Cloning, Sequencing, and Characterization of the Gene Encoding FrpB, a Major Iron-Regulated, Outer Membrane Protein of *Neisseria gonorrhoeae*

MARGARET BEUCHER AND P. FREDERICK SPARLING*

Departments of Medicine and Microbiology and Immunology and Curriculum of Genetics and Molecular Biology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

FrpB (for Fe-regulated protein B) is a 76-kDa outer membrane protein that is part of the iron regulon of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. The *frpB* gene from gonococcal strain FA19 was cloned and sequenced. FrpB was homologous to several TonB-dependent outer membrane receptors of *Escherichia coli* as well as HemR of *Yersinia enterocolitica* and CopB of *Moraxella catarrhalis*. An Ω insertion into the *frpB* coding sequence caused a 60% reduction in ^{55}Fe uptake from heme, but careful analysis suggested that this effect was nonspecific. While FrpB was related to the family of TonB-dependent proteins, a function in iron uptake could not be documented.

Many factors contribute to the virulence of bacterial pathogens. The ability to adhere to and/or invade eukaryotic cells (20), the secretion of toxins-hemolysins (20), the synthesis of capsule (12, 28) and immunoglobulin proteases (20), the sialylation of surface components (52), phase and antigenic variation (37), and the ability to scavenge iron or other essential nutrients from host sources (65) are all associated with virulence. While many factors work in concert to establish infection, the abilities to adhere and to obtain iron may be the only factors common to all pathogenic bacteria.

Iron is essential for many biological processes. Besides electron transport, iron serves at the catalytic center for enzymes involved in the reduction of dinitrogen and ribonucleotides, as well as those involved in the activation and decomposition of peroxides (4). The concentration of free iron in mammalian tissue (10^{-18} M) is far below that required to support bacterial growth (0.4 to 4 μM) (64), and therefore, pathogens have evolved multiple, coordinately expressed mechanisms to scavenge iron from host iron storage and transport compounds. Most bacteria synthesize and secrete high-affinity, low-molecular-weight, iron-chelating compounds called siderophores (39). These compounds are capable of stripping iron from the host glycoproteins transferrin (Tf) and lactoferrin (Lf) (10), and the cell surface of iron-releasing tissues, i.e., macrophages and hepatocytes (11). The ferri-siderophore complex is then bound by a specific receptor on the bacterial cell surface and internalized by an energy-requiring process.

Neisseria gonorrhoeae does not synthesize siderophores (66). Nevertheless, it has been reported to utilize the *Escherichia coli* siderophores aerobactin (67) and enterobactin (45). All gonococci are capable of utilizing Tf-bound iron directly (38). A saturable specific gonococcal Tf receptor is responsible for this activity (9). The Tf receptor appears to be composed of two iron-repressible Tf-binding proteins (50), designated Tbp1 and Tbp2 (21). Tbp1 is essential for utilization of Tf-bound iron in vitro (13). Tbp2, although not essential in vitro, appears to make the process of iron utilization from Tf more efficient (2) and may be essential in vivo. Lf also serves as an iron source

for some strains of *N. gonorrhoeae* and requires direct contact as well (36). Biswas and Sparling have recently isolated a gene that encodes the 105-kDa iron-repressible, outer membrane, Lf-binding protein (Lbp) from gonococcal strain FA19 (6). Citrate, heme, hemoglobin, and hemoglobin bound to haptoglobin but not heme bound to hemopexin or serum albumin also serve as iron sources for gonococci in vitro (17, 38).

The iron uptake systems of *Neisseria meningitidis* are similar to those described for *N. gonorrhoeae* (3, 17). Dyer et al. (16) reported a 70-kDa iron-regulated major outer membrane protein common to *N. gonorrhoeae* and *N. meningitidis*. This protein has been designated FrpB (21). Previous studies showed that FrpB is surface exposed and immunogenic in vivo (1, 16, 41). Polyclonal and some monoclonal anti-FrpB antibodies recognize the denatured protein on Western blots (immunoblots) of nearly all gonococcal and meningococcal isolates tested (reference 16 and this study). Other monoclonal antibodies directed against meningococcal FrpB are bactericidal and strain specific (41). Nevertheless, the size of FrpB appears to be well conserved. Interest in the role of FrpB in iron uptake was promoted by studies of an ethylmethanesulfonate-mutagenized, streptonigrin-enriched, meningococcal mutant (FAM11) that did not express FrpB (16). FAM11 was capable of using heme and hemoglobin normally but was unable to use ferric ions from a variety of sources including Tf and Lf. Nevertheless, revertants that regained the ability to utilize Tf and Lf never regained expression of FrpB. The inability to transform the mutation into a clean genetic background prevented clear interpretation of the role of FrpB in iron utilization.

In this paper, we report the cloning, sequencing, and mutagenesis of *frpB* from gonococcal strain FA19 and discuss experiments designed to determine the physiological function of the FrpB protein.

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains used in this study are described in Table 1. *Neisseria* strains were routinely cultured on gonococcal base (GCB) medium (Difco Laboratories) containing Kellogg's supplements I and II (29) and grown overnight at 35°C in an atmosphere of 5% CO_2. Antibiotic selection employed chloramphenicol at 1 μg/ml for mTn*3*(Cm) (51)-mutagenized strains and streptomycin at 100 μg/ml for Ω (44)-mutagenized strains. For Western blot analysis of total membrane proteins of iron-stressed gonococci, cells were grown in chemically defined medium (CDM) as previously

TABLE 1. Bacterial strains, plasmids, and phage

Strain, plasmid, or phage	Description	Reference or source
Strains		
FA19	Wild type	38,
FA6807	frpB::Ω (FrpB−)	This study
FA6808	frpB::Ω tbpA::mTn3(Cm) (FrpB− Tbp1−)	This study
FA6747	tbpA::mTn3(Cm) (Tbp1−)	13
FA6819	ΔtbpB (Tbp2−)	2
FA6775	lbpA::mTn3(Cm) (Lpb−)	6
UU1008	Wild type	Zell McGee
DH5αMCR	F− mcrA mcrB mrr φ80dlacZΔM15 Δ(argF-lac)U169 recA1 endA1 hsdR hsdM supE44 λ− thi-1 gyrA96 relA1	Bethesda Research Laboratories
BN1071	F− pro trp rslL entA (Ent− FepA+)	30
AN102	BN1071 leu fepA (Ent+ FepA−)	30
KDF541	BN1071 entA fepA (Ent− FepA−)	46
KDF541/pABN6	(Ent− FepA− IutA+ Iuc−)	14
LG1315/pcolV	BN1071 cir (IutA+ Iuc+)	63
RK1065	hemA	R. Kadner
HB101	F− hsd20 (rB mB−) recA13 ara-14 proA2 lacY1 galK2 rpsL20 (Smr) xyl-5 mtl-1 supE44 λ−	47
C386	ompA lpp	53
Plasmids and phage		
pACYC184	ori p15a Cmr Tcr	New England Biolabs
pBluescript II SK+	ori pMB1 Apr	Stratagene
pHP45Ω	Source for the Ω fragment (Smr)	44
pUP1	pHSS6 containing gonococcal uptake sequence (Kanr)	19
pEBH21	pBC II SK+ derivative (Cmr)	22
pUNCH319	pBluescript II SK+ containing 540-bp EcoRI-ClaI fragment from λfrpB.4	This study
pUNCH320	pBluescript II SK+ containing 5.3-kb ClaI-EcoRI fragment from λfrpB.4	This study
pUNCH321	pUP1 containing 540-bp EcoRI-ClaI fragment from pUNCH319	This study
pUNCH324	pUNCH321 containing Ω fragment from pHP45Ω in unique BglI site	This study
pUNCH325	pBluescript II SK+ containing 2.8-kb ClaI-MluI fragment from pUNCH320	This study
pUNCH330	540-bp EcoRI-ClaI fragment from pUNCH319 in pACYC184	This study
pUNCH331	Reassembled gonococcal frpB gene in pACYC184	This study
λ-ZapII	Excisable lambda phage vector	Stratagene

described (13). Cultures were made iron replete as indicated by the addition of 100 μM ferric citrate.

