

Identification of the Iron-Responsive Genes of *Neisseria gonorrhoeae* by Microarray Analysis in Defined Medium†

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Received 24 January 2005/Accepted 11 April 2005

To ensure survival, most bacteria must acquire iron, a resource that is sequestered by mammalian hosts. Pathogenic bacteria have therefore evolved intricate systems to sense iron limitation and regulate gene expression appropriately. We used a pan-*Neisseria* microarray to examine genes regulated in *Neisseria gonorrhoeae* in response to iron availability in defined medium. Overall, 203 genes varied in expression, 109 up-regulated and 94 down-regulated by iron deprivation. In iron-replete medium, genes essential to rapid bacterial growth were preferentially expressed, while iron transport functions, and predominantly genes of unknown function, were expressed in low-iron medium. Of those TonB-dependent proteins encoded in the FA1090 genome with unknown ligand specificity, expression of three was not controlled by iron availability, suggesting that these receptors may not be high-affinity transporters for iron-containing ligands. Approximately 30% of the operons regulated by iron appeared to be directly under control of Fur. Our data suggest a regulatory cascade where Fur indirectly controls gene expression by affecting the transcription of three secondary regulators. Our data also suggest that a second MerR-like regulator may be directly responding to iron availability and controlling transcription independent of the Fur protein. Comparison of our data with those recently published for *Neisseria meningitidis* revealed that only a small portion of genes were found to be similarly regulated in these closely related pathogens, while a large number of genes derepressed during iron starvation were unique to each organism.

Iron plays a prominent role in a variety of metabolic pathways, making it essential for life in most organisms. Iron is a cofactor for proteins such as catalase, cytochromes, hemoglobin, metalloflavoproteins, myoglobin, ribonucleotide reductase, and peroxidase (43). Despite its essential importance to metabolism, iron is paradoxically a difficult nutrient to manage. Under biological conditions (oxygenated, aqueous, neutral pH), iron quickly becomes oxidized to the ferric state (Fe³⁺) (5). Ferric iron reacts rapidly with water to form insoluble oxy-hydroxide complexes that are not metabolizable. Further, the ferric iron is extremely toxic due to the ability to drive free radical production via the Fenton reaction. This can lead to DNA strand breakage, lipid peroxidation, and protein denaturation (73). To avoid these potential problems, mammalian physiology has evolved a variety of mechanisms to sequester iron, suppress its redox reactivity, and maintain solubility. This is achieved by incorporating iron intracellularly in proteins such as ferritin and hemoproteins and binding iron extracellularly by lactoferrin and transferrin (43). This results in free iron concentrations of approximately 10⁻¹⁸ M, a concentration that will not sustain growth for most microorganisms, which typically require an iron concentration of ~10⁻⁶ M to sustain life.

Thus, mammalian physiology nonspecifically suppresses the growth of many potential pathogens by withholding iron, commonly referred to as “nutritional immunity” (78).

To be a successful pathogen, bacteria must therefore evolve methods to acquire iron from the host. The sexually transmitted disease pathogen *Neisseria gonorrhoeae* is typically seen as a pathogen of mucosal surfaces, predominantly associated with symptomatic urethritis in males and an often asymptomatic endocervicitis in women. For women, this can progress to pelvic inflammatory disease and salpingitis, potentially resulting in ectopic pregnancy and sterility (15, 22). Iron is sequestered on the urogenital mucosal surface by lactoferrin and transferrin (1), while during menses hemoglobin is released into the environment (36). The gonococcus, an obligate human pathogen, has evolved several iron transport systems that bind human iron carrier proteins and remove the iron directly from these ligands. Thus, the gonococcus possesses receptors that bind and remove iron from human hemoglobin (HmbR), hemoglobin-haptoglobin complexes (HpuAB), lactoferrin (LbpAB), and transferrin (TbpAB) (58). The gonococcus also can use exogenously produced enterobactin (a siderophore) via the FetA receptor (20); presumably, this may be important for allowing the organism to metabolize catecholate siderophores found in mixed microbial environments, such as the female urogenital tract. The lactoferrin and transferrin receptors are extremely important for gonococcal survival in vivo. In infection of male volunteers, both *lbpAB*⁺ *ΔtbpAB* and *ΔlbpAB* *tbpAB*⁺ strains were capable of initiating disease where a *ΔlbpAB* *ΔtbpAB* strain was not (3, 24).

Thus, it is not surprising that iron availability is often found to be a key environmental signal that controls virulence in a

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

variety of pathogens. For instance, iron availability regulates expression of *Corynebacterium diphtheriae* diphtheria toxin, *Shigella dysenteriae* Shiga toxin, and *Pseudomonas aeruginosa* exotoxin A (43). Microarray analysis of *Helicobacter pylori* revealed iron-regulated expression of the virulence genes *cagA*, *napA*, and *vacA* (47). Early studies demonstrated that iron starvation enhanced capsular polysaccharide biosynthesis in *Neisseria meningitidis* (45) and was associated with increased virulence in mice (14, 37).

In gram-negative and gram-positive bacteria, transcription of genes involved in iron acquisition and virulence are often under the control of the ferric uptake regulator (Fur) protein. Fur, in the presence of ferrous iron, binds as a dimer to DNA regulatory sequences (Fur boxes), which typically results in the repression of transcription of many iron-repressible genes (25). Several gonococcal iron-repressible promoters in the gonococcus, including *fetA*, *hmbR*, *fbpABC*, *hpuAB*, *lbpAB*, and *tbpAB*, have this consensus Fur box (33, 42). Thomas and Sparling found 32 proteins by two-dimensional gel electrophoresis that were directly controlled by the gonococcal *fur* gene (72). However, aside from this little is known about the gonococcal iron response regulon. To begin to address this, we have used a pan-*Neisseria* microarray to analyze the steady-state, mid-log gene expression profiles of gonococci in response to iron availability.

