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Identification of an Iron-Regulated Outer Membrane Protein of *Neisseria meningitidis* Involved in the Utilization of Hemoglobin Complexed to Haptoglobin

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Hemoglobin complexed to the plasma protein haptoglobin can be used by *Neisseria meningitidis* as a source of iron to support growth in vitro. An *N. meningitidis* mutant, DNM2E4, was generated by insertion of the mini-Tn*3*erm transposon into the gene coding for an 85-kDa iron-regulated outer membrane protein. Membrane proteins prepared from DNM2E4 were identical to those of the wild-type strain except that the 85-kDa protein was not produced. This mutant was unable to use hemoglobin-haptoglobin complexes as an iron source to support growth and was also impaired in the utilization of free hemoglobin. The mutant failed to bind free hemoglobin, hemoglobin-haptoglobin complexes, or apo-haptoglobin in a solid-phase dot blot assay. The 85-kDa protein was affinity purified when hemoglobin-haptoglobin complexes were used as a ligand but was not purified when free hemoglobin was used. We hypothesize that the 85-kDa iron-regulated protein is the hemoglobin-haptoglobin receptor and designate this protein Hpu (for hemoglobin-haptoglobin utilization).

The acquisition of iron (Fe), an essential nutrient for bacterial growth, is a crucial determinant of pathogenesis. *Neisseria meningitidis*, the gram-negative diplococcus responsible for causing epidemic meningitis (50), possesses a number of different Fe acquisition systems that may be important at different stages of meningococcal disease. Meningococci initially colonize the nasopharyngeal mucosal epithelium, where lactoferrin (LF) is thought to be the primary Fe source. From this site the organism can enter the bloodstream to cause invasive disease (35, 50, 52). Transferrin (TF) is the major Fe source in the bloodstream (35, 52). However, some studies have suggested that Fe-TF may be limiting in later stages of septicemic disease because of the hypoferremic response (15–17, 43). Meningococci possess outer membrane receptors for both LF and TF which are involved in the removal of Fe from these proteins (18, 36, 39, 44, 45).

In vitro, *N. meningitidis* is also able to acquire Fe from heme (Hm) and hemoglobin (Hb) (31, 59). However, little is known about how Fe uptake from these compounds occurs. Hm is presumably present on the mucosal surface, where meningococci first colonize, since *Haemophilus influenzae*, which requires a porphyrin source, grows well on this nasopharyngeal epithelium (35, 56). Intracellular Hb released as a result of disseminated intravascular coagulation during invasive disease could be used as an Fe source to sustain prolonged septicemia (58). However, haptoglobin (Hp), a plasma glycoprotein, rapidly binds to released Hb, forming a complex (Hb-Hp) that is removed from the circulation by hepatocytes (14, 20, 32). Hp has been shown to prevent *Escherichia coli* from using Hb as an Fe source and can prevent disease in experimentally infected animals (10). We have shown that unlike *E. coli*, *N. meningitidis* is able to acquire Fe from Hb-Hp complexes in vitro (9).

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We previously isolated *N. meningitidis* FAM29, an ethyl methanesulfonate-derived mutant which was unable to use TF as an Fe source (*fud-6* mutation) and was impaired in the ability to use Hb (*hga-1* mutation) (7). This mutant lacked two Fe-repressible proteins (FeRPs) of 95 and 85 kDa. Transformation of the *fud-6* mutation back into wild-type meningococci suggested that the 85-kDa FeRP was not associated with the *fud-6* mutation. Transformants were able to use Hb and produced the 85-kDa FeRP. The 95-kDa FeRP has subsequently been identified as TBP1, a component of the TF receptor (18). Because of the phenotype of the *fud-6 hga-1* double mutant, we suspected that the 85-kDa FeRP was associated with the *hga-1* mutation. To examine the function of this protein, we constructed a mutant containing a transposon insertion in the gene coding for the 85-kDa FeRP. This mutant, which does not produce the 85-kDa FeRP, was impaired in the ability to grow with Hb and was unable to use Hb-Hp complexes as an Fe source. We developed a whole-cell dot assay in which Fe-regulated binding of Hb, Hb-Hp, and apo-Hp was demonstrated in the parent strain. Binding of these ligands to the 85-kDa FeRP mutant was not detected. Although the 85-kDa FeRP did not bind Hb-Hp on a Western blot (immunoblot), we were able to affinity purify the 85-kDa protein by using the Hb-Hp complex. We hypothesize that this protein is an Fe-regulated outer membrane receptor for Hb and the Hb-Hp complex. We have designated this protein Hpu (for hemoglobin-haptoglobin utilization).

MATERIALS AND METHODS

Bacterial strains and plasmids. *N. meningitidis* strains (Table 1) were routinely grown on GC base agar (Difco Laboratories, Detroit, Mich.) at 37°C in a 5.2% CO_2 atmosphere. Strains FAM70 and DNM2 are both derivatives of *N. meningitidis* FAM18, a serogroup C, serotype 2a meningococcal isolate from the bloodstream of an infected patient (7). Conditions for Fe-limited growth in Chelex-treated defined medium (CDM) were described previously (7). CDM agar plates were prepared by the addition of 1% methanol-washed agar and 0.5% potato starch (Difco) (3). All glassware was washed in 3 N nitric acid and then rinsed extensively with high-quality deionized water to remove residual Fe. When used,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Source or reference
N. meningitidis		
FAM18	Serogroup C, serotype 2a	7
FAM20	FAM18 *nal-1*	7
FAM70	FAM20 *frpA*::mTn*3*erm, *frpC*::Ω interposon, Erm^r Str^r Nal^r	49
DNM2	FAM18 *nal-2*	This work
DNM2E4	DNM2 *hpu*::mTn*3*erm, Erm^r Nal^r	This work
E. coli		
DH5αF′	Δ(*lacZYA-argF*) U169	BRL[b]
Y1090	Δ(*lac*U169) *supF* (pMC9) Amp^r	Promega
RDP146	Δ(*lac-pro*)	46
NS2114SM	(λ*cre*$^+$) Str^r	46
Plasmids		
pCR II, version 3	Amp^r Kan^r	Invitrogen
pTA1	850-bp *Apo*I fragment of *hpu* in pCR II; Amp^r Kan^r	This work
pHSS8	Kan^r	46
pSM85k	850-bp *Apo*I fragment of *hpu* in pHSS8; Kan^r	This work
pTCA	tnpA$^+$ Tet^r	46
pOX38::mTn*3*erm	tra$^+$ Erm^r	49
pSM85kE	pSM85k::mTn*3*erm; Kan^r Erm^r	This work

