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A Functional *tonB* Gene Is Required for Both Utilization of Heme and Virulence Expression by *Haemophilus influenzae* Type b

GREGORY P. JAROSIK,[1] JERRY D. SANDERS,[1] LESLIE D. COPE,[1] URSULA MULLER-EBERHARD,[2] AND ERIC J. HANSEN[1]*

Department of Microbiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235,[1] and Departments of Pediatrics, Biochemistry, and Pharmacology, Cornell University Medical School, New York, New York 10021[2]

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***Haemophilus influenzae* is nearly unique among facultatively anaerobic bacteria in its absolute requirement for exogenously supplied heme for aerobic growth. In this study, a mutant analysis strategy was used to facilitate identification of *H. influenzae* cell envelope components involved in the uptake of heme. Chemical mutagenesis was employed to produce a mutant of a nontypeable *H. influenzae* strain unable to utilize either protein-bound forms of heme or low levels of free heme. This mutant was transformed with a plasmid shuttle vector-based genomic library constructed from the same wild-type nontypeable *H. influenzae* strain, and a growth selection technique was used to obtain a recombinant clone that could utilize heme. Analysis of the DNA insert in the recombinant plasmid revealed the presence of several open reading frames, one of which encoded a 28-kDa protein with significant similarity to the TonB protein of *Escherichia coli*. This *H. influenzae* gene product was able to complement a *tonB* mutation in *E. coli*, allowing the *E. coli tonB* mutant to form single colonies on minimal medium containing vitamin B_{12}. When this *H. influenzae* gene was inactivated by insertional mutagenesis techniques and introduced into the chromosome of wild-type strains of *H. influenzae* type b, the resultant transformants lost their abilities to utilize heme and produce invasive disease in an animal model. Genetic restoration of the ability to express this TonB homolog resulted in the simultaneous acquisition of both heme utilization ability and virulence. These results indicate that the *H. influenzae* TonB protein is required not only for heme utilization by this pathogen in vitro, but also for virulence of *H. influenzae* type b in an animal model.**

Haemophilus influenzae remains an important bacterial pathogen. Encapsulated serotype b strains of *H. influenzae* (Hib) cause invasive infections including meningitis, cellulitis, and epiglottitis (11), whereas unencapsulated, nontypeable *H. influenzae* (NTHI) strains produce disease primarily in the upper and lower respiratory tracts (40). The ability of *H. influenzae* to survive and produce pathologic effects in its human host is likely dependent on a multitude of virulence factors, only a few of which have been identified. These include several major surface antigens of the organism, including the polysaccharide capsule (43, 56) and lipooligosaccharide (13, 52) of Hib strains, as well as at least one outer membrane protein (10).

In contrast to the intensive study of these classic virulence factors, little effort has been made to determine which metabolic and physiologic bacterial factors are essential for the survival and growth of *H. influenzae* in vivo. This organism can be distinguished from all other bacterial pathogens by its absolute requirement for both heme and NAD for aerobic growth (17). This requirement for exogenous heme, a protoporphyrin IX (PPIX)-iron complex, is the result of the inability of *H. influenzae* to carry out the sequence of enzymatic reactions necessary to convert δ-aminolevulinic acid to PPIX, the immediate biosynthetic precursor of heme (20). Acquisition of heme by *H. influenzae* in vivo is complicated by the fact that free heme does not exist as such in the body but instead is present in hemoglobin or is tightly bound to carrier proteins, including the serum proteins hemopexin (28) and albumin (4).

Recent studies on possible heme acquisition systems of *H. influenzae* have identified several cell envelope components that may be involved in the utilization of this porphyrin, including a 39-kDa heme-binding protein that is present on the *H. influenzae* cell surface (32), a heme-binding periplasmic lipoprotein (22, 24), and a surface-exposed 100-kDa protein that is required by Hib strain DL42 for the utilization of heme bound to human hemopexin (23). However, mutant analysis studies performed with the 100-kDa heme:hemopexin-binding protein revealed that this protein is not essential for virulence expression by Hib strain DL42, at least in an animal model (23).

To investigate directly which *H. influenzae* cell envelope components are essential for heme acquisition, we used chemical mutagenesis to produce a mutant of *H. influenzae* unable to utilize heme. This mutant was then used for selection of a recombinant plasmid, from an *H. influenzae* genomic library, that complemented this mutation. The relevant *H. influenzae* gene in this plasmid was shown to encode a protein with homology to the TonB protein of *Escherichia coli*. When this gene was inactivated in Hib strains, the resultant mutants could neither utilize heme nor produce invasive disease in an animal model.

MATERIALS AND METHODS

Bacterial strains and culture conditions. NTHI strain TN106 (47), Hib strain DL302 (34), and Hib strain Eagan (37) have all been described and are listed in Table 1. *E. coli* RK5173 and RK5048 (Table 1) were generously supplied by R.

