JOURNAL OF BACTERIOLOGY, Apr. 1996, p. 2145–2149 Vol. 178, No. 7
0021-9193/96/$04.00+0

The *fbpABC* Locus of *Neisseria gonorrhoeae* Functions in the Periplasm-to-Cytosol Transport of Iron

PRATIMA ADHIKARI,[1] SALLY A. BERISH,[2] ANDREW J. NOWALK,[1] KRISTEN L. VERALDI,[1] STEPHEN A. MORSE,[2] AND TIMOTHY A. MIETZNER[1]*

Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261,[1] and Division of AIDS, Sexually Transmitted Diseases and Tuberculosis Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[2]

Received 26 September 1995/Accepted 19 January 1996

We have determined that the DNA sequence downstream of the well-characterized gonococcal *fbp* gene contains two open reading frames: one designated *fbpB*, which encodes a protein proposed to function as a cytoplasmic permease, and one designated *fbpC*, which encodes a protein proposed to function as a nucleotide-binding protein. The *fpbABC* operon composes an iron transport system that is homologous to the *sfu* and *hit* operons previously reported for *Serratia marcescens* and *Haemophilus influenzae*, respectively, and displays elements characteristic of ATP binding cassette transporters. The *fpbABC* operon differs from these loci in that it is lethal when overexpressed in *Escherichia coli*.

A prerequisite for infection by *Neisseria gonorrhoeae* is the ability to multiply within the human host. Many factors contribute to this, including the capacity to compete for host-sequestered iron (13, 14, 16). For *N. gonorrhoeae*, the process of iron acquisition begins in vivo with the binding of an iron source (e.g., human transferrin) by outer membrane protein receptors (12, 16, 21). Previous studies have determined that upon entry into the periplasm, free iron is chelated by the iron-binding protein Fbp (ferric-iron-binding protein), which initiates the transport of iron from the periplasm to the cytosol (9, 16, 17). Molecular details of the transport of Fbp-bound iron from the periplasm to the cytosol have not been previously described. An emerging theme in the biology of import and export processes across membranes is facilitation by the general class of ATP binding cassette (ABC) transporters (the ATP-binding cassette is a hallmark of transport systems of this type) (11). In gram-negative bacteria, active transport of molecules from the periplasm to the cytosol often involves a periplasmic binding protein in conjunction with an ABC transporter (11). We have previously suggested that periplasm-to-cytosol transport of iron by *N. gonorrhoeae* proceeds by an analogous mechanism (9). Therefore, at least two further activities (that have not been previously described), that of a cytoplasmic permease and that of a nucleotide-binding protein, would logically be implicated in gonococcal periplasm-to-cytosol iron transport. This report describes the genetic loci and physical existence of these activities.

Sequence and genetic organization of the neisserial *fbp* operon. The major focus of this study was to demonstrate the existence of the *fbpABC* operon. The *fbp* gene sequence has been well characterized (5, 6, 22) and has been designated *fbpA* for the purpose of this study. Since *fbpA* had already been sequenced (5, 6, 22), only partial sequencing of this gene near its 3′ end was performed. Sequencing downstream of *fbpA* was accomplished by digestion of gonococcal chromosomal DNA with *Rsa*I followed by ligation into the *Rsa*I site of pUC18. This ligation mixture was used as a template for a standard PCR with the oligonucleotides FbpTaa and For, as described previously by Berish et al. (5, 6) (Table 1). This generated a PCR fragment of approximately 1,350 bp that contained one-half of the *fbpB* gene sequence. Similarly, primer WLK1 (Table 1) was used with *Cla*I-digested genomic DNA after ligation into the *Cla*I site of pUC18. This consistently amplified a 2,500-bp fragment that encoded regions overlapping the *fbpB* gene locus to 3′ of the *fbpC* locus. Primers based upon known sequences were developed and used in the preparation of various PCR products for sequencing (Fig. 1), as previously described (7). The accuracy of the sequences obtained was ensured by multiple rounds of sequencing on different PCR templates with a wide panel of different overlapping primers for both strands (Fig. 1). The sequence of the complete *fbpABC* operon obtained from *N. gonorrhoeae* F62 is reported in Fig. 1. Sequence analysis of the *fbpA* locus has been reported previously by Berish et al. (4, 5) and Zhou and Spratt (22). The latter reported the presence of an additional codon that would result in an insertion of Ala at position 231 of the mature Fbp amino acid sequence. Subsequent analysis of our sequencing results has confirmed the presence of this codon. A 60-bp intragenic region follows the *fbpA* stop codon and includes a strong stem-loop structure with the potential to form 38 hydrogen bonds (8). Analogous structures are predicted to be in similar positions for the *hit* and *sfu* operons (data not shown). Since stem-loop structures can function in message stability (19), the presence of this structure may allow Fbp expression at levels much higher than those of the *fbpB* and *fbpC* gene products. This is consistent with our inability to detect proteins corresponding to FbpB and FbpC in iron-stressed gonococcal membranes or soluble extracts or in *Escherichia coli* constructs expressing the *fbpABC* operon, as described below (data not shown).

