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Utilization of Transferrin-Bound Iron by *Haemophilus influenzae* Requires an Intact *tonB* Gene

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Haemophilus influenzae **can utilize iron-loaded human transferrin as an iron source for growth in vitro. *H. influenzae tonB* mutants, containing a chloramphenicol acetyltransferase gene within their *tonB* genes, could bind iron-charged human transferrin to their cell surfaces, but they were unable to utilize this serum glycoprotein as the sole source of iron for growth in vitro. In contrast, these *tonB* mutants were able to utilize an iron chelate (ferric ammonium citrate) for growth. Transformation of a *tonB* mutant with a plasmid encoding a wild-type *H. influenzae tonB* gene restored the ability of a *tonB* mutant to utilize iron-charged human transferrin. These results indicate that the uptake of iron from human transferrin by *H. influenzae* is a TonB-dependent process.**

In order to survive within the human host, both commensal and pathogenic bacteria must acquire iron, an essential cofactor of enzymes involved in many biological processes, including energy production (17). However, these bacteria are faced with the challenge that virtually all of the extracellular iron found in the human host is tightly bound to high-affinity iron-binding proteins (5) such as lactoferrin (2) and transferrin (19). In order to survive and multiply in this iron-restricted environment, bacteria have evolved efficient mechanisms to acquire iron from host sources. Some organisms express siderophores, which are low-molecular-weight, high-affinity iron chelators that compete directly with host iron-binding factors (8, 22, 25). Other bacteria synthesize outer membrane receptors that recognize host molecules or proteins which contain iron (24), including heme (4, 9, 15, 23), lactoferrin (3, 32, 33), and transferrin (1, 6, 7, 12, 16, 21, 30, 31, 34). The best-studied examples of the latter class include both the pathogenic *Neisseria* species and *Haemophilus influenzae*, which bind iron-loaded host proteins directly to their cell surfaces.

It has been established that *H. influenzae* can utilize a number of compounds as iron sources for growth in vitro, including ferric salts, heme, and iron-loaded human transferrin (26, 36). Utilization of the latter serum glycoprotein as a source of iron has been shown to involve two outer membrane proteins, designated Tbp1 and Tbp2, which have apparent molecular masses of approximately 100 and 70 to 90 kDa, respectively, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13, 30, 31). It is also known that these proteins are expressed in vivo (12) and that their expression, at least in vitro, is regulated by iron (11, 30) or heme (20).

Recent analyses of the Tbp1 outer membrane protein expressed by pathogenic *Neisseria* species revealed that this polypeptide is homologous to TonB-dependent proteins (7, 16). This particular class of outer membrane proteins has been shown to be involved in the uptake and utilization of several nutrients or growth factors, including iron bound to siderophores (18, 27). In *Escherichia coli*, the TonB protein is anchored in the cytoplasmic membrane and spans the periplasm to contact outer membrane proteins involved in energy-dependent transport processes. Energy transfer catalyzed by the TonB protein results in the release into the periplasm of the ligand bound by the TonB-dependent outer membrane protein. By analogy with the gonococcal and meningococcal Tbp1 proteins, the Tbp1 protein of *H. influenzae* is also likely a TonB-dependent protein that would function to facilitate the energy-dependent transfer of transferrin-derived iron across the outer membrane.

Direct evidence for the involvement of a TonB protein in the utilization of transferrin-bound iron by *Neisseria gonorrhoeae* and *Neisseria meningitidis* is still lacking because the neisserial *tonB* gene has not been identified to date. In contrast, the availability of the cloned *H. influenzae tonB* gene (14) presented the opportunity to investigate directly the possible involvement of TonB in the utilization of transferrin-bound iron by this pathogen.