* Corresponding author. Mailing address: Department of Microbiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9048. Phone: 214-648-2284. Fax: 214-648-2199.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
H. influenzae		
TN106	Wild-type NTHI strain	47
TN106.16	Chemically induced TN106 mutant unable to grow on media containing various heme sources	This study
DL302	Wild-type Hib strain	34
DL302.3	Cm^r mutant unable to grow on media containing various heme sources; obtained by transforming DL302 with linearized pGJ302	This study
DL302.42	Cm^r mutant unable to grow on various heme sources; obtained by transforming DL302 with linearized pGJ342	This study
DL302.425	Cm^s, *tonB*$^+$ transformant obtained by transforming DL302.42 with DL302 chromosomal DNA	This study
Eagan	Wild-type Hib strain	37
Eagan.15	Cm^r mutant unable to grow on media containing various heme sources; obtained by transforming Eagan with linearized pGJ302	This study
E. coli		
RK5173	F *araD139* Δ(*argF-lac*)*U169 relA1 rpsL150 deoC1 flb-5301 thi gyrA219 non metE70*	R. Kadner (25)
RK5048	As RK5173 but also *recA tonB*	R. Kadner (25)
Plasmids		
pLS88	Plasmid cloning shuttle vector; Kn^r Sulf^r Strep^r	55
pGJ300	pLS88 containing 3.6-kb fragment of NTHI TN106 chromosomal DNA encoding the *H. influenzae tonB* gene	This study
pGJ302	pGJ300 derivative in which an internal 1.2-kb *Bgl*II fragment of the 3.6-kb insert was replaced by a *cat* cartridge; in this construct, part of the *tonB* gene and all of a second gene likely involved in DNA replication/repair were deleted	This study
pGJ303	pGJ300 containing a *Xba*I linker inserted into the *Apa*LI site within the *tonB* gene	This study
pGJ342	pGJ300 containing a *cat* cartridge inserted into the *Xho*I site within the *tonB* gene	This study

Kadner (25). *H. influenzae* strains were grown in brain heart infusion (BHI) broth containing both NAD (10 μg/ml) and a heme source. Free heme was supplied as hemin (heme chloride) at 8.5 μg/ml in all experiments except one, in which heme was incorporated into BHI-NAD agar at a final concentration of 50 μg/ml. PPIX was incorporated into media at a final concentration of 20 μg/ml. Other heme sources included the following: human hemoglobin, 200 μg/ml; heme:human hemopexin, 5 μg/ml; hemoglobin:human haptoglobin, 50 μg/ml; and 10% (vol/vol) Levinthal's base (i.e., a horse blood extract [1]). Media for *E. coli* consisted of LB or M9 minimal medium appropriately supplemented (33, 36). When required, antimicrobial compounds were added at the following concentrations: kanamycin, 20 μg/ml; chloramphenicol, 0.5 μg/ml; sulfonamide, 250 μg/ml.

Genetic techniques. Standard genetic techniques including plasmid isolation, restriction enzyme digestions, DNA modifications, ligation reactions, and transformation of *E. coli* were performed as described elsewhere (33, 46, 53). A genomic DNA library was constructed from the wild-type NTHI strain TN106 in the shuttle vector pLS88 (55), using the *Eco*RI site in this vector for cloning purposes. Transformation of *H. influenzae* with linear (27) or plasmid (51) DNA was performed as described, as was electroporation of NTHI strain TN106 with plasmid DNA (26).

Mutagenesis. Chemical mutagenesis of NTHI strain TN106 was accomplished by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (12). Briefly, TN106 cells in the logarithmic phase of growth were treated with NTG (100 μg/ml) for 30 min, washed, and incubated in BHI-NAD-PPIX broth for 30 min at 37°C. These cells were then plated on BHI-NAD-PPIX agar plates, and the plates were incubated overnight at 37°C in a 95% air–5% CO_2 atmosphere. The resultant colonies were tested for growth on BHI NAD agar plates containing various heme sources. Insertional mutagenesis of cloned genes in recombinant plasmids was accomplished by ligating either a 1.2-kb chloramphenicol acetyltransferase (*cat*) gene cartridge or an *Xba*I linker (New England Biolabs) into the desired restriction site.

DNA sequence and Southern blot analyses. Nucleotide sequence analysis of the 3.6-kb *Eco*RI insert from pGJ300 was performed by using nested deletions and other standard methods (46). DNA sequence information was analyzed using the Intelligenetics Suite package and programs from the University of Wisconsin Genetics Computer Group sequence analysis software package (15). Southern blot analysis of chromosomal DNAs purified from wild-type, mutant, and transformant Hib strains was performed as described elsewhere (26, 47).

