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The Iron-Responsive Regulator Fur Is Transcriptionally Autoregulated and Not Essential in *Neisseria meningitidis*

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Fur is a well-known iron-responsive repressor of gene transcription, which is used by many bacteria to respond to the low-iron environment that pathogens encounter during infection. The *fur* gene in *Neisseria meningitidis* has been described as an essential gene that may regulate a broad array of genes. We succeeded in obtaining an *N. meningitidis* mutant with the *fur* gene knocked out and used it to undertake studies of *fur*-mediated iron regulation. We show that expression of both Fur and the transferrin binding protein Tbp2 is iron regulated and demonstrate that this regulation is Fur mediated for the Tbp2 protein. Footprinting analysis revealed that Fur binds to two distinct sites upstream of its coding region with different affinities and that these binding sites overlap two promoters that differentially control transcription of the *fur* gene in response to iron. The presence of two independently regulated *fur* promoters may allow meningococcus to fine-tune expression of this regulator controlling iron homeostasis, possibly during infection.

Iron is an essential element for almost all living organisms. The human body as a host provides an environment of iron limitation, as iron is complexed to carrier molecules and therefore not readily available. As a consequence, pathogenic bacteria have developed high-affinity iron uptake systems by which they may scavenge iron in vivo. The human pathogen *Neisseria meningitidis* is a common colonizer of the nasopharynx, and in a small percentage of carriers, meningococcus can cross the epithelial barrier to enter the bloodstream, causing septicemia, and then further cross the blood-brain barrier, causing meningitis. The genome sequence of *N. meningitidis* suggests that this bacterium possesses several iron-scavenging strategies(32). Although *N. meningitidis* does not produce siderophores, it possesses outer membrane receptors that have been postulated to scavenge the iron-loaded siderophores secreted by other bacteria colonizing the nasopharyngeal tract (5). Once inside the host, the organism must compete for iron with host iron proteins, and meningococcus possesses receptors for transferrin, lactoferrin, and hemoglobin (24).

The importance of iron for meningococcal pathogenesis is well documented: treatment with inorganic iron enhances *N. meningitidi*s infection in mice (4, 18), and strains with mutations in iron uptake systems are attenuated in animal models (13, 29, 31). The ability of meningococcus to acquire iron has been shown to play an important role in promoting the survival of the organism within the host, both in its ability to replicate within epithelial cells (15) and in its in vivo survival in the bloodstream (29). Given the location of iron receptors on cell surfaces, their role in pathogenicity, and often their interstrain sequence conservation, these types of proteins have been under study as possible candidates for vaccines against meningococcal infection (1, 2, 17).

Iron overload results in toxicity; therefore, iron uptake is tightly regulated and, in many bacteria, this regulation is mediated by the ferric uptake regulator (Fur) protein (8). The Fur protein senses cellular iron concentrations and acts as a transcriptional repressor by binding to sequences in the promoters of iron-regulated genes and blocking the entry of RNA polymerase, thus inhibiting initiation of RNA transcription. A 19-bp consensus Fur binding site (Fur box) has been elucidated, and Fur proteins from different bacteria have been shown to bind this sequence (20, 38). Classically, Fur-regulated promoters are repressed under high-iron conditions. However, it has recently been demonstrated that in *Helicobacter pylori*, Fur regulates iron-repressed and iron-activated promoters (7). Due to Fur's involvement in the regulation of activities as varied as the acid tolerance response, the oxidative stress response, metabolic pathways, and virulence factors, it has been proposed to be a global regulator in response to environmental iron concentration (8).

The *fur* gene of *N. meningitidis* has been cloned and was shown to be capable of regulating *Escherichia coli* iron-regulated promoters (33). Furthermore, sequences resembling those that encode the Fur box have been identified in meningococcal iron-regulated genes (16, 21, 22, 29, 35) as well as in those of gonococci (25). One of the major limitations in the research on the role of Fur in *N. meningitidis* has been the inability to make a *fur* null mutant. Unsuccessful attempts to isolate insertional null mutants of both *N. meningitidis* (33) and *Neisseria gonorrhoeae* (34) have been reported; however, a *fur* mutant containing a point mutation in the *N. gonorrhoeae* gene was subsequently isolated by manganese selection (34).

In the present study, we report the construction of a *fur* null mutant, which suggests that this gene is not essential in *Neisseria* spp., and present initial studies of Fur-mediated iron regulation showing autoregulation of the *fur* gene and demon-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
DH5-α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	10
BL21(DE3)	*hsdS gal* (λcI*ts*857 *ind-1 S*am7 *nin-5 lacUV5*-T7 gene *1*)	30
N. meningitidis strains		
MC58	Clinical isolate; sequenced strain	32
MC-Fko	*fur* mutant; derivative of MC58 in which the *fur* gene is replaced by a kanamycin cassette; *fur* Km^r	This study
MC-Fko-C	Complemented *fur* mutant; derivative of MC-Fko in which the *fur* gene under the control of its own promoter was inserted along with an erythromycin cassette in the noncoding region between the NMB1074 and NMB1075 genes; fur^+ Km^r Ery^r	This study
MC-*furlacZ*	Derivative of MC58 containing 317 bp consisting of the 5′ end of the *fur* gene and the entire upstream region fused to a promoterless *lacZ* gene chromosomally located between the NMB1074 and NMB1075-genes; Ery^r	This study
MC-*smpAlacZ*	Derivative of MC58 containing 277 bp consisting of the 5′ end of the *smpA* gene and the entire upstream intergenic region fused to a promoterless *lacZ* gene chromosomally located between the NMB1074 and NMB1075 genes; Ery^r	This study
Plasmids		
pET15b	Expression plasmid for expression of recombinant proteins with the N-terminal His tag and thrombin site for removal of the tag	Invitrogen
pGem3Z	Cloning vector	Promega
pGemT	Cloning vector	Promega
pILL600	Plasmid containing the kanamycin cassette from *Campylobacter coli*	14
pSL1190	Cloning vector	Pharmacia
pCMVβ	Plasmid containing the *lacZ* gene of *E. coli*	Clontech
pET15*furB*	Derivative of pET15b containing a 435-bp *Nde*I-*Bam*HI fragment of the *fur* coding region obtained by PCR on MC58 DNA using primers Fmb-F and Fmb-R	This study
pGemFkoB:Km	pGem3Z derivative containing a 587-bp *Eco*RI-*Bam*HI region upstream of the *fur* gene obtained by PCR with primers FkoB-1 and FkoB-2, a 1.4-kb *Bam*HI fragment of the kanamycin gene from plasmid pILL600, and a 465-bp *Bam*HI-*Pst*I region downstream of the *fur* gene obtained by PCR with primers FkoB-3 and FkoB-4; this plasmid was selected because it contains the kanamycin cassette oriented in the same direction as that of *fur* gene transcription	This study
pSLFur-C1	pSL1190 derivative containing a 510-bp *Spe*I-*Xho*I PCR fragment of the NMB1074 locus with primers Fla-UP-L and Fla-UP-R, a 1.1-kb *Xho*I-*Pst*I PCR fragment of the *erm* gene obtained with primers Eryt-DO and Eryt-UP, a 658-bp *Nsi*I-*Bam*HI PCR fragment of the *fur* promoter and coding region obtained with primers Fur-N and Fmb-R, and a 909-bp *Bam*HI-*Xma*I PCR fragment of the NMB1075 locus obtained with primers Fla-DO-L and Fla-DO-R	This study
pSL-*furlacZ*	pSL1190 derivative containing a 510-bp *Spe*I-*Xho*I PCR fragment of the NMB1074 locus obtained with primers Fla-UP-L and Fla-UP-R, a 1.1-kb *Xho*I-*Pst*I PCR fragment of the *ermAM* gene obtained with primers Eryt-DO and Eryt-UP, a 317-bp *Nsi*I-*Sph*I fragment of the *fur* promoter region amplified with primers Fur-N and Fur-P2, a 3.4-kb *Sma*I-*Bam*HI fragment carrying the *lacZ* gene from plasmid pCMVβ (Clontech), and a 909-bp *Bam*HI-*Xma*I PCR fragment of the NMB1075 locus obtained with primers Fla-DO-L and Fla-DO-R	This study
pSL-*smpAlacZ*	pSL-*furlacZ* derivative in which the *fur* promoter region was replaced with a 277-bp *Nsi*I-*Sph*I PCR fragment of the *smpA* promoter region with primers Upf-N2 and Upf-S	This study
pGemT-Fur	Derivative of pGemT containing the *fur* promoter region cloned as a 322-bp PCR product with primers Fur-P1 and Fur-P2	This study

