HtaA Is an Iron-Regulated Hemin Binding Protein Involved in the Utilization of Heme Iron in Corynebacterium diphtheriae

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Many human pathogens, including Corynebacterium diphtheriae, the causative agent of diphtheria, use host compounds such as heme and hemoglobin as essential iron sources. In this study, we examined the Corynebacterium hmu heme transport region, a genetic cluster that contains the hmuTUV genes encoding a previously described ABC-type heme transporter and three additional genes, which we have designated htaA, htaB, and htaC. The hmu gene cluster is composed of three distinct transcriptional units. The htaA gene appears to be part of an iron- and DtxR-regulated operon that includes hmuTUV, while htaB and htaC are transcribed from unique DtxR-regulated promoters. Nonpolar deletion of either htaA or the hmuTUV genes resulted in a reduced ability to use heme as an iron source, while deletion of htaB had no effect on heme iron utilization in C. diphtheriae. A comparison of the predicted amino acid sequences of HtaA and HtaB showed that they share some sequence similarity, and both proteins contain leader sequences and putative C-terminal transmembrane regions. Protein localization studies with C. diphtheriae showed that HtaA is associated predominantly with the cell envelope when the organism is grown in minimal medium but is secreted during growth in nutrient-rich broth. HtaB and HmuT were detected primarily in the cytoplasmic membrane fraction regardless of the growth medium. Hemin binding studies demonstrated that HtaA and HtaB are able to bind heme, suggesting that these proteins may function as cell surface heme receptors in C. diphtheriae.

Corynebacterium diphtheriae is a gram-positive bacterium and the etiological agent of diphtheria. Studies of C. diphtheriae have focused primarily on the structural characterization and genetic regulation of diphtheria toxin, a secreted exotoxin that is responsible for much of the morbidity and mortality associated with human infection by this pathogen (9). Expression of diphtheria toxin is repressed by iron, and transcription of the structural gene, tox, is regulated by the diphtheria toxin repressor protein, DtxR, in association with iron (4, 36). DtxR is a global iron-dependent repressor in C. diphtheriae that controls the expression of at least 50 genes at more than 20 different promoters (7, 14, 15, 29, 34, 38). While iron-dependent regulation of bacterial virulence factors has been well studied, it is also known that acquisition of iron from the extracellular environment is often critical for microbial pathogens to cause disease (5, 6). Bacteria have developed a variety of mechanisms for uptake and utilization of iron. These mechanisms include high-affinity siderophore uptake systems (48) and binding protein-dependent transporters that facilitate the acquisition of iron from various host sources, including transferrin, lactoferrin, and heme, which is bound by various host proteins, such as hemoglobin (27, 35, 42). Siderophore transport systems are ubiquitous in bacteria, and it has been known for many years that C. diphtheriae secretes a siderophore and contains a siderophore-specific uptake system (10, 30). The structure of the C. diphtheriae siderophore has not been determined; however, a gene required for siderophore biosynthesis and genes encoding an ABC-type siderophore transporter were recently identified in C. diphtheriae and were shown to be regulated by DtxR and iron (15).

Hemin is the oxidized form of heme that is found in extracellular environments, and it is the form of heme transported by bacterial uptake systems. Hemin iron transport and utilization systems have been identified in numerous bacterial species and were initially described in gram-negative pathogens, where it was shown that an outer membrane receptor and a periplasmic binding protein-dependent ABC-type transporter are required for hemin uptake (13, 18, 41). Certain gram-negative species also obtain heme iron through the use of hemophores, which are low-molecular-weight secreted heme binding proteins that are able to extract hemin from hemoglobin and then transfer the hemin to receptors in the bacterial outer membrane (20).

Hemin uptake systems have recently been described in gram-positive bacteria, including Staphylococcus aureus (24, 46), Streptococcus pyogenes (3, 19), and Corynebacterium species (11, 35). S. aureus binds hemin or hemoglobin to its cell envelope through various surface-anchored proteins, termed iron-regulated surface determinants (Isd), which contain sorside recognition signals at their C termini that are essential for the covalent linkage of these proteins to the peptidoglycan (24, 46). It is believed that heme is transported through the cell envelope via a cascade mechanism in which hemin is transferred between various Isd receptors (22, 50). In S. pyogenes, heme is proposed to bind initially to surface-exposed proteins, designated Shp and Shr, which appear to be anchored to the membrane by a putative transmembrane region in their C termini (21, 49). In both S. aureus and S. pyogenes it is thought that heme is transferred from the surface-anchored proteins...
to a substrate binding component that is associated with a heme-dependent ABC transporter that facilitates the passage of heme into the cytosol.

