A Gene Cluster Involved in the Utilization of Both Free Heme and Heme:Hemopexin by *Haemophilus influenzae* Type b

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The utilization of heme bound to the serum glycoprotein hemopexin by *Haemophilus influenzae* type b (Hib) strain DL42 requires the presence of the 100-kDa heme:hemopexin-binding protein encoded by the *hxaA* gene (M. S. Hanson, S. E. Pelzel, J. Latimer, U. Muller-Eberhard, and E. J. Hansen, Proc. Natl. Acad. Sci. USA 89:1973–1977, 1992). Nucleotide sequence analysis of a 5-kb region immediately upstream from the *hxaA* gene revealed the presence of two genes, designated *hxC* and *hxB*, which encoded outer membrane proteins. The 78-kDa HxC protein had similarity to TonB-dependent outer membrane proteins of other organisms, whereas the 60-kDa HxB molecule most closely resembled the ShlB protein of *Serratia marcescens*. A set of three isogenic Hib mutants with *cat* cartridges inserted individually into their *hxaA*, *hxB*, and *hxC* genes was constructed. None of these mutants could utilize heme:hemopexin. The *hxC* mutant was also unable to utilize low levels of free heme, whereas both the *hxaA* and *hxB* mutants could utilize free heme. When the wild-type *hxC* gene was present in *trans*, the *hxC* mutant regained its ability to utilize low levels of free heme but still could not utilize heme:hemopexin. The *hxaA* mutant could utilize heme:hemopexin when a functional *hxaA* gene from a nontypeable *H. influenzae* strain was present in *trans*. Complementation analysis using this cloned nontypeable *H. influenzae hxaA* gene also indicated that the HxB protein likely functions in the release of soluble HxA from the Hib cell. These studies indicate that at least two and possibly three gene products are required for utilization of heme bound to hemopexin by Hib strain DL42.

The requirement of *Haemophilus influenzae* for exogenously supplied heme and NAD for aerobic growth (13) distinguishes this bacterium from nearly every other facultative anaerobe. While other bacteria can use heme, a protoporphyrin IX-iron complex, as a source of inorganic iron (10, 12, 24, 38–41), *H. influenzae* also requires the porphyrin ring of this molecule because this organism lacks the ability to convert δ -aminolevulinic acid to protoporphyrin IX, the immediate biosynthetic precursor of heme (14, 59).

The identities of all of the *H. influenzae* cell envelope components involved in the uptake and transport of heme remain to be determined, but several macromolecules that interact with heme or affect heme utilization by *H. influenzae* have been described over the past 3 years. These include a periplasmic lipoprotein which binds heme and has structural similarity to an *Escherichia coli* dipeptide permease (20, 22), a 39-kDa heme-binding protein that has been reported to be present on the *H. influenzae* cell surface (31), and a recently identified gene encoding a protein with amino acid sequence similarity to the TonB protein of *E. coli* (28). When expression of this TonB homolog was inactivated in both *H. influenzae* type b (Hib) and nontypeable *H. influenzae* (NTHI) strains, the resultant isogenic mutants were unable to utilize heme in any form (28).

H. influenzae possesses the ability to use not only free heme but also heme bound to serum carrier proteins (51). These latter heme:protein complexes are directly relevant to the growth of this organism in vivo, where free heme as such probably does not exist. It has been shown that *H. influenzae* can readily utilize heme:hemopexin, heme:albumin, and hemo-

globin:haptoglobin complexes in vitro to satisfy its requirement for heme (51). In fact, *H. influenzae* receptors for both heme:hemopexin (21) and hemoglobin (26) have been described recently. A 100-kDa protein has been shown to be required for utilization of heme bound to the serum protein hemopexin by Hib strain DL42 (21). Additional studies have shown that this 100-kDa protein is also synthesized by NTHI strains and is released by growing cells of both Hib and NTHI strains into the culture medium (7). More recently, another laboratory has indicated that *H. influenzae* may contain a second hemopexin receptor that functions independently of the 100-kDa protein (60).

In a previous report, it was noted that the cloned Hib DNA fragment that contains the *hxaA* gene encoding the 100-kDa heme:hemopexin-binding protein also expressed a protein with an apparent molecular mass of approximately 60 kDa (21). Linker insertion mutations in the recombinant plasmid pHX1-6 that eliminated expression of this 60-kDa protein also had an adverse effect on the expression of heme:hemopexin-binding activity by these recombinant *E. coli* cells (21). In this report, we describe our finding that two Hib genes located in tandem immediately upstream from *hxaA* encode 60- and 78-kDa proteins, respectively. The 60-kDa HxB protein is apparently involved in the release of the soluble HxA molecule from the Hib cell, whereas expression of the 78-kDa HxC protein is required for Hib to utilize free heme at low concentrations.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The wild-type Hib strain DL42 used in this study has been described previously (9, 17). The various isogenic mutant and recombinant strains derived from Hib strain DL42 during the course of this study are listed in Table 1. All of these strains were grown routinely in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Hib strain		
DL42	Wild type	8
DL42.61	DL42 with a <i>cat</i> cartridge inserted into the <i>hxaA</i> gene, obtained by transforming DL42 with linearized pHX1-61; does not express HxaA and cannot utilize heme:hemoexin as a source of heme	This study
DL42.62	DL42 with a <i>cat</i> cartridge inserted into the <i>hxaB</i> gene, obtained by transforming DL42 with linearized pHX1-62; does not express either HxaB or HxaA and cannot utilize heme:hemoexin as a source of heme for growth	This study
DL42.63	DL42 with a <i>cat</i> cartridge inserted into the <i>hxaC</i> gene, obtained by transforming DL42 with linearized pHX1-63; does not express HxaC and expresses barely detectable levels of HxaB and HxaA; cannot use low levels of free heme for growth and also cannot utilize heme:hemoexin as a source of heme	This study
DL42.61.T	Chloramphenicol-sensitive transformant obtained by transforming DL42.61 with a <i>Bam</i> HI digest of pHX1-60; exhibits the heme utilization phenotype of the wild-type strain	This study
DL42.62.T	Chloramphenicol-sensitive transformant obtained by transforming DL42.62 with a <i>Bam</i> HI digest of pHX1-60; exhibits the heme utilization phenotype of the wild-type strain	This study
DL42.63.T	Chloramphenicol-sensitive transformant obtained by transforming DL42.63 with a <i>Bam</i> HI digest of pHX1-60; exhibits the heme utilization phenotype of the wild-type strain	This study
DL42(pGJB103)	DL42 containing the shuttle vector pGJB103	This study
DL42.63(pGJB103)	DL42.63 containing pGJB103	This study
DL42.63(pHX1-64)	DL42.63 with the recombinant plasmid pHX1-64 containing the wild-type <i>hxaC</i> gene; can grow on low levels of free heme but cannot utilize heme:hemoexin	This study
DL42(pSET200)	DL42 with the recombinant plasmid pSET200 containing the NTHI <i>hxaA</i> gene	This study
DL42.61(pSET200)	DL42.61 with pSET200 containing the NTHI <i>hxaA</i> gene; can utilize heme:hemoexin	This study
DL42.62(pSET200)	DL42.62 containing pSET200; cannot utilize heme:hemoexin	This study
DL42.63(pSET200)	DL42.63 containing pSET200; cannot utilize heme:hemoexin	This study
Plasmids		
pGJB103	Shuttle vector capable of replication in both <i>E. coli</i> and <i>H. influenzae</i> , Amp ^r Tet ^r	2
pBR322	Cloning vector, Amp ^r Tet ^r	45
pBluescript II	Cloning vector, Amp ^r	45
pHX1-6	pBR322 containing a 13.6-kb <i>Pst</i> I fragment of Hib DL42 chromosomal DNA. This insert contains the <i>hxaA</i> , <i>hxaB</i> , and <i>hxaC</i> genes	21
pHX1-60	pBluescript II SK+ with a 10.5-kb <i>Pst</i> I- <i>Xba</i> I fragment from pHX1-6 containing the <i>hxaA</i> , <i>hxaB</i> , and <i>hxaC</i> genes	This study
pHX1-61	pHX1-6 with a <i>cat</i> cartridge inserted into a <i>Not</i> I linker within the <i>hxaA</i> gene	This study
pHX1-62	pHX1-60 with a <i>cat</i> cartridge inserted into the <i>Mlu</i> I site within the <i>hxaB</i> gene	This study
pHX1-63	pHX1-6 with a <i>cat</i> cartridge inserted into a <i>Not</i> I linker within the <i>hxaC</i> gene	This study
pHX1-64	pGJB103 containing the <i>hxaC</i> gene from DL42. A 2.9-kb <i>Alw</i> NI fragment from pHX1-60 containing the DL42 <i>hxaC</i> gene was excised and, after the addition of <i>Pst</i> I linkers, ligated into pGJB103	This study
pSET200	pGJB103 with an 11-kb insert from NTHI strain N182 that contains a functional NTHI <i>hxaA</i> gene. This insert contains only the terminal one-third of the NTHI <i>hxaB</i> gene and does not contain the NTHI <i>hxaC</i> gene. There is no native promoter preceding the <i>hxaA</i> gene in this insert	7

