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Neisseria meningitidis Expressing Transferrin Binding Proteins of *Actinobacillus pleuropneumoniae* Can Utilize Porcine Transferrin for Growth

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Homologous recombination was used to generate a number of mutants of serogroup B *Neisseria meningitidis* B16B6 with the following characteristics: (i) an inability to bind human or porcine transferrin because of loss of both transferrin binding proteins (Tbp) A and B [strain B16B6(Str^r)/tbpA$^-$B$^-$] and (ii) an ability to bind porcine transferrin but not human transferrin [strain B16B6(Str^r)/tbpA^{ap}B^{ap}] due to replacement of the meningococcal Tbp with the Tbp of *Actinobacillus pleuropneumoniae*. During construction of the B16B6(Str^r)/tbpA^{ap}B^{ap} strain, transformants expressing only TbpA or TbpB of *A. pleuropneumoniae* were isolated [strains B16B6(Str^r)/tbpA^{ap}B$^-$ and B16B6(Str^r)/tbpA$^-$B^{ap}]. Expression of the *A. pleuropneumoniae* Tbp in *N. meningitidis* B16B6 was iron regulated and expressed under the control of the meningococcal promoter. The relative abilities of the meningococcal transformants to bind porcine transferrin were in the order B16B6(Str^r)/tbpA^{ap}B^{ap} > B16B6(Str^r)/tbpA^{ap}B$^-$ > B16B6(Str^r)/tbpA$^-$B^{ap}. Of these transformants, only B16B6(Str^r)/tbpA^{ap}B^{ap} could grow in the presence of porcine transferrin as the sole iron source, achieving a growth rate similar to that of the B16B6 parent strain in the presence of human transferrin.

Neisseria meningitidis, an important cause of bacterial meningitis worldwide (21), is exclusively a pathogen of humans (11). This is reflected in its iron acquisition systems, which exploit iron-sequestering compounds such as human transferrin (hTF), human lactoferrin, and hemoglobin (19, 28, 47). The meningococcal receptors for hTF and human lactoferrin are unable to bind these molecules from other animal species, whereas one of the hemoglobin receptors has been shown to utilize nonhuman sources of hemoglobin, although with reduced efficiencies (19, 47).

The inability of *N. meningitidis* to use nonhuman iron sources such as TF and lactoferrin can be seen in animal models for meningococcal infection. Intraperitoneal injection of large numbers of bacteria in mice and infant rats in the absence of an accessible iron source can cause bacteremia, infection of the central nervous system, and death (31, 43). However, the large doses of organisms required and the speed with which the animals die suggest that mortality is the consequence of toxicity rather than a true infection (31). In contrast, when mice are supplemented with an accessible iron source, such as hTF or iron dextran, consistently high mortality rates are achieved with doses of bacteria several orders of magnitude lower (5, 6, 22, 44). At these doses, the survival and pathogenicity of *N. meningitidis* strains in mice are clearly dependent on the levels of an accessible iron source (5, 6, 22, 44). In an attempt to mimic the route of entry of *N. meningitidis* more realistically, infant mice have been successfully infected via the nasal route, although, as with previous models, a supplement of hTF or iron dextran is necessary (30, 40). If *N. meningitidis* could be engineered to utilize iron from TF from a suitable animal host, an animal model which more closely mimics the course of the human infection might be developed.

N. meningitidis acquires iron from hTF by a receptor-mediated process involving two meningococcal TF binding proteins (Tbp). TbpA is an outer membrane protein that is thought to transfer the iron from hTF across the bacterial periplasmic membrane in a TonB-dependent process (11, 19). TbpB is a lipoprotein anchored in the outer membrane, and its ability to discriminate between iron-loaded and apo-TF has led to the proposition that its role is to enrich the bacterial surface with the iron source (4, 11, 19, 35, 38). Together TbpA and TbpB constitute the bacterial TF receptor; both bind exclusively to hTF (14, 23). There is evidence that both are required for meningococcal growth in vitro when hTF is the sole iron source (23), although Pintor et al. (34) have demonstrated growth when TbpB alone is expressed. There is some dispute as to whether TbpA and TbpB actually form a complex on the surface of *N. meningitidis* and whether they interact with a 1:1 or 2:1 stoichiometry (4, 11, 34, 35). Following its transport into the periplasm, iron is subsequently transported across the inner membrane by the ferric binding protein (Fbp) ABC transporter complex (19, 24). An analogous system of iron uptake from TF has been demonstrated in a range of species from the *Neisseriaceae* and *Pasteurellaceae* families, each restricted to the TF molecule from its particular natural host (19). TbpA and TbpB, encoded by the genes *tbpA* and *tbpB*, respectively, are expressed from a chromosomal operon in response to an iron-restricted growth environment and are probably under the control of the ferric uptake regulation repressor protein, Fur (15, 19). The *tbp* operons from *N. meningitidis*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* (all specific to hTF), *Actinobacillus pleuropneumoniae* (specific to porcine TF [pTF]), and *Pasteurella haemolytica* (specific to bovine, sheep, and goat TFs) have been cloned and sequenced (1, 17, 18, 27,

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TABLE 1. PCR primers used in this study

Primer	DNA sequence[a]	Target sequence
DL1	ATTAACCCTCACTAAAGGGA	T3 promoter site in the pTG3791 multiple-cloning site
DL3	GA**GGATCC**AAACGCGCGAAATGC (*Bam*HI)	Immediately downstream of the *N. meningitidis tbpA* stop codon
DL4	CCTGCAGGTCGAC**TCTAGAGAATTC** (*Xba*I) (*Eco*RI)	Within the multiple-cloning site of pTG2780
DL6	GA**GGATCC**TTAGAATTTCATTTCGAATGAAACAA (*Bam*HI)	Antisense strand immediately downstream of the *A. pleuropneumoniae tbpA* stop codon
DL7	GA**AGATCT**GAAACAGCTATGACCATGATTAC (*Bgl*II)	pUC primer 1201 (New England Biolabs) site in pFLOB4300.
DL8	GA**AGATCT**CGTTGTAAAACGACGGCCAGT (*Bgl*II)	pUC primer 1211 (New England Biolabs) site in pFLOB4300.
DL18	CG**GGATCCAGTACTC**AACAAAAACACAGGCAGC (*Bam*HI) (*Hpa*I)	Antisense strand immediately upstream of the *N. meningitidis tbpB* start codon
DL19	TATATGCATTTTAAACTTAATCCCTAT	Immediately upstream of the *A. pleuropneumoniae tbpB* start codon
DLN5	TGTCTGGGTGGCGGCGGCAGT	Immediately downstream of the signal leader cleavage site in the *N. meningitidis tbpB* gene
DLN6	TTAAAACTTCATTTCCAAGCTAAATG	Antisense strand immediately upstream of the *N. meningitidis tbpA* stop codon

[a] Primers are shown 5′ to 3′. Restriction enzyme sites are in boldface and are described in parentheses.