strating that the iron-dependent expression of the transferrin binding protein Tbp2 is Fur mediated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *N. meningitidis* strains were routinely cultured in GC-based (Difco) agar medium containing Kellogg's supplement I (12) at 37°C in a 5% CO_2–95% air atmosphere at 95% humidity. Strains were stocked in 10% skim milk and stored at −80°C. Each bacterial manipulation was started from an overnight culture of a frozen stock. For liquid cultures, *N. meningitidis* strains were grown overnight on solid medium, diluted in phosphate-buffered saline (PBS) to an optical density at 600 nm (OD_{600}) of 1, and inoculated at a 1:100 dilution into GC broth supplemented with Kellogg's supplement I, 12.5 μM $Fe(NO_3)_3$, and, when required, erythromycin and kanamycin added to achieve final concentrations of 5 and 100 μg/ml, respectively. For transformation by naturally competent *N. meningitidis*, four to five colonies of a freshly grown overnight culture were resuspended in 20 μl of PBS, spotted onto GC medium plates to which 5 to 10 μg of linearized plasmid DNA was added, allowed to dry, and incubated for 6 to 8 h at 37°C. Transformants were then selected on plates containing erythromycin (5 μg/ml) and kanamycin (150 μg/ml), and single colonies were restreaked on selective media for further analysis. Single colonies were diluted in 50 μl of PBS, placed in a boiling water bath for 5 min, and centrifuged in a bench top centrifuge for 5 min at 8,000 × *g*. One microliter of the sample was used as the template for PCR analysis. *E. coli* cultures were grown in Luria-Bertani medium, and when required, ampicillin and kanamycin were added to achieve final concentrations of 100 and 25 μg/ml, respectively.

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DNA techniques. DNA manipulations were carried out routinely as described by Sambrook et al. (23). Small- and large-scale plasmid DNA preparations were carried out with a QIAprep Spin Mini kit and Plasmid Midi kit (QIAGEN, Inc.) according to the manufacturer's instructions. DNA fragments or PCR products for cloning purposes were purified from agarose gels with a QiaEx DNA purification kit (QIAGEN, Inc.). PCR was performed in a Perkin-Elmer 2400 thermal cycler with Platinum *Taq* polymerase (Invitrogen). One microliter of each reaction mixture contained 10 to 50 ng of chromosomal DNA or 1 μl of bacterial sample (see above), 100 pmol of the required primers, and a 200 μM concentration of each deoxynucleotide in a volume of 100 μl of 1× PCR buffer containing $MgCl_2$ (New England Biolabs, Inc.). After the initial denaturing step at 95°C for 5 min, 30 cycles of denaturing at 95°C, annealing at the temperatures appropriate for the specific primers, and elongation at 72°C were carried out. DNA fragments were sequenced according to the dideoxy-chain termination method by using [α-^{32}P]dATP (Amersham) and a T7 sequencing kit (Pharmacia).

Construction and complementation of a *fur* mutant of *N. meningitidis*. In order to generate an *N. meningitidis fur* mutant in which the *fur* gene is deleted and replaced by allelic exchange with a kanamycin gene orientated similarly and lacking transcriptional terminators, the MC58 strain was transformed with plasmid pGemFkoB:Km (Table 1). Kanamycin-resistant colonies were selected and checked by PCR for correct insertion by a double-homologous-recombination event. Primer pairs internal (FkoB-1–FkoB-4) and external (FkoB-5–FkoB-6) to the recombination sites as well as internal to the *fur* gene (Fmb-F–Fmb-R) were used to check transformants, and those with the correct PCR profile were further checked by Western blot analysis. We generated one MC58 isogenic *fur* mutant, MC-Fko. Complementation of the MC-Fko *fur* mutant was achieved by insertion of the *fur* locus, complete with promoter and full coding region, into a noncoding chromosomal location between the two converging open reading frames (ORFs) NMB1074 and NMB1075, flanked on both sides with transcriptional terminators. For complementation by allelic replacement, the MC-Fko *fur* mutant was transformed with the pSLFur-C1 plasmid (Table 1). Transformants were selected on erythromycin and checked by PCR, and complementation of the *fur* mutant strain was verified by Western blot analysis.

Construction of chromosomally located transcriptional *lacZ* fusions. To generate transcriptional *lacZ* fusions of the promoters under study at a chromosomal location between the two converging ORFs NMB1074 and NMB1075, flanked on both sides with transcriptional terminators, plasmids pSL-*furlacZ* and pSL-*smpAlacZ* for allelic exchange in *N. meningitidis* strains were constructed (Table 1). The *erm* erythromycin resistance gene (36) was used as a selection marker. These plasmids were then transformed into MC58, and transformants were selected on erythromycin and verified by PCR with primer pairs (Fla-UP-C–Ery-DO-C and Fla-DO-C2–Lac-DO-C); the resultant strains are listed in Table 1.