MATERIALS AND METHODS

Bacterial strains and growth. *N. gonorrhoeae* strain FA1090 was kept as a freezer stock at -80°C in GC medium (Difco) supplemented with 20% glycerol. Prior to growth experiments, FA1090 was inoculated on GCB agar (Becton Dickinson) supplemented with IsoVitalX (Becton Dickinson) and grown at 37°C , 5% CO_2 for 16 h. Several nonpiliated colonies were transferred to a fresh GCB agar plate and grown for an additional 16 h. Nonpiliated organisms were used in this study, as piliated bacteria clump significantly in broth, making the measurement of growth based on optical density difficult. Piliation was determined based on criteria established by Swanson et al. (71). These bacteria were used to inoculate Chelex-treated (Bio-Rad) chemically defined medium (CDM) broth in acid-washed glassware (27). The organisms were allowed to undergo one doubling in CDM without the addition of $10\ \mu\text{M}$ iron (CDM-0) to deplete the organisms' internal iron pools. The cultures were then diluted threefold (20-ml total volume) in either CDM plus $10\ \mu\text{M}$ FeNO_3 (CDM-10) or CDM-0 and grown to stationary phase. Ten-milliliter samples for microarray experiments were taken at mid-log phase (see Fig. 1, below). Each growth experiment was performed four times.

RNA extraction. An aliquot of bacterial culture was immediately added to an ice-cold 95% phenol-5% ethanol (EtOH) mixture at a 1:10 (vol/vol) ratio and gently inverted to arrest bacterial growth and preserve RNA integrity. Samples were allowed to remain on ice for several minutes and then spun at $5,000 \times g$ for 10 min to pellet the cells. Cells were resuspended in TE (10 mM Tris-HCl, 1 mM EDTA), pH 8.0, lysed by addition of a 1/10 volume of 10% sodium dodecyl sulfate (SDS), and incubated at 64°C for 2 min. A 1/10 volume of 1 M sodium acetate, pH 5.2, was added, and RNA was isolated by extraction, using an equal volume of prewarmed water-saturated phenol followed by an extraction using an equal volume of chloroform. Each extraction mixture was incubated at 64°C for 6 min with inversion every minute, chilled, and centrifuged at $15,000 \times g$ for 10 min, 4°C . After the second extraction, bulk RNA was precipitated from the aqueous layer using a standard EtOH precipitation (80). The RNA was then resuspended in $10\ \mu\text{l}$ distilled water (dH_2O) and treated with 20 U RNase inhibitor (Roche) and 5 U RNase-free DNase (Invitrogen) in a 50- μl volume for 15 min, followed by extraction with the RNeasy Mini kit (QIAGEN) according to the manufacturer specifications. The DNase treatment was repeated, and the RNA concentration and integrity were determined by measuring the optical density at 260 nm and examined by 1% agarose gel electrophoresis using Tris-acetate-EDTA buffer (59). PCR against the *porI* locus of FA1090 was performed to assess DNA contamination. If necessary, DNase treatment was repeated to ensure that no detectable chromosomal DNA was in each RNA preparation.

Construction of pan-*Neisseria* DNA microarray. The detailed description of the pan-*Neisseria* microarray will be published elsewhere (60). Briefly, the published genome sequences of meningococcal strains Z2491 and MC58 and gonococcal strain FA1090 were used to generate a PCR amplicon-based microarray. Open reading frames (ORFs) that shared 90% or greater nucleotide sequence identity between at least two of the three genomes were used to design a set of "core" amplicons using FA1090 DNA. Strain-specific genes were amplified using the respective DNA from each meningococcal or gonococcal strain. Amplified ORF targets ranging from 150 to 450 bp in length were resuspended in 50% (vol/vol) dimethyl sulfoxide and spotted in triplicate onto GAPS slides (Corning). The printed microarray contains 99.6% of all the FA1090 annotated features.

Microarray hybridization and analysis. Equal concentrations (16 μg) of test RNA extracted from cells grown under high- or low-iron conditions were used to set up standard reverse transcription reactions using random nonamers (Integrated DNA Technologies), SuperScript II (Invitrogen), and aminoallyl-dUTP (Sigma) to generate cDNA. The cDNA was purified using Micron 30 spin columns (Millipore) and indirectly labeled with a monofunctional *N*-hydroxysuccinimide ester Cy3- or Cy5-containing dye (Amersham). Reaction mixtures were combined, and unincorporated dye was removed using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer specifications. The labeled cDNA was vacuum concentrated without heat prior to suspension in 35 μl of hybridization buffer (25% formamide, $5 \times \text{SSC}$ [$1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS) containing 1 μl of a 10-mg/ml solution of sheared salmon sperm DNA. The probe was heated to 99°C for 2 min, briefly bench top cooled, added to the back of a prewarmed Hybri-slip (Sigma), placed on a prehybridized array (25% formamide, $5 \times \text{SSC}$, 0.1% SDS, and 1% bovine serum albumin for 45 min at 42°C), and enclosed in a prewarmed glass array hybridization chamber (Ambion). After an 18-h hybridization, the array was removed from the hybridization chamber and stringently washed in 1-liter volumes with stir bar agitation as follows: $2 \times \text{SSC}$ and 0.1% SDS for 5 min at 42°C , $0.1 \times \text{SSC}$ and 0.1% SDS for 10 min at room temperature, and three washes of $0.1 \times \text{SSC}$ for 1 min at room temperature. The slide was briefly washed with dH_2O , dried by spinning in a microcentrifuge with slide adapter (Telechem International), and scanned with a ScanArray ExpressHT microarray scanner (Perkin-Elmer). The brightest spots on each array (rRNA) were used to establish a scan setting (80% of signal maximum) for each individual array. Photomultiplier tube gain and laser settings were varied from array to array to achieve this setting.

After scanning, the data were analyzed using the ArrayVision 7.0 software package (Imaging Research). Actual signal intensity was calculated by subtracting the local mean background intensity of each spot from the mean signal intensity of each spot. Any spot with an adjusted signal intensity lower than its mean background intensity plus two standard deviations in both channels was excluded from further analysis; this removed 445 genes from consideration. Data were formatted for import into GeneSpring version 4.2.1 (Silicon Genetics) and normalized per spot and per chip using Lowess transformation. The data of each spot represent the average of four experiments. The Student *t* test was then applied to the data to find genes with highly reproducible gene expression. Differentially expressed genes were defined as genes in which the normalized ratios were >1.5 -fold (CDM-0/CDM-10 for iron-repressed genes or CDM-10/CDM-0 for iron-responsive genes) (38, 63, 69) with a *P* value of <0.02 . Genes with a *P* value of >0.02 were excluded from consideration, resulting in the removal of 87 additional genes from further analysis. Raw array data are stored at the National Center for Biotechnology Information's GEO database (7) under the series accession number GSE2147.

