

Construction and Characterization of *Moraxella catarrhalis* Mutants Defective in Expression of Transferrin Receptors

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We have previously reported the construction of an isogenic mutant defective in expression of OmpB1, the TbpB homologue, in *Moraxella catarrhalis* 7169. In this report, we have extended these studies by constructing and characterizing two new isogenic mutants in this clinical isolate. One mutant is defective in expression of TbpA, and the other mutant is defective in expression of both TbpA and TbpB. These isogenic mutants were confirmed by using PCR analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and sequencing. In vitro growth studies, comparing all three mutants, demonstrated that the *tbpA* mutant and the *tbpAB* mutant were severely limited in their ability to grow with human holotransferrin as the sole source of iron. In contrast, the *ompB1* (*tbpB*) mutant was capable of utilizing iron from human transferrin, although not to the extent of the parental strain. While affinity chromatography with human holotransferrin showed that each Tbp was capable of binding independently to transferrin, solid-phase transferrin binding studies using whole cells demonstrated that the *tbpA* mutant exhibited binding characteristics similar to those seen with the wild-type bacteria. However, the *ompB1* (*tbpB*) mutant exhibited a diminished capacity for binding transferrin, and no binding was detected with the double mutant. These data suggest that the *M. catarrhalis* TbpA is necessary for the acquisition of iron from transferrin. In contrast, TbpB is not essential but may serve as a facilitory protein that functions to optimize this process. Together these mutants are essential to provide a more thorough understanding of iron acquisition mechanisms utilized by *M. catarrhalis*.

Iron is a critical element essential for the growth of many organisms, and there is little free iron available in the human body (5, 11, 14, 19, 29). The sequestering of iron provides an important line of defense against pathogenic microorganisms. Most of the iron present in the body is intracellular in the ferrous form as hemoglobin, heme, ferritin, or hemosiderin. In addition, extracellular iron is complexed to the high-affinity iron-binding glycoproteins transferrin (Tf) and lactoferrin (5, 14, 19, 29). Tf-bound iron is found predominantly in serum, while it is thought that lactoferrin is the primary carrier of iron on most mucosal surfaces. To survive in and colonize the human host, many mammalian pathogens have evolved numerous mechanisms that allow them to obtain iron in vivo. Both direct iron-binding compounds and specific receptors for iron carrier proteins have been described for many of these bacteria (11, 13, 19, 28).

Previous studies have demonstrated that *Moraxella catarrhalis* expresses proteins that bind human Tf and lactoferrin (Lf) similar to those receptors described for the pathogenic members of the families *Neisseriaceae* and *Pasteurellaceae* (10, 15, 26, 27, 31). The Tf-binding proteins (Tbps) were identified by using affinity chromatography with human holotransferrin coupled to a Sepharose matrix. These proteins are iron repressible and have molecular sizes in the range of 80 to 84 kDa (TbpB) and 115 to 120 kDa (TbpA) (31). Our previous studies identified OmpB1 as an *M. catarrhalis* Tf receptor (6, 20). This outer membrane protein appears to be the homologue of TbpB, which was described by Myers et al. (for clarity, OmpB1 will be referred to as TbpB in this report) (22). *tbpA* and *tbpB* have been cloned and sequenced from various strains of *M.*

catarrhalis, and the proteins encoded by these genes have been compared (22). Similar to the *tbpB* of other bacteria, the *M. catarrhalis* *tbpB* exhibits marked heterogeneity between strains while *tbpA* appears to be highly conserved.

