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Gonococcal Transferrin-Binding Protein 2 Facilitates but Is Not Essential for Transferrin Utilization

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Pathogenic *Neisseria* species have been shown to scavenge iron from transferrin (Tf), although the mechanism is not yet fully understood. Two iron-repressible proteins that exhibit Tf-binding activity have been identified. This work describes the cloning and sequencing of *tbpB*, a 2.1-kb gene in *N. gonorrhoeae* that encodes Tbp2, an 85-kDa iron-repressible lipoprotein. Transcriptional interruption of *tbpB* had a strong polar effect on *tbpA*, the structural gene for Tbp1 that is located immediately downstream from *tbpB*. Such *tbpB* mutants did not express either Tbp2 or Tbp1, did not bind Tf to whole cells, did not grow on Tf plates, and did not take up iron from Tf. A mutant in which most of *tbpB* was deleted, presumably leaving *tbpA* under transcriptional control of the *tbpB* promoter, was constructed. This mutant did not express Tbp2 but expressed wild-type levels of Tbp1 and possessed the phenotype of reduced binding of Tf, decreased iron uptake from Tf, but normal growth on Tf plates. Mutants expressing Tbp2 and not Tbp1 bound less Tf, did not grow on Tf plates, and did not take up iron from Tf. These results suggest that *tbpB* and *tbpA* are polycistronic. Tbp2 apparently facilitates binding of Tf but is not essential for acquisition of iron from Tf under these in vitro conditions.

Iron acquisition is commonly recognized as a virulence factor in human pathogens (49, 52). Many pathogens synthesize siderophores that both solubilize ferric iron and scavenge iron from the soluble iron-binding glycoproteins transferrin (Tf) and lactoferrin (Lf) (29). However, the pathogenic *Neisseria* species do not synthesize siderophores (1, 26, 50). *Neisseria meningitidis* (1, 26, 43, 45), *Neisseria gonorrhoeae* (24, 26), and certain other bacterial species (12, 17, 27, 30, 31, 36) are capable of direct utilization of Tf as their sole source of iron in the apparent absence of siderophore production. Meningococci and most, although not all, gonococcal isolates can also use Lf as a sole iron source (25). When colonizing the urogenital tract, rectum, or pharynx, *Neisseria* spp. presumably obtain iron from Lf that is abundant on these mucosal surfaces (14). Gonococci and meningococci (39, 51) are also apparently capable of using siderophores produced by other organisms for iron acquisition, which may facilitate growth on mucosal sites containing other microorganisms. Upon dissemination to the blood, synovial membranes, or cerebrospinal fluid, these pathogens probably use the more abundant Tf as their iron source (14, 49).

In iron-limited environments, gonococci and meningococci produce multiple outer membrane proteins designated iron-repressible proteins (9, 50). Gonococci (24) and meningococci (45) can bind Tf and Lf directly to their surfaces when grown under iron-limiting conditions and remove iron from Tf and Lf in an energy-dependent manner (24, 45). Tf is not internalized during iron uptake (24, 45). Binding of Tf is saturable and specific (3, 21, 43, 48), suggesting that there is a specific Tf receptor. Biochemical evidence (42, 43) suggests and genetic evidence (3, 6, 18, 48) confirms the existence of iron-repressible specific receptors for both Tf and Lf.

By using Tf affinity purification, two neisserial iron-repressible proteins that specifically bind human Tf have been identified: a 96- to 100-kDa Tf-binding protein designated Tbp1 (6, 33, 41), and a 64- to 85-kDa Tf-binding protein designated Tbp2 (6, 33, 41). The size of Tbp2 varies widely depending on the meningococcal strain examined (41, 43). Analysis of antigenic and genomic features of meningococcal Tbp2 variants has prompted the division of this protein into two classes (37). Both meningococcal (41, 43) and gonococcal (6) Tbp2 bind Tf after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and electroblotting. In contrast, Tbp1 Tf-binding activity apparently requires native conformation and is not evident under denaturing conditions (6, 41, 43).

Cornelissen et al. (6) isolated and characterized the gonococcal gene *tbpA*, which encodes Tbp1. The predicted protein sequence showed that it is a member of the TonB-dependent receptor family. Interruption of *tbpA* by insertional mutagenesis and gene replacement generated mutants that bound less horseradish peroxidase-labeled Tf (HRP-Tf) to whole cells and did not grow on plates containing Tf as the iron source (6). Recombinant *Escherichia coli* strains expressing gonococcal *tbpA* acquired the ability to bind human Tf specifically, although they were not able to utilize Tf as their sole iron source (5). These observations indicate that Tbp1 is a critical component of the gonococcal Tf receptor but suggest that other proteins contribute to the function of the receptor.

Recently, the meningococcal *tbpA* and *tbpB* genes were cloned, sequenced, and mutagenized (18, 22). Mutations in either *tbpA* or *tbpB* abolished the ability of meningococci to grow under conditions in which iron was supplied on Tf (18). In this study, we report the cloning and analysis of gonococcal *tbpB* and show that gonococcal and meningococcal *tbpB* genes are related. Gonococcal *tbpB* mutants which expressed Tbp1 but not Tbp2 bound less HRP-Tf to the cell surface and took up less iron from Tf compared with the wild type but grew on Tf as the sole source of iron.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. gonorrhoeae* strains used in this study are described in Table 1. Bacteria

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TABLE 1. Strains and plasmids used

N. gonorrhoeae strain or *E. coli* plasmid	Description	Source or reference
Strains		
FA19	Wild type (Tbp1$^+$ Tbp2$^+$)	26
FA6747	*tbpA*::mTn*3*(Cm) (Tbp1$^-$ Tbp2$^+$)	6
FA6813	*tbpB*::mTn*3*(Cm) (Tbp1$^{+/-}$ Tbp2$^-$)	This study
FA6814	*tbpB*::mTn*3*(Cm) (Tbp1$^{+/-}$ Tbp2$^-$)	This study
FA6815	*tbpB*::Ω (Tbp1$^-$ Tbp2$^-$)	This study
FA6819	Δ*tbpB* (Tbp1$^+$ Tbp2$^-$)	This study
Plasmids		
pBluescript II SK(+)	Amp^r	Stratagene
pHP45Ω	Source for the Ω fragment, Str^r	35
pUP1	pHSS6 containing the gonococcal uptake sequence, Kan^r	10
pUNCH125	pUP1 containing a 3.4-kb *Ava*I fragment extending from *Sau*3AI in *tbpA* downstream with a mTn*3*(Cm) insertion between *Xba*I sites	2
pUNCH411	pBluescript II SK(+) containing the *Hin*dIII-to-*Xba*I fragment of *tbpA*	7
pUNCH416	pBluescript II SK(+) containing a 2.2-kb PCR-amplified *tbpB* fragment cloned into *Bam*HI-to-*Kpn*I sites	This study
pUNCH421	*Hin*dIII-to-*Xba*I fragment of *tbpA* from pUNCH411 cloned into *Hin*dIII-to-*Xba*I sites of pUNCH416	This study
pUNCH750	pBluescript II SK(+) containing a 1.8-kb *Sau*3AI fragment of *tbpB*	This study
pUNCH751	pUP1 containing a 1.8-kb *Sau*3AI fragment of *tbpB*	This study
pUNCH751a	pUNCH751 with mTn*3*(Cm) insertion	This study
pUNCH751b	pUNCH751 with mTn*3*(Cm) insertion	This study
pUNCH752	pBluescript II SK(+) containing a 0.85-kb *Dra*I fragment of *tbpB*	This study
pUNCH754	pUNCH421 deleted from *Nhe*I to *Hin*dIII	This study
pUNCH755	pUNCH754 with the 2.1-kb *Xba*I fragment from pUNCH125	This study
pUNCH756	pUNCH751 with Ω fragment in the *Xmn*I site	This study