[a] Amp^r, ampicillin resistance (100 μg/ml); Erm, erythromycin resistance (300 μg/ml for *E. coli* and 2 μg/ml for *N. meningitidis*); Kan^r, kanamycin resistance; (40 μg/ml); Nal^r, nalidixic acid resistance (20 μg/ml); Str^r, streptomycin resistance (100 μg/ml); Tet^r, tetracycline resistance (12.5 μg/ml).
[b] BRL, Bethesda Research Laboratories.

antibiotics were added at the concentrations indicated in Table 1, footnote *a*. *E. coli* strains were maintained on Luria-Bertani medium supplemented with antibiotics at the concentrations indicated in Table 1, footnote *a*. Plasmids used in these studies and their relevant properties are listed in Table 1. To detect α-complementation of *lacZ*, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (40 μg/ml) was added to the medium.

Reagents. All chemicals were purchased from Fisher Scientific Co., Pittsburgh, Pa., unless otherwise indicated. DNA-modifying enzymes were purchased from Promega, Madison, Wis., or New England Biolabs Inc., Beverly, Mass., unless otherwise indicated. A random primer DNA labeling system and the Klenow fragment of *E. coli* DNA polymerase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. All radioisotopes were purchased from Dupont, NEN Research Products, Boston, Mass.

Fe-dependent growth. To examine the kinetics of growth with specific Fe sources, meningococci were Fe starved in CDM and then grown in CDM supplemented with the Fe source of interest, as described previously (7–9). In summary, meningococci from 15-h CDM agar plates were suspended in CDM supplemented with 20 mM sodium bicarbonate and grown at 37°C with shaking until cultures reached mid-logarithmic phase. These Fe-depleted cells were used to inoculate fresh CDM, which was supplemented with an Fe source (see below). Growth was monitored with a Klett-Summerson colorimeter with a green filter. *N. meningitidis* DNM2E4, a mutant containing mini-Tn*3*erm (mTn*3*erm) inserted into the 5′ end of the gene for the 85-kDa FeRP, was maintained under erythromycin selection only during growth on CDM agar plates.

Fe sources. Ferric nitrate [$Fe(NO_3)_3$] and ferric dicitrate were used at final concentrations of 100 and 10 μM, respectively. Ferric dicitrate was prepared as described by West and Sparling (55). TF and LF (Sigma Chemical Co., St. Louis, Mo.) were Fe loaded (55) and added to cultures at a final protein concentration of 5 μM. In these assays, TF was saturated to 17% of the total Fe-binding capacity, while LF was saturated to 23% of the total Fe-binding capacity. Fe saturation levels were determined as described previously (1, 11). Bovine Hm, human ferrous Hb-A_o, and human Hp (a mixture of 1-1, 2-1, and 2-2 forms) were purchased from Sigma Chemical Co. These reagents were prepared and filter sterilized on the day of use. Hm was prepared as described previously (9) and added to cultures to a final concentration of 5 μM. Hb was prepared as a 100 μM stock solution in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4) and added to cultures at a final concentration of 1 μM Hb (4 μM Fe). Hb-Hp complexes were prepared as previously described (9) and added to cultures at a final concentration of 4 μM Fe. Desferal (10 μM), a potent Fe chelator which meningococci are unable to use for growth (5, 31), was added to Hm- and Hb-containing cultures to chelate any free Fe. This was done to ensure that growth observed with Hm-containing Fe sources was not due to degradation of Hm in aqueous solution (29). Human serum albumin (Sigma Chemical Co.), when added to Hb-containing cultures, was used at a final concentration of 16 μM. This was done to distinguish growth with Hb from growth with Hm released into the medium as a result of the dissociation of Hb. Meningococci are not able to use Hm complexed to serum albumin as an Fe source (9).

Preparation of membrane proteins. Meningococci were grown to late logarithmic phase in CDM with 100 μM $Fe(NO_3)_3$ or without added Fe and harvested by centrifugation at 8,000 × *g* for 10 min. The resulting cell pellets were stored at −70°C prior to preparation of total and Sarkosyl-insoluble membranes. Cells were suspended in 10 mM HEPES (pH 7.5) and passed though a French pressure cell (16,000 lb/in^2; gauge) three times. Cellular debris was removed by centrifugation at 8,000 × *g* for 10 min, and membrane proteins were pelleted at 100,000 × *g* for 1 h. This crude membrane fraction was washed twice by centrifugation at 100,000 × *g* in 10 mM HEPES (pH 7.5). Membranes were suspended in 200 to 500 μl of 10 mM HEPES (pH 7.5) and stored at −70°C. Sarkosyl-insoluble outer membranes were isolated as previously described (7). The membrane protein concentration was determined by a modified Lowry protein assay (25).

PAGE. Membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the discontinuous buffer system of Laemmli (21). Approximately 40 μg of protein was loaded per lane on gels (16 by 18 cm) ranging from 7.5 to 15% acrylamide; the proteins were separated by electrophoresis at 40 mA for 4 h prior to fixation and staining with silver (57) and/or Coomassie blue R250 (41).

Nondenaturing PAGE, used to monitor the formation of complexes between Hb and Hp, was performed as previously described (9). SDS was omitted from all gel components, and samples were not heated prior to electrophoresis. The electrophoretic mobility of Hb-Hp complexes was retarded compared to that of apo-Hp. No free Hb was visualized on these gels (data not shown).

