JOURNAL OF BACTERIOLOGY, Apr. 2006, p. 2483–2492 Vol. 188, No. 7
0021-9193/06/$08.00+0 doi:10.1128/JB.188.7.2483–2492.2006

Effect of *Neisseria meningitidis* Fur Mutations on Global Control of Gene Transcription†

Isabel Delany,[1] Renata Grifantini,[1] Erika Bartolini,[1] Rino Rappuoli,[1] and Vincenzo Scarlato[1,2]*

Chiron Vaccines, Via Fiorentina 1, 53100 Siena, Italy,[1] and Department of Biology, University of Bologna, Via Selmi 3, 40126 Bologna, Italy[2]

Received 26 October 2005/Accepted 29 December 2005

The ferric uptake regulator 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. In this study we used comparative transcriptome analysis to define the role of the Fur protein in the global control of gene transcription and iron regulation in *Neisseria meningitidis*. By using the Fur-null mutant and its complemented derivative, we identified 83 genes whose transcription is controlled by Fur. We report that Fur may control differential expression of these genes by binding directly to their promoters or through indirect mechanisms. In addition, mutation of the *fur* gene resulted in the induction of the heat shock response, and transcription of these genes does not respond to iron limitation. Furthermore, analysis of the iron starvation stimulon in the Fur-null mutant provided evidences of iron-responsive regulation that is independent of Fur. We began to dissect the regulatory networks of Fur and the heat shock (stress) response in *N. meningitidis*, and the observed interlink between the two circuits is discussed.

It is well established that bacterial pathogenesis is dependent on the ability to acquire iron within the host. Pathogens coordinate important global responses in gene expression to the availability of iron. This allows the maintenance of iron homeostasis in the cell through the modulation of transcription of genes involved in iron acquisition and storage and, furthermore, the coregulation of genes important for survival and pathogenesis in the host (2). The global response to iron has been documented in several different bacterial pathogens, including *Neisseria meningitidis* (17, 30, 32). Moreover, in some cases the comparative transcriptome analysis of regulatory mutants has identified the target genes affected by iron-responsive transcriptional regulators such as the Fur and IdeR proteins (4, 14, 22, 29, 33, 34).

Fur is a well-known repressor of gene transcription in both gram-negative and gram-positive bacteria. Due to its involvement in regulation of functions as varied as iron metabolism, oxidative stress response, acid tolerance response, motility, metabolic pathways and virulence factors, it has been proposed as a global regulator in response to environmental iron concentration (2, 15). Classically, Fur acts as a repressor blocking the entry of RNA polymerase at iron-regulated promoters. Fur senses cellular iron concentrations and in general iron acts as a corepressor activating the DNA-binding activity of Fur for operators in iron-repressed promoters. However, in *Helicobacter pylori*, a mechanism of iron-sensitive repression has also been demonstrated, where Fur has been shown to bind operators in its iron-free form repressing the *pfr* iron-storage ferritin gene, resulting in iron-induced regulation of the gene (7). Fur has been reported to act positively rather than negatively in the expression of certain genes, and the mechanism of positive regulation by Fur for a number of genes in *Escherichia coli* and *Pseudomonas aeruginosa* has been shown to be at the posttranscriptional level through the repression of regulatory small RNAs (27, 40). This mechanism of positive regulation is as yet unknown in *N. meningitidis*; instead, the Fur protein has been implicated in the direct activation of at least one gene promoter, *norB*, where it was shown to bind to upstream sequences, resulting in the activation of RNA transcription in vivo and in vitro (9). Recently, iron-regulated transcriptome analysis allowed the identification of 233 iron-regulated genes in *N. meningitidis*, and half of these genes were found to contain Fur-binding consensus sequences (19). In addition, 32 promoter regions were bound by Fur in gel shift experiments, leading to the conclusion that iron can regulate a broad array of *N. meningitidis* genes through both Fur-dependent and Fur-independent pathways. However, the lack of a Fur mutant of this bacterium has made it impossible to clearly identify Fur-dependent regulation at the genomic level.

To identify Fur-regulated genes in *N. meningitidis*, we carried out comparative transcriptome analysis with a recently available *fur* deletion mutant and its complemented derivative. We identified genes differentially expressed in the presence or absence of the Fur protein and in response to iron limitation and thereby identified the target genes affected directly and indirectly by the Fur transcriptional regulator. Furthermore, we show an interlink between the Fur modulon and the heat shock response.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The meningococcal strains used in the present study are derivatives of the *N. meningitidis* MC58 wild-type strain (38) and correspond to a knockout *fur* deletion mutant, MC-Fko, and its complemented derivatives, MC-Fko-C (8), Fko-Nmf, Fko-D91M, and Fko-H92I, whose construction is described below. *N. meningitidis* strains were cultured in GC-based (Difco) agar medium supplemented with Kellogg's supplement I (24) at 37°C in a 5% CO_2–95% air atmosphere at 95% humidity. Strains were stocked

* Corresponding author. Mailing address: Molecular Immunology Unit, Chiron Vaccines, Via Fiorentina 1, 53100 Siena, Italy. Phone: 39 0577 243565. Fax: 39 0577 243564. E-mail: enzo_scarlato@chiron.com.

† Supplemental material for this article may be found at http://jb.asm.org/.

in 10% skimmed 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, resuspended in phosphate-buffered saline to an optical density at 600 nm of 1, and inoculated with a 1:100 dilution into GC broth supplemented with Kellogg's supplement I and 12.5 μM $Fe(NO_3)_3$ and, when required, erythromycin, kanamycin, and/or chloramphenicol were added to final concentrations of 5, 100, and 5 μg/ml, respectively. *E. coli* DH5α (21) cultures were cultured in Luria-Bertani medium and, when required, ampicillin was added to a final concentration of 100 μg/ml.

Construction of site-directed mutants of Fur. DNA manipulations were carried out routinely as described for standard laboratory methods (35). In order to complement the *fur*-null mutant with a copy of the *fur* gene that expresses the wild-type Fur protein or an iron-blind Fur protein under the control of the P_{tac}-*lacI* IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible system the pPindNmf, pPindD91M, and pPindH92I plasmids were generated as follows. Double-recombination events on transformation of MC-Fko *fur*-null mutant strain with these plasmids allowed the insertion of a chloramphenicol cassette and P_{tac}-*lacI* system expressing the wild-type or mutant *fur* gene into the noncoding region between open reading frames NMB1428 and NMB1429 for complementation. The wild-type *fur* gene was amplified from the MC58 chromosomal DNA with primers FMB-F/FMB-N and cloned as a 435-bp NdeI/NsiI fragment substituting the 910-bp NdeI/NsiI of the *crgA* gene in pPindcrgA plasmid (23) recently used for IPTG-inducible complementation of the *crgA* gene, generating pPindNmf. This plasmid, consisting of the wild-type *fur* gene under the control of the P_{tac}-*lacI*-inducible promoter, was mutagenized with the QuikChange site-directed mutagenesis kit (Stratagene, Inc.), and the primer pairs D91M-f–D91M-r and H92I-f–H92-I-r were designed according to the manufacturer's instructions for incorporation of the two mutations into the regulatory iron-binding site of the protein. This resulted in the generation of two plasmids, pPindD91M and pPindH92I, carrying site-directed mutant *fur* genes. These plasmids, along with pPindNmf, were linearized and transformed into the MC-Fko *fur*-null mutant; transformants were selected for chloramphenicol resistance, correct insertion was verified by PCR, and the resultant strains were named Fko-CII, Fko-D91M, and Fko-H92I. The induction of the protein was achieved by growing the strain in GC broth with IPTG to logarithmic phase, and Western blot analysis of each of the complementing strains confirmed that the mutant or wild-type Fur protein was expressed to similar levels in each (data not shown). The Fko-CII, Fko-D91M, and Fko-H92I strains were further analyzed for restoration of the growth phenotype, and S1 nuclease assay of Fur-regulated genes was carried out.

RNA preparation. *N. meningitidis* strains were grown in liquid culture to logarithmic phase and then split in two and exposed for 15 min to treatment with or without 100 μM 2,2′-dipyridyl (specific iron-chelator; Sigma) or to a temperature of 37 or 42°C. After 15 min, the cultures were added to an equal volume of equivalent frozen medium to bring the temperature immediately to 4°C, and RNA was extracted from the pelleted cells as previously described (8).