33). Recombinant TbpA, recombinant TbpB, and truncated or chimeric TbpB proteins have been expressed and purified from *Escherichia coli* for binding studies (8, 9, 14, 17, 26, 29, 33, 36–38). However, the expression of heterologous or chimeric Tbp in members of the *Neisseriaceae* or *Pasteurallaceae* families, which would be useful for functional studies, has not been reported. The aim of this study was to construct a meningococcal mutant strain in which the *tbpA* and *tbpB* genes were replaced with those from *A. pleuropneumoniae* and to test the ability of the engineered strain to bind to, and utilize iron from, pTF instead of hTF. Demonstration of the ability to change host TF specificity would enable further structure-function studies of the iron uptake system. Additionally, it would open up new possibilities for the development of improved animal models of meningococcal infection and disease.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α and HB101 (41) were grown at 37°C on Luria-Bertani agar or in Luria-Bertani broth (Oxoid) with shaking. Media were supplemented with kanamycin (50 μg/ml), erythromycin (10 μg/ml), or streptomycin (10 μg/ml) as necessary.

The streptomycin-resistant derivative of *N. meningitidis* B16B6 (45) (a gift of A. Schryvers) was grown at 37°C on GC agar (Difco) supplemented with 1% Vitox (Oxoid) in a 5% CO_2 atmosphere or in Mueller-Hinton broth (MHB) (Oxoid) aerobically with shaking. Erythromycin (5 μg/ml) or streptomycin (10 mg/ml) was added as appropriate. To produce iron-restricted growth conditions for *N. meningitidis*, broths were inoculated from agar plates to produce an A_{600} of 0.05 and grown for 2 h, the cells were resuspended to an A_{600} of 0.1 in fresh medium containing 25 μM desferrioxamine (Desferal; Ciba), and growth was continued overnight. For growth with various purified TFs as sole iron sources, bacteria from an overnight iron-restricted culture were used to inoculate MHB containing 100 μg of desferrioxamine per ml and 2 μM iron-loaded TF to an A_{600} of 0.02. Cultures were then grown at 37°C with shaking, and the A_{600} was recorded at intervals. The hTF and pTF used in these experiments were purchased in a partially iron-saturated form from Sigma and First Link (Brierley Hill, United Kingdom), respectively. They were iron loaded essentially as described by Schryvers and Morris (45), by incubation with a fivefold excess of $FeCl_3$ for 1 h followed by purification from the remaining unbound iron with a desalting column (HiTrap; Pharmacia Biotech).

A. pleuropneumoniae H49 (16) (a gift of A. Schryvers) was grown at 37°C on chocolate agar (Difco) in a 5% CO_2 atmosphere or aerobically with shaking in brain heart infusion broth (Difco) supplemented with 5 μg of βNAD (Sigma) per ml. Iron-restricted *A. pleuropneumoniae* was produced overnight as described above for *N. meningitidis*, but with MHB replaced with brain heart infusion broth supplemented with 5 μg of βNAD per ml.

Construction of suicide vectors. The plasmid pCRII(A−), derived from the commercial plasmid pCRII (Invitrogen), contains an engineered deletion in the β-lactamase gene for regulatory reasons. pTG2780 and pTG3791, containing the Tbp operon (*tbp*) from *N. meningitidis* B16B6 (27), were gifts of E. Jacobs (Transgene, Strasbourg, France). pFLOB4300, containing the *rpsL-ermC* antibiotic marker cassette (D. M. Johnston, S. F. Isbey, T. L. Snodgrass, M. Apicella, D. Zhou, and J. G. Cannon, Abstr. 10th Int. Pathog. Neisseria Conf., p. 37–38, 1996), was a gift of D. Johnston (University of North Carolina, Chapel Hill). Genomic DNA from *A. pleuropneumoniae* H49 was prepared by using the Puregene DNA isolation kit (Gentra Systems). DNA sequencing was performed by the chain termination method (42) with a 373 automated sequencer (Applied Biosystems).

PCR was performed with the proofreading *Pfu* DNA polymerase (Stratagene), except in the case of the Nm3′ fragment, for which *Taq* DNA polymerase (Boehringer Mannheim) was used due to primer-template mismatches near the 3′ end of primer DL4. Oligonucleotide primer sequences are based on the known sequences of the *tbp* operon in *N. meningitidis* B16B6 (27) and *A. pleuropneumoniae* H49 (17) and are listed in Table 1. PCRs were performed according to the manufacturer's instructions, and subsequent cloning steps undertaken with *E. coli* DH5α used standard molecular biology techniques (41).

The Nm3′ PCR product was amplified from pTG2780 with primers DL3 and DL4, digested by using the restriction endonucleases *Xba*I and *Bam*HI, and cloned between the *Xba*I and *Bam*HI sites in pCRII(A−), to produce pCRII(A−)/Nm3′ (not shown). The Nm5′ PCR product was amplified from pTG3791 with primers DL1 and DL18, digested with *Hin*dIII and *Bam*HI, and cloned between the *Hin*dIII and *Bam*HI sites in pCRII(A−)/Nm3′ to produce pCRII(A−)/F. This plasmid was the basis for constructing both suicide vectors. The first suicide vector, pCRII(A−)/F/rpsL-ermC′, was constructed by amplifying the *rpsL-ermC* cassette from pFLOB4300 by PCR, using primers DL7 and DL8, digesting the product with *Bgl*II, and ligating it into the *Bam*HI site of pCRII(A−)/F. In order to make the second suicide vector, pCRII(A−)/F/ApORF, the ApORF PCR product was amplified from *A. pleuropneumoniae* H49 genomic DNA by using primers DL19 and DL6, digested with *Bam*HI, and cloned between the *Hpa*I and *Bam*HI sites in pCRII(A−)/F (the blunt end of the PCR product at the DL19 primer sequence was compatible with the digested *Hpa*I site in the vector).

