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MpeR Regulates the *mtr* Efflux Locus in *Neisseria gonorrhoeae* and Modulates Antimicrobial Resistance by an Iron-Responsive Mechanism

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Previous studies have shown that the MpeR transcriptional regulator produced by *Neisseria gonorrhoeae* represses the expression of *mtrF*, which encodes a putative inner membrane protein (MtrF). MtrF works as an accessory protein with the Mtr efflux pump, helping gonococci to resist high levels of diverse hydrophobic antimicrobials. Regulation of *mpeR* has been reported to occur by an iron-dependent mechanism involving Fur (ferric uptake regulator). Collectively, these observations suggest the presence of an interconnected regulatory system in gonococci that modulates the expression of efflux pump protein-encoding genes in an iron-responsive manner. Herein, we describe this connection and report that levels of gonococcal resistance to a substrate of the *mtrCDE*-encoded efflux pump can be modulated by MpeR and the availability of free iron. Using microarray analysis, we found that the *mtrR* gene, which encodes a direct repressor (MtrR) of *mtrCDE*, is an MpeR-repressed determinant in the late logarithmic phase of growth when free iron levels would be reduced due to bacterial consumption. This repression was enhanced under conditions of iron limitation and resulted in increased expression of the *mtrCDE* efflux pump operon. Furthermore, as judged by DNA-binding analysis, MpeR-mediated repression of *mtrR* was direct. Collectively, our results indicate that both genetic and physiologic parameters (e.g., iron availability) can influence the expression of the *mtr* efflux system and modulate levels of gonococcal susceptibility to efflux pump substrates.

Neisseria gonorrhoeae is a strict human pathogen and the causative agent of the sexually transmitted infection (STI) termed gonorrhea. Gonorrhea ranks second in the United States as the most common STI, with a rate of infection reported to be 112 cases per 100,000 people in 2008 (7). Worldwide, it has been estimated that over 90 million cases of gonorrhea occur each year (35). This annual incidence of gonorrhea, the increasing prevalence of antibiotic-resistant clinical isolates (39), especially those expressing decreased susceptibility or clinical resistance to ceftriaxone (6, 18, 38), evidence that repeated gonococcal infections increase host susceptibility to infection by the human immunodeficiency virus (4, 25), the lack of a protective vaccine (5), and the serious consequences of infection on the reproductive health of males and females make gonorrhea a major global health problem.

With respect to antibiotic resistance, the Centers for Disease Control and Prevention placed *N. gonorrhoeae* on the "Super Bugs" list in 2007, and growing concern exists about the future of antibiotic therapy in treating gonorrhea (17, 23). Accordingly, it is important to understand the genetic and physiologic processes that govern antibiotic resistance in the gonococcus. In this respect, energy-dependent efflux of multiple, structurally diverse antimicrobials by the MtrC-MtrD-MtrE (Mtr) system is an important mechanism used by gonococci to resist the bactericidal action of certain antibiotics (e.g., β-lactams and macrolides), topically applied spermicides (e.g., nonoxynol-9), and host-derived compounds that participate in innate host defense (e.g., progesterone and the antimicrobial peptide LL-37 [20, 29]). Not only does the Mtr efflux pump assist gonococci in developing clinically significant levels of antibiotic resistance, it also provides an advantage during infection since its production is required for a sustained lower genital tract infection in a female mouse model (20). Moreover, the ability of gonococci to upregulate its production provides a fitness benefit because certain *cis*- or *trans*-acting mutations that enhance *mtrCDE* expression can significantly increase levels of *in vivo* fitness in the mouse model of infection (36, 37).