E. coli strains were routinely cultured on Luria-Bertani medium (47). Antibiotic selection was 100 μg of ampicillin per ml, 100 μg of streptomycin per ml, 40 μg of kanamycin per ml, and/or 30 μg of chloramphenicol per ml. δ-Aminolevulinic acid was used at 30 μg/ml, and heme was used at 50 μg/ml. *E. coli* cultures were iron stressed by the addition of 200 μM 2,2-dipyridyl (Sigma Chemical Co., St. Louis, Mo.). Deferoxamine mesylate (Desferal) was obtained from Ciba-Geigy (Basel, Switzerland).

SDS-PAGE and Western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 7.5% polyacrylamide resolving gel and a 4.5% polyacrylamide stacking gel. Electrophoresis was carried out at either 40 mA for one gel or 80 mA for two gels in the discontinuous buffer system of Laemmli (32). Transfer and development were as described previously (23, 61).

Preparation of polyclonal antisera and monoclonal antibodies. Preparation of polyclonal antisera was described previously (8). Anti-FrpB monoclonal antibodies were generated by methods described previously (60).

DNA isolation, digestion, and Southern blot analysis. Chromosomal DNA was purified by CsCl-gradient centrifugation according to the method of Stern et al. (54). Plasmids were purified either by CsCl centrifugation or according to the instructions provided in the Magic Miniprep DNA Purification Kit (Promega, Madison, Wis.). Southern blotting and DNA hybridizations were performed as previously described (13). Restriction enzymes, Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) or Bethesda Research Laboratories (Gaithersburg, Md.) and were used according to the manufacturer's specifications. λ-ZapII and pBluescript II SK+ were obtained from Stratagene (La Jolla, Calif.).

DNA sequencing and sequence analysis. CsCl-purified pUNCH319 and pUNCH325 were used as templates for double-stranded DNA sequencing (31) with United States Biochemical Sequenase and the dideoxy chain termination procedure of Sanger et al. (48). Both dG- and dI-labeling reactions were carried out for all primers. Both strands of pUNCH319 were sequenced with vector-specific or insert-specific primers. Exonuclease III-exonuclease VII nested deletions (40) were generated from the MluI end of pUNCH325, and vector-specific primers were used to sequence individual deletion clones. Internal primers were used to sequence gaps between clones as well as the opposite strand. DNA

sequences were analyzed with the Genetics Computer Group software package (15) (University of Wisconsin).

Mutagenesis and gonococcal transformation. pHP45Ω (44) was used to insertionally inactivate *frpB*. pUNCH321 was digested with BglI, and ends were repaired with Klenow fragment. pHP45Ω was digested with SmaI, and the 2.0-kb Ω fragment was isolated from an agarose gel according to the instructions provided in the Geneclean II Kit (Bio 101 Inc., La Jolla, Calif.). Transformation of plasmid DNA into FA19 was as previously described (7).

Preparation of FrpB for amino-terminal sequence analysis. N-Lauroylsarcosine (Sigma)-insoluble membrane fractions were prepared from iron-stressed gonococcal strain UU1008, and protein concentration was determined by a bicinchoninic acid assay (Pierce, Rockford, Ill.). Two hundred micrograms of protein was loaded into a preparative well of an SDS–7.5% polyacrylamide gel, poured 24 h previously to permit TEMED (N,N,N′,N′-tetramethylethylenediamine) and ammonium persulfate to evaporate. Electrophoresis was carried out at 40 mA of constant current with the discontinuous buffer system of Laemmli (32). The gel was soaked for 15 min in transfer buffer (13) before transferring. Polyvinylidene difluoride membrane was placed in 100% methanol for 2 s, transferred to distilled deionized water for 5 min, and soaked in transfer buffer for 10 min prior to transfer. Transfer was for 3.5 h at 90 mA in a submerged trans-blot apparatus (Bio-Rad, Richmond, Calif.). Subsequent to transfer, the polyvinylidene difluoride membrane was stained for 5 min in 0.1% Coomassie brilliant blue–20% methanol–10% acetic acid to visualize proteins and destained for 10 min in distilled deionized H_2O with one change. Filtrate was frozen at −20°C overnight. FrpB was identified by molecular weight, and the amino-terminal amino acid sequence of the protein on the filter was determined by the Protein Microsequencing Facility at University of California, Los Angeles.

⁵⁵Fe uptake assays. Data were compiled from three individual experiments performed in triplicate on separate days. Gonococci were iron stressed as previously reported (2) prior to experimentation. SDS-PAGE and Western blotting of whole-cell lysates were routinely performed to determine that cultures were consistently and equivalently iron stressed, as evidenced by reactivity with anti-FrpB monoclonal antibody and/or anti-Tbp1 antisera. Iron uptake assays were performed as previously reported (9) with the following modifications. Filters were blocked just prior to experimentation with 30 μl of 10-mg/ml bovine serum albumin in 1× CDM. Assays were performed in 200-μl volumes in 96-well filtration plates (MAHV; Millipore, Bedford, Mass.) at 35°C in a 5% CO_2
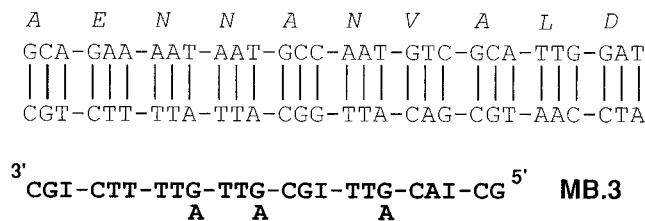
```
      A     E     N     N     A     N     V     A     L     D
     GCA - GAA - AAT - AAT - GCC - AAT - GTC - GCA - TTG - GAT
     |||   |||   |||   |||   |||   |||   |||   |||   |||   |||
     CGT - CTT - TTA - TTA - CGG - TTA - CAG - CGT - AAC - CTA


  3'                                                      5'
     CGI - CTT - TTG - TTG - CGI - TTG - CAI - CG          MB.3
            A     A     A           A
```