Virulence testing. The virulence of wild-type and mutant strains of Hib was assessed by using intraperitoneal (49) and intranasal (38) challenge of infant rats, with subsequent detection of bacteremia as evidence of invasive disease (49).

Vitamin B_{12} growth assay. The *E. coli metE tonB* mutant strain RK5048 was transformed with the vector plasmid pLS88 or with recombinant plasmids containing either the wild-type or mutated versions of the *H. influenzae tonB* gene; the abilities of wild-type, mutant, and transformant strains of *E. coli* to utilize vitamin B_{12} were determined by a plate growth assay as described elsewhere (21). In addition, the ability of the *H. influenzae tonB* gene to complement this *E. coli tonB* mutation was also assessed using a liquid medium identical to the solidified medium described above, with the exception that the liquid medium also contained ferric chloride (0.5 μg/ml) and sodium citrate (5 mM).

Nucleotide sequence accession number. The *H. influenzae tonB* nucleotide sequence has been submitted to GenBank and assigned accession no. U04996.

TABLE 2. Growth of wild-type, mutant, and recombinant *H. influenzae* strains on BHI-NAD agar supplemented with various heme sources

Strain	Supplement[a]					
	PPIX	Lev	Heme (8.5 μg/ml)	Hg	Hg-Hpt	Hm-Hpx
NTHI						
TN106	+	+	+	+	+	+
TN106.16	+	−	−	−	−	−
TN106.16(pGJ300)	+	+	+	+	+	+
TN106.16(pGJ302)	+	−	−	−	−	−
TN106.16(pGJ303)	+	−	−	−	−	−
TN106.16(pGJ342)	+	−	−	−	−	−
Hib						
DL302	+	+	+	+	+	+
DL302.3	+	−	−	−	−	−
DL302.3(pGJ300)	+	+	+	+	+	+
DL302.42	+	−	−	−	NT	NT
DL302.42(pGJ300)	+	+	+	+	NT	NT
DL302.42(pGJ303)	+	−	−	−	NT	NT
DL302.42(pGJ342)	+	−	−	−	NT	NT
DL302.425	+	+	+	+	NT	NT
Hib						
Eagan	+	+	+	+	+	+
Eagan.15	+	−	−	−	−	−
Eagan.15(pGJ300)	+	+	+	+	+	+

[a] Lev, Levinthal's base; Hg, hemoglobin; Hg-Hpt, hemoglobin:haptoglobin; Hm-Hpx, heme:hemopexin; NT, not tested.

RESULTS

Production of an NTHI mutant unable to utilize heme. NTHI strain TN106 was mutagenized with NTG, and the survivors were selected by their abilities to grow on BHI-NAD agar plates containing PPIX instead of heme. *H. influenzae* can synthesize heme by using the enzyme ferrochelatase to insert ferrous iron into PPIX (20, 54), thus allowing growth of the organism in the absence of exogenously supplied heme. These mutagenesis survivors were then tested for their abilities to grow on BHI-NAD agar plates containing free heme (8.5 μg/ml). From 2,500 colonies screened in this manner, one mutant, designated TN106.16, that could not utilize either free or protein-bound forms of heme was identified (Table 2).

Cloning of an NTHI gene that complemented the mutation in TN106.16. This mutant was electroporated with a genomic DNA library from the wild-type NTHI strain TN106 that had been constructed in the plasmid shuttle vector pLS88 (55). The electroporation reaction mixture was plated on BHI-NAD-heme plates containing sulfonamide to select for recombinants possessing the ability to utilize heme. A transformant that was able to grow on this BHI-NAD-heme medium was shown to possess a recombinant plasmid with a 3.6-kb DNA insert; this plasmid was designated pGJ300 (Fig. 1A). The presence of pGJ300 in TN106.16 allowed this mutant to utilize not only free heme, but all of the other heme compounds that could not be utilized by the original mutant (Table 2). In an attempt to localize the DNA encoding this heme utilization ability, the 1.2-kb *Bgl*II fragment within the insert in pGJ300 was excised and replaced with a *cat* cartridge, yielding the mutated plasmid pGJ302 (Fig. 1B). This mutated plasmid could not complement the mutation in strain TN106.16 (Table 2), indicating that the excised DNA region likely contained, at least in part, or had a polar effect on, the gene(s) which complemented the chemically induced mutation in TN106.16.