Expression and purification of the Fur protein. Plasmid pET15*furB* (Table 1) was transformed into the *E. coli* strain BL21(DE3). From an overnight culture of the BL21(DE3)(pET15*furB*) strain, 200 ml of Luria-Bertani medium was inoculated and grown to an OD_{600} of 0.5, and expression of the recombinant Fur protein containing an N-terminal histidine tag was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and further incubation for 3 h. The protein was purified from the harvested cells by Ni-nitrilotriacetic acid (QIAGEN) affinity chromatography under nondenaturing conditions according to the manufacturer's instructions. The purified protein preparation was then diluted to 1 μg/μl and dialyzed overnight in PBS at 4°C. To remove the His tag, the dialyzed protein was then digested at a concentration of 0.5 μg/μl with thrombin (10 U/μg protein; Pharmacia/Amersham) at room temperature for 4 h, and the thrombin was then deactivated by incubation with 1 mM of phenylmethylsulfonyl fluoride at 37°C for 15 min. The digested His tag was removed by twice dialyzing the protein preparation against 1 liter of PBS at 4°C in a 6,000- to 8,000-molecular-weight-cutoff dialysis tube (Membrane Filtration Products, Inc.). The protein preparation was then dialyzed against storage buffer (20 mM Tris [pH 7.9], 50 mM NaCl, 10 mM MgC_{12}, 0.01% NP-40, 1 mM dithiothreitol, 50% glycerol). The purity of the protein was estimated to be 99% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the protein in this preparation was determined by using the Bradford colorimetric assay (Bio-Rad), and the protein was aliquoted and stored at −80°C.

Generation of anti-Fur antiserum and Western blot analysis. To prepare anti-Fur antiserum, 20 μg of purified protein was used to immunize 6-week-old CD1 female mice (Charles River Laboratories), and four mice were used. The protein was given intraperitoneally, together with complete Freund's adjuvant for the first dose and incomplete Freund's adjuvant for the second (day 21) and third (day 35) booster doses. Bleed-out samples were taken on day 49 and used in Western blot analysis. Colonies from freshly grown overnight plate cultures were diluted in 4 ml of PBS until an OD_{600} of 1.0 was reached. One milliliter was then pelleted at 8,000 × *g* and resuspended in 100 μl of SDS-PAGE loading buffer, and 10 μl of each total protein sample was separated on an SDS–15% polyacrylamide gel and transferred onto a nitrocellulose filter by standard methods (23). Filters were blocked for 1 h at room temperature by agitation in blocking solution (3% skim milk and 0.1% Triton X-100 in PBS) and incubated for 1 h more with a 1:1,000 dilution of the Fur protein serum in blocking solution. After being washed, the filters were incubated in a 1:2,000 dilution of peroxidase-conjugated anti-mouse immunoglobulin (Dako) in blocking solution for 1 h and the resulting signal was detected with the Supersignal West Pico chemiluminescent substrate (Pierce).

DNase I footprinting. Probe preparation and DNase I footprinting were carried out as previously described (6, 7) except for the following variations. The plasmid pGemT-Fur was 5′-end labeled with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) at its *Bam*HI site and separated from the vector by PAGE after digestion with *Eco*RI, thereby producing a probe labeled at one extremity only. Binding reactions were performed in binding buffer consisting of 20 mM Tris-HCl (pH 7.9), 50 mM KCl, 10 mM $MgCl_2$, 0.01% NP-40, 100 μM $MnCl_2$, and 10% glycerol containing 1 μg of sonicated salmon sperm DNA as nonspecific competitor DNA. DNase I digestion was carried out by the addition of 1 μl of DNase I (0.02 U/μl) in binding buffer containing 5 mM $CaCl_2$ for precisely 1 min at room temperature. As a molecular weight marker, a G+A sequence reaction (19) was performed for each DNA probe and run in parallel to the corresponding footprinting reactions.

RNA preparation and primer extension analysis. *N. meningitidis* strains were grown in liquid culture to logarithmic phase and then split in three and harvested immediately or after a 15-min treatment of iron limitation (addition of 100 μM 2,2′-dipyridyl; Sigma). To harvest cells, cultures were placed first on ice for 5 min and then centrifuged at 5,000 × *g* in a bench top centrifuge at 4°C. RNA was extracted from the pelleted cells as previously described (28). In each case primer extension was performed as previously reported (7). To ensure correct mapping of the promoter, the sequencing reaction was carried out with a T7 sequencing kit (U.S. Biochemical Corp.) by using the same primer that was used in the primer extension reactions and the plasmid consisting of the relevant cloned promoter.

S1 nuclease mapping. Radioactively labeled DNA probes for quantitative S1 nuclease mapping of each promoter were prepared. A Fur probe, consisting of a 533-bp *Nsi*I-*Eco*RI fragment labeled at the *Eco*RI site was prepared as follows. The pSLFur-C1 plasmid was digested with *Eco*RI, and the ends were dephosphorylated with calf intestinal phosphatase (New England Biolabs). The 5.4-kb vector backbone was then purified from the internal *Eco*RI fragments by extraction from an agarose gel. Approximately 2 pmol of the purified 5.4-kb fragment was labeled with T4 polynucleotide kinase and 4 pmol of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) and digested with *Nsi*I, and the 533-bp *Nsi*I-*Eco*RI probe was purified from a 5% preparative polyacrylamide gel. For the Smp probe, a 325-bp fragment was amplified by PCR with the Us1-Us2 primer pair (Table 2). After purification of the fragment from an agarose gel, 2 pmol of the PCR product was labeled at both extremities with T4 polynucleotide kinase and 4 pmol of [γ-^{32}P]ATP. One labeled extremity was removed by digestion with *Bam*HI, a site for which is incorporated into the Us2 primer, and the resultant Smp probe labeled at one end was extracted from a preparative polyacrylamide gel. Probes extracted from polyacrylamide gels were first eluted overnight in 3 ml of elution buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA, 300 mM Na acetate [pH 5.2], 0.2% SDS) at 37°C with shaking, phenol-chloroform extracted, ethanol precipitated, and resuspended in 100 μl of Tris-EDTA. Approximately 20 fmol of labeled probe was coprecipitated with 15 μg of total RNA and resuspended in 20 μl of hybridization buffer (80% formamide, 60 mM Tris-HCl [pH 7.5], 400 mM NaCl, 0.4 mM EDTA). The mixture was overlaid with 5 μl of paraffin oil, denatured at 100°C for 3 min, and then incubated at an annealing temperature (T_m) calculated on the basis of the following formula: $T_m = 81.5 + 0.5(\%G+C) + 16.6(\text{natural log of Na concentration}) - 0.6(\%\text{formamide})$. After 4 to 16 h of hybridization, 180 μl of ice-cold S1 buffer (33 mM Na acetate [pH 5.2], 5 mM $ZnSO_4$, 250 mM NaCl) and 100 U of S1 nuclease (Invitrogen) were added and S1 nuclease digestion was carried out for 30 min at 37°C. Samples were then extracted once with phenol-chloroform, precipitated with ethanol, resuspended in 5 μl of sequencing loading buffer (23), and subjected to urea–6% PAGE. Quantification of the signals from the digested probes was performed by using a PhosphorImager and ImageQuant software (Molecular Dynamics).

