# The Gonococcal Fur Regulon: Identification of Additional Genes Involved in Major Catabolic, Recombination, and Secretory Pathways

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In this study, we have characterized the in vitro binding of *Neisseria gonorrhoeae* Fur to several well-defined iron transport genes, as well as to additional genes involved in major catabolic, secretory, and recombination pathways of gonococci. The gonococcal Fur protein was recombinantly expressed in *Escherichia coli* HBMV119. Fur was isolated from inclusion bodies and partially purified by ion-exchange chromatography. Gonococcal Fur was found to bind to the promoter/operator region of a gene encoding the previously identified Fur-regulated periplasmic binding protein (FbpA) in a metal ion-dependent fashion, demonstrating that purified Fur is functional. In silico analysis of the partially completed gonococcal genome (FA1090) identified Fur boxes in the promoters of several genes, including *tonB*, *fur*, *recN*, *secY*, *sodB*, *hemO*, *hmbR*, *fumC*, a hypothetical gene (Fe-S homolog), and the *opa* family of genes. By using purified gonococcal Fur, we demonstrate binding to the operator regions of *tonB*, *fur*, *recN*, *secY*, *sodB*, *hemO*, *hmbR*, *fumC*, the Fe-S homolog gene, and the *opa* gene family as determined by an electrophoretic mobility shift assay. While gonococcal Fur was demonstrated to bind to the promoter regions of all 11 *opa* genes (*opaA* through -*K*), we did not detect binding of purified *E. coli* Fur with 8 of the 11 *opa* members, indicating that target DNA sequence specificities between these two closely related proteins exist. Furthermore, we observed differences in the relative strengths of binding of gonococcal Fur for these different genes, which most likely reflect a difference in affinity between gonococcal Fur and its DNA targets. This is the first report that definitively demonstrates the binding of gonococcal Fur to its own promoter/operator region, as well as to the *opa* family of genes that encode surface proteins. Our results demonstrate that the gonococcal Fur protein binds to the regulatory regions of a broad array of genes and indicates that the gonococcal Fur regulon is larger than originally proposed.

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In several gram-negative pathogens, the ferric uptake regulator protein (Fur) functions as a general global regulator and controls the expression of not only genes required for iron transport but also genes required for virulence. The virulence-associated genes include those for the *Shigella* Shiga toxin, *Escherichia coli* hemolysin, the fimbriae of enterotoxigenic *E. coli*, and exotoxin A of *Pseudomonas aeruginosa* (8, 19, 21, 27, 41). Fur has also been demonstrated to repress the transcription of genes induced in response to oxidative stress, including the *E. coli* and *P. aeruginosa sodA* (superoxide dismutase) genes (13, 35). Furthermore, an *E. coli fur* mutant was shown to be sensitive to hydrogen peroxide and showed increased oxidative DNA damage (38). The *P. aeruginosa* Fur protein has been demonstrated to control the expression of other regulatory genes that encode transcriptional activators, which, in turn, regulate the expression of the *toxA* gene (1, 24, 27, 40, 41). In some organisms, Fur may also act as a positive regulator in controlling gene expression. In salmonellae, expression of the acid tolerance response is controlled positively by Fur (11), as is the expression of *E. coli sodB* (6). A recent study has shown that Fur enhances the expression of urease in entero-

hemorrhagic *E. coli*, thus contributing to the acid tolerance of the organism, similar to *Salmonella enterica* serovar Typhimurium (14). Bijlsma et al. have also demonstrated that Fur is involved in the urease-independent acid resistance of *Helicobacter pylori* (4).

Characterization of *fur* mutants isolated by a manganese selection procedure in several gram-negative organisms has also identified both Fur-repressed and Fur-activated genes (12, 21, 30). A Fur titration assay has also been utilized for the detection of novel Fur-regulated promoters in salmonellae and *E. coli* (2, 31, 39). The systematic evolution of ligands by exponential enrichment assay has been used to isolate novel Fur-dependent genes in *P. aeruginosa*. Expression of these new Fur-regulated *Pseudomonas* genes was derepressed under iron-depleted conditions, and this derepression ranged from highly stringent control to only partial control (25).

A Fur homolog has been identified in *Neisseria gonorrhoeae* (3). Although *Neisseria* genes involved in iron transport have been proposed to be regulated by Fur, binding of gonococcal Fur to the promoters of these genes has not been demonstrated. Furthermore, the gonococcal Fur regulon is not well defined; the only genes that appear to be regulated by Fur are those involved in iron transport. The characterization of a missense mutant of *N. gonorrhoeae fur* isolated following manganese selection demonstrated an alteration of the regulation of a broad range of genes (37), supporting the existence of a

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| Strain or plasmid | Genotype or phenotype | Source or reference |
|---|---|---|
| *N. gonorrhoeae* | | |
| F62 | Wild type | Laboratory collection |
| FA1090 | Wild type | Laboratory collection |
| *E. coli* | | |
| QC1732 | *fur::kan* | 36 |
| HBMV 119 | QC1732/λDE3 | 2 |
| Plasmids | | |
| pET11C | Amp$^r$ | Novagen |
| pKASH.1 | Amp$^r$ | This study |

large set of genes that may be under Fur control. Nine iron-regulated genes have been identified in *N. gonorrhoeae*, all of which are involved in iron transport. Although these gonococcal genes have been proposed to be regulated by Fur, the Fur-dependent iron regulation of these proteins has been difficult to test directly because of the inability to isolate a gonococcal *fur* null mutant.

Previous studies in our laboratory have demonstrated that *E. coli* Fur binds to a 42-bp site within the gonococcal *fbpA* (ferric binding protein A) promoter (5). We have also shown 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. In order to determine additional components of the gonococcal Fur regulon, we used a 160-bp gonococcal *fbpA* promoter/operator fragment that has been shown to encompass the Fur box by footprinting analysis with *E. coli* Fur (5) and conducted an in silico search of the gonococcal genome for Fur box-like sequences. Our analysis identified additional gonococcal genes involved in major catabolic, secretory, and recombination pathways that contained putative iron boxes in their promoter regions. By using purified gonococcal Fur, we demonstrated binding to the promoter regions of several of these genes, including the genes that encode the gonococcal Opa (opacity) proteins, a family of antigenic and phase-variable outer membrane proteins known to promote *Neisseria* adherence to and invasion of epithelial and endothelial cells, as well as professional phagocytes. The in vitro analysis reported here demonstrates that the gonococcal Fur protein binds to the regulatory regions of the *fur*, *tonB*, and *opa* genes differentially and has a role similar to that of a global regulatory protein.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and medium.** The *N. gonorrhoeae* and *E. coli* strains and plasmids used in this study are indicated in Table 1. *E. coli* strains were grown on Luria-Bertani agar (Sigma, St. Louis, Mo.) or broth at 37°C. When necessary, ampicillin (100 μg/ml) was added to the medium.

**Nucleic acids.** Double-stranded plasmid DNA was prepared by using the Qiaprep miniprep system (Qiagen, Valencia, Calif.). Restriction endonucleases, the Klenow fragment of DNA polymerase, and *Taq* DNA polymerase (Gibco BRL, Grand Island, N.Y.) were used in accordance with the manufacturer's specifications. Restriction endonuclease fragments were separated by electrophoresis in 1% agarose gels in Tris-acetate-EDTA buffer.