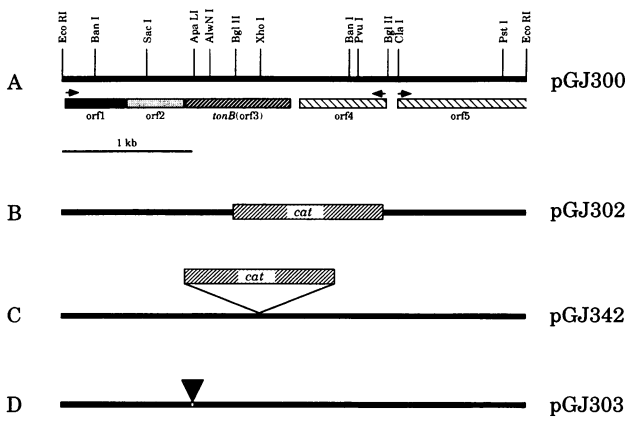

FIG. 1. Partial restriction enzyme map of the 3.6-kb NTHI TN106 chromosomal DNA insert in pGJ300 and its derivatives. (A) ORFs present in the wild-type DNA insert in pGJ300 (the arrows indicate the direction of transcription of each ORF); (B) the mutated insert in pGJ302 in which the 1.2-kb *Bgl*II fragment was excised and replaced with a 1.2-kb *cat* cartridge; (C) the mutated insert in pGJ342 in which the 1.2-kb *cat* cartridge was inserted into the *Xho*I site within the *tonB* ORF; (D) the mutated insert in pGJ303 in which an *Xba*I linker (triangle) was inserted into the *Apa*LI site located near the proximal end of the *tonB* ORF.

Nucleotide sequence analysis of the 3.6-kb insert. Several open reading frames (ORFs) were identified in the 3.6-kb DNA insert in pGJ300. Two of these ORFs (ORF4 and ORF5 in Fig. 1A) had divergent promoters and encoded products that had significant homology with the *E. coli* single-stranded DNA binding protein (35) and the *E. coli* UvrA protein (29), respectively (data not shown). The other three ORFs were arranged in tandem (Fig. 1A). The deduced amino acid sequence of ORF1 was 27% identical to that of the ExbB protein of *E. coli* (16), while that of ORF2 was 28% identical to the ExbD protein of the same organism (16). ORF3 (Fig. 1A) encoded a protein that had 31% identity with the TonB protein of *E. coli* (42).

The gene and putative protein product encoded by ORF3 (Fig. 1A) share several features with the *E. coli tonB* gene and its protein product. First, this ORF encodes a protein with a predicted molecular weight of 28,379 that also has a hydrophobic amino terminus like that of the 26-kDa *E. coli* TonB protein. Second, both this 28-kDa *H. influenzae* protein and the *E. coli* TonB protein contain a proline-rich region in which Glu-Pro repeats are followed by Lys-Pro repeats (Fig. 2 and 3). Third, both this *H. influenzae* protein and the *E. coli* TonB molecule contain a glutamine residue at position 160 (Fig. 2 and 3); this particular glutamine residue has been shown to be important for the function of the *E. coli* TonB protein (5). Finally, similarly to *E. coli*, the region immediately downstream from this *H. influenzae* ORF contains regions of dyad symmetry of unknown function but which could be involved in termination of transcription (Fig. 2).

Complementation of an *E. coli tonB* mutation. The similarities between this 28-kDa *H. influenzae* protein and the *E. coli* TonB molecule prompted us to determine directly whether the former could replace TonB in *E. coli*. It has been well established that the uptake of vitamin B_{12} by *E. coli* requires a functional TonB protein (3). Therefore, to determine whether this 28-kDa *H. influenzae* protein exhibited TonB activity, we performed complementation analysis using an *E. coli* strain (RK5048) which is mutated in both its *metE* and *tonB* genes.

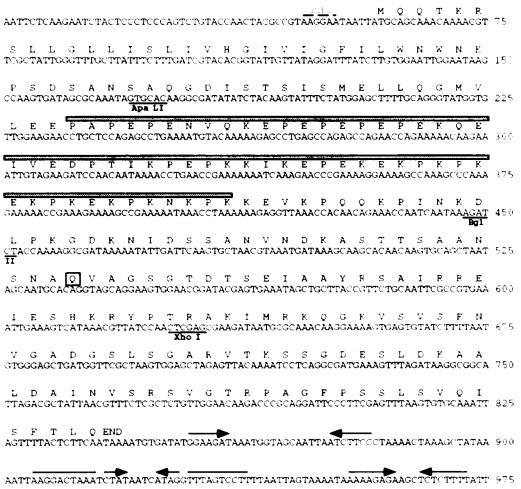

FIG. 2. Nucleotide sequence of the *H. influenzae tonB* gene together with the deduced amino acid sequence. The putative ribosomal binding site is designated S.D. in the first line. The stippled bar denotes the proline-rich region containing the Glu-Pro and Lys-Pro repeats. The box denotes the glutamine residue at position 160. The arrows indicate regions of dyad symmetry immediately downstream from the *tonB* gene. Pertinent restriction enzyme sites are also shown.