Although the Tbps of *M. catarrhalis* specifically bind human Tf, similar to the Tbps expressed by other human mucosal pathogens, there are some differences observed in the arrangement of the genes that code for these proteins. Investigators have previously reported that the *M. catarrhalis* *tbpA* gene precedes *tbpB*, unlike other known *tbp* operons, and there is a third open reading frame (termed *orf3*) located between these genes (22). In addition, the identification of three potential promoter sequences located upstream of each open reading frame suggests that the genes are independently transcribed. Despite these observations, the lack of specific mutants, similar to the *tbp* mutants constructed for the pathogenic *Neisseriaceae*, have precluded studies designed to evaluate the relationship between the regulation and expression of the *M. catarrhalis* genes which code for the Tbps. In this report, we constructed isogenic mutants in *M. catarrhalis* 7169 that are deficient in the expression of either TbpB (previously described), TbpA, or both of these receptors. Comparative studies were performed to characterize each mutant for the ability to bind Tf at the bacterial cell surface, to utilize iron from Tf for in vitro growth, and for the affinity isolation of each receptor from a holotransferrin matrix. These Tbp mutants provide important tools that will be critical in studies designed to provide a more thorough understanding of the Tf system utilized by *M. catarrhalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. catarrhalis* 7169, a clinical isolate obtained by typanocentesis from a child with otitis media, was kindly provided by Howard Faden (Children's Hospital, Buffalo, N.Y.). The Chelex-treated defined growth medium (CDM) and the culturing conditions for iron depletion of *M. catarrhalis* were described previously (7, 21, 30). *M. catarrhalis* strains were

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cultured at 35.5°C on GC plates (without supplements) in a 5% CO₂ atmosphere. Antibiotic-resistant isogenic mutants were cultured in the presence of 20 (7169b12 and 7169*tbpA35*) or 40 (7169*tbpAB-1*) µg of kanamycin per ml. *M. catarrhalis* strains were made competent for electroporation by a previously described method (17). *Escherichia coli* XLI-Blue was cultured at 37°C on Luria-Bertani agar plates or in Luria-Bertani broth in the presence of the appropriate antibiotic (100 µg of ampicillin per ml or 20 µg of kanamycin per ml).

DNA manipulations. The restriction endonucleases, T4 ligase, DNA polymerase I, and molecular biology reagents were purchased from New England Biolabs, Inc., Beverly, Mass., or Promega, Madison, Wis. The QIAprep spin kit was used to isolate plasmid DNA, and electrophoretically separated DNA fragments were gel purified by using the QIAquick PCR purification kit (Qiagen, Santa Clarita, Calif.). *M. catarrhalis* genomic DNA was isolated by standard methods, as previously described (20, 24). Chromosomal DNA was subjected to PCR amplification for 30 cycles with an annealing temperature of 55°C, using oligonucleotide primers based on sequences within *tbpA* (primer 61, 5'-TGATAAG GTGGTCAAACCTG-3' [sense]; primer 62, 5'TGTCATTAGTCCAACCCG C-3' [antisense]) or *ompB1* (primer 42, 5'-CGTCTTATTAACCGCTTGTGG-3' [sense]; primer 43, 5'-TCGACCGCTTTCGTGTTTC-3' [antisense]), which flank the predicted insertional region of the *aphA-3* cassette.

Insertional mutagenesis. The construction of *M. catarrhalis* 7169b12 (*tbpB::kan*) has been previously described (20). To insertional inactivate the *tbpA* gene, PCR primers (described above) were designed to an internal region of the *M. catarrhalis* Q8 *tbpA* sequence obtained from the GenBank database. A 1,772-bp fragment of the *M. catarrhalis* 7169 *tbpA* was amplified and cloned into the pGEM T-tailed vector (Promega Corp., Madison, Wis.). A portion of the amplified gene product was sequenced to confirm that the *tbpA* from *M. catarrhalis* 7169 had been isolated. The *aphA-3* nonpolar cassette of pUC18K was subcloned into a 233-bp internal deletion of the partial *tbpA* gene such that an ATG codon was placed 3' of the resistance gene and in frame with the remainder of the *tbpA* coding region. The disrupted *tbpA::kan* construct was amplified by PCR with primers 61 and 62 and electroporated into *M. catarrhalis* 7169, by using a previously described method (16). The double mutant was constructed essentially as described for the *ompB1* mutant 7169b12, except that the *pdelB1* construct was electroporated into competent *M. catarrhalis* 7169*tbpA35* (20). Strain 7169 Tbp derivatives were selected based on resistance to kanamycin at 20 µg/ml (7169*tbpA35* and 7169b12) or 40 µg/ml (7169*tbpAB-1*) and screened by loss of reactivity to a TbpB-specific antibody, monoclonal antibody (MAb) 11C6 (7169b12 and 7169*tbpAB-1*). DNA sequences of the selected clones were obtained via automated DNA sequencing (CAMBI Nucleic Acid Sequencing Facility, State University of New York at Buffalo, Buffalo) and analyzed with MacVector 6.0 and the Wisconsin Sequence Analysis Packages (Genetics Computer Group, Madison, Wis.).

