria-Bertani medium containing 100 μ g/ml ampicillin. The cells were subcultured into fresh medium and grown at 37 °C to mid-log phase. At this time, target gene expression was induced using 1 mM isopropyl-1-thiol-D-galactopyranoside. Cell growth was continued for three hours at 30 °C, and the cells were harvested by centrifugation (10,000 \times *g* for 15 min). Cells were lysed using a French press in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl containing 100 μ M phenylmethylsulfonyl fluoride. The cell suspension was centrifuged at 100,000 \times *g* for 60 min, and the soluble fraction was applied to a Ni-NTA column, pre-equilibrated with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl. The column was washed with 20 volumes of 50 mM Tris-HCl (pH 7.5) 150 mM NaCl followed by a second washing with 30 volumes of 50 mM Tris-HCl (pH 7.5) 150 mM NaCl containing 10% glycerol and 10 mM imidazole. The protein was then eluted in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl containing 50 mM imidazole, and fractions were dialyzed against 50 mM Tris-HCl (pH 7.5) 150 mM NaCl. The purified proteins were stored at -20 °C.

Fractionation of *Staphylococci*—*S. aureus* were grown to mid-log phase and sedimented by centrifugation at 13,000 \times *g* for 5 min. The supernatant was collected and precipitated with ice-cold trichloroacetic acid and washed with ice-cold acetone (supernatant fraction). The resulting pellet was suspended in TSM (100 mM Tris-HCl (pH 7.0), 500 mM sucrose, 10 mM MgCl₂), and incubated in the presence of 100 μ g of lysostaphin for 60 min at 37 °C. The resulting protoplasts were sedimented at 13,000 \times *g* for 15 min, and the supernatant was collected and precipitated in trichloroacetic acid/acetone (cell wall fraction). The pellet was suspended in membrane buffer (50 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, 60 mM KCl) and subjected to five rounds of freeze-thaw in a dry ice ethanol bath to lyse the protoplasts. The membranes were sedimented by ultracentrifugation at 200,000 \times *g* for 30 min, and the cytoplasm and membrane fractions were precipitated with trichloroacetic acid and acetone. Acetone-washed precipitates were suspended in sample buffer, separated by 15% SDS-PAGE, and analyzed by immunoblotting.

Reconstitution of *IsdG* and *IsdI* with Hemin—The heme-*IsdG* and heme-*IsdI* complexes were prepared as described previously for heme-heme oxygenase complexes (13, 22). Hemin was added to purified protein at a ratio of 3:1 heme:protein. The sample was applied to a nickel-nitrilotriacetic acid-agarose column pre-equilibrated with 50 mM Tris-HCl (pH 7.5) 100 mM NaCl. The column was then washed with the same buffer (30 volumes), and the protein was eluted in 500 mM imidazole. The fractions containing the heme-protein complexes were pooled and dialyzed against 50 mM Tris-HCl (pH 7.5), 100 mM NaCl.

Absorption Spectroscopy—All absorption spectra were obtained using a Varian Cary 50BIO. Hemin binding studies were carried out by difference absorption spectroscopy in the Soret region. Aliquots of heme (0.1–25 μ M) were added to both the sample cuvette (10 μ M either *IsdG* or *IsdI*) and reference cuvettes at 25 °C. Spectra were recorded 5 min after the addition of heme. The millimolar extinction coefficient was determined using the pyridine hemochrome method (23).

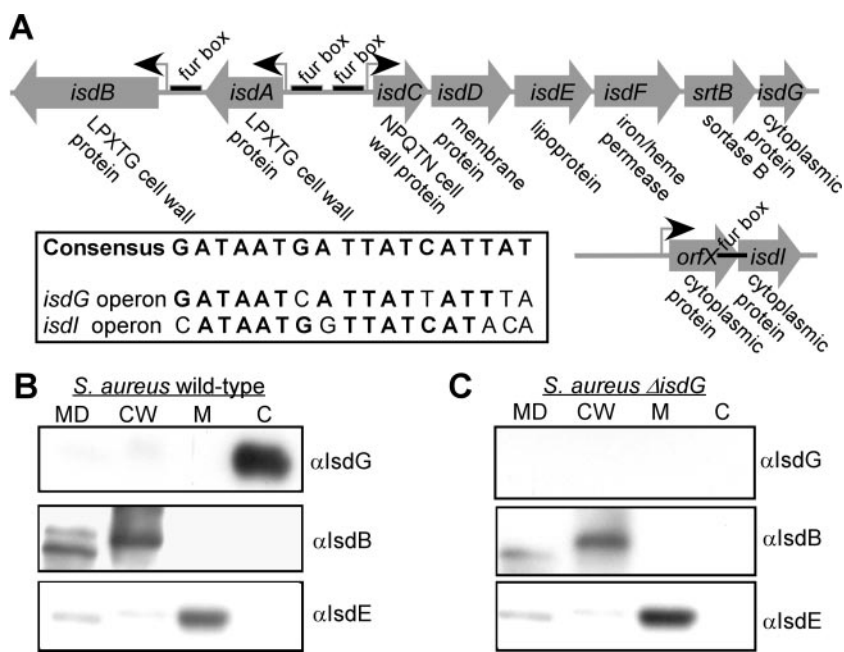
Hemin Degradation Assays—Iron release assay: To measure the release of free iron from heme, 10 μ M of *IsdG* or *IsdI* and 55 heme mixture (0.4 nM [⁵⁵Fe]hemin (RI Consultants) per 50 μ l of 2% bovine serum albumin) were placed into 50 mM HEPES pH 7.4, 1 mM EDTA to a final volume of 1 ml, and incubated at 30 °C for 30 min. 10 μ l of 1 mM unlabeled heme in 2% bovine serum albumin was added to stop the reaction and the sample was vortexed. Trichloroacetic acid was added to the sample at a concentration of 7.5% followed by incubation on ice for thirty minutes. The resulting precipitate was sedimented by centrifugation at 13,000 \times *g* at 4 °C for 15 min. 750 μ l of the supernatant was withdrawn and added to 5 ml scintillation fluid. The amount of [⁵⁵Fe] was determined using a scintillation counter (Beckman LS600K). Reaction with ascorbate: ascorbic acid-dependent degradation of heme was monitored spectrophotometrically as previously described (14). *IsdG*-Hemin (10 μ M) and *IsdI*-Hemin (10 μ M) in 50 mM Tris-HCl (pH 8.0) were incubated with ascorbic acid at a final concentration of 10 mM. The spectral changes between 300 and 800 nm were recorded every 5 min. The products of the reaction were extracted and subjected to HPLC as described below. Reaction with NADPH P450 reductase: the reaction of *IsdG*-Hemin and *IsdI*-Hemin in the presence of human NADPH-cytochrome P450 oxidoreductase (recombinant enzyme from *Spodoptera frugiperda*, Calbiochem) was similar to that previously described (13, 14). Human cytochrome P450 oxidoreductase was added to the *IsdG*-heme and *IsdI*-heme complex (10 μ M) at a ratio of reductase/*Isd* equal to 0.3:1 in a final volume of 1 ml 50 mM Tris-HCl (pH 8.0). Initiation of the reaction was carried out by the addition of NADPH in 10 μ M increments to a final concentration of 100 μ M. The spectral changes between 300 and 800 nm were monitored. Following completion of the reaction, the reaction products were extracted and subjected to HPLC as described below. Reaction in the presence of catalase: Purified recombinant catalase from *Aspergillus niger* (Sigma), was added to all reaction cuvettes at a ratio of catalase:hemo protein equal to 0.5:1 immediately before the addition of either reductant or reductase.