The *metE* mutation results in a requirement for either methionine or vitamin B_{12} for growth on minimal medium (3). However, this strain cannot transport vitamin B_{12} because it has a *tonB* mutation (3).

E. coli RK5048 was transformed with pGJ300, and the resultant transformants were tested for their abilities to grow on a solidified minimal medium containing 5 μM vitamin B_{12}. In addition, this *E. coli tonB* mutant was transformed with the pLS88 vector and with pGJ302, which has a deletion mutation within the ORF encoding the 28-kDa *H. influenzae* protein (Fig. 1B). The *E. coli tonB* mutant strain RK5048 and the transformant strains RK5048(pLS88) and RK5048(pGJ302) all failed to grow on these assay plates. In contrast, both the *E. coli metE* mutant strain RK5173, which has a functional *E. coli* TonB protein (25), and the *E. coli* transformant strain RK5048(pGJ300), containing the gene encoding the *H. influenzae* 28-kDa protein, readily formed single colonies on this medium. These results, together with the amino acid sequence

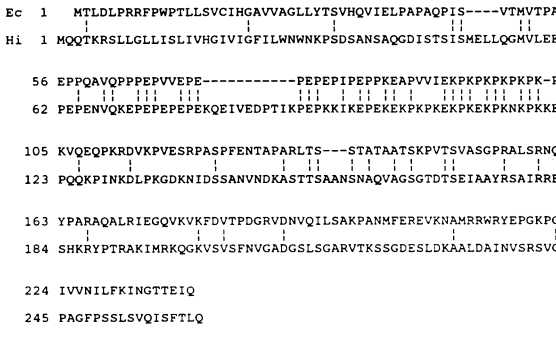

FIG. 3. Comparison of the amino acid sequences of the *H. influenzae* (Hi) and *E. coli* (Ec) TonB proteins. Vertical bars indicate identity between residues. The glutamine residue at position 160 in each protein is indicated by shading.

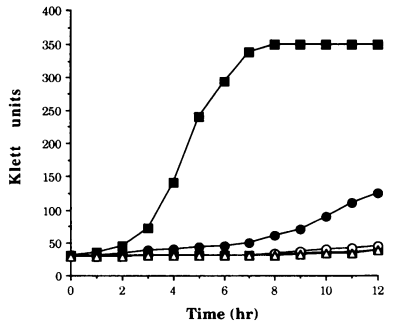

FIG. 4. Growth of wild-type, mutant, and recombinant *E. coli* strains in minimal medium containing vitamin B_{12}. These strains included the *metE* mutant strain RK5173 (■), the *metE tonB* mutant strain RK5048 (□), the recombinant strain RK5048(pLS88) (○), the recombinant strain RK5048(pGJ300) (●), and the recombinant strain RK5048(pGJ303) (△).

similarities described above, indicate that the 28-kDa protein encoded by ORF3 (Fig. 1A) is the *H. influenzae* TonB protein.

Further evaluation of the ability of the *H. influenzae tonB* gene to complement an *E. coli tonB* mutation involved the use of a liquid growth medium to determine whether the *H. influenzae* TonB protein was truly interchangeable with its *E. coli* counterpart. The *E. coli metE* mutant strain RK5173 containing a functional *E. coli tonB* gene grew readily in this medium (Fig. 4). The *E. coli metE tonB* mutant RK5048 could not grow at all in this minimal medium containing vitamin B_{12}, while the presence of the *H. influenzae tonB* gene in the recombinant strain RK5048(pGJ300) permitted only limited growth in this medium (Fig. 4).

Effect of a *tonB* mutation on virulence of Hib. Taken together, the preceding experiments indicated that a functional TonB protein is required by *H. influenzae* for utilization of heme. However, it is likely that a *tonB* mutation may also affect several other transport processes potentially relevant to growth of *H. influenzae* in vivo (e.g., utilization of transferrin-bound iron [14]). To investigate directly the effect of a *tonB* mutation on the virulence of *H. influenzae*, we constructed isogenic Hib *tonB* mutants for evaluation in an animal model for invasive Hib disease. We used a linearized form of pGJ302 to transform the wild-type Hib strains Eagan and DL302 and thereby introduce the deletion construct containing the *cat* cartridge into the chromosomes of these two strains by allelic exchange; the desired transformants were selected on BHI-NAD-PPIX plates containing chloramphenicol. Two Cm^r transformants, DL302.3 and Eagan.15, were shown to be unable to utilize either protein-bound heme or low levels of free heme (Table 2). The presence of the wild-type *H. influenzae tonB* gene in *trans*, in the form of pGJ300, complemented the mutation in each of these strains, allowing them to utilize heme (Table 2).

