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Neisseria meningitidis tonB, *exbB*, and *exbD* Genes: Ton-Dependent Utilization of Protein-Bound Iron in Neisseriae

IGOR STOJILJKOVIC* AND NITHYA SRINIVASAN

Department of Microbiology and Immunology, Emory University, Atlanta, Georgia 30322

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We have recently cloned and characterized the hemoglobin (Hb) receptor gene, *hmbR*, from *Neisseria meningitidis*. To identify additional proteins that are involved in Hb utilization, the *N. meningitidis* Hb utilization system was reconstituted in *Escherichia coli*. Five cosmids from *N. meningitidis* DNA library enabled a heme-requiring (*hemA*), HmbR-expressing mutant of *E. coli* to use Hb as both porphyrin and iron source. Nucleotide sequence analysis of DNA fragments subcloned from the Hb-complementing cosmids identified four open reading frames, three of them homologous to *Pseudomonas putida*, *E. coli*, and *Haemophilus influenzae exbB*, *exbD*, and *tonB* genes. The *N. meningitidis* TonB protein is 28.8 to 33.6% identical to other gram-negative TonB proteins, while the *N. meningitidis* ExbD protein shares between 23.3 and 34.3% identical amino acids with other ExbD and TolR proteins. The *N. meningitidis* ExbB protein was 24.7 to 36.1% homologous with other gram-negative ExbB and TolQ proteins. Complementation studies indicated that the neisserial Ton system cannot interact with the *E. coli* FhuA TonB-dependent outer membrane receptor. The *N. meningitidis tonB* mutant was unable to use Hb, Hb-haptoglobin complexes, transferrin, and lactoferrin as iron sources. Insertion of an antibiotic cassette in the 3′ end of the *exbD* gene produced a leaky phenotype. Efficient usage of heme by *N. meningitidis tonB* and *exbD* mutants suggests the existence of a Ton-independent heme utilization mechanism. *E. coli* complementation studies and the analysis of *N. meningitidis hmbR* and *hpu* mutants suggested the existence of another Hb utilization mechanism in this organism.

All bacterial pathogens have developed highly sophisticated iron assimilation systems as a response to iron-limiting conditions encountered in environment and host's body fluids. Production of siderophores, small nonproteinaceous molecules with extremely high affinity for iron(III), is one of the most successful and widely utilized strategies of iron assimilation (16, 47). Some bacterial pathogens do not produce siderophores but are able to use different host iron-binding proteins like transferrin, lactoferrin, hemoglobin (Hb), and heme as sources of iron (13, 15, 49, 51). Common components of both siderophore-dependent and host iron-binding protein-dependent iron acquisition systems are receptor proteins involved in binding of siderophores and interacting with the host iron-binding proteins. These large outer membrane proteins are responsible for the transport of iron or iron-containing compounds through the otherwise impermeable outer membrane (8, 48).

Neisseriae do not produce siderophores but are able to utilize the host iron-binding proteins transferrin, lactoferrin, and Hb and haptoglobin-Hb complexes as their sole sources of iron (4, 5, 12, 13, 17, 28, 41, 45, 50, 51). These solely human pathogens can also use iron citrate, aerobactin, and heme but not desferrioxamine B and heme-albumin complexes as sources of iron (12, 17, 68, 69).

Two outer membrane proteins of *Neisseria meningitidis* were recently shown to be involved in Hb and haptoglobin-Hb utilization in this pathogen. HmbR is an 89.5-kDa Hb-binding protein essential for Hb utilization in some strains of *N. meningitidis*. This protein recognizes the globin part of the Hb molecule, and its expression is essential for full virulence of *N. meningitidis* in the rat model of meningococcemia (62, 63). HpuB is an 85-kDa protein involved in the utilization of Hb by some *N. meningitidis* isolates (42, 43, 63). In addition to HpuB, the expression of the HpuA lipoprotein is necessary for utilization of Hb-haptoglobin complexes (43). *N. meningitidis hpu* and *hmbR* mutants are still proficient in heme utilization, suggesting the existence of another system for heme utilization in neisseriae (63). Attempts to reconstitute neisserial transferrin and Hb utilization systems in *Escherichia coli* were only partially successful, suggesting that some components of transferrin and Hb utilization system still await isolation and characterization (2, 14, 62). In this report, we present the reconstitution of neisserial Hb utilization system in *E. coli*. At least four neisserial open reading frames (ORFs) in addition to the *hmbR* gene were necessary for complementation of Hb utilization in *E. coli*. Three ORFs have significant amino acid homology with *Pseudomonas putida*, *E. coli*, and *Haemophilus influenzae* TonB, ExbB, and ExbD proteins. These cytoplasmic membrane proteins are known to participate in energization of gram-negative outer membrane siderophore, heme, and vitamin B_{12} receptor proteins (9, 31, 64). The *N. meningitidis tonB* mutant was devoid of ability to utilize Hb, Hb-haptoglobin complexes, transferrin, and lactoferrin, while heme utilization was grossly unimpaired.