HPLC of the *IsdG* and *IsdI* Reaction Products—Following the reaction of the heme-*Isd* complexes with NADPH-cytochrome P450 oxidoreductase or ascorbate, 200 μ l of glacial acetic acid and 200 μ l of 3 M HCl were added to quench the cleavage reaction. Subsequently, the reaction mixture was extracted with 1.5 ml of chloroform. The organic layer was washed three times with 1 ml of distilled water, and the chloroform layer was removed under a stream of nitrogen. The resultant residue was dissolved in 800 μ l of 85:15 (v/v) methanol:water prior to HPLC analysis. The samples were analyzed by reverse-phase HPLC on a Thermo hypersil C18 aquasil column (Keystone Scientific Operations), using a Beckman Coulter System Gold HPLC machine, eluted with 85:15 (v/v) methanol:water at a flow rate of 0.2 ml/min.

In Vivo Heme Utilization Assay—To determine the ability of *C. ulcerans* strains to utilize heme, a plate assay modified from that developed by Michael P. Schmitt (18) was employed. Briefly, *C. ulcerans* strains (CU712(pCM2.6), CU29(pCM2.6), CU29(pCD293), CU29(pEPS10)) were grown in HIBTW at 37 °C overnight under the appropriate antibiotic selection. The following day, ~10⁷ bacteria were plated onto the surface of HIBTW agar medium, HIBTW agar medium containing 200 μ g/ml EDDA, or HIBTW agar medium containing 200 μ g/ml EDDA and 1 μ M heme. In the absence of added heme, 200 μ g/ml EDDA completely inhibits the growth of all strains tested. After 28 h of incubation at 37 °C, the number of colony forming units on HIBTW plates containing EDDA, and heme was divided by the number of colony forming units on HIBTW plates alone, and presented as "Hemin Growth Efficiency."

FIG. 1. Sequence analysis and subcellular localization of IsdG and IsdI.

A, genomic organization of the *isd* locus with three transcriptional units *isdA*, *isdB*, and *isdCDEFsrtBisdG* that are controlled by Fur through conserved DNA sites called *fur*-boxes. A bicistronic operon encoding *isdI* is also controlled by Fur but is located elsewhere on the chromosome of *S. aureus*. Nucleotide sequence of predicted *Fur*-boxes of *isdG* and *isdI* as compared with the *S. aureus* *Fur*-box consensus sequence is shown. Immunoblotting of subcellular fractions of *S. aureus* strains (B) Newman and (C) EPS1 [$\Delta(isdG)$] grown under iron-starved conditions. Culture medium (MD), cell wall (CW), membrane (M), and cytoplasmic (C) fractions are shown. Antibodies raised against purified IsdB, IsdE, and IsdG were used for chemiluminescent detection.



RESULTS

Identification and Genomic Context of IsdG and IsdI—The iron atom of heme is used as a nutrient source by pathogenic bacteria capable of infecting vertebrates (15). Analysis of all available complete and incomplete bacterial genomes using previously identified bacterial heme oxygenase sequences (12–14,18) reveals that putative heme oxygenase enzymes are only identifiable in 5 genera including, *Corynebacterium*, *Neisseria*, *Pseudomonas*, *Agrobacterium*, and *Streptomyces* (data not shown). We have recently identified a cluster of three operons in *S. aureus* containing numerous iron regulated genes, encoding for a heme uptake apparatus (16) (Fig. 1A). The genes involved in the process of transportation of the porphyrin ring of heme into the bacterial cytoplasm have been identified, however the mechanism whereby *Staphylococci* access the iron atom contained within the porphyrin macrocyclic molecule has yet to be determined. We hypothesized that a gene within the *isd* gene cluster, predicted to encode for a cytoplasmic protein, *isdG*, is involved in the degradation of heme (Fig. 1A) (the amino acid sequence of this protein can be accessed through NCBI Protein Data base under NCBI Accession NP_371660). To establish the subcellular location of IsdG, staphylococcal cultures were fractionated into four compartments (extracellular medium, cell wall envelope, plasma membrane, and cytoplasm) and specific polypeptides were detected by immunoblotting (Fig. 1B). As expected, IsdG was found only in the staphylococcal cytoplasm. As a control, the lipoprotein IsdE was observed in the plasma membrane, whereas IsdB, the sortase A anchored surface protein, was located in the cell wall fraction. Small amounts of a faster migrating IsdB species were detected in the medium, suggesting that IsdB degradation products may be released from the staphylococcal surface.

