

## FbpC Is Not Essential for Iron Acquisition in *Neisseria gonorrhoeae*

SHITE SEBASTIAN AND CAROLINE ATTARDO GENCO\*

*The Maxwell Finland Laboratory for Infectious Diseases, Department of Medicine,  
Boston University School of Medicine, Boston, Massachusetts 02118*

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**The *fbpABC* locus of *Neisseria gonorrhoeae* has been proposed to encode a periplasmic protein-dependent iron transport system. Although the function of the gonococcal FbpA protein has been well characterized and its role as a periplasmic binding protein is well defined, little is known about the function of the FbpB and FbpC proteins. To define the function of the gonococcal FbpC protein, an *N. gonorrhoeae* F62 *fbpC* mutant was constructed by insertional inactivation with the kanamycin gene. The *N. gonorrhoeae* F62 *fbpC* mutant was observed to grow with heme, transferrin, or ferric nitrate as the sole exogenous iron source, indicating that the gonococcal FbpC protein is not absolutely required for growth with these iron sources. In previous studies we were unable to detect *fbpB*- or *fbpC*-specific transcripts by Northern analysis. Reverse transcription-PCR analysis with RNA obtained from *N. gonorrhoeae* F62 grown under iron-replete and -depleted conditions detected *fbpA* and *fbpAB* transcripts but failed to detect *fbpC* or *fbpBC* transcripts. These results indicate that FbpC does not play a pivotal role in iron transport in *N. gonorrhoeae* and suggest that additional ABC transport systems are functional in the gonococcus for the acquisition of iron.**

Microorganisms have developed diverse and elaborate systems to obtain iron, which is present in limited quantities in the human host. Some pathogenic bacteria produce siderophores, which efficiently scavenge ferric iron from the environment and facilitate the growth of the organism (27). The pathogenic *Neisseria* species do not produce siderophores (17, 26) but instead produce an array of distinct iron-regulated outer membrane receptors which specifically interact with different iron binding proteins. These include the transferrin (TF) binding proteins, Tbp1 and Tbp2, the lactoferrin (LF) binding proteins, LbpA and LbpB (2, 5, 14, 24), and the hemoglobin receptors, HmbR (22, 23) and HpuB (6, 7, 15), which facilitate the uptake of heme iron from hemoglobin and hemoglobin-haptoglobin complexes, respectively. Although the binding of iron-containing ligands to these receptors is well characterized, the subsequent steps of iron removal and transport into the bacterial cytoplasm are not well defined. Previous studies have determined that iron is removed from TF and LF in an energy-dependent manner but that neither TF nor LF is internalized. Once the iron is removed from TF, it is bound by the ferric binding protein (FbpA). FbpA functions within the periplasm, shuttling iron from TF through the periplasm and to the cytoplasmic membrane. Our studies indicate that iron from heme interacts with FbpA (8); however, the specificity of this interaction and the fate of iron following its interaction with FbpA are not well defined. *Neisseria meningitidis* and *N. gonorrhoeae* *fbpA* mutants were recently demonstrated to be deficient in the ability to use iron from human TF and LF; however, the use of iron from heme and hemoglobin was unimpaired (12, 25). These results, together with our biochemical data obtained with *N. gonorrhoeae*, suggest that more than one periplasmic binding protein may be used for the transport of iron from heme.