Transformation of bacteria with DNA. *E. coli* strains were transformed with plasmid DNA by electroporation (13). *N. meningitidis* strains were transformed with sheared genomic DNA or plasmid DNA by using a method based on that of Robertson and colleagues (39). Meningococci were resuspended in Proteose Peptone Medium (Difco) containing 1% Vitox (Oxoid) and 10 mM $MgCl_2$ to an A_{600} of 0.2. Approximately 250 ng of DNA was added to 250 μl of bacteria, incubated for 4 h at 37°C in a 5% CO_2 atmosphere, and plated onto GC agar plates containing 1% Vitox and the appropriate antibiotic. Sheared genomic DNA from the streptomycin-resistant *N. gonorrhoeae* strain FA1090 was kindly donated by D. Johnston (University of North Carolina).

Southern blotting. Genomic DNAs from *N. meningitidis* and *A. pleuropneumoniae* were prepared by using the Puregene DNA isolation kit (Gentra Systems). Approximately 2 μg of each DNA sample was digested with restriction enzymes and separated on a 0.7% agarose gel. DNA was transferred to Hybond N+ membrane (Amersham) by capillary blotting (41). DNA probes were generated by PCR, radiolabelled with [^{32}P]dATP (Amersham) by random priming (Prime-It II kit; Stratagene), and purified from any unincorporated nucleotides by using NucTrap columns (Stratagene). The NmORF probe was amplified from *N. meningitidis* genomic DNA by using the primers DLN5 and DLN6, the *rpsL-ermC* probe was amplified from pFLOB4300 by using DL7 and DL8, and the ApORF probe was amplified from *A. pleuropneumoniae* genomic DNA by

using DL19 and DL6 (Table 1). Hybridizations were performed by standard methods (41), and the results were detected by autoradiography with RX Medical X ray film (Fuji).

Immunoblotting. Outer membrane proteins were isolated from *N. meningitidis* and *A. pleuropneumoniae* by the rapid microprocedure of Carlone et al. (7). Samples containing extracts equivalent to 0.25 ml of bacteria at an A_{600} of 0.5 were boiled in sample buffer (containing β-mercaptoethanol), and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25). They were then transferred to nitrocellulose (Protran; Schleicher and Schuell) by electroblotting. Blots were incubated in blocking buffer (phosphate-buffered saline [PBS] containing 0.05% Tween 20 and 0.5% skim milk) for 1 h and washed once in PBS containing 0.05% Tween 20. They were then incubated with rabbit polyclonal antiserum raised against purified TbpA or TbpB of *A. pleuropneumoniae* H49, at a dilution of 1/3,000 or 1/30,000, respectively (gifts of A. Schryvers) (17), in blocking buffer for 2 h and washed three times. The blots were then incubated with an alkaline phosphatase-conjugated anti-rabbit secondary antibody (Sigma) at the recommended dilution in blocking buffer for 1 h and washed three more times, and color was developed by using 5-bromo-4-chloro-3-indolylphosphate with nitroblue tetrazolium (Sigma).

TF binding assay. hTF conjugated to horseradish peroxidase (HRP-hTF) was purchased from Jackson ImmunoResearch Laboratories. pTF (First Link) was conjugated to HRP by periodate coupling (20) to generate HRP-pTF. For the TF binding assay, *N. meningitidis* or *A. pleuropneumoniae* strains were grown overnight under iron-restricted conditions and resuspended to the desired A_{600} in PBS containing 25 μM desferrioxamine and 10 mM $MgCl_2$. Non-iron-restricted negative controls were grown overnight in medium without the addition of desferrioxamine and resuspended in PBS containing 10 mM $FeSO_4$ and 10 mM $MgCl_2$. Five-microliter volumes of bacterial suspensions were spotted onto nitrocellulose and air dried briefly. Blots were incubated in blocking buffer (as described above but containing skim milk at 3%) for 1 h, after which HRP-hTF or HRP-pTF was added to 1 μg/ml and incubation was continued for 1.5 h. The blots were then washed three times in PBS containing 0.05% Tween 20, and color was developed by using 4-chloro-1-naphthol (Sigma).

RESULTS

Construction of suicide vectors. Two suicide vectors, pCRII(A−)/F/rpsL-ermC′ and pCRII(A−)/F/ApORF, were constructed by cloning PCR products into the plasmid pCRII(A−), which does not replicate in *N. meningitidis*. Their construction is summarized in Fig. 1a.

Both vectors were derived from the intermediate plasmid pCRII(A−)/F, which contained DNA sequences identical to those flanking the *tbp* operon in the *N. meningitidis* B16B6 genome, in order to facilitate homologous recombination with genomic DNA. A 580-bp DNA fragment, Nm5′, contained genomic DNA upstream of, but not including, the first codon of the *tbpB* open reading frame, and a 2.2-kb fragment, Nm3′, corresponded to the genomic sequence downstream of the *tbpA* open reading frame, starting immediately after the translational stop codon.

To construct the first suicide vector, pCRII(A−)/F/rpsL-ermC′, the *rpsL-ermC′* antibiotic marker cassette was cloned between the meningococcal flanking DNA sequences. Within this cassette, the gonococcal *rpsL* gene confers susceptibility to streptomycin when present in a streptomycin-resistant (Str^r) strain of *N. gonorrhoeae* or *N. meningitidis* (D. Johnston, personal communication, and our unpublished observations). The *ermC′* gene confers resistance to erythromycin (32). Functional integrity of the *rpsL-ermC′* cassette in the pCRII(A−)/F/rpsL-ermC′ vector was confirmed with the Str^r *E. coli* strain HB101 by antibiotic selection following transformation with the plasmid (results not shown).