After deletion of the *isdG* gene by allelic replacement, the $\Delta(isdG)$ mutant strain *S. aureus* EPS1 failed to produce anti-IsdG immunoreactive species (Fig. 1C), indicating that the cytoplasmic protein identified in Fig. 1 indeed represents IsdG. *S. aureus* encodes an *isdG* paralogue, *isdI*, located outside of the *isd* locus (2.756 Mb of the genome) in a bicistronic transcriptional unit at 1.834 Mb (Fig. 1A) (the amino acid sequence of this protein can be accessed through NCBI Protein Data base under NCBI Accession NP_370689). The deduced IsdG protein is a 107 amino acid molecule with a calculated molecular

weight of 12,545 Da, and a calculated pI of 6.61, while the deduced IsdI protein is a 109 amino acid protein with a calculated molecular weight of 12,790 and a calculated pI of 4.8. IsdG and IsdI are 78% similar at the amino acid level, and both proteins demonstrate little identity to the previously identified heme oxygenases, a group of enzymes capable of degrading heme (data not shown). IsdG has a histidine residue at position 27, corresponding to a potential candidate for the established proximal ligand of His-25 in mammalian HO-1 and His-20 in bacterial HmuO (24, 25). Furthermore, Pfam analysis (26) associated IsdG and IsdI with the ABM family of monooxygenases, enzymes from *Streptomyces* that catalyze oxidation of aromatic polyketides. This enzyme family is unique in that its members catalyze the oxygenation of various compounds without the need for prosthetic groups or cofactors typically associated with the activation of molecular oxygen (27). Upstream of the first gene in the *isdG* containing transcriptional unit, and immediately upstream of *isdI* are canonical *Fur* boxes (Fig. 1A), consensus nucleotide sequence to which the *Fur* repressor is capable of binding (28), implying that these genes are iron-regulated. Iron-regulation of IsdG was verified experimentally by immunoblot (data not shown). The genomic context surrounding these two genes reveals *isdG* localized at the end of a heme uptake operon, and *isdI* is immediately downstream of a gene with unknown function. The genetic linkage to a heme uptake system, confirmed iron regulation, cytoplasmic localization, and inclusion in a monooxygenase family of enzymes caused us to hypothesize that IsdG and IsdI might represent a new family of heme-degrading monooxygenases in pathogenic bacteria.

Expression of IsdG and IsdI—Both *isdG* and *isdI* were expressed as six-histidyl-tagged proteins under the control of the T7 polymerase promoter in *E. coli* and purified by affinity chromatography on nickel nitrilotriacetic acid-agarose yielding distinct protein bands migrating at ~ 12.5 kDa on SDS-PAGE (Fig. 2, A and B). This size corresponds well with the predicted sizes of IsdG and IsdI. Typical purifications produced ~ 35 mg of protein per liter of *E. coli* BL21(DE3). Notably, *E. coli* cultures overexpressing IsdG or IsdI exhibited a bright yellow color as compared with the darker yellow color of cultures containing the pET15b expression vector alone (data not shown).

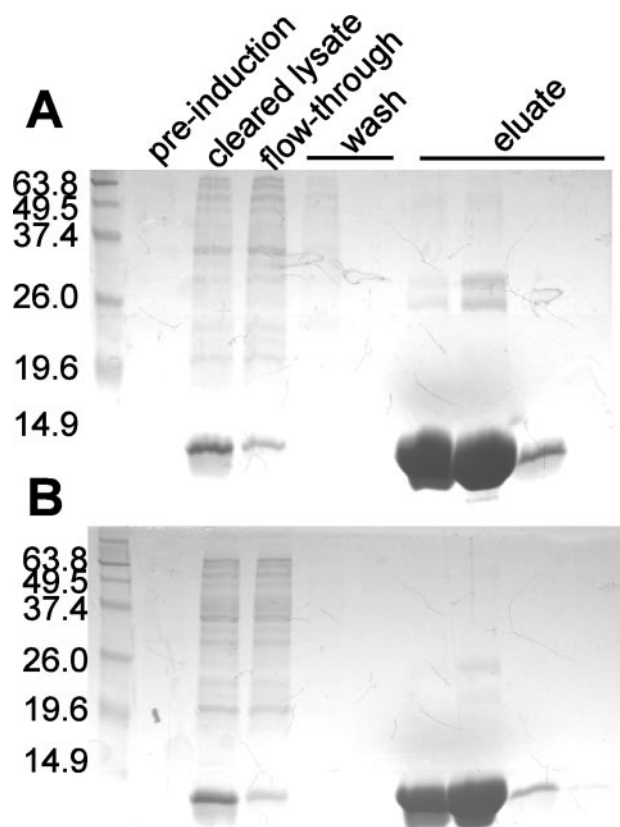


FIG. 2. **Purification of IsdG and IsdI.** SDS-PAGE analysis of the purification of His-IsdG (A) and His-IsdI (B) from recombinant *E. coli* lysed after T7 polymerase induced expression of *isd* genes. Lane 1, marker; lane 2, pre-induction; lane 3, cleared lysate; lane 4, flow through; lane 5, wash 1; lane 6, wash 2; lane 7, eluate 1; lane 8, eluate 2; lane 9, eluate 3; lane 10, eluate 4.