Immunoblotting. Proteins were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H., or Micron Separations Inc., Westboro, Mass.) by using a Genie transfer apparatus (Idea Scientific Co., Minneapolis, Minn.) and Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) containing 20% methanol. Electrotransfer was performed for 30 min at 24 V, according to the manufacturer's instructions. Nitrocellulose membranes were blocked for 1 h with Tris-saline buffer (TSB) (50 mM Tris [pH 7.5] and 150 mM NaCl) containing 0.5% skim milk (Difco) and then probed overnight with monoclonal antibody (MAb) A4.85 (49) or blocking solution (conjugate control). The nitrocellulose was washed three times with TSB (15 min each) to remove unbound MAb and then incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G-immunoglobulin M (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) diluted 1:3,000 in blocking reagent. Finally, the membranes were again washed with TSB and developed with an alkaline phosphatase (nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate tetrazolium) developing kit from Bio-Rad Laboratories, Richmond, Calif.

N-terminal amino acid analysis. Sarkosyl-insoluble outer membrane proteins from Fe-starved *N. meningitidis* DNM2 were separated by SDS-PAGE on 7.5% acrylamide gels. Thioglycolic acid (0.002%) was added to the upper buffer chamber to scavenge free radicals and minimize modification of reactive amino acids residues or amino termini (19). Proteins were transferred, in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS)–10% methanol (pH 11.0), to a polyvinyldene difluoride membrane (Bio-Rad) and visualized by staining with 0.1% Coomassie blue R250 in 50% methanol as described previously (19, 27). The N-terminal amino acid sequence of the 85-kDa FeRP was obtained by the UCLA Protein Microsequencing facility.

DNA isolation and manipulation. Chromosomal DNA from *N. meningitidis* or *E. coli* was prepared as described by McLaughlin and Hughes (28), with the

following modifications. Cells were grown on agar plates instead of in broth, and the CsCl density centrifugation step was omitted. RNase A was added to a final concentration of 0.1 mg/ml and incubated at 37°C for 2 h. Plasmid DNA was isolated by alkaline lysis (40) or by the use of Qiagen plasmid columns (Qiagen Inc., Chatsworth, Calif.). Lambda phage DNA was isolated as described by Sambrook et al. (40) or on a Qiagen lambda column.

Library construction. Chromosomal DNA isolated from *N. meningitidis* FAM70 (49) was used to construct a genomic library in the expression vector λgt11 (38). DNA was digested overnight at 50°C with *Apo*I and ligated (3 h at room temperature) to *Eco*RI-digested, calf alkaline phosphatase-treated λgt11 phage arms. Recombinant λgt11 phage were packaged (Packagene; Promega) as described by the manufacturer and used to infect *E. coli* Y1090. Phage plaques were transferred to a Magnagraph nylon membrane (Micron Separations Inc.) and prepared for plaque hybridization (40).

Southern blotting and DNA hybridization. DNA was digested with restriction enzymes as suggested by the manufacturers, separated on agarose gels (0.6 to 1.0% agarose) in TAE buffer (40 mM Tris and 1 mM EDTA, pH 8.0), and transferred to a Magnagraph nylon membrane by the method of Southern, as described by Sambrook et al. (40). Bacterial colonies were transferred to a Magnagraph nylon membrane and prepared for hybridization as described by Sambrook et al. (40).

The synthetic oligonucleotide 5′-GCCCAAACCYTNAACGAAATCACC GT-3′ (where Y is C or T and N is G, A, T, or C), synthesized by Biosynthesis, Inc. Lewisville, Tex., was designed on the basis of the N-terminal amino acid sequence of the 85-kDa FeRP after consulting the neisserial codon preference data of West and Clark (54). Polynucleotide kinase was used to label the 5′ end of the oligonucleotide with [γ-^{32}P]dATP. Unincorporated nucleotide was removed with a Sephadex G-25 column. Filters to be probed with the oligonucleotide were incubated with prehybridization solution (6× SSC [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate], 1× Denhardt's solution [1% bovine serum albumin, 1% Ficoll, and 1% polyvinylpyrolidine], 0.5% SDS, and 0.05% sodium PP_i [$NaPP_i$]) for 2 h at 55°C. The ^{32}P-labeled oligonucleotide was then hybridized (with 6× SSC, 1× Denhardt's solution, and 0.05% $NaPP_i$) to the filters overnight at 55°C (2×10^6 cpm of end-labeled oligonucleotide per ml), and the filters were washed three times for 15 min each in 4.5× SSC–0.05% $NaPP_i$ at 50°C.

Double-stranded DNA was labeled by using the random primer DNA labeling system (Bethesda Research Laboratories) according to the manufacturer's instructions, with 25 μCi of [α-^{32}P]dCTP. Prior to hybridization, blots were blocked for 2 h at 65°C (with 3× SSC and 1× Denhardt's solution). The probe was added and hybridized overnight with the filters in 6× SSC–1× Denhardt's solution–0.5% SDS, which was followed by washing in 0.1× SSC–0.05% SDS at 52°C as described above. Blots were exposed to Kodak X-OMAT AR-5 film overnight at −70°C, and the film was then developed to identify hybridization products.

To remove radiolabeled probe, blots were treated with 5 mM Tris (pH 8.0)–0.2 mM EDTA–0.05% $NaPP_i$–0.1× Denhardt's solution for 2 h at 75°C (42). This solution was changed once during the incubation. Overnight autoradiography was used to confirm that the probe had been removed.

Cloning. Phage clones that hybridized to the N-terminal oligonucleotide were selected and plaque purified (40) for further analysis. DNA was amplified from one λgt11 phage, λA11-111, by PCR with primers homologous to the λgt11 phage arms (λgt11 3′ primer, 5′-GGTAGCGACCGGCGC-3′; λgt11 5′ primer, 5′-ACTCCTGGAGCCCG-3′). By using phage particles eluted from Luria-Bertani agarose plates, PCR was carried out for 30 cycles (each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and primer extension at 72°C for 2 min) with *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Phage were disrupted by being heated to 70°C for 5 min and chilled on wet ice, prior to amplification. PCR products were ligated (with T4 DNA ligase) into the pCR II version 3 vector (Invitrogen Corporation, San Diego, Calif.) and transformed into competent DH5αF′. *E. coli* was made competent for DNA transformation by the method of Hanahan, as described by Sambrook et al. (40). The resulting plasmid, designated pTA1, contained an 850-bp insert flanked by *Eco*RI restriction enzyme sites.