In *Corynebacterium ulcerans*, the transport of heme involves use of the ABC-type transporter encoded by the *hmuTUV* genes, which share sequence similarity to genes encoding heme uptake systems in other organisms, including *C. diphtheriae* (11, 35). The *hmuTUV* genes in *C. diphtheriae* were shown to complement an *hmuTUV* mutation in *C. ulcerans*, and it was further demonstrated that the HmuT protein in *C. diphtheriae* is a heme binding lipoprotein that is tethered to the cytoplasmic membrane by an N-terminal lipid moiety (11). An insertion mutation in *hmuT* had no effect on heme iron utilization in *C. diphtheriae*; however, it is suspected that the medium conditions used in the study were not optimal for detection of a heme uptake deficiency (16, 35). The completion of the genome sequence of a clinical isolate of *C. diphtheriae* (8) resulted in identification of *htaA*, an iron- and DtxR-regulated gene that is located immediately upstream of *hmaT* (14). No promoter activity was detected in the *htaA-hmaT* intergenic region, and it was suspected that *htaA* and the *hmaTUV* genes may constitute a DtxR-regulated operon (14, 35). Two other genes, designated *htaB* and *htaC*, were also identified in this heme transport gene cluster. The function of the predicted products of *htaA*, *htaB*, and *htaC* has not been determined; however, the linkage of these genes to the *hmaTUV* operon suggests that they have a possible function in heme uptake or heme iron utilization. The use of heme as an iron source in *C. diphtheriae* also involves the *hmaO* gene, which encodes a heme oxygenase that catalyzes the degradation of intracellular heme and the subsequent release of the heme-associated iron (16, 31, 47). The *hmaO* gene in *C. diphtheriae* is not linked to the heme transport gene cluster, but transcription of *hmaO* is regulated by DtxR and iron, as well as by heme (32, 33).

In this study, we extended our characterization of the heme transport locus, designated *hma*, to examine the expression and cellular localization of specific *hma* gene products. We show here that the *hmaTUV* and *htaA* genes are involved in the use of heme as an iron source in *C. diphtheriae*. Protein localization studies revealed that HtaA, HmuT, and HtaB are associated predominantly with the cytoplasmic membrane when bacteria are grown in minimal medium, which suggests that these proteins may function to transport heme. We also demonstrate that HtaA and HtaB are surface-exposed heme binding proteins that may function as hemceptors in *C. diphtheriae*.

**MATERIALS AND METHODS**

**Bacterial strains and media.** *Escherichia coli* and *C. diphtheriae* strains used in this study are listed in Table 1. Luria-Bertani (LB) medium was used for culturing *E. coli*, and heart infusion broth (Difco, Detroit, MI) containing 0.2% Tween 80 (HIBTW medium) was used for routine growth of *C. diphtheriae* strains. Bacterial stocks were maintained in 20% glycerol at −80°C. Antibiotics were added to LB medium at concentrations of 34 µg/ml for chloramphenicol, 50 µg/ml for kanamycin, and 100 µg/ml for ampicillin for *E. coli* cultures and for HIBTW medium at concentrations of 2 µg/ml for chloramphenicol and 50 µg/ml for kanamycin for *C. diphtheriae* cultures. HIBTW medium was made a low-iron medium by addition of ethylenediamine di(iso-hydroxyphenylacetic acid) (EDDA) at a concentration of 12 µg/ml (unless indicated otherwise). Modified PGT (mPGT) medium is a semidefined low-iron medium that has been described previously (44). Antibiotics, EDDA, Tween 80, hemin (bovine), and hemoglobin (human) were obtained from Sigma Chemical Co. (St. Louis, MO).

**TABLE 1. Strains and plasmids used in this study**

<table>
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<th>Strain or plasmid</th>
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**Mutant construction.** A previously described allelic replacement technique (45) was used to construct nonpolar deletion mutations in the *C. diphtheriae* 1737 *htaA, htaB*, and *hmaTUV* genes, as well as in the complete *hma* gene cluster (*hmaT* through *hmaR*). Mutant construction utilized PCR to clone DNA fragments located upstream and downstream of the region targeted for deletion. The deleted regions in the *htaA*, *htaB*, and *hmaTUV* mutants are predicted to encode peptides consisting of approximately 20 amino acids that contain in-frame fusions of residues derived from the N and C termini of the original proteins. Primers used for construction of the mutants are listed in Table 2. PCR was used to confirm the mutations in all of the deletion mutants (not shown). An R47H point mutation was introduced into the *C7 dxrR* gene using a previously described procedure (15).

**Hemoglobin iron utilization assays.** The hemoglobin iron utilization assay has been described previously (16). Briefly, *C. diphtheriae* strains were grown overnight (20 to 22 h at 37°C) in HIBTW medium and then inoculated to an optical density at 600 nm (OD<sub>600</sub>) of 0.2 into fresh HIBTW medium that contained 12 µmol of the iron chelator EDDA. Strains were grown for several hours at 37°C until log phase, at which time bacteria were recovered by centrifugation, resuspended in mPGT medium, and then inoculated at an OD<sub>600</sub> of 0.03 into fresh mPGT medium that contained various supplements, as indicated below. After 20 to 22 h of growth at 37°C, the OD<sub>600</sub> of the cultures were determined.

Experiments that examined the effect of secreted or purified HtaA on hemooglobin iron utilization used HtaA that was either purified from *E. coli* or obtained in native form from *C. diphtheriae* culture supernatant. Culture supernatants were prepared as follows. *C. diphtheriae* strain 1737hmaAΔ containing either the cloned *htaA* gene on pKhtaA or the vector pKN2.6Z, was grown to late log phase in low-iron HIBTW medium, and culture supernatants were concentrated by ammonium sulfate precipitation. One milliliter of a culture supernatant containing 12 µmol of the iron chelator EDDA. Strains were grown for several hours at 37°C until log phase, at which time bacteria were recovered by centrifugation, resuspended in mPGT medium, and then inoculated at an OD<sub>600</sub> of 0.03 into fresh mPGT medium that contained various supplements, as indicated below. After 20 to 22 h of growth at 37°C, the OD<sub>600</sub> of the cultures were determined.