FIG. 1. Amino acid sequence obtained for mature FrpB protein and oligonucleotide MB.3. The FrpB amino acid sequence is shown above the *frpB* coding sequence. Oligonucleotide MB.3 is shown 3′ to 5′ and corresponds to noncoding strand.
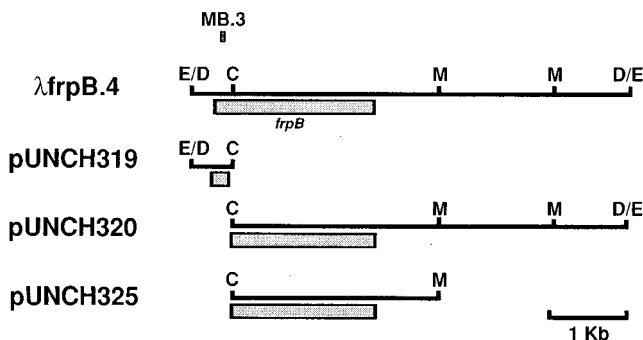

FIG. 2. Restriction map of *frpB* clones. The position of the *frpB* ORF is indicated below the physical map by the stippled box. Only relevant cloning sites are shown: C, *Cla*I; D, *Dra*I; E, *Eco*RI; M, *Mlu*I. Also shown is the position of oligonucleotide MB.3, which was deduced from the amino-terminal amino acid sequence of the mature protein.

atmosphere. Potassium cyanide was dissolved in 1× CDM. The vacuum manifold was from the Millipore Multiscreen Assay System. Heme was used at 0.5 μM, transferrin was used at 6.25 μM, and citrate was used at 100 μM. Membranes were air dried overnight, and the Millipore punch kit was used to separate and collect individual filters prior to counting. Data were expressed as counts per minute per microgram of protein.

Preparation of aerobactin and enterobactin. Purified aerobactin and enterobactin were the generous gift of P. E. Klebba. Aerobactin was ferrated as follows. Ferric sulfate was dissolved to 4 mM in 50 ml of distilled deionized H_2O containing 1.5 μl of HCl. Four hundred microliters of 4 mM aerobactin was added to 400 μl of 4 mM ferric sulfate and 80 μl of 0.5 M Na_2HPO_4. The ferri-aerobactin was run over a carboxymethyl-cellulose (Sigma) column equilibrated in 0.05 M Na_2HPO_4. The final concentration of aerobactin was determined by reading the A_{400} (24).

Iron sources. Human Tf, human Lf, bovine heme, human hemoglobin, and human haptoglobin were obtained from Sigma Chemical Co. ^{55}Fe hemin was purchased from the custom synthesizing facility at NEN Products Dupont (Wilmington, Del.), lot no. FE55.1193RS. Tf, Lf, and citrate were ferrated with ^{55}FeCl as previously described (36).

RNase assay. The RNase assay was performed as previously described (71), except that we used 0.1 N HCl instead of 0.5 N HCl.

Hemin affinity purification. Hemin agarose was purchased from Sigma Chemical Co. The method of affinity purification was previously described by Lee (33).

Bactericidal assays. Bactericidal assays were performed as described previously (18).

Nucleotide sequence accession number. The gonococcal *frpB* sequence presented in this report was deposited with GenBank under the accession number U13980.

RESULTS

Cloning the gonococcal *frpB* gene. Sarcosyl-insoluble membrane fractions from gonococcal strain UU1008 were used to obtain FrpB N-terminal amino acid sequence (see Materials and Methods). A degenerate oligonucleotide containing inosine (designated MB.3, shown in Fig. 1) was deduced from this sequence and used to probe a Southern blot of FA19 chromosomal DNA. Each restriction digest contained a single hybridizing band (data not shown). A 5.8-kb *Dra*I fragment was chosen for further analysis.

A λ-ZapII library containing *Eco*RI-linked FA19 chromosomal *Dra*I fragments (2) was screened with oligonucleotide MB.3. Approximately 1 positive plaque was identified for every 10,000 plaques screened. Attempts to excise the phagemid containing the intact insert consistently resulted in deletion products smaller than pBluescript II SK⁺ alone. Reasoning that such a large chromosomal fragment potentially contained both the *frpB* promoter and entire *frpB* coding sequence and that the expression of FrpB might be toxic in *E. coli*, we decided to subclone smaller fragments into pBluescript II SK⁺.

DNA prepared from one of the positively hybridizing plaques, λfrpB-4 (Fig. 2), was digested with *Eco*RI to release the insert DNA. The expected 5.8-kb fragment was isolated from an agarose gel and further digested with *Cla*I to generate a 540-bp, MB.3-hybridizing fragment and an approximately 5.3-kb fragment which did not hybridize to MB.3. The smaller fragment ligated into pBluescript II SK⁺ was stable in *E. coli*

DH5αMCR and was designated pUNCH319. The larger fragment ligated into pBluescript II SK⁺ generated pUNCH320. pUNCH320 caused *E. coli* DH5αMCR to grow poorly and appeared to be severely restricted in copy number. These data suggested that other sequences located 3′ of *frpB* may also be toxic to *E. coli* and that further subcloning was necessary to obtain stable clones. Digestion of pUNCH320 with *Mlu*I and *Eco*RI released fragments of approximately 1.0 and 1.5 kb, leaving a 2.8-kb *Cla*I-*Mlu*I fragment attached to pBluescript II SK⁺. This 5.8-kb fragment (vector plus 2.8-kb *Cla*I-*Mlu*I insert) was subsequently isolated, treated with Klenow fragment, and religated to itself to generate pUNCH325. DH5αMCR (pUNCH325) transformants were stable, and the plasmid copy number was apparently normal.

Nucleotide sequence analysis of *frpB*. PCR amplification of chromosomal DNA followed by sequence analysis of clones confirmed the *Cla*I junction between pUNCH319 and pUNCH325. The combined nucleotide sequence and deduced amino acid sequence from pUNCH319 and pUNCH325 are shown in Fig. 3. Putative promoter sequences were located upstream of a well-conserved Fur box (4). A string of nine cytosine residues was noted between the putative −10 and −35 RNA polymerase binding sites. A Shine-Dalgarno sequence starting at nucleotide 307 and ending at nucleotide 310 (Fig. 3) was located six bases before an ATG codon, the start of a 1,925-bp open reading frame (ORF). This ORF encoded a protein of 713 amino acids. The predicted protein contained a typical signal sequence and characteristic Ala-X-Ala, signal peptidase I cleavage site. The first 10 amino acids adjacent to the cleavage site were identical to the peptide sequence obtained from the mature FrpB protein. A classical TonB box was noted at residues 32 to 36. The mature protein had a calculated molecular mass of 76.6 kDa and pI of 10.38. The sequence downstream of the ORF revealed an inverted repeat but no string of T residues characteristic of rho-independent transcription termination (69). The protein terminated with an aromatic residue preceded by nine alternating hydrophobic and hydrophilic amino acids. This structure is typical of many bacterial outer membrane proteins sequenced to date (58).