Confirmation of pan-*Neisseria* microarray data. Verification of gene expression levels was measured by real-time quantitative PCR (qRT-PCR). Five derepressed genes (*fetA*, *hemO*, *tbpA*, *tbpB*, and *cah*), four repressed genes (L11, NGO0295, NGO1246, and *secY*), and two stably expressed genes (NGO1996 and *porI*), as well as the four expressed, unidentified ligand TonB-dependent genes (NGO0021, NGO0553, NGO0952, and NGO1205), were chosen for this confirmatory study. Oligonucleotide primers (Table 1) were designed with PRIMER EXPRESS software (PE Biosystems) and were tested prior to qRT-PCR analysis to confirm that they resulted in a single amplicon of the proper size when genomic DNA was used as template. After RNA isolation, 2 μg of total RNA was reverse transcribed using SuperScript II with 250 ng of random nonamers according to the manufacturer's specifications (Invitrogen). After reverse transcription, 5 ng of cDNA along with 250 nM of each primer pair was subjected to qRT-PCR using an ABI Prism 7700 sequence detection system (Applied Biosystems) and SYBR Green master mix, according to manufacturer specifications (PE Biosystems). The relative expression level of each gene was normalized to *porI*, the gene encoding a major outer membrane protein of *N. gonorrhoeae* whose expression level remained stable irrespective of iron availability (data not

TABLE 1. Oligonucleotide primers for RT-PCR microarray validation

Gene	Forward primer	Reverse primer
<i>fetA</i>	ATCCAACACCAATTGGCGTAC	CATCGGCGGAATAGCGTTF
<i>hemO</i>	GAAACTGCAATCCGTGTTC	CATCGTATCGCGCCATGTATT
<i>tbpA</i>	AGACACTTGGGCGGATTATG	GAACAGTGCCTGTGCTGAAA
<i>tbpB</i>	TGGATGCGGTTGAATTGACA	GGACAGGAGCGGAATCATAATG
<i>cah</i>	GCGTATCATGGTTGGTGTG	CACGTGCATTACAGGGCTGTAC
L11	TGATGGAACACGGTGCACCTT	TGCTGCCAGATTGTTCGGT
NGO0295	GCGGTTTATCGGCATTCTGAT	CTGCCTGATTTTCGGTGTGTT
NGO1246	TCTGCCGAAACGCAAAAATC	AAAGCACCGCCGTAATCTCA
<i>secY</i>	TGGTGCCCATATACCCGTACCT	TCTAACGACCCCGGAAAAC
NGO1996	TATCCCGTTGTGCAGATGTCC	ATATGCCTGCCCTGTATCCGT
<i>porI</i>	TGTCGGTACGCTACGATTCTCC	AGCCAACGTGGTAAGATTCGC
NGO0021	GGCAGATACAGGCAGGCTAC	GGTAGGCGGTGAAGAGTTTG
NGO0553	AAAGACGCACAGGCAGTGTAT	GCCGTCTGTAATGTTCGGTTF
NGO0952	GATCAGGACAACGGGCTTTA	GCCCTTTATCGCCCTTAAAC
NGO1205	TCAACGCAAGACTGTACGC	CCGACTTCAAAGGTGTTGGT

shown). Quantitative values were obtained by using the comparative threshold cycle ($\Delta\Delta C_T$) method, as recommended by Applied Biosystems. Each gene was assayed in duplicate from each of the four pools of microarray-isolated RNA, and the mean C_T value was used for further comparison. Gene expression was calculated as $2^{-\Delta\Delta C_T}$, where values for $\Delta\Delta C_T$ ($\Delta C_{T,Fe-} - \Delta C_{T,Fe+}$) were obtained by subtracting the mean C_T value of the specific gene tested from the mean C_T value of the *porI* gene. Controls to ensure that there was no contaminating genomic DNA in the cDNA (cDNA reaction mixtures generated using RNA with no reverse transcriptase) as well as controls to identify any possible primer dimer artifacts (qRT-PCR mixtures containing primers alone) were also run with each set of qRT-PCRs.

Bioinformatics. To examine the potential role of Fur in the transcriptional regulation of each gene, it was first necessary to predict the location of putative operons in the FA1090 genome, so that presumptive promoter regions in the genome could be identified. First, GeSTer was used to predict transcriptional termination sites within the gonococcal genome (75). ORFs were then assigned to a putative operon based on proximity to nearby genes (within 100 bp), the presence of a predicted transcriptional termination site, and the predicted direction of the surrounding ORFs (see Table S3 in the supplemental material). For the first differentially expressed gene of each predicted operon, a Perl script was written to compile the 150 bp immediately upstream of the translational start into a file in FASTA format. These sequences were analyzed by using MEME (6) for both the *Escherichia coli* (GATAATGATAATCATTATC) (70) and *N. gonorrhoeae* (GATAAT-ATAATAATTATC-TTT) (33) Fur-binding consensus sequences. Putative Fur-binding sites were then placed in a multiple sequence alignment using ClustalX, and a consensus sequence logo was generated using Seqlogo (26).

The predicted amino acid sequences of the differentially expressed genes were collected in FASTA format in their unprocessed and processed forms. A Perl script was written to generate both SignalP (50) and TMHMM (65) data on each protein sequence. Additionally, each hypothetical and conserved hypothetical protein was subjected to Pfam analysis by comparison of the protein translation to the Pfam database (64). The Institute for Genomic Research Comprehensive Microbial Resource was consulted for functional classifications (53). To compare gene expression between *N. gonorrhoeae* and *N. meningitidis*, Perl scripts were used to create protein databases of both the entire genomes and the iron-responsive, differentially regulated genes of *N. gonorrhoeae* and *N. meningitidis*. These databases were then compared by BLASTP comparisons (2). Orthologues were identified as having a 70% identity over the entire span of the predicted amino acid sequences of both proteins.