Mutagenesis. Shuttle mutagenesis was performed as described by Seifert et al., using mTn*3*erm (46, 47, 49). The pTA1 insert was subcloned into the *Eco*RI site of the Tn*3*-susceptible vector pHSS8; this construction, designated pSM85k, was used in shuttle mutagenesis. Derivatives of pSM85k containing mTn*3*erm in the 850-bp insert were identified by restriction enzyme digestion and used to transform DNM2. Transformation of *N. meningitidis* DNM2 was done as previously described (2, 47).

Dot blot analysis. Meningococci from 15-h CDM agar plates were grown to mid-logarithmic phase in CDM or CDM supplemented with 100 μM ferric nitrate at 37°C with shaking as described previously (8). Cells (100 μl) were filtered onto nitrocellulose by using the Minifold I (Schleicher and Schuell). Hb-Hp complexes were prepared as described above, except that the buffer used was 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–20 mM $NaHCO_3$. Complex formation was assessed by nondenaturing PAGE as described previously (9), and no free Hb was observed. Apo-Hp was prepared as a 1.5-mg/ml stock solution in the above-described buffer. After drying overnight, filters were blocked (with 100 mM Tris-HCl [pH 7.2], 150 mM KCl, and 0.5% skim milk) and then probed with either the Hb-Hp complex (3.0 μg/ml) or apo-Hp (3.0 μg/ml) diluted in blocking buffer. Unbound complex was removed by washing three times (15 min each) with 100 mM Tris (pH 7.2)–150 mM KCl–0.02% Tween 20. Bound complex was detected by incubating filters with anti-human Hp antiserum generated in rabbits (Sigma Chemical Co.), diluted 1:10,000, followed by goat anti-rabbit immunoglobulin G (heavy plus light chains)–horseradish peroxidase (HRP) conjugate (Bio-Rad), diluted 1:3,000 in blocking buffer. Detection was performed with HRP color development reagent (4-chloro-1-napthol) (Bio-Rad). Control filters were exposed to conjugate alone, conjugate and anti-Hp antiserum, or conjugate and Hb-Hp complexes or apo-Hp. All incubations were performed at room temperature for 1 h. Cell surface binding of Hb (0.3 to 6.0 μg/ml) was detected in a similar assay with TSB and rabbit anti-human Hb antiserum (1:10,000) (Sigma Chemical Co.). LF and TF were biotinylated as described by Schryvers and Morris (44). Meningococcal strains were prepared and filtered onto nitrocellulose as described above. Cells were probed with biotinylated LF or TF as previously described (3), except that 0.02% Tween 20 was added to wash buffers for blots probed with biotinylated LF.

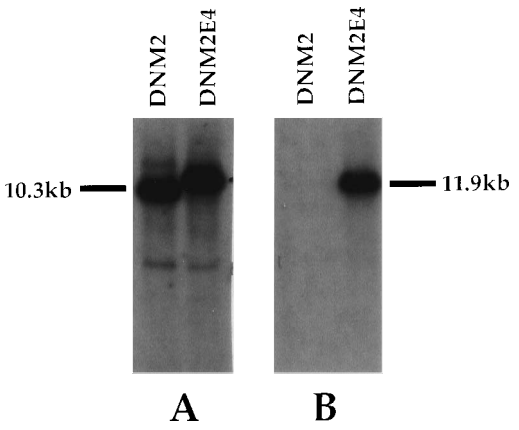


FIG. 1. Southern blot analysis of meningococcal DNA. (A) *Cla*I-digested DNA was probed with an oligonucleotide specific for the N terminus of the 85-kDa FeRP. (B) The DNA was hybridized with a probe specific for the *ermC* determinant on mTn*3*erm. DNM2, wild-type DNA; DNM2E4, *hpu*::mTn*3*erm DNA.

Affinity purification. Affinity purification of the 85-kDa FeRP was performed essentially as described by Schryvers and Morris (44), with the following modifications. Total membranes (750 μg) prepared from *N. meningitidis* strains grown under Fe-starved or Fe-sufficient conditions were incubated with Hb-Hp complexes (100 μg) or apo-Hp (100 μg) for 1 h at room temperature with gentle agitation in 1 ml of TSB. Membranes were pelleted by centrifugation at 16,000 × *g* for 10 min and resuspended in TSB containing anti-Hp antiserum diluted 1:5,000. After incubation and centrifugation as described above, membranes were suspended in 1 ml of TSB containing 10 mM EDTA, 0.75% Sarkosyl, and 25 μl of protein A-agarose (100 μg) (Bethesda Research Laboratories). Following a 1-h incubation at room temperature, the affinity resin was pelleted and washed as described by Schryvers and Morris (44), using wash method 1 but substituting TSB for 50 mM Tris–100 mM NaCl at pH 8.0. Bound proteins were eluted as described by Schryvers and Morris (44), separated on an SDS–7.5% PAGE gel, and stained with silver. Control purifications were with total membranes prepared from Fe-starved meningococci incubated as described above in the absence of ligand (Hb-Hp or Hp); in other control experiments, membranes of strain DNM2E4 were used. Similar affinity purifications with Hb (60 μg) and anti-human Hb antiserum or with TF (20 μg) and anti-human TF antiserum (Boehringer Mannheim Biochemicals) were also conducted.