Tf binding assay. Biotinylated human Tf was purchased from Sigma Chemical Co. (St. Louis, Mo.). Bacteria were cultured in CDM broth under iron limitation, harvested, and resuspended in phosphate-buffered saline to an optical density at 600 nm of 0.3. One hundred microliters of successive 1:1 bacterial dilutions was immobilized onto nitrocellulose. After drying for 15 min at 37°C, the filters were blocked with 3% bovine serum albumin in phosphate-buffered saline and then incubated with biotinylated human Tf (250 ng/ml) for 1 h at room temperature. Horseradish peroxidase-conjugated streptavidin (Zymed Laboratories, Inc., San Francisco, Calif.) was used as the secondary reagent for colorimetric detection of Tf binding.

Protein analysis. Zwittergent-solubilized outer membrane protein preparations (OMPs) were made and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assays were performed as previously described (6). Covalent linkage of human holotransferrin (>98% iron saturated) to CNBr-activated Sepharose 4B was carried out in accordance with the manufacturer's instructions (Sigma). Total membrane fractions of *M. catarrhalis* were prepared by resuspending iron-depleted bacterial cultures in 10 mM HEPES (pH 7.4) containing 1 µM phenylmethylsulfonyl fluoride. The cell suspension was frozen at -70°C, thawed in a room-temperature water bath, and then incubated for 30 min at 37°C with 5 µg of lysozyme/mg (wet weight) of bacteria. The cells were then held on ice and sonicated with four 20-s bursts with a microtip set to the microtip limit. The total membrane preparation was obtained by centrifuging the crude lysate at 100,000 × g for 1 h at 4°C. The resulting pellets were resuspended in 50 mM Tris-HCl-1 M NaCl (pH 8.0) containing 10 mM EDTA and 0.75% Sarkosyl, and any insoluble cellular debris was removed by centrifugation at 10,000 × g for 10 min. Affinity isolation experiments were performed essentially as described previously (31), with several modifications. Solubilized iron-deficient membranes were incubated with 1.5 to 2.0 ml of Tf-Sepharose slurry for 45 min at room temperature or overnight at 4°C. Tf-binding fractions, eluted with 4 M guanidine-HCl, were dialyzed overnight against 50 mM Tris (pH 8.0). Column fractions were concentrated with Centricon concentrators (Amicon, Inc., Beverly, Mass.) and analyzed by SDS-PAGE.

RESULTS

Construction of the isogenic mutants. We have previously reported the use of the kanamycin-resistant determinant from pUC18K to construct a *tbpB* (*ompB1*) isogenic mutant in *M.*