Properties of the Heme-IsdG and Heme-IsdI Complex—Spectral analysis of purified IsdG and IsdI in the range of 400–600 nm did not reveal absorption signals suggestive of heme binding (Fig. 3). Reconstitution of both IsdG and IsdI with heme at pH 8.0 generated optical absorption spectra containing a Soret band at ~412 nm, and α/β bands at ~567 and 532 (Fig. 3, A and B). These spectral properties are consistent with those of known heme-binding proteins, albeit that the Soret band has a slightly higher wavelength than previously identified heme oxygenases in which the proximal ligand is a histidine residue (13, 29–31). The presence of the α/β bands at pH 8.0 implies that heme-IsdG and heme-IsdI complexes exist as a six-carbon low spin system at alkaline pH, which is consistent with the mammalian and bacterial heme oxygenases (32). Incremental addition of heme to both IsdG and IsdI allows for the visualization of the stoichiometric complex of these proteins with heme. Due to the fact that the Soret peak of the heme-protein complexes is different than that of free heme alone at neutral pH, spectrophotometric titration of IsdG and IsdI was carried out utilizing this difference. Incremental addition of heme to both IsdG (10 μM) and IsdI (10 μM) produced a distinct inflection point at 10 μM heme, revealing a 1:1 stoichiometric relationship between protein and heme (Fig. 3). These data were used to calculate molecular affinities using Michaelis-Menten kinetics. IsdG-bound heme with a K_d of $5.0 \pm 1.5 \mu\text{M}$, and IsdI-bound heme with a K_d of $3.5 \pm 1.4 \mu\text{M}$. The pyridine hemochrome method (23) was used to determine the millimolar extinction coefficient for IsdG to be $131 \text{ mM}^{-1} \text{ cm}^{-1}$ and for IsdI to be $126 \text{ mM}^{-1} \text{ cm}^{-1}$. These values for extinction coefficient and dissociation constant are in a range consistent with known

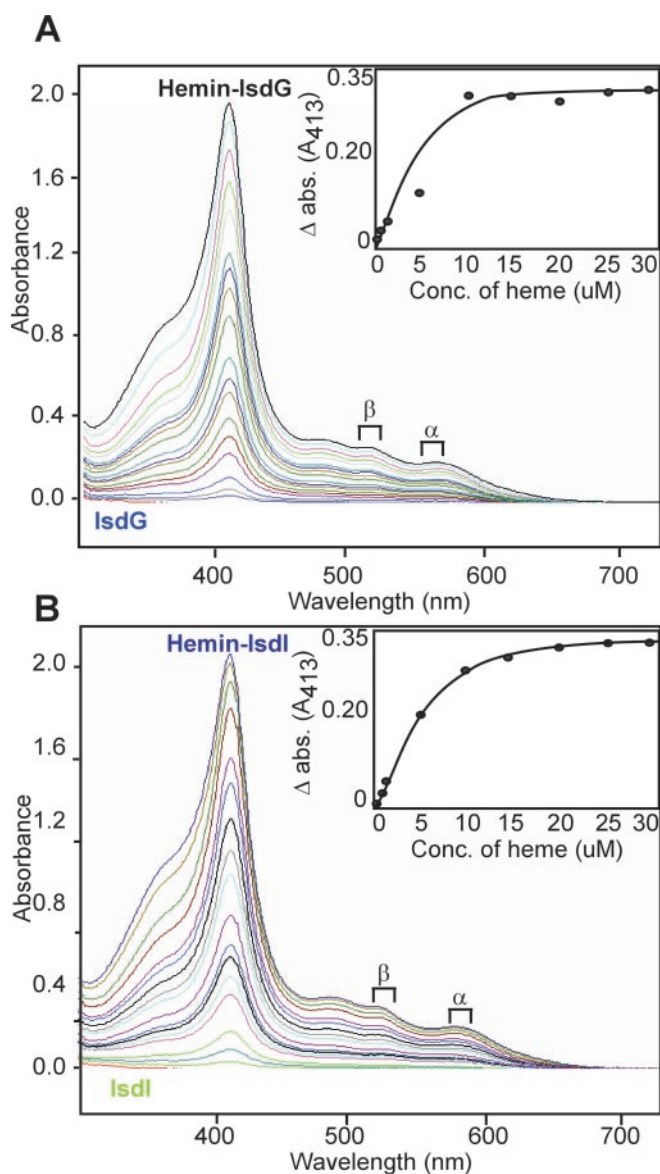


FIG. 3. **Heme binding of IsdG and IsdI.** Absorption difference spectra of heme binding to IsdG (A) or IsdI (B). Increasing amounts of heme (0.5–30 μM) were added to both sample (10 μM) and reference cuvettes. Purified IsdG and IsdI spectra in the absence of heme do not demonstrate absorptive signals between 400 and 600 nm. The Soret band at 412 nm increases with each addition of heme as demonstrated by different colored tracings increasing at 412 nm. The α and β symbols depict the predicted location of the α and β porphyrin bands. Differential spectroscopy revealed saturation at a 1:1 ratio of heme to protein (10 μM), as shown in the insets.

heme degradation enzymes (Table I). Taken together, these results reveal that IsdG and IsdI bind heme in a manner resembling known heme-degrading enzymes.