RESULTS

N-terminal amino acid sequencing. To examine the role of the 85-kDa FeRP in Hb utilization, we set out to construct a mutant which did not produce this protein. Sarkosyl-insoluble outer membrane proteins from strain DNM2 were separated by SDS-PAGE on a 7.5% acrylamide gel, transferred to a polyvinylidene difluoride membrane, and stained with Coomassie blue as described in Materials and Methods. The 85-kDa FeRP was excised from this blot, and the N-terminal amino acid sequence of the protein was determined and found to be ADPAPQSAQTLNEITV(L)(A)TNK. The neisserial codon usage chart published by West and Clark (54) was used to design a 26-mer oligonucleotide probe. This oligonucleotide

probe was found to hybridize to a single DNA fragment in the DNM2 chromosome (Fig. 1), suggesting that the oligonucleotide could be successfully used to identify recombinant clones containing the gene for the 85-kDa FeRP. No hybridization to *E. coli* or λ DNA was detected (data not shown).

Cloning. A genomic library from *N. meningitidis* FAM70 (49) was constructed in the lambda cloning vector λgt11. MAb A4.85 (49), which reacts with FrpA, FrpC, and the Hpu protein, was originally used to screen this library. Strain FAM70, which does not produce FrpA or FrpC, was used to construct the library to increase the odds of cloning Hpu. No *hpu* clones were identified with MAb A4.85, but two recombinant phage were isolated from this library by using the N-terminal oligonucleotide as a probe. These phage were identical on the basis of restriction enzyme mapping (data not shown). The 850-bp insert from one λgt11 phage, designated λA11-111, was amplified by PCR and ligated into the pCR II version 3 vector (pTA1).

Insertional inactivation of *hpu*. The 850-bp insert of pTA1 was excised by *Eco*RI digestion and subcloned into the *Eco*RI site of the Tn*3*-susceptible plasmid pHSS8. The resulting plasmid, pSM85k, was subjected to shuttle mutagenesis as described by Seifert et al. (47). The transposon mTn*3*erm (49), which is a derivative of mTn*3* (47) that carries the *ermC* erythromycin resistance marker from plasmid pE5 (37), was used. A plasmid containing mTn*3*erm inserted within the 850-bp fragment (designated pSM85kE) was identified by restriction enzyme digestion. Plasmid pSM85kE was then used to transform *N. meningitidis* DNM2. Since pHSS8 cannot be maintained as a plasmid in pathogenic *Neisseria* spp., transformants should carry the transposon inserted by homologous recombination into *hpu*. One transformant, designated *N. meningitidis* DNM2E4, was selected for further analysis.

Southern hybridization. Southern analysis of DNM2E4 DNA indicated that recombination resulted in a single insertion of mTn*3*erm into *hpu* and that no gross rearrangements had occurred (Fig. 1). When chromosomal DNA from wild-type strain DNM2 was digested with *Cla*I and probed with the 85-kDa N-terminal oligonucleotide, we observed that the oligonucleotide hybridized with a 10.3-kb *Cla*I fragment (Fig. 1A). When DNM2E4 DNA was digested with *Cla*I and probed with this oligonucleotide, an 11.9-kb band hybridized to the oligonucleotide. *Cla*I does not cut within the 850-bp fragment used to mutagenize DNM2 or within the transposon. The increase in the size of the *Cla*I fragment that hybridized to the oligonucleotide in DNM2E4 was equivalent to the size of mTn*3*erm (1.64 kb), indicating that the mTn*3*erm transposon had recombined by a double crossover into the desired location.

The probe was removed from the above-described Southern blots, and they were reprobed with the *ermC* marker in pUC19 (Fig. 1B). A single fragment of 11.9 kb hybridized to this probe in DNM2E4, while no hybridization was detected in DNM2. These data indicated that only one copy of the transposon was present in DNM2E4. The size of the fragment that hybridized to *ermC* was identical to the size of the band that hybridized to the N-terminal oligonucleotide, further indicating that the transposon had recombined into the gene coding for the 85-kDa FeRP.

SDS-PAGE and Western blot analysis. To determine if Hpu was produced by DNM2E4, total membranes were prepared from cells grown in Fe-sufficient [100 μM $Fe(NO3)_3$] and Fe-limited media. Membranes were analyzed by SDS-PAGE (Fig. 2A) and Western blotting (Fig. 2B). When membrane proteins were stained with either silver or Coomassie blue, Hpu was clearly visible in membranes prepared from Fe-stressed DNM2

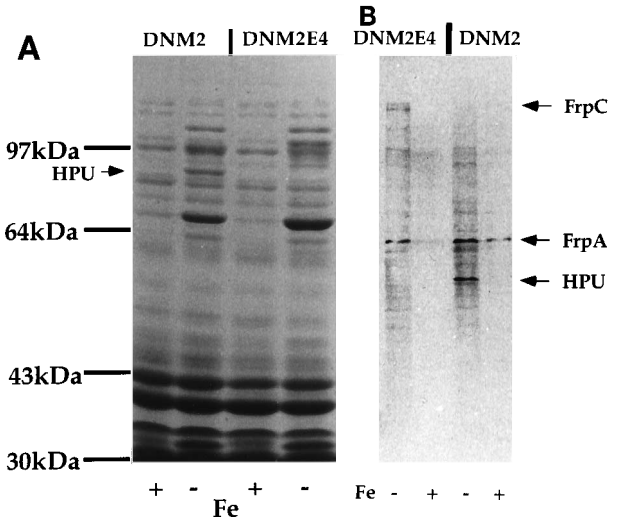


FIG. 2. Comparison of total membrane protein profiles obtained from strains DNM2 and DNM2E4. (A) SDS–7.5% PAGE gel containing membranes isolated from iron-starved (−) or iron-replete (+) DNM2 or DNM2E4. Note the absence of Hpu in the last lane. (B) Western blot of these membranes after incubation with MAb A4.85. The locations of FrpA, FrpC, and Hpu are indicated.