GenBank homologies. Comparison of FrpB with other sequences in GenBank revealed some interesting homologies. Localized homology was found between FrpB and the family of TonB-dependent outer membrane receptor proteins including BtuB (25) and FepA (35) of *E. coli* and between Tbp1 (13) and IroA (42) of *Neisseria* species. This similarity was limited to the highly conserved domains (13) and suggested that FrpB may
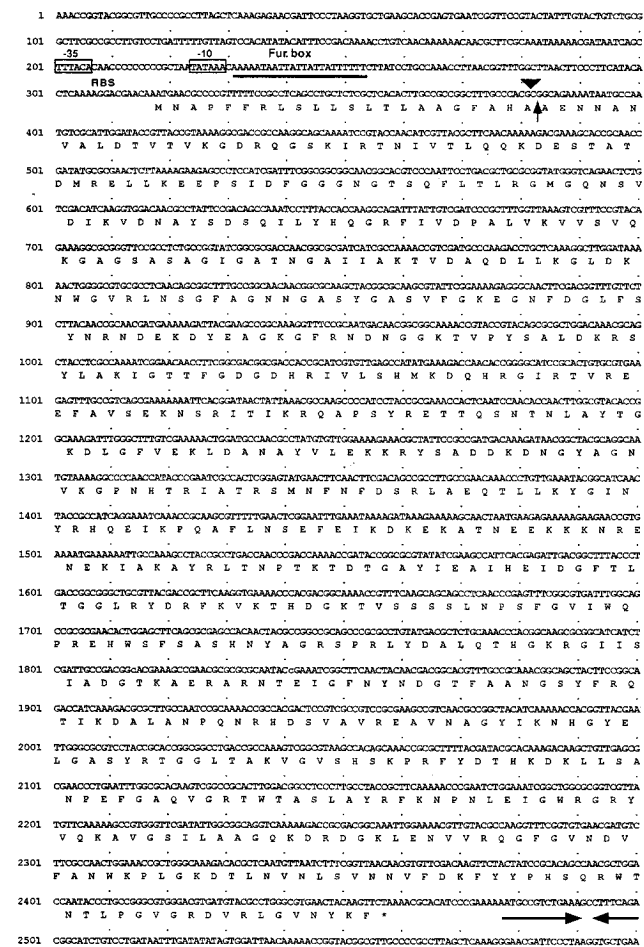
FIG. 3. Nucleotide sequence of the gonococcal *frpB* gene. Single-letter codes for deduced amino acid sequence are shown below the nucleotide sequence. Asterisk indicates termination codon. Solid bar below nucleotide sequence indicates putative Fur box. Putative −10 and −35 sequences are boxed. RBS indicates ribosome binding site. Solid triangle shows *Bgl*I site of Ω insertion. Vertical arrow indicates signal peptidase I cleavage site. Horizontal arrows indicate inverted repeat.

FIG. 4. Southern blot analysis of FA19 and FA6807 DNA. Panel A was probed with pUNCH319-specific fragment. Panel B was probed with the Ω fragment. Lanes 1 contain FA19 DNA digested with *Hinc*II, and lanes 2 contain FA6807 DNA digested with *Hinc*II. Ω fragment is 2 kb. Molecular size markers are shown in kilobases.

also be a TonB-dependent receptor. More similarity was found with HemR, the hemin receptor of *Yersinia enterocolitica* (55). HemR is an iron-regulated, outer membrane protein that is also a member of the family of TonB-dependent receptor proteins. Overall, the two proteins were 26% identical and 48% similar. The most notable similarity was seen with CopB, a major outer membrane protein of *Moraxella catarrhalis* (26). A physiological function for this protein has yet to be determined. Overall, FrpB and CopB were 52% identical and 71% similar.

Transposon mutagenesis of *frpB*. In order to construct FrpB mutants, the gonococcal insert in pUNCH319 was ligated into pUP1 (19), creating pUNCH321. The Ω fragment from pHP45Ω was ligated into a unique *Bgl*I site in pUNCH321 (insertion site shown in Fig. 3). This DNA was reintroduced into the chromosome of gonococcal strain FA19 by transformation and allelic replacement, creating FA6807. Southern blot analysis of chromosomal DNA from FA19 and FA6807 indicated that a 450-bp, MB.3-hybridizing *Hinc*II fragment present in the parent was missing in FA6807 and a new reactive band of approximately 2.5 kb was present (Fig. 4A). An iden-
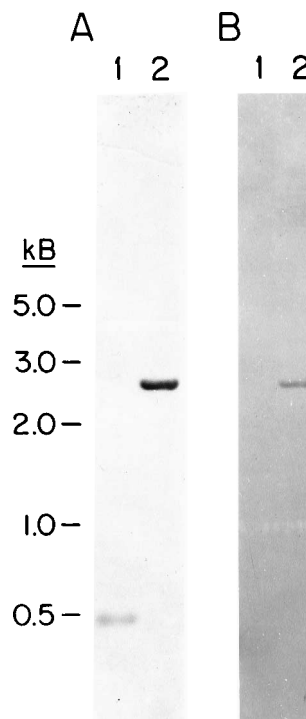
tical blot (Fig. 4B) probed with Ω hybridized only to the 2.5-kb fragment in FA6807. SDS-PAGE and Western blot analysis with anti-FrpB monoclonal antibody W.6 confirmed that FrpB was absent from this strain (Fig. 5).

The Ω insertion in *frpB* was also introduced into FA6747 [*tbpA*::mTn*3*(Cm)] by transformation and allelic replacement, creating FA6808. The FrpB⁻ Tbp1⁻ phenotype of FA6808 was confirmed by SDS-PAGE and Western blot analysis (data not shown). This strain was used for FrpB function analysis as described below.

Utilization of iron sources. In an attempt to determine the function that FrpB plays in iron utilization, FA19 and FA6807 were grown in CDM lacking iron. Aliquots of iron-stressed cultures were plated onto CDM agarose containing 10 μM Desferal and GC base agar containing 50 μM Desferal. Sterile 3-mm discs containing either citrate, Tf, Lf, heme, hemoglobin, or hemoglobin bound to haptoglobin were positioned around each plate. One disc without any added iron source was added as a negative control. After overnight incubation, growth of both strains was evident around all discs except the negative control. These results did not suggest any obvious function for FrpB in iron utilization.

Because it has been previously reported that *N. gonorrhoeae* can utilize aerobactin (67) and enterobactin (45) as iron sources, we decided to determine if FrpB functioned as either an aerobactin or enterobactin receptor. FA19, FA6808, FA6747, KDF541, KDF541/pABN6, and BN1071 (Table 1) were iron stressed in CDM as described above and plated onto CDM agarose containing 2.5 μM 30% iron-saturated Tf. FA6747 and FA6808 could not use Tf as an iron source because they lacked Tbp1; therefore, these strains could grow only in the presence of a functional high-affinity siderophore
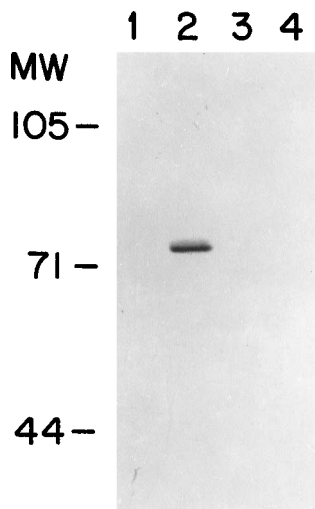
FIG. 5. Western blot of FA19 and FA6807 membranes. Blot was probed with anti-FrpB monoclonal antibody, W.6. Lanes 1 and 2 contain FA19; lanes 3 and 4 contain FA6807. Lanes 1 and 3 contain total membranes prepared from iron-sufficient cultures; lanes 2 and 4 contain total membranes from iron-deficient cultures. Approximate locations of molecular weight standards (MW) are indicated at left in thousands.

receptor. Three sterile discs were positioned around each plate. Either 30% saturated Lf (positive control for gonococcal viability) or filter-sterilized, iron-free supernatant from LG1315 pColV (aerobactin producer) or AN102 (enterobactin hyperproducer) was added to each disc. After overnight incubation, E. coli controls grew as expected (data not shown), suggesting that both siderophores were efficient at stripping iron from Tf, the sole iron source provided in the medium. FA19 grew over the entire Tf plate as expected; however, growth of FA6808 and FA6747 was evident only around the Lf discs, suggesting that the cells were viable but unable to use aerobactin or enterobactin under these conditions.