Construction of a *tonB* mutant. A *tonB* mutant of nontypeable *H. influenzae* (NTHI) strain TN106 (Table 1) was constructed by transforming the wild-type NTHI strain with the 4.9-kb *Eco*RI insert from pGJ342 (14). This cloned NTHI TN106 DNA segment contains a chloramphenicol acetyltransferase (*cat*) gene inserted within the *tonB* gene, and it was previously used to construct *tonB* mutants of *H. influenzae* type b (Hib) strains by transformation and allelic exchange (14). Transformants were selected on brain heart infusion (Difco Laboratories, Detroit, Mich.) agar plates supplemented with NAD (10 μg/ml), protoporphyrin IX (PPIX) (20 μg/ml), and chloramphenicol (0.5 μg/ml), which were incubated overnight at 37°C in an atmosphere of 95% air–5% CO_2. PPIX was used in place of heme because a previous study (14) revealed that *tonB* mutants of *H. influenzae*, which cannot utilize heme for aerobic growth, can satisfy their heme requirement by synthesizing heme from PPIX and iron via the activity of the enzyme ferrochelatase (10, 35). One of the chloramphenicol-resistant transformants, designated TN106.42 (Table 1), was selected for further testing and was shown to be unable to utilize heme or heme-containing compounds for aerobic growth. As seen before with Hib *tonB* mutants (14), this NTHI *tonB* mutant could be complemented by the recombinant plasmid pGJ300, which contains the wild-type NTHI TN106 *tonB* gene.

Effect of the *tonB* mutation on utilization of transferrin-bound iron. In addition to the wild-type strain TN106 and the *tonB* mutant strain TN106.42, three other TN106.42-derived transformant strains were tested for their ability to utilize iron

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
H. influenzae		
TN106	Wild-type NTHI strain	29
TN106.42	Cm^r *tonB* mutant unable to utilize heme; obtained by transforming TN106 with the mutated *tonB* gene from pGJ342	This study
TN106.42(pLS88)	TN106.42 carrying the shuttle vector pLS88	This study
TN106.42(pGJ300)	TN106.42 carrying the recombinant plasmid pGJ300 with a wild-type NTHI TN106 *tonB* gene	This study
TN106.42(pGJ342)	TN106.42 carrying the plasmid pGJ342 with a mutated *tonB* gene	This study
DL302	Wild-type Hib strain	14
DL302.42	Cm^r *tonB* mutant of Hib strain DL302 unable to utilize heme	14
DL302.42(pGJ300)	DL302.42 carrying the recombinant plasmid pGJ300 with the wild-type NTHI TN106 *tonB* gene	14
Plasmids		
pLS88	Shuttle vector (Kan^r Sulf^r Str^r) capable of replication in *E. coli*, *H. influenzae*, and *Haemophilus ducreyi*	37
pGJ300	pLS88 containing a 3.6-kb *Eco*RI insert of TN106 chromosomal DNA carrying the *tonB* gene	14
pGJ342	pGJ300 containing a *cat* cartridge inserted into the *Xho*I site within the *tonB* gene	14

bound to transferrin. These contained the shuttle vector pLS88, the recombinant plasmid pGJ300 with a wild-type *tonB* gene, or plasmid pGJ342 with the mutated *tonB* gene (Table 1).

Each strain was grown into the mid-logarithmic phase (10^9 CFU/ml) in brain heart infusion broth supplemented with NAD (10 μg/ml) and PPIX (20 μg/ml). These cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C and washed twice with unsupplemented brain heart infusion broth, and 10^7 CFU was spread onto the surface of WSJM agar plates prepared as described previously (38) except that this medium lacked ferric nitrate and contained NAD (10 μg/ml), PPIX (2 μg/ml), and the iron chelator ethylenediamine di(*o*-hydroxyphenylacetic acid) (Sigma Chemical Co., St. Louis, Mo.) at a 100 μM final concentration. Sterile filter paper disks (4-mm diameter) were then placed onto the surface of the agar and loaded with 10 μl of phosphate-buffered saline (PBS), ferric ammonium citrate (5.2 mg/ml), or iron-loaded human transferrin (Sigma Chemical Co.) (20 mg/ml). These plates were incubated as described above for 24 to 36 h.

As expected, no growth was visible around the disks that had been loaded with PBS, whereas all five strains were able to utilize ferric ammonium citrate as an iron source for growth (Fig. 1). In contrast, while the wild-type strain (Fig. 1A) was able to grow around the disk containing transferrin as the source of iron, the *tonB* mutant strain TN106.42 (Fig. 1B) was not. When a wild-type *tonB* gene was present in *trans* in this mutant [strain TN106.42(pGJ300); Fig. 1D], the presence of the functional *tonB* gene allowed the utilization of iron bound to transferrin and growth of the mutant. The presence of the shuttle vector pLS88 (Fig. 1C) or a mutated *tonB* gene in *trans* (Fig. 1E) did not allow the utilization of transferrin-bound iron by the *tonB* mutant. This deleterious effect of the *tonB* mutation on utilization of transferrin-bound iron was also observed when a Hib strain (DL302) and its isogenic *tonB* mutant (DL302.42) were tested in this same system, with only the wild-type Hib strain being able to grow around the disk containing the iron-loaded transferrin (data not shown). Again, provision of the wild-type *tonB* gene in *trans* allowed this mutant [DL302.42(pGJ300)] to utilize transferrin-bound iron (data not shown). These results indicate that utilization by *H. influenzae* of transferrin as an iron source requires an intact *tonB* gene.