**Plasmid construction.** Plasmids used in this study are listed in Table 1. PCRam derived DNA fragments were initially cloned into the pCR-Blunt II-TOPO vector (Invitrogen), and genomic DNA derived from *C. diphtheriae* strain 1737 was used as a template for all PCRs (unless otherwise indicated). The promoter probe vector pCM502 (32) was used for construction of all lacZ promoter fusions; this vector contains a promoterless lacZ gene and replicates at a low density.
Protein expression and antibody production. E. coli strain BL21(DE3) carrying the cloned htaA or htaB gene on the pET24a+ expression vector was grown in 100 ml of LB medium at 37°C to mid-log phase, at which time 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the cultures were allowed to grow for an additional 2 to 3 h before they were harvested. The cultures were washed in 10 ml of 20 mM Tris-HCl (pH 7.5), and each pellet was stored at −20°C overnight. The pellet was thawed and resuspended in buffer containing 50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole (pH 8.0) at 4°C, and the bacteria were lysed with a French pressure cell, which was followed by removal of cell debris by centrifugation at 10,000 × g at 4°C. The soluble fraction containing the HtaB protein was collected and purified using Ni-nitrilotriacetic acid affinity resin. However, we observed that HtaA could be significantly enriched after solubilization in 8 M urea, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then excision of the HtaA protein band from the acrylamide gel. The gel-purified HtaA and affinity-purified HtaB proteins were used for production of polyclonal antibodies in guinea pigs by standard methods (Cocalico Biologicals Inc.).

The procedure used for expression of HtaA with the pGEX-6P-1 vector was similar to the procedure described above for the pET expression system. The GST-HtaA protein was found predominantly in the soluble fraction after lysis of the bacteria, and the fusion protein was purified using GST resin (GE Healthcare) by a batch method according to the manufacturer’s instructions. Purified HtaA protein was obtained after removal of the GST tag by cleavage with

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<td>5′-GGGATCCCATGCGAAGTCGAGCTACG-3′</td>
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</table>

**TABLE 1. Primers used in this study**
PreScission protease (GE Healthcare), which was followed by purification steps performed according to the manufacturer’s protocol.

SDS-PAGE and Western blot analysis. Proteins were separated by SDS-PAGE (17) and stained with Bio-Safe Coomassie blue (Bio-Rad) by following the manufacturer’s instructions. Western blot procedures were performed as described previously (12), and anti-HtaA and anti-HtaB antibodies were used at dilutions of 1:10,000 and 1:20,000, respectively. Antibodies raised against HmuT (35), DtxR (39), and diphertheria toxin (a gift from R. K. Holmes) were used at dilutions of 1:40,000, 1:5,000, and 1:10,000, respectively. Binding of the primary antibodies to immobilized proteins was detected by using appropriate alkaline phosphatase-labeled secondary antibodies. Alkaline phosphatase activity was detected by using established procedures (35).

Protein localization studies. To determine whether proteins were secreted or cell associated, 1-ml cultures were grown to mid-log phase at 37°C in HIBTW medium containing 12 μg/ml EDDA (low-iron medium). Cultures were centrifuged briefly to pellet the cells, and the supernatant was recovered and passed through a 0.2-μm filter to remove bacteria. Proteins in the filtered supernatant were precipitated with 10% trichloroacetic acid, washed with ethanol, dried, and then resuspended in Tris-EDTA buffer (pH 7.4). One half of the sample was analyzed after SDS-PAGE and Coomassie blue staining, and the other half of the sample was used for Western analysis. The resulting cell pellet was lyzed and then resuspended in SDS loading buffer, and samples were analyzed as described above.

A previous described procedure was used to determine the cellular location of cell-associated proteins (11), with the following modifications. Briefly, 200-ml cultures of various C. diphtheriae strains were grown to late log phase in HIBTW medium at 37°C. Cells were harvested by centrifugation, washed once with PBS (pH 7.4) (Invitrogen), and then resuspended in 10 ml of PBS, which was followed by lysozyme treatment and lysis of the bacteria using a French pressure cell. Cell debris was removed by centrifugation, and the soluble fraction, which contained both soluble and membrane proteins, was centrifuged at 100,000 × g to pellet the cytoplasmic membrane. The supernatant, which contained the soluble intracellular proteins, was recovered and stored at 4°C, while the membrane fraction was solubilized in 0.1% Triton X-100. C. diphtheriae cultures grown in 50 ml of mPGT medium were prepared similarly.

Proteinase K experiments. C. diphtheriae 1737htaA/pKhtaA was grown as described above for the hemoglobin utilization assay, except that 0.5 μM FeSO₄ was added to mPGT medium (low-iron conditions) in place of hemoglobin. Cultures were harvested after overnight growth and centrifuged at 7,000 × g for 10 min. The cells were resuspended in PBS, which was followed by addition of proteinase K at a final concentration of 50 μg/ml to the cell suspension. The reaction mixture was incubated for 30 min at 37°C, at which time the cells were pelleted and then washed twice with PBS. The bacteria were then treated with lysozyme followed by 0.75% Sarkosyl to lyse the bacteria. Whole-cell protein preparations were boiled under reducing conditions prior to analysis by SDS-PAGE and Western blotting. Control samples were not treated with proteinase K. Plasmid pKhtaA was used to obtain better detection of the HtaA protein.