The second suicide vector, pCRII(A−)/F/ApORF, was generated by cloning a PCR product (ApORF) carrying the *tbp* genes from *A. pleuropneumoniae* H49 between the *N. meningitidis* flanking sequences. This created a hybrid operon containing the *tbp* promoter region and the *tbpB* ribosome binding site from *N. meningitidis*; followed by the *tbpB* open reading frame, the *tbpA* ribosome binding site, and open reading frame from *A. pleuropneumoniae*; followed in turn by the transcriptional terminator from the meningococcal operon (Fig. 1b). It

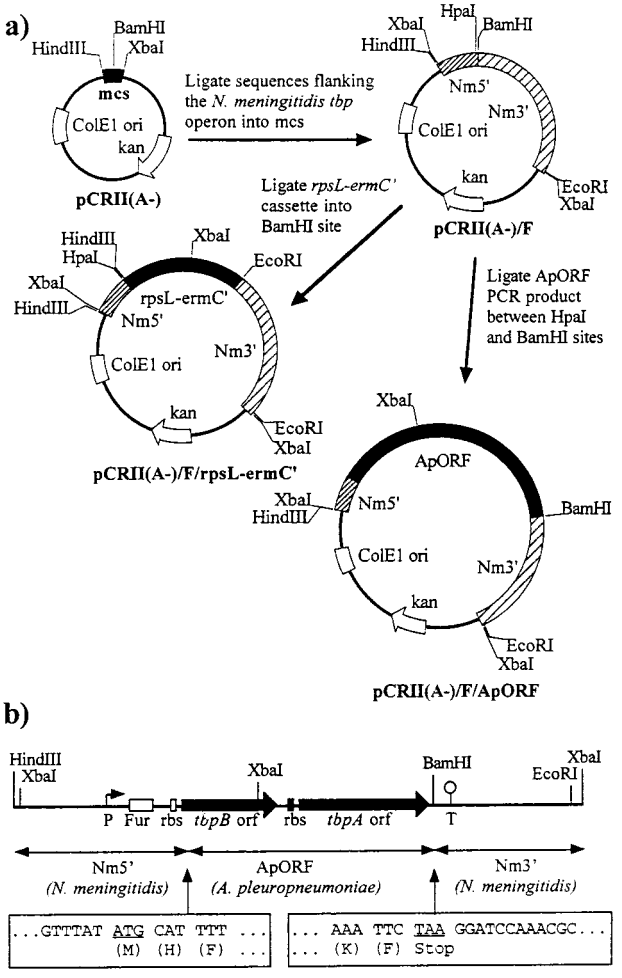

FIG. 1. Construction of the suicide vectors pCRII(A−)/F/rpsL-ermC′ and pCRII(A−)/F/ApORF. (a) Schematic summary of vector construction (ColE1 ori, ColE1 origin of replication; kan, kanamycin resistance gene; mcs, multiple-cloning site). (b) Details of the genetic construct assembled in pCRII(A−)/F/ApORF, showing the relative positions of the *tbp* operon promoter (P), Fur box (Fur), ribosome binding sites (rbs), *tbpA* and *tbpB* open reading frames (orf), and transcriptional terminator (T). The species origin of the DNA sequence in each cloned PCR product is indicated, and the DNA sequences across the genetic fusions are shown.

was noted that the transcriptional terminator region contained two copies of the 10-bp recognition sequence (GCCGTCTGA A), required for the uptake of target DNA by *Neisseria* species (46), as an inverted repeat. Selected regions within the hybrid operon in the vector pCRII(A−)/F/ApORF (the promoter region and beginning of *tbpB* open reading frame, the end of *tbpB* open reading frame and beginning of the *tbpA* open reading frame, and the end of the *tbpA* open reading frame and the transcriptional terminator) were subjected to DNA sequencing in order to confirm the cloning strategy had been successful.

Homologous recombination in a streptomycin-resistant mutant of *N. meningitidis* B16B6. The two-step procedure for the replacement of the *tbpA* and *tbpB* open reading frames in the meningococcal genome required the use of a streptomycin-resistant mutant of *N. meningitidis* B16B6. This strain, B16B6 (Str^r), was created by transforming the parent strain with sheared genomic DNA from an Str^r isolate of *N. gonorrhoeae* followed by selection on streptomycin (results not shown).

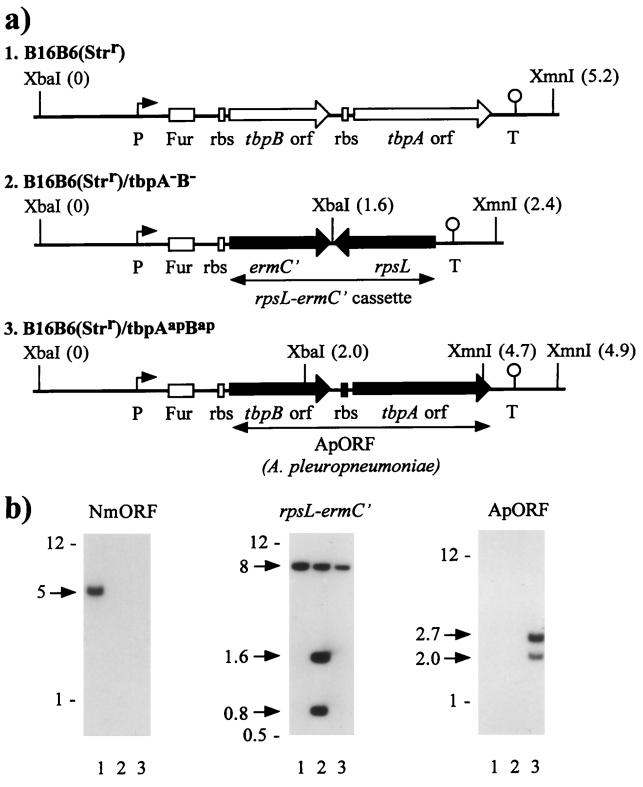

FIG. 2. The *tbp* operon locus within the genomes of *N. meningitidis* B16B6(Str^r), B16B6(Str^r)/tbpA$^-$B$^-$, and B16B6(Str^r)/tbpA^{ap}B^{ap}. (a) Diagrammatic representation of the locus in each strain, highlighting the positions of *Xba*I and *Xmn*I restriction enzyme sites. The relative position of each site, in kilobases, is shown in parentheses. Abbreviations are as in Fig. 1. (b) Results of Southern blotting with genomic DNAs from the three strains. DNA was digested with the restriction enzymes *Xba*I and *Xmn*I, blotted, and probed with radioactively labelled PCR products corresponding to the *tbpB-tbpA* region from *N. meningitidis* B16B6 (NmORF), the *rpsL-ermC′* antibiotic cassette (*rpsL-ermC′*), or the *tbpB-tbpA* region from *A. pleuropneumoniae* (ApORF). The scale of each image is indicated by the relative positions of selected molecular size standards, in kilobases, at the left of each blot. The sizes of the DNA fragments generating a positive signal are shown with arrows.

