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Utilization of Host Iron Sources by *Corynebacterium diphtheriae*: Identification of a Gene Whose Product Is Homologous to Eukaryotic Heme Oxygenases and Is Required for Acquisition of Iron from Heme and Hemoglobin

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***Corynebacterium diphtheriae* was examined for the ability to utilize various host compounds as iron sources. *C. diphtheriae* C7(-) acquired iron from heme, hemoglobin, and transferrin. A siderophore uptake mutant of strain C7 was unable to utilize transferrin but was unaffected in acquisition of iron from heme and hemoglobin, which suggests that *C. diphtheriae* possesses a novel mechanism for utilizing heme and hemoglobin as iron sources. Mutants of *C. diphtheriae* and *Corynebacterium ulcerans* that are defective in acquiring iron from heme and hemoglobin were isolated following chemical mutagenesis and streptonigrin enrichment. A recombinant clone, pCD293, obtained from a C7(-) genomic plasmid library complemented several of the *C. ulcerans* mutants and three of the *C. diphtheriae* mutants. The nucleotide sequence of the gene (*hmuO*) required for complementation was determined and shown to encode a protein with a predicted mass of 24,123 Da. Sequence analysis revealed that HmuO has 33% identity and 70% similarity with the human heme oxygenase enzyme HO-1. Heme oxygenases, which have been well characterized in eukaryotes but have not been identified in prokaryotes, are involved in the oxidation of heme and subsequent release of iron from the heme moiety. It is proposed that the HmuO protein is essential for the utilization of heme as an iron source by *C. diphtheriae* and that the heme oxygenase activity of HmuO is involved in the release of iron from heme. This is the first report of a bacterial gene whose product has homology to heme oxygenases.**

Corynebacterium diphtheriae, a gram-positive aerobic bacterium, is the causative agent of diphtheria. The bacterium colonizes the mucosal surfaces of the upper respiratory tract in humans, where it secretes the potent protein exotoxin diphtheria toxin. In most cases of respiratory diphtheria, the bacterium is rarely found outside the local area of infection. The severe systemic symptoms associated with diphtheria are due to the dissemination of diphtheria toxin, which can cause severe tissue damage throughout the body (4, 9).

With the exception of studies examining diphtheria toxin, very little is known about the molecular mechanisms involved in the pathogenesis of *C. diphtheriae*. Factors involved in the colonization and survival of *C. diphtheriae* on the mucosal surfaces of the human host have not been identified. Diphtheria toxin, a 58,000-Da protein secreted by *C. diphtheriae*, has been well studied both genetically and biochemically (14, 31). The bacteriophage-encoded *tox* gene is the structural gene for diphtheria toxin, and it has been shown to be regulated at the transcriptional level by iron and the diphtheria toxin repressor protein, DtxR (1, 37, 50). DtxR is a global iron-dependent repressor which binds to a sequence 5′ of the *tox* gene that overlaps the −10 region of the *tox* promoter (40, 48). DtxR also binds to the promoter regions for the *C. diphtheriae* iron-regulated *irp1* and *irp2* genes (38) and has been shown to regulate the expression of the *C. diphtheriae* siderophore (33, 34, 37). Expression of *tox* and other *dtxR*-regulated genes is repressed in iron-replete environments and is derepressed in low-iron conditions. Homologs of DtxR have recently been identified in several gram-positive bacteria, including species of *Brevibacterium* (29), *Streptomyces* (15), and *Mycobacterium* (7, 39). Although DtxR is functionally similar to the ferric uptake repressor protein (Fur) present in gram-negative bacteria (17), these proteins have no significant nucleotide or amino acid sequence homologies (1, 37).

Almost all bacteria require iron for growth, and the ability to acquire sufficient iron during infection is essential for many bacterial pathogens to cause disease (51). Much of the extracellular iron in mammalian hosts is associated with transferrin or lactoferrin, while much of the intracellular iron is associated with heme which is bound by various heme-containing proteins such as hemoglobin (30). Heme which can be released into the extracellular medium following cell lysis is bound by the serum proteins hemopexin and serum albumin (30). Since diphtheria toxin expression is derepressed during *C. diphtheriae* infection, it is assumed that the site of colonization is low in available iron. Numerous virulence determinants produced by bacterial pathogens are derepressed in low-iron environments, including toxins (23), hemolysins (43), siderophore-dependent high-affinity iron uptake systems (12, 26, 55), and transport proteins involved in the removal of iron from host compounds, such as transferrin, lactoferrin, heme, hemoglobin, and other heme-containing proteins (22, 27). Although the human host has very little free iron that is readily available to an invading pathogen, bacteria have devised a variety of mechanisms to extract iron from host iron-containing compounds. Many bacteria produce and secrete siderophores, low-molecular-weight iron-chelating compounds, to obtain iron during growth in iron-depleted environments. Several siderophore systems that have been shown to be important for virulence can utilize iron bound to transferrin and lactoferrin (12, 26, 55). Bacteria have also developed