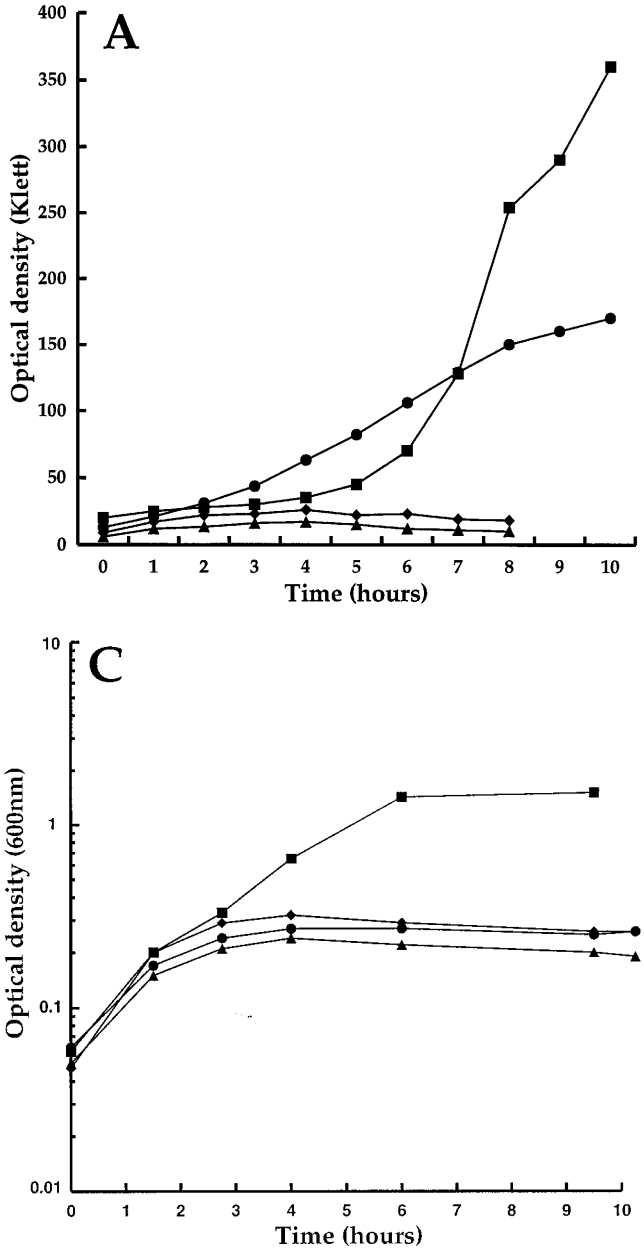
but was not detected in DNM2E4 (Fig. 2A). The gel shown in Fig. 2A is a representative gel chosen from multiple gels analyzed. We did not observe any reproducible differences between membrane proteins isolated from DNM2 and DNM2E4, other than the loss of the 85-kDa FeRP from DNM2E4.

MAb A4.85 (49), which reacts with Hpu and two Fe-regulated RTX-like proteins of *N. meningitidis*, was used to probe DNM2E4 total membranes. This MAb did not detect Hpu in DNM2E4 membranes, although the two RTX-like proteins, FrpA (122 kDa) and FrpC (250 kDa), were apparent (Fig. 2B). The additional bands seen in Fig. 2B are due to degradation products of FrpA and FrpC, which are extremely sensitive to proteolysis (49). These extraneous bands are not present in membranes prepared from strain FAM70, a mutant lacking both FrpA and FrpC (53).

Growth of DNM2E4 with various Fe sources. The growth of DNM2E4 was equivalent to that of the parent strain, DNM2, for all Fe sources tested with the exception of Hb and Hb-Hp complexes (Table 2). Growth with Hb (Fig. 3A) was dramatically reduced in DNM2E4, which consistently grew to only 50% of the final extent of DNM2. This phenotype is similar to that observed earlier with meningococcal mutant FAM29 (7), which is associated with the *hga-1* mutation. The growth of DNM2E4 in cultures containing Hb as the sole source of Fe

TABLE 2. The *hpu* mutation affects growth only with Hb and Hb-Hp complexes

Iron source tested	Growth	
	DNM2	DNM2E4
Ferric nitrate	+	+
Ferric dicitrate	+	+
TF	+	+
LF	+	+
Hm	+	+
Hb	+	+/−
Hb-Hp complexes	+	−



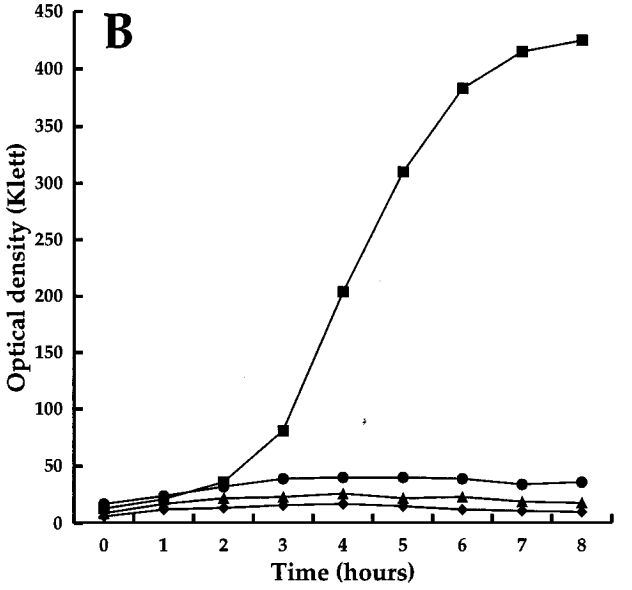


FIG. 3. Growth of meningococci with Hb (A) and Hb-Hp (B). Symbols: ■, DNM2 with Hb or Hb-Hp; ●, DNM2E4 with Hb or Hb-Hp; ◆, DNM2 without Fe; ▲, DNM2E4 without Fe. (C) Effect of serum albumin on growth of DNM2E4 with Hb. Symbols: ■, DNM2 with Hb-serum albumin; ●, DNM2E4 with Hb-serum albumin; ◆, DNM2 without Fe and with serum albumin; ▲, DNM2E4 without Fe and with serum albumin. Desferal (10 μM) was added to all cultures to chelate any free Fe.

was inhibited by the addition of serum albumin, while growth of wild-type strain was not affected (Fig. 3C). *N. meningitidis* is not able to use Hm complexed to serum albumin as an Fe source (9). More striking was the effect on growth with Hb-Hp complexes. *N. meningitidis* DNM2 normally grows better with Hb-Hp complexes (Fig. 3B) than with Hb alone (9). In contrast, DNM2E4 was unable to acquire Fe from Hb-Hp complexes (Fig. 3B), even after prolonged incubation (24 h) (data not shown).