Intraperitoneal challenge of infant rats with the wild-type DL302 and Eagan strains confirmed that, as demonstrated previously (34, 39), both of these Hib strains are virulent in this model (Table 3). In contrast, the mutant strain DL302.3 was avirulent in this model, failing to produce detectable bacteremia in any of the 11 animals that were challenged with this strain (Table 3). Similarly, the mutant strain Eagan.15 was much less virulent than its respective parent strain, producing detectable bacteremia in only 2 of 11 animals challenged with this mutant (Table 3). The greatly reduced virulence of this latter mutant is emphasized by the fact that the mean level of bacteremia in these two infected animals was 3 orders of

TABLE 3. Virulence of wild-type, mutant, and transformant strains of Hib in the infant rat model

Strain	Challenge route[a]	Inoculum (CFU/animal)	No. of bacteremic animals/total no. of animals	Mean CFU/ml of blood
DL302	IP	61	22/22	4.9×10^4
DL302.3	IP	107	0/11	0
DL302.42	IP	131	0/15	0
DL302.425	IP	74	14/14	3.9×10^4
Eagan	IP	4	10/10	3.4×10^4
Eagan.15	IP	39	2/11	63
Eagan	IN	1×10^7	10/10	5.9×10^3
Eagan.15	IN	1×10^9	0/10	0

[a] IP, intraperitoneal; IN, intranasal.

magnitude lower than that present in the animals challenged with the wild-type Eagan strain (Table 3).

It is clear that the *tonB* mutation in Hib strain Eagan.15 greatly reduced but did not totally eliminate the virulence potential of this strain, as assessed by the intraperitoneal challenge route in the infant rat model. To provide a more stringent test of this mutant's virulence potential, we used the intranasal challenge route to evaluate the virulence of the Eagan.15 mutant. This latter model system demands penetration from the nasopharynx into the bloodstream as well as survival and growth in vivo for successful infection (37, 44, 45). When used in the intranasal challenge model, the Eagan.15 mutant was avirulent and failed to cause detectable bacteremia in any of the animals challenged with this strain, even when the challenge inoculum was 2 orders of magnitude greater than that of the wild-type parent strain (Table 3).

The only limitation to the preceding experiments was that the deletion of the *Bgl*II fragment used to construct pGJ302 removed not only part of the *tonB* ORF but also part of ORF4, whose protein product, tentatively identified as the *H. influenzae* homolog of the *E. coli* single-stranded DNA binding protein, is likely involved in DNA replication and repair (Fig. 1A and B). To eliminate any possibility that this latter ORF was somehow involved in heme utilization, we inactivated the cloned *tonB* gene by two different methods. First, we introduced a *cat* cartridge into the *Xho*I site within the *tonB* ORF, yielding the mutated plasmid pGJ342 (Fig. 1C). Second, we inserted an *Xba*I linker into the *Apa*LI site at the 5′ end of the *tonB* gene, yielding the mutated plasmid pGJ303 (Fig. 1D). As expected, neither plasmid could complement the original chemically induced mutation in NTHI TN106.16 (Table 2). Furthermore, neither plasmid could complement the *tonB* mutation in *E. coli* RK5048 on solidified growth medium containing vitamin B_{12}. In addition, the recombinant strain *E. coli* RK5048(pGJ303) could not grow at all in liquid growth medium containing vitamin B_{12} (Fig. 4).

To take advantage of the selection ability permitted by use of the *cat* cartridge, a linearized form of pGJ342 was used to transform Hib strain DL302, and several Cm^r transformants were obtained, none of which could utilize heme. One of these transformants, which was designated DL302.42 (Table 2), was selected for further study. Again, as expected, the mutation in DL302.42 was complemented by pGJ300 but could not be complemented by either pGJ303 or pGJ342 (Table 2). This mutant and the wild-type parent strain exhibited indistinguishable growth rates in BHI-NAD-PPIX medium (data not shown).

To confirm that proper allelic exchange (i.e., replacement of the wild-type *tonB* gene with the mutated *tonB* gene containing the *cat* cartridge) had occurred in this Cm^r mutant, chromosomal DNA preparations from the wild-type strain DL302 and the Cm^r mutant strain DL302.42 were digested with *Nde*I and probed by Southern blot analysis with either an *H. influenzae tonB*-specific probe or the *cat* cartridge. The wild-type parent strain possessed a 5.5-kb *Nde*I fragment reactive with the *tonB* probe (Fig. 5A, lane 1), whereas the Cm^r mutant had a 6.7-kb *Nde*I fragment that hybridized to this same probe (Fig. 5A, lane 2); the 1.2-kb difference in the sizes of these two *tonB*-hybridizing fragments is consistent with the presence of a single 1.2-kb *cat* cartridge in the mutant strain's *tonB* gene. The presence of the *cat* cartridge within the *tonB* gene of this mutant was confirmed by the finding that a 6.7-kb *Nde*I fragment from this strain hybridized to the *cat* probe (Fig. 5B, lane 2); the DNA from the wild-type strain did not bind this probe (Fig. 5B, lane 1).