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siderophore-independent mechanisms for acquiring iron from host compounds. Outer membrane receptors involved in the binding and utilization of iron from transferrin and lactoferrin have been well studied in pathogenic *Neisseria* and *Haemophilus* species (27). Additionally, numerous gram-negative bacterial pathogens are known to possess transport mechanisms involved in the acquisition of iron from heme and heme-containing proteins (22). In most of these bacteria only the outer membrane binding protein has been identified; however, in *Yersinia enterocolitica* the acquisition of iron from heme and hemoglobin involves several proteins, including the outer membrane heme receptor HemR, the periplasmic binding protein HemT, and a cytoplasmic membrane permease composed of HemV and HemU. Additionally, a cytosolic protein, HemS, has been proposed to be involved in the degradation of heme and the release of iron; however, an enzymatic activity for HemS has not been reported, and HemS has no sequence homology with heme oxygenases (44, 45).

In this study, the ability of *C. diphtheriae* to utilize various host iron sources was examined. *C. diphtheriae* acquired iron from heme, hemoglobin, and transferrin, and mutants unable to utilize heme or hemoglobin as an iron source were isolated. A clone from a *C. diphtheriae* genomic library was shown to complement the defect in some of these mutants. The gene required for complementation, *hmuO*, was sequenced and shown to encode a protein that has extensive homology with the human heme oxygenase protein HO-1.

MATERIALS AND METHODS

Bacterial strains, plasmid vectors, and media. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used for the routine isolation and purification of plasmid DNA. *C. diphtheriae* C7(-), a nontoxigenic derivative of C7(β) (20), and HC1 (6) and *Corynebacterium ulcerans* 712 (42) were obtained from the strain collection of Randall K. Holmes (University of Colorado Health Sciences Center, Denver). *C. diphtheriae* 1716 is a clinical strain that was isolated during the current diphtheria outbreak in Russia and was kindly provided by Tanja Popovic (CDC). All strains are stored at −70°C in 20% glycerol. Plasmid pCM2.6 is an *E. coli-C. diphtheriae* shuttle vector which has been described previously (37). The cloning vector pBluescript KS+ (Stratagene, La Jolla, Calif.) was used in the initial stages in the construction of some of the subclones derived from plasmid pCD293. *E. coli* DH5α was grown in Luria-Bertani medium (25), and *Corynebacterium* strains were grown in heart infusion broth (Difco, Detroit, Mich.) with 0.2% Tween 80 (HIBTW). The antibiotics ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml for *E. coli* and 2 μg/ml for *Corynebacterium* spp.) were added to the media as required. To make HIBTW medium low in iron, ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA) was added when needed and at the concentrations indicated, and EDDA was deferrated by the method of Rogers (32). A 1-mg/ml stock solution of streptonigrin was prepared in 10 mM Tris-HCl (7.6) and was added to solid medium at a final concentration of 0.5 μg/ml. Tween 80, antibiotics, EDDA, and streptonigrin were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Utilization of iron sources by *Corynebacterium* species. Approximately 10^7 organisms diluted from an overnight culture grown in HIBTW medium were plated onto the surface of HIBTW agar medium that contained 150 μg of EDDA per ml for C7(-), 1716, and *C. ulcerans* strains or 50 μg/ml for *C. diphtheriae* HC1 strains. These concentrations of EDDA completely inhibit the growth of the bacteria unless an exogenous iron source is added. Wells, 3 mm in diameter, were made in the agar, and 10-μl volumes of the various iron sources were placed into the wells. Plates were incubated at 37° for 24 to 36 h and then examined for the presence of bacterial growth. The following iron sources were used at the indicated concentrations: ferrous sulfate, 1 mM; heme (bovine), 250 μM; hemoglobin (human), 10 μM; protoporphyrin IX, 250 μM; ferritin (bovine), 1 mg/ml; transferrin, 30% iron saturated (human), 30 μM; apotransferrin (human), 30 μM. All iron sources were purchased from Sigma Chemical Co.

The modified PGT-maltose (mPGT) medium is a low-iron medium which has been used previously to detect siderophore and diphtheria toxin production by *C. diphtheriae* strains (46). To test for utilization of iron sources in mPGT liquid medium, bacteria were grown overnight in mPGT medium and then diluted 1:100 into fresh mPGT liquid medium which contained the various iron sources at the concentrations indicated above. Bacteria were grown for 24 h with shaking at 37°C.

Mutagenesis of *C. ulcerans* 712 and *C. diphtheriae* HC1. Bacteria grown overnight in HIBTW medium were diluted 1:100 into 10 ml of fresh HIBTW medium and grown with shaking at 37°C to mid-late log phase. Bacteria were washed two times with 100 mM Tris-HCl (7.5) and then resuspended in 5 ml of the same buffer. A total of 75 μl of methanesulfonic acid ethyl ester (EMS) (Sigma Chemical Co.) was added, and the cells were incubated for 2 h at 37°C with occasional mixing. The cells were then washed two times with 100 mM Tris-HCl (7.5), resuspended in 10 ml of HIBTW medium, and grown overnight at 37°C. As reported previously, a 2-log decrease in cell viability occurred after EMS treatment (6).