In the first step of the *tbpA* and *tbpB* replacement procedure, the *tbpA* and *tbpB* open reading frames were replaced in the genome of the B16B6(Str^r) strain with the *rpsL-ermC′* cassette by transformation with the pCRII(A−)/F/rpsL-ermC′ suicide vector. Following transformation, a meningococcal transformant exhibiting resistance to erythromycin and susceptibility to streptomycin was isolated and referred to as the B16B6(Str^r)/tbpA$^-$B$^-$ strain. The modified *tbp* operon in this strain is shown diagrammatically in Fig. 2a.

In the second step of the replacement procedure, the *rpsL-ermC′* cassette was replaced in the genome of the B16B6(Str^r)/tbpA$^-$B$^-$ meningococcal strain with the *tbpB* and *tbpA* open reading frames from *A. pleuropneumoniae* by transformation with the pCRII(A−)/F/ApORF suicide vector. A double-crossover event between the sequences flanking the *tbp* operon in the vector and the chromosomal locus would create a hybrid chromosomal *tbp* operon expressing the heterologous *tbpB* and *tbpA* genes (Fig. 2a). Following transformation of the B16B6(Str^r)/tbpA$^-$B$^-$ strain with the pCRII(A−)/F/ApORF vector, a transformant which had regained streptomycin resistance, but lost erythromycin resistance, was selected and named the B16B6(Str^r)/tbpA^{ap}B^{ap} strain.

Southern blotting was used to confirm that the meningococcal *tbp* genes from the B16B6(Str^r) strain had been replaced with the *rpsL-ermC′* cassette in the B16B6(Str^r)/tbpA$^-$B$^-$ strain, and that these were then replaced in turn by the *A. pleuropneumoniae tbp* genes in the B16B6(Str^r)/tbpA^{ap}B^{ap} strain (Fig. 2b). Genomic DNAs from the three meningococcal strains were digested by using restriction enzymes for sites flanking the *tbp* operon (*Xmn*I and *Xba*I), and the resulting blots were probed with PCR products corresponding to either the *tbpB-tbpA* region from B16B6 (NmORF), the *rpsL-ermC′* antibiotic marker cassette (*rpsL-ermC′*), or the *tbpB-tbpA* region from *A. pleuropneumoniae* H49 (ApORF).

The NmORF probe hybridized to a single fragment (approximately 5 kb) in the digested DNA from the B16B6(Str^r) parent strain but to nothing in the B16B6(Str^r)/tbpA$^-$B$^-$ or B16B6(Str^r)/tbpA^{ap}B^{ap} strain. This confirmed that the meningococcal *tbp* genes were deleted as the result of the first homologous recombination event. The *rpsL-ermC′* probe highlighted a high-molecular-size fragment (approximately 8 kb) in all of the meningococcal strains, consistent with the continued presence of a mutated *rpsL* gene at an unknown chromosomal locus, which conferred the original Str^r phenotype. However, this probe also highlighted two smaller fragments in the B16B6(Str^r)/tbpA$^-$B$^-$ strain (1.6 and 0.8 kb), which was predicted if the *rpsL-ermC′* cassette had replaced the original *tbpB-tbpA* region (Fig. 2a). The absence of these bands in DNA from the B16B6(Str^r)/tbpA^{ap}B^{ap} strain showed that the cassette was subsequently deleted by the second recombination event. The ApORF probe hybridized to two fragments in DNA from the B16B6(Str^r)/tbpA^{ap}B^{ap} strain (2.0 and 2.7 kb) but not to DNA from the previous two strains, suggesting that the heterologous *tbpB-tbpA* fragment had indeed been inserted into the *tbp* operon during the second recombination procedure.

The sequences of two PCR products generated from genomic DNA, spanning either end of the ApORF insert, confirmed that the ApORF sequence was fused to the expected meningococcal flanking sequences in the B16B6(Str^r)/tbpA^{ap}B^{ap} genome.

During construction of the B16B6(Str^r)/tbpA^{ap}B^{ap} strain, transformants which expressed solely TbpA or TbpB from *A. pleuropneumoniae* (as judged by immunoblotting) were isolated. They were named the B16B6(Str^r)/tbpA^{ap}B$^-$ and B16B6(Str^r)/tbpA$^-$B^{ap} strains, respectively, and were included in subsequent analyses for comparison to the B16B6(Str^r)/tbpA^{ap}B^{ap} strain. Subsequent DNA sequencing analysis revealed that the failure of these individual clones to express one or the other of the Tbp was due to the presence of a frameshift mutation in the *tbpB* or *tbpA* gene (results not shown).

Expression of recombinant TbpA and TbpB in *N. meningitidis*. Expression of *A. pleuropneumoniae* TbpA and TbpB in response to low-iron conditions was assayed by immunoblotting. *N. meningitidis* and *A. pleuropneumoniae* strains were grown overnight under iron-rich or iron-deficient conditions, and bacterial outer membrane proteins were isolated. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with polyclonal antiserum specific to either TbpA or TbpB from the *A. pleuropneumoniae* H49 strain (Fig. 3).

The anti-TbpA antiserum cross-reacted with two proteins in the parent *N. meningitidis* B16B6(Str^r) strain grown under iron-restricted conditions (Fig. 3a, lane 2). The smaller of the two is slightly smaller than the *A. pleuropneumoniae* TbpA (lanes 3 and 4) and was not detected in the B16B6(Str^r)/tbpA$^-$B$^-$ knockout strain (lanes 5 and 6). It is most likely the 100-kDa meningococcal TbpA, which was present only in the parent strain. The higher-molecular-mass protein in lane 2 was assumed to be some unrelated, iron-inducible, meningococcal outer membrane protein, since it was detected in iron-deficient