MATERIALS AND METHODS

Plasmids, bacteria, and media. Strains and plasmids used and constructed in this study are listed in Table 1. The meningococci were grown on GCB (Difco) agar containing supplements as described previously (62) and were incubated at 37°C with 5% CO_2. *E. coli* was grown in LB (Luria broth). When necessary, the following antibiotics were used in work with *E. coli*: chloramphenicol (30 mg/liter), tetracycline (10 mg/liter), and ampicillin (100 mg/liter). For neisseriae, neomycin (100 mg/liter) was used when needed. To test the ability of neisseriae to use various iron-containing proteins as sole iron sources, a suspension of bacteria was plated onto GCB agar containing 75 μM deferoxamine mesylate (Desferal; Ciba Geigy). Filter discs (0.25 in.; Schleicher & Schuell, Inc., Keene, N.H.) impregnated with test compounds (10 μl of 5-mg/ml stock solutions unless otherwise stated) were placed on these plates. Zones of growth around the discs were recorded after overnight incubation at 37°C in the presence of 5% CO_2

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Emory University, 1510 Clifton Rd., 3001 Rollins Research Building, Atlanta, GA 30322. Phone: (404) 727-1322. Fax: (404) 727-3659. E-mail: stojiljk@microbio.emory.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain	Description	Reference or source
N. meningitidis		
IR1072	*N. meningitidis* 8013 isolate 6, serogroup C	Lab collection
IR1074	*N. meningitidis* serogroup B, clinical isolate	Lab collection
IR1075	*N. meningitidis* serogroup C, clinical isolate	Lab collection
IR1078	IR1075 but *hmbR*::Km^r	63
IR1097	*hmbR* deletion derivative of IR1072	63
IR1098	IR1072 *hmbR*::Km^r	63
IR2035/2036	IR1078 but *hpu*::mTn*3*Erm	63
IR2113	IR1072 but *tonB*::Km^r	This study
IR2115	IR1097 but *tonB*::Km^r	This study
IR2162	IR1072 but Δ*Hpa*I-*Eco*RV::Km^r	This study
IR2163	IR1072 but *Hpa*I::Km^r	This study
IR2164	IR1072 but *Eco*RV::Km^r	This study
IR2165	IR1097 but Δ*Hpa*I-*Eco*RV::Km^r	This study
IR2200/2201	IR1075 but *tonB*::Km^r	This study
IR2279/2281	IR1072 but *exbD*::Km^r	This study
E. coli		
DH5α MCR		Stratagene
IR1532	DH-5α MCR *hemA*	This study
EB53	*E. coli hemA aroB* Rif^r	64
Plasmids		
pMLC210	Chl^r, pACYC derivative	46a
pBluescript	Amp^r	Stratagene
pWKS30	Amp^r	67
KIXX	Km^r cassette	Pharmacia
pKD102	Km^r cassette, *aphA* gene	66
pT76.53	*Y. enterocolitica hemPRST′*	64
pSM85kE	*hpu*::mTn*3*Erm	42
pIRS626	pWKS30, *flaX rfaC hmbR* IS*1106*	This study
pIRS1173	pACYC184, carrying 3-kb *Bam*HI-*Hin*dIII, *hmbR*^{wt}	This study
pIRS1099	3.2-kb *Cla*I-*Eco*RI from cos2, 5′ truncated *exbD*	This study
pIRS1120	2.3 kb from cos2, carrying *tonB* and *exbB* genes	This study
pIRS1122	*Cla*I-*Kpn*I subclone of pIRS1120	This study
pIRS1133	*Eco*RI-*Kpn*I subclone of pIRS1120	This study
pIRS1136	pIRS1120 with KIXX in *Kpn*I site (*tonB*)	This study
pIRS1172	pIRS1099 with Km^r cassette in *Hin*dIII site (*exbD*)	This study
pIRS1190	Δ derivative of pIRS1173, affected in Hb use	This study

(62). Hemin (bovine), haptoglobin (human), and Hb (human) were obtained from Sigma. Human iron-saturated transferrin and lactoferrin were obtained from Zehava Eisenbaum (Department of Microbiology, Emory University). Both proteins were saturated with iron and dialyzed to remove unbound iron.

Binding of biotinylated Hb to whole cells. Detection of Hb binding to whole cells was done essentially as described previously (63). Streptavidin-peroxidase complex and a BM blue-peroxidase substrate (Boehringer, Mannheim, Germany) were used for the detection of biotinylated Hb.

Recombinant DNA techniques. Standard methods for plasmid DNA preparation, restriction endonuclease analyses, and ligations were carried out as described by Sambrook et al. (56). Southern blot analysis was done with a digoxigenin nonradioactive DNA labeling and detection kit (Genius system; Boehringer) under high-stringency conditions (60°C, two washes with 2× SSC 0.1% sodium dodecyl sulfate [SDS] at room temperature followed by two washes in 0.1× SSC–0.1% SDS at 60°C).

Construction of *N. meningitidis tonB* and *exbD* mutants. Plasmid pIRS1120 (Table 1) was partially digested with restriction enzyme *Kpn*I and ligated with the 1.5-kb kanamycin resistance (Km^r) cassette from pKD102 (66). Plasmid pIRS1136, containing the Km^r cassette in the *Kpn*I site of the *N. meningitidis tonB* gene, was linearized with restriction enzyme *Eco*RI and transformed into *N. meningitidis* IR1072 and IR1097, producing IR2113 and IR2115, respectively. Both mutations were checked by Southern blotting (Fig. 5a). Plasmid pIRS1099 contained a large part of the *N. meningitidis exbD* gene and a unique *Hin*dIII site at its 3′ end (Fig. 1). This plasmid was partially digested with restriction enzyme *Hin*dIII and ligated with a 1.2-kb *Hin*dIII-digested KIXX (Pharmacia, Sweden) Km^r cassette. Plasmid pIRS1172, containing the KIXX cassette in the *Hin*dIII restriction site of the *exbD* gene, was linearized with restriction enzyme *Hpa*I or *Pst*I and transformed into *N. meningitidis* IR1072. Two putative *exbD* mutants, IR2279 and 2281, contained the Km^r cassette in the *exbD* gene were isolated (data not shown).