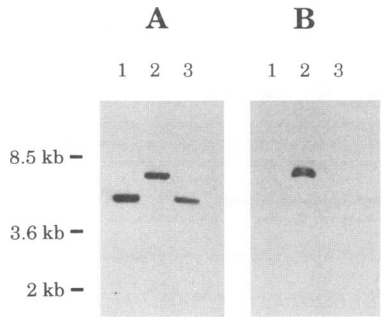

FIG. 5. Southern blot analysis of *Nde*I-digested chromosomal DNAs from the wild-type Hib strain DL302 (lane 1), the isogenic Cm^r *tonB* mutant strain DL302.42 (lane 2), and the Cm^s transformant strain DL302.425 (lane 3) using the 0.5-kb *H. influenzae tonB*-specific *Apa*LI-*Xho*I fragment from pGJ300 (A) and the 1.2-kb *cat* cartridge (B) as probes. Size markers in kilobases are shown on the left side of this figure.

Virulence testing of the isogenic *tonB* mutant strain DL302.42 revealed that this mutant was unable to produce detectable bacteremia in the infant rat model (Table 3). To confirm further that the *tonB* mutation was responsible for the inability of strain DL302.42 to utilize heme and cause invasive disease, we transformed this mutant with chromosomal DNA from the wild-type DL302 strain and selected for growth on BHI-NAD-heme agar plates. Several Cm^s transformants were obtained that could utilize heme, and one of these, which was designated DL302.425 (Table 2), was selected for further study. DL302.425 proved to be fully virulent when tested in the infant rat model (Table 3). Southern blot analysis of chromosomal DNA from this transformant proved that it no longer possessed a *cat* cartridge within its *tonB* gene (Fig. 5A and B, lane 3).

TonB is not required for growth of *H. influenzae* on excess heme. All three *H. influenzae tonB* mutants constructed in this study (TN106.16, DL302.42, and Eagan.15) were unable to grow on BHI-NAD agar plates containing heme at a final concentration of 8.5 μg/ml (Table 2). However, when the heme concentration was increased to 50 μg/ml, all three *tonB* mutants were able to form single colonies on this medium (data not shown).

DISCUSSION

The TonB protein, first identified functionally by its ability to allow *E. coli* to be infected by the bacteriophage T1 (41), is now known to be required by this enteric bacterium for the

uptake and utilization of several different nutrients or growth factors, including iron-siderophore complexes and vitamin B_{12} (41). Recent advances in studies of the function of this protein indicate that TonB is anchored in the cytoplasmic membrane and likely spans the periplasm to contact outer membrane proteins involved in transport processes (41). Moreover, it has been proposed that TonB acts to transduce energy from the cytoplasmic membrane to specific energy-requiring processes in the outer membrane, resulting in the release into the periplasm of ligands bound by these outer membrane proteins (41). These TonB-dependent outer membrane proteins share several conserved regions, including one near the amino terminus called the TonB box which likely interacts directly with the TonB protein (5, 7, 25, 48).

Very recently, genes encoding TonB homologs have been identified in a number of other enteric bacteria (8, 9, 19, 31) and in at least one *Pseudomonas* species (6). In *Neisseria gonorrhoeae*, from which a *tonB* gene has not been cloned to date, the identification of an outer membrane receptor possessing a TonB box indicates the likely presence of a TonB protein in this organism (14). The apparent dependence of *H. influenzae* on a functional TonB protein for utilization of either protein-bound heme or low levels of free heme in vitro, as demonstrated in the present study, allows one to infer that there must exist one or more *H. influenzae* outer membrane proteins that are TonB dependent and involved in the uptake of heme. Indeed, utilization of heme as an iron source by *Yersinia enterocolitica* was recently shown to be TonB dependent, and a heme-binding outer membrane receptor has been identified in this enteric pathogen (31, 50).

Little is known about how *H. influenzae* satisfies its absolute requirement for heme for aerobic growth, especially while growing in vivo where free heme as such does not exist. While at least two *H. influenzae* outer membrane proteins that bind either free heme or heme carrier proteins have been identified recently (i.e., the 39-kDa heme-binding protein [32] and the 100-kDa heme:hemopexin-binding protein [23]), there is no information available concerning the amino acid sequence of the 39-kDa protein, and we have recently determined that the 100-kDa protein does not possess a TonB box (data not shown). However, the fact that the *H. influenzae tonB* mutation prevented utilization of both protein-bound heme and low levels of free heme suggests that there may be a common, TonB-dependent pathway for entry of heme through the outer membrane of *H. influenzae* once the free heme or heme-protein complex has been bound to the cell surface.