Microarray procedures, hybridization, and analysis. DNA microarray analysis was performed as previously described (19). Four microarray experiments were performed: expression ratios were obtained by the direct comparison of RNA obtained from (i) wild-type versus *fur*-null mutant cells grown under iron-replete conditions, i.e., MC58(+) versus MC-Fko(+); (ii) *fur*-null mutant versus complemented mutant cells grown under iron-replete conditions, i.e., MC-Fko(+) versus MC-Fko-C (+); (iii) wild-type cells before and after treatment for 15 min with 2,2′-dipyridyl, i.e., MC58(+) versus MC58(−); and (iv) *fur*-null mutant cultures before and after treatment for 15 min with 2,2′-dipyridyl, i.e., MC-Fko(+) versus MC-Fko(−). For each RNA comparison, cDNA was prepared from a pool of three RNA preparations extracted from three independent cultures as described above. The hybridization probe was made up of a mixture of the differently labeled cDNA derived from each RNA pool. Probe hybridization, washing, and slide scanning were performed as previously described (19). For each image, the signal value of each spot was determined by subtracting the mean pixel intensity of the background value and normalizing the value to the median of all spot signals. The spots, which gave a negative value after background subtraction, were arbitrarily assigned the standard deviation value of negative controls. The data resulting from direct and reverse labeling were averaged for each spot. The accuracy and statistical significance of the expression ratios were determined applying the Student *t* test analysis provided by the Cyber-T program (http://genomics.biochem.uci.edu/genex/cybert/). To process our data with Cyber-T, we calculated a set of *n* repeated $\log_2$ ratios. For each spot we calculated the paired expression value to estimate the mean expression level within the experimental and control data sets. Genes whose expression ratios changed above 1.7-fold and had *P* values of <0.01 were considered up- or downregulated.

Radioactively labeled probe preparation. For DNase I footprinting, upstream promoter regions of genes to be tested were amplified with primers containing EcoRI or BamHI sites (Table S2 in the supplemental material) and cloned into pGemT (Promega). The resulting plasmids were 5′ end labeled with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) at their BamHI sites and separated from the vector by polyacrylamide gel electrophoresis after digestion with EcoRI. Exceptions to this include the NMB0034 probe, which was labeled at the EcoRI site and then separated from the vector by digestion at the SpeI site within the pGemT multicloning site, and the NMB1796 and NMB1988(*frpB*) probes, which were both labeled at the SpeI site and then digested with EcoRI. Probes extracted from polyacrylamide gels were eluted in 3 ml of elution buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA, 300 mM sodium acetate [pH 5.2], 0.2% sodium dodecyl sulfate) overnight at 37°C with shaking, phenol-chloroform extracted, ethanol precipitated, and resuspended in 100 μl of water. Radioactively labeled DNA probes for quantitative S1 mapping of each promoter were prepared by PCR. Each probe was amplified by using specific primer pairs (Table S2 in the supplemental material). 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 BamHI or EcoRI sites for which are incorporated into the upstream primer, and the resultant probe labeled at one end was purified by using Chomaspin TE-100 columns (Clontech).

DNase I footprinting. DNase I footprinting was carried out as previously described (8). 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 addition of 1 μl of DNase I (0.02 U/μl) in binding buffer containing 5 mM $CaCl_2$ for 1 min at room temperature. As a molecular weight marker, a G+A sequence reaction (28) was performed for each DNA probe and run in parallel to the corresponding footprinting reactions.

Primer extension. In each case, primer extension was performed as previously reported (23). To ensure correct mapping of the promoter, a sequencing reaction was carried out with a T7 sequencing kit (USB Corp.) using the same primer as in the primer extension reactions and a plasmid consisting of the relevant cloned promoter.

S1 nuclease mapping. 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 (Tm) calculated for each probe on the basis of the following formula: Tm = 81.5 + 0.5(%GC) + 16.6 (natural log of the sodium concentration) − 0.6 (% formamide). After 4 to 16 h of hybridization, 180 μl of ice-cold S1 buffer (33 mM sodium acetate [pH 5.2], 5 mM $ZnSO_4$, 250 mM NaCl) and 100 U of S1 nuclease (Invitrogen) were added, and S1 digestion was carried out for 30 min at 37°C. Samples were extracted once with phenol-chloroform, ethanol precipitated, resuspended in 5 μl of sequencing loading buffer (35), and subjected to 6% urea polyacrylamide gel electrophoresis. Quantification of the signals from the digested probes was performed by using a PhosphorImager and ImageQuant software (Molecular Dynamics).

RESULTS

Transcriptome analyses. In order to demonstrate the role of Fur in regulation of gene expression at the global level in *N. meningitidis* and to define target genes affected by this transcriptional regulator, we performed comparative transcriptome analyses of the *fur*-null mutant (MC-Fko) of the MC58 strain and its complemented derivative (MC-Fko-C). RNA was prepared from wild-type and mutant cells grown to mid-logarithmic phase and also by iron limitation under iron-replete conditions (+) and after exposure to iron limitation (−) for 15 min by the addition of the iron chelator 2,2′-dipyridyl. Cells were harvested 15 min after treatment to capture the early transcriptional responses elicited by iron limitation and to exclude possible secondary transcriptional responses due to growth adaptation in the new condition. Four independent competitive hybridization experiments were performed to determine relative ratios for each gene by using DNA microar-

rays as described in Materials and Methods. (i) RNA from wild-type cells was compared to RNA from *fur*-null mutant cells to identify genes differentially expressed in the two strains. (ii) RNA from *fur*-null mutant cells was compared to RNA from the complemented mutant cells to verify restoration of Fur regulation. (iii) RNA from wild-type cultures, untreated or treated with iron chelator, was compared to identify genes that were differentially regulated in response to iron limitation. (iv) Finally, RNA from *fur*-null mutant cultures, untreated or treated with iron chelator, was compared to identify genes regulated in response to iron limitation independently of Fur.

Using the data sets from each of the four experiments, we transformed the relative ratios into log units and show relevant experiments in a two-dimensional graphical display in order to investigate global trends of the meningococcal transcriptome (Fig. 1). In particular, groups of differentially expressed genes are highlighted. In Fig. 1A, we plotted the induction caused by iron limitation against the induction (or derepression) caused by the *fur*-null mutation and observed that there is a good correlation between genes regulated by Fur and iron, i.e., the data are clustered around a line with a slope close to 1 (0.93). Two main categories of Fur regulated genes can be distinguished: genes differentially regulated by Fur and iron (indicated by gray boxes) and genes that are differentially regulated by Fur but below the cutoff in response to iron limitation (indicated by diamond symbols). These two categories of genes are indicated by similar symbols in the plots of global responses in the other transcriptome experiments (Fig. 1B and C). Figure 1B shows a plot of the induction caused by the *fur* mutation and the corresponding repression caused by the complementation, and we observed a correlation between genes differentially regulated in the *fur* mutant and also conversely deregulated in the complemented mutant. However, the trend of the data brings the slope closer to the *x* axis since the relative ratios for each gene are higher in the *fur* mutation experiment than in the complementation experiment. This possibly reflects the fact that the expression of Fur in the complemented strain is lower than that of the wild type (8). This is particularly true of the highly Fur-repressed genes (those in the lower right-hand quadrant of Fig. 1B) and may be an indication that these genes respond to Fur in a dose-dependent manner. Finally, in Fig. 1C the response of genes to iron limitation in the wild type (Fur^+) is plotted against the response of genes to iron limitation in the *fur* mutant (Fur^-). We observe that a relatively limited number of genes respond to iron limitation in the Fur^- background, i.e., nine genes show significant differential expression and only one of these (*frpB*) is also differentially expressed in the wild type. The other eight genes (indicated by open circles) that are differentially expressed on treatment of the *fur* mutant strain with iron limitation are neither Fur nor iron regulated in the other experiments, as exemplified in Fig. 1A, where they are clustered around zero on both axes. Furthermore, the group of genes that responded to the *fur* mutation, but not to iron (diamonds), are centered around zero in Fig. 1C, distinguishing them as not significantly regulated in response to iron limitation in the wild type and mutant.