Isolation of *E. coli* outer membranes. Outer membranes were prepared from iron-starved *E. coli* cells as described previously (25). Briefly, *E. coli* spheroplasts were prepared by resuspending the bacterial pellet in 0.5 ml of 0.2 M Tris (pH 8) and sequentially adding 1 ml of 0.2 M Tris, 1 M sucrose, 0.2 mg of lysozyme, and 3.2 ml of water. Spheroplasts were solubilized in 5 ml of solubilizing buffer (2% Triton X-100, 50 mM Tris, 10 mM $MgCl_2$) and 1 mg of DNase (Boehringer) per ml. Bacterial outer membranes were pelleted by centrifugation at 40,000 × *g* for 1 h at 4°C, washed three times in deionized water, and resuspended in water or SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Membranes were separated by SDS-PAGE using a 10% acrylamide gel and stained with Coomassie blue.

DNA sequence determination. The DNA sequence of *tonB*, *exbB*, and *exbD* genes (both strands) was determined by the dideoxy chain termination method using an AutoRead kit and 377 Prizm automatic sequenator (ABI).

Nucleotide sequence accession number. The sequence reported has been assigned GenBank accession number U77738.

RESULTS

Reconstitution of HmbR-dependent Hb utilization in *E. coli* DH5α *hemA*. To identify additional proteins involved in Hb utilization in *N. meningitidis*, we have reconstituted the neisserial Hb utilization system in *E. coli*. An *N. meningitidis* pLAFR2 cosmid DNA library of a clinical *N. meningitidis* serogroup C isolate able to utilize heme, Hb, and Hb-haptoglobin complexes as iron sources was introduced into a heme-requiring mutant of *E. coli* DH5α. The *hemA* mutant of *E. coli* DH5α was isolated by a neomycin-enrichment procedure (64). This strain could not give rise to normal-size colonies on LB plates without supplementation with 5-aminolevulinic acid. The growth defect was not rescued by the introduction of a HmbR receptor-encoding plasmid (pIRS626) or by Hb supplementation. Obviously, additional products were needed to fully complement HmbR-dependent Hb utilization in *E. coli*. A simple complementation strategy was devised to identify genes encoding these products. The *hemA* (*hmbR*$^+$) strain was transformed with the *N. meningitidis* cosmid library and plated on Hb-supplemented medium (LB with 75 μM iron chelator dipyridyl, 250 μg of Hb per ml, and 10 μg of tetracycline per ml). Transformants able to grow on Hb-containing plates can be of two types: they may contain a gene which complements a heme biosynthesis defect (i.e., *hemA* of *N. meningitidis*), or they may allow *E. coli* to utilize Hb as its porphyrin source. After the introduction of the *N. meningitidis* cosmid library into *E. coli hemA* (HmbR$^+$) and incubation for 2 days at 37°C, several tetracycline-resistant (Tc^r) colonies appeared on Hb-dipyridyl plates. After restreaking, some Tc^r colonies were able to grow on unsupplemented LB plates, indicating that these bacteria most probably contain the *hemA* homolog of *N. meningitidis*. These clones were not studied further. Cosmids from five Tc^r colonies able to grow only in the presence of Hb were used for further studies. Cosmid DNA was isolated from these clones and purified from the *hmbR*$^+$-carrying plasmid by transformation into DH5α *hemA*. Two cosmids (cos7 and cos9) allowed growth of DH5α *hemA* on Hb as an iron source, while three cosmids (cos2, cos4, and cos8) required the presence of the *hmbR*$^+$-carrying plasmid to support growth on Hb (Table 2). Hybridization with the *hpu* probe (encoding a second *N. meningitidis* Hb receptor) did not reveal the presence of the corresponding gene on cos7 and cos9 (data not shown) (42, 43, 63).

Phenotypic characterization of Hb-utilizing *E. coli* clones. Several activities may have assisted HmbR-expressing *E. coli* in

TABLE 2. Growth on Hb as both iron and porphyrin source

E. coli hemA plus:	Growth[a]	
	hmbR$^+$ plasmid present	*hmbR*$^+$ plasmid absent
cos2	+++	−
cos4	+++	−
cos7	+++	+++
cos8	+++	−
cos9	+++	+++[b]

[a] Utilization of Hb as a source of iron and porphyrin was tested on NBD plates. Ten microliters of Hb solution (5 mg/ml) was placed on the disc, and the growth zone was measured after an overnight incubation. −, no growth; +++, growth equal or larger than 15 mm.