Dot blot analysis. To further investigate the role of Hpu in utilization of Hb and Hb-Hp complexes, we developed dot blot assays to examine the binding of Hb, Hb-Hp complexes, and apo-Hp to meningococci. Lee and Hill have previously reported the Fe-regulated binding of Hb to intact meningococci (22). We were able to detect this binding in our wild-type strain, but not in DNM2E4, with anti-Hb antiserum (Fig. 4). We used anti-Hp antiserum to detect binding of Hb-Hp complexes and apo-Hp to intact meningococci. As expected, binding of Hb-Hp complexes was detected in the parent strain, DNM2, following growth in Fe-limited medium. No binding to cells grown in Fe sufficient medium was detected (Fig. 4). This suggests that the wild-type strain, DNM2, possesses an Fe-regulated receptor for Hb-Hp complexes. When a dot assay with apo-Hp was performed, binding to DNM2 Fe-starved cells was detected; however, this reactivity was much weaker than that observed with Hb-Hp complexes (Fig. 4). Binding of Hb-Hp complexes or apo-Hp to DNM2E4 was not detected (Fig. 4). Biotinylated LF was used as a positive control to ensure that cells were indeed Fe starved. Both DNM2 and DNM2E4 grown in Fe-depleted medium bound biotinylated LF, while cells grown in Fe-sufficient medium did not (Fig. 4). Similarly, DNM2E4 bound biotinylated TF as well as wild-type strain DNM2 did (data not shown).

Affinity purification. Direct binding of Hpu to Hb-Hp was demonstrated by affinity isolation. Total membranes prepared

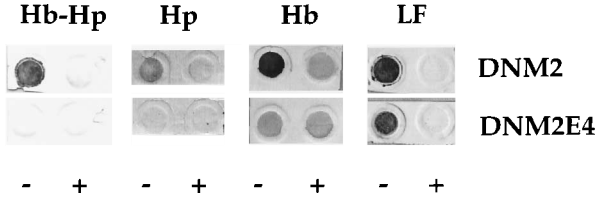


FIG. 4. Dot blot assay for binding of Hb, Hb-Hp complexes, and apo-Hp to intact meningococci. Iron-starved (−) or iron-replete (+) DNM2 or DNM2E4 was deposited onto nitrocellulose, and the filter was blocked and incubated with either Hb, Hb-Hp complexes, or apo-Hp followed by either anti-Hb antiserum (Hb) or anti-Hp antiserum (Hb-Hp and Hp). Goat anti-rabbit immunoglobulin G–HRP conjugate and HRP substrate were used to detect ligand bound to meningococci. To detect binding of LF, meningococci were incubated with biotinylated LF followed by avidin-HRP conjugate and HRP substrate.

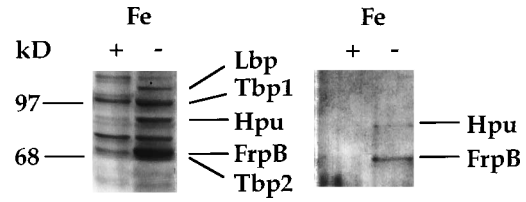


FIG. 5. Affinity purification of Hpu by using the Hb-Hp complex. Total membranes prepared from iron-starved (−) or iron replete (+) DNM2 were incubated with Hb-Hp followed by anti-Hp antiserum and protein A coupled to agarose beads. Proteins recovered from the affinity resin were separated by SDS–7.5% PAGE and stained with silver (right panel). Total membranes isolated from iron-starved (−) or iron-replete (+) DNM2 (left panel) are also shown.

from meningococci grown in Fe-depleted medium were incubated with Hb-Hp complexes and then with anti-Hp antiserum. Protein A coupled to agarose beads was used to isolate bound proteins. Hpu was recovered from the affinity resin when total membranes prepared from DNM2 were used (Fig. 5). As an internal control, the TF receptor proteins TBP1 and TBP2 were purified in a similar assay with TF and anti-TF antiserum (data not shown). Hpu was not recovered from membrane proteins prepared from Fe-sufficient cells (Fig. 5). Likewise, the 85-kDa FeRP could not be affinity purified from DNM2E4 (data not shown). A 70-kDa protein, FrpB, was also recovered in the affinity purification from wild-type Fe-starved membranes (Fig. 5). This protein was also purified in a control experiment in which no Hb-Hp was present, suggesting that this protein was bound by either protein A or the anti-Hp antiserum used in the assay. Furthermore, a mutant strain which does not produce FrpB is not impaired in growth with Hb and displays wild-type binding to apo-Hp (51). The 85-kDa FeRP was also purified when apo-Hp was used as a ligand; however, the yield was meager (data not shown). Affinity isolation with Hb and anti-Hb antiserum was not successful.

DISCUSSION

The role of Hm compounds as Fe sources supporting the in vivo growth of *N. meningitidis* has not been well studied. However, it is likely that Hm-containing compounds are important for supporting meningococcal growth on the nasopharyngeal mucosa and in the bloodstream. As mentioned previously, there is good reason to suspect that biologically significant amounts of Hm may exist on the nasopharyngeal mucosal surface. Further, one potential outcome of meningococcal septicemia is erythrocyte lysis which releases significant amounts of Hb and Hm into the bloodstream during invasive disease. Fulminant meningococcemia is characterized by the development of septic shock, multiple organ failure, and severe disseminated intravascular coagulation (4). During disseminated intravascular coagulation, the normal homeostatic balance between blood coagulation and fibrinolysis (desolution of clots) is severely disturbed (33), leading to activation of the coagulation cascade and the deposition of fibrin clots. Mechanical shearing of erythrocytes as they are forced through the severely clogged vasculature (58) can release significant amounts of Hb, with consequent Hb-Hp formation, during the later stages of invasive meningococcal disease.