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TABLE 2. Primers used in this study

Primer	Sequence[a]	Site[b]
Eryt-UP	*GCAAACTgcAGAGTGTGTTGATAG*	*Pst*I
Eryt-DO	*CCGTAGGCGCTcGaGACCTCTTTA GCTTCTTG*	*Xho*I
Ery-DO-C	*CAGGTTACTAAAGGGAATGGAG*	
Fla-UP-L	GGTTCCGTACTAgTTGTACTGT CTGC	*Spe*I
Fla-UP-R	aatttaactcgagCCACCAATCCCACAC CACCCTTACC	*Xho*I
Fla-DO-L	ATAAATGTAAAGGaTCCGTTTCA TAGCTAAGG	*Bam*HI
Fla-DO-R	CGCCGTCAACCCgGgTGCCGAGC TGGAAAAAGAGC	*Xma*I
Fla-UP-C	CTGAAGCAAAGTCGGAAAACGC CGGC	
Fla-DO-C2	CTCGAAACCGGTTCTGACGG	
FurP-1	cggatgaattcTCACGGAAATGCCTTT CTGTGC	*Eco*RI
FurP-2	attcagggatccCTTCCGCATGCGTCTC GAAC	*Bam*HI
Fmb-F	cggatccatATGGAAAAATTCAACAA TATTGCAC	*Nde*I
Fmb-R	attcagggatccTTAACGTTTGCCCTTG GCCTG	*Bam*HI
Fur-N	attcaggatgcatTCACGGAAATGCCTT TCTGTGC	*Nsi*I
FkoB-1	attcaggaattcGAGCGGTGTCATGTG TGTTCC	*Eco*RI
FkoB-2	attcagggatccGACGTTATAATACGC AATTTCGGCC	*Bam*HI
FkoB-3	attcagggatccCCGGACGGTTTGTTG TTCAGAC	*Bam*HI
FkoB-4	attcagctgcagGAATGCGCGTACCCC ATTTCG	*Pst*I
FkoB-5	CGGGATGGTTGTTGACGGC	
FkoB-6	CGTTTCACCGCTTTCATCGGG	
Lac-DO-C	*CGCTACCATTACCAGTTGGT CTGG*	
LacZ-PE	*TAGCAGGCTCTTTCGATCC*	
Upf-N2	attcagatgcatGGTAACCTTCAGACC GCTGTC	*Nsi*I
Upf-S	attcaggcatgcTCACGGAAATGCCTT TCTGTGC	*Sph*I
Us1	CCAGCGGTCGGTATGGAATGCG	
Us2	CGATGCGCGGACAGGGCggatccC GCTTAATG	*Bam*HI

[a] Capital letters indicate *N. meningitidis*-derived sequences, italicized capital letters indicate *E. coli*-derived sequences, lowercase letters indicate sequences added for cloning purposes, and underlined letters indicate recognition sites.
[b] Restriction enzyme sites added for cloning purposes.

RESULTS

Construction and complementation of a *fur* null mutant. Previous attempts to isolate a *fur* null mutant of *N. meningitidis* by insertional replacement through allelic exchange were unsuccessful (33), and as a result, it has been hypothesized that *fur* is an essential gene of *N. meningitidis*. Seventy-two base pairs downstream of the *fur* gene maps a homolog (ORF NMB0206) of the leucyl, phenylalanyl-tRNA-protein transferase gene *aat* from *E. coli* (27), which is oriented in the same direction. Nucleotide sequence analysis of this region showed no obvious promoter elements, suggesting that the *fur* and *aat* genes may represent an operon. We attempted to insertionally inactivate the *fur* gene by allelic exchange, as shown in Fig. 1A, by replacing it with a kanamycin cassette oriented similarly to

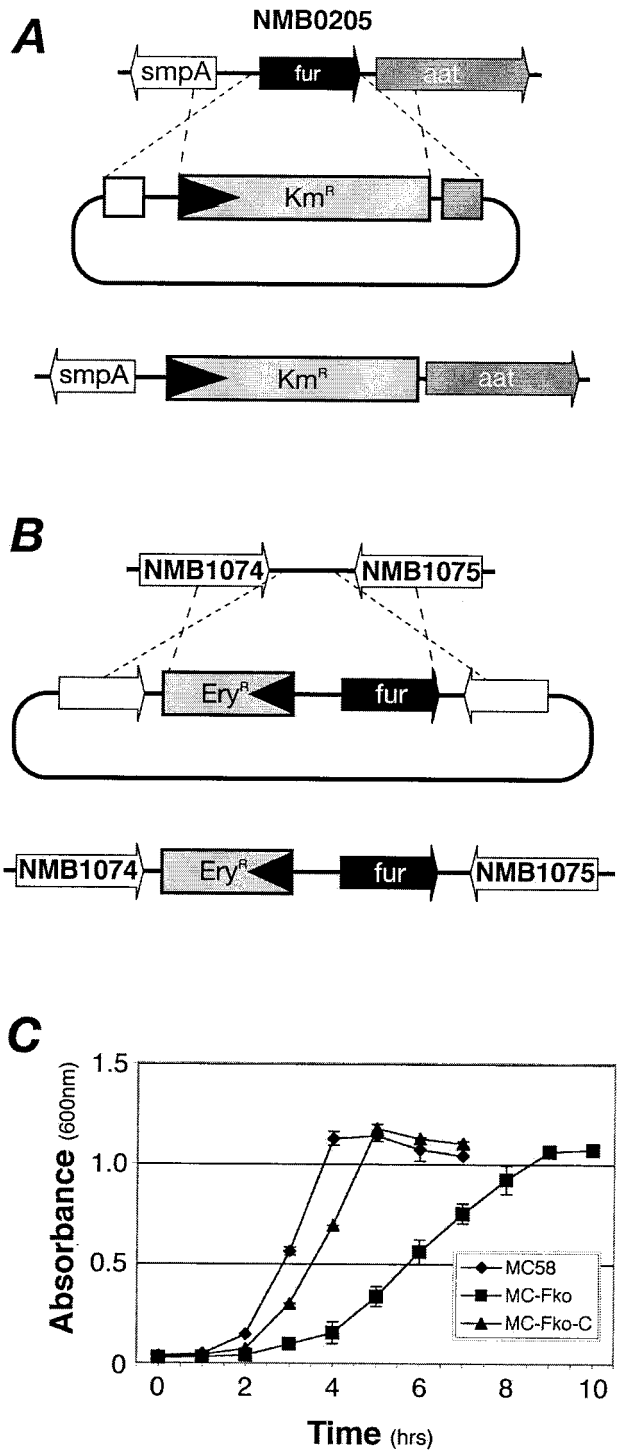

FIG. 1. Mutation and complementation of the *fur* gene of *N. meningitidis*. (A) Schematic representation of the strategy used to construct a *fur* mutant; (B) schematic representation of the strategy used for complementation of Fur; (C) growth curves of the wild-type MC58 strain, the *fur* MC-Fko mutant, and the MC-Fko-C complemented mutant in supplemented GC medium.

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the *fur* gene; this replacement may drive expression of the downstream hypothetical *aat* gene, thereby minimizing the polar effect. On transformation of the MC58 strain with the allelic-replacement construct, pFkoB:Km, only 1 out of 15 Km^r transformants had the correct PCR profile and this transformant, named MC-Fko, was selected for further analysis.