The open reading frames (ORFs) of the *fbpB* and *fbpC* gene sequences were deduced on the basis of homology to those previously reported for *hitBC* (20) and *sfuBC* (2). The predicted product of *fbpB* is compared with the proposed cytoplasmic permeases (HitB and SfuB) in Fig. 2A, and the predicted product of *fbpC* is compared with the nucleotide-binding protein (HitC and SfuC) components of the previously described operons in Fig. 2B.

The predicted FbpB protein is a 511-amino-acid polypeptide

* Corresponding author. Phone: (412) 648-9244. Fax: (412) 624-1401. Electronic mail address: Mietzner@vms.cis.pitt.edu.

TABLE 1. Bacterial strains, plasmids, and primers

Strain, plasmid, or primer	Relevant characteristics	Source
Strains		
N. gonorrhoeae F62	Genomic DNA for PCR sequencing and amplification of *fbpABC* fragment	S. Morse
E. coli DH5αMCR	Host for cloning *fbpABC* operon	BRL
E. coli H-1443	*aroB* mutant; no growth on nutrient agar containing 200, 2,2′-dipyridyl	V. Braun
Plasmids		
pREP4	3.7-kb plasmid derived from a p15A replicon which expresses large quantities of *lac* repressor; Kan^r	Qiagen
pBSKS$^-$	3.1-kb plasmid used as a vector for expression of the *fbpABC* operon in these studies; Amp^r	Stratagene
pUC18	2.6-kb plasmid used as a ligation vector for site-specific PCR in these studies	BRL[a]
pAFbpO	3.6-kb PCR fragment of the *fbpABC* operon ligated into the *Eco*RV and *Bam*HI sites of pBSKS$^-$. This plasmid puts the Fbp operon under the control of the *lac* repressor and is selected for by Amp^r.	This study
Primers		
FBPTaa	Oligonucleotide used in site-specific PCR for *Rsa*I ligation; 5′GAAAAAGAACACGCCACCCGGCTG3′	This study
For	Oligonucleotide used in site-specific PCR for both *Rsa*I and *Cla*I ligations; 5′CCCAGTCACGACGTTGTAA AACG3′	BRL
WLK1	Oligonucleotide used in site-specific PCR for *Cla*I ligation; 5′CGGACACTTCTTTATTTTCAGGAC3′	This study
FbpO-3′*Bam*HI	3′ oligonucleotide used for the amplification of the *fbpABC* operon; 5′CGGGATCCAAGATAAATATCCCG CAGGCATTGTGG3′	This study
F4-*Sca*I	5′ oligonucleotide used for the amplification of the *fbpABC* operon; 5′AAAAAGTACTCGATATGAAAACA TCTATCCGA3′	This study

[a] BRL, Bethesda Research Laboratories, Inc.

with an estimated molecular weight of 56,320. Comparison of FbpB with the predicted HitB sequence by BESTFIT analysis indicates an identity of 64.4% and a similarity of 77.5%. A similar comparison between the *fbpB* and *sfuB* gene products indicates an identity of 34.9% and a similarity of 58.2%. These values are similar to the homologies reported for the FbpA, SfuA, and HitA protein homologs (1, 4). FbpB is proposed to function as a cytoplasmic membrane permease. Optimal alignment of FbpB, HitB, and SfuB proteins identifies 11 regions of primary sequence that, on the basis of the algorithm of Persson and Argos (18), are predictive of transmembrane segments. These segments are commonly associated with membrane permeases (11). In addition, two sequences that match the consensus permease motif EAA---G---------I-LP can be identified (Fig. 2A). These regions are thought to be located on cytoplasmic loops that interact with the ATP-binding protein component (11). This sequence and location are analogous to those previously reported for MalF, MalG, HisQ, HisM, and OppC, all of which are well-characterized cytoplasmic permeases (10, 11).