[b] Single colonies appearing after 36 h of incubation at 37°C.

the utilization of heme from Hb: (i) complementing clones may express outer membrane proteins that assist HmbR in binding of Hb to the surface, similar to Tbp2 proteins involved in transferrin utilization by neisseriae (12, 13, 41); (ii) complementing clones may express protease activity that makes heme available to the HmbR heme transporter; and (iii) complementing clones express the *N. meningitidis* TonB ExbBD machinery that is essential for a proper functioning of any TonB-dependent receptor (8, 31, 54). Outer membrane analysis by SDS-PAGE did not indicate the presence of any major new outer membrane protein expressed from the complementing cos2 (Fig. 2). To completely rule out the possibility that HmbR-dependent complementing cosmids are involved in Hb binding, whole cells were blotted on nitrocellulose and binding of biotinylated Hb was attempted. As can be seen from Fig. 3, *E. coli* strains expressing the *hmbR* gene from plasmids IRS1173 and pIRS626 were able to bind biotinylated Hb, while *E. coli* containing cos2 was not able to bind it. This results clearly shows that HmbR is a major determinant of Hb binding in *E. coli* reconstituted for Hb utilization. The ability of biotinylated Hb to bind to complemented HmbR-expressing cells also ruled out Hb-specific protease activity. These experiments narrowed the activity of complementing clones to the last possibility; these clones encode *N. meningitidis* TonB, ExbB, and ExbD, proteins which optimize the interaction with HmbR and enable efficient Hb utilization.

Nucleotide sequence determination of *N. meningitidis tonB*, *exbB*, and *exbD* genes. To identify genes responsible for complementation phenotype, cos2 was subcloned into pMLC210 (Cm^r [chloramphenicol-resistant]) vector, and subclones were transformed into *E. coli hemA*[pIRS626]. Six of seven cos2 *Cla*I restriction fragments, when subcloned, were unable to complement HmbR-mediated Hb utilization. Subcloning of the 5.5-kb *Cla*I restriction fragment, common to all five complementation cosmids (data not shown), was not successful even when the low-copy-number vector pWKS29/30 was used. This fragment was subcloned in two pieces by using an internal *Eco*RI restriction site (Fig. 1). Plasmids IRS1120 and IRS1133, carrying both parts of the 5.5-kb *Cla*I fragment, were also not able to complement Hb utilization in the presence of HmbR (data not shown). These data suggested that genes responsible for the complementation phenotype may contain internal *Cla*I and/or *Eco*RI restriction sites. Partial nucleotide sequence determination of each subclone was carried out to test this hypothesis. This strategy revealed that the 5.5-kb *Cla*I fragment codes for proteins with a significant homology to *P. putida* and *E. coli* ExbD and ExbB products, respectively.

Nucleotide sequence determination of the 5.5-kb *Cla*I fragment revealed four ORFs; the product of the second one had significant amino acid homology with TonB proteins, while

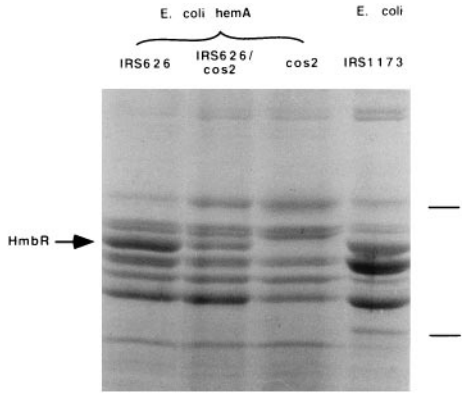

FIG. 2. SDS-PAGE analysis of *E. coli* outer membranes expressing cos2 and HmbR-expressing plasmids pIRS626 and pIRS1173 used in the study. The arrow indicates the position of the HmbR protein; bars indicate molecular mass standards (110 and 46 kDa).

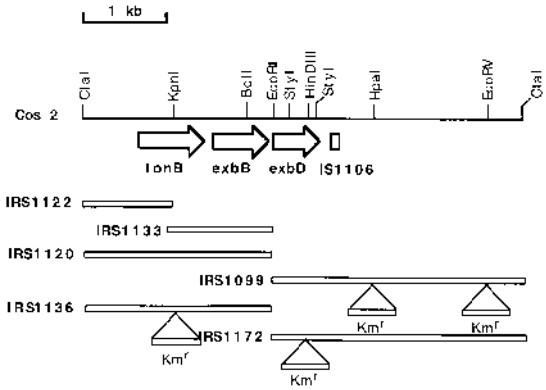

FIG. 1. Restriction maps of the *N. meningitidis tonB exbBD* locus and different plasmids used in subcloning and gene replacement experiments. Two ORFs homologous to unknown cyanobacterial ORFs were identified downstream of the *exbD* gene. Gene knockouts in these ORFs did not have any Hb or heme-related phenotype (data not shown).

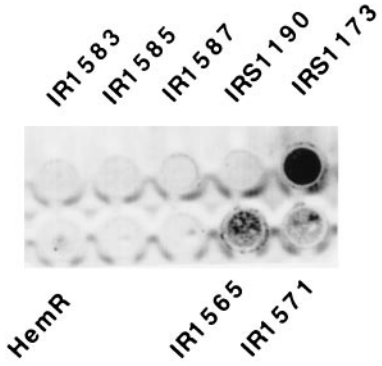

FIG. 3. Hb-binding activities of iron-starved *E. coli* whole cells expressing different plasmids used in this study. IR1583, *E. coli hemA*/cos2 [*tonB exbB exbD*]; IR1585, *E. coli hemA*/cos7 [*tonB exbB exbD*]; IR1587, *E. coli hemA*/cos9 [*tonB exbB exbD*]; IR1190, *E. coli hemA*/cos2+IRS1190 [*tonB exbB exbD hmbR*^{1190}]; IR1173, *E. coli hemA*/cos2+IRS1173 [*tonB exbB exbD hmbR*^{wt}]; HemR, *E. coli hemA*/pT76.53 [*hemRSTU'*]; IR1565, *E. coli hemA*/pIRS626 [*tonB exbB exbD hmbR*^{wt}]; IR1571, *E. coli hemA*/cos2+pIRS626 [*tonB exbB exbD hmbR*^{wt}]. Human biotinylated Hb and streptavidin-peroxidase conjugate were used.