Hemin binding analysis. Purified GST-HtaA and HtaB, from which the leader peptide and the transmembrane region were deleted (HtaB also lacked the six-His tag), were analyzed to determine their hemin binding properties by UV-visible spectroscopy using a Beckman DU 640 spectrophotometer. Proteins at a concentration of 3.5 μM in PBS buffer containing glycerol were assessed to determine their abilities to bind hemin in the concentration range from 0.5 to 20 μM. Proteins were incubated in the presence of hemin for at least 15 min before spectrophotometric analysis. UV-visible absorption scans of HtaA and HtaB were done using wavelengths between 280 and 600 nm. Absorbance spectra for all protein-hemin samples were zeroed against a reference cuvette that contained the same concentration of hemin in PBS buffer in the absence of protein.

Detection of heme-dependent peroxidase activity. The chromogenic compound 3,3′,5,5′-tetramethylbenzidine (TMBZ) turns blue in the presence of heme-dependent peroxidase activity and was used to detect hemin-protein complexes as previously described (43). Prior to separation of proteins by SDS-PAGE, samples were incubated for 30 min at room temperature in the presence or absence of hemin. Hemin was prepared in 0.1 N NaOH and was used at a concentration of 0.625 μM for HtaA detection or at a concentration of 2.5 μM for studies with HtaB. Samples were also incubated in the absence of hemin with 0.1 N NaOH. Proteins were not boiled or exposed to reducing agents prior to SDS-PAGE. Lithium dodecyl sulfate (LDS) was used in place of SDS in the HtaB studies as described previously (43). HtaA in culture supernatants was concentrated by precipitation with 75% ammonium sulfite followed by dialysis in 50 mM Tris (pH 8.0).

Computer analysis. Amino acid sequence similarity searches were done using the BLAST program (1) at the National Center for Biotechnology Information and also using the BLAST server provided at the online site for the Sanger Institute (http://www.sanger.ac.uk/Projects/C_diphtheriae). The annotated genome sequence of C. diphtheriae strain NCTC13129 (8) is accessible in the EMBL/GenBank database under accession number BX248353.

RESULTS

Analysis of the C. diphtheriae hmu hemin transport gene cluster. Previous studies of C. diphtheriae identified three genes, hmuT, hmuU, and hmuV, which encode components of an ABC-type hemin transporter (11). The HmuT protein was shown to be a membrane-anchored hemin binding lipoprotein, while HmuU and HmuV were predicted to be the permease and ATPase components, respectively. In a subsequent study (14), we showed that the hmuTUV genes were part of a larger gene cluster that included the htaA, htaB, and htaC genes (Fig. 1A), and we reported that htaA and htaC were transcribed from divergent DtxR- and iron-regulated promoters (14). In this study, we identified a putative DtxR binding site upstream of htaB that aligns with 12 of the 19 residues in the consensus DtxR binding sequence and matches 9 of the 11 most highly conserved bases (Fig. 1A and C). Analysis of an htaB-lacZ transcriptional fusion construct (phtaub-Z) in the wild type and in a dtxR mutant of C. diphtheriae showed that expression from the htaB promoter is regulated by iron and DtxR (Fig. 1D). Transcription from the htaB promoter was not affected by the divergent metal derivatives Mn and Zn, and the presence of hemoglobin resulted in a slight decrease in expression, since hemin serves as an iron source in C. diphtheriae (data not shown). Western analysis using antibodies raised against HtaA, HtaB, and HmuT demonstrated that the production of each of these proteins is repressed by iron (Fig. 2A to C), which is consistent with the study described above and with previous findings (14). Diphertheria toxin, a well-characterized iron-regulated protein, and DtxR, which is constitutively expressed, were included as controls in this study (Fig. 2D).

BLAST analysis of the predicted amino sequence of HtaA reveals that it has homology (ranging from 20% to 60% identity) with proteins in several bacterial species that are related to C. diphtheriae, including C. ulcerans, Corynebacterium jeikeium, Corynebacterium glutamicum, Propionibacterium acnes, and Streptomyces coelicolor. HtaA also contains approximately 150 to 200 residues in its N-terminal region that share approximately 50% sequence similarity to a segment in its C terminus. These two conserved regions are approximately 35% similar to a region in HtaB (Fig. 3A). HtaA and HtaB both contain predicted signal peptides and a putative transmembrane region in their C termini, which is followed by one (HtaA) or two (HtaB) positively charged residues (Fig. 3A and B). The htcA gene is predicted to encode a putative membrane protein that shares limited amino acid sequence similarity with proteins with unknown functions.