RESULTS AND DISCUSSION

Microarray analysis of *N. gonorrhoeae* FA1090 steady-state mRNA levels in response to iron availability. To study the gene expression profile of the gonococcus in response to iron availability, we used Chelex-treated CDM rather than resorting to the use of chelators to withhold iron. We were concerned that chelators might introduce confounding variables that could

complicate an attempt to analyze iron-specific responses. Chelators such as 2,2-dipyridyl diffuse into the cell and could affect intracellular events by chelating divalent cations other than iron (62). In addition to having a strong affinity for ferrous iron, 2,2-dipyridyl can also chelate Mg^{2+} (46), Mn^{2+} (55), and Zn^{2+} (66). Similarly, although Desferal probably withholds iron from *Neisseria* by binding it extracellularly, it also can bind copper, nickel, and zinc (16). Such effects may be enough to trigger noniron, ion-specific regulatory events or otherwise affect gene expression in such a way as to complicate our analysis of iron-specific responses. CDM is a modification of the Morse and Bartenstein medium (49) developed by Dyer et al. to study transferrin-specific iron uptake in the meningococcus (27). This medium has been successfully utilized in the study of iron acquisition of both pathogenic *Neisseria* species. CDM has been used to study *Neisseria* iron acquisition from aerobactin (79), heme-bound iron (28), lactoferrin (10, 12), and transferrin (13, 23, 44) and the role of three putative TonB-dependent outer membrane proteins in iron transport (74). CDM-0 supported limited growth (Fig. 1). Bacteria grown in CDM-0 versus CDM-10 had a slower doubling time (approximately 90 min versus 60 min) and lower optical density during mid-log phase (0.38 ± 0.03 versus 0.64 ± 0.03). Cells were taken directly from these broth cultures and mixed with buffer-saturated phenol to freeze the mRNA population. Bulk RNA was then prepared and either labeled for microarray hybridization or used in RT-PCR experiments to validate the microarray data.

The microarray data were processed as described in Materials and Methods. We detected an alteration in steady-state mRNA levels of 203 genes, representing slightly over 10% of the total ORF content of FA1090. Of these 203 genes, 109 genes were repressed by high iron (see Table S1 in the supplemental material) and 94 were induced by growth in high iron (see Table S2 in the supplemental material).

Validation of microarray data by dilution RT-PCR. We used qRT-PCR to validate the microarray data (Fig. 2), analyzing transcript levels for 14 genes. These included genes whose transcripts appeared to be derepressed (*fetA*, *hemO*, *tbpA*, *tbpB*, and *cah*), repressed (L11, NGO0295, NGO1246, and *secY*), or unresponsive (NGO1996) in the absence of iron. In

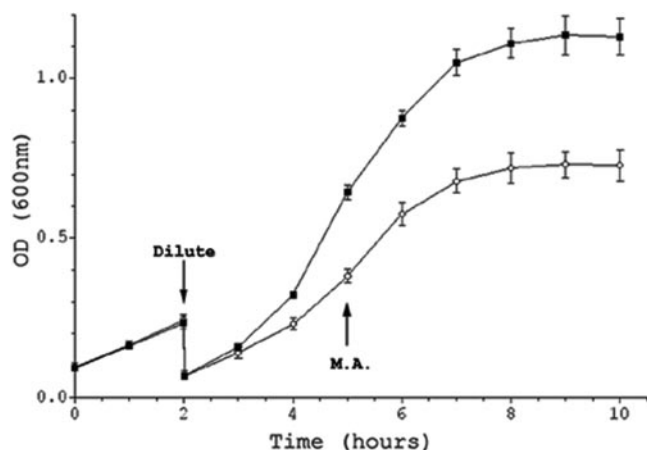


FIG. 1. Growth of *Neisseria gonorrhoeae* FA1090 in CDM grown with (CDM-10 [■]) or without (CDM-0 [○]) 10 μ M ferric nitrate. Cultures were diluted after first doubling, and a 10-ml sample for microarray analysis (M.A.) was taken at mid-log as indicated on the graph. This figure is the average of four independent experiments, with standard deviations shown as error bars; at some time points, the error was so small that it cannot be seen on this figure. OD, optical density.

each instance, the RT-PCR results correlated well ($R^2 = 0.95$) with those obtained from the microarrays, suggesting that the microarray results were an accurate reflection of the gene expression profile in *N. gonorrhoeae* FA1090.

Response to growth under low-iron conditions. Figure 3 represents the number of differentially expressed genes grouped by functional categories. Interestingly, genes encoding transport functions constituted the largest category of genes with known or predicted function (nine genes total) found to be derepressed under iron-depleted growth conditions (Fig. 3, column 7). Of these nine genes, seven encode the proteins ExbB, essential for TonB-dependent energy transduction (11), FbpA, an iron-binding periplasmic protein (8), FetAB, which is involved in the binding and transport of ferric enterobactin (20), the transferrin receptor TbpAB (4), and TdfG, an iron-

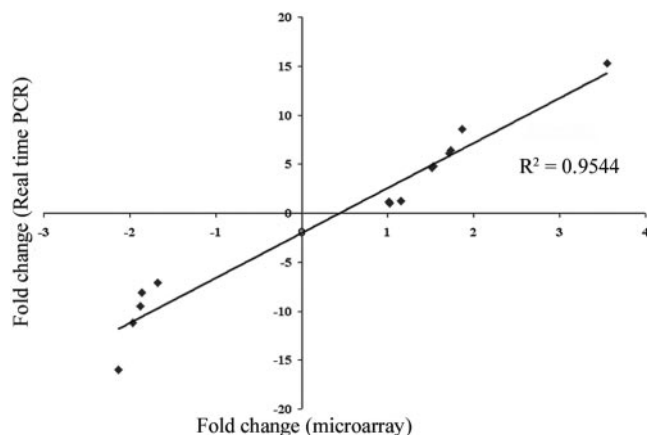


FIG. 2. Validation of microarray results by qRT-PCR. Shown are five genes derepressed, four genes repressed, and one gene unresponsive to iron deprivation, as well as the four expressed, TonB-dependent protein genes of unknown ligand specificity. Fold changes are shown [(iron-depleted expression level)/(iron-replete expression level)].