those of the third and fourth ORFs were similar to ExbB and ExbD proteins of gram-negative bacteria, respectively (Fig. 1). Additional two ORFs, approximately 40% similar to unknown cyanobacterial ORFs, were identified on a *Cla*I-*Hpa*I DNA fragment, downstream of the *exbD* homolog (data not shown). The putative *N. meningitidis tonB* gene was preceded by a short ORF with no obvious homologs in protein data banks. It is not known whether this ORF codes for a polypeptide. The sequence GCAAATAGGAATTGTTGCT starting approximately 40 bp upstream of the *tonB* start codon resembles a Fur-binding site. A short intergenic region (67 bp) was identified between *tonB* and *exbB* genes. At least two potential Fur box sites were identified in the beginning of the *exbB* coding region. However, these sequences contained several "forbidden" nucleotide choices and therefore may be very weak binding sites (61). A start site of the *exbD* gene is located six nucleotides downstream of the last codon of *exbB*. An almost perfect 31-bp inverted repeat (AATGCCGTCTGAAAGTCT TTCAGACGGCAT) was located downstream of *exbD*. This DNA element, found in many neisserial operons, may be involved in the termination of transcription. However, this sequence is also part of the *N. meningitidis* IS*1106* element (35). Indeed, nucleotide sequence downstream of the inverted repeat was 98% identical to part of the IS*1106* element (data not shown). All five complementing cosmids hybridized with the DNA probe derived from *tonB* and *exbB* genes. However, the hybridization pattern indicated that DNA inserts in these five cosmids are not completely identical, which may partially explain the difference in complementation patterns (data not shown).

Attempts to subclone the complete Ton operon on a single plasmid were unsuccessful. Our data implicate *exbD* as a potentially toxic component of the operon, since both the *tonB* and *exbB* genes were successfully subcloned (plasmid pIRS1120 [Table 1]).

Analysis of *N. meningitidis* TonB, ExbB, and ExbD proteins. Comparison of neisserial TonB, ExbB, and ExbD proteins with homologs identified in other gram-negative bacteria is shown in Fig. 4. Overall, neisserial proteins shared approximately 30% identical amino acid residues with other TonB, ExbB, TolQ, ExbD, and TolR proteins. The relatively high degree of conservation between different ExbB, ExbD, TolQ, TolR, and TonB proteins and ORFs identified in this study clearly establishes that the cloned genes encode *N. meningitidis* TonB, ExbB, and ExbD homolog. However, the *N. meningitidis tonB* clone (cos2), when transformed into *E. coli tonB* mutant BR158, could not complement sensitivity to phage ϕ80 and colicin M (data not shown). This result indicates that the neisserial Ton system does not interact with at least one *E. coli* TonB-dependent receptor (FhuA). An attempt to reconstitute Hb utilization in *E. coli* EB53 with *N. meningitidis tonB*, *exbB* (pIRS1120), and *hmbR* (pIRS1173) genes was also not productive, indicating that *E. coli* ExbD or TolR cannot substitute for neisserial ExbD.

Construction of *N. meningitidis tonB* and *exbD* mutants and characterization of their phenotypes. To understand the role that these genes play in iron utilization by neisseriae, *tonB* and *exbD* genes were inactivated by introducing a Km^r cassette into *Kpn*I and *Hin*dIII restriction sites, respectively (Fig. 1). Southern blot hybridization indicated that the wild-type *tonB* gene was inactivated in strains IR2113 and IR2115 (Fig. 5a). *N. meningitidis* chromosomal mutants constructed in the downstream ORFs (*Hpa*I and *Eco*RV restriction sites [Fig. 1]) were also confirmed by this analysis (mutants IR2162 through IR2165 [Fig. 5a]).

A plate assay was used to determine the ability of *tonB* and *exbD* mutants to use different sources of iron (Fig. 6). The *tonB* mutation (IR2113) completely abolished Hb, transferrin, and lactoferrin utilization. Haptoglobin-Hb complexes were also not utilized by *N. meningitidis tonB* mutants. However, both *tonB* mutants (IR2113 and IR2115) were still able to use heme as a source of iron. Moreover, the zones of stimulation around heme discs were significantly larger in *tonB* mutants than in any other strains used in the study. This finding suggest that the TonB-independent heme uptake is derepressed in these mutants. *N. meningitidis exbD* mutants (IR2279 and 2281) were proficient in utilization of all sources of iron. However, zones of growth around different iron sources were smaller than in the wild-type control. For example, while the growth zone of IR1072(*exbD*$^+$) around 50 μg of human Hb was 15 to 16 mm in diameter, the same amount of Hb stimulated the *exbD* mutant only to approximately 10 mm of growth. This result suggest that either neisserial Tol proteins complement lack of ExbD function or a truncated ExbD protein produced by the *exbD* mutant is partially functional (7). Heme utilization was not affected in *exbD* mutants.

N. meningitidis mutants in the *Hpa*I and *Eco*RV restriction sites (IR2162, IR2163, IR2164, and IR2165 [Table 1 and Fig. 5a]) were not affected in Hb or heme utilization and therefore were not studied further. Hybridization data showed the presence of *tonB* and *exbB* homologs in *N. gonorrhoeae*, *N. polysaccharheae*, and serogroup C and serogroup B *N. meningitidis* strains (Fig. 5b).