Mutations in htaA and hmuTUV result in a reduced ability to utilize hemoglobin as an iron source in C. diphtheriae. A function for HtaA in C. diphtheriae has not been determined previously, although the close proximity of htaA to genes involved in hemin transport suggests that HtaA may have a role in hemin uptake or hemin iron utilization. In previous studies (11, 35), the C. diphtheriae hmuTUV genes were shown to complement mutations in the hmuTUV genes in C. ulcerans; however, an insertion mutation in C. diphtheriae hmuT did not
result in a defect in hemin iron use (35). Since the previous report, we have developed a more sensitive method for measuring hemin iron utilization in *Corynebacterium* (16). To determine the functions of proteins encoded in the *hmu* gene cluster in *C. diphtheriae*, we constructed various nonpolar deletions in this region (Fig. 1B). The various mutations in the *hmu* gene cluster were confirmed by PCR (data not shown) and Western analysis (Fig. 2A to C).

*C. diphtheriae* strain 1737 was used to construct the various *hmu* mutants and to assess hemoglobin iron utilization. The 1737 strain is a clinical isolate from the recent Russian diphtheria epidemic (28) and is very

![Western blot analysis of proteins produced by *C. diphtheriae* wild-type strain 1737 (WT) and various 1737 deletion mutants. Strains were grown in 1 ml of iron-replete HIBTW medium (+Fe) or iron-depleted medium (HIBTW medium containing 12 μg/ml EDDA) (−Fe). Samples were normalized using OD600 before SDS gels were loaded. htaAΔ, 1737htaAΔ; TUVΔ, 1737TUVΔ; hmuΔ, 1737hmuΔ; htaBΔ, 1737htaBΔ.](http://jb.asm.org/)

FIG. 2. Western blot analysis of proteins produced by *C. diphtheriae* wild-type strain 1737 (WT) and various 1737 deletion mutants. Strains were grown in 1 ml of iron-replete HIBTW medium (+Fe) or iron-depleted medium (HIBTW medium containing 12 μg/ml EDDA) (−Fe), and proteins present either in culture supernatants (HtaA and diphtheria toxin [DT] in panels A and D, respectively) or in whole-cell extracts (HtaB, HmuT, and DtxR in panels B, C, and D, respectively) were detected by Western blot analysis using antiserum raised against the proteins indicated on the left. The values are the means of three experiments. Each result varied less than 15% from the mean. The difference between high- and low-iron conditions for the *dtxR* mutant was statistically significant (*P* < 0.05), and this suggests that the point mutant (R47H) maintains some low-level Fe-dependent repressor activity. See Materials and Methods for experimental details.
closely related to the strain used for analysis of the genome sequence (8).

The ability to use hemoglobin as an iron source was analyzed using low-iron mPGT medium in the presence of 25 \( \mu \)g/ml hemoglobin after 22 to 24 h of growth at 37°C. All strains grew to similar \( \text{OD}_{600} \) when 1 \( \mu \)M FeSO\(_4\) was added to mPGT medium (the \( \text{OD}_{600} \)s were between 3.0 to 3.5), and the growth of all strains was fully inhibited in low-iron mPGT medium in the absence of an iron source (low-iron mPGT medium contains 10 \( \mu \)M EDDA). Deletion of the \( C. \) diphteriae 1737 htaA gene, the hmuTUV genes, or the complete hmu gene cluster resulted in significantly diminished growth compared to the growth of the wild-type strain when hemoglobin was supplied as the sole iron source (Fig. 4A). Deletion of the htaB gene had no effect on the use of hemoglobin as an iron source in these assays (Fig. 4A). The presence of the cloned htaA and hmuTUV genes restored the ability to utilize hemoglobin as an iron source in the htaA and hmuTUV mutants, respectively (Fig. 4A). These findings suggest that the products of htaA and hmuTUV are involved in hemin transport or in hemin iron utilization in \( C. \) diphteriae. However, the results also suggest that additional genes outside the hmu locus encode products that have the ability to transport hemin, since hemoglobin was still able to stimulate growth of all of the deletion strains. All of the mutants described with mutations in the \( C. \) diphteriae hmu locus that showed reduced growth when hemoglobin was added as the sole iron source exhibited similar reductions in growth compared to the wild-type strain when hemin was used as the only iron source (data not shown).

**Cellular localization of the \( C. \) diphteriae hmu gene products.** To better understand the function of the hmu gene products in hemin iron utilization, studies were done to identify the cellular locations of the various proteins encoded in this gene cluster. Studies were initially done to determine whether various proteins are cell associated or secreted into the culture medium. As shown in Fig. 5A, HtaA was predominantly cell associated when \( C. \) diphteriae was grown in low-iron mPGT minimal medium, although low levels of HtaA were detected in the culture supernatant. Surprisingly, HtaA was found almost exclusively in the supernatant fraction when bacteria were grown in low-iron HIBTW medium, and HtaA exhibited a secretion profile almost identical to that of diphtheria toxin, a well-known secreted protein in \( C. \) diphteriae. Analysis indicate that HtaA is associated primarily with the cytoplasmic membrane when \( C. \) diphteriae 1737 is grown in mPGT medium (Fig. 5C). HtaA and diphtheria toxin were not detected in any cellular fraction when bacteria were grown in HIBTW medium, since these proteins are secreted into the supernatant in this medium (Fig. 5A). HtaB and HmuT are localized primarily to the cytoplasmic membrane, while the transcriptional regulatory protein, DtxR, is found exclusively in the soluble cytosolic fraction (Fig. 5C).