**Expression and purification of gonococcal Fur.** Oligonucleotide primers (Table 2) F1 and R1 were designed to incorporate an *Nde*I restriction site at the 5′ end and a *Bam*HI site at the 3′ end of the gene during the amplification of gonococcal *fur* from *N. gonorrhoeae* F62 (restriction sites are underlined in Table 2). The PCR-amplified fragment was cloned into the *Nde*I/*Bam*HI site of ex-

pression vector pET11c (Novagen, San Diego, Calif.) to generate pKASH.1 (Table 1). The *fur* gene in pKASH.1 is under the control of a T7 promoter. The pKASH.1 construct was transformed into *E. coli* expression strain HBMV119 (Table 1). The overnight cultures of these transformants were used to inoculate 1 liter of fresh Luria-Bertani broth supplemented with ampicillin (100 μg/ml). At an optical density at 600 nm of 0.4, the cells were induced to express Fur by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.) and growth continued at 39°C. Following induction, cells were harvested by centrifugation and resuspended in 20 ml of chilled STET buffer (50 mM Tris-Cl, 0.5 M EDTA, 8% sucrose, 5% Triton X-100). The cells were lysed by the addition of lysozyme (1 mg/ml), followed by sonication. The extract was then clarified by centrifugation at 13,000 rpm in a Sorvall RC5C Plus centrifuge for 25 min. The resulting pellet was enriched in the inclusion body fraction. The gonococcal Fur protein was purified from the inclusion body pellet by dissolving the pellet in 6 M urea in phosphate buffer (pH 7.5) at room temperature (RT) and then centrifuging it. The resulting supernatant, consisting of the solubilized inclusion body fraction, was dialyzed against refolding buffer (50 mM Tris-Cl, 50 mM NaCl, 5 mM reduced glutathione, 1 mM oxidized glutathione) supplemented with 500 μM manganese (Mn$^{2+}$) to renature the protein. The sample was further dialyzed against 10 mM phosphate buffer (pH 7.5) supplemented with 100 μM Mn$^{2+}$. The dialyzed sample was purified on an DEAE-Sepharose ion-exchange column equilibrated with 10 mM phosphate buffer, pH 7.5. The column was developed with a 0 to 0.5 M linear NaCl gradient in phosphate buffer. Fractions were collected, and then a protease inhibitor cocktail (Sigma) was immediately added. The presence of gonococcal Fur was confirmed by N-terminal sequencing. Rabbit polyclonal antibodies against recombinant gonococcal Fur were prepared in accordance with standard protocols (Lampire Biological Laboratories, Pipersville, Pa.) and used for immunoblot analysis.

**Gel electrophoresis and immunoblot analysis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (20), with 12% polyacrylamide gels. Proteins were either stained with Coomassie brilliant blue R-250 (Bio-Rad, Hercules, Calif.) or electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad) for immunoblot analysis. Immunoblots were probed with either *P. aeruginosa* anti-Fur serum (1:1,000) or *N. gonorrhoeae* F62 anti-Fur serum (1:1,000), and then goat anti-rabbit immunoglobulin G antibody (1:10,000; Sigma) was added. Immunoreactive proteins were detected by the addition of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate substrate in accordance with the directions of the manufacturer (Sigma).

**EMSA.** The different promoter fragments for electrophoretic mobility shift assays (EMSA) were PCR amplified from *N. gonorrhoeae* FA1090, with the exception of *fur* and *tonB*, which were amplified from *N. gonorrhoeae* F62 with the oligonucleotides shown in Table 2 (restriction sites are underlined). Restriction-digested and gel-purified DNA fragments were end labeled by a Klenow fill-in reaction with [α-$^{32}$P]dATP and a nucleoside triphosphate mixture (dCTP, dTTP, and dGTP). For the *opa* probes used (see Fig. 8), 100-bp forward and reverse oligonucleotides specific for each *opa* member were generated (see Fig. 7), annealed, and similarly labeled. Unincorporated nucleotides were removed with MicroSpin G-25 columns (Amersham Pharmacia Biotech, Piscataway, N.J.). The fragments were diluted to a final concentration of 0.1 nM and incubated with the indicated amounts of *E. coli* or *N. gonorrhoeae* Fur protein in binding buffer. Where indicated, competitor DNA (a 50- to 1,200-fold excess) was added to the reaction mixture. Samples were incubated at RT for 20 min, and the reaction mixture was loaded immediately onto a native 6% polyacrylamide gel (acrylamide/bisacrylamide ratio, 30:1 [wt/wt]). The gel was subjected to electrophoresis for 2 h at RT and 100 V, placed on filter paper, dried for 2 h at 80°C, and exposed overnight with an intensifying screen at −70°C. Radioactive bands were visualized by autoradiography. To quantitate binding, the gels were exposed to a PhosphorImager screen (Molecular Dynamics) and band intensity was measured with a Phosphorimager. Sigma Plot software was then used, data were graphed, and the $K_d$ values of gonococcal Fur for the *tonB*, *fur*, and *opa* promoters were determined.

## RESULTS

**Overexpression, purification, and functionality of gonococcal Fur.** To study the interaction of gonococcal Fur with the operator regions of gonococcal genes, we overexpressed and purified gonococcal Fur by using an *E. coli* expression strain (HBMV119). *E. coli* strain HBMV119 is a *fur* mutant (QC1732) that was infected with lambda DE3 to create an

TABLE 2. Primers used in this study

| Primer[a] | Description |
| --- | --- |
| F1: ACGTACGTCATATGGAAAAATTCAGCAACATTGCGAA................Amplifies 432-bp gonococcal Fur ORF[b] | |
| R1: ACGTGGATCCTTAACGTTTGCCCTTGGCCTGACAGTC | |
| | |
| F2: CGGGATCCACAGGCGAGGATAGGGTTTGT.......................Amplifies 120-bp promoter fragment of gonococcal *fur* | |
| R2: CCAAGCTTGACAGGGTAAAATACCGCTTA | |
| | |
| F3: CGGGATCCGAATTCCTATCCGATTTGCCG.......................Amplifies 100-bp promoter fragment of gonococcal *rmp* | |
| R3: CCAAGCTTTTATTCCCTCATTAGATTTGTA | |
| | |
| F4: CGGGATCCCACGGTTTGCCTATGAATGG........................Amplifies 100-bp promoter fragment of gonococcal *tonB* | |
| R4: CCGGATCCGGGCATAATAATAGCAACAATTCC | |
| | |
| F5: CGGATCCGTTCCCTTTGAGCCGGGG.........................Amplifies 100-bp promoter fragment of gonococcal *opa* multigene family | |
| R5: CGGATCCCGGCTCCTTATTCGGTTTGACC | |
| | |
| F6: CGGGATCCCGGTAAGGGATAAGGATGCGG ....................Amplifies 132-bp promoter fragment of gonococcal *hemO* | |
| R6: CCGGATCCCCCCGCTTAAAAATATAACGC | |
| | |
| F7: CGGGATCCCAACTCAAGATGTAATGGATTG....................Amplifies 103-bp promoter fragment of gonococcal *hmbR* | |
| R7: CGGGATCCCACAGATTTATTTGTACGGG | |
| | |
| F8: CGGGATCCGGCAGAGTCGGCATCCTGAATG....................Amplifies 175-bp promoter fragment of hypothetical gonococcal Fe-S gene | |
| R8: CCGGATCCTACTTTAAGTAATTGACCGTTGC | |
| | |
| F9: CGGGATCCGAATCGGCGGCTCACGCATTC.....................Amplifies 184-bp promoter fragment of gonococcal *sod* | |
| R9: CCGGATCCCCGCCTCCATCGAATGCGGAG | |
| | |
| F10: CGGGATCCCTTTTCAGACGGCATTTTT.......................Amplifies 109-bp promoter fragment of gonococcal *fumC* | |
| R10: CCAAGCTTCGTTTCTCCTTTTGAAATGT | |
| | |
| F11: CGGGATCCCGCCTACGGATGAAGAAGAGC...................Amplifies 219-bp promoter fragment of gonococcal *recN* | |
| R11: CCAAGCTTGGAATTCTACCGACAAAAGCAA | |
| | |
| F12: CCGGATCCCGTGTTGGTGGTAAGATTGAAA .................Amplifies 193-bp promoter fragment of gonococcal *secY* | |
| R12: CCAAGCTTGGCGCTTTCGTATAATTTAGC | |

[a] F, forward; R, reverse. Restriction sites are underlined.
[b] ORF, open reading frame.

expression strain (Table 1). Expression of the gonococcal Fur protein was examined by SDS-PAGE. As shown in Fig. 1A, a 16-kDa protein representing gonococcal Fur was overexpressed by using the T7 expression system and subsequently purified from the inclusion body pellet by dissolving the pellet in 6 M urea in phosphate buffer. The solubilized inclusion body fraction was dialyzed against refolding buffer supplemented with manganese (100 μM) to renature the protein, and the dialyzed sample was purified by ion-exchange chromatography. Figure 1B shows the $A_{280}$ profile of fractions obtained by 0 to 0.5 M NaCl gradient elution. As shown in Fig. 1C, most of the gonococcal Fur protein was found in fractions 15 and 16. Western blot analysis with polyclonal anti-*P. aeruginosa* Fur sera (data not shown) and anti-*N. gonorrhoeae* Fur sera (Fig. 1D) confirmed that the purified protein with an apparent molecular mass of 16 kDa was indeed gonococcal Fur. N-terminal sequencing confirmed the identity of the first eight amino acids of gonococcal Fur (Fig. 1E).