were cultured in GC broth or on GC agar (Difco) or chemically defined medium (CDM) as previously described (6). Iron was removed from CDM by using Chelex-100 (Bio-Rad) (50). For Western blot (immunoblot) analysis of total membrane proteins, gonococci were grown in GC broth or CDM as described previously (6). GC broth was depleted of iron, when indicated, by addition of 50 μM Desferal (deferoxamine mesylate; CIBA-GEIGY, Summit, N.J.). CDM-grown bacteria were made iron replete by the addition of 100 μM ferric nitrate.

Growth on plates containing either Tf or Lf as an iron source was assayed on CDM agarose containing Tf or Lf as previously described (6).

DNA isolation, digestion, and library construction. Chromosomal DNA was purified on CsCl gradients as described by Stern et al. (46). *E. coli* plasmids were amplified, isolated, and digested with restriction endonucleases by standard methods (23). To clone pUNCH750 (Fig. 1), a library was constructed from FA19 chromosomal DNA digested to completion with *Sau*3AI. Size-fractionated fragments (from 1 to 3 kb) of this *Sau*3AI digest were ligated into the *Bam*HI site in pBluescript II SK(+) (Stratagene, La Jolla, Calif.). This library was transformed into $CaCl_2$-treated (23) *E. coli* DH5αMCR (Bethesda Research Laboratories, Gaithersburg, Md.) competent cells and screened with a 107-bp probe (GS3′; Fig. 1) generated by PCR. This probe, amplified from the 5′ end of pUNCH405 (6) that contains most of the *tbpA* gene, was labeled with digoxigenin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) during the PCR.

To clone pUNCH752 (Fig. 1), a total genomic library was constructed from FA19 DNA completely digested with *Dra*I, followed by the addition of *Eco*RI linkers and ligation into *Eco*RI-digested λ ZAPII (Stratagene). The library was packaged with the Packagene system (Promega, Madison, Wis.) and plated on *E. coli* DH5αMCR (Bethesda Research Laboratories). This library was screened with a 142-bp probe (GS5;

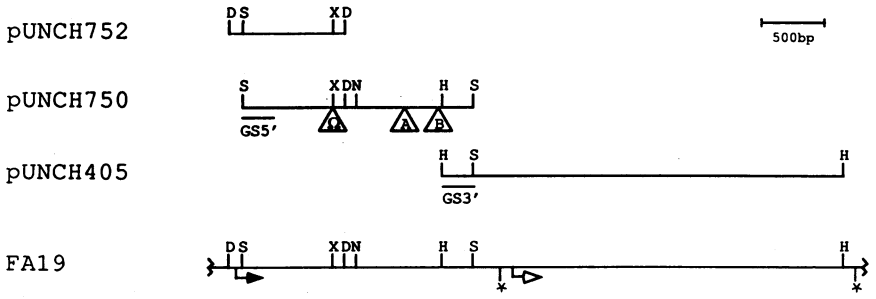

FIG. 1. Cloning map for *tbpB*. Symbol: ⧋, position of Ω insertion strain FA6815; ⧋, position of mTn*3*(Cm) insertion strain FA6813; ⧋, position of mTn*3*(Cm) insertion strain FA6814. The solid arrow indicates the beginning and the direction of the ORF of the *tbpB* gene; the open arrow indicates the beginning and direction of the ORF of the *tbpA* gene. Asterisks indicate the ends of the *tbpB* and *tbpA* ORFs. The underlined areas represent the probes used to screen genomic libraries. Designations for sites of restriction endonuclease recognition: D, *Dra*I; H, *Hin*cII; N, *Nhe*I; S, *Sau*3AI; X, *Xmn*I. ⟩ reflects the continuation of the chromosome.

Fig. 1) constructed by PCR amplification from the 5′ end of pUNCH750 and labeled as described above for GS3′.

Southern blot analysis, Western blot analysis, and whole-cell HRP-Tf binding assays. Southern blotting, Western blotting, and whole-cell HRP-Tf binding assays were done as previously described (6).

Sequence analysis of clones. CsCl gradient-purified DNA was used to generate exonuclease III deletions (32) of both strands as described by Stratagene. DNA from the deletion clones was purified by using the Magic Miniprep DNA purification kit (Promega) and sequenced by using the Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio). Any remaining gaps between deletion clones were sequenced by using oligonucleotides designed to these specific regions. DNA and protein sequences were analyzed by using the University of Wisconsin Genetics Computer Group DNA analysis programs (8).

Mutagenesis. The insert from pUNCH750 was subcloned into the *Bam*HI site in pUP1 (10) and designated pUNCH751 (Table 1). The insert in pUNCH751 was mutagenized by using the minitransposon mTn*3*(Cm) to generate *tbpB* mutants according to the shuttle mutagenesis procedure described by Seifert et al. (44). Insertion sites were determined by sequence analysis using oligonucleotides designed to the regions flanking the repeats on each end of the minitransposon (data not shown). These constructs (designated pUNCH751a and pUNCH751b) containing insertion mutations were transformed into FA19 and by homologous recombination used to replace the wild-type *tbpB* gene, creating FA6813 and FA6814.

Another *tbpB* mutant was constructed by insertion mutagenesis using the 2.0-kb Ω fragment from pHP45Ω (35) (Fig. 1). The Ω insert contains transcriptional and translational termination signals as well as a selectable streptomycin resistance (Str^r) marker. The Ω fragment was excised from pHP45Ω with *Sma*I and ligated into the *Xmn*I site in pUNCH751. This construct, designated pUNCH756, was recombined into the chromosome of FA19, creating FA6815.

Labeling of gonococcal proteins with ^{14}C-palmitic acid. Bacteria were grown overnight on GC agar plates, resuspended in iron-depleted CDM to 15 Klett units, and grown through one doubling in a 5% CO_2 atmosphere at 37°C. Five milliliters of the cell culture was transferred to a 50-ml conical tube containing 5 μCi of ^{14}C-palmitic acid (New England Nuclear, Boston, Mass.), grown for an additional 2 h, and then harvested by centrifugation. Cells were washed in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), centrifuged, and resuspended in Laemmli solubilizing buffer (20).