**FIG. 4. Utilization of hemoglobin as an iron source by \( C. \) diphteriae wild-type strain 1737 and mutant strains.** (A) \( \text{OD}_{600} \)s of cultures grown for 20 to 22 h in mPGT medium containing 10 \( \mu \)M EDDA and 25 \( \mu \)g/ml hemoglobin (human). The plasmids carried by the various strains included pKhtaA containing the cloned htaA gene (pA), vector pKN2.6Z (pK), vector pCM2.6 (pCM), and pCD842 containing the cloned hmuTUV genes (p842). wt, wild-type strain 1737; \( \Delta \), 1737htaA; \( \Delta \) TUV; 1737TUV; hmuA, 1737hmua; htaB\( \Delta \), 1737htaB\( \Delta \); TUV\( \Delta \), 1737TUV\( \Delta \); hmuTUV\( \Delta \); htaB\( \Delta \) hmuTUV\( \Delta \); htaA hmuTUV\( \Delta \); htaB\( \Delta \) hmuTUV\( \Delta \); htaA hmuTUV\( \Delta \) pKhtaA. (B) Results of experiments performed like the experiments described for panel A, except that cultures of 1737htaA\( \Delta \) were supplemented with 50 \( \mu \)l of concentrated culture supernatant from 1737hmua\( \Delta \) that contained either the vector pKN2.6Z (SupK) or the cloned htaA gene on pKhtaA (SupA). The presence of HtaA in culture supernatants in 1737hmua\( \Delta \)pKhtaA\( \Delta \) was confirmed by SDS-PAGE analysis (not shown). The results are the averages and standard deviations of three independent experiments. The values for 1737htaA\( \Delta \) pKN2.6Z are significantly different from the values for 1737/pKN2.6Z and 1737htaA\( \Delta \)pKhtaA\( \Delta \) (\( * \), \( P < 0.05 \)), and the values for 1737TUV/ pCM2.6 are significantly different from the values for 1737/pKN2.6Z and 1737TUV/pCD842 (\( ** \), \( P < 0.05 \)).
that are exposed outside the cell wall. As shown in Fig. 5D, HtaA and HtaB were fully digested by proteinase K, suggesting that these proteins are exposed on the surface of the bacteria. The levels of HmuT were reduced after protease treatment, but this protein was not fully digested like HtaA and HtaB. This finding suggests that HmuT is not as accessible to proteinase K as the other two proteins and may be partially protected by the cell wall; a similar observation was reported previously for the lipoprotein component of the ABC-type hemin transporter in S. aureus (24). DtxR, which is an intracellular protein, was not digested by proteinase K (Fig. 5D).

**HtaA and HtaB are hemin binding proteins.** UV-visible spectroscopy was used to analyze the hemin binding properties of the purified GST-HtaA and HtaB proteins. The GST-HtaA and HtaB proteins at a concentration of 3.5 μM were incubated at room temperature for at least 15 min with hemin at concentrations ranging from 0.5 μM to 10 μM. UV-visible absorption scans of the protein-hemin samples indicated that both GST-HtaA and HtaB showed strong absorption peaks at 406 nm (Fig. 6A and B), which is an absorption maximum consistent with the binding of hemin (24). Absorption measurements for HtaA in the absence of the GST tag could not be obtained due to proteolytic degradation of HtaA, which occurred after cleavage of the GST tag and the subsequent dialysis that was needed to remove the GST-protease cleavage buffer. The GST-protease cleavage buffer was found to interfere with absorbance measurements for the HtaA-hemin complex. When the GST protein alone was incubated with 5 μM hemin, it showed no absorption peak in the 400-nm region (Fig. 6C), indicating that it does not bind hemin, as previously reported (24). Measurements of absorption at 406 nm for GST-HtaA and HtaB with increasing hemin concentrations were used to determine dissociation constants of 1.9 ± 0.4 μM for GST-HtaA and 4.9 ± 0.7 μM for HtaB (Fig. 6D and E).

The chromogenic substrate TMBZ, which has been used previously to detect heme-dependent peroxidase activity that is associated with heme binding proteins (43), was used to analyze the ability of the native form of the HtaA protein to bind hemin. TMBZ staining of secreted proteins from C. diphtheriae 1737 in the absence of any plasmid (not shown). No peroxidase activity was uniquely associated with the HtaA protein (Fig. 6F, lane 4). Hemin gel, lane 5). Peroxidase activity was also associated with HtaA that was present in culture supernatants of wild-type strain 1737 in the absence of any plasmid (not shown). No proteins were stained with TMBZ in supernatants from C. diphtheriae 1737 carrying plasmid pKhtaA grown in low iron MB medium due to heme-dependent peroxidase activity was uniqueley associated with the HtaA protein (Fig. 6F, lane 5). Peroxidase activity was also associated with HtaA (without the GST tag) and the GST-HtaA fusion protein (lacking the signal peptide and the putative C-terminal membrane-spanning region) exhibited heme-dependent peroxidase activity in the presence of TMBZ (Fig. 6F, lane 5). Compared to HtaA, the purified HtaB protein showed very weak, nonspecific, heme-dependent peroxidase activity in the presence of TMBZ (data not shown). This observation suggests that the HtaB-hemin complex is not stable

**FIG. 5.** (A) Western blot analysis to measure secretion or cell association of proteins expressed from C. diphtheriae wild-type strain 1737. One-milliliter cultures were grown for 20 to 22 h at 37°C in iron-depleted HIBTW medium or in mPGT medium (only results for HtaA are shown for growth in mPGT medium). Polyclonal antiserum (specific to the proteins indicated on the left) was used for detection of proteins associated with the cell pellet (Cell) and the supernatant fraction (Sup). Doublet bands for HtaA and HtaB indicate breakdown products, which exhibited some variability between protein preparations. See Materials and Methods for a description of the method used for sample preparation. DT, diphtheria toxin. (B) Western blot detection of HtaA in culture medium from various C. diphtheriae clinical isolates after growth in iron-depleted HIBTW medium. The antisera used for detection are indicated on the left and the concentration of experimental details.