The ORF corresponding to *fbpC* encodes a 357-residue peptide with a predicted molecular weight of 38,173. A comparison of FbpC with the *hitC* and *sfuC* gene products indicates 51 and 40% identity and 68 and 58% similarity, respectively. In contrast to FbpB, which is composed of 62% hydrophobic amino acids (indicative of an integral membrane protein), FbpC is composed of 50.5% hydrophobic residues. FbpC is proposed to interact with FbpB to supply the energy for the transport of iron across the cytoplasmic membrane through the binding and hydrolysis of a nucleotide triphosphate. A common ATP binding domain is characteristic of the nucleotide-binding protein components of ABC transporters. These domains are about 200 residues in length and have considerable sequence identity. Short consensus sequences designated the Walker A and B motifs are specifically positioned within this 200-residue region (11). Comparison of the *fbpC*, *hitC*, and *sfuC* gene products demonstrates highly conserved Walker A and B motifs (Fig. 2B).

Comparison of the ORFs derived from *fbpBC* with those from *hitBC* and *sfuBC* illustrates sequence similarities conserved among the general class of ABC transporters (11). This observation, coupled with the homology across this operon, argues that the *fbp*, *hit*, and *sfu* operons have evolved separately but function similarly in the periplasm-to-cytosol transport of free iron.

Cloning of the intact *fbpABC* operon. The cloning of the *fbpABC* operon was initially attempted with high- and medium-copy-number vectors such as pUC19 and pBR322. These cloning attempts led to constructs in which all or portions of the *fbpABC* operon were spontaneously deleted (data not shown), suggesting that expression of the complete operon was lethal in *E. coli*. To overcome this problem, expression of the *fbpABC* operon was placed under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *lac* promoter on pBSKS$^-$. In order to clone the operon into this vector, primers with engineered restriction sites were synthesized on the basis of the *fbpABC* operon DNA sequence (Table 1). PCR amplification with these primers yielded a 3.6-kb fragment that was subsequently ligated into the *Eco*RV and *Bam*HI sites of pBSKS$^-$, creating a fusion with *lacZ*. The ligation mixture was then used to transform *E. coli* DH5αMCR carrying pREP4 (a p15A replicon derived from pACYC184) (3). The plasmid pREP4 overexpresses LacI, and in the absence of IPTG, genes driven from the *lac* promoter are not transcribed. From this ligation mixture, 86 transformants were obtained; they were transferred to Luria-Bertani (LB) agar with ampicillin and kanamycin, with or without 32 mg of IPTG per ml. Nine of 86 transformants grew poorly on LB agar containing IPTG whereas all grew well on medium containing no IPTG, suggesting that upregulation of this operon is inhibitory. Plasmid isolation and restriction enzyme analysis indicated that of the nine transformants that grew poorly in the presence of IPTG, six contained pREP4 and pBSKS$^-$ with an intact *fbpABC* operon (data not shown).

Toxicity associated with expression of the intact *fbpABC* operon in *E. coli*. *E. coli* DH5αMCR(pREP4) transformed with either pAFbpO or pBSKS$^-$ was inoculated into LB broth and grown to mid-log phase, and the *fbpABC* operon was