We further examined the question of aerobactin utilization

by FA19 and FA6807 in liquid CDM, employing various concentrations of purified ferri-aerobactin (Fig. 6). Aerobactin receptor-negative E. coli KDF541 and aerobactin receptor-positive E. coli KDF541(pABN6) were used as controls (data not shown). These data suggested that N. gonorrhoeae FA19 and FA6807 used ferri-aerobactin similarly and in a concentration-dependent fashion analogous to the aerobactin receptor-negative E. coli control. Growth stimulation of gonococci by ferri-aerobactin required relatively high concentrations (3 μM) and never attained a density equivalent to that of the Tf or citrate controls. These experiments confirmed the ability of gonococci to utilize ferri-aerobactin as an iron source in vitro but showed that this ability was not dependent upon a high-affinity receptor-mediated event.

^{55}Fe uptake from hemin, Tf, and citrate. Because of the high degree of similarity between HemR, a known hemin receptor in Y. enterocolitica, and FrpB, we decided to determine if a quantitative difference in ^{55}Fe uptake from hemin could be detected between FA19 and FA6807. Uptakes of ^{55}Fe from Tf by FA19, FA6807, and the Tbp1 mutant FA6747 were used as controls. The results indicated that while ^{55}Fe uptake from Tf was approximately wild type in FA6807 ($P = 0.826$), ^{55}Fe uptake from hemin was reduced by approximately 60% ($P < 0.001$) (Fig. 7). Surprisingly, ^{55}Fe uptake from hemin was also significantly reduced in FA6747 ($P < 0.001$). To determine whether the inability to use ^{55}Fe from hemin was specific to FA6807 (FrpB$^-$) and FA6747 (Tbp1$^-$), we assayed ^{55}Fe uptake from hemin in other well-characterized gonococcal mutants specifically altered in the expression of other iron-repressible proteins. The Tbp2$^-$ and Lbp$^-$ strains, FA6819 and FA6775, respectively, were also reduced in ^{55}Fe internalization from hemin ($P < 0.001$) (data not shown). These data suggested that either more than one protein was involved in the internalization of hemin iron or the notable decrease in hemin-iron uptake in these mutants resulted from unanticipated, nonspecific effects of each of these mutations on a separate membrane-bound, heme-iron-uptake system.

Reconstruction of frpB in pACYC184 and functional complementation of RK1065 (hemA). In an attempt to deter-
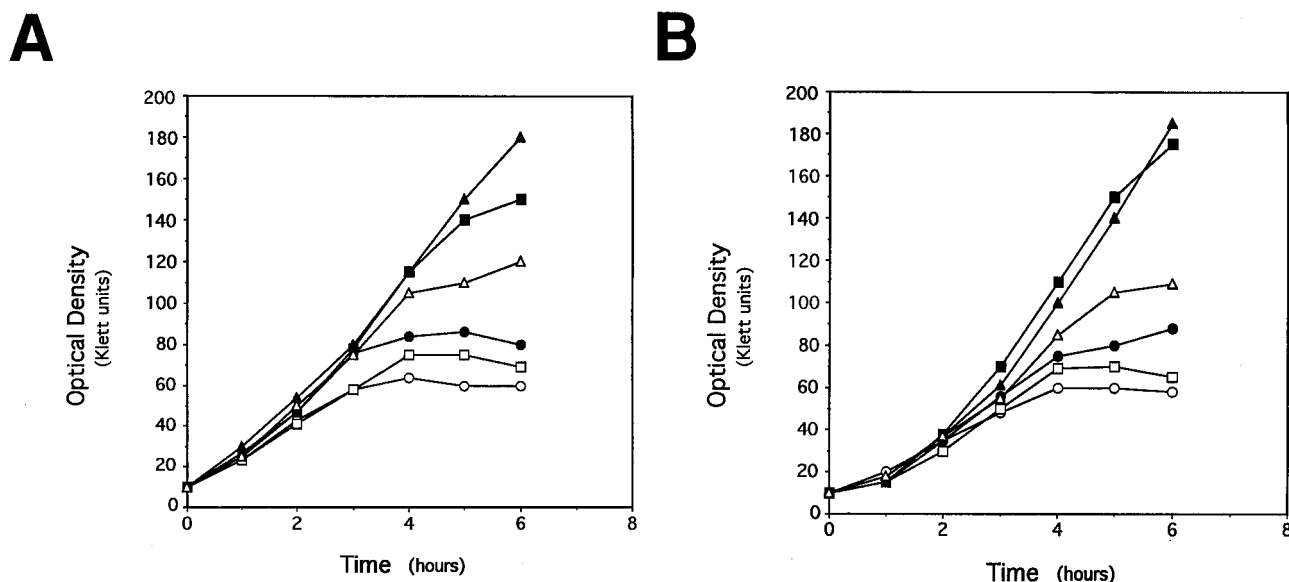

FIG. 6. Growth of FA19 and FA6807 in CDM in the presence of variable concentrations of aerobactin. Graph A represents FA19; graph B represents FA6807. ▲, 100 μM citrate; ■, 2.5 μM Tf; △, 3 μM aerobactin; ●, 1 μM aerobactin; □, 0.3 μM aerobactin; ○, no iron source.
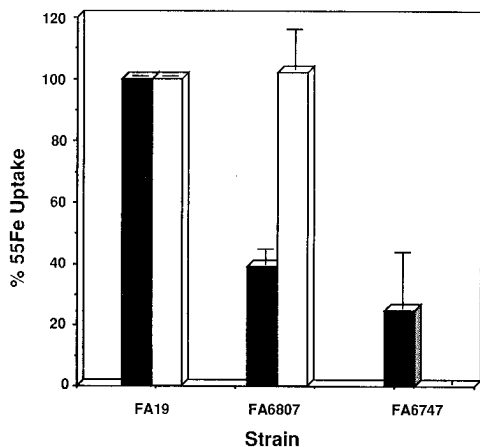
FIG. 7. ^{55}Fe uptake from ^{55}Fe-heme and ^{55}Fe-Tf. Solid columns represent mean uptake from heme, and open columns represent mean uptake from Tf. One hundred percent uptake was determined from average FA19 uptake experiment. Standard deviations are indicated by error bars. The strains and genotypes are FA19 (wild type), FA6807 (*frpB*), and FA6747 (*tpbA*).
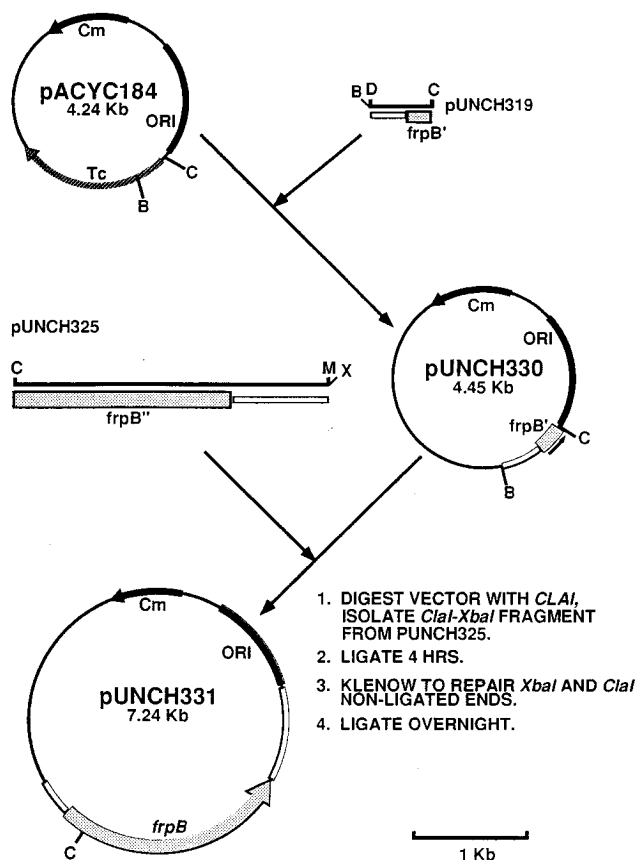