IsdG- and IsdI-mediated Heme Degradation and Iron Release—Initially, to determine if IsdG and IsdI release iron from heme via cleavage of macrocyclic porphyrin, we measured the ability of these enzymes to liberate radiolabeled [^{55}Fe] from [^{55}Fe]hemin. Incubation of IsdG or IsdI with [^{55}Fe]hemin led to a 848.5% ($\pm 134\%$) and 465.9% ($\pm 65\%$) increase in the amount of [^{55}Fe] liberated from the porphyrin molecule as compared with IsdB, a protein shown previously to bind heme (Table I) (16). To determine if iron release from heme occurred concomitantly with opening of the porphyrin macrocycle, we utilized optical absorption spectroscopy to monitor the IsdG- and IsdI-mediated degradation of heme. In the presence of a suitable

TABLE I
Comparison of heme-degrading enzymes from various species

Enzyme	Organism	K_d	Extinction coefficient	Released [55]Fe ^a	Ref.
		μM	$\text{mM}^{-1}\text{cm}^{-1}$		
HO-1	<i>Homo sapiens</i>	0.84 +/- 0.2	140	N/A ^b	54, 55
HmuO	<i>Corynebacterium diphtheria</i>	2.50 +/- 1.0	121	N/A	13, 31
			150		
HemO	<i>Neisseria meningitidis</i>	N/A	179	N/A	14
PigA	<i>Pseudomonas aeruginosa</i>	N/A	129	N/A	12
IsdG	<i>Staphylococcus aureus</i>	5.00 +/- 1.5	131	848.5 +/- 134	This work
IsdI	<i>Staphylococcus aureus</i>	3.50 +/- 1.4	126	465.9 +/- 65	This work

^a Released iron reported as percent increase over sample containing control protein lacking heme oxygenase activity.

^b N/A, not available.

electron donor such as ascorbate or NADPH-cytochrome P450 reductase (22), heme oxygenases catalyze the oxidative degradation of heme first to α -meso-hydroxyheme, followed by verdoheme, and finally biliverdin, carbon monoxide (CO), and iron (5, 6). Incubation of the IsdG-heme complex with NADPH-cytochrome P450 reductase in the presence of NADPH produced a UV spectrum for hemin cleavage products indistinguishable from those produced by the heme oxygenase from rat (HO-1) (Fig. 4, A and B). To characterize this reaction further, IsdG-heme and IsdI-heme were incubated with NADPH-cytochrome P450 reductase, and upon the addition of NADPH, the degradation of heme was monitored spectrophotometrically every 5 min over the course of 1 h. This reaction led to a slight increase in wavelength of the Soret peak from 412 to 414, and an almost complete elimination of the Soret and α/β peaks of the protein-heme complex consistent with degradation of the heme tetrapyrrole (Fig. 4, C and E). The 340-nm band of NADPH increases upon addition of 10- μM increments of NADPH and subsequently decreases in proportion to the decrease of the Soret band. Over time, the Soret maximum decreased further and shifted back toward 400 nm with complete disappearance of the α/β bands. Furthermore, visualization of the reaction-containing cuvette revealed a change in color from brown-red to bright yellow-green over time. Substitution of ascorbate for NADPH-cytochrome P450 reductase as an electron donor led to similar color changes in the cuvette, and a more pronounced decrease in the Soret band of the protein-heme complex (Fig. 4, D and F). In addition, optical absorption spectroscopy demonstrated the formation of different reaction products upon ascorbate-catalyzed heme degradation as compared with the reaction performed with reductase. More specifically, a shoulder at 395 nm appears early in the reaction, likely indicative of verdoheme formation. In the later time points, broad bands centered near 380 nm and 600–700 nm appear, consistent with biliverdin formation. The discontinuity of the tracings in the early time points suggests that the ascorbate-driven reaction initiates quickly, with later steps in the reaction occurring less rapidly.

Non-enzymatic oxidation of heme has been reported for certain heme-binding proteins such as myoglobin, cytochrome b_5 , and cytochrome b_{562} (33–35). The coupled binding and oxidation of heme requires exogenous hydrogen peroxide, and therefore can be inhibited by catalase (34, 36). To distinguish between coupled oxidation of heme, and IsdG- and IsdI-mediated enzymatic degradation of heme, we repeated the above heme degradation reactions in the presence of purified catalase. Purified catalase did not inhibit the ability of IsdG or IsdI to degrade heme using either NADPH-cytochrome P450 reductase or ascorbate as an electron donor (Fig. 4). To further distinguish IsdG- and IsdI-mediated heme degradation from coupled oxidation, pyridine was added to the reaction products. Coupled oxidation of heme will produce a verdoheme end product, whose regioisomers have distinct peaks between 640–680

nm in the presence of pyridine (37, 38). Pyridine extraction of the reaction products from all IsdG- and IsdI-mediated heme degradation reactions did not produce a spectrum typical of a pyridine-verdohemochrome (data not shown). Thus, IsdG and IsdI appear to catalyze enzymatic cleavage of the heme tetrapyrrole ring in the presence of NADPH-cytochrome P450 reductase. If so, IsdG, IsdI, or rat heme oxygenases should generate similar reaction products. Upon completion of the NADPH-cytochrome P450 reductase catalyzed degradation of heme, the reaction products were extracted with chloroform and subjected to high-performance liquid chromatography on a C18 column. For chromatography calibration, hemin eluted at 20 min (85:15 MeOH:H₂O) from the column, whereas biliverdin, the reaction product of rat heme oxygenase-mediated heme degradation, eluted at 10 min. The reaction products of both IsdG- and IsdI-mediated cleavage of hemin eluted at 10 min following HPLC analysis (Fig. 5), suggesting that the staphylococcal heme oxygenases indeed generate similar reaction products as the mammalian enzyme.

In Vivo Activity of IsdI—Complementation experiments using *isdG* are complicated by the location of this gene at the end of a large transcriptional unit (Fig. 1A), therefore *isdI* was chosen for complementation experiments. To demonstrate *in vivo* heme degradation by IsdI, we measured the ability of IsdI expressed *in trans* to complement the heme utilization deficiency of the previously characterized *C. ulcerans* heme oxygenase mutant, CU29 (18). To ensure appropriate expression of *isdI*, *orfXisdI* and ~100-bp upstream of *orfX* were cloned into a corynebacterial expression vector, creating pEPS10. Expression of pEPS10 encoded IsdI in *C. ulcerans* led to a decrease in colony size as compared with wild-type (data not shown) on HIBTW medium, implying that expression of *orfXisdI* in *C. ulcerans* causes low level toxicity. However, pEPS10 restores the ability of CU29 to utilize heme as a sole iron source in the presence of iron chelator (Fig. 6, A and B). This restoration of growth reaches levels similar to that of the previously characterized *C. ulcerans* heme oxygenase mutant complemented with the *Corynebacterium diphtheriae* heme oxygenase gene, *hmuO*[CU29(pCD293)] (18). These results demonstrate that IsdI has an *in vivo* role in heme degradation and subsequent iron release for use by the bacterium as a nutrient source.