In summary, upon analysis of the global trends of Fur-responsive genes in *N. meningitidis*, we conclude that genes with transcriptional responses to iron limitation also respond to the *fur* mutation and complementation in a similar and inverse way, respectively. In addition, in the Fur mutant background these genes no longer respond to iron. There is only one gene, *frpB* (*fetA*), which also responds to iron limitation in the mutant, and eight genes that exhibit differential regulation are not normally iron nor *fur* regulated and are probably examples of adaptive responses in the Fur mutant, which are distinct from the Fur-mediated regulatory network in meningococcus. However, this was not investigated further in the present study. Interestingly, there appears to be a group of genes that may respond to the Fur mutation but not significantly to iron limitation, and this putative category of genes is identified here for the first time.

Fur modulon of *N. meningitidis*. The first transcriptome experiment in which we compared relative transcript levels in the Fur mutant and the wild type allowed us to identify 83 genes that respond significantly, either positively (44 genes) or negatively (39 genes), to the absence of the Fur protein. We call these genes members of the Fur modulon. In Table S1 in the supplemental material we present a summary of the results of the four transcriptome experiments for each of the 83 genes of the Fur modulon, and Fig. 2 shows a graphic output of them. In general, there is an inverse relationship between the response to the *fur* mutation in the wild type and the complementation in the mutant, thus confirming that the differential transcription of these genes is dependent on the Fur protein. A clear exception to this is nmb1988, which codes for the FrpB (FetA) iron receptor protein, in which the transcript appears to be upregulated in the mutant compared to the wild type and further upregulated on restoration of Fur expression in the complementing strain, and this was selected for further investigation (see below). We can then further classify the positively and negatively affected genes by their transcriptional response to iron limitation in the wild-type strain.

Classically regulated Fur- and iron-repressed genes can be separated into three main functional families. As expected, a large number of classically regulated genes belong to known iron-regulated transport systems involved in iron uptake, including the transferrin-binding receptor, the lactoferrin-binding receptor, and ExbBD, Fbp, and FrpB(FetA) iron transport proteins, as well as three genetic loci—nmb0034-36, nmb0175, and nmb0744—showing homologies to iron metabolism and assimilation genes (Table S1 in the supplemental material). The *frpC* operons and associated genes encoding for iron-regulated secreted proteins related to the RTX family of cytotoxins (39), as well as other secreted or cell envelope proteins, represent a second family of classically Fur-regulated genes. A third family includes some genes involved in energy metabolism such as lactate dehydrogenase, alcohol dehydrogenase, and fumarase (*fumC*). Fur- and iron-induced genes are represented in two main families: genes coding for iron-containing proteins involved in iron homeostasis and oxidative stress resistance such as those encoded by the *bfrAB* and *sodB* genes, respectively. The other major family contains protein complexes involved usually in electron transfer and energy metabolism, such as NADH dehydrogenase I of the respiration chain and cytochromes.

Within the Fur modulon we observed one family of genes whose expression is altered by Fur but not by iron limitation, and these consist of the chaperones and proteases that govern the

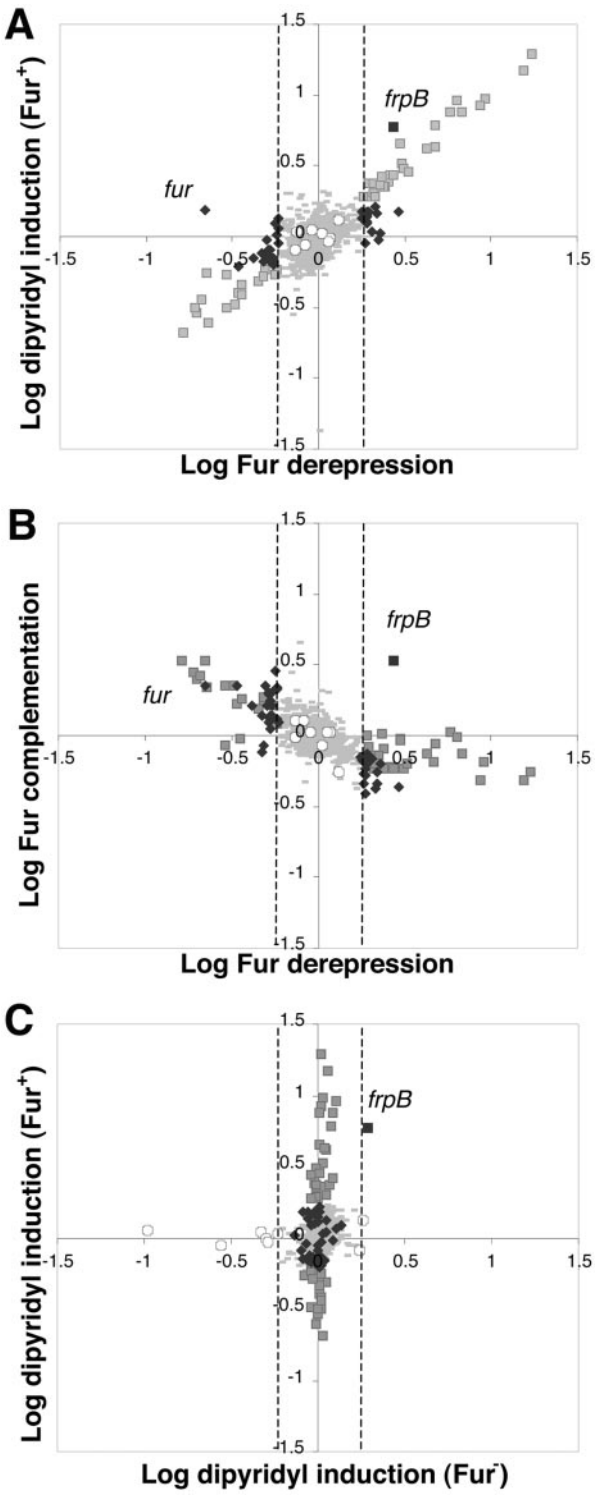

FIG. 1. Global responses of meningococcal genes to Fur and iron by transcriptome analysis. (A) The induction of genes by the addition of the iron chelator 2,2′-dipyridyl in the wild type (log[MC58(−) versus MC58(+)]) is plotted against the derepression of genes due to the Fur$^-$ mutation (log[MC-Fko(+) versus MC58(+)]). Genes showing differences in expression due to the Fur mutation of <1.7-fold (dashed lines) in our analysis were considered unchanged. The genes significantly altered in expression due to the Fur mutation and also significantly altered due to iron chelation are indicated (▨). *frpB* is the only gene whose expression was significantly altered in all of the four transcriptome experiments and is highlighted in each plot with a black box. Genes that show a significant difference in expression due to the Fur mutation but are not altered more than 1.7-fold due to iron chelation are indicated (◆). The *fur* gene is indicated and appears significantly downregulated in the knockout mutant, has a moderate induction due to iron limitation (8) that is not above the cutoff of these experiments, and serves as a good internal control. (B) The differential expression of genes by complementation of the mutation (log[MC-Fko(+) versus MC-Fko-C(+)]) is plotted against the derepression of genes due to the Fur$^-$ mutation (log[MC-Fko(+) versus MC58(+)]). (C) The induction of genes by dipyridyl in the wild type (Fur$^+$) (log[MC58(−) versus MC58(+)]) is plotted against the induction of genes by dipyridyl in the Fur$^-$ mutant (log[MC-Fko(−) versus MC-Fko(+)]). Genes whose expression showed differences in the Fur$^-$ mutant greater than 1.7-fold on addition of the dipyridyl chelator but that did not undergo a significant change in response to iron chelation in the wild type or in response to Fur are indicated (○) and were not investigated further in the present study. The expression of *frpB* was also induced by dipyridyl in the Fur mutant and is indicated with a black box.

general stress or heat shock response, including *dnaK*, *clpB*, and *lon*, which are upregulated in the Fur mutant and, conversely, the nmb0838 cold shock gene is downregulated in the mutant. This category of genes was selected for further investigation.