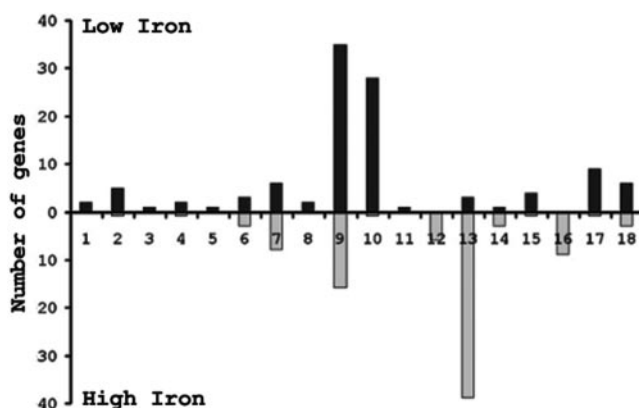


FIG. 3. Differentially expressed genes categorized by functional classification according to TIGRFAM designations. Columns: 1, amino acid biosynthesis; 2, biosynthesis of cofactors; 3, cell envelope; 4, cellular processes; 5, central intermediary metabolism; 6, DNA metabolism; 7, energy metabolism; 8, fatty acid metabolism; 9, conserved hypothetical proteins; 10, hypothetical proteins; 11, other; 12, protein fate; 13, protein synthesis; 14, purines, pyrimidines, nucleosides, and nucleotides; 15, regulatory functions; 16, transcription; 17, transport; 18, unknown function. The solid bars and shaded bars represent genes up-regulated and down-regulated in the absence of iron, respectively.

repressible TonB-dependent family protein of as-of-yet-undetermined function (74). FbpA, FetAB, TbpAB, and TdfG have been previously reported to be iron-repressible proteins (9, 31, 68, 74). While we observed a derepression of expression of many gonococcal iron-repressible proteins, we did not observe all that may have been anticipated. For instance, we did not observe differences in expression of the *hmbR* or the *hpuAB* receptors in response to iron availability. This was expected; the *hpuAB* operon is phase variable by slipped-strand mispairing in a poly(G) tract in the *hpuA* gene (41) and is phase varied "off" in FA1090. Similarly, the FA1090 *hmbR* gene has multiple frameshifts that inactivate protein expression. Since mRNA from untranslated frameshifted genes is less stable than transcripts being translated (51), the apparent lack of *hpuAB* and *hmbR* expression was probably masked by mRNA instability.

A number of other genes derepressed under low-iron conditions are predicted to encode potential virulence determinants or to be involved in DNA transformation. NGO0788 encodes a putative macrophage infectivity potentiator protein (*mip*), similar to one found in *Legionella pneumophila*. Knockouts of *mip* attenuate *Legionella* mutants infecting human mononuclear phagocytes and lung epithelial cells (39). NGO1718 encodes a putative MviN-like protein. Deletion of this gene in *Salmonella enterica* serovar Typhimurium attenuates mouse infectivity (19). The four identical *comE* genes within the FA1090 genome also were derepressed under low-iron conditions, although we could not discriminate between these in this assay. The *comE* loci are necessary for DNA-mediated transformation (21). Our observations are consistent with those of Serkin and Seifert, who showed that iron-starved gonococci have an increased transformability and increased, RecA-independent DNA recombination and repair activity (61). Also consistent with this observation is the derepression

TABLE 2. SignalP and Pfam analysis of gonococcal iron-regulated conserved hypothetical proteins^a

Pfam family	Special characteristic	<i>N. gonorrhoeae</i> CHP ORF(s)
NA	Contain a signal peptide	NGO0165, NGO0449, NGO0757, NGO0787, NGO0853, NGO0895, NGO1063, NGO1168, NGO1237, NGO1471, NGO1559, NGO1622, NGO1656, NGO1686
PF01551	Zinc metalloproteinase	NGO1686
PF01430	Molecular chaperone	NGO1189
PF00582	Universal stress protein	NGO0959
PF01126	Heme oxygenase	NGO1318
PF02498	BRO family protein	NGO1652
PF00691	OmpA family protein	NGO1559
PF01722	BolA family protein	NGO1657

^a Abbreviations: NA, not applicable; CHP, conserved hypothetical protein.

of NGO0173, which encodes a putative very short patch DNA repair endonuclease.

The largest number of iron-repressed loci encoded hypothetical and conserved hypothetical proteins, accounting for 58% (63 of 109) of the total. This also was observed in *H. pylori*, *N. meningitidis*, and *Pasteurella multocida* (35, 47, 52). SignalP analysis (50) predicted that 13 conserved hypothetical and 2 hypothetical proteins could be exported. Further examination of these 15 genes for the presence of α -helices using TMHMM analysis (65) revealed that NGO0449 and NGO1471, both conserved hypothetical proteins, were predicted to contain three and two transmembrane helices, respectively. This indicates that these genes probably encode proteins that localize to the inner membrane. The remaining 13 genes may possibly encode proteins that are either periplasmic or associated with the outer membrane. In addition to those observations mentioned above, this suggests that iron starvation may significantly affect the antigenic mosaic on the surface of the gonococcus. Pfam analysis of these hypothetical proteins (Table 2) (64) revealed four conserved hypothetical proteins with functional domains. In one particular instance, the conserved hypothetical gene NGO1318 encodes a PigA-like protein which in *Pseudomonas aeruginosa* functions as a heme oxygenase (56). PigA is responsible for liberating iron from heme, yielding biliverdin and carbon monoxide. NGO1318 shares 84% identity with the experimentally proven heme oxygenase (*hemO*) of *N. meningitidis* (81). Cleavage of heme to release iron serves as an iron source for the gonococcus (81).