DISCUSSION

Work in Gram-negative bacteria has revealed numerous ways whereby these organisms capture heme and heme-containing molecules and transport these compounds across the double membrane envelope. Receptors for heme (39–41) and heme-containing proteins such as hemoglobin (42, 43), haptoglobin (44), and hemopexin (45) have been identified. Additionally, numerous Gram-negative microbes are capable of producing extracellular-binding proteins that shuttle molecules containing heme to outer membrane receptors. These molecules, also known as hemophores, are capable of extracting

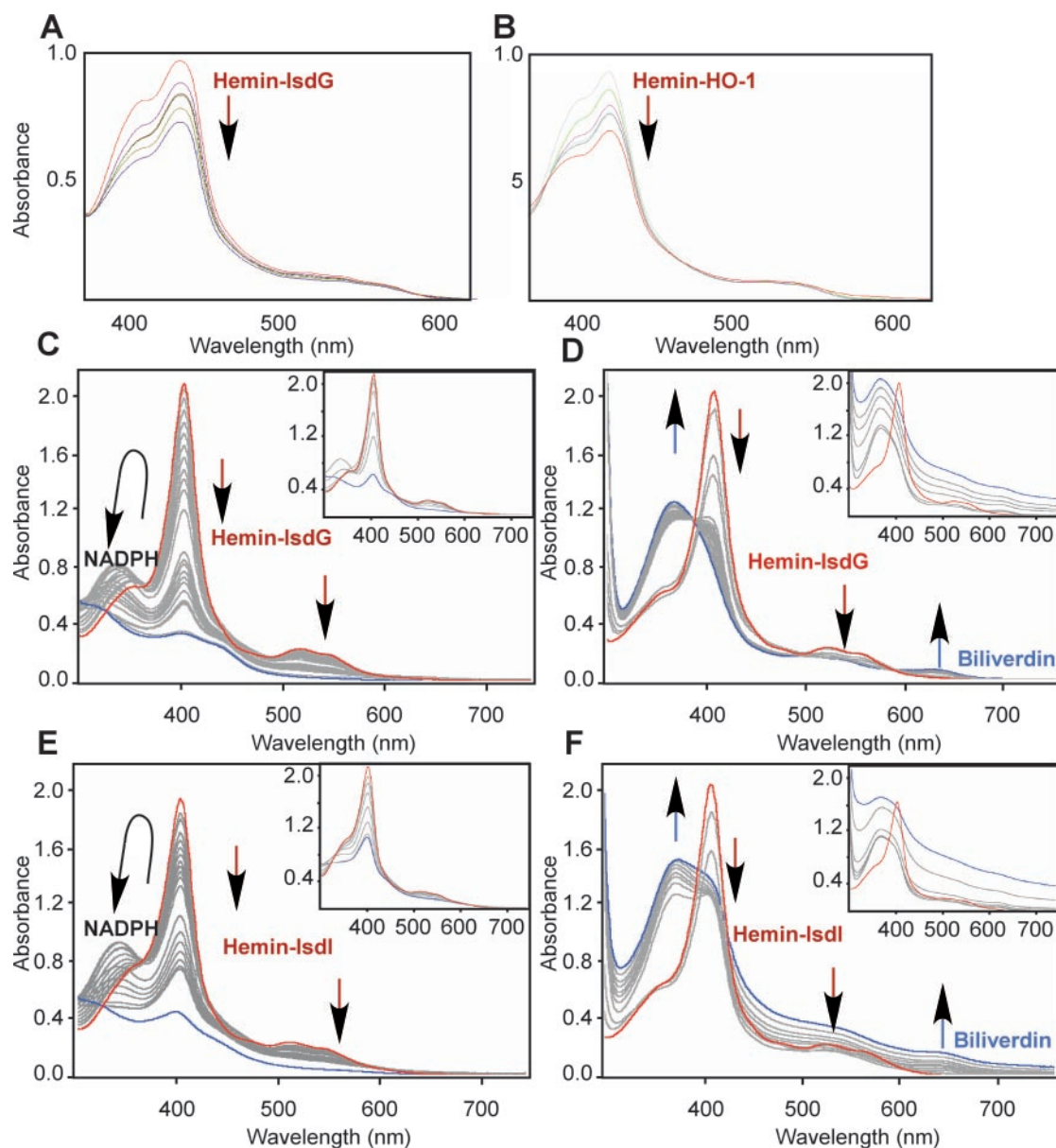


FIG. 4. UV spectral analysis of the reaction of IsdG and IsdI with ascorbate or NADPH P450-reductase. Comparison of short term heme degradation in the presence of NADPH cytochrome P450-reductase in the presence of NADPH between (A) IsdG and (B) Rat HO-1. C, ferric heme-IsdG or (E) ferric heme-IsdI complex in the presence of NADPH-P450 cytochrome reductase after the addition of NADPH in $10\text{-}\mu\text{M}$ increments, spectra taken at 5-min intervals represented by gray lines. Spectrum at time = 0 shown as red line and spectrum at time = final shown as blue line, with intermediate time points shown as gray lines. D, ferric heme-IsdG or (F) ferric heme-IsdI after the addition of ascorbate ($10\text{-}\mu\text{M}$), spectra were taken at 10-min intervals. Spectrum at time = 0 shown as red line and spectrum at time = final shown as blue line, with intermediate time points shown as gray lines. Spectral peaks are labeled with the predicted corresponding compounds. Arrows indicate the direction of movement over the course of 1 h. Reactions were also performed in the presence of catalase at a molar ratio of 0.5:1.0 (catalase:hemo-protein). Catalase-containing reactions are shown as insets in the appropriate graphs.