Biochemical verification of direct Fur regulation: the Fur regulon. A consensus binding sequence (GATAATGATAAT CATTATC) has been identified that is known as the Fur box, and Fur proteins from different bacteria have been shown to bind this sequence (3, 31). Footprinting experiments on six promoters of the operons in Table S1 in the supplemental material have been performed previously in this lab and reported in other studies, including promoter regions of the *fur* (nmb0205) gene (8) and the *tbp2* (nmb0460), *norB-pan1* (nmb1622-nmb1623), *nuoA* (nmb0261) (9), and nmb1436 (18) genes. These binding experiments have shown that the meningococcal Fur protein results in protection at these promoters with high affinity in vitro and recognizes sequences similar to the Fur box consensus. To further extend biochemical analysis of Fur binding to promoters of genes of the Fur modulon, we cloned a number of promoter regions and performed in vitro footprinting analysis with the purified meningococcal Fur protein. Since the binding of Fur to iron-induced promoters has been the subject of a previous report (9), we focused in the present study on the classical iron- and Fur-repressed genes that are likely to be members of the Fur regulon of meningococcus. We prepared radioactively labeled probes of candidate genes, and Fig. 3 shows the footprints on the probes that exhibited high-affinity operators for Fur out of the promoters selected for analysis. Eleven of the probes in Fig. 3 represent promoters of genes that are classically Fur repressed and iron repressed (Table S1 in the supplemental material). We also included the promoter of the hemoglobin receptor *hmbR*; in the transcriptome analysis this gene resulted 1.66-fold induced by iron limitation and is a well-known iron-regulated gene (36). Protection was achieved upon addition of 34 nM Fur in the nmb0175, nmb1796, *lbpB*, nmb1879, and *frpB* probes, at 68 and 137 nM Fur in the nmb0034, nmb1377, *hmbR*, and *tonB* probes and the *recN*, *fumC* probes, and finally at 275 nM in the nmb1395 probe. In addition, each of the protected regions corresponding to high-affinity binding sites is proximally up-

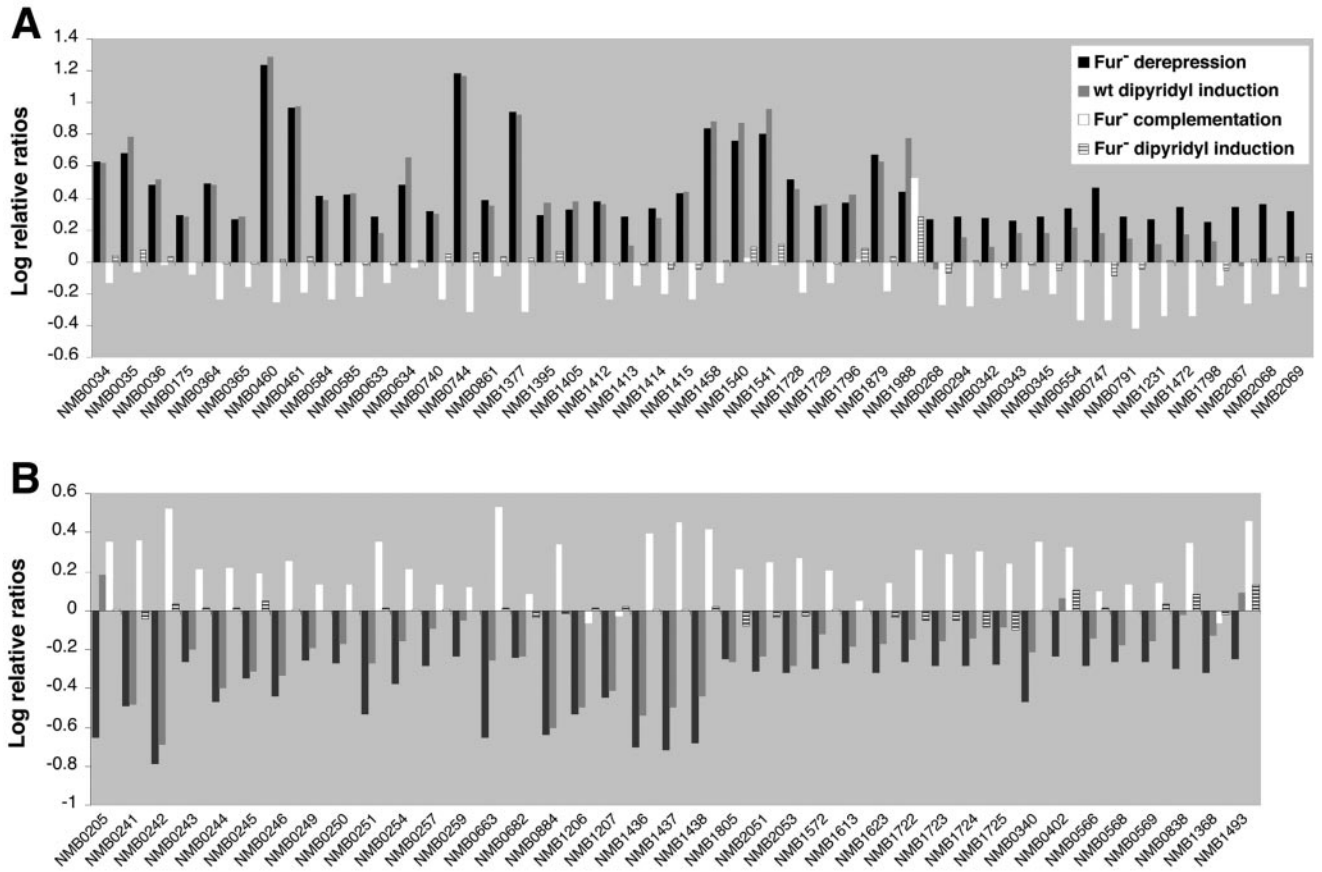

FIG. 2. Fur modulon: genes whose expression was significantly altered in the presence or absence of the Fur protein. The differential expression in each of the four transcriptome experiments of the 44 Fur-repressed genes (A) and the 39 Fur-induced genes (B) of the Fur modulon is presented graphically. The relative ratios of the transcriptome competitive hybridizations are shown for Fur-responsive expression [MC-Fko(+) versus MC58(+)] as black bars (■), Fur-complemented expression [MC-Fko(+) versus MC-Fko-C(+)] as white bars (□), iron limitation-responsive expression in the wild type [MC58(−) versus MC58(+)] as gray bars (▨), and iron limitation-responsive expression in the *fur* mutant [MC-Fko(−) versus MC-Fko(+)] as hatched bars (▤).

stream of the coding region of the gene or of the first gene in the putative operon. Moreover, the transcriptional start sites of the *recN*, nmb1796, and *frpB* genes were mapped within high-affinity binding sites (shown in Fig. 3), demonstrating that these Fur operators overlap promoter elements. The nmb1796 and *frpB* probes exhibit two regions of protection, and the second binding site in each case is directly downstream or upstream of the promoter, respectively. In the case of three other probes (nmb1377-1378, nmb1395-1396, and nmb1879-1880) corresponding to two divergently oriented genes, two regions of protection were identified in the footprinting experiments and may correspond to regions overlapping two divergent promoters, although the genes upstream were not regulated in this microarray study and more study is needed to understand the positions and regulation of the promoters therein. Nonetheless, as reported previously with the *tbpB* and *fur* iron-repressed promoters (8, 9), in each case where the transcriptional start sites have been determined, the Fur operators are overlapping the classically regulated promoter elements. Furthermore, nucleotide sequence analysis of the Fur-protected sites showed conservation with respect to the *E. coli* consensus Fur box.