The MC-Fko *fur* mutant when grown on solid medium has an obvious small-colony phenotype; i.e., freshly grown overnight cultures result in pinpoint colonies on GC plates. In order to verify that this phenotype was indeed due to the lack of the Fur protein, we complemented the MC-Fko mutant by inserting the *fur* gene with its own promoter region and an upstream erythromycin cassette as a selection marker between ORFs NMB1074 and NMB1075 as described in Materials and Methods and shown in Fig. 1B. The size of the colonies of the complemented mutant, named MC-Fko-C, is intermediate between that of the pinpoint colonies of the mutant and that of the normal round colonies of the wild type. We determined the growth curves of the three strains to further analyze the apparent growth defect of the mutant, and the results are shown in Fig. 1C. The doubling time of the mutant at 63 (±3) min is considerably longer than that of the wild type at 40 (±2) min. Nevertheless, the fast-growth phenotype is restored almost to wild-type levels in the complemented strain whose doubling time is 51 (±3) min. In growth experiments with supplemented GC medium with low [1 to 10 μM $Fe(NO_3)_3$] or trace [no $Fe(NO_3)_3$ added] concentrations of iron, the slow-growth phenotype did not match that of the wild type and, therefore, the slow-growth phenotype appears not to be a result of possible cellular iron overload due to the derepression of iron uptake systems in the mutant (data not shown).

In order to investigate the expression of the Fur protein in these strains, we purified the Fur protein and raised antibodies against Fur in mice. The *fur* gene was cloned into an expression vector in *E. coli*, and the protein was expressed and purified by Ni^{2+} affinity chromatography by virtue of an N-terminally located His tag, which was then cleaved and removed after purification. Samples from the expression and purification steps of the recombinant Fur protein are shown in Fig. 2A. After SDS-PAGE of the purified tagged and untagged Fur proteins, we clearly observed the major protein bands migrating close to the expected positions and minor slowly migrating bands in each case (lanes 3 and 4). The nature of these slowly migrating bands has not been investigated and may probably correspond to dimeric forms of the proteins.

Figure 2B shows the immunoblot of total protein extracts from *N. meningitidis* wild-type MC58, the MC-Fko mutant, and the MC-Fko-C mutant strains. The antibodies recognize a protein band migrating to a position corresponding to approximately 15 kDa in the wild-type MC58 and in the complemented MC-Fko-C mutant but not in the MC-Fko deletion mutant (Fig. 2B). This result verifies that, indeed, the Fur protein is not expressed in the MC-Fko mutant and confirms the complementation of the *fur* mutant. The signal from the Fur protein is weaker in the MC-Fko-C complemented mutant, however, than in the wild-type strains, indicating that the level of expression of *fur* from the heterologous location on the chromosome is not at the level of expression of the wild type. This observation may account for the fact that the complemented mutant has a lower growth rate than the wild-type

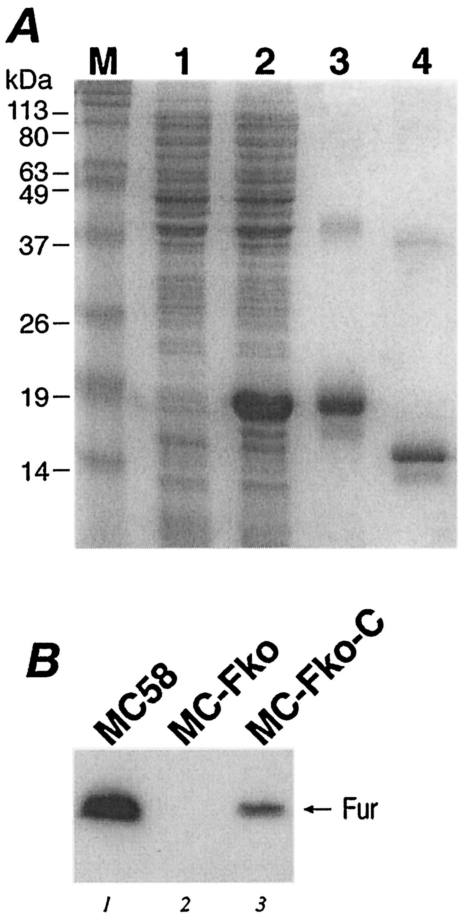

FIG. 2. (A) Expression and purification of the Fur protein. Lane 1, protein extracts from noninduced *E. coli* cells harboring plasmid pET15*furB*; lane 2, protein extracts from cells induced for 3 h with IPTG; lane 3, purified His-tagged Fur protein; lane 4, untagged Fur protein. (B) Western blot analysis showing Fur expression in the wild-type MC58 (lane 1), the MC-Fko *fur* mutant (lane 2), and the MC-Fko-C complemented mutant (lane 3) strains. Lane M, molecular size standards.

strain. In conclusion, we successfully constructed a *fur* null mutant, which strongly suggests that the *fur* gene is not essential in *N. meningitidis* as it is currently considered to be. The growth rate of the *fur* null mutant of *N. meningitidis* is affected.

Iron- and Fur-mediated regulation of protein expression. The Tbp2 protein is a subunit of the transferrin receptor of *N. meningitidis* and is known to be iron regulated (2). To establish whether this iron regulation is Fur mediated and to gain insight into the regulation of the *fur* gene in response to iron, we decided to monitor the fates of the Tbp2 and Fur proteins in total protein extracts of *N. meningitidis* strains cultured under iron-replete and iron-limiting conditions. We grew parallel cultures of the MC58, MC-Fko, and MC-Fko-C strains under iron-replete [GC medium with 12 μM $Fe(NO_3)_3$] and iron-limiting (GC medium with 25 μM desferal) conditions to an OD_{600} of 0.4 to 0.6 and analyzed the expression of the Fur and Tbp2 proteins by Western blot analysis. Equal quantities of total protein from each strain under iron-replete and iron-limiting conditions were separated by SDS-PAGE, blotted

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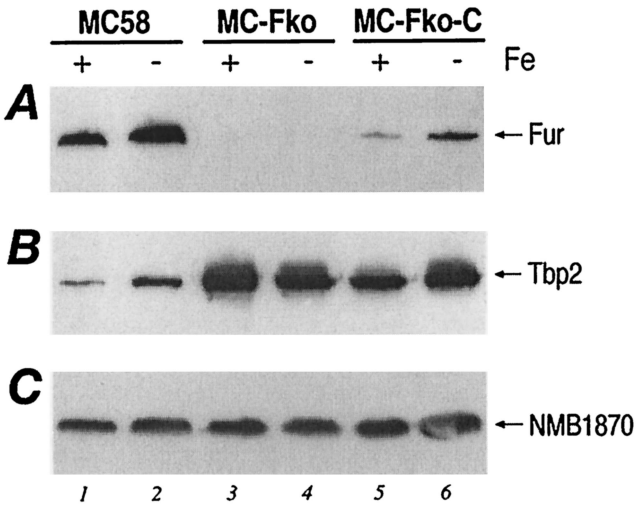

FIG. 3. Iron and Fur regulation of gene expression. Western blot analysis showing iron-regulated protein expression. Wild-type MC58, the MC-Fko *fur* mutant, and the MC-Fko-C complemented mutant were grown under iron-replete (+) (supplemented GC medium) and iron-limiting (−) (supplemented GC medium with 25 μM desferal) conditions and were harvested at an OD_{600} of 0.4 to 0.6. Equal amounts of total protein from each culture were fractionated by SDS-PAGE, blotted onto nitrocellulose filters, and stained with antiserum raised against the Fur protein (A), the Tbp2 protein (B), and constitutive protein NMB1870 as the negative control (C).