We have previously reported on the cloning and sequencing

of the gonococcal *fbpA* gene (4). DNase footprinting studies indicate that *E. coli* Fur binds to a 42-bp site within the *fbpA* promoter (9). We have also established that the *fbpA* promoter is regulated by Fur and iron in *E. coli* and that the level of transcriptional regulation of *fbpA* in the gonococcus is directly related to the degree of iron restriction. Two open reading frames downstream of the gonococcal *fbpA*, designated *fbpB* and *fbpC*, have been identified (1). The proteins encoded by these loci are homologous to the products of portions of previously described operons expressed by *Haemophilus influenzae* (*hitABC*), *Serratia marcescens* (*sfuABC*), and *Yersinia enterocolitica* (*yfuABC*) (3, 19, 20). The *fbpABC* locus has been proposed to encode for a periplasmic protein-dependent transport system, with FbpA functioning as the periplasmic binding protein, FbpB (511 amino acids) functioning as the hydrophobic membrane protein, and FbpC (352 amino acids) functioning as the cytoplasmic membrane-associated nucleotide binding protein. However, attempts to detect proteins corresponding to FbpB and FbpC in iron-stressed gonococcal membranes or in *Escherichia coli* constructs expressing the *fbpABC* operon have failed. Likewise, in previous studies we were unable to detect *fbpB*- or *fbpC*-specific transcripts by Northern blot analysis (10), and thus the exact role of these proteins in iron transport remains to be defined. In this study, we have constructed and characterized a gonococcal *fbpC* mutant and have further defined the transcription of the *N. gonorrhoeae* *fbpABC* locus.

**Bacterial strains and growth conditions.** *N. gonorrhoeae* F62 (obtained from R. P. Williams, Baylor College of Medicine, Houston, Tex.) was maintained on complex medium (gonococcal base; Difco Laboratories, Detroit, Mich.) agar containing 1% IsoVitaleX (BBL, Baltimore, Md.) and grown aerobically under 5% CO<sub>2</sub> at 37°C. Broth cultures were grown in chemically defined medium (CDM) (16) supplemented with 4.2% NaHCO<sub>3</sub>. All glassware was washed with 10% nitric acid and thoroughly rinsed in deionized water to remove residual iron. Cold heme (Na plus K salt) was purchased from Porphyrin Products Inc. (Salt Lake City, Utah), and apotransferrin and ferric nitrate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Cold heme was dissolved in distilled H<sub>2</sub>O and

\* Corresponding author. Mailing address: The Maxwell Finland Laboratory for Infectious Diseases, Boston University School of Medicine, Department of Medicine, Boston, MA 02118. Phone: (617) 414-5282. Fax: (617) 414-5280. E-mail: caroline.genco@bmc.org.

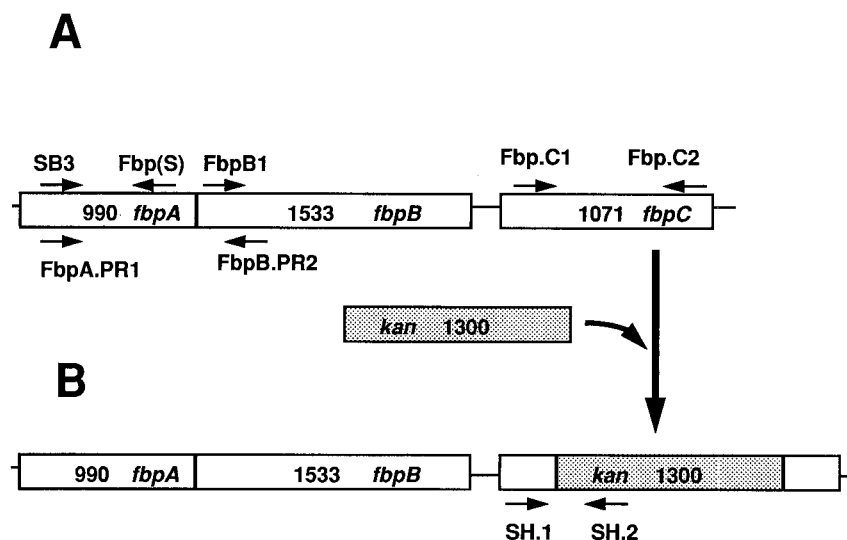


FIG. 1. Schematic diagram of the *Neisseria fbpABC* operon. (A) Positions of the oligonucleotide primers used in RT-PCR. (B) Positions of the oligonucleotide primers used to confirm the presence of the kanamycin cassette (indicated by shading) in the recombinant plasmid construct and the *fbpC* mutant *N. gonorrhoeae* F62C.1.

prepared fresh for each experiment. *N. gonorrhoeae* F62 was grown in CDM plus 25  $\mu$ M Desferal (Ciba-Giegy) (CDM/25D) for 3 h aerobically at 37°C, washed, and resuspended in CDM/25D. This culture served as the inoculum into fresh CDM/25D with a starting absorbance at 660 nm ( $A_{660}$ ) of 0.06. To this was added either iron-loaded human TF (5  $\mu$ M, 30% iron saturated), ferric nitrate (10  $\mu$ M), or hemin (4  $\mu$ M). Growth was monitored by measuring the  $A_{660}$  hourly for 5 h.