Binding of transferrin by an *H. influenzae tonB* mutant. By analogy with the pathogenic *Neisseria* species (1, 7, 16), utilization of transferrin-bound iron by *H. influenzae* likely involves the binding of this serum glycoprotein to the bacterial cell surface via the Tbp1 and Tbp2 proteins (31). To determine whether the *tonB* mutation might have affected this binding ability, a dot blot transferrin binding assay was performed (30, 31). Cell paste from cultures of the five strains described above grown on brain heart infusion agar plates supplemented with NAD (10 μg/ml), PPIX (2 μg/ml), and the iron chelator Desferal (desferoxamine mesylate; Ciba-Geigy Ltd., Basel, Switzerland) (30 μM) was spotted onto Whatman No. 40 filter paper and dried at 37°C for 1 h. *E. coli* HB101 (28) was utilized as a negative control and was grown on Luria-Bertani agar plates (28). After incubation with Tris-buffered saline, pH 7.4,

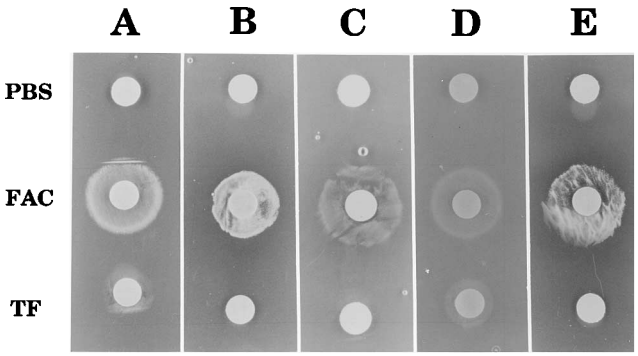

FIG. 1. Utilization of transferrin-bound iron as the sole iron source for growth by wild-type, mutant, and transformant strains of *H. influenzae*. Logarithmic-phase cells of the wild-type NTHI strain TN106 (A), the *tonB* mutant strain TN106.42 (B), the transformant strain TN106.42(pLS88) (C), the transformant strain TN106.42(pGJ300) (D), and the transformant strain TN106.42(pGJ342) (E) were spread onto WSJM agar plates supplemented as described in the text. Paper filter disks placed onto the surfaces of the plates were loaded with PBS (a negative control), ferric ammonium citrate (FAC), or iron-loaded human transferrin (TF), and the plates were incubated as described in the text.

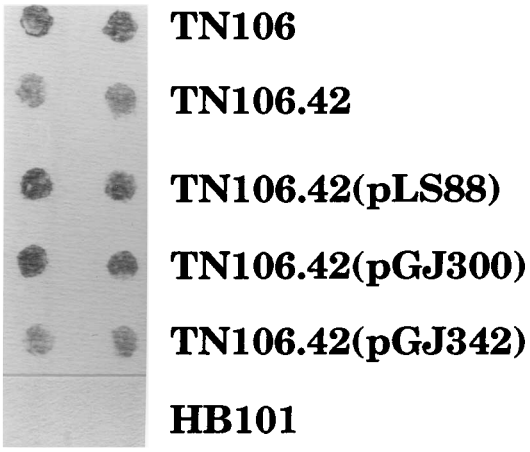

FIG. 2. Binding of transferrin by wild-type, mutant, and transformant strains of *H. influenzae*. Cell paste from each strain was spotted in duplicate on filter paper, which was then incubated with horseradish peroxidase-conjugated human transferrin. Strain designations are listed on the right.

containing 0.5% (wt/vol) nonfat dry milk for 1 h, the filters were incubated with horseradish peroxidase-conjugated human transferrin (Jackson Immunoresearch Labs, West Grove, Pa.) for 4 h at room temperature. Following three 15-min washes with Tris-buffered saline, the blots were developed by incubating the filters in Tris-buffered saline containing 0.01% hydrogen peroxide and 4-chloro-1-naphthol (2 mg/ml).