FIG. 8. Reconstruction of *frpB* in pACYC184. Relevant sites are *Bam*HI (B), *Cla*I (C), *Dra*I (D), *Mlu*I (M), and *Xba*I (X). Solid arrow represents chloramphenicol acetyltransferase (Cm), striped arrow represents tetracycline resistance gene (Tc), solid bar represents pACYC184 origin of replication (ORI), stippled boxes represent *frpB* coding sequences, stippled arrow indicates entire *frpB* coding regions, and open boxes represent DNA 5′ and 3′ of *frpB*. *frpB*′ and *frpB*″ represent partial *frpB* coding sequences.

mine if FrpB could function as a heme receptor, we tried to complement an *E. coli hemA* mutant with FrpB. Although expression of FrpB from the high-copy-number vector pBluescript II SK$^+$ was toxic to *E. coli*, expression from the low-copy-number vector pACYC184 was tolerated. The *frpB* reconstruction strategy is outlined in Fig. 8. Briefly, the insert from pUNCH319 was ligated into the *Cla*I and *Bam*HI sites of pACYC184, generating pUNCH330. pUNCH330 was digested with *Cla*I, and the gel-purified *Cla*I-*Xba*I fragment from pUNCH325 was ligated into this site as follows. After ligating for 4 h, Klenow fragment was added to the ligation mixture for 30 min at room temperature to repair nonligated *Cla*I and *Xba*I ends. The reaction mixture was further ligated overnight. The *frpB* clone in pACYC184 was designated pUNCH331. FrpB expression from pUNCH331 was iron repressible, suggesting regulation by *E. coli* Fur (data not shown).

RK1065 is an *E. coli hemA* mutant which is unable to synthesize or internalize heme (27). Growth stimulation requires either δ-aminolevulinic acid or heme and a functional heme receptor. Transformation of pUNCH331 into RK1065 supported growth on heme plates, whereas pACYC184 alone did not (Fig. 9). We performed an RNase leakage assay, to determine if FrpB expression altered the *E. coli* outer membrane, thereby allowing heme to simply diffuse into the cell (71). *E. coli* C386 and HB101 containing pEBH21 were used as positive and negative controls, respectively. No difference in leakiness was detected between RK1065(pACYC184) and RK1065(pUNCH331) (data not shown), suggesting that growth of RK1065(pUNCH331) on heme plates was not due to a membrane perturbation gross enough to permit leakage of the periplasmic protein RNase H. Nevertheless, RK1065(pUNCH331) was more sensitive to several hydrophobic antibiotics than the same strain with pACYC184 alone (Fig. 9). This experiment suggested that the presence of FrpB in *E. coli* probably allowed heme to enter nonspecifically either by creating a pore or by perturbing the integrity of the outer membrane. No KCN-inhibitable uptake of ^{55}Fe from hemin could be attributed to expression of FrpB in RK1065(pUNCH331) (data not shown).

Bactericidal assay. In *M. catarrhalis*, CopB, the protein with the greatest similarity to FrpB, appears to play a major role in serum resistance. Mutants which are missing CopB have de-

creased serum resistance and survival in a mouse model (26). We performed standard bactericidal assays with normal human serum on FA19 and FA6807 grown under iron-limiting conditions and were unable to detect any difference in survival; both strains were completely serum resistant (data not shown).
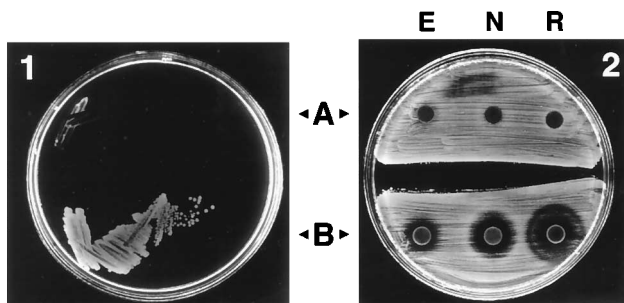

FIG. 9. Growth of RK1065(pACYC184) and RK1065(pUNCH331) on heme plates. Plate 1 contains heme only. Plate 2 contains heme and δ-aminolevulinic acid. A is RK1065(pACYC184) and B is RK1065(pUNCH331). Antibiotic discs are erythromycin (E), novobiocin (N), and rifampin (R).

DISCUSSION

FrpB is a major iron-regulated, outer membrane protein in many gonococcal and meningococcal strains. Its surface exposure (1, 16, 41), partial antigenic conservation (8, 16), and susceptibility to attack by bactericidal antibodies (41) warrant its study as a potential vaccine candidate. We have cloned and sequenced *frpB* from gonococcal strain FA19 and studied its possible role in iron uptake. Several regions of the predicted FrpB protein shared similarity with regions identified in other proteins as potentially important for membrane localization and/or TonB interaction. TonB in association with ExbB and ExbD is believed to transduce the energy from the proton motive force generated in the cytoplasmic membrane to receptors in the outer membrane (43). FrpB also shared a significant region of similarity with a 104-amino-acid domain of HemR, the heme receptor of *Y. enterocolitica*. While this region included one of the highly conserved TonB domains (13), it also extended a significant distance upstream and downstream of this region. These homologies suggested that FrpB may be a TonB-dependent receptor.

In order to assess the function of FrpB in iron utilization, we constructed an insertional mutant of the cloned gene in *E. coli* and introduced it by transformation and allelic replacement into the chromosome of FA19. This mutant, FA6807, was able to grow in the presence of all tested iron sources including Tf, Lf, citrate, heme, hemoglobin, and hemoglobin bound to haptoglobin. ^{55}Fe uptake from Lf (data not shown), Tf, and citrate by FA6807 was similar to that by wild type, while ^{55}Fe uptake from hemin was approximately 60% reduced. These data opened the possibility that FrpB was involved in heme iron uptake. However, other gonococcal mutants missing either Tbp1, Tbp2, or Lbp were also reduced in ^{55}Fe from heme. Thus, the heme uptake-deficient phenotype was not specific to FrpB. It is possible that Tbp1, Tbp2, and Lbp form an outer membrane protein complex which is essential to the configuration of proteins involved in heme-iron uptake. Inactivation of one member of this complex may alter nearby proteins, thus affecting the cell's ability to utilize heme-bound iron. We did not investigate the possible effects of FrpB, Tbp1, Tbp2, or Lbp mutants on the surface accessibility of other iron-repressible proteins such as those isolated by hemin-affinity purification.