**Construction and characterization of a gonococcal *fbpC* mutant.** Plasmid pAFbpO, which contains a 3.6-kb DNA fragment corresponding to the *fbpABC* locus ligated into the *EcoRV* and *BamHI* sites of pBSKS (1) was used to construct an *fbpC* mutant. To disrupt the *fbpC* gene, a 600-bp *BstEII-AccI* internal fragment of the *fbpC* gene was deleted and the linearized plasmid was blunt ended with T4 DNA polymerase and subsequently dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim) to reduce the religation background. A 1.3-kb *EcoRI* fragment encompassing the Tn903 kanamycin cassette was excised from pUC4K-KSAC (18) and blunt ended with T4 DNA polymerase. The kanamycin cas-

sette was ligated with the linearized pAFbpO $\Delta$ C vector in the presence of T4 DNA ligase overnight at 14°C, and the ligation mix was used to transform high-efficiency JM109 cells (Promega Corp., Madison, Wis.). Transformants were selected on Luria-Bertani agar plates containing kanamycin (50  $\mu$ g/ml).

Three transformants were obtained, and one was used in further studies. Plasmid DNA was isolated from this transformant (designated pRSC.1), and the presence of the *kan* gene in the pRSC.1 construct was confirmed by the ability of *PstI* (unique restriction enzyme flanking the *kan* gene) to excise a 1.3-kb fragment corresponding to the kanamycin cassette. *PstI* digestion was also performed with pUC4K-KSAC (positive control) and pAFbpO (negative control) to confirm the presence of the kanamycin cassette in the plasmid construct pRSC.1 (data not shown). The presence of the kanamycin cassette in the *fbpC* gene was also confirmed by amplification and sequencing of the *fbpC-kan* junction (data not shown) with a *fbpC* gene-specific forward primer (SH.1) (Fig. 1B and Table 1) and a *kan* gene-specific reverse primer (SH.2) (Fig. 1B). After confirmation of the disruption of the *fbpC* gene in *E. coli*,

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence	Product
SH.1	5' AAATACCAACCTTCCCGTCC 3'	281-bp <i>fbpC-kan</i> junction
SH.2	5' CGATAGATTGTCGCACCTGA 3'	
SB.3 FBP(S)	5' CAGACATTACCGTGTACAACGGCCAAC 3' 5' TTATTCATACCGGCTTGCTCAAGC 3'	933-bp <i>fbpA</i> gene
FbpA.PR.1 FbpB.PR.2	5' AAAAGGATCCCGATATGAAAACATCTATCCGA 3' 5' CGGGATCCGAGGCCGGTAAGCCAAATGGTAT 3'	1.1-kb <i>fbpAB</i> gene
FbpB1 Fbp.C2	5' CGATGAAAAACACTATGTCTCCTAA 3' 5' ATAAATATCCGCAGGCTTGTTGGATG 3'	2.5-kb <i>fbpBC</i> gene
Fbp.C1 and Fbp.C2	5' TTGAGGAAGCACCGCTATGACCGCC 3'	1.1-kb <i>fbpC</i> gene
RMP F1 RMP R1	5' CATGTTTCTACAGCGGCTG 3' 5' CGGCAAGATATTACCTAGCCT 3'	800-bp <i>rmp</i> gene