Anti-*N. gonorrhoeae* Fur sera cross-reacted weakly with the *E. coli* Fur protein (Fig. 1D), indicating that *N. gonorrhoeae* Fur harbors at least one immunodominant domain that differs from that in *E. coli* Fur. Gonococcal Fur lacks the potential metal binding motif present in the C-terminal portion of *E. coli* Fur (HCX$_4$-C-X$_4$HXH) (15). Western blot results shown here

are in agreement with similar observations obtained with anti-*P. aeruginosa* Fur sera (24) and *E. coli* Fur.

To demonstrate the functionality of a partially purified repressor, we examined the interaction of gonococcal Fur with the gonococcal *fbpA* promoter/operator (5), a well-established *fur*-regulated gene, by EMSA. Following incubation of gonococcal Fur with the *fbpA* probe, we detected a shift in the DNA probe (Fig. 2). These results demonstrated that the recombinant gonococcal Fur protein was able to bind to the *fbpA* probe. To define the role of divalent metal ions in the activity of gonococcal Fur, we preincubated gonococcal Fur with either 1 mM EDTA or 1 mM 2,2′-dipyridyl and then examined the ability of the repressor to bind to the *fbpA* promoter by EMSA. As expected, preincubation with either of these chelators resulted in a lack of shifting of the *fbpA* probe (Fig. 2). In the same set of experiments, the mobility of an internal fragment of the gonococcal *fur* gene was not altered (Fig. 2B). These results demonstrate that the partially purified gonococcal Fur protein was functional and that binding of the repressor to the *fbpA* promoter was metal ion dependent and specific.

**Identification of gonococcal contigs containing putative Fur boxes in their promoters.** Potential Fur boxes that have previously been identified by in silico analysis in neisseriae include those in the *fur*, *tbpB*, *lbpB*, *fbpA*, *frpC*, *hmbR*, and *fetA* gene
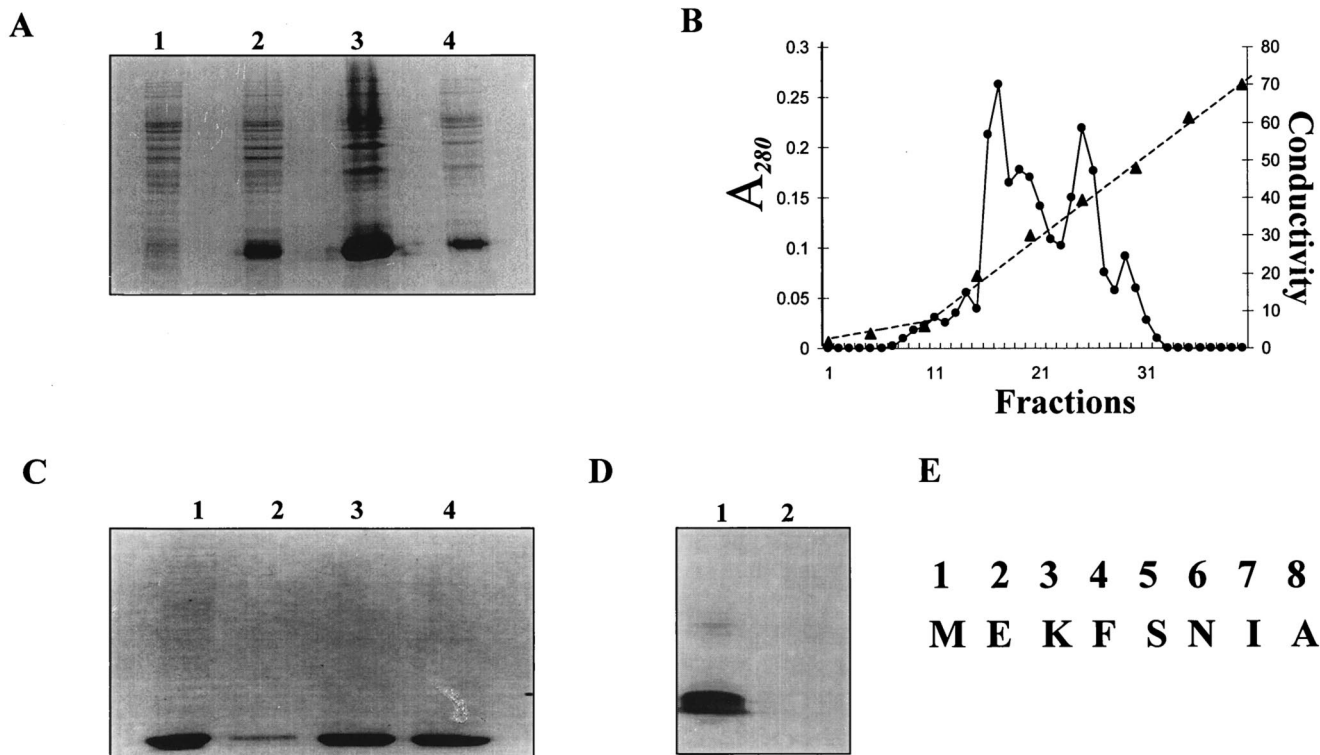
FIG. 1. Expression of gonococcal Fur in *E. coli* HBMV119. (A) SDS-PAGE analysis of recombinant Fur. Lanes: 1, before IPTG addition; 2, 2 h after IPTG addition; 3, inclusion body fraction; 4, supernatant fraction. (B and C) Purification of gonococcal Fur by ion-exchange chromatography. (B) $A_{280}$ (●) of fractions obtained from the NaCl gradient (▲). (C) SDS-PAGE analysis of fractions 14 (lane 2), 15 (lane 3), and 16 (lane 4). Lane 1, inclusion body fraction used for purification. (D) Western blot analysis. The *E. coli* and gonococcal Fur proteins (5 μg) were subjected to SDS-PAGE, transferred to polyvinylidene difluoride, and probed with *N. gonorrhoeae* Fur antiserum. Lanes: 1, *N. gonorrhoeae* Fur; 2, *E. coli* Fur. (E) N-terminal sequence of gonococcal Fur.

promoter regions (9). Alignment of these promoters with the ClustalW multiple-sequence alignment program yields a putative *Neisseria* Fur box consensus sequence that has ~80% homology with the *E. coli* Fur box consensus binding sequence (9). An initial analysis of the *N. gonorrhoeae* FA1090 and *N. meningitidis* MC58 genome databases with the *Neisseria* Fur box consensus sequence in untranslated regions within 200 bp upstream of an open reading frame revealed matches to several gene promoters. Some of these were upstream of the previously identified iron transport genes. However, other regions were identified in the promoter regions of genes that have not previously been reported to be iron or Fur regulated in gonococci (data not shown). Recognizing that this analysis could provide new information on the gonococcal Fur regulon, we reexamined the meningococcal MC58 and gonococcal FA1090 databases by using a 160-bp sequence comprising the *fbpA* Fur binding sequence as defined by DNase I footprinting with *E. coli* Fur (5). This analysis identified several gonococcal and meningococcal genes that contain putative Fur boxes in their promoter regions and included genes encoding well-defined gonococcal virulence factors (Table 3). Our analysis revealed several genes that we anticipated could contain the Fur box sequence in the promoter/operator region. This included the *tonB* gene, which encodes a cytoplasmic membrane protein that, in association with the ExbB and ExbD proteins, provides energy for the transport of iron from host iron binding recep-

tors across the outer membrane into the periplasmic space in a number of gram-negative organisms. The Fur box in the *tonB* promoter is 61% identical to the gonococcal Fur consensus binding sequence and overlaps the −10 region of the promoter (Table 3; Fig. 3A). The presence of a Fur box-like sequence 40 bp upstream of the *tonB* start codon has previously been suggested (33). A Fur binding sequence located in the promoter region of the gonococcal *fur* gene, and overlapping the −10 region of the *fur* promoter (Table 3; Fig. 3A), was also iden-
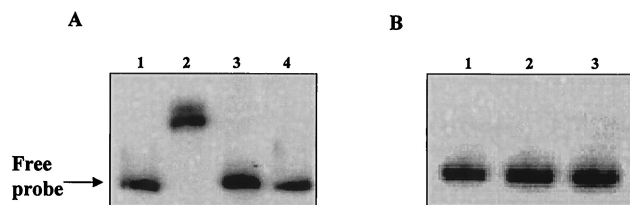


FIG. 2. EMSA analysis of gonococcal Fur binding. (A) *fbpA* operator. Lanes 1 through 4 contained the 160-bp *fbpA* operator. Lanes: 1, free DNA; 2 through 4, 250 nM gonococcal Fur protein without preincubation (lane 2) or preincubated with 1 mM EDTA (lane 3) or 1 mM 2,2′-dipyridyl (lane 4). (B) Internal *fur* fragment. Lanes 1 through 3 contained the 200-bp internal fragment of *fur*. Lanes: 1, free DNA; 2, DNA plus 250 nM gonococcal Fur protein without preincubation; 3, DNA plus 250 nM gonococcal Fur protein preincubated with 1 mM EDTA.