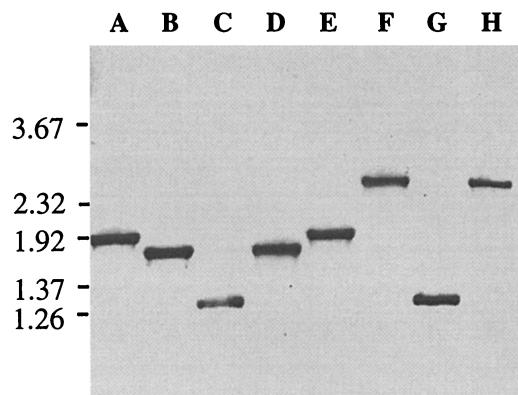


FIG. 1. PCR analysis of mutants. Chromosomal DNA from strain 7169 and the isogenic mutants 7169b12 (TbpA⁺ TbpB⁻), 7169*tbpA35* (TbpA⁻ TbpB⁺), and 7169*tbpAB-1* (TbpA⁻ TbpB⁻) was subjected to PCR analysis, and the products were separated by electrophoresis. Products in lanes A, C, E, and G were obtained with *tbpB*-specific primers 42 and 43 (described in Materials and Methods). Lanes B, D, F, and H were obtained with primers 61 and 63, which are specific for sites within *tbpA*. Both primer sets flank the predicted site of the *aphA-3* cassette insertion within the mutants. Lanes: A and B, strain 7169; C and D, 7169b12; E and F, 7169*tbpA35*; G and H, 7169*tbpAB-1*. A negative image of an ethidium bromide-stained agarose gel is shown with molecular size standards, in kilobases, to the left.

catarrhalis 7169 (7169b12) (20). A *tbpA* isogenic mutant and a *tbpAB* double mutant were constructed by insertional mutagenesis essentially as described for construction of the *tbpB* mutant (20). Linearized DNA, containing the *aphA-3* gene inserted within an internal deletion of *tbpA* or *tbpB*, was electroporated into competent *M. catarrhalis*. Mutated clones were selected by the ability to grow on kanamycin, and *tbpB* mutants were screened for the loss of reactivity to MAb 11C6, a TbpB-specific MAb (20). Clones 7169*tbpA35*, a TbpA⁻ mutant, and 7169*tbpAB-1*, a TbpA⁻ TbpB⁻ double mutant, were selected for further analysis.

Verification of the isogenic mutants. Chromosomal DNA was isolated from the three isogenic mutants and the parental strain. PCR amplifications, using primers flanking the predicted sites of mutagenesis cassette insertion, were used to verify the identity of the isogenic mutants. Agarose gel analysis of these fragments confirmed that the kanamycin resistance cassette was inserted into the *tbpA* and/or *tbpB* gene of *M. catarrhalis* 7169 (Fig. 1). Primers 42 and 43, recognizing sites within *tbpB*, amplified a 1.93-kb PCR product from the parental strain 7169 (lane A) as well as from 7169*tbpA35*, the TbpA mutant (lane E). A 1.28-kb PCR product was obtained from both 7169b12, the TbpB mutant (lane C), and 7169*tbpAB-1*, the double mutant (lane G). This result is consistent with the insertion of the 850-bp *aphA-3* kanamycin-resistant determinant into a 1.5-kb internal deletion of *tbpB*. Primers 61 and 62, specific for sites within *tbpA*, amplified a 1.77-kb PCR product from strain 7169 (lane B) and from 7169b12 (lane D). In contrast, a 2.4-kb fragment was obtained from PCR amplification of 7169*tbpA35* (lane F) and 7169*tbpAB-1* (lane H), which is consistent with the insertion of the 850-bp kanamycin cassette into a 233-bp internal deletion of *tbpA*. Subsequent DNA sequence analysis of these PCR products confirmed that the *aphA-3* nonpolar mutagenesis cassette had been inserted into *tbpB* or *tbpA* in the predicted frame (data not shown).

To confirm the loss of TbpA and/or TbpB expression, OMPs from wild-type strain 7169 and the isogenic mutants 7169b12, 7169*tbpA35*, and 7169*tbpAB-1* were prepared from bacteria grown under iron limitation and analyzed by SDS-PAGE. Fig-