DISCUSSION

We have recently cloned and characterized the Hb receptor gene, *hmbR*, from *N. meningitidis* serogroup C isolate (62, 63). The *N. meningitidis hmbR* mutant was unable to use Hb as an iron source but was still proficient in heme utilization (62). HmbR-expressing *E. coli* cells were not proficient in utilization of Hb, indicating that additional proteins are involved in Hb utilization in neisseriae. Our complementation strategy identified cosmids from an *N. meningitidis* DNA library that enabled HmbR-dependent Hb utilization in *E. coli*. Further analysis determined that three ORFs, encoding proteins highly homologous to TonB, ExbB, and ExbD proteins of different gram-negative bacteria, were present on all complementing cosmids.

The *N. meningitidis* TonB protein is 28.8 to 33.6% identical to other gram-negative TonB proteins (Fig. 4a). However, all parts of TonB protein thought to be important for its structure and/or function are present in the neisserial protein. These include (i) an amino-terminal SV-H-L motif (34, 37, 39, 65); (ii) a structurally but not functionally important proline-, glutamic acid-, and lysine-rich region that spans the periplasmic space (19, 24, 38, 65); (iii) a highly conserved PXYP motif around amino acid residue 208 (residue 160 in *E. coli* TonB) thought to interact with the outer membrane TonB boxes (22, 26, 31, 65); and (iv) two putative amphiphilic β-strands (first, 219-QGTVVLSVLV-228 followed by a β-turn [SPGG]; second, 269-FKVPVKFELN-278) containing highly conserved glycine residues (underlined) (3, 10, 11). The amino acid sequence of a putative α-helical region positioned between two amphiphilic β-strands is more similar to the same regions of *H. influenzae* and *P. putida* TonB proteins than to other enterobacterial TonB proteins (Fig. 4a). Analysis of 10 TonB proteins revealed also the conservation of an amino-proximal arginine residue, conservation of an aromatic residue in the SV-H-L motif, and absence of the conserved C-terminal glycine residue.