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**Surface exposure of hmu gene products.** To determine whether HtaA, HtaB, or HmuT is exposed on the cell surface and therefore may be accessible to bind hemin or possibly hemoglobin, C. diphtheriae was grown in low-iron mPGT medium, which was followed by treatment of the intact bacteria with proteinase K. This protease is predicted to cleave proteins

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under the conditions used to detect TMBZ-dependent peroxidase activity.

**DISCUSSION**

While hemin transport systems were first described in gram-negative bacteria (13, 41), the Corynebacterium HmuTUV system was the first hemin transporter to be identified in a gram-positive species (11). Over the last several years, hemin transport systems have been described in several additional gram-positive organisms, including the important human pathogens *S. aureus* (24) and *S. pyogenes* (3, 19). While both gram-negative and gram-positive bacteria employ ABC-type transporters and an associated substrate binding protein to move hemin through the cytoplasmic membrane, these organisms utilize distinctly different components to bind hemin or hemoglobin to the cell surface. While gram-negative bacteria bind hemin or hemoglobin to large outer membrane proteins, gram-positive bacteria, which do not have distinct outer membranes, bind hemin or hemoglobin to proteins anchored to the cell envelope. These surface receptors either are covalently linked to the cell wall peptidoglycan via sortases, as observed with the Isd proteins in *S. aureus* (24, 46, 50), or are tethered to the cytoplasmic membrane through a transmembrane C-terminal tail region, as proposed for the Shp and Shr proteins in *S. pyogenes* (3, 19, 21, 49) and for HtaA and HtaB in *C. diphtheriae*.

Both HtaA and HtaB contain N-terminal signal sequences and have putative transmembrane regions in their C termini, structural features that are also present in the *S. pyogenes* heme binding proteins Shp and Shp (3, 19). Although HtaA and HtaB share structural and possibly functional similarities with these *S. pyogenes* proteins, BLAST analysis shows that HtaA and HtaB have no significant sequence similarity to either Shp...
or Shr. A comparison of the C-terminal regions of these four proteins reveals that they all contain a putative membrane-spanning region that is followed by a positively charged tail sequence, which in \textit{S. pyogenes} is proposed to be involved in anchoring the proteins to the cytoplasmic membrane (Fig. 3B) (3, 19). HtaA contains only a single charge in this tail region, and membrane prediction models suggest that the HtaA transmembrane segment is relatively weak compared to the membrane-spanning regions in HtaB, Shp, and Shr (http://www.ch.embnet.org/software/TMPRED_form.html).

In this study, the \textit{hmu} hemin transport system in \textit{C. diphtheriae} was analyzed and shown to include genes whose products are involved in the utilization of hemin and hemoglobin as iron sources. Deletion of \textit{htaA}, \textit{hmuTUV}, and the complete \textit{hmu} locus resulted in a reduction in hemin iron use; however, each of the mutants maintained the ability to use hemin iron for growth, which indicates that additional uptake and/or utilization systems for hemin iron acquisition are active in \textit{C. diphtheriae}. The presence of more than one hemin iron utilization and uptake system has been described or proposed for several bacteria, including the gram-negative organisms \textit{Vibrio cholerae} (26) and \textit{Yersinia enterocolitica} (41) and the gram-positive organisms \textit{S. pyogenes} (3) and \textit{S. aureus} (24). For many of these organisms, it was reported that mutations in the hemin-specific ABC transporters did not eliminate the ability of these species to use hemin as an iron source, which suggested that alternative transporters were active. Analysis of the genome of \textit{C. diphtheriae} has revealed several ABC-type iron or siderophore transporters (8), all of which show some sequence similarity to previously identified hemin transporters. However, the products encoded by the \textit{hmuTUV} genes show the highest levels of sequence similarity to previously described hemin uptake systems (11). Genes that are predicted to encode proteins with sequence similarity to HtaA (DIP0522 and ChtA) and HtaB (ChtB) are present in the \textit{C. diphtheriae} genome, and all of these factors contain putative N-terminal secretion signals, as well as C-terminal transmembrane regions (8, 14). It is possible that DIP0522 and/or ChtA may contribute to the hemin iron uptake activity observed in an \textit{htaA} mutant, and similarly, the product of \textit{chtB} may complement an \textit{htaB} mutant. Further studies are needed to determine whether DIP0522, ChtA, ChtB, HtaB, or any of the other related iron uptake systems in the \textit{C. diphtheriae} genome is involved in hemin iron utilization.