Levinthal's base (BHIs) (1) and with tetracycline (5 µg/ml) or chloramphenicol (2 µg/ml) as necessary. For preparation of outer membrane vesicles and culture supernatants, *H. influenzae* strains and mutants were grown in BHI broth containing NAD (10 µg/ml) and hemin (10 µg/ml), with antimicrobial supplementation as necessary. Testing of the ability of *H. influenzae* strains and mutants to utilize various heme sources was accomplished by using BHI agar plates containing NAD (10 µg/ml) and the heme source at various concentrations, again with antimicrobial supplementation when necessary. Broth cultures were grown at 37°C with aeration; agar plates were incubated at this same temperature in an atmosphere of 5% CO₂-95% air. The *E. coli* cloning host HB101 was grown in LB medium (34) with appropriate antimicrobial supplementation.

Plasmids. Plasmid pBluescript II SK+ was obtained from Stratagene (La Jolla, Calif.). The plasmid shuttle vector pGJB103 (2, 54) was obtained from Gerard J. Barcak. The recombinant plasmid pHX1-6 possesses a 13.6-kb insert of Hib strain DL42 chromosomal DNA that contains the *hxaA* gene encoding the 100-kDa heme:hemoexin-binding protein (21). The recombinant plasmid pSET200 has an 11-kb insert of NTHI strain N182 chromosomal DNA that contains the gene encoding the 100 kDa heme:hemoexin-binding HxaA protein of this NTHI strain (7). This intact NTHI *hxaA* gene is preceded by a 0.8-kb region that represents the 3' half of the NTHI *hxaB* gene and which terminates at the *Pst*I cloning site in the vector; there is apparently no promoter in the chromosomal DNA preceding the NTHI *hxaA* gene in pSET200.

Recombinant DNA methods. Standard recombinant DNA methods including restriction enzyme digestions, ligation reactions, agarose gel electrophoresis, and plasmid purifications were performed as described previously (34, 45). Restriction enzymes and DNA polymerase Klenow fragment were purchased from New England Biolabs (Beverly, Mass.), as were *Not*I and *Pst*I linkers. T4 DNA ligase was purchased from GIBCO-BRL (Bethesda, Md.). Random linker insertion

mutagenesis was performed as described previously (20) with 8-bp *Not*I linkers (5'-GCGGCCGC-3'). The *cat* gene encoding chloramphenicol acetyltransferase and lacking an internal *Eco*RI site was derived from plasmid pUC4DEcat, which was kindly provided by Bruce A. Green. After excision from pUC4DEcat with *Eco*RI, *Not*I linkers were added to the *cat* cartridge (19).

***H. influenzae* genetic transformation methods.** The glycerol-lactate shock method (52) was used to transform Hib strain DL42 with the shuttle vector pGJB103 and recombinant plasmids derived from this vector. Transformants were selected on BHIs agar plates containing tetracycline (5 µg/ml). For transformation of Hib with linear DNA molecules, the M_{IV}-based method of Herriott and colleagues (25) was used.

Preparation of culture supernatants for detection of heme:hemoexin-binding activity. Logarithmic-phase cultures (25 ml) of *H. influenzae* grown in BHI-NAD medium containing heme and *E. coli* grown in LB medium were harvested by centrifugation at 12,000 × *g* for 10 min. The supernatant from this first centrifugation step was decanted and then centrifuged at 200,000 × *g* for 1 h. The final supernatant was drawn off without disturbing the membrane pellet and was concentrated 40-fold by the use of a Centriprep concentrator (Amicon Inc., Beverly, Mass.). The concentrated supernatant was shown to be free of contaminating outer membrane blebs or fragments by Western blot (immunoblot) analysis using a monoclonal antibody (MAb 9F5) reactive with the P2 major outer membrane protein of Hib (6). The concentrated supernatant was then precipitated with 10% (vol/vol) trichloroacetic acid, washed in acetone, and suspended in 50 µl of digestion buffer (15). After incubation at 37°C for 15 min, this material was stored at -20°C until used.

Detection of heme:hemoexin-binding activity. Purified heme:human hemoexin (21) was radioiodinated to a specific activity of 40 µCi/µg of protein by

using Iodo-Beads (Pierce Chemical Co., Rockford, Ill.) and a protocol provided by the manufacturer.

(i) Detection of heme:hemoexin-binding activity expressed by bacterial cells was accomplished by a colony blot method in which bacterial colony paste was spotted onto a Whatman no. 40 filter paper with an applicator stick and dried at 37°C for 1 h. The filter was then incubated for 1 h with gentle agitation in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (Sigma Chemical Co., St. Louis, Mo.) and 0.1% (wt/vol) bovine serum albumin (Sigma) (PBS-T-BSA). Next, the filter was incubated in PBS-T-BSA containing 0.5 µCi of radioiodinated heme:hemoexin overnight at 4°C. The filter then was washed three times (15 min each) in PBS-T-BSA and then dried and processed for autoradiography.

(ii) Detection of heme:hemoexin-binding activity in culture supernatant was accomplished by use of a direct binding assay that was a modification of a Western blot method (21). Briefly, quantities of culture supernatant equivalent to 0.5 ml of the original culture were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (15) except that a Mini-Protein II system (Bio-Rad, Hercules, Calif.) with a 10% (wt/vol) polyacrylamide separating gel was used. The gel-resolved proteins were electrophoretically transferred to nitrocellulose by using a Mini-Protein II Trans-Blot system (Bio-Rad). The nitrocellulose membrane was then incubated in PBS-T-BSA for 1 h at 4°C, after which it was transferred to PBS-T-BSA containing 1 µCi of radioiodinated heme:hemoexin. After overnight incubation at 4°C, the membrane was washed twice (for 15 min each time) in PBS-T-BSA, dried, and processed for autoradiography.