TABLE 1. Utilization of iron sources by *Corynebacterium* spp.

Strain	Iron source[a]						
	Fe	Heme	PPIX	Hb	Tf	aTf	Ft
C. diphtheriae C7(-)	+	+	−	+	+	−	−
C. diphtheriae 1716	+	+	−	+	+	−	−
C. diphtheriae HC1 Sid-	+	+	−	+	−	−	−
C. ulcerans 712	+	+	−	+	+	−	−

[a] Concentrations of iron sources are given in Materials and Methods. Fe, ferrous sulfate; Hb, hemoglobin; PPIX, protoporphyrin IX; Tf, transferrin; aTf, apotransferrin; Ft, ferritin; +, ability to utilize iron source for growth; −, unable to utilize iron source. Utilization of iron sources was determined using HIBTW-EDDA agar plates and in mPGT liquid medium; see Materials and Methods for details.

Construction of a plasmid library with *C. diphtheriae* chromosomal DNA. Chromosomal DNA from *C. diphtheriae* C7(-) was isolated and purified as described previously (36). An *Msp*I partial digest of the chromosomal DNA was performed following standard procedures (25), and the DNA was separated on a 0.8% agarose gel. DNA fragments of 3 to 8 kb were excised from the gel and purified with a gel extraction kit (Qiagen, Chatsworth, Calif.). The size-fractionated chromosomal DNA was ligated to the pCM2.6 shuttle vector which had been digested with *Cla*I and treated with alkaline phosphatase.

Construction of plasmid subclones in pCM2.6. Plasmid pCD293.7 was generated by isolating the 1,273-bp *Nsi*I fragment from pCD293 and ligating this fragment into the *Pst*I site in pCM2.6. Plasmid pCD293.3 was produced by ligating the 1.3-kb *Bam*HI-*Sal*I fragment from pCD293 into the *Bam*HI-*Sal*I sites in pCM2.6. Plasmids pCD293.8 and pCD293.10 were constructed by first ligating the 878-bp *Hin*cII and 1-kb *Stu*I-*Hae*III fragments from pCD293 into the *Eco*RV site of KS+. The 878-bp and 1-kb inserts in KS+ were then excised with *Bam*HI and *Sal*I and ligated into pCM2.6, creating pCD293.8 and pCD293.10, respectively. Plasmid pCD293.9 was constructed by first ligating the 900-bp *Pst*I-*Sal*I fragment into the *Pst*I-*Sal*I site in KS+, followed by excision of the insert in KS+ with *Bam*HI and *Sal*I and ligation of this fragment into the *Bam*HI and *Sal*I sites of pCM2.6.

DNA manipulations. Plasmids were isolated and purified from *E. coli* strains by using a plasmid purification kit purchased from Qiagen Inc., and plasmids were isolated from *Corynebacterium* strains as described previously (36). DNA was transformed into *Corynebacterium* species by electroporation according to Haynes and Britz (18), and transformation into *E. coli* was by the method of Hanahan (16). Restriction endonucleases (Bethesda Research Laboratories), T4 DNA ligase (Amersham, Cleveland, Ohio), and shrimp alkaline phosphatase (Amersham) were used following procedures suggested by the suppliers.

DNA sequencing was performed with alkaline-denatured double-stranded templates by the chain termination method of Sanger et al. (35) by using a DNA sequencing kit purchased from Amersham.

Nucleotide sequence accession number. The sequence of the 1,273-bp chromosomal insert in plasmid pCD293.7 was assigned the GenBank accession number U73860.

RESULTS

Utilization of host iron sources by *Corynebacterium* species. No previous studies have examined whether *C. diphtheriae* or related *Corynebacterium* spp. can use eukaryotic iron compounds as sources of iron. To determine if *Corynebacterium* spp. are capable of utilizing host compounds, three different strains of *C. diphtheriae* and *C. ulcerans* 712 were examined for their ability to acquire iron from a variety of sources. All of the strains were capable of using ferrous sulfate, heme, and hemoglobin for growth when plated on low-iron agar medium or grown in low-iron mPGT liquid medium (Table 1). Transferrin was used by all strains except the *C. diphtheriae* siderophore uptake mutant HC1, and none of the strains were able to grow in the presence of apotransferrin or protoporphyrin IX, which were used as negative controls. The inability of HC1 to utilize

transferrin as an iron source, while maintaining the ability to acquire iron from heme and hemoglobin, suggests that the acquisition of iron from transferrin by *C. diphtheriae* is dependent on a functional siderophore uptake system while the ability to utilize iron from heme and hemoglobin employs a transport mechanism that is distinct from the siderophore system. Several other *C. diphtheriae* siderophore transport mutants were also unable to use transferrin as an iron source but were fully capable of acquiring iron from both heme and hemoglobin (data not shown).