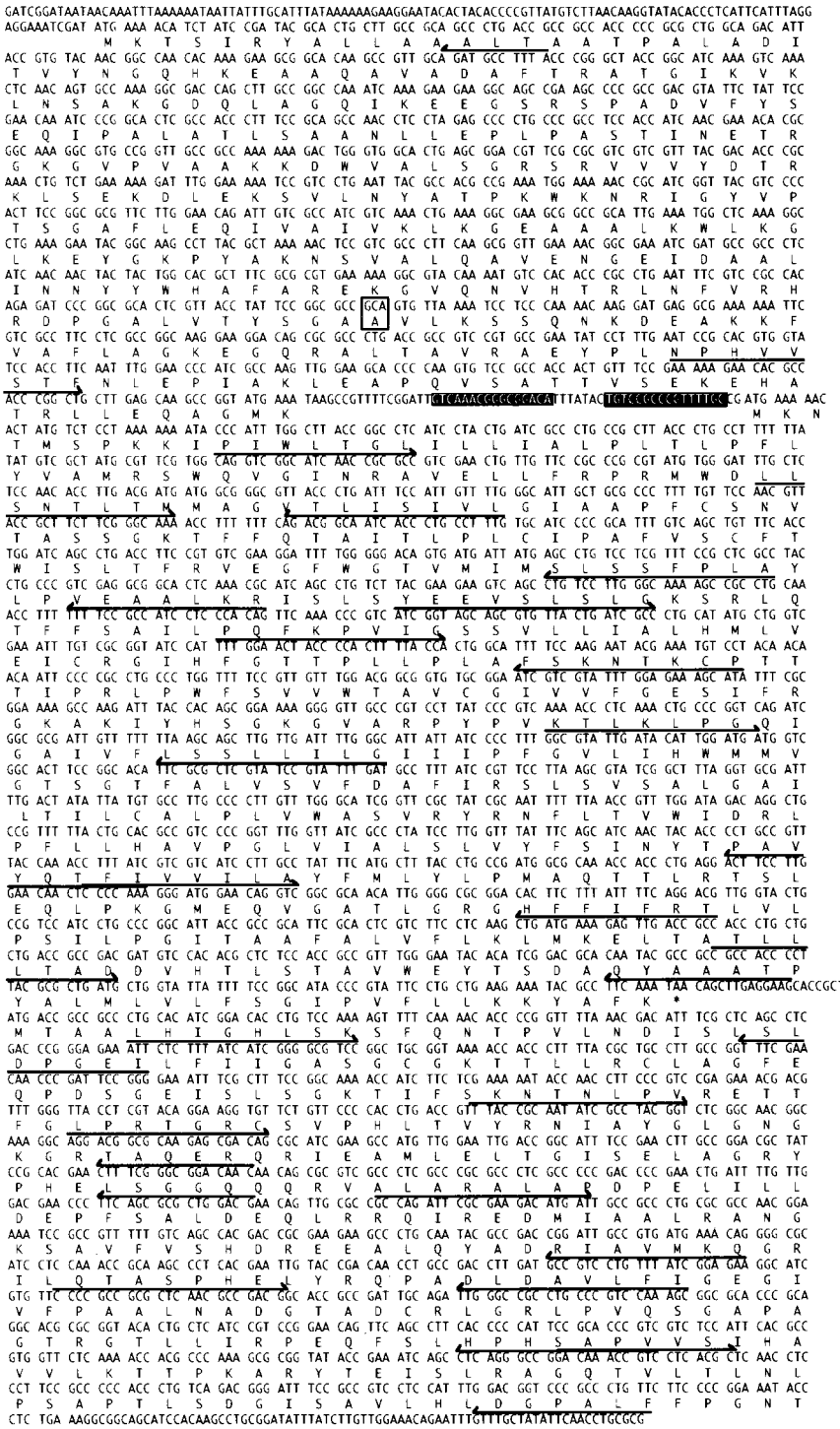

FIG. 1. Sequence of the gonococcal *fbpABC* operon. DNA sequence of the 3,862-bp *fbpABC* operon is depicted with predicted ORFs. Primers used in the sequencing of the operon are shown as arrows above the appropriate sites. ←, primer corresponding to complementary strand; →, primer corresponding to coding strand. A predicted stem-loop structure within the intragenic region between *fbpA* and *fbpB* is indicated by black boxes. An Ala at position 231 has been added to the mature FbpA sequence previously reported (6) and is denoted by an open box.

induced by the addition of 1 mM IPTG. Upon the addition of IPTG, the growth of *E. coli* DH5αMCR(pREP4/pAFbpO) was rapidly inhibited, whereas the growth of *E. coli* DH5αMCR (pREP4/pBSKS$^-$) was not (Fig. 3). Comparison of viable counts of IPTG-induced organisms on LB agar indicated that increased expression of the *fbpABC* operon in *E. coli* was bactericidal rather than bacteriostatic (data not shown). This observation may explain the general difficulty previously encountered in attempts to clone *fbp* and linked sequences (6).