MAbs. MAb 4C11 is directed against the 100-kDa heme:hemoexin-binding HxuA protein from Hib strain DL42 and has been described elsewhere (7). MAb 2B7 was shown to be reactive with the 60-kDa HxuB protein. To obtain this MAb, mice were immunized with outer membrane vesicles prepared from NTHI strain TN106 cells. Spleen cells from these immunized mice were fused with SP2/0-Ag14 plasmacytoma cells, and lymphocyte hybridomas were selected as described previously (44). Culture supernatants from these lymphocyte hybridomas were screened in Western blot analysis using TN106 outer membrane vesicles as the antigen. MAb 2B7 was identified as an immunoglobulin G1 MAb that reacted with an antigen with an apparent molecular mass of approximately 60 kDa in SDS-PAGE. The reactivity of this MAb with the 60-kDa HxuB protein was confirmed by the use of this MAb in Western blot analysis against whole cell lysates from the recombinant strain *E. coli* RR1(pHX1-6), which expresses the Hib 60-kDa HxuB protein.

N-terminal amino acid sequence analysis. Outer membrane vesicles of the wild-type Hib strain DL42 were solubilized and subjected to SDS-PAGE as described previously (29). The 60-kDa HxuB protein band was excised from these gels and then subjected to SDS-PAGE by the method of Hunkapiller et al. (27). The gel-resolved protein band was transferred to a polyvinylidene difluoride membrane by the method of Matsudaira (35) and subjected to N-terminal amino acid analysis as described previously (18).

Western blot and colony blot analyses. Detection of the reactivity of murine MAbs with *H. influenzae* antigens in Western blot analysis was accomplished as described previously (29). Detection of the binding of radioiodinated MAbs to bacterial colonies was performed as described elsewhere (15).

Nucleotide sequence analysis. *H. influenzae* DNA inserts contained in pBlue-script II SK+ were sequenced by standard techniques, including the use of nested deletions (45). In all cases, both strands were sequenced in their entirety. DNA sequence information was analyzed by using the Intelligenetics Suite package and programs from the University of Wisconsin Genetics Computer Group software sequence analysis package (11).

Southern blot analysis. Chromosomal DNA purified from wild-type, mutant, and transformant *H. influenzae* strains was digested to completion with appropriate restriction enzymes and probed in Southern blot analysis by using a nonradioactive enhanced chemiluminescence random prime labeling and detection system (Amersham, Arlington Heights, Ill.) as described by the manufacturer. The DNA probes used in these experiments included the 1.2-kb *cat* gene from pUC4DEcat and several derived from Hib strain DL42: a 1.2-kb *SpeI*-*HindIII* fragment representing the 5' half of the *hxC* gene, a 1.4-kb *AlwNI* fragment containing nearly all of the *hxB* gene, and a 2.2-kb *PstI*-*PvuII* fragment comprising the majority of the *hxA* gene.

Nucleotide sequence accession number. The nucleotide sequences of the *hxC* and *hxB* genes have been deposited at GenBank and assigned accession number U09840.

RESULTS

Identification of genes located upstream from *hxA*. A 10.5-kb *PstI*-*XbaI* fragment derived from the 13.6-kb *PstI* fragment in pHX1-6 (21) was subcloned into pBluescript II SK+, yielding the recombinant plasmid pHX1-60 (Fig. 1). Nucleotide sequence analysis of a 5-kb region located immediately upstream from the Hib strain DL42 *hxA* gene (7) in this plasmid revealed the presence of two open reading frames (ORFs) arranged in tandem. These encoded proteins with

predicted molecular masses of approximately 62 and 80 kDa, and these genes were tentatively designated as *hxB* and *hxC*, respectively (Fig. 1). In contrast, sequence analysis of the 500-bp region immediately downstream from *hxA* did not detect any other ORFs.

Features of the *hxC* sequence. The likely translational start site for this ORF is located at nucleotides 112 to 114 (Fig. 2). This ATG codon is located 7 nucleotides downstream from a sequence (5'-AGGA-3') with homology to ribosomal binding sites (48). Putative -35 and -10 consensus sequences were also identified upstream from the start codon (Fig. 2). A 19-nucleotide sequence (5'-GATTATTATTGTAATCATC-3') with weak similarity (13 of 19 matches) to the Fur binding site consensus sequence (32) was also located upstream from the start codon (Fig. 2). Sequence analysis of the 500-bp region immediately upstream from *hxC* did not reveal any additional ORFs.

Features of HxuC. The ORF initiated at nucleotide 112 began with a hydrophobic amino acid sequence similar to those of leader peptides (49, 56). Two possible signal peptidase I cleavage sites (i.e., A-X-A) were identified at amino acid residues 17 to 19 and 19 to 21 (Fig. 2). The HxuC precursor had a calculated molecular weight of 80,843; after processing, the calculated molecular weight of the mature HxuC protein would be either 78,852 or 78,668, depending on which of the two possible signal peptidase I cleavage sites is actually used in the processing of this macromolecule. The HxuC protein has a phenylalanine residue as its C-terminal amino acid; this finding is consistent with HxuC being present in the Hib outer membrane (described below) (53).

When the amino acid sequence of HxuC was compared with those of other proteins in the available databases, HxuC was found to be similar to the class of outer membrane proteins which are described as being TonB dependent (16, 43). HxuC was found to possess the classic TonB box near its N terminus as well as the other six regions that are common to this type of outer membrane protein (Fig. 3), which is typically involved in the binding and transport of certain low-molecular-weight ligands (3, 33, 43).

Features of the *hxB* gene. Two possible translational start sites for this ORF were located 74 and 81 nucleotides, respectively, downstream from the termination codon for *hxC* (Fig. 2). Possible -35 and -10 consensus sequences were also located in appropriate positions 5' from these two closely grouped ATG codons, while two possible ribosomal binding sites (5'-AGGA-3') were identified immediately upstream from these two ATG codons (Fig. 2). A 20-nucleotide sequence (5'-CTTTGAAAACGCCCTAAAG-3') with weak similarity to the Fur box consensus sequence was also identified in the same region as the possible -35 consensus sequences. The start codon for the *hxB* gene was located 12 nucleotides downstream from the stop codon for the *hxC* gene (Fig. 2).

Features of HxuB. The ORF beginning at nucleotide 2364 (Fig. 2) encoded a protein with a calculated molecular weight of 62,664. Inspection of the deduced amino acid sequence at the N-terminal end of this protein revealed that it was mainly hydrophobic and resembled a leader peptide. A putative signal peptidase I cleavage site (i.e., V-M-A) was located at amino acid residues 24 to 26, such that the predicted molecular weight of the mature HxuB protein was 59,832. N-terminal amino acid sequence analysis of gel-purified HxuB protein from Hib strain DL42 outer membranes confirmed the existence of the proposed leader peptide (Fig. 2). The carboxyl-terminal residue of the HxuB polypeptide was phenylalanine, a finding consistent with the presence of HxuB in the Hib outer

FIG. 1. Partial restriction enzyme maps of the DNA inserts in the recombinant plasmids used in this study. Plasmids pHX1-61 and pHX1-63 were derived from the pBR322-based recombinant plasmid pHX1-6 (21), whereas pHX1-60 and pHX1-62 are pBluescript II SK+ based plasmids. Plasmid pHX1-64 is a pGJB103-based recombinant. The extents of the ORFs for the *hxC*, *hxB*, and *hxA* genes are depicted by the boxes beneath pHX1-60. The insertion sites for the *cat* cartridges in pHX1-61, pHX1-62, and pHX1-63 are indicated for each construct. The pairs of diagonal marks on the right side of the pHX1-61 and pHX1-63 maps indicate that these insert maps were truncated for the purpose of clarity.

membrane (described below) (53). When the available protein databases were searched, the protein with the greatest similarity to HxB (i.e., 25% identity) was the ShIB protein of *Serratia marcescens*. This 62-kDa outer membrane protein is required for secretion and activation of the ShIA hemolysin of *S. marcescens* (4, 42, 46).