Transferrin failed to stimulate growth of C7(-), 1716, and *C. ulcerans* in the HIBTW-EDDA agar medium; however, transferrin was utilized as an iron source by these strains in low-iron mPGT liquid medium. The reason for the inability of transferrin to stimulate growth in HIBTW-EDDA medium is unclear, although it is possible that the EDDA in the medium may have interfered with the ability of the siderophore to extract iron from transferrin. HC1 and other siderophore-deficient strains were unable to utilize transferrin as an iron source regardless of the medium.

Construction and characterization of *Corynebacterium* mutants that are deficient in acquiring iron from heme and hemoglobin. The results presented in Table 1 indicate that the ability of *C. diphtheriae* to acquire iron from heme and hemoglobin is independent of the high-affinity siderophore uptake system and suggest that *C. diphtheriae* may possess a novel transport system for the acquisition of iron from heme and hemoglobin. A streptonigrin enrichment procedure was developed for the purpose of isolating *Corynebacterium* mutants unable to utilize heme or hemoglobin as an iron source.

The antibiotic streptonigrin has been used by numerous investigators to isolate iron uptake mutants (3, 8, 13). Streptonigrin interacts with intracellular iron pools, causing the formation of reactive oxygen species, which results in DNA damage and eventually cell death (5, 52). Bacterial strains grown in high-iron medium have greater sensitivity to streptonigrin than bacteria grown in iron-depleted medium, since the organisms in iron-replete environments accumulate larger intracellular iron pools (53). Bacteria capable of growing in moderately low-iron medium in the presence of streptonigrin are inhibited for growth if an exogenous iron source, such as heme or hemoglobin, is added to the medium. However, mutants unable to utilize the iron source will maintain their resistance to streptonigrin since they are unable to accumulate intracellular iron. In the mutagenesis experiments, *C. diphtheriae* HC1 was used in place of wild-type *C. diphtheriae* C7(-) due to the inability to establish optimal growth conditions for the wild-type C7(-) strain in medium containing EDDA and streptonigrin. Additionally, *C. ulcerans*, an organism closely related to *C. diphtheriae*, was utilized in the mutagenesis experiments and in later studies since it is more amenable to genetic manipulation than is *C. diphtheriae* (37).

C. ulcerans was examined for its sensitivity to streptonigrin in the presence of various concentrations of EDDA and in the presence or absence of heme or hemoglobin (Table 2). The results from Table 2 indicate that *C. ulcerans* failed to grow or grew poorly at EDDA concentrations higher than 50 μg/ml, which is likely due to low iron concentrations in the medium. However, since *C. ulcerans* is able to utilize heme and hemoglobin as iron sources, the addition of heme or hemoglobin at all EDDA concentrations restored growth. Growth of *C. ulcerans* in the presence of streptonigrin was inhibited at concentrations of EDDA higher than 17.5 μg/ml and lower than 10 μg/ml. The inability of *C. ulcerans* to grow at the higher EDDA concentrations is likely due to the derepression of the siderophore uptake system, as reported by others (56). The inability of *C. ulcerans* to grow in the presence of streptonigrin at concentrations of EDDA lower than 10 μg/ml is due to an adequate iron supply in the medium. Only EDDA concentrations between 12.5 and 15 μg/ml permitted optimal growth of *C. ulcerans* in the presence of 0.5 μg of streptonigrin/ml (Table 2). *C. diphtheriae* HC1 also exhibited optimal growth under identical conditions (data not shown). Both strains failed to grow when heme or hemoglobin was added to the medium containing streptonigrin (Table 2).

TABLE 2. Streptonigrin sensitivity of *C. ulcerans* in the presence of various concentrations of EDDA[a]

EDDA (μg/ml)	Supplement(s) added to HIBTW/EDDA medium			
	None	Sng[b] (0.5 μg/ml)	He/Hb[c]	He/Hb + Sng
0	+	−	+	−
5	+	−	+	−
10	+	+/−	+	−
12.5	+	+	+	−
15	+	+	+	−
17.5	+	+/−	+	−
20	+	−	+	−
50	+/−	−	+	−
150	−	−	+	−

[a] Bacteria were plated or streaked on HIBTW agar plates. +, wild-type colony size; +/−, small pinpoint colonies; −, no growth.
[b] Sng, streptonigrin.
[c] He/Hb, heme or hemoglobin added as iron source.