Overexpression of either FbpA (4) or FbpC (unpublished

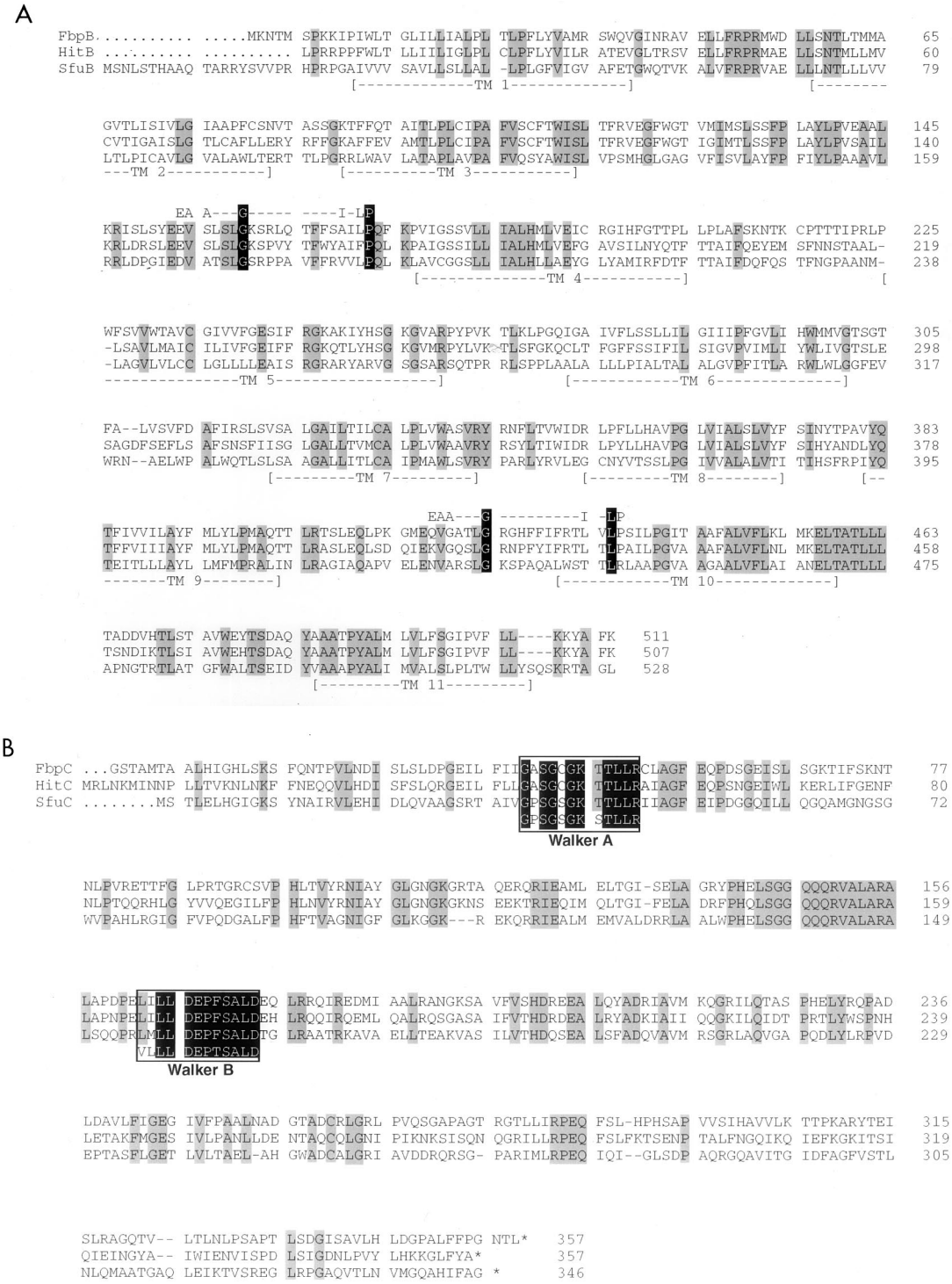

FIG. 2. Comparison of the predicted amino acid sequences of the proposed ABC transporters encoded by the *fbp*, *hit*, and *sfu* operons. (A) Comparison of the predicted amino acid sequences of FbpB, HitB, and SfuB demonstrates the predicted transmembrane regions which are characteristic of cytoplasmic permeases. The predicted transmembrane regions are indicated as TM 1 through TM 11. A sequence motif common to cytoplasmic permeases can be found twice within this sequence and is indicated by text beginning "EAA" above the amino acid sequences. (B) Comparison of predicted amino acid sequences of FbpC, HitC, and SfuC, the proposed nucleotide-binding components. The Walker motifs conserved across the predicted FbpC, HitC, and SfuC protein sequences are indicated with boxes. For both panels, gaps in the alignment are designated with a dash.

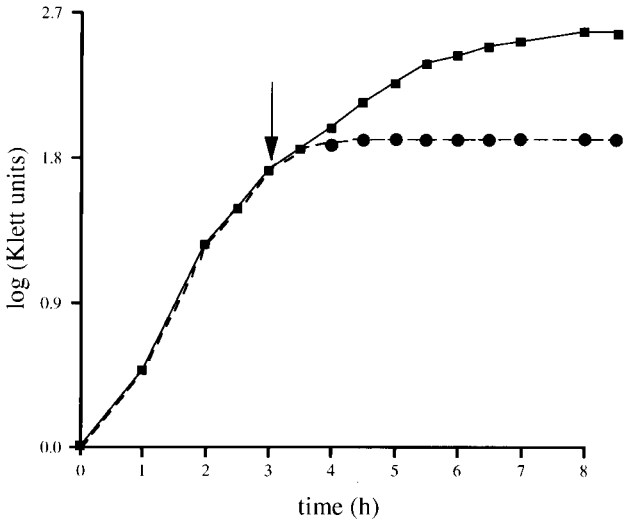

FIG. 3. Toxicity of the *fbpABC* operon for *E. coli* DH5αMCR(pREP4). To demonstrate lethality due to increased expression of the gonococcal *fbpABC* operon in *E. coli*, *E. coli* DH5αMCR(pREP4/pAFbpO) (closed circles) and DH5αMCR(pREP4/pBSKS$^-$) (closed squares) were grown as described in the text. At mid-log phase the cultures were divided and IPTG was added to the culture (indicated by the arrow). Growth was monitored turbidometrically into stationary phase (>5 h).