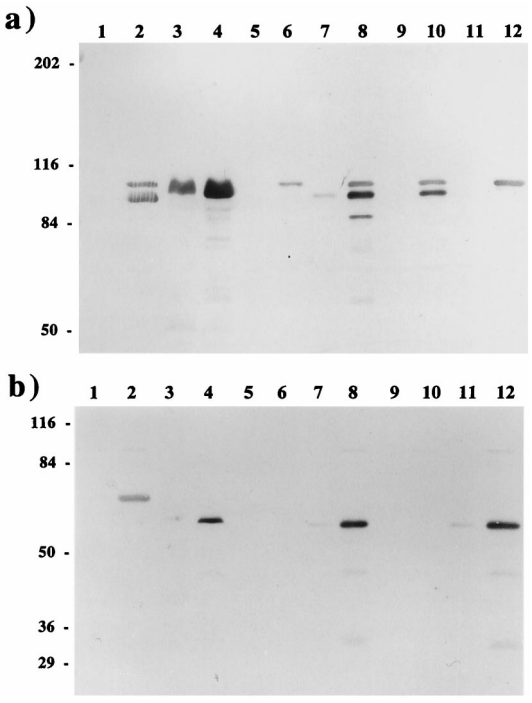

FIG. 3. Immunoblotting of outer membrane extracts from *N. meningitidis* and *A. pleuropneumoniae* strains. Proteins were separated by SDS-PAGE and probed with a polyclonal rabbit antiserum specific to TbpA (a) or TbpB (b) of *A. pleuropneumoniae* H49. Samples: lanes 1 and 2, *N. meningitidis* B16B6(Str^r); lanes 3 and 4, *A. pleuropneumoniae* H49; lanes 5 and 6, *N. meningitidis* B16B6(Str^r)/tbpA$^-$B$^-$; lanes 7 and 8, *N. meningitidis* B16B6(Str^r)/tbpA^{ap}B^{ap}; lanes 9 and 10, *N. meningitidis* B16B6(Str^r)/tbpA^{ap}B$^-$; lanes 11 and 12, *N. meningitidis* B16B6(Str^r)/tbpA$^-$B^{ap}. Odd-numbered samples were prepared from bacteria grown under iron-rich conditions, and even-numbered samples were prepared from bacteria grown under iron-restricted conditions. The relative positions of molecular mass standards, in kilodaltons, are indicated at the left of each gel.

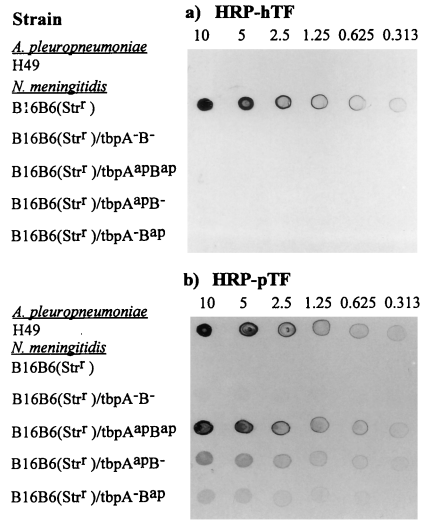

FIG. 4. Ability of *A. pleuropneumoniae* H49 and *N. meningitidis* B16B6(Str^r), B16B6(Str^r)/tbpA$^-$B$^-$, B16B6(Str^r)/tbpA^{ap}B^{ap}, B16B6(Str^r)/tbpA^{ap}B$^-$, and B16B6(Str^r)/tbpA$^-$B^{ap} to bind HRP-hTF and HRP-pTF. Iron-restricted bacteria were resuspended to an A_{600} of 10, 5, 2.5, 1.25, 0.625, or 0.313 and spotted onto nitrocellulose. Blots were then probed with HRP-hTF (a) or HRP-pTF (b), and color was developed by using a chromogenic HRP substrate.

samples from all of the meningococcal strains (lanes 6, 8, 10, and 12). As expected, the anti-TbpA antiserum highlighted a band corresponding to the 102-kDa TbpA in *A. pleuropneumoniae* H49. This was weakly expressed following growth under iron-rich conditions and strongly expressed under iron-restricted conditions (lanes 3 and 4). It also detected a similar-sized band in the B16B6(Str^r)/tbpA^{ap}B^{ap} and B16B6(Str^r)/tbpA^{ap}B$^-$ recombinant strains when they were grown under iron restriction (lanes 8 and 10), with the band being stronger in the B16B6(Str^r)/tbpA^{ap}B^{ap} strain (lane 8). This protein was also detected faintly following iron-rich growth (lane 7). The results suggest that in the B16B6(Str^r)/tbpA^{ap}B^{ap} and B16B6(Str^r)/tbpA^{ap}B$^-$ strains, the heterologous TbpA protein was expressed under the control of the meningococcal promoter and transported into the bacterial outer membrane. Its expression appeared to be higher in the strain which also expressed the foreign TbpB. It is unknown whether the smaller band present in the B16B6(Str^r)/tbpA^{ap}B^{ap} extract (lane 8) was a truncated form of TbpA.

The anti-TbpB antiserum cross-reacted weakly with a protein of approximately 63 kDa in the iron-restricted version of the parent *N. meningitidis* B16B6(Str^r) strain (Fig. 3b, lane 2). Due to its molecular mass and its absence in the B16B6(Str^r)/tbpA$^-$B$^-$ knockout strain (lanes 5 and 6), it was assumed to be meningococcal TbpB from the parental *N. meningitidis* strain. The anti-TbpB antiserum also produced a strong band of approximately 60 kDa in outer membrane extracts from *A. pleuropneumoniae* H49 and the meningococcal B16B6(Str^r)/tbpA^{ap}B^{ap} and B16B6(Str^r)/tbpA$^-$B^{ap} strains when grown under iron-deficient conditions (lanes 4, 8, and 12). The results imply that the B16B6(Str^r)/tbpA^{ap}B^{ap} and B16B6(Str^r)/tbpA$^-$tbpB^{ap} strains express the heterologous TbpB protein under the control of the meningococcal promoter and transport it into the bacterial outer membrane.

Binding of recombinant bacteria to hTF and pTF. The ability of whole cells of the different meningococcal strains to bind hTF or pTF under iron-restricted conditions was determined by dot blotting (Fig. 4). *N. meningitidis* and *A. pleuropneumoniae* strains were grown overnight under low-iron conditions, immobilized by being spotted onto nitrocellulose, and probed with horseradish peroxidase conjugates of partially saturated hTF or pTF.

The parent meningococcal strain, B16B6(Str^r), bound hTF but not pTF, as expected. Similarly, *A. pleuropneumoniae* H49 bound pTF but not hTF. The double knockout meningococcal mutant, B16B6(Str^r)/tbpA$^-$B$^-$, bound neither species of TF. The meningococcal strains expressing one or both of the *A. pleuropneumoniae* Tbp showed no binding to hTF but bound pTF to various degrees. The strain expressing both Tbp bound pTF the most strongly, that expressing TbpA alone bound less strongly and that expressing TbpB alone bound even more weakly [strains B16B6(Str^r)/tbpA^{ap}B^{ap}, B16B6(Str^r)/tbpA^{ap}B$^-$, and B16B6(Str^r)/tbpA$^-$B^{ap}, respectively]. None of the bacterial strains tested bound to hTF or pTF when grown under iron-rich conditions (not shown).

