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Identification and Characterization of Genes Encoding the Human Transferrin-Binding Proteins from *Haemophilus influenzae*

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***Haemophilus influenzae*, a strict human pathogen, acquires iron in vivo through the direct binding and removal of iron from human transferrin by an as yet uncharacterized process at the bacterial cell surface. In this study, the *tbpA* and *tbpB* genes of *H. influenzae*, encoding the transferrin-binding proteins Tbp1 and Tbp2, respectively, were cloned and sequenced. Alignments of the *H. influenzae* Tbp1 and Tbp2 protein sequences with those of related proteins from heterologous species were analyzed. On the basis of similarities between these and previously characterized proteins, Tbp1 appears to be a member of the TonB-dependent family of outer membrane proteins while Tbp2 is lipid modified by signal peptidase II. Isogenic mutants deficient in expression of Tbp1 or Tbp2 or both proteins were prepared by insertion of the Tn*903* kanamycin resistance cassette into cloned sequences and reintroduction of the interrupted sequences into the wild-type chromosome. Binding assays with the mutants showed that a significant reduction in transferrin-binding ability resulted from the loss of either of the Tbps and a complete loss of binding was evident when neither protein was expressed. Loss of either Tbp2 or both proteins correlated with an inability to grow on media supplemented with transferrin-bound iron as the sole source of iron, whereas the Tbp1$^+$ Tbp2$^-$ mutant was able to grow only at high transferrin concentrations.**

Although iron is abundant within mammalian tissues, the environment which confronts bacterial pathogens possesses free iron at levels far below that which is required to support bacterial growth (51). Virtually all iron within the mammalian body is held intracellularly as ferritin or as heme compounds, pools which are generally inaccessible to invading microorganisms. Additionally, the small amount of iron present in extracellular spaces is effectively chelated by high-affinity iron-binding host glycoproteins: transferrin (Tf) is present in serum and lymph, while lactoferrin functions to bind free iron present in secretory fluids and in milk (32). To further combat microbial invasion, a rapid shift in iron metabolism occurs early in infection, leading to a decrease in serum iron levels held by Tf to as much as two-thirds below that present in the uninfected state (52). To overcome this important means of nutritional immunity, invading microorganisms must possess some mechanism by which they may gain access to the host iron stores.

Haemophilus influenzae is an important human pathogen and a strict parasite of humans. Although it is commonly carried asymptomatically in the upper respiratory tract mucosa of infants, symptomatic disease may result when the bacterium spreads from this site. Encapsulated type b *H. influenzae* is an etiologic agent of epiglottitis and pneumonia and is a common cause of bacterial sepsis and meningitis in children worldwide (28). Although rarely responsible for systemic infections, the contiguous spread of nonencapsulated (nontypeable) strains of *H. influenzae* within the upper respiratory tract can lead to the development of sinusitis or otitis media. Additionally, movement of nontypeable strains into the lower respiratory tract can exacerbate chronic pulmonary disease (28).

During growth in an aerobic environment, *H. influenzae* has an absolute requirement for a source of porphyrin, and heme can supply both porphyrin and iron requirements for the cell (9). Although levels of free heme are negligible in the extracellular spaces because of the presence of an effective system of scavenging proteins within the host, *H. influenzae* is able to acquire heme from these heme-carrier protein complexes (33, 48). The importance of heme as a source of iron in vivo is uncertain because iron requirements for optimal growth of *H. influenzae* exceed the porphyrin requirements (9). This, together with the fact that porphyrin is not required for anaerobic growth of *H. influenzae* (53), suggests that an alternate mechanism of acquiring iron would prove important to ensure that iron does not become a limiting nutrient during infection of the host.

H. influenzae can use human Tf (hTf) as a sole source of iron for growth (15, 33), and the ability to utilize iron from glycoprotein of only human origin may at least partially explain the narrow host range of this organism (26). In accordance with growth studies, hTf-binding activity was detected in membranes isolated under iron-deficient conditions (38), and an affinity isolation procedure was subsequently developed and used to isolate and identify two Tf-binding proteins (Tbps) from the membrane of these bacteria (39). Although the specificity of the *H. influenzae* Tf receptor system has been questioned (27), a recent study characterized its binding specificity to be such that only those species of transferrins which occur in the hominoid lineage of primates could interact with the Tbps (14).

Isolation of Tbps from a variety of type b and nontypeable strains of *H. influenzae* showed the larger Tbp (Tbp1) to have a molecular mass of approximately 100 kDa. The lower-molecular-mass protein (Tbp2) is more variable in size but is generally between 75 and 85 kDa. In contrast to Tbp1, Tbp2 retains Tf-binding activity even after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting (39).

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In the present study, importance of the Tbps for growth of *H. influenzae* on Tf was probed. The genes encoding Tbp1 and Tbp2 have been cloned and sequenced, and isogenic mutants deficient in Tbp1, Tbp2, or both have been constructed and characterized. Additionally, an alignment of the predicted sequence of these proteins with that of analogous proteins is examined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. influenzae* type b strain DL63 was obtained from E. Hansen, University of Texas Southwestern Medical Center, Dallas, Tex. Cells stored at −70°C in 30% glycerol were streaked onto chocolate agar plates and grown overnight in an atmosphere containing 5% CO_2. Freshly grown *H. influenzae* cells were then used to inoculate supplemented liquid brain heart infusion (Difco Laboratories) to a starting optical density at 650 nm of 0.05, and cultures were grown at 37°C with shaking. For iron-replete growth, the liquid cultures were supplemented with 3.32 μg of NAD per ml and 10 μg of hemin per ml (BHINH medium). Iron-restricted conditions were obtained by growth in the absence of hemin (BHIN medium) and by the addition of ethylenediamine di-*O*-phenylacetic acid (EDDA; BHINE medium).

Growth assays were performed essentially as outlined previously (30), except that NAD was included on the assay plates. Also, because *H. influenzae* DL63 cannot use protoporphyrin IX as a porphyrin source (data not shown), the growth assay mixtures were incubated in an anaerobic environment to take advantage of the fact that *H. influenzae* does not require porphyrin when growing anaerobically. To accomplish this, plates were incubated at 37°C in an anaerobic environment created by an Anaerocult A package (Merck) in a sealed jar as outlined by the manufacturer. Sterile 0.25-in. (0.64-cm) concentration disks (Difco) containing the indicated supplements were placed on the surface of the preseeded and dried plates.

Before use in growth assays, commercial Tf preparations were dialyzed against three changes of 0.1 M sodium acetate–0.1 M sodium phosphate monobasic (pH 5.5) containing 25 mM EDTA to remove any insoluble iron complexes associated with the glycoproteins. The apo-Tf was then iron loaded by using $FeCl_3$ dissolved in 100 mM sodium citrate–100 mM sodium bicarbonate buffer (pH 8.6). The resultant Tf preparation was subjected to gel filtration to remove unbound and/or insoluble iron complexes before being added to the disks. *Pasteurella haemolytica*, a bacterial species possessing a Tf receptor system specific for bovine Tf (bTf) (30), was used as a control in growth experiments to ensure the iron-free status of growth supplements and media and the iron-loaded status of bTf (data not shown).