The ability of pathogenic *Neisseria* spp. to use Hm, Hb, and Hb-Hp complexes to support growth in vitro has been well documented (9, 30, 31). However, little is known about the mechanisms involved in acquiring Fe from these compounds. Several observations suggest that Fe uptake from Hm compounds occurs via a receptor that is distinct from the meningococcal TF and LF receptors. Mutants lacking the TF receptor (26) or the LF receptor (39) are not impaired in growth with Hm Fe sources. Lee and Hill have identified Fe-regulated Hb binding activity in outer membrane preparations from *N. meningitidis* (22). Those authors suggested that the meningococcus possesses two Hb receptors with different affinities for the ligand. However, no Hb-binding proteins were identified. Two Hm-binding proteins (97 and 44 kDa) have been purified from *Neisseria gonorrhoeae* by hemin affinity chromatography (23), but the role of these Hm-binding proteins in Hm utilization has not been established. Desai et al. have presented evidence that *N. gonorrhoeae* removes the Fe from the Hm ring prior to internalization and that this Fe is shuttled to the periplasmic Fe-binding protein Fbp (6). In addition, those authors have isolated Hm uptake mutants that are impaired in growth with both Hm and Hb. Here we have reported the initial characterization of Hpu, an 85-kDa FeRP involved in the acquisition of Fe from Hb and Hb-Hp complexes. Hpu is apparently not involved in the acquisition of Fe from Hm.

By constructing an *N. meningitidis* mutant that does not produce the 85-kDa FeRP, we have demonstrated that this protein is required for acquisition of Fe from Hb and from Hb complexed to Hp. We hypothesize that the 85-kDa FeRP is an Fe-regulated receptor for Hb and/or the Hb-Hp complex and have designated this protein Hpu (for hemoglobin-haptoglobin utilization). In our dot assays we confirmed earlier reports that *N. meningitidis* possesses an Fe-regulated surface-exposed receptor for Hb (23). Mutant strain DNM2E4 does not appear to possess this receptor, since surface binding to Hb was not detected. It is not clear if the 85-kDa protein directly binds Hb or if this phenotype is secondary. We were unable to affinity purify the 85-kDa protein by using Hb as a ligand; however, this result must be interpreted cautiously, since the stability of Hb under the assay conditions is questionable. We also cannot rule out the possibility that a polar mutation occurred as a result of the transposon insertion in DNM2E4 and is responsible for the observed phenotype.

The Fe-repressible binding of Hb-Hp complexes to wild-type meningococci in a dot assay suggests that DNM2 possesses a surface-exposed receptor for Hb-Hp complexes. Interestingly, we also detected Fe-regulated binding of apo-Hp to intact meningococci. The eukaryotic receptor for Hb-Hp complexes does not bind apo-Hp (24). The lack of binding to mutant strain DNM2E4 strongly suggests that Hpu is responsible for binding of these ligands to the meningococcal cell surface. Hpu was affinity purified from membrane proteins when Hb-Hp complexes were used as a ligand and, less well, when apo-Hp was used. This direct binding strongly suggests that the 85-kDa protein is indeed the receptor for Hb-Hp and apo-Hp. Preliminary sequence analysis of the 5′ region of *hpu* indicates that Hpu belongs to the TonB-dependent high-affinity receptor family, supporting the above-described hypothesis. The inability of DNM2E4 to bind Hb in a dot assay suggests that Hpu is also responsible for binding Hb, although we were unable to demonstrate this directly. To our knowledge there is no precedence for Hb receptors which bind Hb-Hp or apo-Hp. For that matter, no Hb-Hp receptors or Hp receptors have been cloned from prokaryotic organisms. In *Vibrio vulnificus* an exocellular Hm-inducible protease capable of releasing Hm from Hb-Hp is involved in utilization of Hb-Hp (34). Studies to better define the binding specificity of Hpu are in progress.

How might Hpu participate in the removal of Fe from Hb and/or Hb-Hp complexes? Hanson et al. (13) identified a hemopexin-binding protein in *H. influenzae* and showed that a mutant lacking this protein could not use Hm complexed to

hemopexin for growth. Hm utilization by this mutant was normal. This suggests that the Hm associated with Hm-hemopexin complexes is transported via an Hm uptake system. By analogy, Hpu most likely channels Hm from Hb and Hb-Hp into a central Hm uptake pathway. Hpu may act merely to bring Hm-containing compounds into close proximity to the bacterial cell, where Hm is removed by other proteins, or alternatively, Hpu may be directly involved in removal of Hm-Fe. For instance, binding of Hb to Hp is thought to increase the exposure of the Hm moiety of Hb to the external solvent (12). Hpu may act to extract the exposed Hm ligand from the Hb-Hp complex and present this Hm to an Hm transport pathway. An extracellular metalloprotease has been shown to be important for the removal of Hm from Hb and Hb-Hp complexes by *V. vulnificus* (34). Hpu may be a membrane-bound protease which cleaves the Hb-Hp complex, releasing Hm. It will be interesting to determine if Hpu has any proteolytic activity. In this context, MAb A4.85, which reacts with Hpu, also binds to the RTX-like proteins of *N. meningitidis* as well as several other RTX proteins (49). These RTX proteins share a common structural feature: the presence of a tandem array (9 to 14 copies) of a nine-amino-acid repeat located in the carboxy-terminal portion of the protein (53). MAb A4.85 may recognize the RTX repeat region that characterizes these toxins (48). The significance of the cross-reactivity of MAb A4.85 with Hpu is not known, but proteases which contain four copies of the RTX tandem repeat have also been described (53). It is not yet known whether Hpu contains the RTX repeat region.

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