heme from heme-containing proteins such as hemoglobin, with subsequent delivery to specific outer membrane receptors (39, 46). Furthermore, extracellular proteases that degrade heme-containing host proteins are capable of releasing heme and making it available to the bacteria (47, 48). Little is known about how and to what extent Gram-positive pathogens utilize heme and how this compound is transported into bacterial cells. Recently, the first Gram-positive heme transport system involving the utilization of hemoglobin as an iron source was identified in *C. diphtheriae* (49). Additionally, a *Streptococcus pyogenes* cell surface protein that associates with heme has been identified (50). We have recently described a system of cell wall anchored proteins involved in the binding and transport of heme in *S. aureus* (16). Although systems involved in heme uptake in Gram-positive bacteria are beginning to be identi-

fied, little characterization has been performed on the mechanism and proteins involved in this process. In addition, the mechanism by which most bacteria capable of utilizing heme as a sole iron source access the iron atom of the heme porphyrin ring remains a mystery. Published reports describe heme oxygenases in *C. diphtheriae* (13), *Pseudomonas aeruginosa* (12), and the pathogenic *Neisseria* (14), however, searches of all available finished and unfinished microbial genomes reveals potential homologues to these enzymes in only two other genera, *Agrobacterium* and *Streptomyces* (data not shown). This is surprising since over 20 different pathogenic species of bacteria are reportedly capable of utilizing heme as a sole iron source (15).

In this study we describe two proteins from *S. aureus* that have sequence signatures placing them in a family of monooxy-

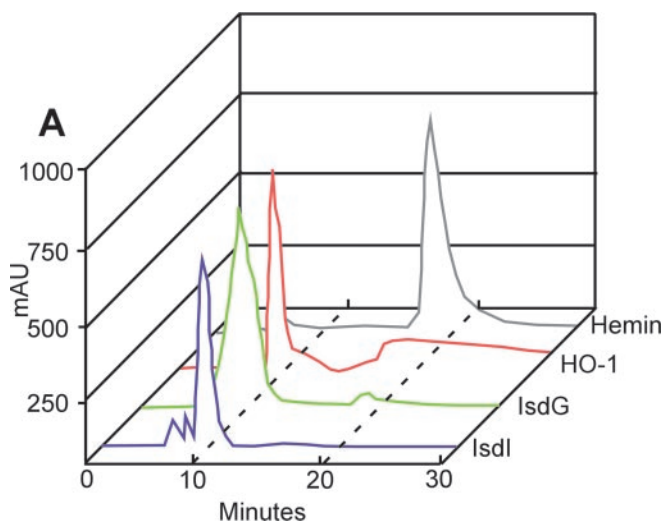


FIG. 5. Chromatographic analysis of heme cleavage products generated by IsdG and IsdI. HPLC chromatograms of hemin alone, or purified products from IsdG, IsdI, and HO-1 catalyzed reactions after extraction into chloroform. Samples were separated on a C18 column and were chromatographed in methanol:water (85:15). Spectra were taken over 30 min at 380 nm.

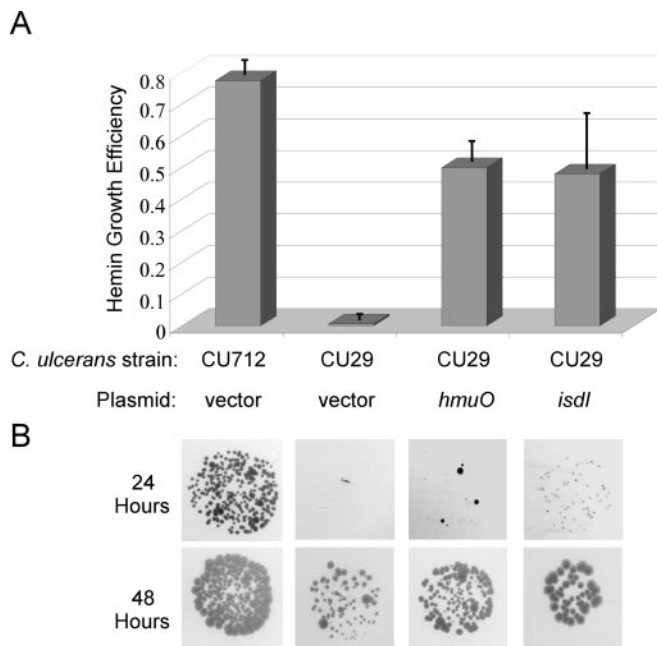


FIG. 6. IsdI complements the heme utilization defect of the *C. ulcerans* heme oxygenase mutant. Comparison of plasmid-containing strains of *C. ulcerans* wild-type (CU712) and *C. ulcerans* *hmuO* mutant (CU29) to utilize heme as an iron source for growth. Strains carry either vector alone (vector), or vectors expressing *C. diphtheria* *hmuO* (*hmuO*) or *S. aureus* *isdI* (*isdI*) as listed. A, ratio of visible colonies at 28 h on HIBTW medium containing 1 μ M hemin and 200 μ g/ml of the iron-chelator EDDA, compared with the number of colony forming units on HIBTW alone. Ratio expressed as Hemin Growth Efficiency. B, photographs of representative strains taken after 24 or 48 h of growth on HIBTW medium containing 1 μ M hemin and 200 μ g/ml EDDA.

genases involved in oxidation of aromatic intermediates (27), a process consistent with heme degradation. Purified IsdG and IsdI are both able to bind heme in a 1:1 ratio, exhibiting binding characteristics consistent with known heme oxygenases. IsdG was shown to be cytoplasmically localized, suggesting that heme degradation in *S. aureus* occurs in the cytoplasmic compartment. Both enzymes are capable of degrading the heme macrocyclic ring in the presence of a suitable electron

donor, as observed spectrophotometrically. This degradation has characteristics similar to the heme degradation reaction carried out by mammalian heme oxygenases. Furthermore, the reaction products produced upon IsdG- and IsdI-mediated heme oxidation appear to include biliverdin, the reaction product of the paradigmatic heme oxygenase reaction (5, 7). We have shown that incubation with IsdG or IsdI leads to a significant increase in the amount of free iron released from heme, likely providing *S. aureus* with a source of iron during infection. Finally, expression of *isdI* *in trans* complements the heme utilization defect of a *C. ulcerans* heme oxygenase mutant.