The ability of the pathogenic *Neisseria* spp. to acquire iron from heme, hemoglobin, and hemoglobin bound to haptoglobin has been well documented (3, 17, 38). However, the mechanism(s) of iron uptake from these sources has never been defined. Recently, Lee (33) isolated two iron-repressible gonococcal proteins of 97 and 44 kDa by hemin-affinity chromatography. The function of these proteins in iron uptake from heme was not addressed. We used the hemin-affinity purification procedure employed by Lee (33) to isolate hemin-binding proteins from FA19. While we were able to isolate a protein of approximately 97 kDa and several smaller-molecular-mass proteins, we were unable to purify FrpB by this procedure (data not shown).

The homology between FrpB and HemR of *Y. enterocolitica* prompted experiments to determine if FrpB could complement an *E. coli* mutant deficient in heme synthesis. Stojiljkovic and Hantke (55) originally reported that two gene products, HemR and HemP, from *Y. enterocolitica* were required to complement an *E. coli hemA* mutant, but recently, it was shown that just HemR expressed on a low-copy-number plasmid was sufficient (56). FrpB expressed on a low-copy-number vector also allowed RK1065*hemA* to grow on heme plates as the sole source of porphyrin. However, expression of FrpB in RK1065 increased the susceptibility of this strain to hydrophobic antibiotics that are normally impermeable to the *E. coli* outer membrane, suggesting that the heme utilization phenotype could result from the creation of a nonspecific pore or other alteration in the outer membrane generated by the presence of FrpB.

Fur is a 17-kDa protein that in the presence of iron binds to the Fur box in the promoter region of iron-regulated genes and represses transcription (4). Both gonococcal (5) and meningococcal (59) Fur homologs have been characterized. An inverted repeat located just upstream of the *frpB* coding sequence matched the *E. coli* consensus Fur box in 13 of 19 bases, including 100% identity with the seven highly conserved bases (4) of the *E. coli* Fur box. Expression of FrpB by *N. gonorrhoeae* and *E. coli* was iron regulated, and it is likely that this regulation was mediated by Fur.

Expression of FrpB in vitro can vary significantly between different gonococcal strains (data not shown). We have also observed considerable variability of FrpB expression during in vitro passage of single gonococcal strains (data not shown). A string of cytosine residues located between the putative -10 and -35 promoter elements of *frpB* may be responsible for this alteration. Slipped-strand mispairing is known to occur readily at repeated sequence elements (34, 57), and loss or gain of a single base pair in a promoter region may alter the affinity of RNA polymerase for a promoter. In *Mycoplasma hyorhinis*, changes in the number of adenine residues between -10 and -35 elements are believed to regulate the variable expression of a set of surface-exposed lipoproteins (*vlp*) (70). In *Bordetella pertussis*, alterations in the number of cytosines in the promoter region of the fimbrial genes (*fim*) are implicated in phase variation (68). In *Haemophilus influenzae*, a string of TA repeats in the overlapping promoter region of the fimbrial subunit (*hifA*) and a fimbrial chaperone protein (*hifB*) mediates the phase variation of these proteins (62). Recently, variation in expression of the Opc protein of *N. meningitidis* was shown to be due to changes in the number of cytosines in the promoter element of this gene (49). Additional studies are required to determine if alteration in the cytosine tract preceding *frpB* results in changes in expression levels of FrpB and to understand the physiological role of FrpB in the biology of gonococcal infection.

REFERENCES

1. **Ala' Aldeen, D. A., H. A. Davies, and S. P. Borriello.** 1994. Vaccine potential of meningococcal FrpB: studies on surface exposure and functional attributes of common epitopes. Vaccine **12:**535–541.
2. **Anderson, J. E., P. F. Sparling, and C. N. Cornelissen.** 1994. Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization. J. Bacteriol. **176:**3162–3170.
3. **Archibald, F. S., and I. W. DeVoe.** 1980. Iron acquisition by *Neisseria meningitidis* in vitro. Infect. Immun. **27:**322–334.
4. **Bagg, A., and J. B. Neilands.** 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51:**509–518.
5. **Berish, S. A., S. Subbarao, C. Y. Chen, D. L. Trees, and S. A. Morse.** 1993.

Identification and cloning of a *fur* homolog from *Neisseria gonorrhoeae*. Infect. Immun. **61:**4599–4606.

6. **Biswas, G., and P. F. Sparling.** Unpublished data.

7. **Biswas, G. D., J. Graves, R. Schwalbe, and P. F. Sparling.** 1986. Construction of isogenic gonococcal strains varying in the presence of a 4.2-kilobase cryptic plasmid. J. Bacteriol. **167:**685–694.

8. **Black, J. R., D. W. Dyer, M. K. Thompson, and P. F. Sparling.** 1986. Human immune response to iron-repressible outer membrane proteins of *Neisseria meningitidis*. Infect. Immun. **54:**710–713.

9. **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.

10. **Briat, J. F.** 1992. Iron assimilation and storage in prokaryotes. J. Gen. Microbiol. **138:**2475–2483.

11. **Brock, J. H., P. H. Williams, J. Liceaga, and K. G. Wooldridge.** 1991. Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of *Escherichia coli* and to other microorganisms. Infect. Immun. **59:**3185–3190.

12. **Brook, I.** 1994. The role of encapsulated anaerobic bacteria in synergistic infections. FEMS Microbiol. Rev. **13:**65–74.

13. **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.

14. **de Lorenzo, V., S. Wee, M. Herrero, and J. B. Neilands.** 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. **169:**2624–2630.

15. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.

16. **Dyer, D. W., E. P. West, W. McKenna, S. A. Thompson, and P. F. Sparling.** 1988. A pleiotrophic iron-uptake mutant of *Neisseria meningitidis* lacks a 70-kilodalton iron-regulated protein. Infect. Immun. **56:**977–983.

17. **Dyer, D. W., E. P. West, and P. F. Sparling.** 1987. Effects of serum carrier proteins on the growth of pathogenic neisseriae with heme-bound iron. Infect. Immun. **55:**2171–2175.

18. **Elkins, C., N. H. Carbonetti, V. A. Varela, D. Stirewalt, D. G. Klapper, and P. F. Sparling.** 1992. Antibodies to N-terminal peptides of gonococcal porin are bactericidal when gonococcal lipopolysaccharide is not sialylated. Mol. Microbiol. **6:**2617–2628.

19. **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.

20. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53:**210–230.

21. **Gotschlich, E. C., C. Cornelissen, S. A. Hill, J. M. Koomey, C. Marschal, S. A. Morse, S. Normark, A. B. Schryvers, H. S. Seifert, P. F. Sparling, and J. Swanson.** 1991. The mechanism of genetic variation of gonococcal pili. Iron-inducible proteins of *Neisseria*. A consensus, p. 405–414. *In* M. Achtman (ed.), Neisseriae 1990: Proceedings of the Seventh International Conference on Pathogenic Neisseria. Walter de Gruyter, Berlin.