Construction of *hxC*, *hxB*, and *hxA* mutants. The close proximity of the *hxB* and *hxC* genes to the *hxA* gene encoding the 100-kDa heme:hemoexin-binding protein raised the possibility that these two upstream ORFs express proteins involved in some aspect of heme utilization. To test this hypothesis directly, isogenic *hxB* and *hxC* mutants were constructed by means of insertion mutagenesis and allelic exchange. In addition, a new *hxA* mutant was constructed for comparison purposes.

***hxA* mutant.** In a previous study, *NotI* linker insertion mutagenesis was used to produce a mutant, designated DL42.107d, that was unable to utilize heme:hemoexin as a source of heme for growth (21). More recently, nucleotide sequence analysis proved that this particular linker insertion was located within the *hxA* gene in the recombinant plasmid pHX1-6 (7). A *cat* cartridge with *NotI* ends was ligated into this

site, yielding the mutated plasmid pHX1-61 (Fig. 1). This plasmid was linearized by digestion with *Bam*HI and used to transform the wild-type Hib strain DL42. Chloramphenicol-resistant transformants were screened for the ability to express the HxA protein by using the HxA-specific MAb 4C11 as a probe in the colony blot-radioimmunoassay. Transformants unreactive with this MAb also were shown to be unable to bind heme:hemoexin in the colony blot system, and one of these, designated DL42.61, was selected for further study. Southern blot analysis confirmed that proper allelic exchange, involving the replacement of the wild-type *hxA* gene with the mutated *hxA* gene, had occurred in DL42.61 (Fig. 4e and f).

***hxB* mutant.** To inactivate the *hxB* gene, the recombinant plasmid pHX1-60 (Fig. 1) was digested to completion with *Mlu*I and then the ends were filled in by treatment with DNA polymerase I Klenow fragment. *Bgl*II linkers were then added to the linearized molecule, and a *cat* cartridge with *Bam*HI ends was ligated onto these *Bgl*II ends, yielding the mutated plasmid pHX1-62 (Fig. 1). This plasmid was linearized by digestion with *Bam*HI and used to transform the wild-type Hib strain DL42. Transformants were selected for chloramphenicol resistance, and whole cell lysates of several of these were