data) has been readily achieved. Clones expressing even the 5′ one-third of FbpB exhibited growth kinetics analogous to that of the intact *fbpABC* operon in *E. coli*, suggesting that increased expression of the hydrophobic *fbpB* gene product was specifically responsible for the lethality associated with this operon. This toxicity remained associated with *fbpB*, even when only a partial gene product was produced (data not shown).

The functionality of the *fbpABC* operon in iron transport was demonstrated in a fashion similar to that reported for the analogous operons in *Serratia marcescens* (23) and *Haemophilus influenzae* (1). Briefly, this entailed demonstrating that the presence of pAFbpO enabled an *aroB E. coli* strain to grow on nutrient agar containing inhibitory concentrations of the iron chelator 2,2′-dipyridyl. Similar to what has been previously described (1, 23), growth of single microcolonies could be observed (data not shown), indicating that like HitABC and SfuABC, FbpABC could complement the periplasm-to-cytosol transport of iron in an *E. coli* background.

The presence of *fbp* operon homologs in *H. influenzae* and *S. marcescens* suggests that the function of this operon is conserved across species boundaries. For *Neisseria* spp., the presence of *fbpABC* correlates with the ability of a strain to obtain iron from transferrin or lactoferrin (13, 14) and with the ability of these organisms to cause disease (15). These observations underscore the importance of an efficient iron acquisition system for the pathogenesis of bacterial infection.

Nucleotide sequence accession number. The sequence of *fbpABC* described in the report is listed in GenBank under the accession number U33937.

This investigation was supported in part by a grant from the National Institute of Allergy and Infectious Diseases (1R29AI32226-01) to T.A.M.