22. **Hardham, J. M., and L. V. Stamm.** 1994. Identification and characterization of the *Treponema pallidum tpn50* gene, an *ompA* homolog. Infect. Immun. **62:**1015–1025.

23. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual, p. 471–510. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

24. **Harris, W. R., C. J. Carrano, and K. N. Raymond.** 1979. Coordination chemistry of microbial compounds. 16. Isolation, characterization, and formation constants of ferric aerobactin. J. Am. Chem. Soc. **101:**2722–2727.

25. **Heller, K. J., R. J. Kadner, and K. Gunther.** 1988. Suppression of the *btuB451* mutation by mutations in the *tonB* gene suggests a direct interaction between TonB and TonB-dependent receptor proteins in the outer membrane of *Escherichia coli*. Gene **64:**147–153.

26. **Helminen, M. E., I. Maciver, M. Paris, J. L. Latimer, S. L. Lumbley, L. D. Cope, G. H. McCracken, and E. J. Hansen.** 1993. A mutation affecting expression of a major outer membrane protein of *Moraxella catarrhalis* alters serum resistance and survival of this organism *in vivo*. J. Infect. Dis. **168:**1194–1201.

27. **Henderson, D. P., and S. M. Payne.** 1993. Cloning and characterization of the *Vibrio cholerae* genes encoding the utilization of iron from haemin and haemoglobin. Mol. Microbiol. **7:**461–469.

28. **Jann, K., and B. Jann.** 1992. Capsules of *Escherichia coli*, expression and biological significance. Can. J. Microbiol. **38:**705–710.

29. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. J. Bacteriol. **85:**1274–1279.

30. **Klebba, P. E., M. A. McIntosh, and J. B. Neilands.** 1982. Kinetics of biosynthesis of iron-regulated membrane proteins in *Escherichia coli*. J. Bacteriol. **149:**880–888.

31. **Kraft, R., J. Tardiff, K. S. Krauter, and L. A. Leinwand.** 1988. Using miniprep plasmid DNA for sequencing double stranded templates with Sequenase. BioTechniques **6:**544–546.

32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.

33. **Lee, B. C.** 1992. Isolation of haemin-binding proteins of *Neisseria gonorrhoeae*. J. Med. Microbiol. **36:**121–127.

34. **Levinson, G., and G. A. Gutman.** 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Mol. Biol. Evol. **4:**203–221.

35. **Lundrigan, M. D., and R. J. Kadner.** 1986. Nucleotide sequence of the gene for the ferrienterochelin receptor FepA in Escherichia coli. Homology among outer membrane receptors that interact with TonB. J. Biol. Chem. **261:**10797–10801.

36. **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.

37. **Meyer, T.** 1987. Molecular basis of surface antigenic variation in *Neisseria*. Trends Genet. **3:**319–324.

38. **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.

39. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:**715–731.

40. **Ozkaynak, E., and S. D. Putney.** 1987. A unidirectional deletion technique for the generation of clones for sequencing. BioTechniques **5:**770–773.

41. **Pettersson, A., B. Kuipers, M. Pelzer, E. Verhagen, R. H. Tiesjema, J. Tommassen, and J. T. Poolman.** 1990. Monoclonal antibodies against the 70-kilodalton iron-regulated protein of *Neisseria meningitidis* are bactericidal and strain specific. Infect. Immun. **58:**3036–41.

42. **Pettersson, A., P. van der Ley, J. T. Poolman, and J. Tommassen.** 1993. Molecular characterization of the 98-kilodalton iron-regulated outer membrane protein of *Neisseria meningitidis*. Infect. Immun. **61:**4724–4733.

43. **Postle, K.** 1990. TonB and the gram-negative dilemma. Mol. Microbiol. **4:**2019–2025.

44. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.

45. **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.

46. **Rutz, J. M., J. Liu, J. A. Lyons, J. Goranson, S. K. Armstrong, M. A. McIntosh, J. B. Feix, and P. E. Klebba.** 1992. Formation of a gated channel by a ligand-specific transport protein in the bacterial outer membrane. Science **258:**471–475.

47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

48. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

49. **Sarkari, J., N. Pandit, E. R. Moxon, and M. Achtman.** 1994. Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. Mol. Microbiol. **13:**207–217.

50. **Schryvers, A. B.** 1989. Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. J. Med. Microbiol. **29:**121–130.

51. **Seifert, H. S., E. Y. Chen, M. So, and F. Heffron.** 1986. Shuttle mutagenesis: a method of transposon mutagenesis for *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83:**735–739.

52. **Smith, H.** 1991. The influence of the host on microbes that cause disease. Proc. R. Soc. Lond. B Biol. Sci. **246:**97–105.

53. **Sonntag, I., H. Schwarz, Y. Hirota, and U. Henning.** 1978. Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J. Bacteriol. **136:**280–285.

54. **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.

55. **Stojiljkovic, I., and K. Hantke.** 1992. Hemin uptake systems of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. EMBO J. **11:**4359–4367.

56. **Stojiljkovic, I., and K. Hantke.** 1994. Transport of hemin across the cytoplasmic membrane through a hemin-specific, periplasmic-binding-protein-dependent transport system in *Yersinia enterocolitica*. Mol. Microbiol. **13:**719–732.

57. **Streisinger, G., and J. E. Owen.** 1985. Mechanisms of spontaneous and induced frameshift mutations in bacteriophage T4. Genetics **109:**633–659.

58. **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.

59. **Thomas, C. E., and P. F. Sparling.** 1994. Identification and cloning of a *fur* homologue from *Neisseria meningitidis*. Mol. Microbiol. **11:**725–737.

60. **Thompson, S. A., L. L. Wang, A. West, and P. F. Sparling.** 1993. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. J. Bacteriol. **175:**811–818.

61. **Towbin, J., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of

proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.

62. **van Ham, S. M., L. van Alphen, and F. Mooi.** 1993. Phase variation of *H. influenzae* fimbriae: transcriptional control of two divergent genes through a variable combined promoter region. Cell **73:**1187–1196.

63. **Warner, P. J., P. H. Williams, A. Bindereif, and J. B. Neilands.** 1981. ColV plasmid-specified aerobactin synthesis by invasive strains of *Escherichia coli*. Infect. Immun. **33:**540–545.

64. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42:**45–66.

65. **Weinberg, E. D.** 1984. Iron withholding: a defense against infection and neoplasia. Physiol. Rev. **64:**65–102.

66. **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.

67. **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.

68. **Willems, R., A. Paul, H. G. J. van der Heide, A. R. ter Avest, and F. R. Mooi.** 1990. Fimbrial phase variation in Bordetella pertussis: a novel mechanism for transcriptional regulation. EMBO J. **9:**2803–2809.

69. **Yager, T. D., and P. H. von Hippel.** 1987. Transcription elongation and termination in *Escherichia coli*, p. 1241–1275. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.

70. **Yogev, D., R. Rosengarten, R. Watson-McKown, and K. S. Wise.** 1991. Molecular basis of *Mycoplasma* surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5′ regulatory sequences. EMBO J. **10:**4069–4079.

71. **Young, K., and L. L. Silver.** 1991. Leakage of periplasmic enzymes from *envA1* strains of *Escherichia coli*. J. Bacteriol. **173:**3609–3614.