Escherichia coli SURE was obtained from Stratagene Cloning Systems, La Jolla, Calif., and all *E. coli* strains were routinely cultured on Luria-Bertani medium (35).

Chemicals. hTf, prestained protein molecular weight markers, peroxidase-conjugated goat anti-rabbit immunoglobulin G, cyanogen bromide-activated Sepharose CL4B, and the antimicrobial agents used were obtained from Sigma Chemical Co., St. Louis, Mo. NAD, hemin, protoporphyrin IX, EDDA, and 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) were also purchased from Sigma. Horseradish peroxidase (HRP)-conjugated hTf was obtained from Jackson Immunoresearch Laboratories, Avondale, Pa., while HRP-conjugated bTf was prepared by the method of Wilson and Nakane (54). Restriction endonucleases and molecular biological reagents were obtained from Pharmacia, Baie d'Urfe, Quebec, Canada, or from Betheseda Research Laboratories, Bethesda, Md.

Isolation of Tbps and preparation of polyclonal antisera. The *H. influenzae* hTf receptor proteins (Tbp1 and Tbp2) used for the production of polyclonal antisera were isolated on the basis of the method outlined previously for an avian *Haemophilus* sp. (31). Briefly, a 20-ml hTf-Sepharose column was prepared as described by the manufacturer's protocol for coupling protein ligands to CNBr-activated Sepharose. The resulting matrix was washed with 3 column volumes of 50 mM Tris-HCl–1 M NaCl (pH 8.0) buffer with 6 M guanidine-HCl to remove noncovalently attached hTf. The column was then equilibrated in 50 mM Tris-HCl (pH 8.0) buffer, and bound hTf was iron loaded by using 1 ml of $FeCl_3$ (10 mg/ml) in buffer containing 100 mM each sodium citrate and sodium bicarbonate (pH 8.6) followed by 2 column volumes of 50 mM Tris-HCl–1 M NaCl (pH 8.0).

Bacterial total membrane (300 mg of total protein) prepared from *H. influenzae* grown on iron-deficient medium as outlined previously (39) was diluted to 2 mg/ml in 50 mM Tris-HCl–1 M NaCl (pH 8.0) buffer and solubilized by the addition of EDTA to 15 mM and Sarkosyl NL97 to a final concentration of 1.5%. After centrifugation at 40,000 × *g* for 1 h, the supernatant was applied to the hTf column; the column was then washed with 10 column volumes of 50 mM Tris-HCl–1 M NaCl (pH 8.0) buffer containing 10 mM EDTA and 0.5% Sarkosyl. Bound receptor proteins were eluted with 2.0 M guanidine-HCl in the same buffer, and eluted fractions were dialyzed extensively into 25 mM ammonium bicarbonate buffer, lyophilized, and stored at −20°C. Isolated Tbps were used to produce polyclonal antiserum in female New Zealand White rabbits by standard immunization techniques.

The small-batch affinity isolation procedure used to compare the hTf-Tbp binding interactions of wild-type and mutant *H. influenzae* was performed essentially as described previously (18), except that binding was done overnight at 4°C.

Introduction of insertion constructs into *H. influenzae*. Insertion constructs HIT1KFH and HIT1KFP were introduced into the *H. influenzae* wild-type genome by transformation with M-IV medium as described previously (6), and transformants were selected on BHINH agar containing 20 μg of kanamycin per ml. Kanamycin-resistant colonies were screened for introduction of the Tn*903* Kan cassette into chromosomal sequences corresponding to the HIT1 and HIT2 clones by direct amplification of DNA sequences from single colonies (37). PCR used to screen transformants involved oligonucleotide primer pairs as outlined in the Results.

Tf-binding assays. The solid-phase binding assay used to determine Tf-binding activity of the *H. influenzae* strains was as outlined previously by Schryvers and Morris (43), except that bound peroxidase was detected with the chromogenic HRP substrate tetramethylbenzidine plus H_2O_2.

Electrophoresis and electroblotting. All SDS-PAGE gels were run with the buffer system of Laemmli (20). Immunological analysis of electroblotted proteins was performed as described previously (14), except that the rabbit anti-*H. influenzae* Tbp polyclonal antiserum generated in this study was used. The blots were developed with luminol as outlined by Ausubel et al. (4).

Nucleotide sequence accession number. The *H. influenzae* DL63 *tbpA* and *tbpB* gene sequences presented here have been deposited into GenBank under accession number U10882.

RESULTS

Isolation and characterization of gene fragments encoding *H. influenzae* Tbp1 and Tbp2. A lambda ZAPII expression library containing random *H. influenzae* chromosomal DNA fragments was plated on *E. coli* SURE, and plaques were screened with the rabbit anti-Tbp polyclonal antiserum. Immunopositive plaques were purified through repeated plating, and plasmid was rescued by in vivo excision (45). Restriction endonuclease digestion analysis of recovered plasmids revealed two clones with apparently overlapping regions (pBHIT1 and pBHIT2; Fig. 1). Western blot analysis of whole cells possessing pBHIT1 and pBHIT2 detected proteins of 80 and 60 kDa, respectively, which reacted with anti-*H. influenzae* Tbp1 and Tbp2 polyclonal antisera (data not shown).

The nucleotide sequence of both clones was obtained in both strands by using the chain termination method of Sanger et al. (36) and a combination of vector-specific primers and primers synthesized to correspond to sequences throughout the clones (data not shown). Translation of the nucleotide sequences obtained and comparison of these with that of the Tbps previously characterized (8, 11, 22) revealed that the clones did not contain the complete coding sequences for these genes. Two methods were used to obtain gene sequences not contained within the HIT1 and HIT2 clones. The region upstream of the HIT1 clone was obtained by ligating *Eco*RI-digested chromosomal DNA isolated from the isogenic mutant strain DL63KFH (see below) into the unique *Eco*RI site in the pBluescript polylinker. Competent *E. coli* DH5α cells transformed with this ligation mixture were then screened for kanamycin resistance by growth on Luria-Bertani agar containing 40 μg of kanamycin per ml. Kanamycin-resistant clones were screened first by restriction mapping and comparison with the HIT clones, and final verification was obtained by sequencing regions overlapping HIT1. The upstream clone was designated as pBHIT4 (Fig. 1).

The region downstream of the HIT2 clone was obtained by ligation of *Eco*RI-digested chromosomal DNA from wild-type *H. influenzae* DL63 into the *Eco*RI site of pBluescript. PCR amplification of this crude ligation mixture with a vector-specific oligonucleotide primer plus a primer created with known sequence from near the 3′ end of the HIT clones (oligo 86; Table 1) allowed amplification in the downstream direction. A *Hin*dIII site introduced near the 5′ end of oligo 86 along with the *Hin*dIII site from the vector polylinker allowed the amplified product to be cloned into the *Hin*dIII site of pBluescript.