To enrich for mutants that are unable to utilize heme or hemoglobin as an iron source, EMS-mutagenized cultures of *C. ulcerans* and HC1 were plated onto HIBTW medium that contained 15 μg of EDDA/ml, 0.5 μg streptonigrin/ml, and either heme or hemoglobin. Streptonigrin-resistant colonies appeared at a frequency of 10^{-6} for both strains, regardless of whether heme or hemoglobin was used as the iron source. To determine whether the streptonigrin-resistant mutants could utilize heme or hemoglobin as a source of iron, the *C. ulcerans* and HC1 mutants were examined for their ability to grow on HIBTW-EDDA medium containing either heme or hemoglobin. Four *C. ulcerans* mutants and three *C. diphtheriae* HC1

TABLE 3. Utilization of iron sources by *Corynebacterium* mutants[a]

Strain[b]	Iron source added to HIBTW-EDDA agar plates			
	Heme	Hb[c]	Fe	None
C. ulcerans				
Wild type	+	+	+	−
CU29	−	−	+	−
CU21	+/−	+/−	+	−
CU24	+/−	+/−	+	−
CU84	−	−	+	−
CU56	−	−	+	−
CU44	−	−	+	−
C. diphtheriae HC1	+	+	+	−
HC1-2	−	−	+	−
HC1-35	−	−	+	−
HC1-99	−	−	+	−

[a] Utilization of iron sources was determined by the plate assay described in Materials and Methods. +, heavy confluent growth around iron source; +/−, weak or faint growth; −, absence of growth.
[b] Strains designated CU are *C. ulcerans* mutants, and strains designated HC1 are *C. diphtheriae* HC1 mutants.
[c] Hb, hemoglobin.

TABLE 4. Complementation of *Corynebacterium* heme utilization mutants[a]

Strain	Plasmids		
	None	pCM2.6	pCD293
CU29	−	−	+
CU21	+/−	+/−	+
CU24	+/−	+/−	+/−
CU84	−	−	−
CU56	−	−	−
CU44	−	−	−
HC1-2	−	−	+
HC1-35	−	−	+
HC1-99	−	−	+

[a] Symbols are the same as those used in Table 3. "+" indicates ability to utilize both heme and hemoglobin as iron sources as determined by the plate assay described in Materials and Methods.

mutants were unable to use either heme or hemoglobin as a source of iron (Table 3). *C. ulcerans* mutants CU21 and CU24 exhibited diminished ability to utilize heme and hemoglobin for growth on the low-iron medium. All of the bacteria tested utilized ferrous sulfate as an iron source and were inhibited for growth in the absence of any iron supplement (Table 3).

Complementation of the *C. ulcerans* heme utilization mutant CU29 by *C. diphtheriae* chromosomal DNA. *C. diphtheriae* strains, including C7 and HC1, are very poorly transformed by plasmid DNA, whereas *C. ulcerans* 712 can be transformed as efficiently as highly competent *E. coli* strains (37). Therefore, complementation experiments were initially attempted using a *C. ulcerans* heme utilization mutant.

A plasmid library carrying *C. diphtheriae* C7(-) chromosomal DNA was transformed by electroporation into the *C. ulcerans* mutant CU29. Transformants were plated onto HIBTW-EDDA medium that contained either heme or hemoglobin. Strain CU29 is unable to grow under these conditions due to its inability to utilize heme or hemoglobin as an iron source. One colony that exhibited robust growth on this medium was isolated and shown to harbor a plasmid designated pCD293. To determine if plasmid pCD293 could restore the ability to utilize heme and hemoglobin to the various mutants, pCD293 was transformed back into CU29 and into the other heme utilization mutants isolated from *C. ulcerans* and HC1. Plasmid pCD293 fully restored the ability of the *C. ulcerans* mutants CU29 and CU21 and all three of the *C. diphtheriae* HC1 mutants to utilize both heme and hemoglobin as iron sources, while the cloning vector pCM2.6 had no effect (Table 4).

Mapping and complementation studies with subclones of plasmid pCD293. A restriction endonuclease map of the chro-