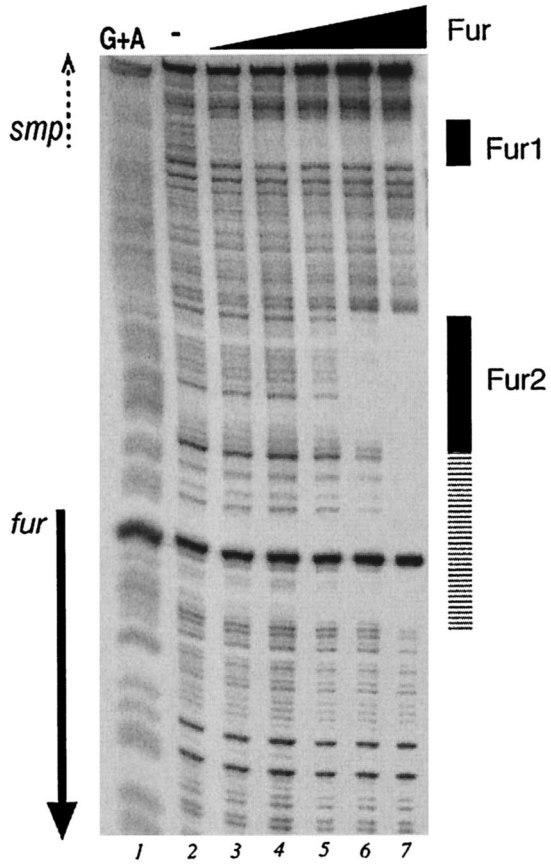

FIG. 4. Footprinting analysis of purified Fur on the NMB204-*fur* intergenic probe. The probe was labeled at one extremity and prepared as described in Materials and Methods. Lanes 2 to 7 contain reaction mixtures to which purified Fur protein at 13.4 nM, 40 nM, 122 nM, 366 nM, 1.1 μM, and 3.2 μM, respectively, was added. Lane 1 represents the G+A sequence reaction (19) obtained with the same probe and used as a molecular weight marker. The solid arrow shows the position and the orientation of the *fur* coding region. The dashed arrow indicates the orientation of the *smpA* gene, which is not contained in the probe.

onto nitrocellulose filters, and tested with antiserum raised against the Fur protein, the Tbp2 protein, and a constitutive protein as a negative control (NMB1870). Figure 3 shows the results of the analysis. As shown in Fig. 2B, there is no signal recognized by the Fur antiserum in the *fur* mutant, as this gene has been deleted (Fig. 3A, lanes 3 and 4) and the relative quantities of Fur in the complemented mutant are less than in the wild type (Fig. 3A, lanes 5 and 6 versus lanes 1 and 2). In the MC58 and MC-Fko-C strains, however, there is an approximately threefold difference in the signal intensities of the Fur protein between the corresponding cultures grown under iron-replete and iron-limiting conditions (Fig. 3A, lane 1 versus lane 2 and lane 5 versus lane 6), indicating that the expression of Fur is iron regulated in both these backgrounds. Moreover, the expression of the Fur protein is down-regulated by iron. In the MC58 strain, the band corresponding to Tbp2 represents approximately threefold less protein in iron-replete culture than in the iron-limited culture, thus confirming that the Tbp2 protein is similarly regulated by iron (Fig. 3B, lane 1 versus lane 2). In the *fur* mutant the expression of Tbp2 is derepressed under both iron conditions (lanes 3 and 4), and in the complemented mutant, although the expression is not repressed to wild-type levels, iron regulation can once more be detected, as the amount of protein expressed is less under iron-replete conditions (lanes 5 and 6). Figure 3C shows the results of the Western blot analysis with anti-NMB1870 antiserum, a protein whose amount is not affected by the iron content of the culture or by the Fur background; thus, its expression is neither regulated by iron nor by Fur and the protein serves as a negative control.

In summary, through Western blot analysis we can detect iron regulation of both the Fur and the Tbp2 protein. Furthermore, iron regulation of the Tbp2 protein is Fur mediated.

Binding of Fur to the *fur* promoter region. In order to investigate whether the meningococcal Fur protein interacts directly with its promoter, we carried out in vitro binding assays with the purified Fur protein (Fig. 2A). We prepared a probe of the *fur* upstream region and carried out DNase I footprinting analysis, with the results shown in Fig. 4. Two binding sites were identified in the *fur* upstream region: with the addition of 40 nM Fur (lane 3), protection occurs at the Fur1 binding site, and with the addition of a 1.1 μM concentration of the protein (lane 6), protection occurs at the Fur2 binding site. Interestingly, with increased amounts of the Fur protein in the reaction mixture, the Fur2 binding site extends towards the translational initiation site of the *fur* gene. The binding ability of the Fur protein was found to be dependent on the presence of a divalent metal ion when footprinting was performed with the addition of 100 μM $MnCl_2$, and no protection was observed when EDTA or 2,2′-dipyridyl was added to the binding reac-