Besides the *mtrCDE* efflux pump-encoding operon, two additional genes within the *mtr* locus are important in the ability of gonococci to export antimicrobials recognized by the Mtr efflux pump. The *mtrR* gene, which encodes a transcriptional repressor (MtrR) of *mtrCDE*, is closely linked to the *mtrF* gene, which encodes a putative inner membrane protein (MtrF); both genes are upstream of *mtrCDE* (15). MtrR, a member of the QacR/TetR family of DNA-binding proteins, dampens *mtrCDE* expression by binding within the promoter region of *mtrCDE* (15, 24). MtrR can also repress *mtrF* (12) and can positively or negatively control the expression of over 65 genes outside the *mtr* locus that are scattered throughout the chromosome (11). Although the precise function of MtrF in gonococci remains to be determined, it seems to function as an accessory protein for the Mtr efflux pump, needed for high levels of antimicrobial resistance mediated by the Mtr efflux pump (12, 34). In addition to being under the negative control of MtrR (11, 12), *mtrF* is subject to repression by MpeR, which is an AraC-like transcriptional regulator (12). The *mpeR* gene is re-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain	Relevant genotype[a]	Source or reference
Neisseria gonorrhoeae		
FA19	Wild type	P. F. Sparling
JF1	Δ*mtrR* in FA19	12
JF4	Inactivation of *mtrF* with insertion of *aphA-3* in FA19 (Km^r)	12
JF5	Inactivation of *mpeR* with insertion of *aphA-3* in FA19 (Km^r)	12
FA140	Mutation in promoter region of *mtrR*, missense mutation at codon 45 (Gly-45 to Asp-45) of *mtrR*	P. F. Sparling
WV16	Inactivation of *mtrF* with insertion of *aphA-3* in FA140 (Km^r)	34
FA19 *mtrF-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrF* in FA19 (Cm^r)	12
JF5 *mtrF-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrF* in JF5 (Cm^r), Km^r	12
AD1	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrF* (Cm^r), *mpeR*$^+$ (Ery^r) in JF5 (Km^r)	This study
AD2	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mpeR* in FA19 (Cm^r)	This study
FA19 *mtrR-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrR* in FA19 (Cm^r)	11
JF5 *mtrR-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrR* in JF5 (Cm^r), (Km^r)	This study
AD3	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrR* (Cm^r), *mpeR*$^+$ (Ery^r) in JF5 (Km^r)	This study
FA19 *mtrC-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrC* in FA19 (Cm^r)	12
JF1 *mtrC-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrC* in JF1 (Cm^r)	This study
JF5 *mtrC-lacZ*	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrC* in JF5 (Cm^r), Km^r	12
AD4	Chromosomal insertion of a translational fusion of *lacZ* to the promoter region of *mtrC* (Cm^r), *mpeR*$^+$ (Ery^r) in JF5 (Km^r)	This study
AD5	Chromosomal complementation of *mpeR* (Ery^r) in strain JF5 (Km^r)	This study
AD6	Inactivation of *terC* with insertion of *aphA-3* in FA19 (Km^r)	This study
AD7	Chromosomal complementation of *terC* (Ery^r) in strain AD6 (Km^r)	This study
AD8	Chromosomal complementation of *mpeR* (Ery^r) in strain AD7 (Km^r)	This study
AD9	Inactivation of *mpeR* with insertion of *aphA-3* in FA140 (Km^r)	This study
Escherichia coli		
DH5α	F^- *ϕ*80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ^- *gyrA96 relA1*	Invitrogen
TOP10	(F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*)*ϕ*80 *lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*) *7697 galU galK rpsL* (Str^r *endAI nupG*)	Invitrogen
Plasmids		
pMal-c2X	Vector for expression of protein fusions to maltose-binding protein	New England Biolabs
pLES94	pUC18-derived cloning vector for fusion of gonococcal genes to a promoterless *lacZ* and chromosomal insertion between *proA* and *proB* (Ap^r)	30
pGCC3	NICS vector for cloning *mpeR* and *terC* under the control of their own promoters and chromosomal complementation between *lctP* and *aspC*	31

[a] Cm^r, chloramphenicol resistance; Ery^r, erythromycin resistance; Km^r, kanamycin resistance; Ap^r, ampicillin resistance; NICS, neisserial insertion complementation system.

stricted to pathogenic *Neisseria* (9) and is negatively regulated by Fur (ferric uptake regulator) and iron (19). Interestingly, MpeR has recently been found by Hollander et al. (16) to directly activate the *fetA* gene of *N. gonorrhoeae* strain FA1090; FetA is a surface-exposed receptor for enterobactin-like siderophores (2). MpeR may also be linked to iron acquisition systems in *N. meningitidis*, as Fantappie et al. (9) reported that it can bind near the promoter for a gene (annotated as NMB1880) that encodes a FetB-like lipoprotein thought to bind siderophores in the periplasmic space.