Comparison of different ExbB proteins and their distant

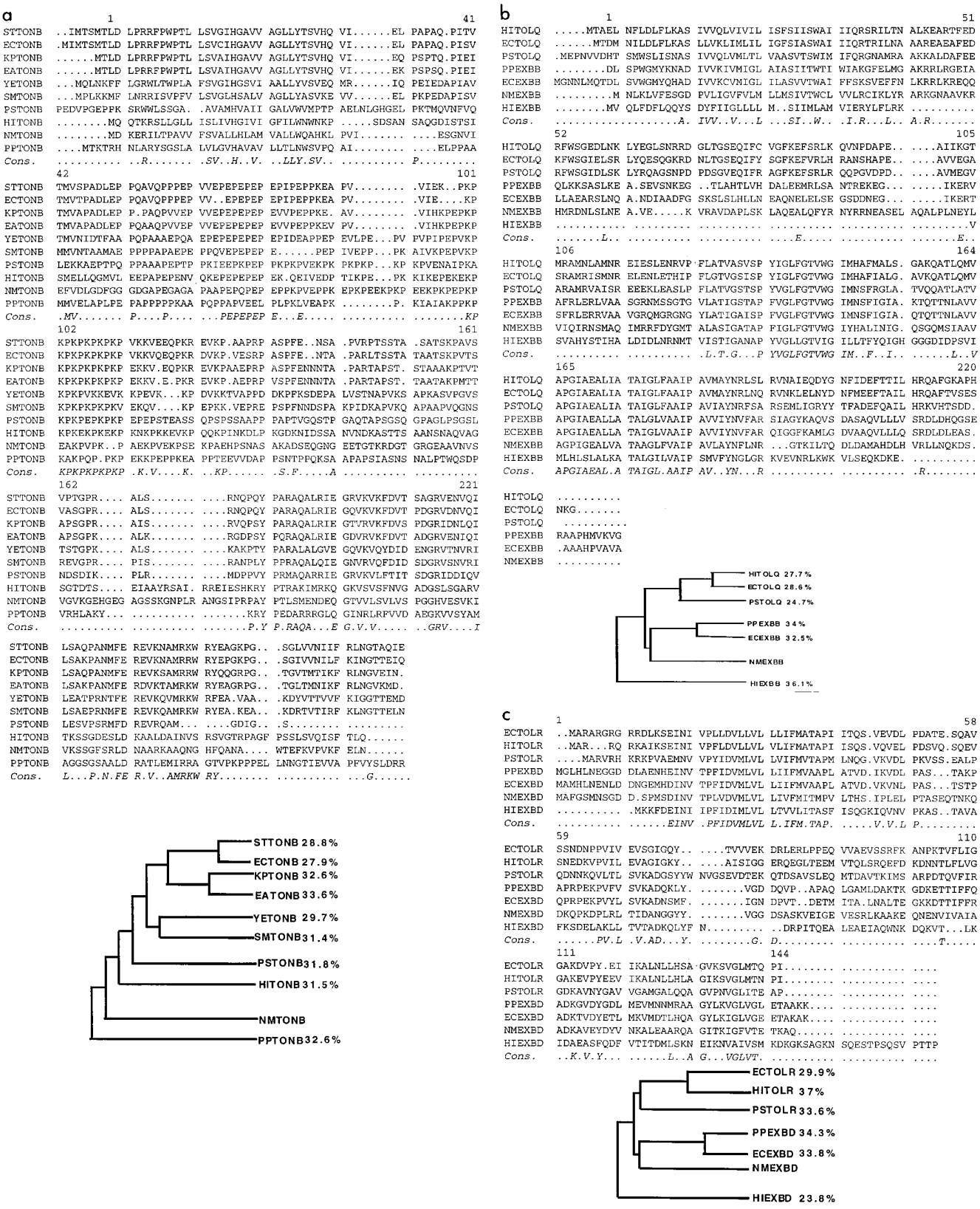

FIG. 4. Comparison of TonB (a), ExbB (b), and ExbD (c) proteins from different gram-negative bacteria. ST, *Salmonella typhimurium* (24); EC, *E. coli* (18, 53); KP, *Klebsiella pneumoniae* (11); EA, *Enterobacter aerogenes* (10); YE, *Y. enterocolitica* (36); SM, *Serratia marcescens* (21); PS, *P. aeruginosa* (52); HI, *H. influenzae* (29); NM, *N. meningitidis* (this study); PP, *P. putida* (6).

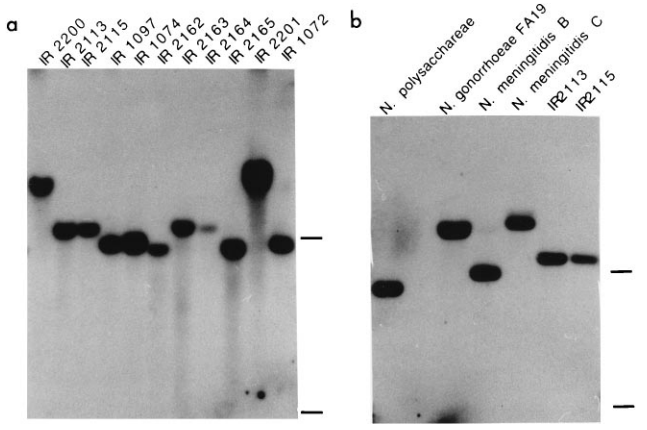

FIG. 5. (a) DNA hybridization analysis of *N. meningitidis tonB* mutants constructed in different genetic backgrounds (IR2113 constructed in IR1072, IR2115 constructed in IR1097, and IR2000 and IR2001 constructed in IR1075 [*N. meningitidis* serogroup C] backgrounds); (b) DNA hybridization analysis of *tonB exbB* genes present in different *Neisseria* species. The *Eco*RI-*Cla*I DNA probe carrying the *tonB* and *exbB* genes (Fig. 1) was used in all experiments.

TolQ relatives revealed a relatively high degree of conservation: the *N. meningitidis* ExbB protein shared between 24.7 and 36.1% identical amino acid residues with other ExbB and TolQ proteins (Fig. 4b). Overall amino acid similarity was much higher, ranging between 48.9% (*P. aeruginosa* TolQ) and 61.8% (*H. influenzae* ExbB). A large number of studies suggested that ExbB directly interacts with the TonB and ExbD proteins (1, 9, 20, 30, 34, 39, 40). This interaction occurs between their transmembrane α-helical domains (33, 37, 39, 65). A highly conserved, transmembrane α-helical $VX_3VX_3LX_3ShX_2W$ motif identified in the ExbB amino terminus (37, 39, 65) is also present in *N. meningitidis* ExbB protein (Fig. 4b). Two additional, strongly conserved regions of ExbB and TolQ proteins were also found in the *N. meningitidis* ExbB protein: a transmembrane, hydrophobic, glycine-rich α-helical region (amino acid residues 127 to 150 in *N. meningitidis* ExbB) and an alanine-rich α-helix. The alanine-rich α-helix also contains fully conserved asparagine (residue 190 in *N. meningitidis* ExbB, present in both TolQ and ExbB proteins) and arginine residues (residue 194, only in ExbB proteins) (18, 33, 34). Genetic evidence suggests that the last transmembrane α-helix of TolQ interacts with the only transmembrane helix of the tolR protein (40).

The *N. meningitidis* ExbD protein shares between 23.3 and 34.3% identical amino acids with other ExbD and TolR proteins (Fig. 4c). Overall amino acid similarity is highest with the *P. putida* ExbD protein (59.4%) and lowest with the *H. influenzae* ExbD protein (50%). The most conserved regions between all ExbD and TolR proteins are the α-helical transmembrane region in the N-terminal part of the protein (32) and the C-terminal, periplasmicly oriented region thought to interact with ExbB and TonB proteins. Mutations (D25N and L132Q) located in these conserved regions were found to severely affect the activity of ExbD (9). While the aspartic acid residue at position 25 is completely conserved in all studied proteins, leucine at position 132 is replaced with phenylalanine and isoleucine in *N. meningitidis* and *H. influenzae* ExbD proteins, respectively.

Chromosomal knockouts of *N. meningitidis tonB* and *exbD* homologs demonstrated the involvement of their products in Hb utilization in neisseriae. Inability to subclone the neisserial *tonB exbBD* operon prevented the determination of minimal set of genes necessary for Hb utilization. There is still a possibility that in addition to *tonB*, *exbB*, and *exbD*, other cosmid ORFs participate in HmbR-dependent Hb utilization. However, only the HmbR protein was shown to possess a strong Hb-binding activity, indicating that additional products, if they exist, are not involved in Hb binding. cos7 and cos9 did not need the *hmbR*-expressing plasmid for complementation of Hb utilization. This finding could be explained by postulating the existence of a new Hb utilization system or receptor encoded by these cosmids. Indeed, *hmbR*, *hpu*, and *hpu hmbR* double mutants of some neisserial strains gave rise to Hb^+ revertants, suggesting the existence of a third Hb uptake mechanism which is under a phase variation control (Fig. 6) (63). However, further study is needed to resolve this issue.