TABLE 3. Neisserial genes with putative Fur boxes[a]

| Gene | Function | Comparison with neisserial consensus (GATAATATAATAA-TTATCTTT)[b] | No. of nucleotides identical | Binding of Gc/Ec Fur[c] |
|---|---|---|---|---|
| *tonB* | Energy transducer | ACAACAAATTATAAAAAATAGG | 13 | Yes/Yes |
| *opa* | Intergonococcal adherence | TTCAACATCAGTGAAAATCTTT | 13 | Yes/No[d] |
| *fur* | Global regulator | **GATAATCATACGCTTAAGCGC** | 12 | Yes/Yes |
| *sod* | Superoxide dismutase | **TATAATAGAAGATTGCAATTT** | 13 | Yes/Yes |
| *hemO* | Heme oxygenase | AAAAATATCATTTGCGTTATATTT | 15 | Yes/Yes |
| *hmbR* | Hemoglobin receptor | **GTTAATTCATAATAAATTTTCTGA** | 17 | Yes/Yes |
| *secY* | Preprotein translocase | GAGATCTTAAGAAACGTCTTT | 13 | Yes/Yes |
| *recN* | Recombination protein | AAAAATCTTAGTTATAATTCGTAT | 14 | Yes/Yes |
| *fumC* | Fumarate hydratase | **GCGAATCACTCTTATTCACATTT** | 13 | Yes/Yes |
| | Hypothetical gene | TGTAATGATAATTATTATCGAA | 15 | Yes/Yes |

[a] Partial listing of *Neisseria* genes containing putative Fur binding sequence (Fur box) in the promoter/operator. Consensus homology was determined by comparison with the previously published 21-bp gonococcal Fur box consensus (9).

[b] Identical nucleotides are in boldface type.

[c] Binding of gonococcal (Gc) and *E. coli* (Ec) Fur proteins to the promoter regions of these genes was determined by EMSA with 500 ng of Fur protein.

[d] *opaH, opaI,* and *opaE* do bind to *E. coli* Fur.

tified, in agreement with a previous report (36). Fur box-like sequences were also identified upstream of the iron-regulated outer membrane hemoglobin binding receptor gene *hmbR* (32) and the heme oxygenase-encoding gene *hemO* (42). Comparison of the promoter regions of *hmbR* and *hemO* with the gonococcal consensus Fur box revealed identities of 80% (17 of 21 bp) and 71% (15 of 21 bp), respectively (Table 3). In silico analysis further revealed 61% identity in the Fur binding site upstream of the gonococcal *fumC* gene, which encodes a tricarboxylic acid cycle fumarase, and 66% identity in the putative Fur binding site upstream of the gonococcal *recN* gene.

The gonococcal RecN protein has recently been demonstrated to be involved in DNA repair and DNA transformation (29).

From a pathogenesis standpoint, it was of interest that we identified putative Fur binding sequences in the promoter regions of the *opa* gene family. In *N. gonorrhoeae*, the *opa* family of genes encodes 11 related outer membrane proteins (OpaA through -K). ClustalW multiple-sequence alignment results revealed that 8 of the 11 *N. gonorrhoeae* FA1090 *opa* genes have a highly conserved promoter region, with the exception of *opaE, opaH,* and *opaI* (Fig. 3B). We observed 61% identity between the putative Fur box upstream of the *opa* promoter/

**A**

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tonB      -35                                -10              +1
AGGCGTTCACATGACAACAAATTATAAAAAATAGGTTGCAAATAG

