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

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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 Verruicola enterocococila 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 glycophores 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 (immuno- blots) 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-mu- tagenized, 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. Never- theless, 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 muta- tagenesis 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 de- scribed 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% CO₂. Antibiotic selection employed chloramphenicol at 1 μg/ml for mTn3(Cm) (51)-mu- tagenized strains and streptomycin at 100 μg/ml for Ω (44)-mutagenized strains. For Western blot analysis of total membrane proteins of iron-stressed gono- cocci, cells were grown in chemically defined medium (CDM) as previously

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described (13). Cultures were made iron replete as indicated by the addition of 200 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. β-Amine-sul-vulnic 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 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 antiserum and monoclonal antibodies. Preparation of polyclonal antiserum 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 the 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. pHP451 (44) was used to insertionally inactivate frpB. pUNCH321 was digested with BglII and EcoRI, and ends were repaired with Klenow fragment. pHP451 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 micromolars 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 transfer-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 destined for 10 min in distilled deionized H2O with one change. Filter was frozen at –20°C overnight. FrpB was identified by molecular weight, and the aminoterminal amino acid sequence of the protein on the filter was determined by the Protein Microsequencing Facility at University of California, Los Angeles.

Fe 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-TbpB 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% CO2.

| Strains, plasmid, or phage | Description | Reference or source |
|---------------------------|-------------|--------------------|
| FA19                      | Wild type   | 38                 |
| FA6807                    | frpB::Ω (FrpB') | This study |
| FA6808                    | frpB::Ω-lpaC::Mn3(Cm) (FrpB' Tbp1') | This study |
| FA6747                    | lpaC::Mn3(Cm) (Tbp1) | 13          |
| FA6819                    | ΔtbpB (Tbp2') | 2               |
| FA6775                    | lpaC::Mn3(Cm) (Lpb') | 6             |
| UU11008                   | Wild type   | 8                 |
| DH5×MCR                  | F- mcrA mcrB mrr Δ808lacZAM15 ΔargF-lac U169 recA1 endA1 hsdR | Bethesda Research Laboratories |
|              | hsdM supE44 k thi-1 gyrA96 relA |            |
| BN1071                   | F' pro trp rIL entA (Ent' FepA') | 30          |
| AN1012                    | BN1071 leuB44 (Ent' FepA') | 30          |
| KDF541                   | BN1071 entA fepl4 (Ent' FepA') | 46          |
| KDF541/pABN6              | (Ent' FepA' IutA' Iuc') | 14          |
| LG1315/pCIV              | BN1071 cir (IutA' Iuc') | 63          |
| KK1065                   | hemA         | R. Kadner         |
| HB101                    | F' hsdR20 (mB') recA13 ara-14 proA2 lacY1 galK2 rpsL20 (Sm') xyl-5 | 47          |
| ml1-4 supE44 λ            | ompA4 lpp   | 53               |

| Plasmids and phage         | Description | Reference or source |
|---------------------------|-------------|--------------------|
| pACYC184                  | ori p15a Cm' Tc' | New England Biolabs |
| pBluescript II SK*        | ori pMBI Ap' | Stratagene          |
| pHP451                   | Source for the Ω fragment (Sm') | 44          |
| pUP1                      | pHSS6 containing gonomococal uptake sequence (Kan') | 19          |
| pEBH21                   | pBC II SK* derivative (Cm') | 22          |
| pUNCH319                  | pBluescript II SK* containing 540-bp EcoRI-ClaI fragment from XfrB4 | This study |
| pUNCH320                  | pBluescript II SK* containing 5.3-kb Clal-EcoRII fragment from XfrB4 | This study |
| pUNCH321                  | pUP1 containing 540-bp EcoRI-ClaI fragment from pUNCH319 | This study |
| pUNCH324                  | pUP1 containing Ω fragment from pH451 in unique BglI site | This study |
| pUNCH325                  | pBluescript II SK* containing 2.8-bp Clal-Mulu fragment from pUNCH320 | This study |
| pUNCH330                  | 540-bp EcoRII-CIaI fragment from pUNCH319 in pACYC184 | This study |
| pUNCH331                  | Reassembled gonococcal frpB gene in pACYC184 | This study |
| λ-ZapII                  | Excisable lambda phage vector | Stratagene        |
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 H2O 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 Na2HPO4. The ferri-aerobactin was run over a carboxymethyl-cellulose (Sigma) column equilibrated in 0.05 M Na2HPO4. The final concentration of aerobactin was determined by reading the A280 (24).

Iron sources. Human Tf, human Lf, bovine heme, human hemoglobin, and human haptoglobin were obtained from Sigma Chemical Co. 55Fe hemin was purchased from Sigma Chemical Co. 55FeCl3 was used at 6.25 μM, and citrate was used at 100 μM.

Bactericidal assays. Bactericidal assays were performed as described previously (71).

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 DraI fragment was chosen for further analysis.

A λ-ZapII library containing EcoRI-linked FA19 chromosomal DraI 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 EcoRI to release the insert DNA. The expected 5.8-kb fragment was isolated from an agarose gel and further digested with ClaI 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 MluI and EcoRI released fragments of approximately 1.0 and 1.5 kb, leaving a 2.8-kb ClaI-MluI fragment attached to pBluescript II SK+.

This 5.8-kb fragment (vector plus 2.8-kb ClaI-MluI 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 ClaI 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
alsobeTonB-dependentreceptor. 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 V fragment from pHP45 V was ligated into a unique BglI site in pUNCH321 (insertion sites 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 \( \text{HincII} \) fragment present in the parent was missing in FA6807 and an new reactive band of approximately 2.5 kb was present (Fig. 4A). An identical blot (Fig. 4B) probed with \( \Omega \) 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).

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 \( \mu \)M Desferal and GC base agar containing 50 \( \mu \)M Desferal. Sterile 3-mm discs containing either citrate, Tf, 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, FA6807, FA6747, KDF541, KDF541/pABN6, and BN1071 (Table 1) were iron stressed in CDM as described above and plated onto CDM agarose containing 2.5 \( \mu \)M 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.
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.

55Fe 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 55Fe uptake from hemin could be detected between FA19 and FA6807. Uptakes of 55Fe from Tf by FA19, FA6807, and the Tbp1 mutant FA6747 were used as controls. The results indicated that while 55Fe uptake from Tf was approximately wild type in FA6807 (P = 0.826), 55Fe uptake from hemin was reduced by approximately 60% (P < 0.001) (Fig. 7). Surprisingly, 55Fe uptake from hemin was also significantly reduced in FA6747 (P < 0.001). To determine whether the inability to use 55Fe from hemin was specific to FA6807 (FrpB2) and FA6747 (Tbp12), we assayed 55Fe uptake from hemin in other well-characterized gonococcal mutants specifically altered in the expression of other iron-repressible proteins. The Tbp2 and Lbp2 strains, FA6819 and FA6775, respectively, were also reduced in 55Fe 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, non-specific 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-
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 pBlue-script 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 55Fe 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 decreased 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. 7.](image-url) **55Fe uptake from 55Fe-heme and 55Fe-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.](image-url) **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.

![FIG. 9.](image-url) **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 bacterial 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 transude 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. E. coli constructed an insertional mutant of the cloned gene in this region. These homologies suggested that FrpB maybe 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, heme, hemoglobin, and hemoglobin bound to haptoglobin, 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, and 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 heme iron uptake 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. Stojilkovic and Hantke (55) originally reported that two gene products, HemR and HemF, from Y. enterocolitica were required to complement an E. coli hema4 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 RK1065hema4 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 antimicrobics 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.

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