The *E. coli* strain did not bind transferrin at detectable levels, as expected (Fig. 2). In contrast, all five of the *H. influenzae* strains tested, including the *tonB* mutant TN106.42, were able to bind transferrin (Fig. 2). These results indicate that although the *tonB* mutation eliminated the ability of *H. influenzae* to utilize transferrin as a source of iron for growth in vitro (Fig. 1), this phenotype was not the result of an inability of the *tonB* mutant to bind transferrin to its cell surface.

In *H. influenzae*, an intact *tonB* gene is required for acquisition of heme from a variety of sources in vitro (14). In this report, we have demonstrated that a functional *H. influenzae tonB* gene is also required for *H. influenzae* to grow with transferrin as the sole source of iron. Thus, the utilization of transferrin-bound iron by *H. influenzae* appears to be a TonB-dependent process, from which it can be inferred that one or more TonB-dependent outer membrane proteins (e.g., Tbp1 or Tbp2) involved in this process likely exist. This likelihood is reinforced by the fact that the Tbp1 outer membrane protein involved in the utilization of transferrin-bound iron by *N. gonorrhoeae* and *N. meningitidis* has been shown to have the characteristics of a TonB-dependent protein (7, 16). Similarly, we have recently identified a 79-kDa *H. influenzae* outer membrane protein that is involved with the utilization of heme and which appears to be a member of the TonB-dependent class of outer membrane proteins (5a). Final determination of whether the *H. influenzae* Tbp1 protein is also a TonB-dependent outer membrane protein will necessarily have to await the cloning and subsequent analysis of the genes encoding the transferrin-binding proteins of *H. influenzae*.

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REFERENCES

1. **Anderson, J. E., P. F. Sparling, and C. N. Cornelissen.** 1994. Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization. J. Bacteriol. **176:**3162–3170.
2. **Bezkorovainy, A.** 1987. Iron proteins, p. 69–137. *In* J. J. Bullen and E. Griffiths (ed.), Iron and infection. Molecular, physiological and clinical aspects. Wiley, Chichester, United Kingdom.
3. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172:**5225–5235.
4. **Bramanti, T. E., and S. C. Holt.** 1993. Hemin uptake in *Porphyromonas gingivalis*: Omp26 is a hemin-binding surface protein. J. Bacteriol. **175:**7413–7420.
5. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. **80:**1–35.

5a. **Cope, L. D., R. Yogev, U. Muller-Eberhard, and E. J. Hansen.** A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. Submitted for publication.