The fact that TonB is not required by *H. influenzae* when heme is present in great excess (e.g., 50 μg/ml) is reminiscent of the ability of *E. coli tonB* mutants to utilize vitamin B_{12} when this latter compound is present in the growth medium at high levels (3). How high concentrations of free heme permit utilization of heme by *H. influenzae* in the absence of TonB is not known, and the physiological relevance of this process is questionable when one considers that free heme does not exist in vivo.

Our results on the TonB dependence of low-level heme utilization by *H. influenzae* also raise an interesting question concerning the physiology of porphyrin uptake by this organism. If utilization of heme by *H. influenzae* is dependent on the presence of a functional TonB protein, then how does PPIX enter the *H. influenzae* cell? Clearly, the *H. influenzae tonB* mutants can satisfy their heme requirement by synthesizing heme from PPIX and iron via the activity of ferrochelatase (Table 2). Preliminary testing revealed that these *tonB* mutants, which cannot utilize heme provided at 8.5 μg/ml, can readily form single colonies on BHI-NAD agar containing PPIX at only 2 μg/ml (data not shown). While heme and PPIX differ only by the presence of an iron atom in the heme molecule, it now appears that these two porphyrin compounds enter *H. influenzae* by distinct mechanisms. Investigation of this interesting dichotomy has been initiated in our laboratory.

It is worthwhile pointing out that, while the presence of the *H. influenzae* TonB protein permitted an *E. coli metE tonB* mutant to readily form single colonies on minimal medium agar plates containing vitamin B_{12}, this *H. influenzae* molecule did not allow either a wild-type rate or extent of growth of this mutant in a liquid medium (Fig. 4). In view of the fact that the identity between the *E. coli* and *H. influenzae* TonB proteins is only 31%, this lack of complete complementation of the *E. coli tonB* mutation by the *H. influenzae tonB* gene is not surprising. In fact, when *tonB* genes from other organisms (i.e., *Enterobacter aerogenes* [9], *Serratia marcescens* [19], and *Pseudomonas putida* [6]) were placed in *trans* in *E. coli tonB* mutants, the degree of complementation varied directly with the extent of identity between the *E. coli* and the individual, heterologous TonB proteins.

We have also identified two ORFs (ORF1 and ORF2 in Fig. 1A) immediately upstream of the *H. influenzae tonB* gene that encode products that have significant similarity to the ExbB and ExbD proteins, respectively, of *E. coli* (16). These last two proteins are thought to form a complex that stabilizes the TonB protein in the cytoplasm of *E. coli*, acting as a chaperone prior to and during translocation of TonB (18, 30). If these two ORFs do indeed encode the *H. influenzae* homologs of ExbB and ExbD, then the positioning of the *H. influenzae tonB* gene immediately downstream from these ORFs resembles closely the arrangement of the *exbB*, *exbD*, and *tonB* genes of *P. putida* (6). In contrast, the *tonB* gene of *E. coli* is located in a locus distant from the *exbB* and *exbD* genes (2). Work is currently in progress to determine whether these ORFs upstream of the *tonB* gene in *H. influenzae* actually encode functional ExbB and ExbD homologs.

It should be noted that, to the best of our knowledge, this is the first report of a *tonB* mutation affecting virulence expression by a pathogen as well as the first description of an *H. influenzae* mutant unable to utilize heme. Clearly, the lack of a functional TonB protein precluded the utilization of either protein-bound heme or low levels of free heme by these *H. influenzae* mutants (Table 2). The Hib *tonB* mutants were also essentially unable to produce invasive disease in the infant rat model, and this deleterious effect of the *tonB* mutation on virulence expression was shown not to be Hib strain specific (Table 3). What effect, if any, the *tonB* mutation may exert on the ability of Hib strains to colonize the nasopharynxes of rats was not directly assessed in this study. However, the dramatic effect of the *tonB* mutation on the virulence potential of these Hib strains was clearly evident regardless of the route of infection (Table 3).

While it is tempting to conclude that this inability to use heme is responsible for the failure of these Hib *tonB* mutants to produce invasive disease, we cannot formally exclude the possibility that the *tonB* mutation affected transport of another nutrient(s) essential for the survival of *H. influenzae* in vivo. Attempts to prove a causal relationship between the heme utilization defect and the avirulence of the Hib *tonB* mutants would necessarily involve the provision of large quantities of both PPIX and soluble iron compounds in vivo to circumvent this bacterium's requirement for heme; these experiments have been precluded by physiological limitations inherent in the infant rat model. Therefore, resolution of this issue will require identification of the TonB-dependent outer membrane protein(s) that must be involved in heme utilization by *H. influ-*

enzae. Subsequent mutational analysis of the responsible gene(s) should allow us to determine conclusively the relationship between heme acquisition and virulence expression by this pathogen in an animal model.

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