We also identified low-affinity binding sites in some promoter regions of some other Fur-modulated genes (data not shown) where protection is detected upon the addition of high concentrations of protein. In general, these resulted in promoter regions of the Fur-repressed (iron-independent) genes, including *dnaK*, *clpB*, and the Fur- and iron-induced genes, including *sodB* and *fumB*. Upon the addition of 1.1 μM, 3.3 μM, or higher concentrations of Fur protein, some areas of protection could be detected within these promoters generally in AT-rich regions (data not shown). These represent a third class of operators for the meningococcal Fur protein in vitro, i.e., operators of low affinity and whose positioning is atypical of Fur-regulated promoters reported to date (8, 9). The biological significance of these weak in vitro bindings is not fully understood. However, a similar phenomenon has been described for the *sodB* promoter in *E. coli* (11, 12), which exhibits in vitro weak binding of Fur to operators downstream of the +1 position of transcription, and it has subsequently been verified that Fur mediates an indirect positive regulation by repression of a small regulatory RNA (sRNA), which in turn negatively regulates a number of Fur- and iron-induced genes in *E. coli* at the posttranscriptional level (16, 27). Therefore, although we cannot exclude that these low-affinity operators in

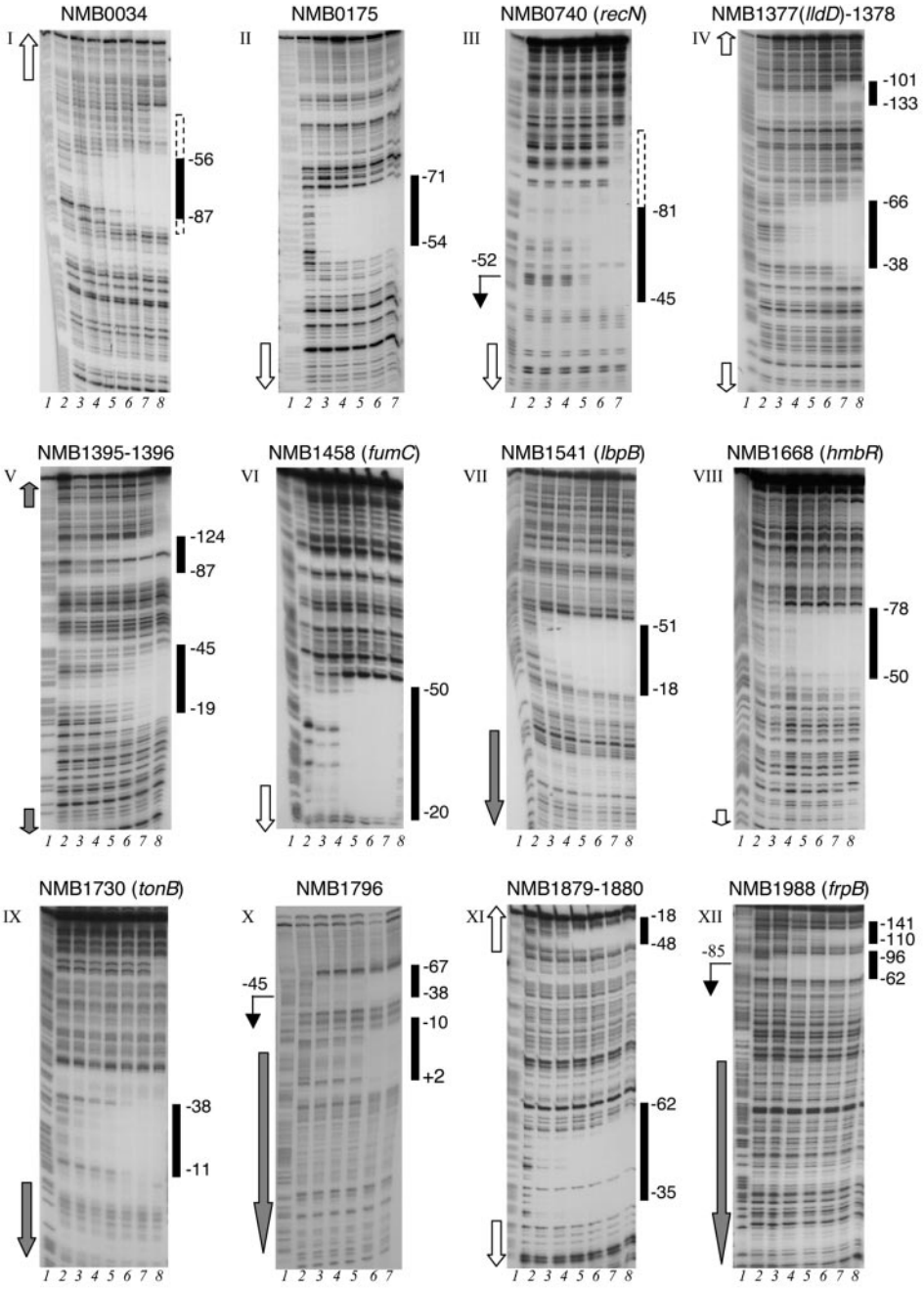

FIG. 3. Biochemical verification of Fur regulation at the promoters of iron-repressed genes. DNase I footprinting analysis on 12 classically regulated promoter regions of meningococcal Fur-regulated genes. DNA probes radioactively labeled at one end were prepared as described in Materials and Methods and correspond to the intergenic regions upstream of Fur-regulated genes indicated above each panel. The gray arrows show the position and the direction of the indicated genes within the probe in each panel, and the empty arrows denote the direction of the gene indicated, although the coding region is not contained within the figure shown. Lane 1 in each panel corresponds to the G+A sequence reaction for each DNA probe run as the molecular weight marker in parallel to the footprinting reactions. Lanes 2 to 8 correspond to the in vitro binding reactions to which increasing amounts of Fur protein were added to final concentrations of 0, 34, 68, 137, 275, 550, and 1,100 nM, respectively. Fur-protected regions are indicated to the right as a vertical black bar, and the numbers indicate the boundaries of the binding site with respect to the ATG translational start site of the downstream gene, with dashed boxes indicating the regions of protection expanding from the binding site upon the addition of higher protein concentrations. A bent arrow to the left of some panels indicates the positions of the initiation of transcription of the downstream gene and the number maps it with respect to the ATG translational start site.

vitro may correspond to direct Fur regulation at the promoters of these genes, we favor the hypothesis that Fur may modulate the expression of these genes indirectly.

Therefore, we conclude that the classically regulated Fur and iron-repressed promoters with highly conserved Fur boxes and/or biochemically verified high-affinity binding sites are members of the Fur regulon. In addition, apart from the previously reported Fur- and iron-induced promoters with high-

affinity binding sites upstream of their promoters, the majority of the promoters from the other classes in the modulon are likely to be regulated in an indirect way through intermediary mechanisms.

Fur-modulated genes contain the heat shock response genes. One of the most striking groups of genes from the transcriptome analysis are those that are Fur repressed but iron independent in that they are upregulated in the *fur* mutant. These include some of the general stress response or heat shock genes such as *dnaK*, *lon*, and *clpB*, which were selected for further studies. Radioactively labeled probes were prepared for S1 nuclease protection assays of the *dnaK*, *lon*, and *clpB* genes in order to map their promoters and also to quantify their specific transcripts in the wild type, *fur* mutant, and the complemented mutant in response to iron. Experiments were repeated on the unpooled RNA preparations that constituted the pools used for the transcriptome analysis, and representative results are shown in Fig. 4A. Transcripts from all three heat shock genes are significantly upregulated in the *fur* mutant (lanes 3 and 4) and conversely restored to almost wild-type levels in the complemented strain MC-Fko-C (lanes 5 and 6), demonstrating that the differential expression of these genes is modulated by the Fur protein (Fig. 4A). Furthermore, there is no significant response of these genes to 15 min treatment with iron-limitation (lanes 2, 4, and 6 versus lanes 1, 3, and 5). Mapping of the respective 5′ S1-protected end revealed sequences similar to the σ^{32} consensus promoter of *E. coli* (Fig. 4B). These results suggest that *rpoH*-dependent promoters are upregulated in the Fur mutant. In support of this, we also included the S1 mapping experiment of the *groESEL* promoter that we expect to be coregulated since it was previously reported in gonococcus as one of the *rpoH*-dependent genes (26, 37). Indeed, in *N. meningitidis* the *groESEL* genes are transcribed from a σ^{32} promoter, which is also upregulated in the *fur* mutant (Fig. 4A and B). From this finding we conclude that the heat shock genes are upregulated in the Fur mutant but not in response to iron limitation.