Western analysis of ^{14}C-palmitic acid-labeled gonococcal proteins. To determine if Tbp2 could be labeled with ^{14}C-palmitic acid, approximately 10^9 CFU of the ^{14}C-palmitic acid-labeled culture was solubilized and separated on an SDS–7.5% polyacrylamide gel (20). The proteins were then transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.) in 20 mM Tris–150 mM glycine–20% methanol–0.1% SDS. Nitrocellulose was air dried and exposed to β-max film (Amersham, Arlington Heights, Ill.). The filter was probed with both polyclonal antisera raised against Tbp1 (6) and HRP-Tf (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) to identify Tbp1 and Tbp2, respectively. A monoclonal antibody (H.101.1.3; a gift from Janne Cannon) raised against the gonococcal lipoprotein Lip (4) was used to identify a known lipoprotein.

^{55}Fe uptake from human Tf and citrate. The iron uptake protocol was similar to that previously described (3). Data were collected from nine individual experiments on nine different days. Briefly, cells were grown overnight on GC agar plates, resuspended in CDM to an optical density of 20 Klett units, and incubated with shaking at 37°C in a 5% CO_2 atmosphere to induce iron starvation. Iron-starved cells were harvested during late exponential phase and diluted 1:1 in fresh CDM in duplicate. Potassium cyanide (KCN) was added to one set of tubes (controls) at a final concentration of 40 μM, and cell suspensions were incubated at 37°C for 10 min. ^{55}Fe-labeled Tf (0.83 μM and either 5 or 25% saturated with iron) or ferric citrate (67 μM) was added, and cell suspensions were incubated for an additional 30 min at 37°C. The gonococcus has been shown to take up iron from 5 and 25% saturated Tf with the same efficiency (24). KCN was added to the remaining cell suspensions to a final concentration of 40 μM, and both sets of cell suspensions were vacuum blotted onto nitrocellulose filters (Gelman, Ann Arbor, Mich.). Filters were allowed to air dry overnight and counted with a 1212 RACKBETA liquid scintillation counter (LKB Wallac, Elkhart, Ind.). Data were expressed as counts per minute per 10^8 CFU. Net uptake was defined as counts per minute determined from the set which received KCN after the 30-min iron uptake assay minus counts per minute for the set that received KCN prior to addition of Tf or ferric citrate.

Nucleotide sequence accession number. The DNA sequence of *tbpB* has been deposited in the GenBank data base under accession number U05205.

RESULTS

Cloning of *tbpB*. During the characterization of clones containing *tbpA* (6), an open reading frame (ORF) was noted in pUNCH405 directly upstream of *tbpA*. An mTn*3*(Cm) insertion into this ORF created a mutant that showed no Tbp2 expression, reduced Tbp1 expression, and reduced HRP-Tf binding on whole-cell dot blots (7). These data suggested that the upstream ORF was either *tbpB* (the gene encoding Tbp2) or a regulatory gene affecting both *tbpA* and *tbpB* expression. Southern blots of FA19 chromosomal DNA probed with GS3′ (Fig. 1) revealed a 1.8-kb *Sau3*A1 fragment. A *Sau3*AI-digested, size-fractionated FA19 chromosomal library was constructed and probed with GS3′. Several putative clones were isolated, and one was characterized. This clone was designated pUNCH750 (Fig. 1). Both sequence analysis and restriction mapping (Fig. 1) showed that the insert in pUNCH750 overlapped and extended 1.7 kb upstream of pUNCH405. The *Sau3*AI insert in pUNCH750 comigrated with a similar genomic fragment when probed with GS3′ (data not shown). When induced, pUNCH750 expressed a fusion protein with the α peptide of the β-galactosidase gene. By Western blot analysis, this ca. 70-kDa fusion protein bound HRP-Tf, confirming that the upstream ORF was *tbpB* (data not shown). Sequence analysis of pUNCH750 indicated that another overlapping clone was needed to obtain the entire coding sequence and promoter.

A second PCR fragment, GS5′ (Fig. 1), was used as a probe to clone, on the basis of genomic Southern data, an overlapping upstream *Dra*I fragment. From a *Dra*I library, three positive clones were identified and excised. One plasmid, designated pUNCH752, was further characterized. The insert in pUNCH752 comigrated with a similar genomic fragment in Southern analysis (data not shown).

Transposon mutagenesis of *tbpB*. To determine the function of Tbp2, the insert in pUNCH750 was insertionally mutagenized with both the minitransposon mTn*3*(Cm) and the Ω fragment. Two *tbpB* mutants with mTn*3*(Cm) insertions at different positions were characterized and through transforma-

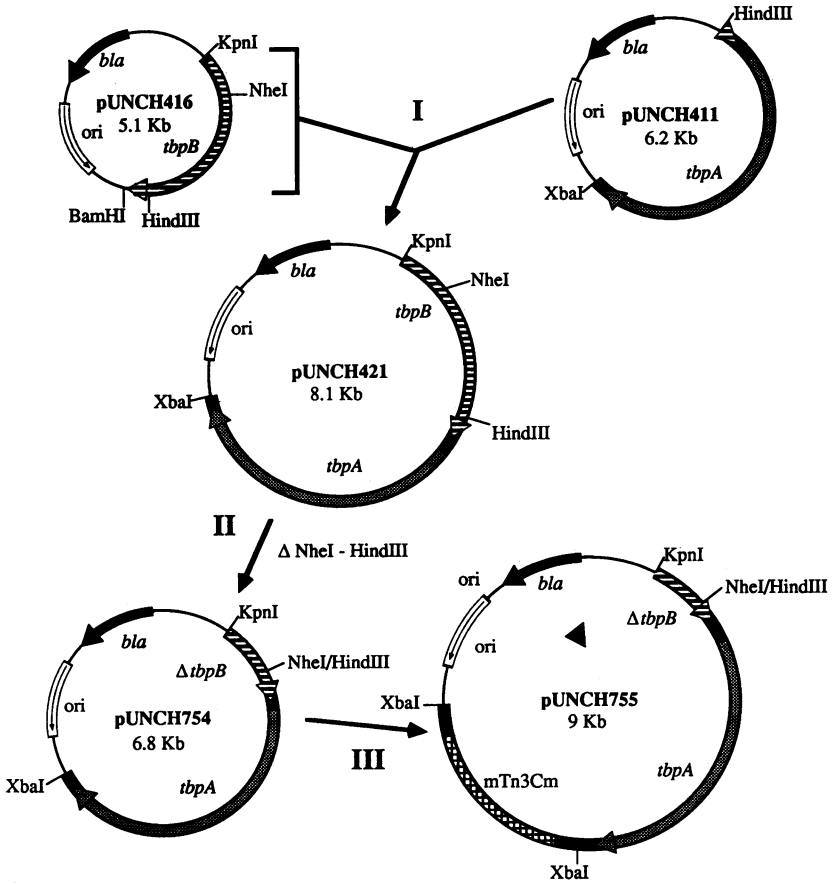

FIG. 2. Construction of the *tbpB* deletion clone. Light stippled arrows represent *tbpA*, which encodes Tbp1. Cross-hatched arrows represent *tbpB*, which encodes Tbp2. The checkered area represents the mTn*3*(Cm) insertion. Black areas represent gonococcal chromosomal sequences contiguous with *tbpA* and *tbpB*. Solid arrows represent the β-lactamase gene, *bla*. Thin lines represent pBluescript II SK(+) vector sequence, and the open box represents the pBluescript origin of replication (ori).