The ability of complemented *E. coli* cells to use Hb as a sole porphyrin and iron source corroborated our hypothesis that HmbR functions by removing heme from Hb and transporting it into the periplasm (62). The mechanism of heme removal from Hb is currently unknown, although successful labeling of HmbR-expressing cells and outer membranes with biotinylated Hb makes protease involvement unlikely. The most likely scenario is that a TonB-dependent conformational change in HmbR strips heme from bound Hb. How heme is used as an iron source is currently unknown, but experiments done with the heme system of *Yersinia enterocolitica* implicate a heme ABC transporter in heme utilization in gram-negative bacteria (60).

Results presented in this study indicate the existence of a TonB-independent heme uptake mechanism (HemX) in neisseriae. A similar system where the inactivation of a lipoprotein makes the strain unable to use heme was recently identified in *H. influenzae* (55). An alternative explanation would be that the hydrophobic heme molecule passes through the relatively leaky outer membrane of neisseriae without the help of a protein transporter. Our modified model of utilization of protein-bound iron by neisseriae is presented in Fig. 7 (62). The main features of the model are as follows: (i) all receptors that utilize protein-bound iron (i.e., transferrin, lactoferrin, Hb, and haptoglobin-Hb) use the cytoplasmic membrane energy provided by the TonB ExbBD machinery to transport iron and/or heme into the periplasm; (ii) some neisserial isolates

	HEME	HB	HB-HPT	HTF	HLF
IR1072 (hmbR$^+$)	++	++	-	+	+
IR1097 (ΔhmbR)	++	-*	-	+	+
IR1098 (hmbR$^-$)	++	-*	-	+	+
IR2113 (tonB$^-$)	++	-	-	-	-
IR2115 (tonB$^-$ hmbR$^-$)	++	-	-	-	-
IR1075 (hmbR$^+$ hpu$^+$)	++	++	++	+	+
IR1078 (hmbR$^-$ hpu$^+$)	++	++	++	+	+
IR2035 (hmbR$^-$ hpu$^-$)	++	-*	-	+	+
IR2200 (tonB$^-$)	++	-	-	-	-
IR2281 (exbD$^-$)	++	+	ND	ND	ND

FIG. 6. Phenotypic characterization of *N. meningitidis* mutants in the Ton operon. Bacteria were seeded onto GCB plates containing Desferal as an iron chelator. Ten microliters of each of the following solutions was applied onto paper discs and placed onto GCB-Desferal plates: human Hb (5 mg/ml), Hb-haptoglobin (HPT) (5 mg/ml); iron-saturated human lactoferrin (HLF; 10 mg/ml); iron-saturated human transferrin (HTF; 10 mg/ml); and bovine hemin chloride (5 mg/ml). Growth was recorded after overnight incubation. −, no growth around the disc; + or ++, growth around the disc; ND, not done; *, satellite colonies appearing around Hb discs after 2 days of incubation.

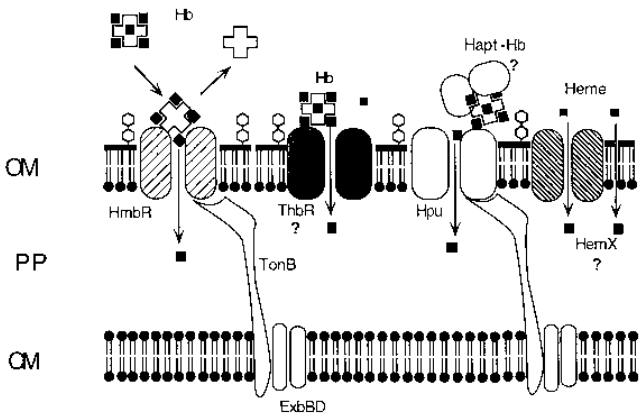

FIG. 7. Hypothetical model of utilization of heme-containing compounds by neisseriae. The HmbR protein is involved in Hb utilization and binds to the globin moiety of the Hb molecule. The Hpu protein is necessary for the utilization of Hb-haptoglobin complexes and Hb alone (42, 43). Hpu probably interacts with the heme moiety of the Hb molecule or Hb-haptoglobin complex. Both proteins function by transporting the heme moiety into the periplasm (PP), using the cytoplasmic membrane energy delivered by the TonB ExbBD machinery. The ThbR protein is a hypothetical third Hb receptor whose expression is under phase variation. The TonB-independent mechanism of heme utilization either involves an unknown outer membrane (OM) protein (HemX) or is the result of leakiness of neisserial outer membrane. Question marks indicate proteins and/or functions that have not been identified in neisseriae. IM, inner membrane.

possess at least two and perhaps three different outer membrane proteins that are involved in Hb utilization; (iii) HmbR binds globin residues of Hb and extracts heme from bound Hb; (iv) Hpu recognizes heme on the globin molecule and extracts it from Hb (this receptor is also involved in utilization of Hb-haptoglobin complexes [42, 43, 63]); (v) the expression of a third, presently unknown Hb utilization system or receptor is under phase variation control; and (vi) neisseriae possess an unknown system involved in the TonB-independent utilization of heme.

The existence of multiple systems for the utilization of heme-containing compounds reflects the importance of heme for survival of neisseriae in their natural habitat, human mucosal surfaces.

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