fur       -35                                -10              +1
CACTGCTTGAATATTTTATAAAAGCGAACGATAATCATACGCTTAAGCGGTA

opa       -35                                -10              +1
TTGTGTTGAAACACCGCCCGGAACCCGATATAATCCGCCCTTCAACATCAGTGAAAATCTTTTTTT
```
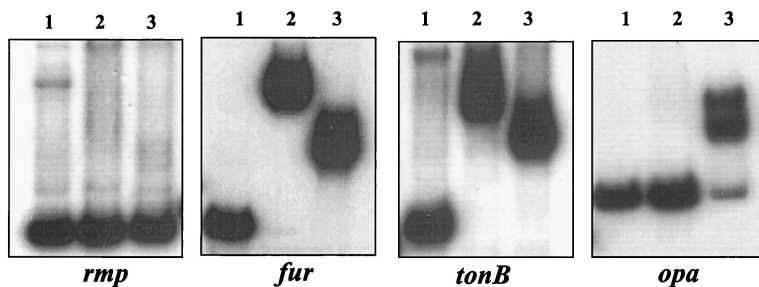
**B**



FIG. 3. (A) Schematic representation of the relative position of the putative iron box (underlined) with respect to the promoter/operator regions of the *tonB, fur,* and *opa* genes. (B) Differential binding of *E. coli* and gonococcal Fur proteins to gonococcal *rmp, tonB, fur,* and *opa* operator DNA probes. Lanes: 1, free probe; 2, with *E. coli* Fur; 3, with gonococcal Fur. A 500-ng sample of each Fur protein and 0.1 nmol of each probe were used. The probe used is indicated at the bottom of each panel.
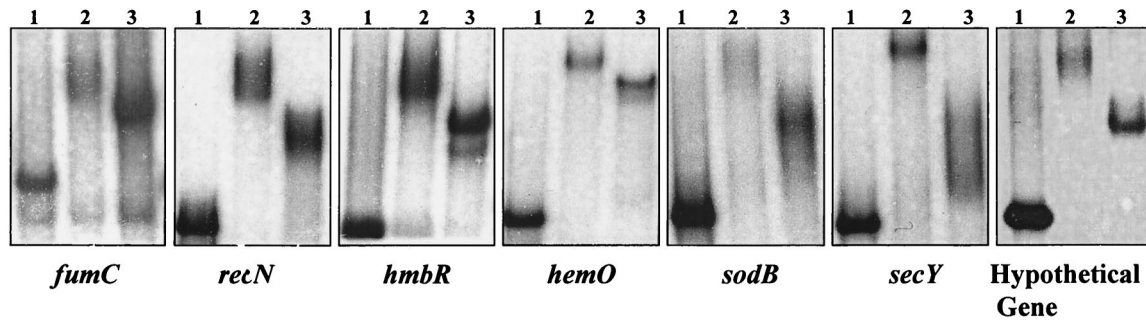
FIG. 4. Differential binding of *E. coli* and gonococcal Fur to gonococcal *fumC*, *recN*, *hmbR*, *hemO*, *sodB*, and *secY* and a hypothetical gene. Lanes: 1, free probe; 2, with *E. coli* Fur; 3, with gonococcal Fur. A 500-ng sample of each Fur protein and 0.1 nmol of each probe were used. The probe used is indicated at the bottom of each panel.

operator regions and the gonococcal consensus sequence (Table 3; Fig. 3A). In contrast to the positions of the *fur* and *tonB* Fur boxes, it was interesting that the Fur box-like sequence in the *opa* multigene family was positioned downstream of the −10 site of the *opa* promoter (Fig. 3A).

**Differential binding of the *E. coli* and gonococcal Fur proteins to the operator regions of *fur*, *tonB*, and *opa*.** Gonococcal *fur* is 50% identical to *E. coli fur* and has been shown to partially complement an *E. coli fur* mutant (3). In order to determine whether DNA binding sequence specificities between the two *fur* homologs exist, we compared the abilities of the purified *E. coli* and gonococcal Fur proteins to bind to the operator regions of the *fur*, *tonB*, *opa*, *recN*, *secY*, *sodB*, *hemO*, *hmbR*, and *fumC* genes and that of a hypothetical gene (Fe-S homolog) by EMSA. Both the *E. coli* and gonococcal Fur proteins resulted in a shift of the *fur*, *tonB*, *recN*, *secY*, *sodB*, *hemO*, *hmbR*, *fumC*, and hypothetical gene (Fe-S homolog) probes (Fig. 3B and 4). Greater retardation of the complex was observed when *E. coli* Fur was used than when gonococcal Fur was incubated with the *fur* gene probe. In marked contrast, when the *opa* probe was used (the *opa* probe used in these assays represents the entire *opa* multigene family), a shift was observed when the gonococcal Fur protein was used but not when the *E. coli* Fur protein was used (Table 3; Fig. 3B). No shift was observed when either of the Fur proteins was incubated with the promoter of the constitutively expressed gonococcal *rmp* gene (10) (Fig. 3B).

**Relative strengths of binding of gonococcal Fur to the *tonB*, *fur*, and *opa* promoter/operators.** A hallmark of a global regulator is differential regulation of genes that the regulator controls. For gonococcal Fur to function as a global regulator, we postulated that the protein should recognize both high- and low-affinity operator sites. To begin to understand the relative strengths of binding of gonococcal Fur to different *Neisserial* iron-regulated genes, we performed EMSA with candidate genes from different categories (regulatory protein, transport protein, and adhesion-invasion protein), including the *fur*, *tonB*, and *opa* genes. We first observed a shift in the *fur* probe with 4 nM gonococcal Fur, and this was complete at 62 nM (Fig. 5A). Interestingly, we found that gonococcal Fur bound to the *tonB* promoter in several steps. At low Fur concentrations (1.5 to 3.0 nM), a type I complex was formed (Fig. 5B). When higher concentrations of Fur were used (5 to 13 nM), two slowly migrating complexes (types I and II) accumulated.

At a Fur concentration of 39 nM, complex II was the major Fur-DNA complex observed (Fig. 5B). An apparent complex was observed with the *opa* multigene family probe at 78 nM gonococcal Fur, and shifting of the probe was complete at 312 nM Fur (Fig. 5C). Two distinct complexes were also identified with the *opa* probe. The two complexes formed with the *tonB* and *opa* probes may correspond to one or two dimers of Fur bound to DNA at one or multiple binding sites. We did not observe a shift with the gonococcal *rmp* probe, even with 416 nM Fur (Fig. 5D). The apparent repressor-operator dissociation constants [$K_{d(app)}$] for the *tonB*, *fur*, and *opa* probes were calculated to be approximately 6, 10, and 100 nM, respectively (Fig. 6). Collectively, these results suggest that gonococcal Fur has a lower affinity for the *opa* promoters than for the *tonB* or *fur* promoters. These differences most likely reflect the different affinities of gonococcal Fur-target operator interactions. To demonstrate the specificity of binding of gonococcal Fur to the putative Fur boxes identified upstream of the *tonB*, *fur*, and *opa* promoter regions, EMSA were performed with an excess of unlabeled probes (unlabeled probe contained the same sequence as the labeled probe). Gradual loss of each of the complexes was observed with a concomitant fold increase in unlabeled probes (data not shown). It is important to note that the *opa* probe used in this assay is representative of the entire *opa* multigene family and that, thus, the $K_d$ of *opa*, as shown here, would represent a range of responses for the *opa* promoters.

To further delineate specifically which *opa* gene of the multigene family could be bound by gonococcal Fur, we performed EMSA with 100-bp oligonucleotides based on the promoter sequence of each specific *opa* gene (Fig. 7). ClustalW analysis of the 100-bp promoter regions of the 11 *opa* genes revealed a high degree of homology among 8 of the 11 *opa* promoter regions. The promoter regions of *opaE*, *opaH*, and *opaI* were more homologous to each other than to other *opa* promoters (Fig. 7). Gonococcal Fur was demonstrated to bind to the promoter regions of all of the *opa* genes tested (Fig. 8). However, when *E. coli* Fur was used in these studies, we only observed a shift with the *opaE*, *opaH*, and *opaI* promoter/operator-specific probes. It was interesting that in studies performed with the *opa* multigene family probe, we did not observe a shift with *E. coli* Fur. One explanation would be that the *opa* multigene family probe did not represent the *opaE*, *opaH*, and *opaI* promoter probes, since the reverse oligonucle-
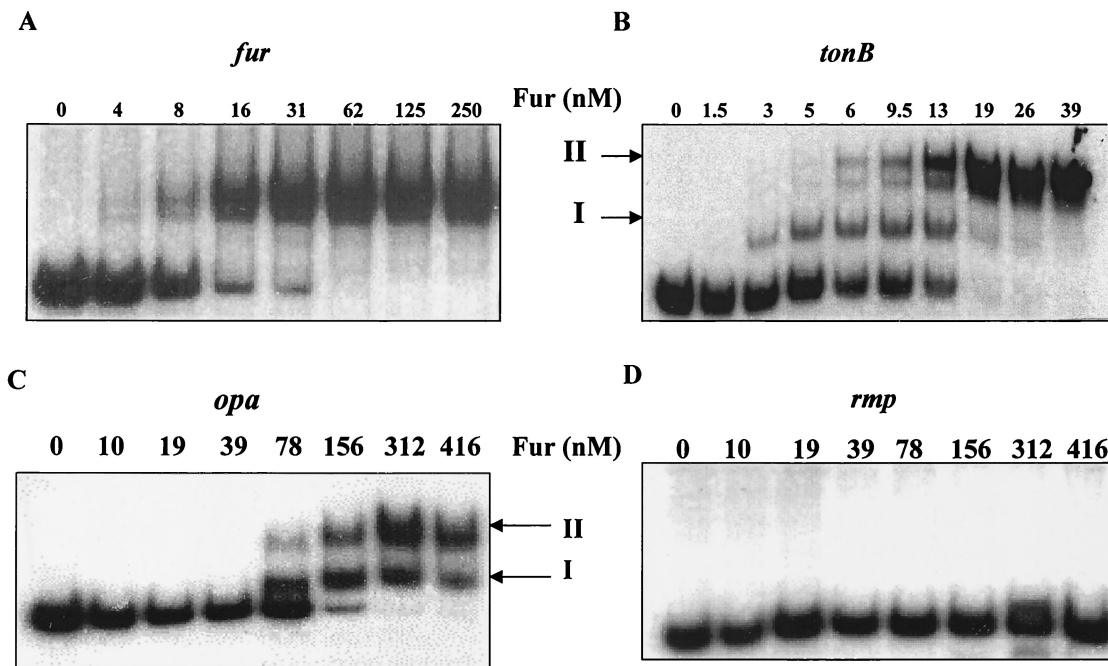
FIG. 5. EMSA analysis of gonococcal Fur binding to the gonococcal *fur*, *tonB*, *opa*, and *rmp* operator DNA probes. A 0.1-nmol sample of each probe was used in each reaction mixture with the indicated increasing concentrations of gonococcal Fur. I, high-affinity complex; II, low-affinity complex. The probe used is indicated above each EMSA.

otide used in the generation of this probe differed at 10, 11, and 12 nucleotides in the *opaH*, *opaI*, and *opaE* promoter regions, respectively, compared to the remainder of the *opa* family members (Fig. 7).

## DISCUSSION

It has been hypothesized that the expression of a number of gonococcal iron-regulated proteins is under the control of the transcriptional regulatory protein Fur. However, conclusive demonstration that the expression of these iron-regulated genes is controlled by Fur has been hampered by the inability to construct a gonococcal *fur* null mutant. In this study, we took an in vitro approach and demonstrated binding of purified gonococcal Fur to the promoter regions of the *fur*, *opa*, *recN*, *secY*, *tonB*, *sodB*, *hemO*, *hmbR*, and *fumC* genes and the gene encoding a hypothetical protein (homolog of Fe-S protein). In a separate study, we have shown that the expression of *fur*, *tonB*, *recN*, *hemO*, *fumC*, and *hmbR* in neisseriae is down regulated during growth with a high iron concentration while the expression of *secY* and *sodB* is upregulated under these conditions (unpublished data). Taken together, these results indicate that the gonococcal Fur regulon is larger than originally proposed and encompasses a broad array of genes.

The association of Fur and iron in regulation of the expression of genes involved in acid tolerance has been previously reported in *S. enterica* serovar Typhimurium (11) and *E. coli* (6, 7). Niederhoffer et al. have reported that the expression of *sodB* in *E. coli* is modulated by Fur, although not in a classical manner of Fe-dependent repression (23). In this study, we identified a Fur box-like element in the promoter region of *sodB* and demonstrated binding of Fur to the *N. gonorrhoeae*

*sodB* promoter region. Furthermore, the transcript level of *sodB* was upregulated under iron-sufficient conditions in neisseriae (unpublished data), and this is in agreement with the positive regulation of *sodB* in *E. coli* (6). The physiological purpose of the Fur-mediated increase in gonococcal *sodB* transcription may be to prevent the damaging effects of iron overload, eventually leading to oxidative stress. A putative Fur consensus sequence was also identified in the promoter region
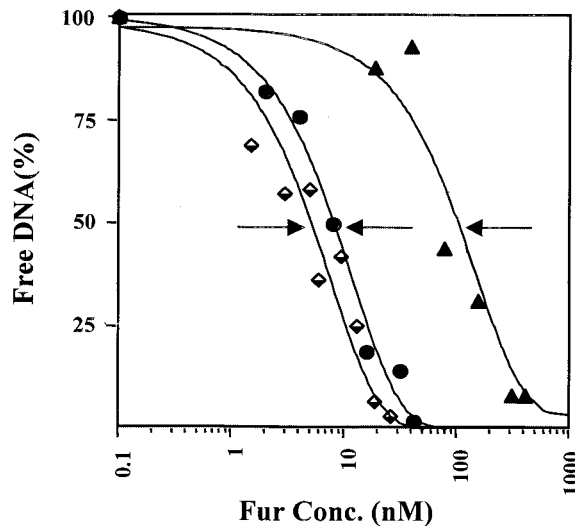


FIG. 6. Determination of the $K_{d(\text{app})}$ of the Fur repressor for the *tonB*, *fur*, and *opa* operators. Binding reactions were performed with 0.1 nmol of DNA and increasing amounts of the Fur repressor. Symbols: ◆, *tonB*; ●, *fur*; ▲, *opa*. Each arrow points to the position of half-maximal binding and corresponds to the $K_{d(\text{app})}$.

***Neisseria* consensus**

GAT**AA TATAA-TAATTATCTTT**