Given the likely importance of MpeR in determining levels of gonococcal resistance to antimicrobials and a possible connection of this regulatory protein with iron acquisition and regulatory processes, we sought to determine the number and types of genes controlled by MpeR. In this context, we now report that the expression of the gene encoding the major transcriptional repressor (MtrR) of the *mtrCDE* efflux pump operon is controlled by MpeR and levels of free iron.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains TOP10 (Invitrogen, Carlsbad, CA) and DH5α mcr were used in all cloning experiments. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates and incubated at 37°C. *N. gonorrhoeae* strain FA19 was used as the primary gonococcal strain (28), and all strains were grown on gonococcal medium base (GCB) agar (Difco Laboratories, Detroit, MI) containing defined supplements I and II at 37°C under 3.8% (vol/vol) CO_2 or in GCB broth with supplements I and II and sodium bicarbonate as previously described (28); except for transformation experiments, nonpiliated, transparent colonies were selected for growth. Iron-depleted cultures were grown in GCB broth containing the above-described supplements and 200 μM deferoxamine mesylate (Desferal). The plasmids and oligonucleotide primers used in this study are listed in Tables 1 and 2. All chemicals were purchased from Sigma Biochemical (St. Louis, MO.).

RNA isolation, microarray design, cDNA labeling and hybridization, and array analysis. Strains FA19 and JF5 (as FA19 but *mpeR*::Km [12]) were grown in 50 ml of GCB broth, and samples of each strain were

TABLE 2 Oligonucleotides used in this study

Oligonucleotide used	Sequence (5′→3′)
5′pMpeRpac	GGTTAATTAAGCAAACAACCTGCAGAAACC
3′GC4MpeR2	GGTTTAAACTCAGCACTTTTTCACATCCGA
5′AterC	GCAACACATAGGGAGACGCTTT
3′BterCSma	CATAATCATGTCCTTCCCGGGAAC
5′CterCSma	GTTCCCGGGAAGGACATGATTATG
3′DterC	GCTTTCGCGTATGCCCATCATAC
5′pterCPac	GGTTAATTAATCTCGCCGAAGGGGAGGA
3′GC4terC	GGTTTAAACTTCTTCGGGCAATTTGGTGAT
5′mpeR	ATGAACACCGCCGCCATCT
3′mpeR	GCACTTTTTCACACTCGAAGG
5′malEmpeR-F	CACTGGGGATCCATGAATACCGCCGCCATCT
3′malEmpeR-R	CACTGGCTGCAGTCAGCACTTTTTCACATCCGA
5′misR−205	CACCGCTGCTGCCCGAACTGCTC
3′misR+104	AGCAGGGCATCGTCATCTACGAG
5′KH9#1	GTCGCAGATACGTTGGAACAACG
3′CEL2A	GCTTTGATACCCGAATGTTCG
5′pmtrF-F	TTAATTTCCCCTATCATCGCA
3′pmtrF-R	TAAATTTTTGAATTTAACATGAAG
5′pmpeR	TAGGATCCGCAAACAACCTGAAGAAACC
3′pmpeR	ATGGATCCCGGTTCATGATTGGATAGGAAC

harvested at both the mid-log (optical density at 600 nm [OD_{600}] = 0.6) and late log (OD_{600} = 0.9) phases of growth for RNA isolation using the hot-phenol method as previously described (8). Following DNase treatment (Qiagen DNase kit), RNA recovery (Qiagen RNeasy minikit), and quantification by a NanoDrop 1000 (NanoDrop Technologies), microarray analysis was performed (11). The microarray design, cDNA labeling and hybridization, and data analysis of the array were all conducted as previously described (11). Genes that showed expression differences of 1.5-fold ($P \leq 0.05$) were considered to be subject to MpeR regulation. Gene numbers were designated using FA1090 genome annotation (http://www.genome.ou.edu).