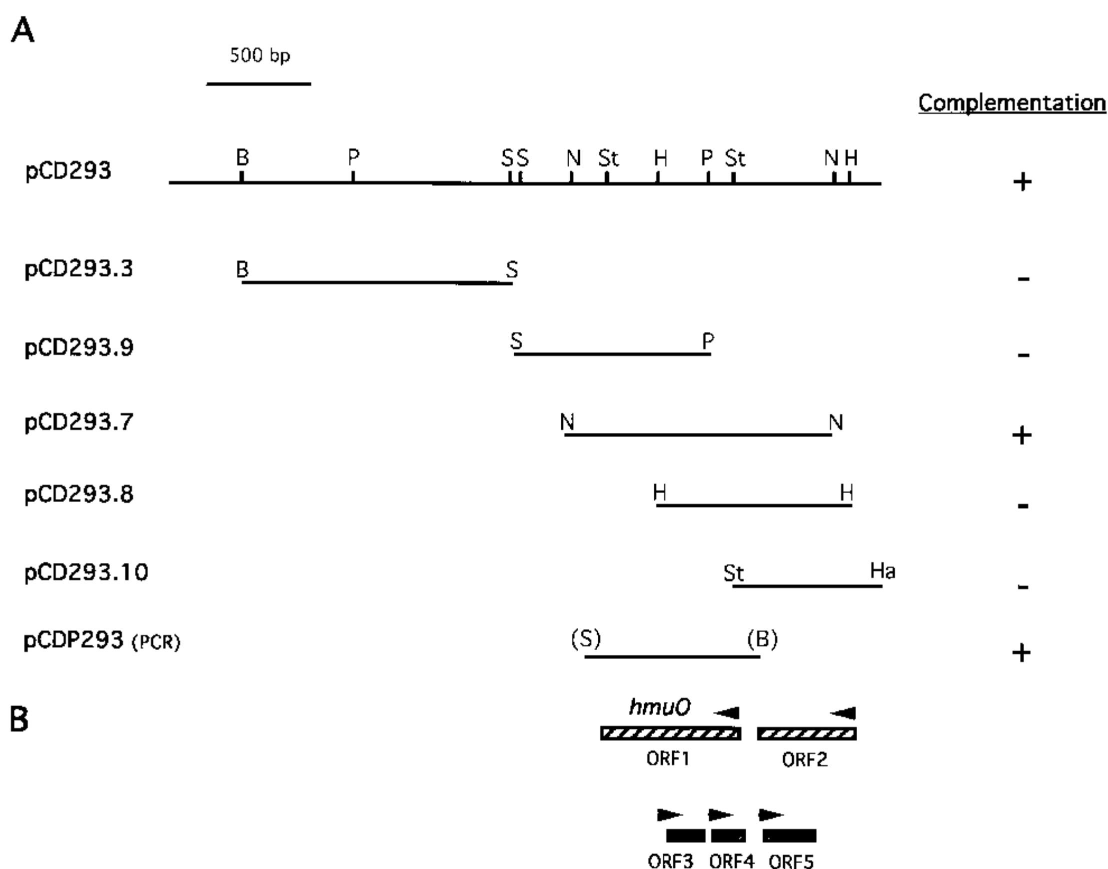

FIG. 1. (A) Endonuclease restriction map of the chromosomal insert in plasmid pCD293. Shown below the restriction map are the inserts present in the various subclones derived from pCD293. To the right of the restriction maps are the results of complementation experiments done with the various plasmids: "+" indicates the plasmid was able to complement the defect in CU29 and HC1-2, and "−" indicates the inability to complement these strains. B, *Bam*HI; H, *Hin*cII; Ha, *Hae*III (located in vector); N, *Nsi*I; P, *Pst*I; S, *Sal*I; St, *Stu*I. *Bam*HI (B) and *Sal*I (S) sites in the PCR-derived fragment in plasmid pCDP293 were placed in the oligonucleotide primers used for PCR and are not present in the chromosome at those locations. Partial restriction maps are shown for *Hin*cII and *Hae*III. (B) ORFs present in plasmid pCD293.7. The arrows indicate the locations of putative ATG start codons. The ORFs are aligned with the restriction maps shown in Fig. 1A.

FIG. 2. Nucleotide sequence of the 1,273-bp chromosomal insert in plasmid pCD293.7. DtxR b. s. indicates the location of the putative 19-bp DtxR binding site, which is highlighted in boldface and has a line drawn over the sequence. Possible −10 and −35 promoter elements are indicated, and three possible ATG start codons are shown in boldface, with the most likely start site indicated with an asterisk. A ribosome binding site, rbs, is located upstream of the putative ATG start codon. Primers used for PCR, 29h25b and 29h24s, are indicated by an arrow over the sequence. The sequence of primer 29h24s is the complement of the sequence shown. Nucleotide changes that were made in order to introduce restriction sites into the oligonucleotide primers are shown above the sequence. The numbers to the right of the sequence indicate the numbering of the nucleotide and amino acid sequences.

mosomal insert in plasmid pCD293 is shown in Fig. 1A. The chromosomal insert, which is approximately 3.5 kb in size, was subcloned in an attempt to identify the smallest region within pCD293 that is required for complementation of strains CU29 and HC1-2. Five separate subclones were constructed in the shuttle vector pCM2.6 and tested for their ability to complement CU29 and HC1-2 (Fig. 1A). Only plasmid pCD293.7, which carries a 1,273-bp *Nsi*I fragment, was able to complement CU29 and HC1-2.

Nucleotide sequence of the chromosomal insert in plasmid pCD293.7. The nucleotide sequence of the 1,273-bp *Nsi*I fragment in plasmid pCD293.7 is shown in Fig. 2. Five open reading frames (ORFs) were identified within this sequence (Fig. 1B), and ORF1, designated *hmuO*, was the only ORF required for complementation of CU29 and HC1-2. The deduced amino acid sequence of the HmuO protein initiating from the ATG codon at position 482 is shown below the nucleotide sequence in Fig. 2. The putative HmuO protein contains 215 amino acids and has a predicted molecular weight of 24,123.