		-35	-35	-10	-10	SD	
1	TTTACGCAATTAATAAATGCAAAATGATTATAAATAAACCAATGATTATATGTAATCATCGTTATTATTATCGCCATTCTCTCTTTTAAATTATTACAATAAGGAAAAACAG						111
112	ATG CGA TTT TCT AAA CTT TCC CTT GCA ATT GCA ACA ACC TTA GTG ACA GCA AAT GCG CTA GCG CAA TCC GTT GAA TTA GAC TCT						195
196	ATC AAC GTT ATT GCG ACA CGA GAT CCA AGT AGG TTT GCT TAT ACG CCA GAA AAA CAA TCT AAA GAT AGT CTT TCC AAG CAA						279
280	GCC ACT AGT GTT GCA GCA GCG TTA GAA GAC ATT CCC AAT GTT GAT ATT AGA GCG GGT TCA AGA AGC ATT GCT CAA AAA CCT AAT						363
364	ATT CGA GGG TTA AGT GAT AAT CGT GTT GTG CAA GTC ATT GAT GCG GTG AGA CAA AAT TTT GAT TTA GCA CAT AGA GGT TCT TAT						447
448	TTT CTT CCA ATG TCA CTC ATC CAA GAA ATT GAA GTA ATC AAA GGA CCA AGT AGC TCC TTA TGG GGT AGC GGT GCA ATG GGT GGT						531
532	GTG GTG GCA ATG CGT ACG CCA AAT GCT TTG GAC TTA TTG AAA AAT AAT GAC AAA TTC GGA GTT AAA ATT CGC CAA GGT TAT CAA						615
616	ACT GCT AAT AAT TTA TCG GAA AGG GAT GTT TCT GTA TTT GCG GCA AAT GAC AAA TTC GAT GTT CTT ATT AGT GGT TTC TAT AAT						699
700	AAT GCG GAT AAT TTA GCG ACT GGT AAA GGC AAT AAG CTG AAT AAT ACC GCG TAT AAA CAG TTT GGG GCG TTA GCA AAA TTC GGT						783
784	TGG CAA ATT AAT GAT GCT AAC GCG GTG GAA TTA TCC CAC GCG GAA ACT CGT TTT AAA CAA ACA GCA CCA AGC AAT AAT GAG GTG						867
868	GAA AAC GAA CTT ACC AAT GAA CAA ATT ACA GAT CAA ATC AGA GAG TTC CAC AAA CCA AAC AAC GGT TCT CCA CCG AAA GCC AAA						951
952	CCA TCA CAA GAA GAG TTT TAC TCT GCG GTG AAA ACA CGT TTT GGT AGT GTC AGT TAT TTA ACT GAT CAA CAA AIT CCT GAT CAA						1035
1036	AGC ACG GTA TTT AAC TAT TAT TTA ACG CCA GAT AAT CCT TAT CTA AAT ACG CAT ATC GCA CTG TAT AAC AAT AAA ACT ATT GAG						1119
1120	AAA GAA CAG CGT AAA GTC AGT GGT GTG AAA GAT CAG ACT AAA TTG ACT ACC CGA GGT ATA AAT TTA CGT AAT TCT TCC GAA TTA						1203
1204	TCT CAC ATT TCC TTT GTT TAT GGG GTG GAT TAT ATG CGA GAT AAA ACT CGT ACC GAA CCA GCG ACA AAC AAT AAA GAT GCG CAG						1287
1288	TTT CGA GCG GAC CCC TAT AAT GCG AAT TCA AAC ACT ACA GGC GTT TAT TTA ATC GCC CAT AIT CCG CTA TTT GGG GAA AAA TTG						1371
1372	CTA CTT TCG CCA AGT GTA CGT TAT GAC CAC TAC GAT ACC TCA AGT AAA ACT GTA AAA TAC AAG GAT AAT CAT TTA TCT CCT GCC						1455
1456	ACA AAA TTA ACT TGG ATA GTG ACC AAT TGG TTA GAT TTT ACT GCG AAA TAT AAT GAA GCT TTC CGA GCA CCA TCT AAT CCA GAG						1539
1540	CGG TTT GTC AGT GGT TCG CAC TTT GGG ACA AGT ATT CTA GCG GCA AAT GAA ATC AAT AAA TTT GTA GCA AAT CCA AAT TCT CGC						1623
1624	CCT GAA ACA GCG AAA AAT AAA GAA ATT ACC GCA AAT CTA CAT TTT GAT AGT CTG TTT AAA CAA GCG GAT AAA TTC AAT ATT GAA						1707
1708	GCG ACT TAT TTC CGT AAT GAT GTG AAA GAT TTT ATT AAC TTA AAA ATA TTT AAT GAT GCA AAG ACA AAT ACA AAT GAT GCG						1791
1792	AGT GCA GGT GCA GGT GCA GGT GCA AAT CCA AAT GGA GCA TTG TTG CCA ACA AAA TCC CAG TAT CAA AAC ATA ACT AAT GCC CGT						1875
1876	TTA AGC GGT ATT GAA TTG CAA GCT CAA TAC CAA ACA GAA CGT TTA ACA CTG TTT ACT AAC TAT GCG AGC ACC AAA GGT AAA GAT						1959
1960	AAA GAT AGT GCG GAA GCT TTA TCA AAC ATT GCG GCA AGC AAA ATC GCG GTA GGG GTA AAT TAT GCT TTA GTA AAA GAC AAA TTC						2043
2044	ACG GTG GGA GCG ACA GTT ACC CAT TAC GCT GCT CAA GCG GCA GTG CCT AAA GAT CAT AGT GTT ACC TAC CCA AGT TAT ATA CTG						2127
2128	ACC GAT CTT CGT GCT ACC TAT GCA CCA TTA AAA GCG GAA TGG AAA AAC CTG CGT TTA GAT TTT GCG CTG GAA AAC TTG TTT GAT						2211
2212	AGA AAA TAT CAA CCG GCA TTT AGT TTA ATG GAA GGC ACT GGT CGT AAT GCG AAA ATT AGT GCG GTT TAT AGC TTT TAA ATTACT						2296
2297	TTGGAACACCCCTTAAGCTGTTTTCCTCAATTTTTGTAATTTTATCAATTAATAGACATAAGGA						2396
2397	AGT GCG GTC AGT TTA GCG TTT GTT TTG AGT AAA TCA GTG ATG GCA TTA GGT CAG CCA GAT ACT GGA TCA TTG AAC CCG GAA CTA						2480
2481	GAA CAA CGT CAA ATT CAA TCA GAA GCG AAA CCT AGT GCG GAA TTA TTT AAT CAA ACG GCA AAT TCG CCT TAT ACT GCA CAG TAT						2564
2565	AAA CAA GAA CTG AAA TTT CCA CTT ACG CAA GTA CAG ATT TTA GAT CGA AAC AAT CAA GAA GTC GTC ACA GAC GAG CTT GCA YAT						2648
2649	ATT TTA AAA AAT TAT GTA GGA AAA GAG GTA TCG CTG TCA GAT TTA AGT AAT TTA GAT AAT GAG ATC AGC GAA TTT TAT CGT CAT						2732
2733	AAT AAT TAT TTA GTG GCA AAA GCG ATT TTA CCA CCG CAG GAA ATT GAG CAA GGT ACG GTA AAA ATT CTG TTA CTT AAA GGT AAT						2816
2817	GTG GCG GAA ATA CCG TTG CAA AAT CAC AGC GCA TTA AGC AAT AAA TTT GTA TCC CGT CTC TCT AAT ACA ACA GTA AAT ACG TCC						2900
2901	GAA TTT ATT TTG AAA GAT GAG TTA GAA AAA TTT GCT TTA ACA ATA AAT GAT GTT CCA GCG GTA AAT GCT GGT TTG CAA CTC TCC						2984
2985	GCA GCG AAA AAA GTC GCG GAA GCG AAT TTA CTG ATT AAG ATT AAC GAT GCA AAA CGT TTT TCT AGT TAT GTT TCA GTA GAT AAC						3068
3067	CAA GCG AAC AAA TAT ACG GCG GGT TAC CGT TTG GCT GCG GCG GCA AAA GTC AGT AAT TTG AAT GCG TGG GCG GAT GAA TTG AAA						3152
3153	CTG GAT TTA ATG AGT TCT AAT CAG GCT AAT TTA AAA AAT GCT CGT ATA GAT TAT TCC TCT TTA ATT GAT GGT TAT TCT ACG CGT						3236
3237	TTT GGT GTA ACT GCT AAC TAT CTC GAC TAC AAA TTA GGT GGT AAT TTT AAA TCA TTA CAA TCT CAA GGG CAT TCC CAT ACC TTA						3320
3321	GGG GCT TAT TTG TTG CAC CCA ACA ATC CGT ACA CCA AAT TTT CGT TTA AGT ACT AAA GTG AGC TTT AAT CAT CAA AAC CTT ACG						3404
3405	GAT AAA CAA CAA GCG GTT TAT GTA AAA CAA AAA CCG AAA ATA AAT GAT TTA ACT GCA GGG ATT GAC GGT TCT TGG AAT CTT ATT						3488
3489	AAA GAT GGG ACA ACT TAT TTT TCA TTA TCT ACC TTG TTG GGT AAT TTA GCG AAT CAA ACT AGT GAA AAA AAA CAT AAT GCA GTA						3572
3573	GAG AAT TTT CAA CCA AAA TCG CAT TTT ACG GTA TAT AAC TAC GCG TTG TCT CAC GAG CAA ATT TTA CCA AAA TCT TTT GCT TTT						3656
3657	AAT ATT GGT ATA AAC GGT CAG TTT GCG GAT AAA ACC CTT GAA AGC TCA CAG AAA ATG TTG CTT GGT GGT TTG TCT GGA GTA CGT						3740
3741	GGA CAT CAA GCT GGT GCG GCT TCT GTG GAT GAA GGG CAT TTG ATA CAA GAA GAA TTC AAA CAT TAT TTA CCA GTC TTT TCC CAA						3824
3825	AGT GTG CTA GTT AGT TCA TTG TTC TAT GAC TAT GGT TTG GGA AAA TAT TAT AAA AAT AGC CAA TTT CTC GAA CAA GCG GTG AAA						3908
3909	AAT AGC GTG AAA TTA CAA AGC GTT GGT GCA GGA CTT TCT CTT TCA GAT GCT GCG AGC TAT GCC ATC AAT GTC AGT GTC GCC AAA						3992
3993	CCG CTT GAT AAC AAT ATT AAT AAT GCG GAT AAA CAC CAA TTT TGG CTT TCT ATG ATT AAA ACT TTC TAA GGAGGTATGCT ATG TAT						4078
4079	AAA TLA AAT GTA ATT TCT CTT ATC ATT TTG ACC ACT TGC AGT GGT GCG GCA TAT GCC TCT ACA CCG GAT TTT CCA CAA CAT CAT						4162

FIG. 2. Nucleotide sequences of the *hxc* and *hxb* genes of Hib strain DL42, together with the deduced amino acid sequences of the HxCu and HxBu proteins. Putative -35 and -10 regions are indicated by underlining, as are the putative ribosomal binding sites (SD). The two nucleotide sequences that have some weak similarity to the Fur box consensus sequence are indicated by shading. The beginning of the *hxcA* gene, which starts at nucleotide 4073, is included at the end of the sequence, together with the deduced amino acid sequence. The putative leader peptide for the HxCu protein is underlined up to the first possible signal peptidase I cleavage site at amino acid residue 19; double underlining continues on to residue 21, the second possible signal peptidase I cleavage site. The leader peptide of the HxBu protein (confirmed by N-terminal amino acid sequence analysis of the mature HxBu protein) is underlined.