Growth with hTP or pTP as the sole iron source. In order to determine whether the *N. meningitidis* strains expressing one or both of the *A. pleuropneumoniae* Tbp could use iron from pTF, their growth in medium containing iron-saturated hTP or pTP as the sole iron source was studied (Fig. 5). Desferrioxamine was added to the growth medium to chelate free iron. Without the addition of extraneous iron, none of the meningococcal strains grew (Fig. 5a). When hTF was added to the medium, the parent B16B6(Str^r) strain grew, but there was no growth of the meningococcal derivatives with the meningococ-

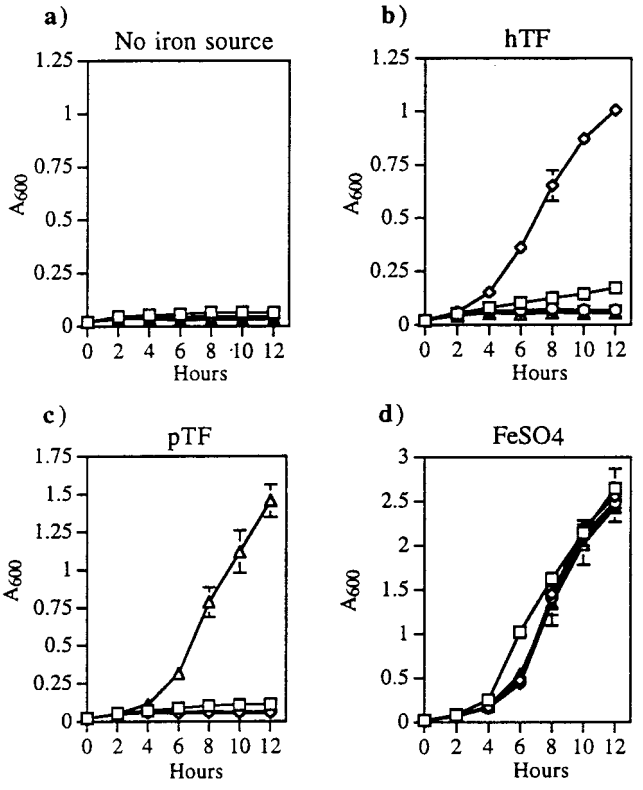

FIG. 5. Growth of *N. meningitidis* B16B6(Str^r) (diamonds), B16B6(Str^r)/tbpA$^-$B$^-$ (squares), B16B6(Str^r)/tbpA^{ap}B^{ap} (triangles), B16B6(Str^r)/tbpA^{ap}B$^-$ (circles), and B16B6(Str^r)/tbpA$^-$B^{ap} (inverted triangles) in vitro with various iron sources. Bacteria were grown overnight under iron-restricted conditions and diluted to an A_{600} of 0.02 in medium containing the iron chelator desferrioxamine plus no iron source (a), iron-loaded hTF (b), iron-loaded pTF (c), or $FeSO_4$ (d), and their growth was recorded for up to 12 h. Each data point represents the mean from three independent experiments. Error bars show one standard deviation.

cal *tbp* gene deleted or expressing the *A. pleuropneumoniae tbp* gene (Fig. 5b). However, the B16B6(Str^r)/tbpA^{ap}B^{ap} strain grew in the presence of pTF (Fig. 5c). Thus, expression of both TbpA and TbpB from *A. pleuropneumoniae* in *N. meningitidis* allowed the use of pTF as an iron source. The B16B6(Str^r)/tbpA^{ap}B$^-$ and B16B6(Str^r)/tbpA$^-$B^{ap} strains, expressing solely *A. pleuropneumoniae* TbpA or TbpB, did not grow under any of these free-iron-limited conditions, showing that expression of both Tbp was necessary for iron utilization. When the medium was supplemented with free iron in the form of iron sulfate, all of the strains grew, including the B16B6(Str^r)/tbpA$^-$B$^-$ knockout strain, confirming that lack of growth of any strain was due to a lack of available iron (Fig. 5d). The *A. pleuropneumoniae* H49 parent strain was not included in these experiments, as it requires different growth conditions. Its ability to use pTF, but not hTF, as its sole iron source has been demonstrated by others, however (16).

A summary of the results of the TF binding and growth studies for the meningococcal and *A. pleuropneumoniae* strains used in this study is given in Table 2.

DISCUSSION

Using a counterselectable antibiotic marker cassette (*rpsL-ermC′*), developed by Johnston and colleagues (Abstr. 10th Int. Pathog. Neisseria Conf., 1996) for use in *N. gonorrhoeae*, we have selectively replaced the *tbpA* and *tbpB* open reading frames in the chromosome of *N. meningitidis* with analogous genes from the pig pathogen *A. pleuropneumoniae*. A two-step procedure of homologous recombination was used, which enables precise changes to be made at a chromosomal locus, using positive antibiotic selection, but without the need to leave a resistance marker at the site of genetic manipulation. This method was also recently used with *N. gonorrhoeae* to insert transcriptional and translational stop codons into the *tbpB* gene in order to generate a mutant which is unable to express TbpA and TbpB (10). Our report shows that the *rpsL-ermC′* cassette can also be used in *N. meningitidis*. The use of a counterselectable marker such as *rpsL-ermC′* for homologous recombination in the meningococcal chromosome is attractive for a number of reasons. First, because the *rpsL-ermC′* cassette is excised from the chromosome during the second recombination step, it could also be used repeatedly to engineer changes at different loci in the same experimental bacterial strain. Second, no antibiotic markers remain at the site of recombination. The only restriction on use of the *rpsL-ermC′* cassette is the requirement that the target bacterium must contain a chromosomal streptomycin resistance marker.

TbpA and TbpB from *A. pleuropneumoniae* H49 were successfully expressed in *N. meningitidis* B16B6, under the control of the native meningococcal iron-regulated promoter, without the need to alter codon usage in the foreign genes. Both proteins were also translocated into the bacterial outer membrane by using their own signal leader peptides. The expression level of the foreign TbpA appeared to be lower in the mutant which failed to coexpress TbpB [the B16B6(Str^r)/tbpA^{ap}B$^-$ strain], suggesting that the failure to translate *tbpB* exerted a polar effect on the translation of *tbpA*.