The four other ORFs on pCD293.7 either are only partially encoded on pCD293.7 or are also encoded on plasmids which failed to complement strains CU29 and HC1-2. ORF2 is present on plasmid pCD293.10, and ORF3, -4, and -5 are located on plasmid pCD293.8; both of these plasmids were unable to complement either CU29 or HC1-2 (Fig. 1A). To further localize the region required for complementation, PCR was used to amplify a DNA sequence which contained the entire coding region of *hmuO* and a small amount of flanking sequence. The two primers used for the PCR amplification are indicated above the sequence in Fig. 2 (29h24s and 29h25b contain *Sal*I and *Bam*HI recognition sites, respectively). The 890-bp PCR-derived DNA product was digested with the appropriate restriction enzymes and ligated into pCM2.6, and the resulting plasmid, pCDP293, was found to complement strains CU29 and HC1-2 (Fig. 1A), thus providing additional evidence that *hmuO* encodes the protein required for complementing the mutations present in both CU29 and HC1-2.

Located upstream of *hmuO* are putative −10 and −35 transcription initiation sequences, and between these sequences is a region that is highly homologous to the DtxR consensus binding site (Fig. 2) (38, 49). An alignment of the sequence located upstream of *hmuO* with the 19-bp DtxR consensus sequence is shown in Fig. 3. The sequence upstream of *hmuO* is identical to 15 of the 19 bases within the DtxR consensus binding site and aligns with 8 of the 10 most highly conserved

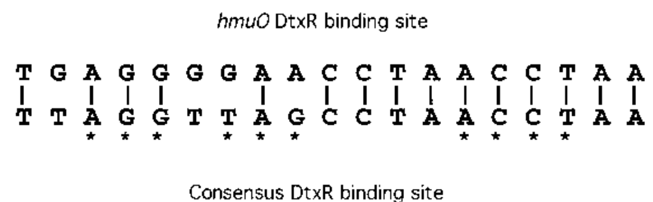

FIG. 3. Alignment of the 19-bp DtxR consensus binding site with the putative DtxR binding site located upstream of the *hmuO* gene. Asterisks indicate the most highly conserved sequences within the consensus binding site.

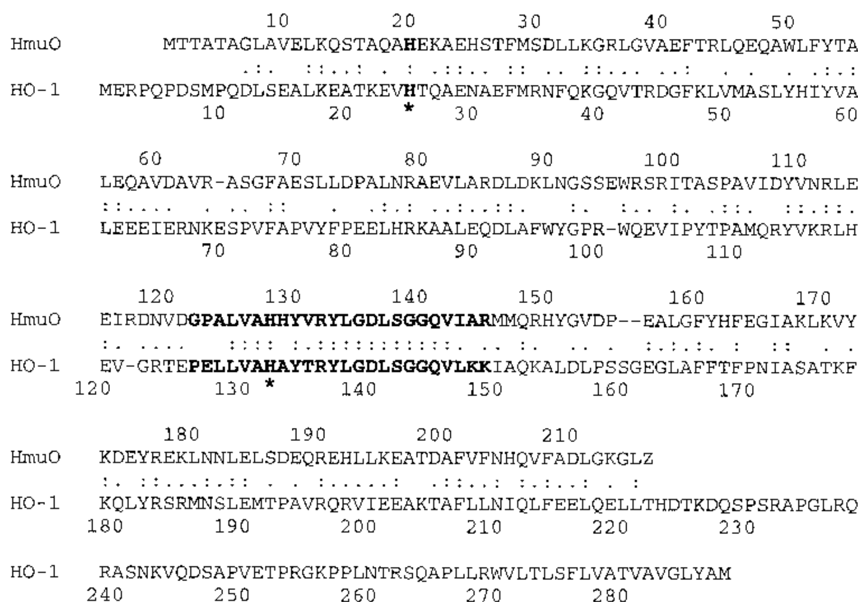

FIG. 4. Amino acid alignment between HmuO and the human heme oxygenase protein HO-1. Conserved histidine residues at positions 25 and 132 in HO-1 are indicated in boldface and by an asterisk. A 24-amino-acid sequence (126 to 149 in HO-1) that is highly conserved among all heme oxygenases is also indicated in boldface. Gaps were introduced into the sequences to obtain an optimal alignment. Colons indicate identical residues, and dots indicate similar residues.

residues within the 19-bp sequence (Fig. 3). While these results suggest that *hmuO* is regulated by DtxR and iron, additional studies are required to confirm this possibility.

hmuO **has homology with eukaryotic heme oxygenases.** A FASTA search of the SwissProt protein database revealed that HmuO had significant homology to the family of eukaryotic heme oxygenases. Shown in Fig. 4 is an amino acid sequence alignment between HmuO and the human heme oxygenase protein HO-1 (57). An alignment of the 215 amino acids of HmuO with the N-terminal 221 amino acids of HO-1 revealed that the proteins are 33% identical and have 70% similarity if conserved residues are included in the comparison. The C-terminal 70 amino acids of eukaryotic heme oxygenases, including HO-1, share very little sequence homology (24), and this poorly conserved region appears to be absent in HmuO. Biochemical analysis with HO-1 revealed that histidine residues located at positions 25 and 132 were essential for full catalytic activity of HO-1 and that the histidine at position 25 was one of the primary heme iron ligands (24, 54). The histidine residues at positions 25 and 132 in HO-1 are conserved at the corresponding location in HmuO (Fig. 4), which suggests that these histidine residues may have a similar role in the activity of the protein encoded by *hmuO*. Other significant sequence similarities between HmuO and HO-1 include the lack of cystine residues and the conservation of 16 of the 24 amino acids within a 24-amino-acid sequence (126 to 149 in HO-1) that is highly conserved among all heme oxygenases and is thought to play an important role in the catalytic activity of the protein (24, 54).