Expression profiles for genes encoding TonB-dependent proteins. In addition to FetA, HmbR, HpuB, and TbpA (see above), strain FA1090 harbors five additional genes encoding TonB-dependent proteins. These ORFs include NGO0021 (*tdfF*), NGO0553 (*tdfG*), NGO0560, NGO0952 (*tdfH*), and NGO1205. Typically, gram-negative TonB-dependent proteins are high-affinity receptors for iron-carrying ligands, and the transcription of these iron transport proteins is generally repressed by iron (58). The lone exception to this is the *E. coli* BtuB protein, which transports vitamin B₁₂ and whose expression is not iron responsive (18). Recently, Turner et al. examined expression of *tdfF*, *tdfG*, and *tdfH* by Western blotting and found that only *TdfG* expression was iron repressible. Similarly, we noticed an increase in the transcript levels of the *tdfG* gene in CDM-0 (see Table S1 in the supplemental material [microarray fold change of 1.58]) (Fig. 2, qRT-PCR fold change of 4.8), and expression of *tdfF* and *tdfH* was unaltered

in response to iron availability. Assuming that derepression of a TonB-dependent protein in response to iron starvation implies a role in iron transport, then our data conversely suggest that *tdfF* and *tdfH* may be high-affinity receptors for important ligands other than iron carriers, since their expression was unresponsive to iron availability. Strikingly, expression of NGO1205 was significantly enhanced by growth under high-iron conditions (see Table S2 in the supplemental material [microarray fold change of -1.67]) (Fig. 2, qRT-PCR fold change of -7.1), suggesting that this protein also is a high-affinity transporter for a noniron ligand. We could not detect expression of NGO0560; NGO0560 contains multiple frameshifts that block translation, most likely rendering the transcript unstable and difficult to detect (51).

Response to growth under high-iron conditions. In general, genes encoding proteins involved in protein synthesis, energy metabolism, and transcription were preferentially expressed under high-iron conditions, suggesting an overall increase of cellular metabolism (Fig. 3). The expression profile of these genes is consistent with an increased growth rate in iron-containing medium compared to iron-restricted medium (Fig. 1). Members of the translational apparatus accounted for the largest functional category induced in high iron, accounting for almost half of the genes regulated in this manner. This included ribosomal proteins and translation factors, such as initiation factors IF-2 (*infB*) and IF-3 (*infC*) and elongation factors G (*fusA*) and TS (*tsf*). Consistent with this, *secE* and *secY*, encoding components of the protein translocation machinery, and *dnaJ*, encoding a protein chaperone, were also induced.

This increased level of protein synthesis would dictate a higher energy requirement for the organism. This is reflected in the microarray data. The putative membrane-associated proteins involved in electron transport, *nqrC* (NGO1415) and *nqrF* (NGO1418), along with *nqrA* (NGO1413) were induced by growth in CDM-10. The NQR complex is a sodium pump coupling electron transfer (NADH to ubiquinone) to the transport of Na⁺ across the periplasmic membrane (54). Complex I, another proton-pumping NADH:ubiquinone oxidoreductase, is a similar system (77) and is likewise regulated in an iron-dependent manner, as seen by the induction of *nuoD* (NGO1748). *NqrF*, in addition to containing binding motifs for NADH and FAD, also contains an iron-sulfur center (67). In complex I, NuoB has been shown to contain an iron-sulfur complex (30).

In contrast to the iron-repressible gene set, the number of conserved hypothetical and hypothetical genes induced by high

iron was limited. Only 1 hypothetical and 16 conserved hypothetical proteins were induced by high iron. SignalP analysis suggested that only one of these genes, NGO1686, encoded a protein with a putative signal sequence. None of these proteins was predicted to contain transmembrane α -helices by TM-HMM analysis. Pfam analysis did reveal that NGO1686, encoding a conserved hypothetical protein, contains a motif similar to other zinc metallopeptidases. Another conserved hypothetical protein, encoded by NGO1189, putatively contains an Hsp33 motif. Hsp33, a redox-regulated chaperone, contains four cysteines (all present in NGO1189) which are oxidized to form disulfide bonds, resulting in its activation. Again, this may be consistent with the increased protein synthetic capacity of the organism under high-iron conditions. Notably, genes involved in amino acid and cofactor biosynthesis were not appreciably induced under high-iron conditions. This is likely due to the fact that CDM is an extremely rich, defined medium that supplies every cofactor and amino acid necessary for growth.

Mechanisms of iron regulation. We assigned the 203 iron-responsive gonococcal genes to 153 putative operons (mono- or polycistronic) (see Table S3 in the supplemental material). Of these, 64 operons were preferentially expressed under high-iron conditions, while 89 were iron repressed. The predicted promoter regions of these operons were examined for the presence of putative Fur boxes. We used the Fur box consensus derived from analysis of both the *E. coli* (GATAATGATAA TCATTATC) (70) and the *Neisseria* (GATAAT-ATAATAA TTATC-TTT) (33) Fur-responsive genes. This was based on the observation that the DNA-binding regions of the *E. coli* and *N. gonorrhoeae* Fur proteins appear to be highly conserved. The DNA-binding motif of the *E. coli* Fur protein is an atypical helix-turn-helix motif, with amino acid residues 20 to 24 and 55 to 61 responsible for protein-DNA interaction (34). A sequence alignment between the Fur proteins of *E. coli* and *N. gonorrhoeae* reveals a high similarity throughout the protein and the DNA-binding motifs (data not shown). Amino acids 20 to 24 (KILEV) of the *E. coli* Fur protein are 60% identical to *N. gonorrhoeae* Fur (KILDL) with strong conservation between the last two amino acids. The *E. coli* Fur protein amino acids 55 to 61 (YRVLNQF) are identical to the gonococcal Fur (YRVLQTF) except for a weakly conserved asparagine-to-threonine difference. The two amino acids absolutely necessary for DNA binding (R56 and F61) are identical in the two Fur proteins (34).