6. **Cornelissen, C. N., G. D. Biswas, and P. F. Sparling.** 1993. Expression of gonococcal transferrin-binding protein 1 causes *Escherichia coli* to bind human transferrin. J. Bacteriol. **175:**2448–2450.
7. **Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson, and P. F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J. Bacteriol. **174:**5788–5797.
8. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53:**517–530.
9. **Daskaleros, P. A., J. A. Stoebner, and S. M. Payne.** 1991. Iron uptake in *Plesiomonas shigelloides*: cloning of the genes for the heme-iron uptake system. Infect. Immun. **59:**2706–2711.
10. **Granick, S., and H. Gilder.** 1946. The porphyrin requirements of *Haemophilus influenzae* and some functions of the vinyl and propionic acid side chains of heme. J. Gen. Physiol. **30:**1–13.
11. **Hardie, K. R., R. A. Adams, and K. J. Towner.** 1993. Transferrin-binding ability of invasive and commensal isolates of *Haemophilus* spp. J. Med. Microbiol. **39:**218–224.
12. **Holland, J., P. R. Langford, K. J. Towner, and P. Williams.** 1992. Evidence for in vivo expression of transferrin-binding proteins in *Haemophilus influenzae* type b. Infect. Immun. **60:**2986–2991.
13. **Holland, J., K. J. Towner, and P. Williams.** 1991. Isolation and characterisation of *Haemophilus influenzae* type b mutants defective in transferrin-binding and iron assimilation. FEMS Microbiol. Lett. **77:**283–287.
14. **Jarosik, G. P., J. D. Sanders, L. D. Cope, U. Muller-Eberhard, and E. J. Hansen.** 1994. A functional *tonB* gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* type B. Infect. Immun. **62:**2470–2477.
15. **Lee, B. C.** 1992. Isolation of an outer membrane hemin-binding protein of *Haemophilus influenzae* type b. Infect. Immun. **60:**810–816.
16. **Legrain, M., V. Mazarin, S. W. Irwin, B. Bouchon, M.-J. Quentin-Millet, and A. B. Schryvers.** 1993. Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. Gene **130:** 73–80.
17. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. **6:**137–149.
18. **Lundrigan, M. D., and R. J. Kadner.** 1986. Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in *Escherichia coli*: homology among outer membrane receptors that interact with TonB. J. Biol. Chem. **261:** 10797–10801.
19. **Morgan, E. H.** 1981. Transferrin. Biochemistry, physiology, and clinical significance. Mol. Aspects Med. **4:**1–123.
20. **Morton, D. J., J. M. Musser, and T. L. Stull.** 1993. Expression of the *Haemophilus influenzae* transferrin receptor is repressible by hemin but not elemental iron alone. Infect. Immun. **61:**4033–4037.
21. **Morton, D. J., and P. Williams.** 1990. Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. J. Gen. Microbiol. **136:**927–933.
22. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:** 715–731.
23. **Otto, B. R., M. Sparrius, A. M. J. J. Verweij-van Vught, and D. M. MacLaren.** 1990. Iron-regulated outer membrane protein of *Bacteroides fragilis* involved in heme uptake. Infect. Immun. **58:**3954–3958.
24. **Otto, B. R., A. M. J. J. Verweij-van Vught, and D. M. MacLaren.** 1992. Transferrins and heme-compounds as iron sources for pathogenic bacteria. Crit. Rev. Microbiol. **18:**217–233.
25. **Payne, S. M.** 1988. Iron and virulence in the family *Enterobacteriaceae*. Crit. Rev. Microbiol. **16:**81–111.
26. **Pidcock, K. A., J. A. Wooten, B. A. Daley, and T. L. Stull.** 1988. Iron acquisition by *Haemophilus influenzae*. Infect. Immun. **56:**721–725.
27. **Postle, K.** 1990. TonB and the Gram-negative dilemma. Mol. Microbiol. **4:**2019–2025.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sanders, J. D., L. D. Cope, G. P. Jarosik, I. Maciver, J. L. Latimer, G. B. Toews, and E. J. Hansen.** 1993. Reconstitution of a porin-deficient mutant of

Haemophilus influenzae type b with a porin gene from nontypeable *H. influenzae*. Infect. Immun. **61:**3966–3975.
30. **Schryvers, A. B.** 1988. Characterization of the human transferrin and lactoferrin receptors in *Haemophilus influenzae*. Mol. Microbiol. **2:**467–472.
31. **Schryvers, A. B.** 1989. Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. J. Med. Microbiol. **29:**121–130.
32. **Schryvers, A. B., and B. C. Lee.** 1989. Comparative analysis of the transferrin and lactoferrin binding proteins in the family *Neisseriaceae*. Can. J. Microbiol. **35:**409–415.
33. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the human lactoferrin-binding protein from *Neisseria meningitidis*. Infect. Immun. **56:**1144–1149.
34. **Tsai, J., D. W. Dyer, and P. F. Sparling.** 1988. Loss of transferrin receptor activity in *Neisseria meningitidis* correlates with inability to use transferrin as an iron source. Infect. Immun. **56:**3132–3138.
35. **White, D. C., and S. Granick.** 1963. Hemin biosynthesis in *Haemophilus*. J. Bacteriol. **85:**842–850.
36. **Williams, P., D. J. Morton, K. J. Towner, P. Stevenson, and E. Griffiths.** 1990. Utilization of enterobactin and other exogenous iron sources by *Haemophilus influenzae*, *H. parainfluenzae* and *H. paraphrophilus*. J. Gen. Microbiol. **136:**2343–2350.
37. **Willson, P. J., W. L. Albritton, L. Slaney, and J. K. Setlow.** 1989. Characterization of a multiple antibiotic resistance plasmid from *Haemophilus ducreyi*. Antimicrob. Agents Chemother. **33:**1627–1630.
38. **Wong, T. P., R. K. Shockley, and K. H. Johnston.** 1980. WSJM, a simple chemically defined medium for growth of *Neisseria gonorrhoeae*. J. Clin. Microbiol. **11:**363–369.