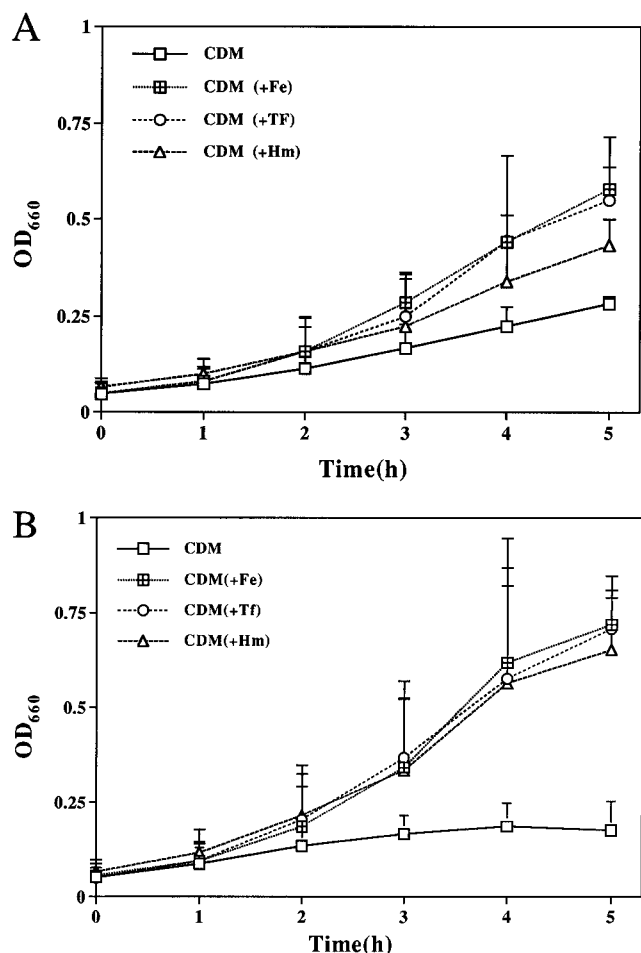


FIG. 2. Growth of *N. gonorrhoeae* F62 (A) and *N. gonorrhoeae* F62C.1 (B). *N. gonorrhoeae* was grown in CDM/25D for 3 h, washed, and resuspended in CDM/25D. This culture served as the inoculum into fresh CDM/25D with a starting  $A_{660}$  of 0.06. To this was added either iron-loaded human TF (5  $\mu$ M, 30% iron saturated), ferric nitrate (Fe, 10  $\mu$ M), or hemin (Hm, 4  $\mu$ M). Cultures without added iron served as negative controls (-Fe). Growth was monitored hourly by  $A_{660}$  for 5 h. Results are from one experiment and are representative of three separate experiments. Mean values are plotted, and the standard deviations are included for each datum point.  $OD_{660}$ , optical density at 660 nm (equivalent to  $A_{660}$ ).

pRSC.1 was used to transform pilated *N. gonorrhoeae* F62 (21) and transformants were selected on gonococcal base medium supplemented with kanamycin (50  $\mu$ g/ml). Inactivation of the gonococcal chromosomal *fbpC* gene in the resulting transformants was confirmed by PCR with primers SH.1 and SH.2 (Fig. 1B). The expected DNA fragment of 281 bp was amplified from *N. gonorrhoeae* F62C.1 as well as from the plasmid construct pRSC.1 (positive control) (data not shown). We did not observe a PCR product with wild-type *N. gonorrhoeae* F62 chromosomal DNA as a template (data not shown). Sequencing of the 281-bp PCR product with primer SH.1 or SH.2 individually confirmed that proper allelic exchange had occurred (data not shown). To examine the ability of the *fbpC* mutant to grow with various iron sources, broth cultures of *N. gonorrhoeae* F62C.1 and wild-type *N. gonorrhoeae* F62 were grown in CDM/25D supplemented with hemin, TF, or ferric nitrate. As shown in Fig. 2B, *N. gonorrhoeae* F62C.1 was capable of growth with heme, TF, or ferric nitrate as the sole exogenous iron source. Although the gonococcal *fbpC* mutant exhibited slightly better growth with the different iron sources,

these differences were not significant compared to the wild-type strain as determined by the Student *t* test with two-tailed analysis, ( $P > 0.1$ ) (InStat 2.00; Graph Pad Software, Paul Stannard Soft Engine). These results indicate that gonococcal FbpC protein is not absolutely required for growth with these iron sources.