Inactivation of *mpeR* in strain FA140 and hydrophobic agent susceptibility testing. Chromosomal DNA from strain JF5 was used as a template to PCR amplify *mpeR*::Km with primers 5′mpeR and 3′mpeR (Table 2). The resulting PCR product was gel purified and used to transform piliated colony variants of strain FA140. Transformants were selected on GCB agar with 50 μg/ml of kanamycin (Km); a representative transformant (see strain AD9 in Table 1) was chosen for further study, and the presence of an inactivated *mpeR* gene was confirmed by PCR and DNA sequence analyses. The MICs of selected antimicrobial agents were determined as previously described (14).

Complementation of *mpeR*::Km. In order to complement the *mpeR*::Km mutation in strain JF5 (Table 1), primers 5′pMpeRpac and 3′GC4MpeR (Table 2) were used to PCR amplify the wild-type *mpeR* gene and 250 bp of upstream sequence from FA19 chromosomal DNA. The resulting DNA fragment was cloned into pGCC3 (31) at the PmeI and PacI (New England BioLabs) sites to produce pGCC3-*mpeR*. Following DNA sequencing to confirm the correct orientation, this construct was transformed into JF5 *mtrF-lacZ*, JF5 *mtrR-lacZ*, JF5 *mtrC-lacZ*, and JF5 gonococcal strains (Table 1) as previously described (13). Transformants in which *mpeR* recombined into the chromosome between the *lctP* and *aspC* genes were selected on GCB agar containing 1 μg/ml of erythromycin (Ery), and the presence of the inserted DNA was confirmed by PCR analysis using primers 5′mpeR and 3′mpeR. The resulting strains were named AD1 (JF5 *mtrF-lacZ mpeR*$^+$), AD3 (JF5 *mtrR-lacZ mpeR*$^+$), AD4 (JF5 *mtrC-lacZ mpeR*$^+$), and AD5 (JF5 *mpeR*$^+$) (Table 1).

Construction of *lacZ* fusion strains in gonococci, preparation of cell extracts, and β-galactosidase assays. All translational *lacZ* fusions were constructed as previously described (30). In order to construct the FA19 *mpeR-lacZ* strain (Table 1), the 161-bp sequence upstream of *mpeR* was PCR amplified from strain FA19 using primers 5′pmpeR and 3′pmpeR (Table 2) and inserted into the BamHI site of pLES94. This construct was transformed into DH5α TOP10; the correct insertion and correct orientation were confirmed by DNA sequencing. The plasmid construct was then transformed into FA19, which allowed for insertion of the translational fusion between the *proAB* genes. Transformants were selected on GCB agar with 1 μg/ml of chloramphenicol (Cm). The JF5 *mtrR-lacZ* strain was constructed by PCR amplifying *mpeR*::Km from strain JF5 using primers 5′mpeR and 3′mpeR (Table 2). This DNA fragment was used to transform the FA19 *mtrR-lacZ* strain. Transformants were selected on GCB agar with 50 μg/ml of Km, and sequencing of a PCR-amplified product was performed to confirm disruption of *mpeR*. In order to construct the JF1 *mtrC-lacZ* strain, the promoter sequence of *mtrC* (15) was cloned into pLES94 as previously described (12), and this plasmid construct was transformed into JF1. Insertion and selection of transformants were performed as described for construction of the FA19 *mpeR-lacZ* strain. Strains harboring *lacZ* fusions were grown in GCB broth, and 15 ml of culture was harvested by centrifugation at 5,000 × *g* for 10 min. The supernatant was discarded, and the remaining pellet was washed once with phosphate-buffered saline (pH 7.4) and resuspended in lysis buffer (0.25 mM Tris [pH 8.0]). Cells were lysed, and β-galactosidase assays were performed as previously described (12, 32).