```
opaC   1 -----------ACCGCCCGGAACCCGATATAATCCGCCCTTCAA-CATCAGTGAAAATCTTT
opaJ   1 ----TGAAACACCGCCCGGAACCCGATATAATCCGCCCTTCAA-CATCAGTGAAAATCTTT
opaA   1 -------------CCCGGAACCCGATATAATCCGCCTTTGAAGCATCAGTGAAAATCTTT
opaD   1 ------------GCCCGGAACCCGATATAATCCGCCCTTCAA-CATCAGTGAAAATCTTT
opaB   1 ----TGAAACACCGCCCGGAACCCGATATAATCCGCCCTTCAA-CATCAGTGAAAATCTTT
opaF   1 -----GAAACACCGCCCGGAACCCGATATAATCCGCCCTTCAA-CATCAGTGAAAATCTTT
opaG   1 GTATTGAAACACCGCCCGGAACCCGATATAATCCGCCCTTCAA-CATCAGTGAAAATCTTT
opaK   1 -----GAAACACCGCCCGGAACCCGATATAATCCGCCCTTCAA-CATCAGTGAAAATCTTT

opaH   1 -----GGCACATCGCTCCAAACCTGATATAATCCGCCTTTCAA-CATCAGTGAAAATCGTT
opaI   1 -----GGCAAAAC-----AAATCTGATAAAAT--GCTTGGCGT---TTATTAACAATCTTT
opaE   1 -------------------ATCTGATAAAAT--ACTTGGCGT---TTATTAACAATCTTT

opaC  51 -TTTTTAACCGGTTAAACCGA-ATAA--GGAGCCGAAAATGAATC--------------
opaJ  57 -TTTTTAACCGGTTAAACCGA-ATAA--GGAGCCGAAAA-------------------
opaA  48 -TTTT-AACCGGTCAAACCGA-ATAA--GGAGCCGAAAATGAATCCAGCCC--------
opaD  48 -TTTT-AACCGGTTAAACCGA-ATAA--GGAGCCGAAAATGAATCCAGC----------
opaB  57 -TTTT-AACCGGTTAAACCGA-ATAA--GGAGCCGAAAAT-------------------
opaF  56 -TTTT-AACCGGTCAAACCGA-ATAA--GGAGCCGAAAATG-----------------
opaG  61 -TTTT-AACCGGTTAAACCGA-ATAA--GGAGCCGA---------------------
opaK  56 -TTTTTAACCGGTCAAACCGATATAA--GGAGCCGAAAA-------------------