tion and gene replacement were introduced into the FA19 chromosome, creating FA6813 and FA6814 (Fig. 1). The two mTn*3*(Cm) mutants showed identical phenotypes. Neither FA6813 nor FA6814 expressed Tbp2 in Western blots, as assayed by binding of HRP-Tf (data not shown). FA6813 and FA6814 expressed low levels of Tbp1 in immunoblots probed with antisera raised against Tbp1 and grew slightly on CDM-Tf plates (data not shown). Another *tbpB* mutant was constructed by cloning the Ω fragment from pHP45Ω into the *Xmn*I site in the insert in pUNCH751, followed by transformation and allelic replacement, creating FA6815. Unlike mTn*3*(Cm), the Ω fragment has both transcriptional and translational terminators and should abolish transcriptional read-through that might occur with mTn*3*(Cm) mutants. By Western blot analysis, FA6815 expressed no detectable Tbp1 or Tbp2 (see Fig. 3), suggesting that the low level of Tbp1 expressed in FA6813 and FA6814 was due to transcriptional read-through of the mTn*3*(Cm) insert or the presence of a promoter within the mTn*3*(Cm) insert.

Construction of the *tbpB* deletion mutant. A deletion construct that expressed Tbp1 in the absence of Tbp2 was created in three stages. Using PCR primers GC-530 and GC-531 (see Fig. 7), designed with *Kpn*I and *Bam*HI restriction sites, respectively, *tbpB* was amplified from FA19 chromosomal DNA. This PCR product was then cloned into the *Kpn*I-to-*Bam*HI sites in pBluescript II SK(+) and designated pUNCH416 (Fig. 2). In step I, the *Kpn*I-to-*Hin*dIII fragment from pUNCH416 was cloned into the *Kpn*I-to-*Hin*dIII sites in pUNCH411 and designated pUNCH421. Plasmid pUNCH411 contains a *Hin*dIII-to-*Xba*I fragment which encodes the entire *tbpA* gene cloned into pBluescript II SK(+) (Fig. 2). Plasmid pUNCH421 thus contains the entire coding sequence for both *tbpA* and *tbpB*. In step II, the 1,200-base *Nhe*I-to-*Hin*dIII fragment in pUNCH421 was deleted; the ends were repaired with Klenow enzyme and religated, creating pUNCH754 (Fig. 2). Plasmid pUNCH754 contains an intact copy of *tbpA* and a deleted *tbpB*. In step III, an *Xba*I fragment from pUNCH124 that contains a mTn*3*(Cm) insert was ligated into the *Xba*I site in pUNCH754, creating pUNCH755 (Fig. 2). The final *tbpA*$^+$ *tbpB* isogenic mutant was constructed by replacing the *tbpA* and *tbpB* genes in FA6815 with those in pUNCH755 by homologous recombination. Plasmid pUNCH755 was transformed into FA6815 by selection for Cm^r and scored for loss of the Ω Str^r marker. The *tbpB* deletion mutant was designated FA6819.

Southern blot analysis confirmed that the insertions and deletions in strains FA6813, FA6814, FA6815, and FA6819 had occurred as expected (data not shown).

Expression of Tbp1 and Tbp2 by mutants. Total membranes from mutants FA6747, FA6815, and FA6819, as well as wild-type FA19, were analyzed for the production of Tbp1 and Tbp2 by SDS-PAGE and immunoblotting. These immunoblots

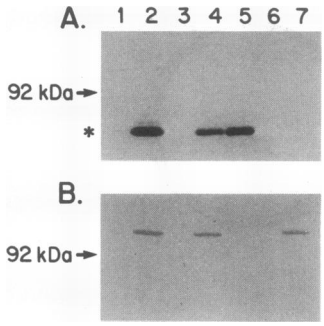

FIG. 3. Tbp1 and Tbp2 expression by *tbpA* and *tbpB* mutants. (A) Western blot probed with HRP-Tf. (B) Western blot probed with anti-Tbp1 antiserum. Lanes: 1 to 4, FA19; 5, FA6747; 6, FA6815; 7, FA6819. Lanes 1 and 3 contain total membranes from iron-sufficient strains, and lanes 2, 4, 5, 6, and 7 contain total membranes from iron-starved strains. Lanes 1 and 2 contain total membranes from bacteria grown in CDM, and lanes 3 and 4 contain total membranes from bacteria grown in GC broth.

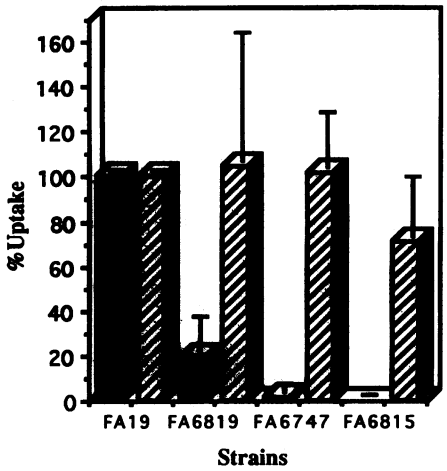

FIG. 5. Uptake of ^{55}Fe from ^{55}Fe-Tf and ^{55}Fe-citrate by *tbpA* and *tbpB* mutants. The filled columns represent percent uptake from Tf, and the hatched columns represent percent uptake from citrate, both normalized to uptake from FA19 (100%). Error bars indicate ±1 standard deviation.

were probed with HRP-Tf, which binds to Tbp2, and with antisera raised against gonococcal Tbp1. Iron-starved FA19 and FA6747 (Tbp1$^-$ Tbp2$^+$) expressed comparable levels of Tbp2, whereas the *tbpB* mutants, FA6819 (Tbp1$^+$ Tbp2$^-$) and FA6815 (Tbp1$^-$ Tbp2$^-$), expressed no Tbp2 (Fig. 3A). FA19 and FA6819 (Tbp1$^+$ Tbp2$^-$) expressed comparable levels of Tbp1, while FA6747 (Tbp1$^-$ Tbp2$^+$) and FA6815 (Tbp1$^-$ Tbp2$^-$) expressed no detectable Tbp1 (Fig. 3B). FA19 grown in CDM was compared with FA19 grown in GC broth with Desferal and showed no appreciable differences with respect to levels of expression of Tbp1 and Tbp2 (Fig. 3).