Not only were *A. pleuropneumoniae* TbpA and TbpB expressed and successfully targeted to the meningococcal outer

TABLE 2. Properties of the *N. meningitidis* and *A. pleuropneumoniae* strains used in this work with respect to Tbp expression

Strain	Receptors expressed	Binding to:		Utilization of iron bound to:	
		pTF	hTF	pTF	hTF
N. meningitidis					
B16B6(Str^r)	*N. meningitidis* TbpA and TbpB	−	+++	−	+++
B16B6(Str^r)tbpA$^-$B$^-$	None	−	−	−	−
B16B6(Str^r)tbpA^{ap}B^{ap}	*A. pleuropneumoniae* TbpA and TbpB	+++	−	+++	−
B16B6(Str^r)tbpA^{ap}B$^-$	*A. pleuropneumoniae* TbpA only	++	−	−	−
B16B6(Str^r)tbpA$^-$B^{ap}	*A. pleuropneumoniae* TbpB only	+	−	−	−
A. pleuropneumoniae H49	*A. pleuropneumoniae* TbpA and TbpB	+++	−	+++[a]	−[a]

[a] Reported by others (16).

membrane in response to iron restriction, but they both specifically bound to pTF. As whole bacterial cells were used in this experiment, the results suggest that the foreign Tbp not only were located in the bacterial outer membrane but also were exposed on the cell surface. This cell surface exposure was confirmed by the observation that meningococcal cells expressing the *A. pleuropneumoniae* Tbp could utilize iron from pTF for growth. When each Tbp was singly expressed, the *A. pleuropneumoniae* TbpB bound less pTF than the TbpA, analogous to the result seen with meningococcal Tbp isogenic mutants and hTF (23). As partially iron-saturated pTF was used in this assay, the observation may simply reflect the fact that TbpB preferentially binds to iron-loaded (holo-) TF, whereas TbpA does not show the same distinction, an observation which has been reported for *N. gonorrhoeae*, *Moraxella catarrhalis*, and *N. meningitidis* (4, 12, 36, 38, 49). However, lack of discrimination for meningococcal TbpA between apo- and holo-TF has been disputed (35). Alternative explanations are that *A. pleuropneumoniae* TbpA has a greater affinity for pTF than TbpB and/or that more TbpA than TbpB is expressed. Further studies will be needed to clarify this issue.

The meningococcal mutant expressing both TbpA and TbpB from *A. pleuropneumoniae* was capable of growing in vitro with pTF instead of hTF as its sole iron source. Its growth rate was comparable to that of the wild-type meningococcal parent strain, suggesting that the heterologous Tbp functioned as well as the native proteins in the meningococcal host. It has been shown that in *N. gonorrhoeae* and *N. meningitidis* utilization of iron from TF is an energy-dependent process requiring the TonB periplasmic protein (3, 48), and that in *N. gonorrhoeae* mutations within the TbpA-TonB box prevent iron usage from human transferrin (8). The ability of the meningococcal mutant expressing *A. pleuropneumoniae* Tbp to utilize iron from pTF indicates that the TonB box motif present in the *A. pleuropneumoniae* TbpA was recognized by the meningococcal TonB protein. This was surprising, since the putative TonB box on the *A. pleuropneumoniae* TbpA does not closely match the TonB consensus sequence, whereas that from meningococcus does (2, 17, 27). The cross-species recognition between TonB and TbpA implied by this study complements recent evidence that TbpA and TbpB from different species share conserved sites for interacting with each other (14). This also suggests that engineering a meningococcal TonB box motif into the foreign TbpA protein may not be necessary to express functional TbpA proteins from other *Neisseria* or *Pasteurella* species in *N. meningitidis*.

The meningococcal strains which expressed only *A. pleuropneumoniae* TbpA or TbpB could not grow with pTF as the sole iron source, even though both strains could bind pTF. This suggests that both TbpA and TbpB from *A. pleuropneumoniae* are required for growth on pTF by a meningococcal mutant and probably, by extrapolation, by the native *A. pleuropneumoniae* host too. An equivalent conclusion was reached for *N. meningitidis* in a similar assay with hTF (23). However, by using different growth assays (solid media), it was found that *N. gonorrhoeae* and *H. influenzae* mutants expressing only TbpA could grow in the presence of hTF (1, 18). The study of the properties of TbpA and TbpB in *A. pleuropneumoniae* by using isogenic mutants has not been reported. If this is due to difficulties involved in manipulating the organism, then the use of *N. meningitidis* as an alternative host could be valuable for functional studies on Tbp.

This study provides proof of the concept that TbpA and TbpB proteins from another member of the *Neisseriaceae* or *Pasteurellaceae* families can be expressed in their functional form in *N. meningitidis*. This has several potential applications in the study of Tbp. As mentioned above, it may expand the study of Tbp from species for which genetic manipulation is difficult or for which the expression of recombinant Tbp in *E. coli* has proven difficult (17). It may also permit the direct comparison of Tbp from different species to determine whether the ability of TbpA on its own to permit some species to use hTF as a sole iron source is the result of some difference between TbpA from different organisms (1, 19). Furthermore, truncated or chimeric TbpB proteins have been expressed and purified from *E. coli* in order to map binding sites for different TF molecules or for TbpA (8, 9, 14, 17, 26, 29, 33, 36–38). Expression of such chimeras, or combinations of TbpA and TbpB from different sources, in *N. meningitidis* would facilitate investigations into the effects of different regions of the molecules on utilization of iron from different sources and would facilitate studies on identification of functional conservation between components of the iron acquisition systems in different species.

Animal models of meningococcal infections which are capable of causing bacteremia and meningitis have been developed mostly with mice, and they require the injection of an iron source such as iron salts or hTF into the animals in order to sustain bacteremia and generate high mortality rates (5, 6, 22, 30, 40, 44). The generation of an *N. meningitidis* mutant capable of growth with pTF as its sole iron source introduces the possibility of generating an animal model of meningococcal infection in the pig, which would not require injection of an iron source. It is anticipated that such a strain, with the meningococcal *tbpA* and *tbpB* genes having been replaced, rather than complemented, would present a significantly reduced safety risk to humans than the wild-type *N. meningitidis*, as expression of the native Tbp has been shown to be essential for virulence of the related species *N. gonorrhoeae* in humans (10).

The work presented in this study has shown that host species-specific TF interactions and associated iron utilization can be altered and raises the prospect of development of animal models of natural infection and vaccine evaluation for human-specific infectious agents. In addition to those on *N. meningitidis*, studies on *N. gonorrhoeae* may also benefit from such an approach.

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