Three ATG codons were present at the 5′ region of *hmuO*; however, only two of these ATG codons (at positions 482 and 566) had upstream Shine-Dalgarno sequences. Based on the sequence homology with the HO-1 protein, the ATG codon at 482 appears to be the most likely start codon for HmuO, especially since the conserved histidine at position 20 in HmuO (position 25 in HO-1) would be deleted if the ATG codon at 566 served as the translational start site.

DISCUSSION

Transport systems for the acquisition of iron from heme and hemoglobin or any heme-containing protein have not been identified in gram-positive bacteria, although some species of *Staphylococcus* and *Streptococcus* can utilize heme and hemoglobin as sources of iron (11, 47). The results of this study indicate that the ability of *C. diphtheriae* to acquire iron from heme and hemoglobin is independent of its siderophore uptake system and may involve a novel transport mechanism. Numerous pathogenic gram-negative bacteria are known to acquire iron from heme and heme-containing proteins. Proteins involved in the binding and utilization of iron from hemoglobin, hemoglobin-haptoglobin complexes, and hemopexin have been described (22). Outer membrane heme-binding proteins that are essential for the utilization of iron from heme and hemoglobin have been identified in *Vibrio cholerae* (19), *Shigella dysenteriae* (28), and *Y. enterocolitica* (44, 45). In these systems, the entire heme moiety has been shown to be transported into the cell, where presumably heme and iron can serve as nutrients for the bacteria. While most of these systems are regulated by iron and Fur, the hemoglobin binding protein in pathogenic *Haemophilus* species is regulated solely by heme (10, 21).

The utilization of heme by gram-negative bacteria appears to share a number of similarities with the mechanism used to transport ferric siderophore complexes. Both the heme and siderophore transport systems (2) utilize iron-regulated, TonB-dependent outer membrane receptors, and, at least in *Y. enterocolitica*, both systems use a periplasmic binding protein-dependent transport system. It has been proposed that heme uptake also requires a heme oxygenase-like protein that would release iron from the heme moiety (22).

The molecular mechanisms used by *C. diphtheriae* or any gram-positive bacterium to acquire iron from heme or hemoglobin are not known. However, based on the findings with *Y. enterocolitica* and other gram-negative bacteria, the transport

of heme in gram-positive bacteria may be similar to the transport of ferric siderophores. *Bacillus subtilis* is the only gram-positive bacterium in which a siderophore uptake system has been studied at the molecular level. Two genes proposed to be involved in the transport of the siderophore ferrichrome have been characterized in *B. subtilis*, and the uptake system appears to require a surface-exposed lipoprotein receptor and a cytoplasmic membrane permease (41). The ferrichrome receptor is anchored to the cytoplasmic membrane by a lipid moiety at its amino terminus and has sequence homology to periplasmic binding proteins involved in siderophore uptake in gram-negative bacteria. Therefore, to compensate for the lack of an outer membrane, *B. subtilis*, and possibly other gram-positive bacteria, utilizes as its primary cell surface siderophore receptor a lipoprotein that has homology to gram-negative periplasmic binding proteins. The cytoplasmic membrane permeases in gram-positive and gram-negative bacteria are presumed to be homologous.

If heme transport in gram-positive bacteria mimics siderophore transport, then heme or heme-containing proteins would initially bind a cell surface lipoprotein receptor that shares homology with periplasmic binding proteins from gram-negative bacteria. Transport of heme into the cytosol presumably would be facilitated by a cytoplasmic membrane permease, followed by the removal of iron from the porphyrin by a heme-degrading enzyme, such as that proposed for HemS in *Y. enterocolitica* (45) or a heme oxygenase-like enzyme (22).

A protein database search revealed that HmuO from *C. diphtheriae* has significant homology to eukaryotic heme oxygenases. Heme oxygenases have been extensively studied both genetically and biochemically in numerous mammalian species (24). The enzymatic activity of heme oxygenase results in the oxidation of heme to biliverdin and subsequent release of iron and CO. The relatively high degree of homology between the amino acid sequence of the human heme oxygenase enzyme HO-1 and the bacterial HmuO protein is remarkable and suggests that many of the residues that are conserved between these proteins are important for the activity or conformation of the protein.

This is the first report of a bacterial gene, *hmuO*, whose product has homology with heme oxygenases. The data suggest that HmuO is required for the utilization of iron from heme in *C. diphtheriae* and that HmuO functions in the removal of iron from heme by means of a heme oxygenase activity. The identification of additional components involved in the transport and utilization of heme iron by *C. diphtheriae* awaits further studies.

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