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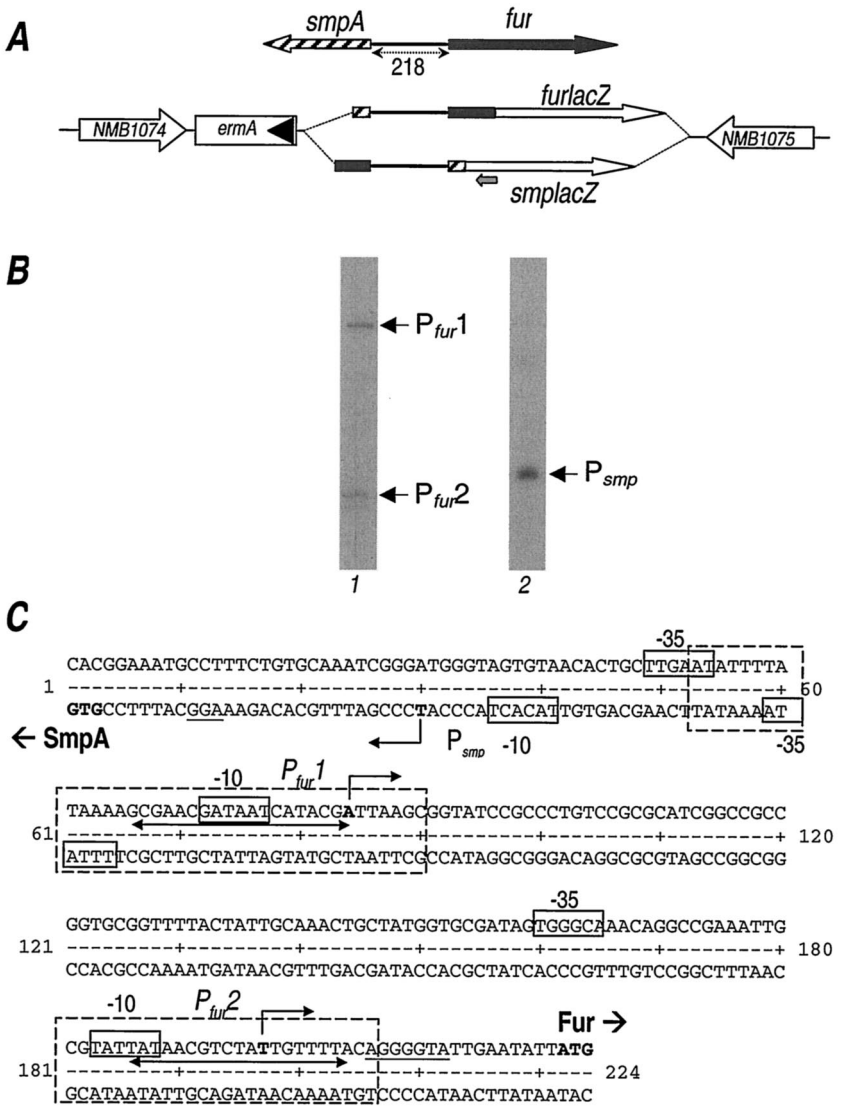

FIG. 5. Mapping the promoters. (A) Schematic representation (not to scale) of the *fur* locus (top) and chromosomal promoter fusions to the *lacZ* gene (bottom) inserted between the NMB1074 and NMB1075 loci. Hatched and filled arrows and boxes indicate all or part of the *smpA* and *fur* genes, respectively. The 218-bp intergenic region is highlighted. Open arrows indicate the *lacZ* gene. The position of the *lacZ*-PE primer is indicated with a small grey arrow. (B) Primer extension reactions with total RNA extracted from strains MC-*furlacZ* (lane 1) and MC-*smpAlacZ* (lane 2). Arrows mark the elongated primer products, $P_{fur}1$, $P_{fur}2$, and P_{smp}. (C) Nucleotide sequence of the *smpA-fur* intergenic region. Deduced initiations of RNA transcription are in boldface and marked with bent arrows. Putative promoter DNA elements are boxed and marked −10 and −35 at $P_{fur}1$, $P_{fur}2$, and P_{smp}. Nucleotides protected by Fur in DNase I protection assays are boxed with broken lines. Double-ended arrows indicate the core Fur box sequences. The translation start sites of the *smpA* and *fur* genes are in boldface and labeled accordingly.

tion mixtures (data not shown). On analysis of the nucleotide sequences corresponding to the protected regions of the Fur1 and Fur2 binding sites, we can identify a Fur box-like element in both binding sites (Fig. 5C) corresponding to seven and eight mismatches, respectively, from the Fur box consensus from *E. coli*.

We conclude that Fur binds to two distinct sites of the *fur* upstream region with different affinities.

Mapping of the promoters. Within the 218 bp of DNA upstream of the *fur* gene, there is a possibility of at least two promoter elements controlling the transcription of the divergent genes *fur* and *smpA*, which codes for a hypothetical lipoprotein (Fig. 1A). To identify the positions of these promoters with respect to the two binding sites identified, we decided to map the 5′ end of the RNA in both directions on both strands. We incurred difficulties in finding primers, within the two divergent genes, which in primer extension experiments gave specific reactions and clear results. Therefore, we generated chromosomally located genes transcriptionally fused to the *lacZ* gene by cloning the promoter region of each gene upstream of a promoterless *lacZ* gene of *E. coli* and introducing this into the chromosome between the NMB1074 and NMB1075 ORFs as described in Materials and Methods and schematically represented in Fig. 5A. The resulting strains,

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namely, MC-*furlacZ* and MC-*smpAlacZ*, carry the chromosomally located transcriptional *lacZ* fusion of the *fur* and *smpA* genes, respectively. RNA was prepared from logarithmic-phase cultures of each of these strains, and primer extension was performed on the RNA preparations with a *lacZ*-specific primer (Fig. 5B). Two elongation products from the primer extension reaction of RNA prepared from the MC-*furlacZ* strain mapping were observed at 138 and 25 bp upstream from the ATG start codon of the *fur* gene, defining the position of the $P_{fur}1$ and $P_{fur}2$ promoters, respectively. One elongation product was observed from reactions of the RNA that was prepared from the MC-*smpAlacZ* strain and that mapped at 27 bp upstream from the ATG start of translation of the *smpA* gene, defining the position of the P_{smp} promoter. The nucleotide sequences in each case upstream of the elongated primers showed the presence of elements similar to the −10 and the −35 hexamers of sigma 70-dependent promoters from *E. coli*, and these are shown in Fig. 5C. The P_{smp} promoter maps divergently upstream from $P_{fur}1$ so that their respective −35 hexamers overlap by 16 bp. The Fur binding sites identified by footprinting, Fur1 and Fur2, overlap the $P_{fur}1$ and $P_{fur}2$ promoter elements, respectively. The P_{smp} promoter maps in a position proximal to the Fur1 binding site (Fig. 5C).

Iron regulation of *fur* gene transcription. To study the iron-mediated regulation of transcription at the $P_{fur}1$, $P_{fur}2$, and P_{smp} promoters, we monitored the accumulation of specific transcripts in total RNA extracted from cells exposed to iron-replete or iron-limiting conditions by carrying out S1 nuclease protection experiments. The MC58 and MC-Fko strains were grown under iron-replete conditions (supplemented GC medium) to logarithmic phase and treated for 15 min with 100 μM 2,2′-dipyridyl (iron limitation), and total RNA was extracted from the cells before and after treatment. Radioactive probes were prepared as described in Materials and Methods for S1 nuclease protection assays of the *fur* and *smpA* transcripts. The labeled probes were hybridized to equal amounts of total RNA and digested with S1 nuclease, and the RNA-protected digestion products were separated on a denaturing gel, the results of which are shown in Fig. 6.