**Analysis of *fbpABC* transcription.** In previous studies we demonstrated by Northern blot analysis that the gonococcal *fbpA* gene is transcribed as a monocistronic mRNA independently of *fbpB* and *fbpC* (10). In this same study we could not detect *fbpB*- or *fbpC*-specific polycistronic or monocistronic transcripts by Northern blot analysis. To precisely define the mechanism of regulation of *fbpABC*, we examined transcription of the *fbpABC* locus by reverse transcription-PCR (RT-PCR) analysis. RT-PCR amplification was performed with the Titian one-tube RT-PCR system (Boehringer Mannheim) as specified by the manufacturer. To eliminate DNA contamination, RNA samples were treated with RNase-free DNase (Promega Corp., Madison, Wis.) and an RT-PCR negative control experiment with DNase-treated RNA samples was performed in parallel with the RT-PCR (Fig. 3, lane 9). RT-PCR analysis was performed with several sets of primers (Table 1 and Fig. 1) and with RNA isolated from *N. gonorrhoeae* F62 grown under iron-replete (CDM with 50  $\mu$ M ferric nitrate) and iron-depleted (CDM with 20  $\mu$ M Desferal) conditions. Constitutive expression of the gonococcal *rmp* gene (11) was confirmed by RT-PCR and used as a positive control (Fig. 3, lane 8).

As predicted, a 900-bp *fbpA*-specific transcript was amplified with *fbpA*-specific oligonucleotides SB3 (forward primer) and FbpS (reverse primer). We also amplified a 1.1-kb *fbpAB* transcript, encompassing most of the *fbpA* gene and the 5' region of the *fbpB* gene, with the *fbpA*-specific primer FbpA.PR1 (F.P) and the *fbpB*-specific primer FbpB.PR2 (R.P) (Table 1). Both the *fbpA* and *fbpAB* transcripts were detected only in RNA obtained from gonococci grown under iron-depleted conditions (Fig. 3). No *fbpC*- or *fbpBC*-specific transcript was detected in RNA samples from *N. gonorrhoeae* cultures grown under iron-replete or -depleted conditions (Fig. 3 and data not

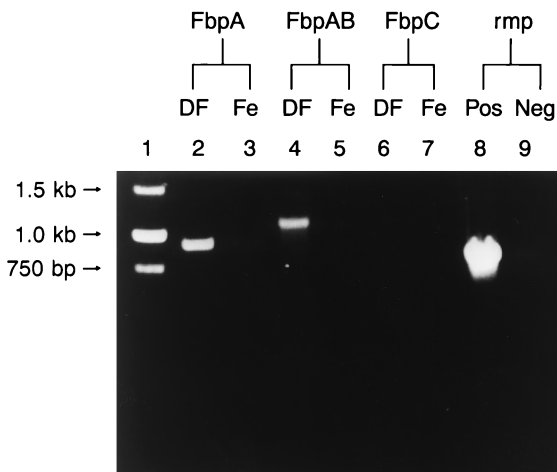


FIG. 3. RT-PCR analysis for detection of *fbpA*, *fbpAB*, and *fbpC* transcripts in *N. gonorrhoeae* grown under iron-depleted and -replete conditions. Lanes: 1, DNA molecular size standards; 2, 4, 6, and 8, RNA obtained from *N. gonorrhoeae* under iron-depleted conditions; 3, 5, 7, and 9, RNA grown under iron-replete conditions; 2 and 3 RT-PCR with primer pairs SB3 and FBP(S) specific for *fbpA*; 4 and 5, RT-PCR with primer pairs FbpA.PR1 and FbpB.PR2 to amplify *fbpA* and the 5' end of *fbpB*; 6 and 7, RT-PCR with primer pairs Fbp.C1 and Fbp.C2 to amplify *fbpC*; 8, RT-PCR with primer pairs RMP F1 and RMP R1 to amplify the *rmp* gene (positive control); 9, DNA control.



shown). The absence of *fbpC* and *fbpBC* transcripts under both iron-depleted and -replete conditions indicates that *fbpC*-specific RNA is not transcribed, or, alternatively, that if *fbpC*-specific RNA is transcribed, it may be highly unstable so that it is not detected by either Northern blot or RT-PCR analysis. From these results, we conclude that only the *fbpA* and *fbpB* genes are cotranscribed in *N. gonorrhoeae*.