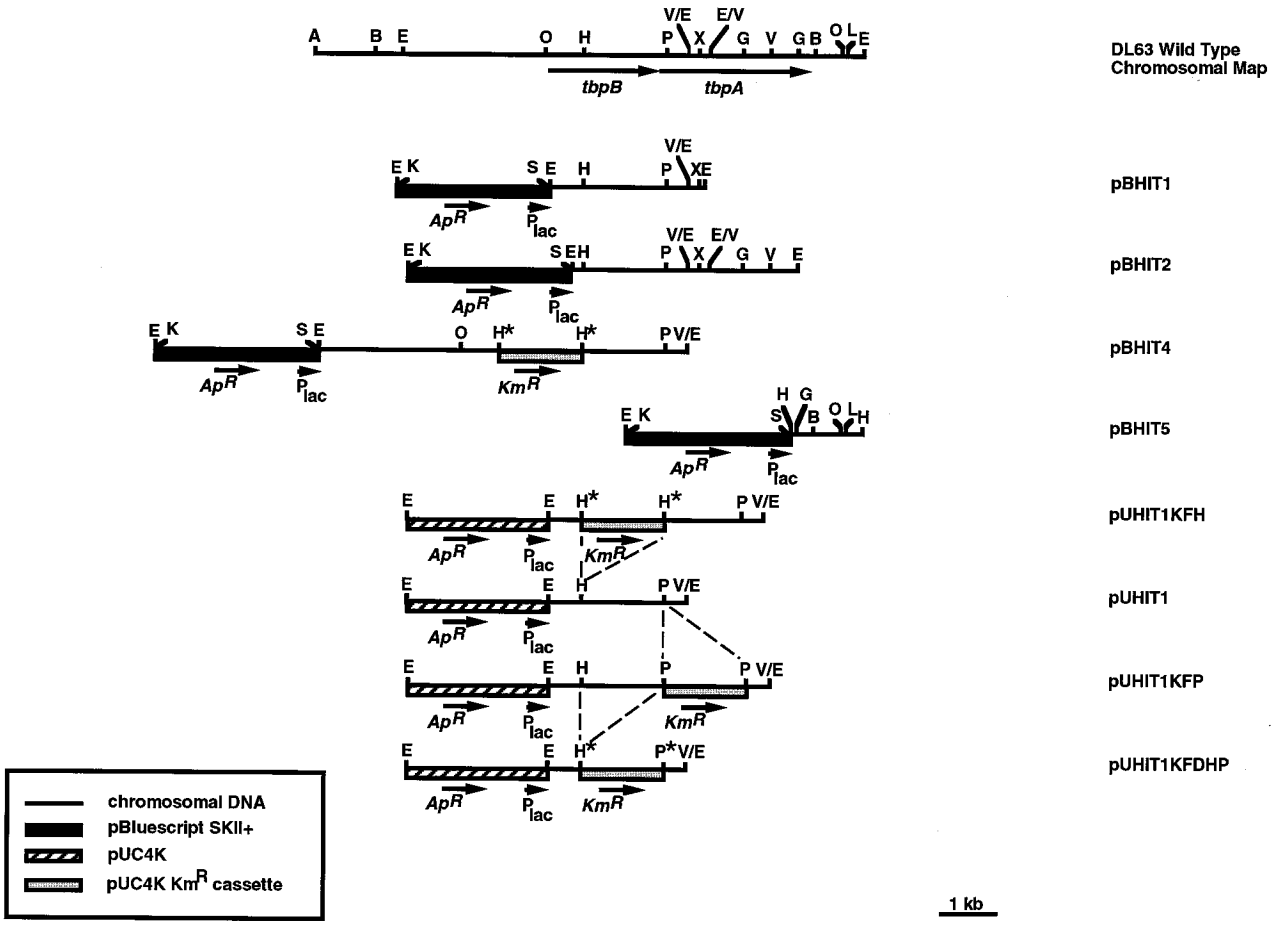


FIG. 1. Physical maps of the *H. influenzae* DL63 *tbpB*/*tbpA* locus and of recombinant plasmids containing cloned fragments from this region. Arrows denote selected promoters or open reading frames encoding the proteins as follows: *tbpB*, Tbp2; *tbpA*, Tbp1; Ap^R, β-lactamase; Km^R, Tn*903* aminoglycoside 3′-phosphotransferase; P_{lac}, β-galactosidase operon promoter. The following designations indicate the site of restriction endonuclease recognition and cleavage: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; G, *Bgl*I; H, *Hin*dIII; K, *Kpn*I; L, *Sal*I; O, *Xho*I; P, *Pst*I; S, *Sac*I; V, *Eco*RV; X, *Xba*I. * denotes sites which have been destroyed by treatment with Klenow fragment or T4 DNA polymerase.

The downstream clone selected was designated as pBHIT5 (Fig. 1).

Organization of the *tbpA*/*tbpB* locus. The *tbpA* and *tbpB* coding sequences were found to lie in close association with each other, with the putative start codon for *tbpA* following the *tbpB* stop codon by only 13 nucleotides (nt; Fig. 2). The sequence ATGAGTATAT lying 7 to 16 nt upstream of the putative *tbpB* start codon (Fig. 2, boldface sequence) shows homology (5 of 10 nt) to the consensus ribosome-binding site sequence (AAGGAGGTGATC) (44). Strong homology exists between sequences AATAAT and TTGACA (Fig. 2, underlined sequence) and the consensus bacterial promoter −10 and −35 sequences (TATAAT and TTGACA, respectively). The sequence AATAATGCGAATTATTATC which overlaps the putative −10 sequence (Fig. 2, outlined sequence) shares 16 of 19 nt with the consensus ferric uptake regulator (Fur) protein-binding site [GAT(AT)ATGAT(AT)AT(CT)ATTATC (10)]. Because the assignment of promoter functions to these sequences is presently based only on sequence comparisons, verification of their function as elements of the promoter regulating transcription of the *tbp* genes requires further study.

A sequence corresponding to 10 of 11 nt of the *H. influenzae*

TABLE 1. Oligonucleotide primers used for PCR[a]

Oligonucleotide	Region	Sequence
20	Tn*903b* (Kan^r), 3′ terminus	ACTCACCGAGGCAGTTCCAT
83	DL63 *tbpA*, 5′ terminus	ACTACATATGACTAAAAAACCCTATTTTCGC
84	DL63 *tbpB*, 3′ terminus	AAAAAAGCTTCCATTACTTGGTTGTTTCTAC
86	DL63 *tbpB*, 5′ reverse	ACTGAATCTAGATCCGCTACAAGCACTTAG
185	DL63 *tbpB*, 5′ terminus	TATATTCATATGAAATCTGTACCTCTTATC
188	DL63 *tbpA*, 3′ terminus	CATCTGAAGCTTTTTAATTTAGAATTTCATTTC

[a] Oligonucleotide primers were synthesized on an Applied Biosystems 391 DNA synthesizer as described by the manufacturer's basic protocol. Newly synthesized oligonucleotides were desalted with C_{18} Plus Sep-Pak Cartridges (Millipore Corp., Milford, Mass.).