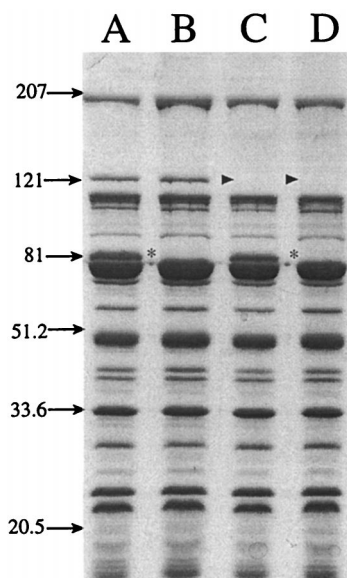


FIG. 2. An SDS-PAGE gel, stained with Coomassie blue, showing the OMP profile of strain 7169 (lane A) and the isogenic mutants 7169b12 (lane B), 7169tbpA35 (lane C), and 7169tbpAB-1 (lane D) grown under iron-depleted conditions. Asterisks (lanes B and D) denote the loss of TbpB expression, and the lack of TbpA expression is indicated by arrowheads (lanes C and D). Molecular size standards are shown in kilodaltons.

ure 2 illustrates that TbpB is expressed by both the wild-type strain (lane A) and the *tbpA* isogenic mutant 7169tbpA35 (lane C) but is absent from both the *tbpB* mutant 7169b12 and the double mutant 7169tbpAB-1. Both the wild-type strain (lane A) and the *tbpB* isogenic mutant 7169b12 (lane B) express TbpA, but this protein is not expressed by the *tbpA* isogenic mutant 7169tbpA35 or by the double mutant 7169tbpAB-1. Further comparison of the SDS-PAGE OMP profiles did not reveal any difference between these four strains. Western blot analysis, using MAb 11C6, also confirmed the loss of TbpB expression from 7169b12 and 7169tbpAB-1 (data not shown).

Characterization of the isogenic mutants. (i) Tf binding assays. The three mutants were evaluated for their ability to bind Tf, in comparison to that of the wild type, in a whole-cell solid-phase binding assay. Aliquots of cells grown under iron limitation were immobilized onto nitrocellulose and incubated with biotinylated human Tf (Fig. 3). There was a slight observable difference in Tf binding between the parental 7169 and

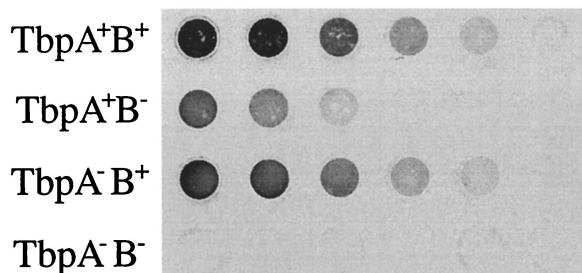


FIG. 3. Solid-phase Tf binding assay. Equal amounts of iron-depleted whole cells from the parental and mutant Tbp derivatives were dotted onto nitrocellulose. The blots were incubated with biotinylated human holotransferrin and developed by using horseradish peroxidase-streptavidin to detect Tf binding. TbpA⁺B⁺, 7169; TbpA⁺B⁻, 7169b12; TbpA⁻B⁺, 7169tbpA35; TbpA⁻B⁻, 7169tbpAB-1.

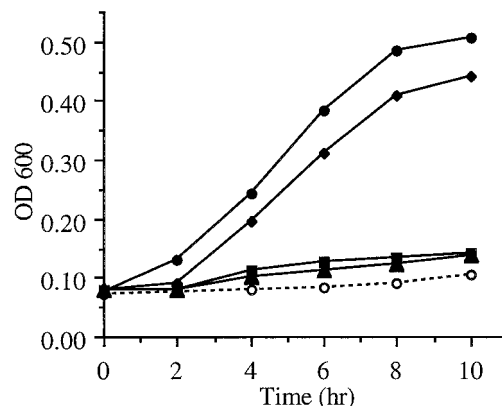


FIG. 4. Growth of parental strain 7169 and the isogenic mutants with Tf as the sole source of iron. Iron-depleted overnight cultures of each strain were used to inoculate fresh CDM 0 or CDM containing 5 μ M human holotransferrin. All cultures contained 10 μ M desferoxamine. The optical density at 600 nm (OD₆₀₀) of each culture was measured in 2-h intervals for 10 h. Symbols: ●, 7169-Tf; ◆, 7169b12-Tf; ■, 7169tbpA35-Tf; ▲, 7169tbpAB1-Tf; ○, 7169-0.

the TbpA mutant 7169tbpA35. In contrast, the Tf binding activity of the TbpB mutant (7169b12) was noticeably reduced but not eliminated. The inactivation of both TbpA and TbpB (7169tbpAB-1) completely eliminated Tf binding activity.