Comparison of putative promoters to these consensus Fur-binding sites demonstrated that 50 operons (33%) were predicted to be under the direct regulatory control of Fur (see Table S3 in the supplemental material). Of these operons, 31 were derepressed in the absence of iron, including *tbpAB*, *fetA*, *fetB*, and *fbpABC*, as well as *tdfG*. The remaining 19 were up-regulated in the presence of iron. The predicted Fur boxes contained within the promoters of the iron-regulated operons were used to generate a consensus logo sequence (Fig. 4). This logo reveals an AT-rich sequence common to previously studied Fur boxes (29). Significantly, putative Fur boxes were found in the promoter regions for three of the four regulatory proteins derepressed in low iron, including two AraC-like regulators, NGO0025 and NGO2115, and an unclassified phage-like regulator, NGO1013. This suggests that low-iron condi-

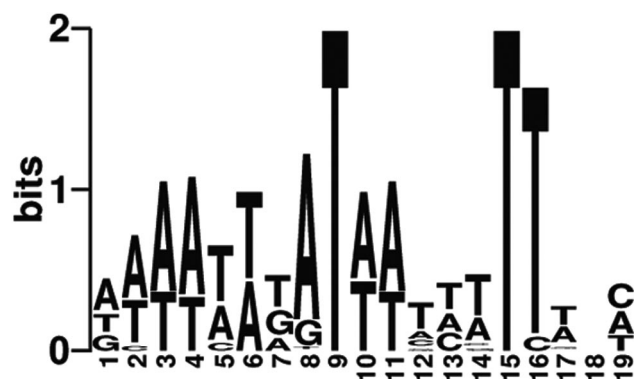


FIG. 4. Sequence logo of the Fur box of *N. gonorrhoeae* FA1090. Fur boxes found in the promoters of genes regulated by iron were used to determine nucleotide frequencies. Regulatory sequence determinations were done using the *E. coli* and *N. gonorrhoeae* Fur box consensus sequences.

tions may activate a regulatory cascade where some effects of Fur are indirect.

AraC-like transcriptional regulators vary in the types of functions that they control, being involved in controlling carbon metabolism, protein synthesis, regulation of virulence factors, and response to environmental stressors (32). One of the putative AraC-like regulators, NGO0025, shares significant identity (99%) with NMB1879, which is similarly derepressed under low-iron conditions in the meningococcus (35). Unfortunately, the diversity of this regulatory family results in an extremely degenerate DNA-binding motif (57), and so we were unable to reliably predict promoter regions that might contain binding sites for these regulators (data not shown). The phage-like regulator NGO1013 may be responsible for control of some or all of the 19 phage-associated hypothetical proteins derepressed under low-iron conditions (see Table S2 in the supplemental material).

The lone iron-repressible regulator that does not have a putative Fur box in the promoter encodes a MerR-like protein. Some MerR-like proteins respond to heavy metals, such as the product of the *E. coli* gene *zntR*, which is responsive to zinc (17). This suggests that two potential regulators, Fur and the MerR-like regulator, may be directly involved in the gonococcal response to iron availability, while three additional regulators indirectly control gene expression in a potential Fur-directed transcriptional cascade.

Comparative genomics of *N. gonorrhoeae* and *N. meningitidis* grown in iron-depleted and iron-replete environments. Grifantini et al. recently published a microarray analysis of the response of meningococcal strain MC48 to iron availability (35). While Grifantini et al. used a chemically defined medium, the formulation of this medium was not explicitly described, and so comparison of the meningococcal data to ours is somewhat problematic. In addition, Grifantini et al. employed 12.5 μ M Desferal to iron limit *N. meningitidis* MC58; as described above, this might complicate the interpretation of these data. Nevertheless, the comparison of our data set with theirs still appears to be instructive. While *N. gonorrhoeae* and *N. meningitidis* are closely related at the genetic level, they cause significantly different diseases. Unlike the gonococcus, *N. men-*

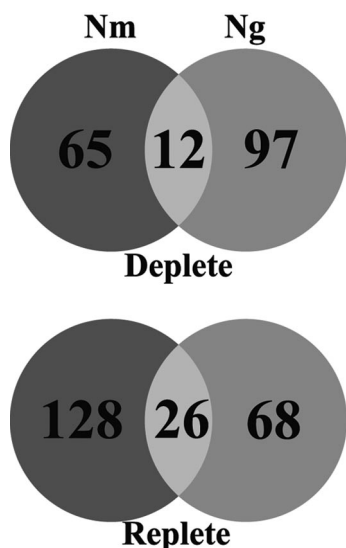


FIG. 5. Venn diagram comparing differentially expressed iron-regulated genes of *N. gonorrhoeae* FA1090 and *N. meningitidis* MC58.

ingitidis typically attaches to the nasopharyngeal mucosa (48) and is transmitted person-to-person via aerosolized respiratory droplets or oral secretions (76). Of the 1,999 gonococcal ORFs, 1,487 (74%) are shared between FA1090 and MC58, 535 of which are conserved hypothetical proteins (40). Approx-

imately 20% of iron-regulated gonococcal genes had iron-regulated orthologues in the meningococcal data set (Fig. 5 and Tables 3 and 4). The majority of genes preferentially expressed in both organisms under high-iron conditions are involved in protein synthesis. Additionally, the three predominant functional categories differentially expressed under high-iron conditions are identical for both organisms, including hypothetical proteins and those involved in protein synthesis and energy metabolism. Of the 26 genes coregulated under high-iron conditions (Table 3), 10 encode ribosomal proteins, with only 2 encoding conserved hypothetical proteins. The genes encoding SecY, NuoD, and Rho also were similarly regulated in both organisms.

Under low-iron conditions (Table 4), expression of genes encoding four conserved hypothetical proteins was shared by the two organisms, as well as the AraC family transcriptional regulator noted above. Not surprisingly, the genes encoding FbpA, FetA, HemO, TbpA, and TbpB were also regulated similarly in each pathogen. In MC58, the paralogous lactoferrin receptor genes *lbpA* and *lbpB* were also derepressed. In FA1090, the *lbpAB* operon has been inactivated by a large deletion that removes the promoter and a significant portion of the *lbpB* ORF (40).