To verify whether the upregulation of heat shock genes in the Fur mutant is directly linked to the functioning Fur protein, we needed a mutant expressing a Fur protein but not responding to iron (a Fur-blind protein). To do this, we constructed two site-directed mutants with amino acid substitutions in the highly conserved amino acids that are thought to constitute the iron-binding site of the Fur protein (1, 6). We generated two mutant *fur* genes in which the histidine at position 92 or the arginine at position 91 had been substituted (H92I or D91M, respectively) and inserted these mutant genes or the wild-type gene under the control of the P_{lac} promoter into a heterologous location of the chromosome as described in Materials and Methods, generating Fko-H92I, Fko-D91M, and Fko-CII, respectively. Introduction of the wild-type gene or the D91M mutant gene restored the fast-growth phenotype of the mutant to almost wild-type levels as in the previous complemented mutant strain MC-Fko-C (8), whereas introduction of the H92I mutant gene did not change the small pinpoint colony phenotype of the *fur*-null mutant. We then analyzed the transcription of the P_{dnaK} and P_{groES} heat shock promoters and of the P_{tbp} iron-regulated promoter in these strains. As can be observed from Fig. 4C, the introduction of the wild-type gene or the D91M mutant gene restored repres-

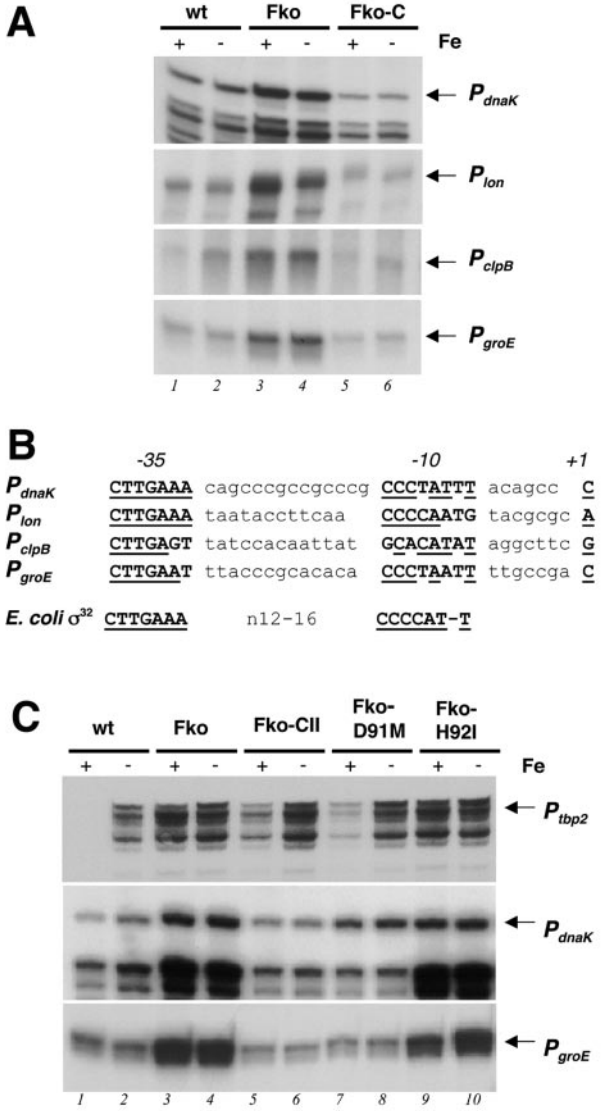

FIG. 4. (A) Upregulation of σ^{32} promoters in the MC-Fko *fur*-null mutant. An S1 nuclease assay on DNA probes corresponding to the *dnaK*, *lon*, *clpB*, and *groE* heat shock genes was conducted as described in Materials and Methods. Total RNA from MC58 (wt), *fur*-null mutant MC-Fko (Fko), and its complemented derivative MC-Fko-C (Fko-C) cells exposed to iron-replete conditions and (+) and iron-limiting conditions (−) was hybridized with radioactively labeled probes and digested with S1 nuclease. Bands corresponding to S1-resistant products were fractionated on denaturing gel and are indicated by arrows and labeled according to the respective promoters. (B) The promoter sequences inferred from experimentally mapped +1 transcriptional start site in the present study are reported and reveal −10 and −35 promoter sequences that share a high similarity to the *E. coli* σ^{32} promoter consensus. (C) Complementation of the *fur* mutant with site-directed mutants of the Fur protein. S1 nuclease assay on DNA probes corresponding to the *tbp2* classically iron-regulated gene and the *dnaK and groE* heat shock genes. Total RNA from MC58 (wt), MC-Fko (Fko), the Fko-CII, Fko-D91M, and Fko-H92I complemented derivative cells treated as described above was prepared and hybridized with radioactively labeled probes and digested with S1 nuclease. Bands corresponding to S1-resistant products were fractionated on denaturing gel and are indicated by arrows and labeled according to the respective promoters.

sion of the heat shock gene promoters and the iron-regulated repression of the P_{tbp} promoter, whereas the H92I mutant gene (lanes 9 and 10) did not. These results indicate that the mutation of arginine at position 91 results in a Fur protein which behaves like the wild type, whereas the mutation of the histidine at position 92 results in an iron-blind (-insensitive) Fur protein, which can no longer repress the *tbp* promoter and that no longer modulates the negative regulation of the heat shock promoters. Therefore, the ability of the Fur protein to sense and respond to internal concentrations of iron is a characteristic that can be directly linked to the slow-growth phenotype of the null mutant and also to the upregulation of the σ^{32}-dependent heat shock promoters.

To investigate further on the role of Fur in heat shock response, we analyzed transcription of the P_{dnaK} promoter by S1 mapping on RNA extracted from the wild type, the Fur-null mutant, and complementing cultures exposed to 37 or 42°C for 15 min. Although transcription from the P_{dnaK} promoter increases at 42°C in the wild-type and complementing strains, no apparent induction was detectable in the Fur mutant under the conditions tested (data not shown). This suggests that the Fur mutation leads to an altered heat shock response.

The *frpB* promoter as either representative or anomaly of classical Fur-regulated gene. The *frpB* (*fetA*) gene in our transcriptome experiments was the only gene to be significantly regulated in all four experiments, suggesting that it was upregulated in the *fur* mutant but, surprisingly, also upregulated on complementation. In addition, it was iron responsive in the wild type and also in the Fur mutant. Figure 5A shows the quantitative primer extension of the *frpB* transcript in the wild-type, *fur* mutant, and complemented mutant strains. As suggested by the transcriptome analysis, there is an upregulation of the *frpB* transcript upon exposure to iron limitation in the wild type (lanes 1 and 2), and the transcript is higher under iron-replete conditions in the *fur* mutant with respect to the wild type (lanes 3 versus 1). As described above, we also mapped two high-affinity Fur operators spanning from positions −62 to −96 and from positions −110 to −141 with respect to the translational start site of the gene. Mapping of the promoter by primer extension (Fig. 5A and B) identified the transcriptional start site, indicated as P_{frpB}, 85 bp upstream of the ATG start site of translation and, therefore, the Fur operators overlap the promoter elements of the *frpB* promoter. This result, along with upregulation in the mutant and in the iron chelation, suggests that it is a classically regulated promoter. In Fig. 5A we also include the S1 nuclease protection of the *tbp2* transcript, which is a classically regulated promoter, as positive control.

What is clearly apparent in Fig. 5A is that the *frpB* promoter is iron responsive in each strain tested and, furthermore, the overall amount of transcript in the complemented mutant Fko-C is significantly higher than in the other strains. However, the promoter sequence contains a tract of 11 C's between the −10 and −35 hexamers similar to the phase-variable promoter reported in the closely related *N. gonorrhoeae* (5). We therefore, sequenced the promoter regions in the different backgrounds and identified that indeed the length of the C tract was variable, with 10 instead of 11 C's in the Fko-C strain. This resulted in an optimal N_{17} spacing between the −10 and −35 hexamers of the σ^{70} promoter, which may account for a

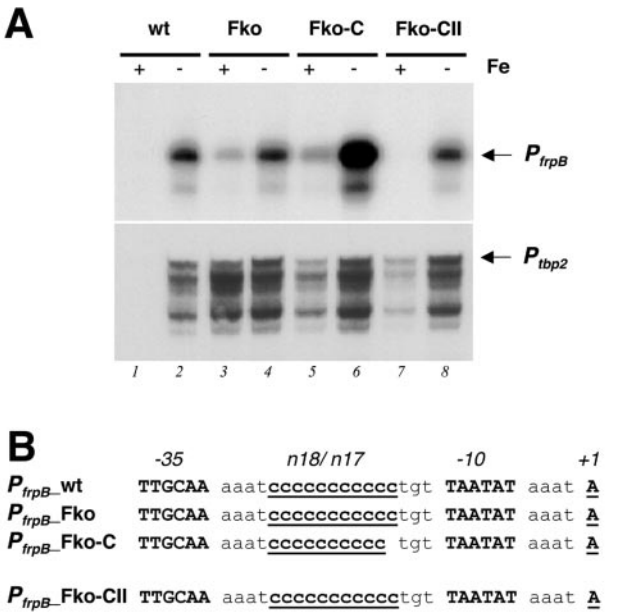

FIG. 5. Fur and iron regulation of *frpB* promoter. (A) Total RNA from MC58 (wt), *fur*-null mutant MC-Fko (Fko), and MC-Fko-C (Fko-C) and Fko-CII complemented derivative cells exposed to iron-replete conditions (+) and iron-limiting conditions (−) was prepared. Quantitative primer extension with an *frpB*-specific primer (frp-s2; Table S2 in the supplemental material) or an S1 nuclease protection assay on the *tbp2* gene probe was performed, and the elongated primer or S1-resistant products are indicated by arrows and labeled according to the respective promoters. (B) The *frpB* promoter regions were amplified from each of the strains described above and sequenced, and the sequences and elements inferred from the experimentally mapped +1 transcriptional start site in the present study are reported and reveal a phase-variable C tract between the −10 and −35 promoter elements in the strains.