BLAST analysis (1; http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) has revealed that bacterial species related to \textit{C. diphtheriae}, including \textit{C. ulcerans}, \textit{C. glutamicum}, \textit{C. jeikeium}, and \textit{Streptomyces coelicolor}, all contain genes whose predicted products have significant sequence similarity to HtaA, and all of these putative proteins contain transmembrane regions in their C termini followed by positively charged residues, an observation that indicates that there is a common anchoring mechanism in this group of related proteins. However, some differences in the anchoring of these proteins to the cell envelope have been observed. A recent study of \textit{Propionibacterium acnes}, an organism related to \textit{Corynebacterium}, identified a putative hemin iron transport protein (PA-21693) that has sequence similarity to HtaA but contains a sortase anchoring motif in its C terminus, suggesting that PA-21693 is covalently anchored to the cell wall through the action of a sortase (23). It is not clear why certain hemin or hemoglobin binding proteins in gram-positive bacteria are covalently anchored to the cell wall by sortases, while others, such as HtaA and Shp, are tethered to the surface by a single membrane-spanning region. A covalent attachment to the cell wall appears to be more secure than binding to the cell by a single transmembrane region. All of the gram-positive species that are known to contain surface-anchored hemin binding proteins produce sortases and numerous sortase substrates, indicating that a lack of sortases is not the reason for the differences in the anchoring of these proteins. It is possible that differences in how these proteins are attached to the cell envelope may be attributed to the different environments that these organisms inhabit and/or to additional functions that may be associated with these surface proteins, such as adhesins.

An unexpected finding in this study was the observation that HtaA is secreted during growth in nutrient-rich HIBTW medium but is predominantly cell associated during growth in mPGT medium, a semidefined minimal medium that is commonly used to culture \textit{C. diphtheriae} strains (44). The presence of a C-terminal transmembrane region in HtaA predicts that the protein is associated with the cytoplasmic membrane, which is observed for HtaB when \textit{C. diphtheriae} is grown in either HIBTW or mPGT medium. However, analysis of the C-terminal amino acid sequence of HtaA indicates that HtaA has a relatively weak transmembrane region compared to the membrane-spanning region in HtaB (http://www.ch.embnet.org/software/TMPRED_form.html). Although a less-than-optimal membrane-spanning sequence may indicate a weaker association with the membrane, it would not account for the differences in localization observed between organisms grown in HIBTW and mPGT media. The reason for the difference in localization of HtaA between organisms grown in the two media is not known, although differences in membrane composition, permeability, cellular metabolism, or the presence of proteases may contribute to the observed differences in the location of HtaA. While it is not known if HtaA is secreted or membrane bound in vivo, studies reported here showed that when the HtaA protein was added to mPGT culture medium, there was no stimulation of growth of the \textit{C. diphtheriae} 1737 \textit{htaA} deletion mutant when hemoglobin was the sole iron source (Fig. 4B). This finding suggests that the secreted form of HtaA may not be involved in hemin iron uptake.

In a previous report (35), we were unable to demonstrate a hemin iron utilization deficiency in a \textit{C. diphtheriae} \textit{hmuT} vector integration mutant (RT5), but we observed that a similarly constructed \textit{hmuT} mutant of \textit{C. ulcerans} was defective in hemin iron use. This previous study was performed using nutrient-rich HIBTW medium, whereas mPGT medium was used as the growth medium in the studies described in this report. The \textit{C. diphtheriae} RT5 mutant does exhibit a hemin iron utilization defect similar to that observed with the \textit{hmuTUV} deletion mutant when cells are grown in mPGT medium (M. P. Schmitt, unpublished observation). The reason for the lack of a phenotype for the \textit{C. diphtheriae} \textit{hmuT} mutant in HIBTW medium is not known; however, as noted above, \textit{C. diphtheriae} \textit{hmu} mutants are able to use hemin iron even in mPGT medium, which suggests that there are alternate hemin uptake or utilization systems. It is possible that these systems (or others) may have more robust activity during growth in HIBTW medium than
during growth in mPGT medium and can fully substitute for the hmu system during growth in HIBTW medium. Differences in metabolism or in heme or iron requirements may also contribute to the observed difference in heme iron use between the two media.

Work in our laboratory has demonstrated that hemin and hemoglobin are equally capable of supplying iron for growth of C. diphtheriae strains (16, 31, 35). While it is not known how C. diphtheriae acquires the heme moiety from hemoglobin, a recent study has suggested that one mechanism by which hemin iron is obtained for use by bacteria is through the spontaneous release of heme from hemoglobin (25). It is also possible that secreted or surface-exposed proteases in C. diphtheriae contribute to the breakdown of hemoglobin and subsequent release of heme. In the model shown in Fig. 7, we propose that hemin initially binds to HtaA at one or both of the conserved domain regions. Hemin binding sites have not been identified for HtaA and future studies should determine if these conserved regions are involved in heme binding or transport. It is predicted that heme bound to HtaA is transferred to HtaB, which may function in an intermediate step in the movement of heme from HtaA to HmuT. This mechanism of action for HtaB would be similar to the function proposed for some of the Isd proteins from S. aureus, which are involved in the movement of heme through the cell wall (22, 46, 50). HmuT is proposed to deliver heme to the HmuU permease, a component of the ABC transporter (HmuU and HmuV), which facilitates the uptake of heme into the cytosol, where it is thought to be degraded by the heme oxygenase enzyme HmuO, releasing iron for cellular metabolism.

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