FIG. 2. Genomic arrangement of the *H. influenzae tbpB/tbpA* locus. The open reading frame encoding Tbp1 (*tbpA*) follows closely behind that which encodes Tbp2 (*tbpB*), with an intergenic region of 13 nt separating these genes. The region upstream of *tbpB* contains regions with high homology to consensus −35 and −10 promoter sequences (underlined) and to a ribosome-binding site (boldface type). Additionally, a sequence overlapping the putative −10 promoter region possesses 16 of 19 nt which have been described as the consensus Fur-binding site (outlined). An *H. influenzae* uptake sequence positioned downstream of *tbpA* is shown (boldface italic type). The sequences corresponding to the putative signal sequences at the N terminus of Tbp2 and Tbp1 (see text for explanation) and the C-terminal two amino acids of the proteins are shown.

uptake sequence (AAGTGCGGTCA) is present downstream of the *tbpA* coding sequence (Fig. 2, boldface italic sequence). This sequence, in particular the first 9 nt of it, is postulated to facilitate the binding and uptake of DNA segments via the natural transformation system of competent *Haemophilus* cells (13).

Analysis of the *H. influenzae tbpB* gene. Analysis of the *tbpB* open reading frame reveals a sequence of 1,938 nt encoding a protein of 645 amino acids with a calculated molecular mass of ~71 kDa (Fig. 3), a value which agrees very well with that estimated by SDS-PAGE (72 kDa; see Fig. 7). The N-terminal region of this protein sequence shows high homology to the leader sequence of lipoprotein precursors (55).

Alignment of the *H. influenzae* Tbp2 sequence with the Tbp2 sequences of *Actinobacillus pleuropneumoniae* and *Neisseria meningitidis* published previously (11, 22) reveals an amino acid identity of 20.6% between the Tbp2s cloned from *N. meningitidis* B16B6 and M982 and *N. gonorrhoeae* and that of *H. influenzae* (Fig. 3, underlined sequence). A 14.9% identity is seen between these and the Tbp2s of *Actinobacillus pleuropneumoniae* serotypes 1 and 7 (Fig. 3, outlined sequence). There is, however, little difference in the level of identity seen between the human pathogens compared with that between *H. influenzae* and *A. pleuropneumoniae* or between *Neisseria meningitidis* and *A. pleuropneumoniae* (data not shown).

Analysis of the *H. influenzae tbpA* gene. The 2,748-bp *H. influenzae tbpA* open reading frame encodes a protein product of 915 amino acids and a predicted molecular mass of 103 kDa (Fig. 4), a value very close to the ~100 kDa predicted by SDS-PAGE (see Fig. 7). N-terminal sequence analysis of Tbp1 confirms the identity of this sequence and indicates that a leader peptide of 22 amino acids is cleaved to produce the mature Tbp1 (Fig. 4, italic sequence).

Amino acid sequence alignment of the *H. influenzae* Tbp1 with those previously published shows a much higher level of homology between these proteins than was seen with the

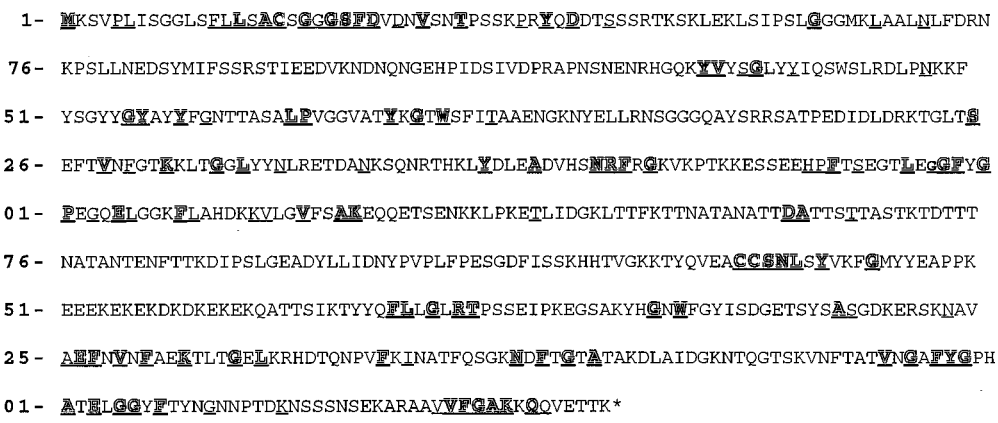


FIG. 3. Analysis of the *H. influenzae* DL63 Tbp2 sequence. The predicted amino acid sequence of DL63 Tbp2 based on the nucleotide sequence of the *tbpB* gene is shown. A comparison of the Tbp2 sequences which have been published to date show amino acid sequence identity both within the Tbp2s of the human pathogens *H. influenzae* (this study), *N. meningitidis* (22), and *N. gonorrhoeae* (1) (underlined) and between these and that of the porcine pathogen *A. pleuropneumoniae* (11) (outlined). Sequence comparisons were performed with automatic and manual alignments by using DNASIS V2.0 (Hitachi Software Engineering Co.).