HRP-Tf binding to whole cells. To measure receptor function at the cell surface, an HRP-Tf binding assay was used (3, 41, 43). Whole cells from iron-starved FA19 bound approximately four- to sixfold more HRP-Tf than did FA6747 (Tbp1$^-$ Tbp2$^+$) (Fig. 4), as described previously (6). Strain FA6819 (Tbp1$^+$ Tbp2$^-$) showed a two- to fourfold reduction in HRP-Tf binding compared with wild-type FA19, and FA6815 (Tbp1$^-$ Tbp2$^-$) showed virtually no binding of HRP-Tf (Fig. 4).

^{55}Fe uptake from Tf by mutants. Iron uptake from Tf was measured in iron-starved bacteria of strains FA6747, FA6819, FA6815, and FA19 (Fig. 5). While strains FA6747 (Tbp1$^-$ Tbp2$^+$) and FA6815 (Tbp1$^-$ Tbp2$^-$) showed negligible levels of uptake of ^{55}Fe from ^{55}Fe-labeled Tf compared with the wild type, FA6819 (Tbp1$^+$ Tbp2$^-$) acquired ^{55}Fe from Tf at ca. 20% of wild-type levels ($P < 0.0005$). Although the strains were reduced in the ability to take up ^{55}Fe from ^{55}Fe-labeled Tf, strain FA6819 acquired significantly more ^{55}Fe than did either strain FA6747 or strain FA6815 ($P = 0.003$ and 0.005, respectively). Strains FA6819 (Tbp1$^+$ Tbp2$^-$) and FA6747 (Tbp1$^-$ Tbp2$^+$) were unimpaired ($P = 0.806$ and 0.845, respectively) in iron uptake from ferric citrate compared with wild-type FA19, while FA6815 (Tbp1$^-$ Tbp2$^-$) showed a reduced ($P = 0.011$) ability to obtain iron from ferric citrate compared with wild-type FA19.

Growth assay. FA6747 (Tbp1$^-$ Tbp2$^+$) and FA6815 (Tbp1$^-$ Tbp2$^-$) did not grow on CDM agarose plates supplemented with human Tf, but FA6819 (Tbp1$^+$ Tbp2$^-$) grew to levels approximating those reached by FA19 (Fig. 6). All three mutants showed growth on CDM-Lf agarose plates comparable to that of wild-type FA19 (data not shown).

DNA sequence analysis and protein structure. The double-stranded sequence data from overlapping clones pUNCH752, pUNCH750, and pUNCH405 (Fig. 7) contained a single ORF of 2.1 kb that overlapped the previously described sequence

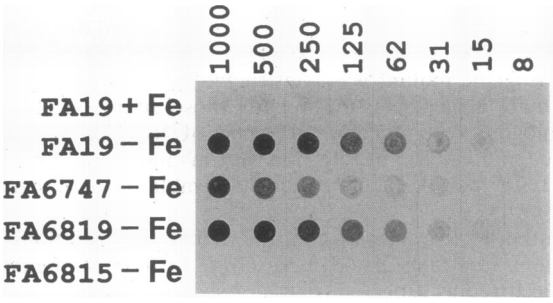

FIG. 4. Binding of HRP-Tf to iron-replete (+Fe) and iron-starved (−Fe) whole cells of *tbpA* and *tbpB* mutants. Strain and iron conditions are indicated at the left, and the concentration of HRP-Tf (nanograms per milliliter) is shown above each column.

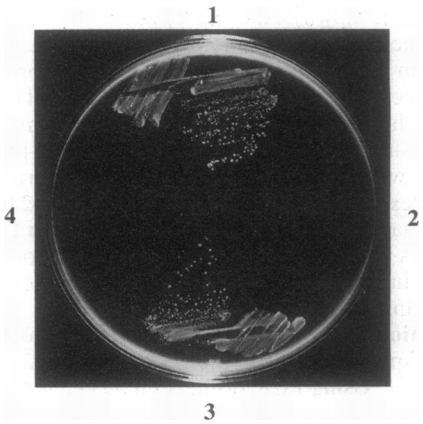

FIG. 6. Growth phenotypes of *tbpA* and *tbpB* mutants on CDM-Tf plates. Quadrants 1 to 4 were streaked with FA19, FA6747, FA6819, and FA6815, respectively.