Comparative growth studies. The ability of these mutants to utilize human holotransferrin as a sole source of iron for in vitro growth was analyzed. The growth rates of *M. catarrhalis* 7169 and the Tbp derivatives 7169b12 (TbpA⁺ TbpB⁻), 7169tbpA35 (TbpA⁻ TbpB⁺), and 7169tbpAB-1 (TbpA⁻ TbpB⁻) were evaluated by culturing the bacteria in iron-depleted CDM supplemented with 5.0 μ M human holotransferrin (Fig. 4). In comparison to the parental strain 7169, mutants 7169tbpA35 and 7169tbpAB-1 were unable to grow with human Tf as the sole source of iron. The TbpB mutant 7169b12 exhibited growth in the presence of Tf, albeit less efficiently than the wild-type strain 7169. All of the strains grew equally well with iron-loaded lactoferrin as a sole source of iron (data not shown). These results were consistently reproducible, confirming that the observed growth differences with Tf are due to specific mutations in TbpA or TbpB.

Column chromatography. Affinity chromatography of Tbps from the isogenic mutants was performed by utilizing a human holotransferrin-Sepharose matrix. Figure 5 is a composite SDS-PAGE gel analyzing the Tf-binding fraction eluted from each column. These data demonstrate that both TbpB and TbpA were isolated from wild-type 7169 total membranes. TbpB could be isolated from 7169tbpA35, and only TbpA was isolated from 7169b12. As expected, TbpA was not isolated from 7169tbpA35 nor was TbpB purified from 7169b12, and no Tbps were purified from the double mutant 7169tbpAB-1. These results were consistent with those predicted from the PCR analysis of the isogenic mutants, confirming the insertional inactivation of the *tbpB* and/or *tbpA* genes.

DISCUSSION

Previous reports have identified and isolated both Tf-binding proteins from various strains of *M. catarrhalis* (22, 23, 31). In addition, the genes that code for each of these proteins have been cloned and sequenced (20, 22). These data, together with our earlier studies, strongly suggest that these proteins are Tf receptors (6, 20). However, these observations were based primarily on sequence homology, binding studies, and affinity

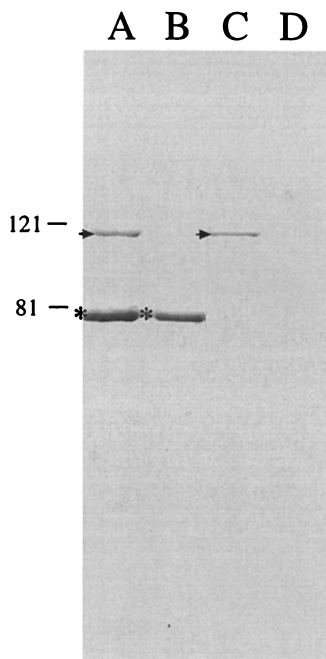


FIG. 5. Human holotransferrin affinity purification. The Tf-binding fractions from solubilized total membranes of 7169 (lane A), 7169*tbpA35* (lane B), 7169b12 (lane C), and 7169*tbpAB-1* (lane D) were analyzed by SDS-PAGE and Coomassie blue staining. Asterisks are placed next to affinity-purified TbpB, and arrows denote affinity-purified TbpA. Molecular size standards, in kilodaltons, are indicated on the left.

chromatography. Although we have previously reported data describing the construction of an *M. catarrhalis* mutant defective in expression of TbpB, these studies did not analyze the growth characteristics of this mutant (20). The construction of the isogenic mutants deficient in either or both of the Tbps in *M. catarrhalis* 7169 and the subsequent characterization of these constructs provide conclusive proof that these proteins function as Tf receptors. In addition, these isogenic mutants provide the means to more thoroughly characterize the relationship between TbpA and TbpB as they function in the *M. catarrhalis* system of iron acquisition from human Tf.