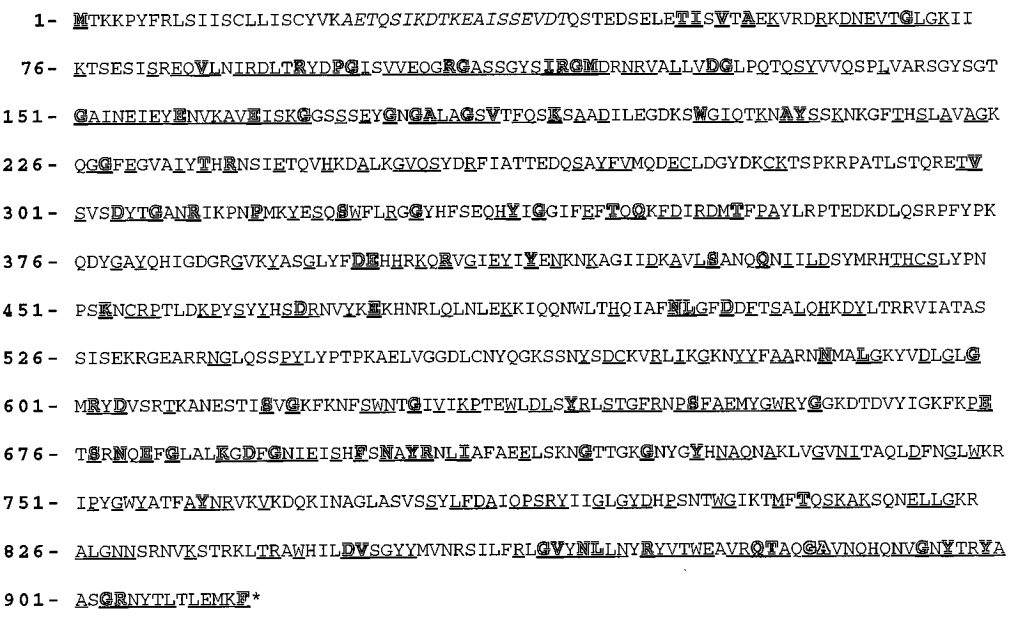


FIG. 4. Analysis of the *H. influenzae* DL63 Tbp1 sequence. The predicted amino acid sequence of DL63 Tbp1 based on the nucleotide sequence of the *tbpA* gene is shown. The sequence in italics denotes the sequence verified by N-terminal sequence analysis of the mature protein. A comparison of the predicted *H. influenzae* Tbp1 sequence with that of *N. gonorrhoeae* (8) and *N. meningitidis* (22) shows a large degree of sequence identity (underlined). Additionally, significant amino acid sequence identity is seen when these Tbp1s are aligned with the sequence of the *E. coli* ferric enterochelin siderophore receptor FepA (outlined). Sequence comparisons were performed with automatic and manual alignments by using DNASIS V2.0.

Tbp2s, with ~43% amino acid identity between the *H. influenzae* and neisserial proteins (Fig. 4, underlined sequence). Also, as with the neisserial proteins (8, 22), homology is seen between the Tbp1s and the ferric enterochelin receptor of *E. coli*, with greater than 11% identity seen when all five of these sequences are compared (Fig. 4, outlined sequence).

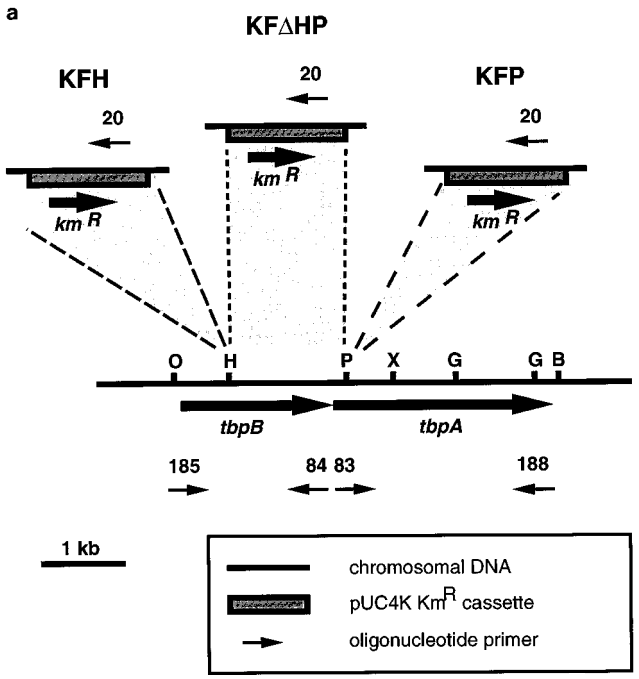
Insertional mutagenesis to interrupt cloned gene sequences. A 2.55-kb *Eco*RI fragment of the insert from pBHIT1 was subcloned into the *Eco*RI sites of pUC4K (50), resulting in removal of the Tn*903* kanamycin resistance (Kan) cassette from this vector (pUHIT1; Fig. 1). This subcloning step facilitated the subsequent insertion of either a *Hin*cII or *Pst*I pUC4K fragment containing the Kan cassette into either the *Hin*dIII or *Pst*I site of pUHIT1, because both are unique sites in this construct (pUHIT1KFH and pUHIT1KFP; Fig. 1). Additionally, a deletion mutant was produced by the insertion of the *Hin*cII-digested Kan cassette between the *Hin*dIII and *Pst*I sites of pUHIT1 (pUHIT1KFΔHP; Fig. 1). These constructs and all intermediate steps in subcloning were maintained by transformation into competent *E. coli* DH5α (23). Following digestion with *Eco*RI to remove the interrupted gene sequences, the constructs were introduced into the *H. influenzae* wild-type genome by transformation with M-IV medium as described previously (6), and transformants were selected on BHINH agar containing 20 μg of kanamycin per ml. Kanamycin-resistant colonies were designated as either *H. influenzae* DL63KFH, DL63KFP or DL63KFΔHP depending on the construct with which they were transformed.

Transformant colonies were screened by direct amplification of DNA sequences from single colonies by PCR. Oligonucleotide primers used in the screening PCR were of two types: reactions involving the oligonucleotide primer pairs 185/84 and 83/188 allowed amplification of intact *tbpB* or *tbpA*, respectively, while those involving primer pairs 185/20 and 83/20 were chosen so that product would be produced only if the Kan cassette was inserted in the proper orientation into the chromosomal region corresponding to the HIT clones (Fig. 5a). This was done by using one primer corresponding to a sequence within the Kan cassette and the second to sequences within the HIT clones.

A comparison of the PCR which amplify the whole gene sequences in Fig. 5b illustrates that an insertion of 1.2 kb into the *tbpB* gene of DL63KFH (oligonucleotides 185/84) and into the *tbpA* gene of DL63KFP (oligonucleotides 83/188) has occurred, as expected. No product was obtained when using DL63KFΔHP chromosomal DNA as template in these reactions, because the region corresponding to the 3′ end of *tbpB* and the 5′ end of *tbpA* has been deleted in this strain and sequences complementary to oligonucleotides 83 and 84 are absent (Fig. 5a).

In reaction 185/20, the 1.4-kb product obtained from DL63KFH and DL63KFΔHP indicates that the Kan cassette has been inserted at the *Hin*dIII site in the same orientation as *tbpB* (Fig. 5b). Similarly, the 1.2-kb product obtained with DL63KFP in reaction 83/20 shows the Kan cassette to be inserted into the *Pst*I site in the same orientation as the *tbpA* gene sequence (Fig. 5b). Again, the absence of product obtained when using KFΔHP chromosomal DNA in reaction 83/20 results from the deletion of the region corresponding to oligonucleotide 83 in the chromosome of this mutant (Fig. 5a). Together, these results indicate that the Kan cassette insertion constructs HIT1KFH, HIT1KFP, and HIT1KFΔHP have recombined appropriately into the wild-type DL63 genome to produce strains DL63KFH, DL63KFP, and DL63KFΔHP, respectively.