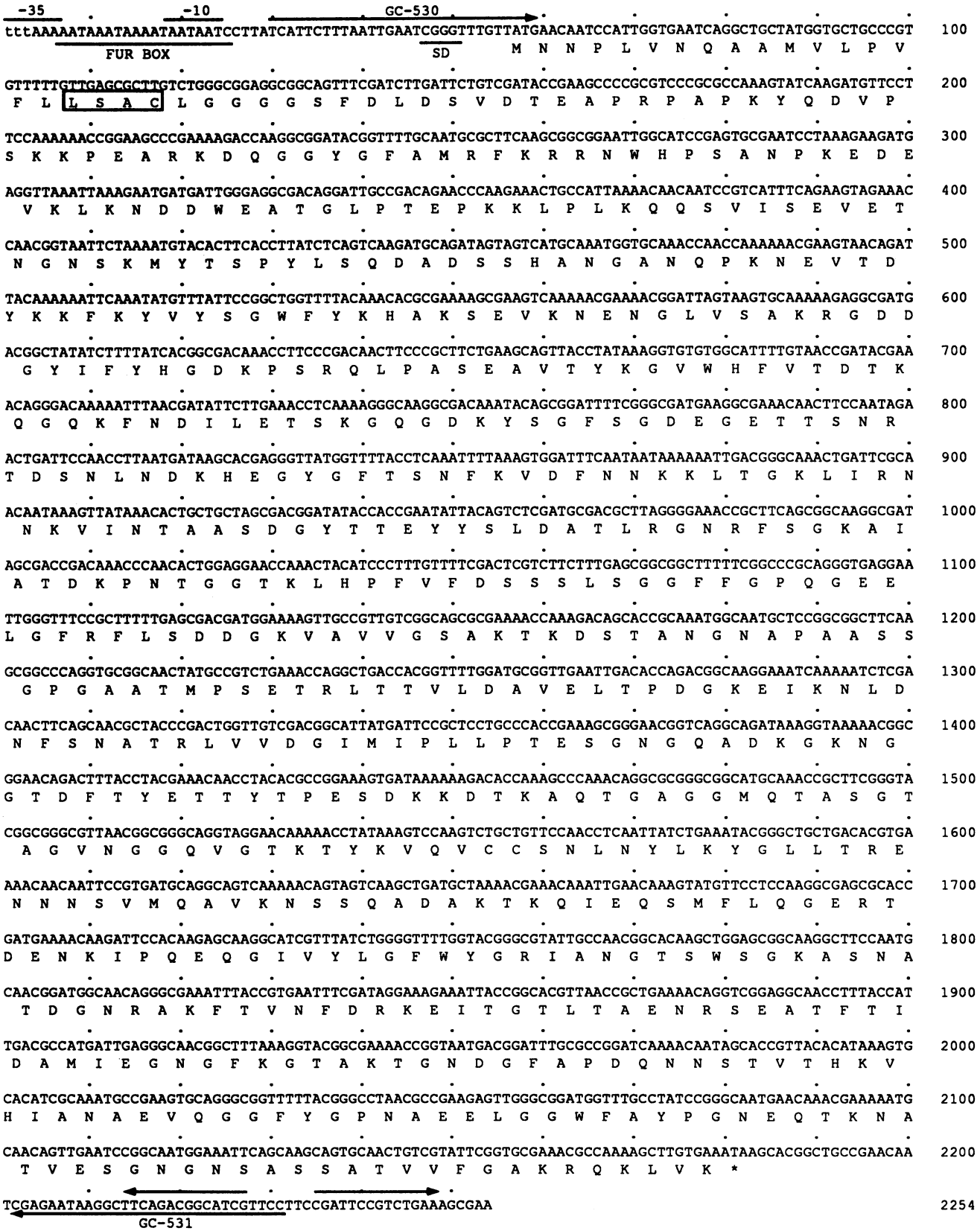

FIG. 7. Nucleotide sequence of the gonococcal *tbpB* gene. Single-letter codes for the deduced amino acid sequence are shown below the nucleotide sequence. An asterisk indicates the termination codon. The inverted arrows indicate the inverted repeat regions capable of forming a stem-loop structure. Putative −35 and −10, Fur-binding site, and the Shine-Dalgarno (SD) consensus sequences are indicated by straight lines. The boxed area indicates the proposed processing site for signal peptidase II. The single arrows labeled GC-530 and GC-531 represent PCR primers used to amplify the *tbpB* structural gene.

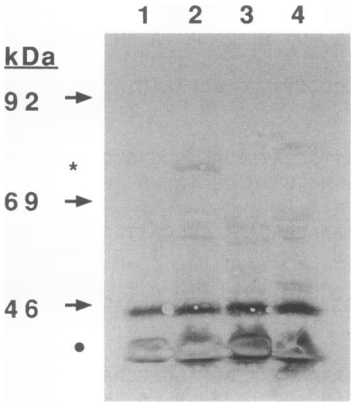

FIG. 8. ^{14}C-palmitic acid labeling of Tbp2. Lanes 1 and 2, FA19; lanes 3 and 4, FA6814. Lanes 1 and 3 contain whole-cell lysates from iron-sufficient cells, and lanes 2 and 4 contain whole-cell lysates from iron-starved cells. The asterisk indicates the position of Tbp2; the dot indicates the position of Lip.

which included *tbpA* located immediately downstream (6). Putative promoter elements were identified on the basis of *E. coli* consensus sequences (Fig. 7). A Shine-Dalgarno consensus sequence which preceded an ATG codon was also identified (Fig. 7). This ORF encoded a polypeptide (designated Tbp2) of 708 residues with a calculated molecular mass of the mature polypeptide of 74.3 kDa and an isoelectric point of 7.98. Located downstream of the *tbpB* coding sequence was a 14-base inverted repeat (Fig. 7), which also was previously noted as being upstream of *tbpA* (6). This repeat element does not contain the poly(T) signature of rho-independent transcriptional terminators; it does contain the 10-bp gonococcal uptake sequence (10, 13).

The University of Wisconsin Genetics Computer Group DNA analysis programs (8) were used to evaluate the sequence data for homology between the two different classes of meningococcal Tbp2 (22, 37) and gonococcal Tbp2. The program Bestfit generated an identity score of 48% and a similarity score of 65% between Tbp2 in FA19 and meningococcal strain B16B6 (22) and scores of 71% identity and 79% similarity between FA19 and meningococcal strain M982 (22). Using the same parameters, only 48% identity was found between Tbp2 in B16B6 and Tbp2 in M982, consistent with published data (22). Less closely related were the Tf-binding proteins from two strains of *Actinobacillus pleuropneumoniae* (11) that shared 36% identity and 55 to 56% similarity with Tbp2 from gonococcal strain FA19.

Intrinsic labeling of Tbp2 with ^{14}C-palmitic acid. The DNA sequence of *tbpB* predicted that Tbp2 contained a bacterial signal peptidase II consensus sequence, LSAC (Fig. 7) (16). To determine if Tbp2 is a lipoprotein, wild-type FA19 and FA6814 (Fig. 8) were grown in the presence of ^{14}C-palmitic acid, and whole-cell lysates were analyzed by SDS-PAGE and autoradiography. After autoradiography, the filters were probed with HRP-Tf to identify Tbp2, polyclonal anti-Tbp1 to identify Tbp1, and monoclonal antibody H.101.1.3 (4) to identify the gonococcal lipoprotein Lip (data not shown). Several iron-regulated proteins, including Tbp2, were lipid modified (Fig. 8). As expected, Lip was also lipid modified. The 100-kDa Tbp1 was not lipid modified, consistent with the observation that the predicted Tbp1 sequence was not preceded by a bacterial signal peptidase II consensus sequence (6).

DISCUSSION

The precise roles that Tbp1 and Tbp2 play in iron acquisition from Tf are unclear. Both proteins are iron regulated and specifically bind to human Tf under various assay conditions, and thus presumably both are components of a Tf-iron utilization system. Since *tbpA* mutants cannot take up iron from Tf or grow on Tf, Tbp1 appears to be required for iron acquisition from Tf. Homology between Tbp1 and TonB-dependent outer membrane receptors (6) and the presence of multiple predicted amphipathic β sheets (7) suggest that Tbp1 is an integral outer membrane protein that is required for iron transport from Tf across the outer membrane. The purpose of constructing *tbpA* and *tbpB* isogenic mutants in this study was to determine the role of Tbp2 in iron utilization from Tf.

In both the meningococcal (22) and gonococcal chromosomes, *tbpB* lies directly upstream of *tbpA*. Although *tbpA* does not contain a canonical promoter (6, 22) upstream of the start codon, *tbpB* is preceded by a consensus promoter, ribosome-binding site, and Fur-binding site (reference 22 and this study). Insertional mutagenesis of *tbpB* with the Ω fragment had a polar effect on *tbpA* expression, suggesting that these genes are transcribed from a single promoter located upstream of *tbpB*. The inverted repeat between the two genes (6) apparently did not serve as a transcriptional terminator under the growth conditions used in these experiments, since Tbp1 and Tbp2 were expressed and coregulated in FA19. However, this putative structure might serve some regulatory role as an antiterminator or attenuator under other growth conditions. To our knowledge, this is the first clear demonstration of an operon arrangement of genes in *Neisseria* species. Cotranscription of *tbpA* and *tbpB* was suggested by analysis of the DNA sequence in the meningococcus (22), but transposon mutagenesis of meningococcal *tbpB* with a cassette containing a promoter oriented in the same direction as the downstream *tbpA* gene did not establish polarity (18).

The two classes of meningococcal Tbp2 are defined by their molecular weights, antigenic heterogeneity (37), and sequence similarity (22). Tbp2 from gonococcal strain FA19 is most similar to Tbp2 from the meningococcal strain M982 (22), sharing 71% identity and 79% similarity. Although these proteins are similar in apparent molecular mass (85 kDa in FA19 and 87 kDa in M982), their isoelectric points are somewhat different (7.98 in FA19 and 6.18 in M982).