opaH  56 CTTTTTAGTCAGTT                                   ---------
opaI  47 --T---AATAATTT                                   ---------
opaE  37 --TT--AATAATTTAATCTTAGATATTCGGAGTTGATATGCATACGGCCGACAAAAAAC
```
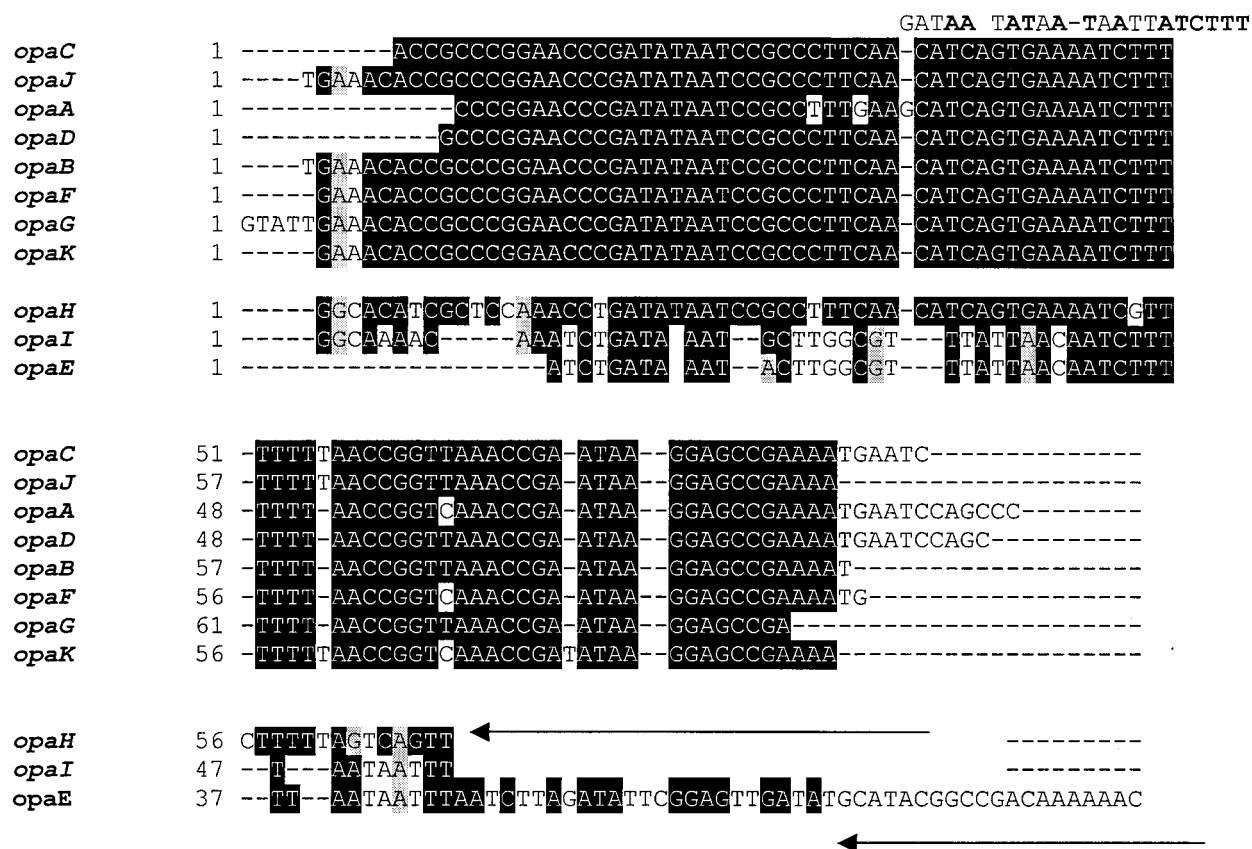
FIG. 7. ClustalW multiple-sequence alignment of the 100-bp *opa* promoter region from *N. gonorrhoeae* FA1090. Sequences encompassing the *opa* promoters were provided by Janne Cannon (University of North Carolina at Chapel Hill). The gonococcal consensus sequence, as previously defined, is shown (9). Identical nucleotides in the putative Fur box in the *opa* promoters and the neisserial consensus are in bold. The reverse oligonucleotide binding site used in the generation of the *opa* multigene family probe is indicated by the arrows.

of *secY*, a gene encoding the preprotein translocase subunit. In vitro analysis further confirmed the binding of gonococcal Fur to the *secY* promoter. As observed with *sodB*, the transcript level of *secY* was found to be upregulated in neisseriae under iron-sufficient conditions (manuscript in preparation), suggesting a positive regulatory role of Fur, as proposed for *sodB*.

A recent report demonstrated that growth of *N. gonorrhoeae* under iron limitation resulted in increased recombinational events, resulting in an increased frequency of pilin antigenic variation (28). Studies to understand the role of recombination genes that might contribute to pilin antigenic variation have revealed conflicting results. Siefert et al. (29) have shown that *recJ*, but not *recN*, in *N. gonorrhoeae* FA1090 and *N. gonorrhoeae* MS11 is involved in pilin antigenic variation. However, studies by Hill et al. showed that an *N. gonorrhoeae* MS11 *recJ* knockout did not alter the antigenic variation at the pilin (*pilE*) locus (16). Nevertheless, the observation that iron limitation resulted in an increased frequency of pilin antigenic variation, together with our identification of a Fur box upstream of *recN* that was shown to bind gonococcal Fur, is noteworthy, since this may allow insights into the role of iron and Fur in recombination of *N. gonorrhoeae*.

As expected, we identified a putative Fur binding sequence

in the promoter regions of several gonococcal genes required for iron transport. Although putative Fur binding sequences have been previously identified in the promoter regions of the *hemO*, *hmbR*, and *tonB* genes, experimental evidence of gonococcal Fur binding to these sequences has not been provided. That iron and Fur may modulate the level of the *tonB* transcript in gram-negative bacteria has been further substantiated by the findings that the level of *tonB* is upregulated 2.5-fold under low-iron conditions in *Pasteurella multocida* (26). Furthermore, expression of HmbR-mediated hemoglobin binding activity has been previously demonstrated to be repressed by iron (32). In vitro studies, such as those described here, with purified gonococcal Fur further support the role of Fur and iron in the regulation of *hmbR*, *hemO*, and *tonB* in *N. gonorrhoeae*.

Our results obtained with a *tonB* probe, a *fur* probe, and an *opa* multigene family probe indicate that a hierarchy exists in the binding affinities of gonococcal Fur for different operators. The $K_{d(app)}$ of gonococcal Fur for the *tonB* and *fur* promoter/operator is consistent with the high-affinity binding range of *E. coli* Fur previously reported for the *fhuA* and *fbpA* operators (3). The $K_{d(app)}$ of gonococcal Fur protein to the *opa* multigene family probe was indicative of a lower binding affinity, similar
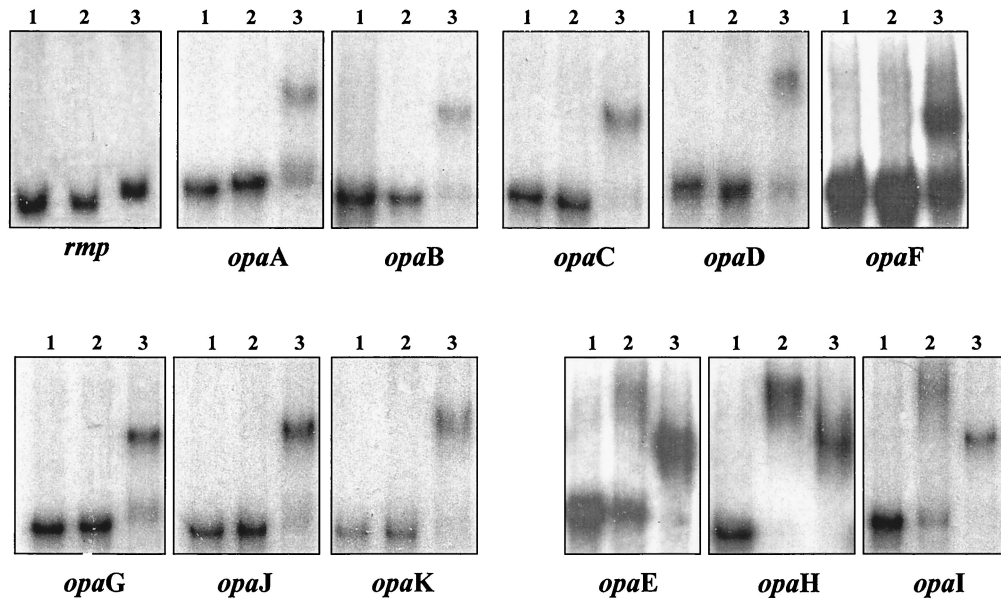
FIG. 8. Differential binding of *E. coli* and gonococcal Fur proteins to the gonococcal *opa* multigene family (*opaA* to *-K*). Lanes: 1, free probe; 2, with *E. coli* Fur; 3, with gonococcal Fur. A 500-ng sample of each Fur protein and 0.1 nmol of each probe were used. The probe used is indicated at the bottom of each panel.

to the reported $K_{d(\text{app})}$ of *E. coli* Fur protein binding to the *sodB* operator (22). The DNA sequence of a given operator is likely to determine the affinity of Fur for a specific target. This would allow for differential expression of Fur-sensitive genes that is dependent, in part, on the ratio of apo-Fur to the active Fur-$Fe^{2+}$ complex. These types of Fur-DNA interactions would allow for differential repression in response to iron among promoters to which Fur binds. Our in vitro analysis shows that gonococcal Fur binds the gene promoters analyzed in this study with various degrees of affinity and thus suggests that Fur may be involved in fine tuning of the expression of the genes under its control.

The finding that gonococcal Fur binds to the *opa* promoters may have direct implications for pathogenesis. The importance of Opa expression for neisserial infection is suggested by the finding that gonococci recovered after urogenital, cervical, or rectal infection are typically Opa$^+$, as are bacteria recovered after the inoculation of human volunteers with transparent (Opa$^-$) bacteria (18, 34). Interestingly, the only exception to such in vivo selection is the observation that Opa$^-$ bacteria predominate during menses (17). Our in vitro and in silico analyses suggest the potential of the entire FA1090 *opa* multigene family to be regulated by Fur. ClustalW analysis of the promoter regions of the 11 members of the *N. gonorrhoeae* FA1090 *opa* multigene family revealed that 8 of the 11 *opa* members have similar levels of identity in the Fur box (13 of 21 bp). A 12- of 21-bp level of identity was revealed in the putative Fur box of the *opaH* promoter region, while *opaE* and *opaI* had an identity level of 9 of 21 bp. EMSA analysis revealed that gonococcal Fur bound to the promoters of all of the 11 *opa* genes. In contrast to the binding of gonococcal Fur, only the mobility of *opaE*, *opaH*, and *opaI* promoter probes was altered by *E. coli* Fur binding. ClustalW alignment of the *opaE*, *opaH*, and *opaI* promoter sequence with the previously published *E.*

*coli* Fur box consensus revealed an identity level of 10 of 21 bp in these promoter regions. Interestingly, the putative Fur box of the *opa* multigene family was positioned downstream of the $-10$ promoter box and found to be AT rich, similar to the Fur box found within the promoter of the *E. coli sodB* gene. Furthermore, the $K_{d(\text{app})}$ of the *opa* multigene family probe was more closely related to the $K_{d(\text{app})}$ of the *sodB* promoter. In *E. coli*, the expression of *sodB* has been shown to be activated by Fur and iron (6). We cannot speculate about whether the *opa* multigene family is positively or negatively regulated by Fur and iron. While the repressive mechanism of Fur has been thoroughly investigated, the mechanism of positive regulation by this protein has not been well elucidated. Studies are under way to define the mechanism of regulation of the *opa* multigene family in vitro and in vivo.

## REFERENCES

1. **Barton, H. A., Z. Johnson, C. D. Cox, A. I. Vasil, and M. L. Vasil.** 1996. Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. Mol. Microbiol. **21:**1001–1017.