difference in the basal promoter strength within the three backgrounds (Fig. 5B). In order to verify this, a second complementation strain Fko-CII, whose promoter sequence was identical to those of the wild type and the Fko mutant, was assessed for the expression of the P_{frpB}, and in this strain the transcription of P_{frpB} is restored to the wild-type levels (Fig. 5B, lanes 7 and 8), confirming that the promoter activity in the Fko-CI complementing strain is dependent on its phase status.

Under iron limitation, the P_{frpB} transcript is comparable in the wild-type, Fko, and Fko-CII strains (Fig. 5A, lanes 2, 4, and 8, respectively); however, whereas in the presence of iron the promoter is repressed to almost undetectable amounts in the wt and the Fko-CII complemented mutant (lanes 1 and 7), there is still an iron repression effect of P_{frpB} in the Fur-null mutant (lane 3 versus lane 4), albeit derepressed with respect to the Fur-expressing strains. This is the first evidence suggesting that there is a regulatory response to iron that is not Fur dependent in *N. meningitidis*.

DISCUSSION

Recent gene expression profiling studies identified sets of genes in *N. meningitidis* that respond to environmental signals such as heat shock, iron, and contact with host cells (10, 17, 19,

20, 25). In the present study, we used comparative transcriptome analysis of the Fur-null mutant to identify target genes affected directly and indirectly by this regulator, and as such we have begun to dissect the regulatory networks of the Fur protein and its role in global responses in *N. meningitidis*. We identified groups of genes whose expression is differentially modulated by the Fur protein, including genes previously reported to be iron regulated in *N. meningitidis* (19). Moreover, using the current approach we were able to distinguish genes that are regulated by iron in a Fur-independent manner and by Fur in an iron-independent manner.

We identify 83 genes in the Fur modulon, and 54 of these are in agreement with the iron-responsive genes reported in a previous microarray study (19) with a further 15 genes of the Fur modulon as additional members of operons of the first 54 and most likely cotranscribed and coregulated. In the previous study in *N. meningitidis*, Grifantini et al. (19) reported results obtained from a 5-h time course experiment of iron-starved cells treated with iron in which 233 genes responded at some point along the time course. Interestingly, of the above-mentioned Fur-modulated genes identified in our study using the Fur knockout, 88% (61 of 69) are members of two iron-responsive clusters, including 39 genes that were repressed continuously along the time course and 40 that were induced continuously by iron and were proposed to be most likely Fur-dependent genes. This highlights the strength of the current approach with the *fur*-null mutant rather than monitoring transcriptional responses during the growth cycle, which would lead not only to the identification of iron-regulated genes but also to a general response, as well as stress provoked by a prolonged exposure time of the cells to iron. Very recently, 203 iron-regulated genes have been identified in *N. gonorrhoeae* (13), and comparison of them to the 233 previously reported for *N. meningitidis* (19) showed that only 12 genes are similarly repressed by iron and 26 are commonly induced during growth by iron in these types of two bacteria. Although direct comparison of the results reported in the present study with previously reported investigation in *N. meningitidis* and *N. gonorrhoeae* is problematic since the experimental conditions used were different in each case, the general responses common to both previous studies as envisaged by the induction of ribosomal genes and the transcriptional machinery in the analyses are nonetheless absent from our list of genes. A comparative analysis using a Fur mutant of *N. gonorrhoeae* would prove a powerful tool to detect the core Fur-regulated genes that may be common to both pathogens.

We demonstrated that genes that are iron regulated in the wild type are also affected by the Fur mutation; furthermore, they no longer respond in the absence of the Fur protein and, consequently, are truly Fur mediated (Fig. 1). The one exception to that is the *frpB* gene, whose expression, being a phase-variable promoter, responded in an unorthodox fashion (Fig. 5). Furthermore, we were able to identify a small number of genes whose regulation on iron limitation is detectable only in the null mutant and may be due to general conditions in the cell that are affected by the Fur mutation, such as intrinsic iron levels, although this was not further investigated in the present study.

Moreover, we investigated biochemically the hierarchy of direct and indirect Fur-mediated mechanisms of control. Fur-mediated regulation may be at the direct level through high-affinity operators in the promoter regions as we demonstrated with the identification of 12 Fur operators in classical iron-repressed promoters through footprinting analysis (Fig. 3). In addition, the lack of evidence for direct high-affinity binding to some Fur-regulated promoters indicates that Fur may mediate indirect regulation of these genes. We strongly suspect that indirect mechanism of Fur regulation may be present in meningococcus either (i) at the posttranscriptional level through as-yet-unidentified sRNA systems analogous to that of *E. coli* and *P. aeruginosa* and/or (ii) through regulatory proteins that may be members of the *fur* regulon, for example, the AraC-like regulator nmb1879, which is shown to be Fur regulated (Table S1 in the supplemental material and Fig. 3).

Finally, in addition to iron-regulated genes, we identified a new set of genes that respond also to the Fur protein. We have determined that the upregulation of the heat shock σ^{32}-dependent promoters in the MC-Fko-null mutant is indeed connected to the fundamental role of Fur. This highlights the requirement for the linkage of the circuits of a global repressor and the heat shock genes. These are the chaperones and proteases, which constitute the "so-called" heat shock response and may be upregulated in bacteria in response to general stress conditions such as upshifts in temperature, osmotic shock, and other environmental stresses, which result in the accumulation of misfolded proteins (41). Our data suggest that the mutation of the Fur protein results in a large number of derepressed genes with constitutive high expression of many proteins, a large number of which are destined for the cell envelope and secretion. In the mutant, this may result in a large translational load on the cell, which in turn results in the induction of the heat shock response, albeit through a signal transduction pathway separate from that of the iron response. The interlinkage of circuits and subcircuits of gene regulatory networks, each performing a different biological function, is a logical and essential concept for any biological system and exemplifies the delicate balance that living organisms have evolved to achieve for each of their niches.

ACKNOWLEDGMENTS

We thank Guido Grandi for essential collaborations with microarrays analyses and Raffaele Ieva for invaluable help in carrying out some experiments and for useful discussions and criticisms. We are grateful to Catherine Mallia for editing and Giorgio Corsi for artwork.

This study was supported by Chiron and in part by a grant from the Italian Ministry of Education, University, and Research and by a grant from the University of Bologna (ex. 60%) to V.S.