1		2	
HxuC 6	DSINVIATRDPSR	HxuC 46	IPNVDIRGGS RSIAQLPNIRGLSDNRVQVIDGVFRN
HemR 16	DTMVVTATGNERS	HemR 59	IPGLTVTGSG RVNGQDVTLRGYKQGVLTLDGIRQG
BtuB 6	DTLVVTANRFEQP	BtuB 49	LPGVDITQNGSGQLSSIFIRGTNASHVLVLIDGVRIN
Cir 6	ETMVTASSVEQN	Cir 49	VPGVQLTNEG DNRKGVSIIRGLDSSYTLILVDGKRVN
3		4	
HxuC 100	EIEVIKGPSSSLWCSGALGGVAMRTPN	HxuC 482	LRPETAKNKEITAN
HemR 113	RVEIVRGPSALLYSGALGGVSYETVD	HemR 451	LKPFETNETQYEGFG
BtuB 106	RVEYIRGPRSAVYGSDAIGGVNIITTR	BtuB 410	LDPEKSKQWE GAF
Cir 107	RIEVVIRGPMSSLYGSDALGGVNIITKK	Cir 422	LKPFETSESWEGLY
5		6	
HxuC 563	TNARLSGIE	HxuC 670	LDFALENLFDRKY
HemR 520	DRAKIWGWD	HemR 624	TTVVLGNADFDRGY
BtuB 457	GKARIKIE	BtuB 561	VRGKIANLFDKDY
Cir 499	NKARNQGE	Cir 604	LRAGVLNLGDKDL
7			
HxuC 693	GRNAKISAVYSF		
HemR 646	GRNAKFFVSYQW		
BtuB 583	GREYTLGSGSYTF		
Cir 627	GRRYFMAVDYRF		

FIG. 3. Peptide alignments between TonB-dependent outer membrane proteins of other organisms and HxuC. The other TonB-dependent proteins include HemR (*Y. enterocolitica* heme-binding protein [50]), BtuB (*E. coli* vitamin B₁₂ receptor [23]), and Cir (*E. coli* colicin I receptor [37]). Shading indicates residues that are similar or identical in at least three of the four sequences.

shown to lack reactivity with the HxuB-specific MAb 2B7 in Western blot analysis. One of these was selected for further study and designated DL42.62 (Table 1). Southern blot analysis was used to confirm that proper allelic exchange had occurred in DL42.62 (Fig. 4c and d).

***hxc* mutant.** A *NotI* linker insertion in the recombinant plasmid pHX1-6, originally characterized as preventing expression of heme:hemoexin-binding activity by the recombinant *E. coli* strain containing this mutated plasmid (21), was localized to the 5' half of the *hxc* gene (Fig. 1). A *cat* cartridge with *NotI* ends was ligated into this site, yielding the mutated plasmid pHX1-63, which was digested with *Bam*HI and used to transform the Hib wild-type strain DL42. Transformants were selected for resistance to chloramphenicol and screened for an inability to bind heme:hemoexin. One of these transformants, designated DL42.63, was selected for further study. Southern blot analysis was used to confirm that allelic exchange had occurred properly in DL42.63 (Fig. 4a and b).

Expression of the HxuA, HxuB, and HxuC proteins by the wild-type and mutant strains. Expression of HxuA was detected by use of the direct heme:hemoexin-binding assay in which culture supernatants were processed by SDS-PAGE,

transferred to nitrocellulose, and incubated with radioiodinated heme:hemoexin (Fig. 5). Expression of HxuB and HxuC was detected by Coomassie blue staining of proteins present in outer membrane vesicles, as resolved by SDS-PAGE (Fig. 6a). HxuB was also identified by Western blot analysis using the HxuB-specific MAb 2B7 (Fig. 6b).

The wild-type parent strain DL42 expressed the HxuA protein (Fig. 5a, lane A) as well as both HxuB (Fig. 6a and b, lane A) and HxuC (Fig. 6a, lane A). Insertion of the *cat* cartridge into the *hxc* gene in the mutant strain DL42.63 eliminated detectable expression of HxuC (Fig. 6a, lane B). Barely detectable amounts of HxuB (Fig. 6a and b, lane B) and HxuA (Fig. 5a, lane B) were expressed by this *hxc* mutant. The *hxc* mutant strain DL42.62 could still express wild-type levels of the HxuC protein (Fig. 6a, lane D) but could express neither the HxuB molecule (Fig. 6a and b, lane D) nor HxuA (Fig. 5a, lane C). Finally, the *hxc* mutant DL42.61 expressed both HxuC (Fig. 6a, lane F) and HxuB (Fig. 6a and b, lane F) but did not express HxuA (Fig. 5a, lane D).

FIG. 4. Southern blot analysis of insertion mutations in the Hib *hxc*, *hxb*, and *hxa* mutants. Chromosomal DNA from the wild-type Hib strain DL42 (lanes A), the respective mutant with a *cat* cartridge inserted into the *hxc*, *hxb*, or *hxa* genes in the bacterial chromosome (lanes B), and a chloramphenicol-sensitive transformant derived from each of these three mutants by transformation with linearized pHX1-60 (lanes C) were digested with various enzymes and then probed with either a fragment of the respective structural gene or the *cat* cartridge. (a and b) chromosomal DNA from DL42, DL42.63, and DL42.63.T digested with *Pst*I and probed with either a 1.4-kb *Alw*NI fragment representing the 5' half of *hxc* (a) or with the *cat* cartridge (b). (c and d) chromosomal DNA from DL42, DL42.62, and DL42.62.T digested with *Pst*I and probed with either a 1.4-kb *Alw*NI fragment containing nearly all of the *hxb* gene (c) or the *cat* cartridge (d). (e and f) chromosomal DNA from DL42, DL42.61, and DL42.61.T digested with both *Pst*I and *Eco*RV and probed with either a 2.2-kb *Pst*I-*Pvu*II fragment containing most of the *hxa* gene (e) or the *cat* cartridge (f). Size markers are shown at the left.

FIG. 5. Release of HxuA into the culture supernatant by wild-type, mutant, and recombinant Hib strains as revealed by detection of heme:hemoexin-binding activity. Proteins in culture supernatants were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with radioiodinated heme:hemoexin prior to processing for autoradiography. Lanes: A, wild-type DL42; B, the *hxc* mutant DL42.63; C, the *hxb* mutant DL42.62; D, the *hxa* mutant DL42.61. In panel a, each strain lacks the recombinant plasmid pSET200 containing the NTHI *hxc* gene. In panel b, each strain is a recombinant containing pSET200. The arrows indicate the position of the HxuA protein in each panel. Molecular weight position markers are shown at the right.

FIG. 6. Detection of HxuB and HxuA proteins in outer membrane vesicles of wild-type, mutant, and transformant Hib strains. Proteins present in outer membrane vesicles were resolved by SDS-PAGE and either stained with Coomassie blue (panel a) or transferred to nitrocellulose and probed in Western blot analysis with the HxuB-specific MAb 2B7 (panel b). Lanes: A, wild-type strain DL42; B, the *hxcA* mutant DL42.63; C, the transformant DL42.63.T; D, the *hxcB* mutant DL42.62; E, the transformant DL42.62.T; F, the *hxcA* mutant DL42.61; G, the transformant DL42.61.T. Only the upper half of the gel is shown in panel a, and only the region of the nitrocellulose pad containing the HxuB protein is shown in panel b. The arrow indicates the position of the 60-kDa HxuB protein in the wild-type strain DL42. Similarly, the triangle indicates the position of the 78-kDa HxuA protein in the wild-type strain. Molecular weight position markers are shown at the left.