A 533-bp *Nsi*I-*Eco*RI *fur*-specific probe was used to study RNA transcription in RNA extracted from the wild-type strain under different iron conditions; however, we did not perform S1 nuclease experiments with the *fur* probe and the mutant, as the *fur* gene and, hence, the specific transcript are not synthesized. The results (Fig. 6A) show an increase (almost threefold) in the *fur* transcript initiating at $P_{fur}1$ but no significant increase in the transcript initiating at $P_{fur}2$ after treatment of the MC58 strain with the iron chelator. It therefore appears that the two *fur* promoters are differentially regulated: $P_{fur}1$ responds to iron in that it is repressed with high concentrations of iron and induced under iron limitation; however, there was no evidence of the iron regulation of $P_{fur}2$. An S1 protection experiment carried out with RNA extracted from wild-type and mutant strains grown under high- and low-iron conditions and a 325-bp *Nsi*I-*Eco*RI *smpA* probe is shown in Fig. 6B. Results show that, independent of the growth conditions and of the *fur* background, the amount of transcripts at the P_{smp} promoter is not changed, suggesting that under the experimental conditions used, neither iron nor Fur affects transcription from this promoter.

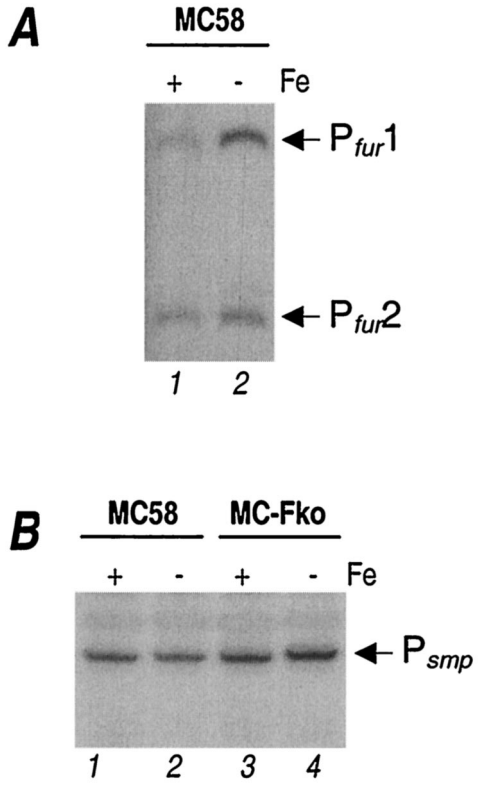

FIG. 6. Analysis of gene transcription by S1 nuclease mappings. (A) Regulation of *fur* transcripts. Total RNA extracted from wild-type MC58 cells exposed to iron-replete (+) or iron-limiting (−) conditions was hybridized to a 533-bp *Nsi*I-*Eco*RI probe labeled at the *Eco*RI site and digested with S1 nuclease. Bands corresponding to S1 nuclease-resistant products were fractionated on denaturing gel and are indicated by arrows and labeled $P_{fur}1$ and $P_{fur}2$. (B) Regulation of *smpA* transcripts. A 325-bp *Nsi*I-*Eco*RI *smpA* probe labeled at the *Eco*RI site was used to hybridize total RNA extracted from wild-type MC58 and MC-Fko strains exposed to iron-replete (+) or iron-limiting (−) conditions and processed as described for panel A. An arrow marks the S1 nuclease-resistant product, P_{smp}.

DISCUSSION

In the present study, we initiate the characterization of the regulatory role of the Fur protein of *N. meningitidis*. The most surprising result is the ability to construct a *fur* null mutant. It has generally been accepted, due to unsuccessful attempts to make *fur* null mutants of *Neisseria* spp. (33, 34), that the *fur* gene was essential in this species. The reason for this remains unclear. The strategy that we attempted was to replace the *fur* gene with a similarly oriented kanamycin cassette, possibly to reduce the polar effects on the downstream *aat* gene (NMB0206), which codes for a putative leucyl, phenylalanyl-tRNA-protein transferase (27, 32). *E. coli* strains containing mutant versions of this gene are viable (11), and we did not investigate whether this gene was essential in meningococcus. Many unsuccessful attempts have been made to replace the *fur* gene of neisserial strains with an allele containing a point mutation that strongly suggests that severe selection against the *fur* mutation takes place (26). It may be that the MC58 strain is sufficiently different to allow the survival of the mutant under the in vitro

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culture conditions used. The *fur* mutant was, however, affected in its growth rate, having a doubling time significantly longer than that of the wild type. We found that this effect was not solely a result of cellular iron overload due to derepression of iron uptake systems as had been reported for the slow-growth phenotype that was also observed for the gonococcal point mutant (34). We complemented the MC-Fko mutant by inserting the entire *fur* gene along with its two promoters into a heterologous chromosomal locus, but this strain does not express Fur to wild-type levels. The incomplete complementation does not seem to be dependent on chromosomal location as insertion of the *fur* locus at another location in the mutant downstream of PorA (NMB1429) resulted in similarly reduced expression of Fur (data not shown), and the reason for this phenomenon was not further investigated. We speculate that it may be due to changes in the stability of the non-wild-type *fur* mRNA.

Although we did not get full complementation of Fur in the MC-Fko-C strain, the slow-growth phenotype is strongly suppressed in the complemented mutant (Fig. 1C). This result, furthermore, supports the hypothesis that secondary mutations have not been selected. Nevertheless, the generation of a *fur* null mutant allowed us to initiate transcriptional studies of strongly suspected iron-regulated and *fur*-mediated genes, the *tbp2* and *fur* genes. Moreover, we present a valuable tool for regulatory studies and the definition of the Fur regulon in meningococcus.

The Tbp2 protein is known to be iron regulated, and here we demonstrate that this regulation is Fur mediated, as its expression is derepressed in the *fur* mutant. We also demonstrate that the *fur* gene itself is classically iron regulated, and we provide strong evidence that this is through a mechanism of autoregulation. Through primer extension and S1 nuclease experiments, we discovered that transcription of the *fur* gene is controlled by two independent promoters, the $P_{fur}1$ distal and the $P_{fur}2$ proximal promoters, which are differentially regulated in response to iron, and sequence analysis implies that recognition occurs by the vegetative sigma of RNA polymerase. Fur binds with high affinity to a site directly overlapping the $P_{fur}1$ promoter, and this binding may be responsible for the observed regulation by iron at this promoter. Although this binding site is proximal to the P_{smp} promoter, we were unable to detect iron regulation of this promoter. The core Fur box of the Fur1 binding site lies well within the total protected region observed in in vitro footprinting and is upstream of the P_{smp} promoter elements (Fig. 5C). Therefore, binding of Fur to this site in vivo may not be sufficient to occlude RNA polymerase entry at the P_{smp} promoter, which has been demonstrated as the mechanism of repression of this protein (9).

A low-affinity binding site was identified over the $P_{fur}2$ promoter, and although no iron regulation was observed under the conditions of the experiments, Fur binds to Fur2 in the footprinting experiment at a concentration that is possibly physiologically significant. Fur is known to be a highly expressed and cellularly abundant regulator protein, and its concentration within the bacterial cell has been estimated as approximately 4 μM (37, 39). This fact suggests that this promoter may also respond to Fur under different environmental conditions, perhaps when concentrations of Fur itself reach a high threshold level. The presence of two differentially regulated *fur* promoters possibly allows meningococcus to fine-tune expression of this important regulator in response to cellular iron as well as to other signals, such as oxidative stress and acid stress, as has been reported for other bacteria (3, 6, 39).

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