The primary sequence of both gonococcal and meningococcal (22) Tbp2 predicts that this protein is processed and lipid modified. Gonococcal Tbp2 was shown to be modifiable with ^{14}C-palmitic acid, verifying this hypothesis. Lipid modification presumably explains the discrepancy between apparent molecular mass in SDS-PAGE (85 kDa) and predicted molecular mass (73.4 kDa). Other lipoproteins which participate in iron acquisition have been described recently. In *Haemophilis influenzae*, a 60-kDa lipoprotein, HbpA, was identified as a heme-binding protein and thus potentially a component of a heme uptake system (15). HbpA shares homology with a periplasmic protein from *E. coli* and fractionates with the detergent-soluble proteins, which led Hanson and Hansen (15) to conclude that HbpA is a periplasmic protein, possibly anchored in the inner membrane. Another lipoprotein in *Bacillus subtilis* is required for uptake of the siderophore ferrichrome and is suggested to be tethered to the cytoplasmic membrane by a lipid anchor (40). This protein also shares homology with periplasmic proteins from *E. coli*. Gonococcal Tbp2 is lipid modified and shares homology only with Tf-binding proteins from *N. meningitidis* and *A. pleuropneumoniae*. Gonococcal Tbp2 fractionates with detergent-soluble membrane proteins (7) like HbpA,

indicating that it could be inner membrane or periplasmically located. However, immunization with an *A. pleuropneumoniae* Tbp2 homolog was protective, indicating surface exposure in this organism (38). Much remains to be done to assess the localization and surface accessibility of Tbp2.

The predicted protein sequence of Tbp2 shows none of the hallmarks of bacterial outer membrane proteins, characterized by the porins (34, 47) and siderophore receptors (19, 28, 47). Tbp2 neither shares homology with TonB-dependent receptors nor ends in an amphipathic domain that is terminated by an aromatic residue, a motif that has been recognized in outer membrane proteins (47). There are few predicted amphipathic β-sheet structures as well as a lack of predicted α-helical structure in the Tbp2 protein sequence. Thus, if Tbp2 is an outer membrane protein, it appears to have novel structural features.

A gonococcal mutant (FA6747) that expressed Tbp2 without Tbp1 bound four- to sixfold less HRP-Tf in a whole-cell dot blot than did the wild type, while in a mutant (FA6819) that expressed Tbp1 without Tbp2, binding was decreased by two- to fourfold. The Ω insertion mutant (FA6815) that did not make Tbp1 or Tbp2 bound no detectable Tf in this assay. Thus, gonococcal Tbp1 and Tbp2 bound Tf independently of one another, and all Tf binding in this assay was attributable to either Tbp1 or Tbp2. Although Irwin et al. (18) found that meningococcal Tbp1 and Tbp2 bound Tf independently in a similar whole-cell dot blot assay, HRP-Tf binding to the meningococcal isogenic mutants was much less than was seen with the gonococcal mutants (>16-fold decreased binding compared with the wild type). Since Irwin et al. detected significantly more Tf binding in membrane preparations containing Tbp1 than in whole cells, the binding to whole cells detected in Fig. 4 might be overestimated if membrane fragments were present in the gonococcal whole-cell HRP-Tf assay. Alternatively, accessibility of these two proteins to Tf in this assay may account for the disparate results for different species.

Gonococcal Tbp1 mutants could not take up iron from Tf or grow on Tf as an iron source (reference 6 and this study), indicating that this protein is absolutely required for Tf utilization. A Tbp2 mutant that expressed Tbp1 normally (FA6819) could take up iron from Tf and grow on Tf as an iron source, although iron uptake was decreased by about fivefold. This finding suggests that the efficiency of Tf utilization is diminished in the absence of Tbp2. A meningococcal Tbp2 mutant, in contrast, could not grow on Tf in a short-term liquid medium assay (18). Irwin et al. (18) concluded that both Tbp1 and Tbp2 are required for growth of the meningococcus on Tf. However, the data presented here indicate that gonococcal Tbp2 is not strictly necessary for growth on Tf but makes the process of iron utilization from Tf more efficient. Differences in growth between gonococcal and meningococcal *tbpB* mutants may be a consequence of species divergence, surface accessibility of Tbp1 to Tf, or the method by which growth was determined. The localization of Tbp2 on the gonococcal cell surface, its proximity to Tbp1, and roles in Tf binding and iron uptake from Tf requires further study before models can be discussed seriously.

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REFERENCES

1. **Archibald, F. S., and I. W. DeVoe.** 1980. Iron acquisition by *Neisseria meningitidis* in vitro. Infect. Immun. **27**:322–334.
2. **Biswas, G. D., and P. F. Sparling.** Unpublished data.
3. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172**:5225–5235.
4. **Cannon, J. G., W. J. Black, I. Nachamkin, and P. W. Stewart.** 1984. Monoclonal antibody that recognizes an outer membrane antigen common to the pathogenic *Neisseria* species but not to most nonpathogenic *Neisseria* species. Infect. Immun. **43**:994–999.
5. **Cornelissen, C. N., G. D. Biswas, and P. F. Sparling.** 1993. Expression of gonococcal transferrin-binding protein 1 causes *Escherichia coli* to bind human transferrin. J. Bacteriol. **175**:2448–2450.
6. **Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson, and P. F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J. Bacteriol. **174**:5788–5797.
7. **Cornelissen, C. N., and P. F. Sparling.** Unpublished data.
8. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
9. **Dyer, D. W., W. McKenna, J. P. Woods, and P. F. Sparling.** 1987. Isolation by streptonigrin enrichment and characterization of a transferrin-specific iron uptake mutant of *Neisseria meningitidis*. Microb. Pathog. **3**:351–363.
10. **Elkins, C., C. E. Thomas, H. S. Seifert, and P. F. Sparling.** 1991. Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence. J. Bacteriol. **173**:3911–3913.
11. **Gerlach, G. F., S. Klashinsky, C. Anderson, A. A. Potter, and P. J. Willson.** 1992. Characterization of two genes encoding distinct transferrin-binding proteins in different *Actinobacillus pleuropneumoniae* isolates. Infect. Immun. **60**:3253–3261.
12. **Gonzalez, G. C., D. L. Caamano, and A. B. Schryvers.** 1990. Identification and characterization of a porcine-specific transferrin receptor in *Actinobacillus pleuropneumoniae*. Mol. Microbiol. **4**:1173–1179.
13. **Goodman, S. D., and J. J. Scocca.** 1988. Identification and arrangement of the DNA sequence recognized in specific transformation of *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA **85**:6982–6986.
14. **Griffiths, E.** 1987. The iron-uptake systems of pathogenic bacteria, p. 69–137. *In* J. J. Bullen and E. Griffiths (ed.), Iron and infection: molecular, physiological, and clinical aspects. John Wiley & Sons, Ltd., Chichester, England.
15. **Hanson, M. S., and E. J. Hansen.** 1991. Molecular cloning, partial purification, and characterization of a haemin-binding lipoprotein from *Haemophilus influenzae* type b. Mol. Microbiol. **5**:267–278.
16. **Hayashi, S., and H. C. Wu.** 1990. Lipoproteins in bacteria. J. Bioenerg. Biomembr. **22**:451–471.
17. **Herrington, D. A., and P. F. Sparling.** 1985. *Haemophilus influenzae* can use human transferrin as a sole source for required iron. Infect. Immun. **48**:284–251.
18. **Irwin, S. W., N. Averil, C. Y. Cheng, and A. B. Schryvers.** 1993. Preparation and analysis of isogenic mutants in the transferrin receptor protein genes, *tbpA* and *tbpB*, from *Neisseria meningitidis*. Mol. Microbiol. **8**:1125–1133.
19. **Koebnik, R., and V. Braun.** 1993. Insertion derivatives containing segments of up to 16 amino acids identify surface- and periplasm-exposed regions of the FhuA outer membrane receptor of *Escherichia coli* K-12. J. Bacteriol. **175**:826–839.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lee, B. C., and A. B. Schryvers.** 1988. Specificity of the lactoferrin and transferrin receptors in *Neisseria gonorrhoeae*. Mol. Microbiol. **2**:827–829.
22. **Legrain, M., V. Mazarin, S. W. Irwin, B. Bouchon, M.-J. Quentin-Millet, E. Jacobs, and A. B. Schryvers.** 1993. Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-