**Concluding remarks.** The gonococcal *fbpC* gene has been predicted to encode a cytoplasmic membrane-associated nucleotide binding protein which functions together with FbpA in the transport of iron into the cell (1). However, the exact role of the gonococcal FbpC in iron transport has not been previously defined. Characterization of the gonococcal *fbpC* mutant described here indicates that the gonococcal FbpC is not essential for iron transport from TF, heme, or inorganic iron (ferric nitrate). Our studies also indicate that the gonococcal *fbpABC* locus is not a functional equivalent of the ABC transport system described in *H. influenzae* (*hitABC*) (19). Previous studies with *H. influenzae* *hitA* and *hitC* mutants have demonstrated that the absence of either the *hitA* or *hitC* gene abolishes the function of the ABC transport system (13, 19). *N. meningitidis* *fbpA* and *N. gonorrhoeae* *fbpA* mutants were recently shown to be unable to use iron supplied from human TF, LF, or inorganic iron, supporting the pivotal role of FbpA in iron transport (12, 25). The observed phenotype of the meningococcal *fbpA* mutant could not be attributed to a specific gene of the operon, since a polar effect on downstream *fbpB* and *fbpC* genes was not ruled out by complementation analysis studies. However, characterization of a specific *N. gonorrhoeae* *fbpC* mutant, as described in the present study, indicates that FbpC is not essential for transport of iron in the gonococcus.

In this study, using RT-PCR of RNA obtained from *N. gonorrhoeae* F62 grown under iron-depleted conditions, we demonstrated that the *fbpA* and *fbpB* genes are cotranscribed. The inability to detect the *fbpAB* transcript in our previous studies (10) may be due to the instability of the *fbpAB* transcript such that the *fbpAB* transcript is rapidly degraded and not detected by Northern blot analysis. Sequencing data has confirmed the presence of a strong stem-loop structure which precedes the *fbpA* stop codon and may function to stabilize the *fbpA* transcript (1). Such a structure is absent in the *fbpB* gene. We were also unable to detect *fbpC* or *fbpBC* transcripts in RNA obtained from gonococcal cultures grown under iron-depleted or -replete conditions, indicating that these transcripts are either highly unstable or not transcribed at detectable levels.

Sequence analysis of the *H. influenzae* *hitBC* (19), *S. marcescens* *sfuBC* (3), and *Y. enterocolitica* *yfuBC* (20) genes suggests a potential for translational regulation of the *C* gene. In these systems, the translational start of the downstream *C* gene either overlaps or precedes the translational stop of the previous open reading frame (*B* gene), thus allowing an efficient translation of the downstream genes. In contrast, the translational start of the *N. gonorrhoeae* *fbpC* gene is 22 bp downstream of the translational stop of *fbpB*. These observations suggest that in the unlikely event that the *fbpC* transcript is produced, *fbpC* may not be efficiently translated. Previous attempts by other investigators to isolate FbpC proteins in iron-stressed gonococcal membrane or soluble extracts or in *E. coli* constructs expressing the *fbpABC* operon have been unsuccessful (1). Based on these observations, as well as on our characterization of the gonococcal *fbpC* mutant and our transcriptional analysis of the *fbpABC* locus, we propose that *N. gonorrhoeae* *fbpC* is a cryptic gene and that alternative ATPases may function together with the periplasmic binding protein FbpA in the transport of iron.

In summary, we have constructed a gonococcal *fbpC* mutant and have demonstrated that FbpC does not play a pivotal role in iron transport in *N. gonorrhoeae*. Our studies indicate that the gonococcal *fbpABC* locus is not a functional equivalent of the ABC transporter described in *H. influenzae* and suggest that other ABC systems may be functional in the gonococcus for the acquisition of iron.

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