REFERENCES

1. **Adrait, A., L. Jacquamet, L. Le Pape, A. Gonzalez de Peredo, D. Aberdam, J. L. Hazemann, et al.** 1999. Spectroscopic and saturation magnetization properties of the manganese- and cobalt-substituted Fur (ferric uptake regulation) protein from *Escherichia coli*. Biochemistry **38:**6248–6260.
2. **Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones.** 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27:**215–237.
3. **Baichoo, N., and J. D. Helmann.** 2002. Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. J. Bacteriol. **184:**5826–5832.
4. **Baichoo, N., T. Wang, R. Ye, and J. D. Helmann.** 2002. Global analysis of the *Bacillus subtilis* Fur regulon and the iron starvation stimulon. Mol. Microbiol. **45:**1613–1629.
5. **Carson, S. D., B. Stone, M. Beucher, J. Fu, and P. F. Sparling.** 2000. Phase variation of the gonococcal siderophore receptor FetA. Mol. Microbiol. **36:**585–593.
6. **Delany, I., G Spohn, A. B. F. Pacheco, R. Ieva, C. Alaimo, R. Rappuoli, and**

V. Scarlato. 2002. Autoregulation of *Helicobacter pylori* Fur revealed by functional analysis of the iron-binding site. Mol. Microbiol. **46:**1107–1122.
7. **Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato.** 2001. The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. Mol. Microbiol. **42:**1297–1309.
8. **Delany, I., R. Ieva, C. Alaimo, R. Rappuoli, and V. Scarlato.** 2003. The iron-responsive regulator Fur is transcriptionally autoregulated and not essential in *Neisseria meningitidis*. J. Bacteriol. **185:**6032–6041.
9. **Delany, I., R. Rappuoli, and V. Scarlato.** 2004. Fur functions as an activator and as a repressor of putative virulence genes in *Neisseria meningitidis*. Mol. Microbiol. **52:**1081–1090.
10. **Dietrich, G., S. Kurz, C. Hubner, C. Aepinus, S. Theiss, M. Guckenberger, U. Panzner, J. Weber, and M. Frosch.** 2003. Transcriptome analysis of *Neisseria meningitidis* during infection. J. Bacteriol. **185:**155–164.
11. **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.
12. **Dubrac, S., and D. Touati.** 2002. Fur-mediated transcriptional and posttranscriptional regulation of FeSOD expression in *Escherichia coli*. Microbiology **148:**147–156.
13. **Ducey, T. F., M. B. Carson, J. Orvis, A. P. Stintzi, and D. W. Dyer.** 2005. Identification of the iron-responsive genes of *Neisseria gonorrhoeae* by microarray analysis in defined medium. J. Bacteriol. **187:**4865–4874.
14. **Ernst, F. D., S. Bereswill, B. Waidner, J. Stoof, U. Mader, J. G. Kusters, E. J. Kuipers, M. Kist, A. H. van Vliet, and G. Homuth.** 2005. Transcriptional profiling of *Helicobacter pylori* Fur- and iron-regulated gene expression. Microbiology **151:**533–546.
15. **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.
16. **Geissmann, T. A., and D. Touati.** 2004. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. EMBO J. **23:**396–405.
17. **Grifantini, R., E. Bartolini, A. Muzzi, M. Draghi, E. Frigimelica, J. Berger, G. Ratti, R. Petracca, G. Galli, M. Agnusdei, M. M. Giuliani, L. Santini, B. Brunelli, H. Tettelin, R. Rappuoli, F. Randazzo, and G. Grandi.** 2002. Previously unrecognized vaccine candidates against group B meningococcus identified by DNA microarrays. Nat. Biotechnol. **20:**914–921.
18. **Grifantini, R., E. Frigimelica, I. Delany, E. Bartolini, S. Giovinazzi, S. Balloni, S. Agarwal, C. Genco, and G. Grandi.** 2004. Characterization of a novel *Neisseria meningitidis* Fur and iron-regulated operon required for protection from oxidative stress: utility of DNA microarray in the assignment of the biological role of hypothetical genes. Mol. Microbiol. **54:**962–979.
19. **Grifantini, R., S. Sebastian, E. Frigimelica, M. Draghi, E. Bartolini, A. Muzzi, R. Rappuoli, G. Grandi, and C. A. Genco.** 2003. Identification of iron-activated and -repressed Fur-dependent genes by transcriptome analysis of *Neisseria meningitidis* group B. Proc. Natl. Acad. Sci. USA **100:**9542–9547.
20. **Guckenberger, M., S. Kurz, C. Aepinus, S. Theiss, S. Haller, T. Leimbach, U. Panzner, J. Weber, H. Paul, A. Unkmeir, M. Frosch, and G. Dietrich.** 2002. Analysis of the heat shock response of *Neisseria meningitidis* with cDNA- and oligonucleotide-based DNA microarrays. J. Bacteriol. **184:**2546–2551.
21. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
22. **Holmes, K., F. Mulholland, B. M. Pearson, C. Pin, J. McNicholl-Kennedy, J. M. Ketley, and J. M. Wells.** 2005. *Campylobacter jejuni* gene expression in response to iron limitation and the role of Fur. Microbiology **151:**243–257.
23. **Ieva, R., C. Alaimo, I. Delany, G. Spohn, R. Rappuoli, and V. Scarlato.** 2005. CrgA, a member of the LysR family of transcriptional regulator of *Neisseria meningitidis*, is an inducible regulator acting as both a repressor and an activator of gene transcription. J. Bacteriol. **187:**3421–3430.
24. **Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle.** 1963. *Neisseria gonorrhoeae.* I. Virulence genetically linked to clonal variation. J. Bacteriol. **85:**1274–1279.
25. **Kurz, S., C. Hubner, C. Aepinus, S. Theiss, M. Guckenberger, U. Panzner, J. Weber, M. Frosch, and G. Dietrich.** 2003. Transcriptome-based antigen identification for *Neisseria meningitidis*. Vaccine **21:**768–775.
26. **Laskos, L., C. S. Ryan, J. A. Fyfe, and J. K. Davies.** 2004. The RpoH-mediated stress response in *Neisseria gonorrhoeae* is regulated at the level of activity. J. Bacteriol. **186:**8443–8452.
27. **Masse, E., and S. Gottesman.** 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **99:**4620–4625.
28. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74:**560–564.
29. **McHugh, J. P., F. Rodriguez-Quinones, H. Abdul-Tehrani, D. A. Svistunenko, R. K. Poole, C. E. Cooper, and S. C. Andrews.** 2003. Global iron-dependent gene regulation in *Escherichia coli*: a new mechanism for iron homeostasis. J. Biol. Chem. **278:**29478–29486.
30. **Merrell, D. S., L. J. Thompson, C. C. Kim, H. Mitchell, L. S. Tompkins, A. Lee, and S. Falkow.** 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. Infect. Immun. **71:**6510–6525.
31. **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.
32. **Ochsner, U. A., P. J. Wilderman, A. I. Vasil, and M. L. Vasil.** 2002. GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. Mol. Microbiol. **45:**1277–1287.
33. **Palyada, K., D. Threadgill, and A. Stintzi.** 2004. Iron acquisition and regulation in *Campylobacter jejuni*. J. Bacteriol. **186:**4714–4729.
34. **Rodriguez, G. M., M. I. Voskuil, B. Gold, G. K. Schoolnik, and I. Smith.** 2002. *ideR*, an essential gene in *Mycobacterium tuberculosis*: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. Infect. Immun. **70:**3371–3381.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
36. **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.
37. **Tauschek, M., C. W. Hamilton, L. A. Hall, C. Chromvarin, J. A. Fyfe, and J. K. Davies.** 2004. Transcriptional analysis of the *groESL* operon of *Neisseria gonorrhoeae*. Gene **189:**107–112.
38. **Tettelin, H., N. J. Saunders, J. Heidelberg, A. C. Jeffries, K. E. Nelson, J. A. Eisen, K. A. Ketchum, D. W. Hood, J. F. Peden, R. J. Dodson, W. C. Nelson, M. L. Gwinn, R. DeBoy, J. D. Peterson, E. K. Hickey, D. H. Haft, S. L. Salzberg, O. White, R. D. Fleischmann, B. A. Dougherty, T. Mason, A. Ciecko, D. S. Parksey, E. Blair, H. Cittone, E. B. Clark, M. D. Cotton, T. R. Utterback, H. Khouri, H. Qin, J. Vamathevan, J. Gill, V. Scarlato, V. Masignani, M. Pizza, G. Grandi, L. Sun, H. O. Smith, C. M. Fraser, E. R. Moxon, R. Rappuoli, and J. C. Venter.** 2000. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science **287:**1809–1815.
39. **Thompson, S. A., L. L. Wang, A. West, and P. F. Sparling.** 1993. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. J. Bacteriol. **175:**811–818.
40. **Wilderman, P. J., N. A. Sowa, D. J. FitzGerald, P. C. FitzGerald, S. Gottesman, U. A. Ochsner, and M. L. Vasil.** 2004. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc. Natl. Acad. Sci. USA **101:**9792–9797.
41. **Yura, T., and K. Nakahigashi.** 1999. Regulation of the heat-shock response. Curr. Opin. Microbiol. **2:**153–158.