2. **Baumler, A. J., R. M. Tsolis, A. W. van der Velden, I. Stojiljkovic, S. Anic, and F. Heffron.** 1996. Identification of a new iron regulated locus of *Salmonella typhi*. Gene **183:**207–213.
3. **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.
4. **Bijlsma, J. J., B. Waidner, A. H. Vliet, N. J. Hughes, S. Hag, S. Bereswill, D. J. Kelly, C. M. Vandenbroucke-Grauls, M. Kist, and J. G. Kusters.** 2002. The *Helicobacter pylori* homologue of the ferric uptake regulator is involved in acid resistance. Infect. Immun. **70:**606–611.

5. **Desai, P. J., A. Angerer, and C. A. Genco.** 1996. Analysis of Fur binding to operator sequences within the *Neisseria gonorrhoeae fbpA* promoter. J. Bacteriol. **178:**5020–5023.

6. **Dubrac, S., and D. Touati.** 2000. Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. J. Bacteriol. **182:**3802–3808.

7. **Dubrac, S., and D. Touati.** 2002. Fur-mediated transcriptional and post-transcriptional regulation of FeSOD expression in *Escherichia coli*. Microbiology **148:**147–156.

8. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. J. Bacteriol. **181:**6223–6229.

9. **Genco, C. A., and P. J. Desai.** 1996. Iron acquisition in the pathogenic *Neisseria*. Trends Microbiol. **4:**179–184.

10. **Gotschlich, E. C., M. Seiff, and M. S. Blake.** 1987. The DNA sequence of the structural gene of gonococcal protein III and the flanking region containing a repetitive sequence. Homology of protein III with enterobacterial OmpA proteins. J. Exp. Med. **165:**471–482.

11. **Hall, H. K., and J. W. Foster.** 1996. The role of *fur* in the acid tolerance response of *Salmonella typhimurium* is physiologically and genetically separable from its role in iron acquisition. J. Bacteriol. **178:**5683–5691.

12. **Hantke, K.** 1987. Selection procedure for deregulated iron transport mutants (*fur*) in *Escherichia coli* K 12: *fur* not only affects iron metabolism. Mol. Gen. Genet. **210:**135–139.

13. **Hassett, D. J., M. L. Howell, U. A. Ochsner, M. L. Vasil, Z. Johnson, and G. E. Dean.** 1997. An operon containing *fumC* and *sodA* encoding fumarase C and manganese superoxide dismutase is controlled by the ferric uptake regulator in *Pseudomonas aeruginosa*: *fur* mutants produce elevated alginate levels. J. Bacteriol. **179:**1452–1459.

14. **Heimer, S. R., R. A. Welch, N. T. Perna, G. Posfai, P. S. Evans, J. B. Kaper, F. R. Blattner, and H. L. Mobley.** 2002. Urease of enterohemorrhagic *Escherichia coli*: evidence for regulation by *fur* and a trans-acting factor. Infect. Immun. **70:**1027–1031.

15. **Hennecke, H.** 1990. Regulation of bacterial gene expression by metal-protein complexes. Mol. Microbiol. **4:**1621–1628.

16. **Hill, S. A.** 2000. *Neisseria gonorrhoeae recJ* mutants show defects in recombinational repair of alkylated bases and UV-induced pyrimidine dimers. Mol. Gen. Genet. **264:**268–275.

17. **James, J. F., and J. Swanson.** 1978. Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. Infect. Immun. **19:**332–340.

18. **Jerse, A. E., M. S. Cohen, P. M. Drown, L. G. Whicker, S. F. Isbey, H. S. Seifert, and J. G. Cannon.** 1994. Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. J. Exp. Med. **179:**911–920.

19. **Karjalainen, T. K., D. G. Evans, D. J. Evans, Jr., D. Y. Graham, and C. H. Lee.** 1991. Iron represses the expression of CFA/I fimbriae of enterotoxigenic *E. coli*. Microb. Pathog. **11:**317–323.

20. **Laemmli, U. K., F. Beguin, and G. Gujer-Kellenberger.** 1970. A factor preventing the major head protein of bacteriophage T4 from random aggregation. J. Mol. Biol. **47(1):**69–85.

21. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. **6:**137–149.

22. **Niederhoffer, E. C., and J. A. Fee.** 1990. Novel effect of aromatic compounds on the iron-dependent expression of the *Escherichia coli* K12 manganese superoxide dismutase (*sodA*) gene. Biol. Met. **3:**237–241.

23. **Niederhoffer, E. C., C. M. Naranjo, K. L. Bradley, and J. A. Fee.** 1990. Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. J. Bacteriol. **172:**1930–1938.

24. **Ochsner, U. A., A. I. Vasil, and M. L. Vasil.** 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. J. Bacteriol. **177:**7194–7201.

25. **Ochsner, U. A., and M. L. Vasil.** 1996. Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc. Natl. Acad. Sci. USA **93:**4409–4414.

26. **Paustian, M. L., B. J. May, and V. Kapur.** 2001. *Pasteurella multocida* gene expression in response to iron limitation. Infect. Immun. **69:**4109–4115.

27. **Prince, R. W., C. D. Cox, and M. L. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. **175:**2589–2598.

28. **Serkin, C. D., and H. S. Seifert.** 2000. Iron availability regulates DNA recombination in *Neisseria gonorrhoeae*. Mol. Microbiol. **37:**1075–1086.

29. **Skaar, E. P., M. P. Lazio, and H. S. Seifert.** 2002. Roles of the *recJ* and *recN* Genes in homologous recombination and DNA repair pathways of *Neisseria gonorrhoeae*. J. Bacteriol. **184:**919–927.

30. **Staggs, T. M., J. D. Fetherston, and R. D. Perry.** 1994. Pleiotropic effects of a *Yersinia pestis fur* mutation. J. Bacteriol. **176:**7614–7624.

31. **Stojiljkovic, I., A. J. Baumler, and K. Hantke.** 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a fur titration assay. J. Mol. Biol. **236:**531–545.

32. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic *Neisseria* spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:**4670–4678.

33. **Stojiljkovic, I., and N. Srinivasan.** 1997. *Neisseria meningitidis tonB, exbB,* and *exbD* genes: Ton-dependent utilization of protein-bound iron in neisseriae. J. Bacteriol. **179:**805–812.

34. **Swanson, J., O. Barrera, J. Sola, and J. Boslego.** 1988. Expression of outer membrane protein II by gonococci in experimental gonorrhea. J. Exp. Med. **168:**2121–2129.

35. **Tardat, B., and D. Touati.** 1993. Iron and oxygen regulation of *Escherichia coli* MnSOD expression: competition between the global regulators Fur and ArcA for binding to DNA. Mol. Microbiol. **9:**53–63.

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

37. **Thomas, C. E., and P. F. Sparling.** 1996. Isolation and analysis of a *fur* mutant of *Neisseria gonorrhoeae*. J. Bacteriol. **178:**4224–4232.

38. **Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Despied.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in delta *fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. **177:**2305–2314.

39. **Tsolis, R. M., A. J. Baumler, I. Stojiljkovic, and F. Heffron.** 1995. Fur regulon of *Salmonella typhimurium*: identification of new iron-regulated genes. J. Bacteriol. **177:**4628–4637.

40. **Vasil, M. L., and U. A. Ochsner.** 1999. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. Mol. Microbiol. **34:**399–413.

41. **Vasil, M. L., U. A. Ochsner, Z. Johnson, J. A. Colmer, and A. N. Hamood.** 1998. The Fur-regulated gene encoding the alternative sigma factor PvdS is required for iron-dependent expression of the LysR-type regulator PtxR in *Pseudomonas aeruginosa*. J. Bacteriol. 180:6784–6788.

42. **Zhu, W., D. J. Hunt, A. R. Richardson, and I. Stojiljkovic.** 2000. Use of heme compounds as iron sources by pathogenic neisseriae requires the product of the *hemO* gene. J. Bacteriol. **182:**439–447.