Inactivation of *terC* and complementation of the *terC* null mutant. The *terC* gene (NGO1059) was inactivated as previously described (12, 22). Briefly, the primer sets 5′AterC and 3′BterCSma along with 5′CterCSma and 3′DterC (Table 2) were used to PCR amplify *terC* from FA19 chromosomal DNA in order to engineer an SmaI site within the gene. The products from these two PCRs were then used as a template to PCR amplify the entire gene using primers 5′A*terC* and 3′D*terC*. This 1,500-bp PCR product was inserted into pBAD-TOPO-T/A and transformed into *E. coli* TOP10 as described in the manufacturer's protocol (Invitrogen). The nonpolar kanamycin resistance cassette *aphA-3* (26) was digested from pUC18K with SmaI and cloned into the engineered SmaI site of *terC*. This construct was transformed into DH5α TOP10, and following plasmid purification, the inactivated *terC*::Km sequence was PCR amplified using primers 5′AterC and 3′DterC. This plasmid was used to transform FA19, and transformants harboring the disrupted gene (see strain AD6 in Table 1) were selected on GCB agar containing 50 μg/ml of Km. The presence of the inactivated *terC* gene was confirmed by PCR and sequencing analysis. Complementation of *terC* in strain AD6 was done as described for *mpeR* (see above) except that primers 5′ pterCPac and 3′GC4terC (Table 2) were used to amplify *terC* from FA19 chromosomal DNA. The resulting complemented strain was termed AD7 (Table 1).

Immunodetection of MtrR. Strain FA19 was grown in GCB broth under iron-replete or -depleted conditions as described above. At the late logarithmic phase of growth, samples were harvested by centrifugation, cell extracts were solubilized, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western immunoblotting was conducted as described previously (10). MtrR was detected using rabbit anti-MtrR antiserum (1:1,000) and goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase (1:1,000) as described previously (11). This analysis was conducted using three independent pairs of samples, and digital images of the immunoblots were used to quantify differences in pixel intensity for the MtrR band in lanes containing separated whole-cell lysates from strain FA19 grown under iron-replete or iron-depleted conditions. Quantification was done using the Adobe Photoshop program at fixed identical dimensions for all blots. The statistical significance of the difference in protein expression was calculated using a paired Student *t* test.

Construction and purification of the MBP-MpeR fusion protein. MpeR was fused in frame at its N terminus to maltose-binding protein (MBP) by using the pMal-c2x fusion vector (New England BioLabs, Beverly, MA). Primers 5′malEmpeRF and 3′malEmpeRR (Table 2) were used

in PCR to obtain a product for subsequent cloning as previously described (16). Growth of the *E. coli* transformant bearing the plasmid construct, induction of expression, and purification of MBP-MpeR were performed as previously described (21).

Electrophoretic mobility shift assay (EMSA). The *mtrR* promoter region was PCR amplified from FA19 chromosomal DNA using primers 5′KH9#1 and 3′cel2A (Table 2) to generate a 360-bp product and purified using a QIAquick PCR purification kit (Qiagen). The PCR product was radiolabeled and purified as previously described (16). The labeled DNA fragment (10 ng) was incubated with 15 μg of fusion protein (MBP-MpeR). Specificity of binding was shown by incubating 15 μg of MBP-MpeR and 10 ng of the radiolabeled *mtrR* probe with increasing concentrations of a 360-bp cold specific competitor (nonradiolabeled *mtrR*) or increasing concentrations of a 309-bp cold nonspecific competitor (nonradiolabeled *misR*); the latter was PCR amplified using 5′misR−205 and 3′misR+104 (Table 2). All reaction mixtures were incubated along with DNA-binding buffer [10 mM Tris-HCl, 200 mM NaCl, 1 mM dithiothreitol, 1 μg/ml of poly(dI-dC)] for 30 min at room temperature. Samples were subjected to electrophoresis on a 5% (wt/vol) native polyacrylamide gel at 4°C followed by autoradiography.

Primer extension of *mtrF*. In order to determine the start site for *mtrF* transcription, total RNA was prepared from a mid-logarithmic culture of strain FA19 as described above. The primer 3′mtrF-R was radiolabeled using [γ-^{32}P] and T4 polynucleotide kinase and used to reverse transcribe 10 μg of total RNA. The primers 5′pmtrF-F and 3′pmtrF-R (Table 2) were used along with FA19 chromosomal DNA as a template to amplify the *mtrF* promoter region in order to generate reference sequence products using a SequiTherm Excel II DNA sequencing kit (Epicentre). Both the primer extension product and the reference sequence were subjected to electrophoresis on a 6% (wt/vol) sequencing gel that was dried. Autoradiography was performed for visualization of the primer extension product.