Tf-binding assays. To assess the effect of the Kan cassette insertions on the ability of these strains to bind Tf, a binding assay with serial dilutions of wild-type and mutant whole cells was used. Incubation with HRP-conjugated hTf followed by development with the chromogenic TMB substrate mixture allowed bound Tf to be visualized. A significant loss in hTf-binding activity compared with that in the wild-type strain was



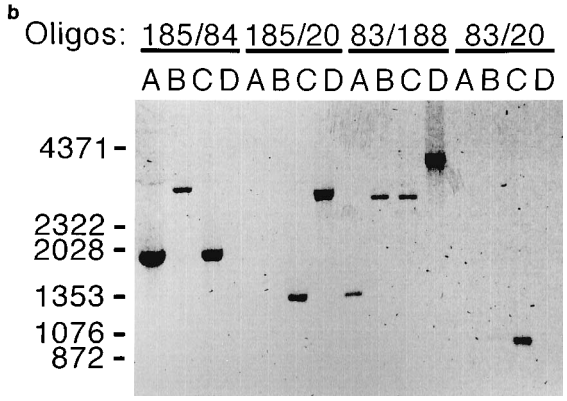


FIG. 5. PCR analysis to confirm genetic insertions into the *tbpB*/*tbpA* locus of the *H. influenzae* chromosome. (a) Schematic diagram illustrating the sites of insertion into the *tbpB*/*tbpA* locus and showing localization of oligonucleotide primers used in screening reactions. Mutant strains depicted here were constructed by transformation with their corresponding plasmids illustrated in Fig. 1. Small arrows localize oligonucleotide primers used in PCR, and large arrows denote open reading frames of the *tbp* or Kan^r genes as indicated. (b) Agarose gel electrophoresis of PCR used to verify genetic insertions into the *H. influenzae* *tbpB*/*tbpA* locus. Reactions involving the oligonucleotide primer pairs indicated were performed as outlined in Materials and Methods. Reaction products were separated by standard agarose gel electrophoresis and visualized with ethidium bromide. Template DNAs used in the reactions are designated as follows: A, DL63; B, DL63KFH; C, DL63KFP; D, DL63KFΔHP. Oligonucleotide primers used are described in Table 1. Figures were imaged by using a Hewlett-Packard ScanJet IIp and labelled by using Adobe Photoshop 2.5.

seen in the mutant strains lacking expression of either Tbp1 or Tbp2, and no binding activity was detected in the double mutant (Fig. 6). HRP-conjugated bTf, used as a negative control in this experiment, was not bound by any of the four membrane preparations (data not shown).

Expression of receptor proteins in insertion mutants. To compare levels of Tbp1 and Tbp2 expression in the mutants with that seen in wild-type membranes, component proteins from bacterial total membranes were separated by electrophoresis, electroblotted onto polyvinylidene difluoride membranes and probed with anti-Tbp polyclonal antiserum (Fig. 7a). While Tbp1 and Tbp2 are both apparent in the wild-type membrane, strains DL63KFH and DL63KFP lack observable expression of Tbp2 and Tbp1, respectively, and DL63KFΔHP lacks expression of both proteins. Importantly, expression levels of Tbp1 or Tbp2 in the membranes of the isogenic mutants DL63KFH and DL63KFP, respectively, are not significantly reduced from that seen in the wild-type membranes (Fig. 7a). Although Tbp1 levels appear to be slightly reduced, this difference would not by itself be expected to severely affect function as determined by the studies described in this paper.

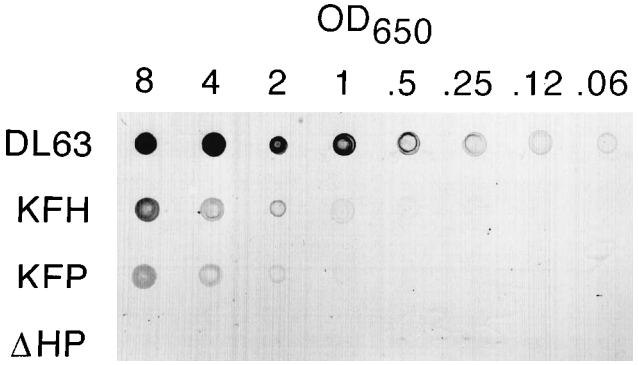


FIG. 6. Direct binding assays with HRP-conjugated hTf. Serial dilutions of whole cells from wild-type and mutant *H. influenzae* strains were spotted onto HA paper and blocked with skim milk prior to incubation with peroxidase-conjugated hTf. Binding was detected by the chromogenic development of tetramethylbenzidine and H_2O_2 by bound peroxidase as outlined in Materials and Methods. OD_{650}, optical density at 650 nm. Figures were imaged by using a Hewlett-Packard ScanJet IIp and labelled by using Adobe Photoshop 2.5.

Use of a small-batch procedure to affinity purify the receptor proteins resulted in the isolation of Tbp1 and Tbp2 from wild-type membranes and of Tbp1 from strain DL63KFH. No protein was isolated when membranes isolated from either strain DL63KFP or DL63KFΔHP were used (Fig. 7b). Interestingly, both Tbp1 and Tbp2 were isolated when a mixture of the membranes prepared from strains DL63KFH and DL63KFP was subjected to the affinity isolation procedure (Fig. 7b; H+P), indicating that Tbp2 is present and does maintain Tf-binding activity in the Tbp1$^-$ mutant.

Growth assays. To correlate the loss of receptor expression with the ability of *H. influenzae* to utilize various iron sources, studies evaluating growth of iron-stressed cultures on defined

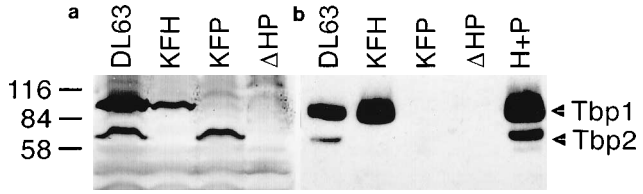


FIG. 7. Expression and isolation of Tbps. Component proteins of total membranes prepared from wild-type and mutant *H. influenzae* strains (a) or proteins affinity isolated from these proteins (b) were separated by SDS-PAGE and subjected to Western blot. Tbp1 and Tbp2 (arrowheads) were identified by incubation with rabbit anti-Tbp polyclonal antisera followed by HRP-conjugated goat anti-rabbit immunoglobulin antibody. Bound peroxidase was developed with the chemiluminescent HRP substrate luminol as outlined in Materials and Methods. Total membrane preparations used are designated as follows: DL63, *H. influenzae* DL63; KFH, strain DL63KFH; KFP, strain DL63KFP; ΔHP, strain DL63KFΔHP; H+P, a mixture of total membranes from strains DL63KFH and DL63KFP. Figures were imaged by using a Hewlett Packard ScanJet IIp and labelled by using Adobe Photoshop 2.5.

TABLE 2. Growth of strains on iron supplements

Strain	Zone size on supplement[a]:				
	hTf	bTf	$FeCl_3$	Hemin	PPIX[b]
DL63	+++	−	++++	++++	−
DL63KFH	+	−	++++	++++	−
DL63KFP	−	−	++++	++++	−
DL63KFΔHP	−	−	++++	++++	−

[a] Concentration disks containing 2.5 nmol of the indicated supplements or 250 nmol of $FeCl_3$ were placed onto BHIN plates which had been preseeded with iron-deficient cultures of the indicated *H. influenzae* strains and incubated as described in Materials and Methods. Zone sizes of growth outside the disks are indicated as follows: −, no growth; +, <1 mm; ++, 1 to 4 mm; +++, 5 to 9 mm; ++++, 10 to 15 mm.