The Tf binding studies using whole bacterial cells showed that the *M. catarrhalis* TbpA mutant (7169*tbpA35*) had slightly less ability to bind iron-loaded human Tf than the wild-type strain. However, the TbpB mutant (7169b12) exhibited noticeably less binding activity. This is in direct contrast with the results of the in vitro growth studies. When human holotransferrin was used as the sole source of iron, growth of 7169*tbpA35* was severely limited. However, 7169b12 exhibited appreciable growth under the same conditions, although not at a rate equivalent to that of the wild type. In addition, affinity purification assays using the isogenic mutants demonstrated that both TbpB and TbpA were capable of high-affinity, independent Tf binding. Based on these results, we hypothesize that the *M. catarrhalis* TbpA is capable of binding Tf, removing the iron, and facilitating the transport of this element into the cell. In contrast, while the *M. catarrhalis* TbpB retains the ability to bind holotransferrin, the growth studies suggest that the Tf-bound iron cannot be utilized in the absence of TbpA.

It is interesting to note that the *M. catarrhalis* Tbps have a unique genomic arrangement, with *tbpA* preceding *tbpB*, with a third open reading frame termed *orf3*, in the intergenic region (22). However, the significance of this observation is currently

unknown. It was reported that the cloned *orf3* encodes an approximately 58-kDa recombinant protein, but the authors of that report were unable to produce antibodies to this protein and no further studies were performed (22). The function of this protein is currently unknown, and the studies described in this report do not provide any additional information related to *orf3*.

The mechanisms of iron acquisition from Tf have been well characterized for the pathogenic *Neisseriaceae*. In these organisms, TbpA has been described as a transmembrane protein with extracellular exposed loops that may act as a gated pore allowing for the transport of iron into the bacterial cell (8, 25). TbpB has been characterized as a surface-exposed lipoprotein that is anchored to the bacterial membrane via the lipid moiety (4, 15). A meningococcal mutant defective in TbpB expression was incapable of utilizing Tf for in vitro growth (18). In addition, TbpB could not be affinity isolated from a TbpA mutant, suggesting that both proteins were necessary for avid binding of the Tf molecule. In contrast, an *N. gonorrhoeae* mutant defective in expression of TbpB was still capable of utilizing Tf for in vitro growth, indicating that TbpA could perform all the necessary functions required to obtain iron from Tf (4). Affinity purification assays, using Sarkosyl-solubilized membrane proteins, demonstrated that both Tbps were capable of independently binding to Tf. These results are in contrast to those reported for the Tbps of the pathogenic *Neisseriaceae* (12, 18).

The isogenic mutants presented in this report provide unique tools that will be valuable in many aspects of future research involving colonization and infection with *M. catarrhalis*. First, these mutants can be used to define the regulation of expression and the relationship between each Tf receptor since these proteins function to obtain iron from human Tf. Second, recent studies have demonstrated that the Tf receptor of *N. gonorrhoeae* is an important virulence factor since mutants defective in expression of this receptor were avirulent in an experimental human infection model (9). Although there is currently no animal model which mimics *M. catarrhalis* infection in humans, our mutants will provide the most effective means of evaluating TbpA and TbpB as virulence factors when an effective model becomes available.

Finally, recent in vitro studies have shown that TbpB is capable of stimulating antibodies that elicit complement-mediated bactericidal activity against *M. catarrhalis* (20, 22). These data are consistent with previous reports of antibodies directed to the TbpB of the pathogenic *Neisseriaceae* (1–3). Although the focus of this report does not encompass vaccine studies, our isogenic mutants will also be useful in future studies designed to evaluate the effect of human antibodies to TbpA and TbpB, providing important data as to the vaccine potential of these proteins versus *M. catarrhalis* infection.

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