While hypothetical proteins and conserved hypothetical proteins were the largest functional category of both organisms derepressed under low-iron conditions, only four of these iron-repressible proteins were shared by *N. gonorrhoeae* and *N.*

TABLE 3. *N. gonorrhoeae* and *N. meningitidis* orthologs differentially expressed under high-iron conditions

<i>N. gonorrhoeae</i>		<i>N. meningitidis</i>		% ID ^a	E value ^b
ORF	Gene product	ORF	Gene product		
NGO0037c	Conserved hypothetical protein	NMB1866	Conserved hypothetical protein	98.87	0
NGO0043c	50S ribosomal protein L11	NMB1862	50S ribosomal protein L11	94.92	8.00E-165
NGO0057	Putative thioredoxin	NMB1845	Thioredoxin	93.49	3.40E-079
NGO0191	30S ribosomal protein S15	NMB0609	30S ribosomal protein S15	100	4.60E-048
NGO0199c	Transcription termination factor rho	NMB0617	Transcription termination factor rho	98.81	0
NGO0260	Putative GTPase	NMB0687	GTP-binding protein Era	98.39	2.00E-156
NGO0295	Threonyl-tRNA synthetase	NMB0720	Threonyl-tRNA synthetase	98.12	0
NGO0296	Translation initiation factor IF-3	NMB0721	Translation initiation factor IF-3	99.35	9.70E-086
NGO0297	50S ribosomal protein L35	NMB0722	50S ribosomal protein L35	100	2.10E-034
NGO0584	50S ribosomal protein L9	NMB1320	50S ribosomal protein L9	99.25	2.00E-059
NGO0905	Conserved hypothetical protein	NMB1437	Conserved hypothetical protein	97.42	2.00E-132
NGO1246c	Serine protease	NMB1587	Putative protease	96.86	1.00E-171
NGO1413	Na ⁺ -translocating NADH-ubiquinone	NMB0569	Na ⁺ -translocating NADH-ubiquinone	96.2	0
NGO1748c	NADH dehydrogenase I chain D	NMB0244	NADH dehydrogenase 1 chain D	98.56	0
NGO1776	Glyceraldehyde 3-phosphate	NMB0207	Glyceraldehyde 3-phosphate	99.42	0
NGO1818c	DNA-directed RNA polymerase alpha	NMB0168	DNA-directed RNA polymerase alpha	98.78	0
NGO1822c	Preprotein translocase SecY subunit	NMB0162	Preprotein translocase SecY subunit	99.08	0
NGO1824.1c	50S ribosomal protein L18	NMB0158	50S ribosomal protein L18	100	3.30E-045
NGO1826.1c	30S ribosomal protein S14	NMB0155	30S ribosomal protein S14	100	5.40E-043
NGO1828c	50S ribosomal protein L24	NMB0153	50S ribosomal protein L24	98.13	6.30E-045
NGO1829c	50S ribosomal protein L14	NMB0152	50S ribosomal protein L14	99.18	1.80E-065
NGO1830c	30S ribosomal protein S17	NMB0151	30S ribosomal protein S17	98.85	2.00E-045
NGO1836c	50S ribosomal protein L23	NMB0144	50S ribosomal protein L23	91.76	1.70E-039
NGO1851c	DNA-directed RNA polymerase, beta subunit	NMB0132	DNA-directed RNA polymerase, beta subunit	99.28	0
NGO1867c	Two-component signal sensor kinase	NMB0114	Putative nitrogen regulation protein NtrY	93.77	0
NGO1870c	Methionyl-tRNA formyltransferase	NMB0111	Methionyl-tRNA formyltransferase	97.4	1.00E-162

^a Abbreviation: % ID, percent identity.

^b E value as calculated by BLASTP.

TABLE 4. *N. gonorrhoeae* and *N. meningitidis* orthologs differentially expressed under low-iron conditions

<i>N. gonorrhoeae</i>		<i>N. meningitidis</i>		% ID ^a	E value ^b
ORF	Gene product	ORF	Gene product		
NGO1690c	Conserved hypothetical protein	NMB0311	Hypothetical protein	96.3	1.60E-042
NGO1496c	Transferrin-binding protein B	NMB0460	Transferrin-binding protein B	61.59	0
NGO1495c	Transferrin-binding protein A	NMB0461	Transferrin-binding protein A	93.99	0
NGO0217c	ABC transporter, periplasmic-binding protein	NMB0634	Iron(III) ABC transporter, periplasmic-binding protein	99.09	3.00E-178
NGO0322	Conserved hypothetical protein	NMB0744	Hypothetical protein	96.23	7.70E-087
NGO0385	Delta-aminolevulinic acid dehydratase	NMB0801	Delta-aminolevulinic acid dehydratase	98.2	1.00E-177
NGO0165c	Conserved hypothetical protein	NMB0861	Hypothetical protein	97.79	6.20E-091
NGO1442	Alcohol dehydrogenase	NMB1395	Alcohol dehydrogenase	70.5	5.50E-023
NGO0108	Conserved hypothetical protein	NMB1796	Conserved hypothetical protein	99.38	4.10E-093
NGO1066	MafI protein	NMB1798	IS1016 transposase fragment	96.55	4.50E-011
NGO0025	AraC family transcriptional regulator	NMB1879	Hypothetical protein	99.03	6.20E-059
NGO2093c	Ferric enterobactin receptor FetA	NMB1988	Iron-regulated outer membrane protein FrpB	93.28	0

^a Abbreviation: % ID, percent identity.

^b E value as calculated by BLASTP.

meningitidis. Twenty of the derepressed meningococcal hypothetical and 30 of the gonococcal hypothetical protein-encoding genes are unique to their respective genomes. The gonococcus causes a sexually transmitted disease and primarily inhabits the urogenital tract, whereas the meningococcus colonizes the upper respiratory tract and often invades the bloodstream of susceptible individuals. So, while these two organisms share a high degree of genetic similarity, the diseases they cause are very different; the iron-regulated hypothetical proteins of each organism probably play a significant role in the specific pathogenesis of these diseases.

In summary, use of microarray analysis to examine differences in gene expression of *N. gonorrhoeae* in response to iron availability resulted in demonstrating that roughly 10% of gonococcal ORFs are involved in this response. Under high-iron conditions, the predominance of up-regulated genes encode proteins involved in protein synthesis, while under low-iron conditions, the predominance of derepressed genes encode proteins of unknown function. Of these ORFs roughly 30% are putatively controlled by the Fur protein, with additional control being provided by a MerR-like regulator. Likewise, a putative Fur-directed transcriptional cascade is suggested. Comparison of the gonococcal data to similar meningococcal data has revealed a surprisingly low degree of overlap between the iron regulons of the two organisms, suggesting that specific responses of each organism to iron availability dictate in part the different diseases that each pathogen causes.

ACKNOWLEDGMENTS

We thank A. F. Gillaspay for critical review of the manuscript and J. K. Davies for printing the pan-*Neisseria* arrays.

This work was supported by USPHS grants 5P20-RR-15564 (COBRE) and 5P20-RR-016478 (INBRE).

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