[b] PPIX, protoporphyrin IX.

iron sources were initiated. Since *H. influenzae* DL63 is unable to use protoporphyrin IX as a porphyrin source, sustained aerobic growth requires hemin and optimal iron limitation of growth is therefore not possible. When aerobic broth culture experiments involving limiting concentrations of hemin were performed, the magnitude of iron-dependent growth was therefore not dramatic. Under these conditions, the wild-type strain did, however, consistently grow more rapidly and to a higher culture density than did the three Tbp mutants (data not shown). There was no obvious difference in growth of the three mutants under the conditions of this experiment. Subsequent attempts to take advantage of the fact that *H. influenzae* does not require a source of porphyrin when growing anaerobically led to the development of a microaerophilic broth culture assay system. As seen with the hemin limitation growth assays, the wild-type strain consistently grew better than did the three mutants; however, the low culture density attained made more precise interpretation of the results difficult.

In an attempt to determine whether the mutants could acquire iron from Tf to support growth, an anaerobic plate assay system was developed (Table 2). This system had the inherent benefits that the anaerobic incubation again alleviated the porphyrin requirements for growth and that the plate assay system allowed exposure of the cells to a range of Tf concentrations. As expected, no growth was seen on iron-deficient media alone or around disks impregnated with protoporphyrin IX. The addition of hemin to the media enhanced growth of all four *H. influenzae* strains, indicating that the mechanism of hemin assimilation is distinct from the Tf-mediated iron acquisition pathway and that it remains intact in the three mutants constructed in this study. Wild-type strain DL63 showed a growth zone of nearly 9 mm around the hTf disk, whereas strain DL63KFH showed growth in a zone of only ~1 mm (Table 2), suggesting that this mutant could grow only under high Tf concentrations. hTf did not support the growth of either DL63KFP or DL63KFΔHP.

DISCUSSION

Two distinct mechanisms by which microorganisms gain access to host Tf iron pools have been described to date. The better understood of these involves the production and secretion of low-molecular-mass (500- to 1,000-Da), high-affinity, ferric-specific ligands which are secreted by the microorganism into the environment under iron-restricted conditions. These molecules, called siderophores, can efficiently compete with Tf pools for iron and, upon becoming ferrated, are reabsorbed via a corresponding receptor system in the bacterial envelope (5).

A more direct approach to gaining access to Tf iron pools has been identified for a number of pathogens which have become adapted to life within a single host species. The ability of *N. meningitidis* to acquire iron from human transferrin was first recognized by Archibald and DeVoe (2). Since then, the human pathogens *Neisseria gonorrhoeae* (24) and *H. influenzae* (26) and veterinary pathogens including *A. pleuropneumoniae* (porcine) (12) and *P. haemolytica* (bovine) (30) have been found to possess similar activity. This assimilatory process has been found to require bacterial cell surface contact with the mammalian glycoprotein and occurs in the absence of siderophore production (2, 3, 12, 15, 26, 30). Tf binding appears to be mediated by the activity of two proteins present in the bacterial outer membranes, Tbp1 and Tbp2 (12, 29, 30, 39, 42, 43).

Recently, the genes encoding Tbp2 from *A. pleuropneumoniae* (11), Tbp1 (8) and Tbp2 (1) from *N. gonorrhoeae*, and Tbp1 and Tbp2 from *N. meningitidis* (22) have been cloned and sequenced. In *H. influenzae*, the close association of *tbpB* and *tbpA*, along with the lack of any promoter-like sequences adjacent to *tbpA*, is reminiscent of the situation reported for *N. meningitidis* (22) and *N. gonorrhoeae* (1) and suggests that the genes are expressed as a single transcriptional unit (Fig. 2). Interestingly, a region with high homology to the binding site for the ferric uptake regulatory protein (Fur) was found to overlap the putative −10 promoter sequence of the *tbp* transcript (Fig. 2). In *E. coli*, Fur binds to ferric iron within the cell and represses transcription from the promoters of iron-regulated operons (5). Although no Fur homolog has previously been characterized in *H. influenzae*, it would not be surprising to see a similar regulatory system, because the expression of a number of proteins, including that of the Tbps (39) and the putative outer membrane hemin-binding protein of *H. influenzae* (21), is repressed by iron. Morton et al. (25) have, however, reported that expression of the Tbps is repressible by hemin and not by elemental iron and that a novel regulatory system in which hemin functions as a corepressor instead of iron may exist. Future studies aimed at determining if a novel regulatory mechanism is in place or if hemin is, instead, functioning as an efficient source of intracellular iron by which a Fur homolog can regulate transcription in the conventional manner will therefore prove insightful.

A comparison of published Tbp1 sequences shows a much higher level of conservation within these proteins than is seen between the Tbp2s. Interestingly, the highly conserved carboxy-terminal sequences of the Tbp1s are characteristic of proteins which are integral components of the bacterial outer membrane (47). Localization of Tbp1 to the outer membrane is further supported by its sequence homology with the *E. coli* outer membrane protein FepA (Fig. 4). The surface accessibility of Tbp1 and therefore its localization to the outer membrane would seem to be a necessity, given its ability to interact with Tf (Fig. 6 and 7b) and the lack of Tf uptake into the cell during the iron assimilatory process (24).

As suggested previously (8, 22), the homology seen between Tbp1 and FepA implies that Tbp1 may belong to the TonB-dependent family of outer membrane proteins, a family which includes the ferric siderophore receptor proteins from *E. coli*. Amino acids 50 to 54 (ETISV) downstream of the *H. influenzae* Tbp1 start codon have good homology with the TonB box, a pentapeptide sequence found near the N terminus of the mature protein, which is conserved among the proteins of this family (7). The cytoplasmic membrane-bound TonB protein is thought to span the periplasmic space and physically interact with the periplasmic side of these outer membrane proteins at the region corresponding to the TonB box. This interaction is believed to function in the transduction of energy from the

electrochemical gradient of the cytoplasmic membrane out to the level of the outer membrane in order to power energy-requiring processes which occur there (34). A model in which TonB acts to energize iron acquisition via the *H. influenzae* Tbps is supported by the work of Jarosik et al. (19), who recently cloned and characterized the gene encoding a TonB homolog from *H. influenzae*.

Sequence alignments show no obvious correlation between the relationships of Tbp2 sequences and either the phylogenetic relationship of these bacterial species or their host species specificity. On the basis of the specificity of the bacterial receptor systems and the hypothesis that the receptor systems all interact with analogous regions on the surface of Tf (14), regions which directly interact with Tf should be conserved between receptors specific for the same Tf species but should be distinct between receptors which interact with different Tf species. The lack of obvious stretches of amino acids which are conserved in such a manner within the Tbp2s compared here is, however, inconsistent with this hypothesis. Although the possibility that Tf-receptor interactions are not conserved cannot yet be ruled out, it is more likely that folding patterns of the protein provide the necessary juxtaposition of such sequences. Further knowledge of the tertiary structure of the protein and the identity of sites of interaction both on transferrin and on the receptor proteins will be required before this hypothesis can be conclusively tested.