binding proteins Tbp1 and Tbp2. Gene **130:**73–80.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **McKenna, W. R., P. A. Mickelsen, P. F. Sparling, and D. W. Dyer.** 1988. Iron uptake from lactoferrin and transferrin by *Neisseria gonorrhoeae*. Infect. Immun. **56:**785–791.
25. **Mickelsen, P. A., E. Blackman, and P. F. Sparling.** 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from lactoferrin. Infect. Immun. **35:**915–920.
26. **Mickelsen, P. A., and P. F. Sparling.** 1981. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. Infect. Immun. **33:**555–564.
27. **Morton, D. J., and P. Williams.** 1990. Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. J. Gen. Microbiol. **136:**927–933.
28. **Murphy, C. K., V. I. Kalve, and P. E. Klebba.** 1990. Surface topology of the *Escherichia coli* K-12 ferric enterobactin receptor. J. Bacteriol. **172:**2736–2746.
29. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:**715–731.
30. **Ogunnariwo, J. A., J. Alcantara, and A. B. Schryvers.** 1991. Evidence for non-siderophore-mediated acquisition of transferrin-bound iron by *Pasteurella multocida*. Microb. Pathog. **11:**47–56.
31. **Ogunnariwo, J. A., and A. B. Schryvers.** 1990. Iron acquisition in *Pasteurella haemolytica*: expression and identification of a bovine-specific transferrin receptor. Infect. Immun. **58:**2091–2097.
32. **Ozkaynak, E., and S. D. Putney.** 1987. A unidirectional deletion technique for the generation of clones for sequencing. BioTechniques **5:**770–773.
33. **Padda, J. S., and A. B. Schryvers.** 1990. N-linked oligosaccharides of human transferrin are not required for binding to bacterial transferrin receptors. Infect. Immun. **58:**2972–2976.
34. **Pauptit, R. A., T. Schirmer, J. N. Jansonius, J. P. Rosenbusch, M. W. Parker, A. D. Tucker, D. Tsernoglou, M. S. Weiss, and G. E. Schulz.** 1991. A common channel-forming motif in evolutionarily distant porins. J. Struct. Biol. **107:**136–145.
35. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
36. **Redhead, K., T. Hill, and H. Chart.** 1987. Interaction of lactoferrin and transferrins with the outer membrane of *Bordetella pertussis*. J. Gen. Microbiol. **133:**891–898.
37. **Rokbi, B., V. Mazarin, G. Maitre-Wilmotte, and M.-J. Quentin-Millet.** 1993. Identification of two major families of transferrin receptors among *Neisseria meningitidis* strains based on antigenic and genomic features. FEMS Microbiol. Lett. **110:**51–58.
38. **Rossi-Campos, A., C. Anderson, G.-F. Gerlach, S. Klashinsky, A. A. Potter, and P. J. Willson.** 1992. Immunization of pigs against *Actinobacillus pleuropneumoniae* with two recombinant protein preparations. Vaccine **10:**512–518.
39. **Rutz, J. M., T. Abdullah, S. P. Singh, V. I. Kalve, and P. E. Klebba.** 1991. Evolution of the ferric enterobactin receptor in gram-negative bacteria. J. Bacteriol. **173:**5964–5974.
40. **Schneider, R., and K. Hantke.** 1993. Iron-hydroxamate uptake systems in *Bacillus subtilis*: identification of a lipoprotein as part of a binding protein-dependent transport system. Mol. Microbiol. **8:**111–121.
41. **Schryvers, A. B., and B. C. Lee.** 1989. Comparative analysis of the transferrin and lactoferrin binding proteins in the family *Neisseriaceae*. Can. J. Microbiol. **35:**409–415.
42. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the human lactoferrin-binding protein from *Neisseria meningitidis*. Infect. Immun. **56:**1144–1149.
43. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the transferrin receptor from *Neisseria meningitidis*. Mol. Microbiol. **2:**281–288.
44. **Seifert, H. S., M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of introducing transposons into transformable organisms, p. 123–134. *In* A. Hollaender and J. K. Setlow (ed.), Genetic engineering, principles and methods. Plenum, New York.
45. **Simonson, C., D. Brener, and I. W. DeVoe.** 1982. Expression of a high-affinity mechanism for acquisition of transferrin iron by *Neisseria meningitidis*. Infect. Immun. **36:**107–113.
46. **Stern, A., P. Nickel, T. F. Meyer, and M. So.** 1984. Opacity determinants of *Neisseria gonorrhoeae*: gene expression and chromosomal linkage to the gonococcal pilus gene. Cell **37:**447–456.
47. **Struyvé, M., M. Moons, and J. Tommassen.** 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J. Mol. Biol. **218:**141–148.
48. **Tsai, J., D. W. Dyer, and P. F. Sparling.** 1988. Loss of transferrin receptor activity in *Neisseria meningitidis* correlates with inability to use transferrin as an iron source. Infect. Immun. **56:**3132–3138.
49. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42:**45–66.
50. **West, S. E. H., and P. F. Sparling.** 1985. Response of *Neisseria gonorrhoeae* to iron limitation: alterations in expression of membrane proteins without apparent siderophore production. Infect. Immun. **47:**388–394.
51. **West, S. E. H., and P. F. Sparling.** 1987. Aerobactin utilization by *Neisseria gonorrhoeae* and cloning of a genomic DNA fragment that complements *Escherichia coli fhuB* mutations. J. Bacteriol. **169:**3414–3421.
52. **Williams, P. H., and P. J. Warner.** 1980. CoIV plasmid-mediated, colicin V-independent iron uptake system of invasive strains of *Escherichia coli*. Infect. Immun. **29:**411–416.