It has previously been reported that some heme is degraded when bound to certain hemoproteins through a process known as coupled oxidation (33, 34, 51). This process is a non-enzymatic degradation of the heme molecule, whose biological relevance is unknown. Coupled oxidation of heme by myoglobin utilizes an exogenous peroxide source, and as such can be inhibited by the addition of catalase. When added at a molecular ratio at, or greater than, one-tenth of an equivalent of hemoprotein, catalase was shown to inhibit myoglobin-mediated coupled oxidation (34). IsdG- or IsdI-mediated heme degradation in the presence of NADPH-cytochrome P450 reductase or ascorbate was not inhibited by catalase at a ratio of 0.5:1.0 (catalase to hemoprotein). Furthermore, coupled oxidation of heme typically leads to the formation of verdoheme, which remains associated with the hemoprotein (35). Upon addition of pyridine to the hemoprotein-verdoheme complex, verdoheme becomes dislodged from the hemoprotein and associates with pyridine, forming a strong pyridine-verdohemochrome spectrum. This spectrum was not observed upon pyridine extraction of the reaction products from the IsdG- or IsdI-mediated heme degradation. Taken together, these results imply that the observed degradation of heme in our study is likely enzymatic, and not due to coupled oxidation of the heme molecule.

Previously, we have presented the first description of a heme uptake system identified in *S. aureus* known as the iron-regulated surface determinant system (Isd) (16). These genes are found clustered together in the *S. aureus* genome in three iron-regulated transcriptional units. IsdA and IsdB are individually transcribed, and sorted to the cell wall in a sortase A (*srtA*) dependent manner. In a third operon is IsdC, a protein sorted to a distinct portion of the cell wall by sortase B (*srtB*). Downstream from IsdC in the same transcriptional unit is a membrane transport system consisting of IsdD (a membrane protein), IsdE (a heme-binding lipoprotein), and IsdF (a heme-permease), followed by SrtB and IsdG. The gene encoding IsdI, a paralogue to IsdG, exists at an alternate location in the chromosome of *S. aureus*. Finally, IsdH, a separate iron-regulated cell-wall sorted protein with significant identity to IsdB has also been identified in a region outside of the *isd* cluster (16). IsdH, also known as HarA, has been recently reported to be a haptoglobin-hemoglobin receptor (44). Our model envisions an elaborate iron acquisition system in *S. aureus*, involving numerous gene products. Initially upon infection, *S. aureus* produces a number of toxins, including hemolysins capable of lysing red blood cells. This leads to the release of hemoglobin from erythrocytes for use as a potential iron source. The lack of available iron in humans leads to transcription of the *isd* genes upon liberation of Fur-mediated repression. IsdB and IsdA are then sorted to the cell wall by sortase A, concomitant with IsdC sorting to a different portion of the cell wall by SrtB. Free hemoglobin binds to IsdB, with subsequent removal of the heme molecule in an IsdB- and IsdA-dependent manner. The heme molecule is then passed to the IsdC cell wall transport protein, with subsequent movement through the membrane

transport system composed of IsdDEF. Upon entry to the cytoplasm, IsdG and IsdI are capable of carrying out oxidative degradation of the heme molecule, leading to the release of free iron for use as an iron source. As inactivation of individual components of the Isd heme transport system do not inhibit growth on heme as a sole iron source as efficiently as a cell wall sorting mutant, it is likely that remaining heme utilization proteins are yet to be identified in the genome of *S. aureus*. This finding underlines the redundant nature of iron acquisition in *S. aureus*, and reveals the ability of this organism to acquire iron through various iron sources.

Numerous bacterial pathogens are capable of utilizing heme iron as a sole iron source, and many of the uptake systems responsible for acquiring heme have been described (39). Comparatively fewer descriptions exist of enzymes responsible for the removal of the iron atom from the porphyrin ring of heme (12–14, 18). In fact, there is a marked absence of identifiable heme degradation enzymes in the sequenced genomes of bacteria. The identification of IsdG and IsdI from *S. aureus* adds to the list of heme degrading enzymes present in bacterial pathogens. A lack of identifiable sequence identity between IsdG or IsdI and other bacterial heme-degrading enzymes implies that IsdG and IsdI are members of a novel family of heme-degrading enzymes. The presence of homologues to IsdG and IsdI in the genomes of *Bacillus anthracis*, *Staphylococcus epidermidis*, and *Listeria monocytogenes*, suggests a conserved method of heme degradation in these organisms. Furthermore, the unique nature of these enzymes, combined with their presence in important human pathogens, and the vital nature of iron acquisition to successful infection implies that this new family of heme-degrading enzymes may represent a novel target for antimicrobial therapy. This idea is further supported by the antimicrobial potential of porphyrin compounds against staphylococci (52, 53).

Acknowledgments—We thank Drs. Chuan He and Piotr Gornicki for reagents, help, and technical assistance. We would especially like to thank Dr. Michael Schmitt for providing all *Corynebacterium* strains and vectors. We would also like to thank Drs. Caroline Philpott, Olga Protchenko, Mario Rivera, Mahin Maines, Angela Wilks, and Sam Beale for thoughtful discussion.

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