Utilization of heme and heme:carrier protein complexes by the wild-type and mutant strains. The wild-type parent strain DL42 was able to utilize free heme as well as all of the heme:protein complexes tested (Table 2). The *hxcA* mutant DL42.61 was able to utilize free heme and all of the other heme compounds except heme:hemopectin (Table 2), as would be expected from the previous demonstration that expression of the HxuA protein is essential for utilization by Hib strain DL42 of heme bound to hemopectin (21). The heme utilization phenotype of the *hxcB* mutant strain DL42.62 was identical to that of the *hxcA* mutant (Table 2); the inability of this *hxcB* mutant to utilize heme bound to hemopectin correlated with the lack of expression of HxuA by this mutant (Fig. 5a, lane C).

The *hxcC* mutant DL42.63 was unable to utilize heme:hemopectin (Table 2). However, the *hxcC* mutant was also unable to utilize free heme (10 μ g/ml) for growth on a solidified medium (Table 2). This mutant could still utilize both hemoglobin and hemoglobin:haptoglobin (Table 2). More extensive testing revealed that the *hxcC* mutant formed single colonies

FIG. 7. Overexpression of the HxuB protein by a recombinant Hib strain. Outer membrane vesicles from the wild-type Hib strain DL42 (lane A), the recombinant strain DL42.63(pHX1-64) (lane B), and the recombinant strain DL42.63(pGJB103) (lane C) were resolved by SDS-PAGE and stained with Coomassie blue. Only the relevant top half of the separating gel is shown. The arrow indicates the position of the 78-kDa HxuB protein, and the square marks the position of the 60-kDa HxuB protein in lane A. Note the apparent absence of the HxuB protein in lanes B and C. Molecular weight position markers are shown at the left.

when heme was provided at a concentration of 50 μ g/ml. During growth in broth-based medium containing heme at 10 μ g/ml, the wild-type parent strain and the *hxcC* mutant grew at similar rates. However, the mutant was unable to grow when the heme concentration in the broth medium was reduced to 0.1 μ g/ml, whereas the wild-type strain grew readily at this heme concentration (data not shown). Therefore, the *hxcC* mutation adversely affected the ability of strain DL42 to utilize low levels of free heme, in a manner similar to the inability of *H. influenzae tonB* mutants to utilize free heme at low concentrations (28).

To confirm directly the role of the HxuC protein in the utilization of low levels of free heme, a recombinant plasmid which contained only the *hxcC* gene was constructed. To accomplish this, the 2.9-kb *Alw*NI fragment containing the wild-type *hxcC* gene was excised from pHX1-60 and, after the addition of *Pst*I linkers, was ligated into the *Pst*I site within pGJB103, yielding the recombinant plasmid pHX1-64 (Fig. 2). The *hxcC* mutant was transformed with this plasmid, yielding the transformant strain DL42.63(pHX1-64), which greatly overexpressed HxuB (Fig. 7, lane B), by comparison with the levels of HxuB expressed by both the wild-type strain DL42 (Fig. 7, lane A) and the same mutant containing only the

TABLE 2. Utilization of free heme and heme:protein complexes by wild-type and mutant Hib strains

Strain	Growth ^a				
	Heme		Hemoglobin (200 μ g/ml)	Hemoglobin: haptoglobin (9 μ g/ml)	Heme:hemopectin (5 μ g/ml)
	10 μ g/ml	50 μ g/ml			
DL42	+	+	+	+	+
DL42.61 (<i>hxcA</i> mutant)	+	+	+	+	—
DL42.61.T	+	+	+	+	+
DL42.62 (<i>hxcB</i> mutant)	+	+	+	+	—
DL42.62.T	+	+	+	+	+
DL42.63 (<i>hxcC</i> mutant)	—	+	+	+	—
DL42.63.T	+	+	+	+	+
DL42.63(pHX 1-64)	+	+	+	+	—
DL42(pSET200)	+	+	+	+	+
DL42.61(pSET200)	+	+	+	+	+
DL42.62(pSET200)	+	+	+	+	—
DL42.63(pSET200)	—	+	+	+	—

^a As evidenced by development of single colonies on BHI-NAD agar supplemented with the indicated heme compounds.

plasmid vector pGJB103 (Fig. 7, lane C). The presence of the wild-type *hxC* gene in *trans* permitted growth of the transformant strain DL42.63(pHX1-64) on solidified media containing heme at 10 µg/ml (Table 2). The presence of this plasmid did not allow DL42.63(pHX1-64) to utilize heme:hemoexin (Table 2).

Genetic restoration of the abilities to utilize free heme and heme:hemoexin. The recombinant plasmid pHX1-60 containing the wild-type *hxA*, *hxB*, and *hxC* genes was digested with *Bam*HI, yielding a 14-kb fragment containing the entire Hib strain DL42 DNA insert and flanking DNA from the vector. This large DNA fragment was used to transform the chloramphenicol-resistant *hxA*, *hxB*, and *hxC* mutants, and the resultant transformants were all screened for reactivity with the HxA-specific MAb 4C11. Those transformants that bound MAb 4C11 were also tested for sensitivity to chloramphenicol to identify those transformants in which allelic exchange had eliminated the mutated gene containing the *cat* cartridge.

One chloramphenicol-sensitive and MAb 4C11-reactive transformant from each of the three transformation experiments involving the *hxA*, *hxB*, and *hxC* mutants was selected for further testing and designated DL42.61.T, DL42.62.T, and DL42.63.T, respectively. Southern blot analysis confirmed the occurrence of proper allelic exchange in each of these transformants (Fig. 4, lanes C). Each of these transformants also exhibited the wild-type phenotype with regard to outer membrane protein profile (Fig. 6, lanes C, E, and G) and utilization of low levels of free heme (Table 2).

Effect of the *hxB* mutation on release of soluble HxA from Hib. The HxA protein has been shown to be released in soluble form by both Hib and NTHI strains (7). To determine whether either of the two genes immediately upstream from *hxA* was involved in this process, the recombinant plasmid pSET200 containing a wild-type *hxA* gene from a NTHI strain (7) was used to transform Hib strain DL42 and the *hxA*, *hxB*, and *hxC* mutants. It should be noted that the DNA insert in this recombinant plasmid does not contain a NTHI *hxC* gene and contains only the 3' half of the NTHI *hxB* gene immediately preceding the *hxA* gene (Table 1) (7). The resultant transformants were studied for the ability to produce a soluble HxA protein that was released into the culture supernatant.

The presence of pSET200 in the wild-type Hib strain DL42 (Fig. 5b, lane A) resulted in expression of a greater amount of heme:hemoexin-binding activity than expressed by DL42 (Fig. 5a, lane A); this activity represented the two different (i.e., Hib and NTHI) HxA proteins expressed by the recombinant strain. A soluble HxA protein also was detected in the culture supernatant from the recombinant strain DL42.61 (pSET200) (Fig. 5b, lane D), indicating that the NTHI HxA protein was released from this Hib *hxA* mutant carrying the NTHI *hxA* gene in *trans*. The low level of expression of the NTHI HxA protein by DL42.61(pSET200) is likely due to the lack of a native promoter upstream from the NTHI *hxA* gene in pSET200 (Table 1). However, culture supernatant from the recombinant strain DL42.62(pSET200) did not contain the NTHI HxA protein (Fig. 5b, lane C), indicating that expression of the HxB protein was apparently required for release of the NTHI HxA protein into the culture supernatant. Similarly, the recombinant strain DL42.63(pSET200), which expressed barely detectable levels of HxB (Fig. 6, lane B), did not release any detectable NTHI HxA into its culture supernatant (Fig. 5b, lane B).