REFERENCES

1. **Adhikari, P., S. D. Kirby, A. J. Nowalk, K. L. Veraldi, A. B. Schryvers, and T. A. Mietzner.** 1995. Biochemical characterization of a *Haemophilus influenzae* periplasmic iron transport operon. J. Biol. Chem. **270:**25142–25149.
2. **Angerer, A., S. Gaisser, and V. Braun.** 1990. Nucleotide sequences of the *sfuA*, *sfuB*, and *sfuC* genes of *Serratia marcescens* suggest a periplasmic-binding-protein-dependent iron transport mechanism. J. Bacteriol. **172:**572–578.
3. **Anonymous.** 1995. QIAexpress expression system, p. 69. Qiagen product guide. Qiagen, Chatsworth, Calif.
4. **Berish, S. A., C.-Y. Chen, T. A. Mietzner, and S. A. Morse.** 1991. Expression of a functional neisserial Fbp gene in *Escherichia coli*. Mol. Microbiol. **6:** 2607–2615.
5. **Berish, S. A., D. Kapczunski, and S. A. Morse.** 1990. Sequence of the meningococcal Fbp gene. Nucleic Acids Res. **18:**4596.
6. **Berish, S. A., T. A. Mietzner, L. W. Mayer, C. W. Genco, B. P. Holloway, and S. A. Morse.** 1990. Molecular cloning and characterization of the structural gene for the major iron-regulated protein expressed by *Neisseria gonorrhoeae*. J. Exp. Med. **171:**1535–1546.
7. **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.
8. **Brendel, V., and E. N. Trifonov.** 1984. A computer algorithm for testing potential prokaryotic terminators. Nucleic Acids Res. **12:**4411–4427.
9. **Chen, C.-Y., S. A. Berish, S. A. Morse, and T. A. Mietzner.** 1993. The ferric iron-binding protein of pathogenic *Neisseria* spp. functions as a periplasmic transport protein in iron acquisition from human transferrin. Mol. Microbiol. **10:**311–318.
10. **Dassa, E., and M. Hofnung.** 1985. Sequence of gene *malG* in *E. coli* K12: homologies between integral membrane components from binding protein-dependent transport systems. EMBO J. **4:**2287–2293.
11. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
12. **Lee, B., and A. B. Schryvers.** 1988. Specificity of the lactoferrin and transferrin receptors in *Neisseria gonorrhoeae*. Mol. Microbiol. **2:**827–829.
13. **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.
14. **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.
15. **Mietzner, T. A., G. H. Luginbuhl, E. Sandstrom, and S. A. Morse.** 1984. Identification of an iron-regulated 37,000-dalton protein in the cell envelope of *Neisseria gonorrhoeae*. Infect. Immun. **45:**410–416.
16. **Mietzner, T. A., and S. A. Morse.** 1994. The role of iron-binding proteins in the survival of pathogenic bacteria. Annu. Rev. Nutr. **14:**471–493.
17. **Nowalk, A. J., S. B. Tencza, and T. A. Mietzner.** 1994. Coordination of iron by the ferric iron-binding protein of pathogenic *Neisseria* is homologous to the transferrins. Biochemistry **33:**12769–12775.
18. **Persson, B., and P. Argos.** 1994. Prediction of transmembrane segments in proteins utilising multiple sequence alignments. J. Mol. Biol. **237:**182–192.
19. **Petersen, C.** 1991. Multiple determinants of functional mRNA stability: sequence alterations at either end of the *lacZ* gene affect the rate of mRNA inactivation. J. Bacteriol. **173:**2167–2172.
20. **Sanders, J. D., L. D. Cope, and E. J. Hansen.** 1994. Identification of a locus involved in the utilization of iron by *Haemophilus influenzae*. Infect. Immun. **62:**4515–4525.
21. **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.
22. **Zhou, J., and B. G. Spratt.** 1992. Sequence diversity within the *argF*, *fbp* and *rec*A genes of natural isolates of *Neisseria meningitidis*: interspecies recombination within the *arg*F gene. Mol. Microbiol. **6:**2135–2146.
23. **Zimmermann, L., A. Angerer, and V. Braun.** 1989. Mechanistically novel iron(III) transport system in *Serratia marcescens*. J. Bacteriol. **171:**238–243.