In *E. coli*, lipoproteins are generated by the addition of a lipid moiety to the sulfhydryl group of the N-terminal cysteine of the mature protein, which lies approximately 20 amino acids downstream of the ATG start site. Cleavage of the polypeptide chain at the N-terminal side of the lipid-modified cysteine is then catalyzed by signal peptidase II to yield the mature lipoprotein (55). A comparison of the signal sequences of known bacterial lipoproteins shows the presence of a predominantly hydrophobic signal sequence followed by the tetrapeptide sequence Leu-X-Y-Cys, where X and Y are neutral small amino acids such as alanine, glycine, and serine (55). The N terminus of *H. influenzae* Tbp2 conforms exactly with this consensus sequence (Fig. 2), suggesting that *H. influenzae* Tbp2 may be similarly modified and may provide the means by which Tbp2 is anchored into the outer membrane. In agreement with this, [^{14}C]palmitic acid labelling has previously shown the Tbp2s of *A. pleuropneumoniae* (11) and *N. gonorrhoeae* (1) to be lipid modified.

Previous studies have been able to detect HRP-hTf binding by Tbp2 which has been separated from total membrane by SDS-PAGE and Western blotting (27, 39). The inability to affinity isolate Tbp2 from total membrane prepared from strain DL63KFH unless Tbp1 is exogenously applied (Fig. 7b) therefore suggests either that this protein interacts with both Tf and Tbp1 or that a Tbp1-induced conformational change in Tf may function to increase the avidity of the Tbp2-transferrin interaction. On the basis of this hypothesis, the increased affinity of Tbp2 for the Tf-Tbp1 complex conferred by one or both of these phenomena would be required to detect Tbp2 by the affinity isolation procedure.

Further support for the existence of a complex of Tf, Tbp1, and Tbp2 stems from the fact that loss of either Tbp results in a significant reduction in the ability of Tf to be bound at the cell surface (Fig. 6). These data suggest a positive cooperativity in which Tf binding by the two receptor proteins together is greater than the sum of binding by the two proteins on their own. One model which could explain such a phenomenon pictures Tbp1 as an integral outer membrane protein and Tbp2 as being anchored to the outer membrane by its lipid moiety. The more distally located Tbp2 could therefore function to "reach out" and bind Tf in the external milieu, thus allowing for the juxtaposition of Tf and Tbp1 directly on the cell surface. A resultant complex of Tbp2, Tbp1, and Tf would therefore represent the high-affinity binding visualized in the wild-type membranes (Fig. 6).

In this model, the primary function of Tbp2 is to help bring the large host glycoprotein to the cell surface, at which point iron can be removed. The effect of such a system would be to increase the effective concentration of Tf at the cell surface, and it provides an explanation for the importance of Tf concentration to bacterial growth seen in this study (Table 2). On the basis of such assumptions, it is therefore interesting to speculate that the difference seen in growth of the neisserial Tbp1$^+$ Tbp2$^-$ mutants may stem from the fact that the meningococcal mutants are encapsulated (18) whereas the gonococcal mutants are unencapsulated (1, 8). The construction and characterization of similar Tbp1 and Tbp2 mutants in wild-type and isogenic capsule-deficient strains of *H. influenzae* may therefore provide interesting information regarding the relative importance of Tbp2 in the absence of a polysaccharide capsule and the effect of encapsulation on Tf binding and utilization.

The importance of the receptor proteins for bacterial growth was initially based on the correlation between the specificity of Tf binding and the specificity observed in both in vitro and in vivo growth (30, 40, 43). Additionally, uncharacterized mutants defective in Tf-binding activity had lost the ability to utilize Tf as an iron source (16, 49). Characterization of isogenic Tbp mutants constructed in *N. gonorrhoeae* (1, 8) and *N. meningitidis* (18) has confirmed the importance of both receptor proteins in Tf-mediated iron acquisition and growth. As seen with the neisserial Tbp1$^-$ Tbp2$^+$ mutants, *H. influenzae* DL63KFP is unable to grow on media supplemented with Tf as the sole iron source. The three Tbp1$^+$ Tbp2$^-$ mutants do, however, appear to differ in their ability to grow on hTf. *N. gonorrhoeae* (1) and *H. influenzae* DL63KFH (Table 2) Tbp1$^+$ Tbp2$^-$ mutants are both able to grow on hTf, while the meningococcal mutant cannot do so (18). A comparison of the growth assays used in these studies does show obvious differences, including the use of a fivefold difference in hTf concentration in the media in the two neisserial studies (5 versus 25 μM [1, 18]). It is unclear whether the differences in the growth of these organisms can be explained by methodological differences alone or whether species and/or strain differences in Tf-receptor interactions contribute to this disparity. Differences in the structure and function of expressed Tbp1s, in host strain genetic background, and/or in the local conditions present at the cell surface may potentially influence Tf binding and therefore affect iron acquisition via this pathway. Anderson et al. (1) have, however, recently shown that a Tbp1$^+$ Tbp2$^-$ gonococcal mutant which could grow on hTf-bound iron did obtain this iron at a level approximately 20% that of the wild-type strain. It is therefore clear that the loss of Tbp2 does impair Tf receptor function, suggesting that iron acquisition may become a limiting factor for growth of these mutants.

In summary, the genes encoding Tbp1 and Tbp2 have been cloned and sequenced, and isogenic mutants created by the interruption of these genes have been characterized. Mutants created by insertions into *tbp* lacked the expression of Tbp1, Tbp2, or both. The loss of either protein correlated with a significantly decreased ability to bind Tf and an inability to grow on media containing hTf as the sole iron source. As seen previously in *N. meningitidis* (22) and *N. gonorrhoeae* (1), the *tbp* genes appear to be organized into a single transcriptional unit, and both proteins showed a significant degree of conservation with receptor genes previously cloned from both human

and veterinary pathogens (1, 8, 11, 22). Also, homologies between *H. influenzae* Tbp1 and *E. coli* FepA support previous suggestions that Tbp1 is a member of the TonB-dependent family of outer membrane receptor proteins (8, 22).

The facts that the Tf-mediated mechanism of iron assimilation can function in vitro to support *H. influenzae* growth on media containing Tf as the sole iron source (Table 2) (15) and that the Tbps are expressed in vivo (17) suggest that this pathway is functioning during infection. The importance of Tbp1 and Tbp2 for Tf binding and iron acquisition seen in this study suggests that loss of either protein would severely impair the ability of the microorganism to acquire iron in vivo and would therefore impair its chances to successfully invade and establish an infection within human tissues. Further studies are needed to conclusively define the role of the Tf receptor proteins in vivo, but the present findings do support suggestions that they provide ideal vaccine candidates because of their inherent surface accessibility to large proteins and their vital role in the pathogenic process (41, 46).

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