DISCUSSION

The exact mechanism(s) utilized by *H. influenzae* for the acquisition and transport of heme remains to be identified. The first *H. influenzae* protein clearly shown to be involved in a heme acquisition system was the 100-kDa HxA protein, which is required for utilization of heme bound to hemoexin by Hib strain DL42 (21). The subsequent detection of the HxA protein in Hib culture supernatants led to the finding that most Hib and NTHI strains express a 100-kDa heme:hemoexin-binding protein which is released from the bacterial cell (7). Nucleotide sequence analysis of *hxA* genes from both a Hib strain and an NTHI strain revealed that the two proteins were 87% identical, and this similarity at the amino acid level was reinforced by the finding that a NTHI HxA protein could complement a *hxA* mutation in a Hib strain (7).

Subsequent analyses revealed that utilization not only of heme:hemoexin but also of other heme:protein carrier complexes as well as low concentrations of free heme by both Hib and NTHI strains was dependent on the presence of a functional *tonB* gene (28). From this finding, it could be inferred that there must exist one or more TonB-dependent *H. influenzae* proteins that would be directly involved with heme transport. In support of this hypothesis, recent studies of the utilization of heme as an iron source by *Yersinia enterocolitica* indicated that both TonB and a TonB-dependent outer membrane protein (i.e., HemR) were essential for this process (30, 50).

Nucleotide sequence analysis of the 5-kb region immediately upstream from the Hib DL42 *hxA* gene revealed the presence of two ORFs in tandem (*hxB* and *hxC*) which encoded outer membrane proteins. Moreover, the HxC protein had amino acid sequence homology with outer membrane proteins (from other bacteria) that are considered to be TonB dependent (Fig. 3), including the HemR protein from *Y. enterocolitica* (50). To the best of our knowledge, this is the first description of a possible TonB-dependent outer membrane protein in *H. influenzae*.

To determine directly whether the HxB and HxC proteins were involved in heme utilization, these two genes were mutated by insertion of a *cat* cartridge into their respective ORFs. The mutated genes were introduced independently into the chromosome of the wild-type Hib strain by transformation and subsequent allelic exchange. Comparison of the outer membrane protein profiles of these isogenic *hxB* and *hxC* mutants with that of the wild-type parent strain confirmed that both of these proteins were present in the Hib outer membrane (Fig. 6).

The presence of the *cat* cartridge in the *hxB* gene eliminated expression of detectable levels of both HxB (Fig. 6, lane D) and HxA (Fig. 5a, lane C). In DL42.63, the presence of the *cat* cartridge in the *hxC* gene eliminated expression of HxC (Fig. 6, lane B); however, barely detectable amounts of the HxB (Fig. 6, lane B) and HxA (Fig. 5a, lane B) proteins were synthesized by this *hxC* mutant, a finding which raised the possibility that the *hxB* gene may have its own, independent promoter. The fact that the start codon for the *hxB* gene is located nearly 100 nucleotides downstream from the termination codon for the *hxC* gene, together with the identification of putative -35 and -10 consensus sequences in this intergenic region, lends credence to this hypothesis. Alternatively, the very modest expression of the *hxB* and *hxA* genes in the *hxC* mutant may have resulted from the presence of the *cat* cartridge in the *hxC* gene; the *cat* gene in this cartridge does not have a transcriptional terminator (5).

Mutations in the *hxB* and *hxC* genes had very different

effects on the ability of Hib to utilize heme. The *hxB* mutant, unable to synthesize HxB and HxA, had a heme utilization phenotype indistinguishable from that of a *hxA* mutant. Neither the *hxB* mutant nor the *hxA* mutant could utilize heme bound to hemopexin (Table 2). This finding confirms the previously reported dependence of Hib strain DL42 on the presence of a functional HxA protein for growth on heme:hemopexin as the sole source of heme (21). The *hxC* mutant, unable to express HxC while synthesizing barely detectable amounts of HxB and HxA, was also unable to utilize heme:hemopexin (Table 2). However, the *hxC* mutant also could not grow on a solidified medium containing low levels (10 µg/ml) of free heme. When a functional *hxC* gene was supplied in *trans*, the *hxC* mutant was able to grow readily on the solidified medium containing this low level of heme (Table 2), a finding which confirms the involvement of the HxC protein in the utilization of free heme. From this finding together with the fact that HxC has the characteristics of a TonB-dependent protein, it can be inferred that HxC is likely a component of a high-affinity heme transport system.

It is interesting that increasing the concentration of free heme in the solidified growth medium to 50 µg/ml permitted growth of the *hxC* mutant (Table 2). Similar results were obtained in studies with the *H. influenzae tonB* mutants, which could not grow when the heme concentration was 8.5 µg/ml but which grew readily on solidified media containing heme at 50 µg/ml (28). These two sets of results suggest that *H. influenzae* also may possess a TonB-independent mechanism for utilization of relatively high levels of free heme. It is difficult to predict whether such a TonB-independent heme acquisition system would function or be relevant in vivo, where free heme, especially at high concentrations, is unlikely to be present.

While the HxC protein is clearly involved in the utilization of low levels of free heme, the HxB protein, like HxA, appears to be essential for the utilization of heme:hemopexin. When a functional NTHI *hxA* gene was provided in *trans* in a Hib *hxB* mutant, the presence of the NTHI HxA protein did not allow utilization of heme:hemopexin in the absence of HxB (Table 2). However, when this same NTHI *hxA* gene was present in *trans* in a Hib *hxA* mutant that expressed HxB, this recombinant strain was able to utilize heme:hemopexin (Table 2) and also released a functional NTHI HxA protein from the cell (Fig. 5). It is also noteworthy that a database search revealed that the protein with the most similarity to HxB is the ShIB protein of *S. marcescens*. This outer membrane protein and the similar HpmB outer membrane protein of *Proteus mirabilis* (55, 57) have been shown to be essential for the activation and secretion of the *S. marcescens* ShIA and *P. mirabilis* HpmA hemolysins, respectively (4, 46, 55, 57). In contrast to the well-studied RTX cytotoxins (57, 58), both of these hemolysins have leader peptides (4, 46), as does HxA (7). In addition, both the Hib and NTHI HxA proteins possess the amino acid sequence motif A-N-P-N (7) which has been shown to be critical for the activation and secretion of the ShIA hemolysin (4, 47). By analogy with ShIA and ShIB in *S. marcescens*, the role of HxB in the utilization of heme:hemopexin is likely indirect, in that HxB probably functions in the release of HxA from the bacterial cell.

While the HxA, HxB, and HxC proteins are clearly important in heme utilization, nothing is known about the regulation of the expression of these macromolecules. Recent studies involving the expression of transferrin-binding activity by *H. influenzae* indicate that heme concentrations, independent of free iron levels, may control expression of certain outer membrane proteins involved in obtaining iron from transferrin (36). Determination of whether expression of the *hxA*, *hxB*, and

hxC genes can be affected by iron or heme levels will require additional studies.

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