Statistical analysis. Except where indicated, statistical significance was determined using a two-tailed, paired Student *t* test. *P* values for specific comparisons are listed in the figure legends.

Microarray data expression number. Gene expression data for all microarray experiments can be retrieved from the Gene Expression Omnibus (GEO) database at NCBI (http://www.ncbi.nih.gov/geo/) under accession number GSE32717.

RESULTS

MpeR regulation of *mtrF* and influence of iron availability. Prior to determining the MpeR regulon in *N. gonorrhoeae* strain FA19 (see below), we confirmed that *mtrF* expression is negatively controlled by MpeR (12) and that such regulation is influenced by iron availability, as suggested by previous studies (8, 19). For this purpose, we constructed a complemented derivative (strain AD1) of strain JF5 (as FA19 but *mpeR*::Km) (Table 1), which expressed *mpeR* ectopically from its own promoter when placed between the *lctP* and *aspC* genes (31). In order to monitor *mtrF* expression, we used a previously described (12) *mtrF*::*lacZ* translational fusion that harbors 161 bp of the DNA sequence upstream of *mtrF* (Fig. 1A) containing the promoter element for *mtrF* transcription. This promoter was assigned as such based on results from primer extension analysis that showed a transcriptional start point (data not shown) 9 nucleotides downstream of a near-consensus −10 hexamer sequence (Fig. 1A). This putative −10 element was separated from a potential −35 hexamer sequence by an optimal 17 nucleotides. When the *mtrF-lacZ* fusion was introduced into strains FA19, JF5, and AD1, we found that the expression of *mtrF-lacZ* was significantly higher in the *mpeR*::Km mutant (JF5) than in either the wild-type or complemented strain (Fig. 1B).

We next tested whether conditions of iron depletion would

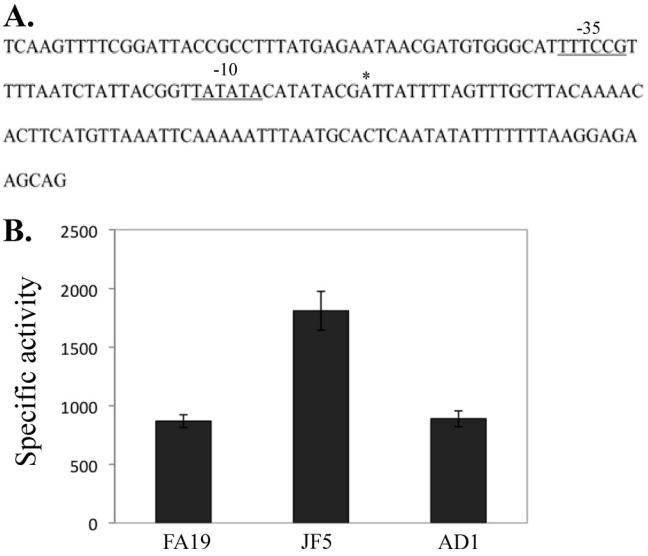

FIG 1 Regulation of *mtrF* by MpeR. (A) DNA sequence of a 161-bp fragment used in *mtrF-lacZ* expression analysis, with an asterisk marking the start of transcription. (B) The specific activities (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per mg of protein) for measuring *mtrF-lacZ* expression in the FA19 *mtrF-lacZ*, JF5 *mtrF-lacZ*, and AD1 (JF5 *mtrF-lacZ* *mpeR*$^+$ [Table 1]) strains are shown. Samples were harvested from gonococci to the mid-log phase of growth. The above-described experiment was done in triplicate and is a representative example of three independent experiments. Error bars represent one standard deviation. The difference in expression of *mtrF-lacZ* between FA19 and JF5 was significant ($P = 0.001$), as was the difference in expression of *mtrF-lacZ* between JF5 and AD1 ($P = 0.001$).

have an impact on *mpeR* expression in strain FA19 as previously reported by Ducey et al. (8) and later found by Jackson et al. (19) to involve a Fur-dependent mechanism. As is shown in Fig. 2, the peak of *mpeR* expression in strain AD2 (as FA19 but *mpeR-lacZ*) grown in iron-replete GCB broth occurred at the late logarithmic phase of growth when free iron levels would be reduced due to consumption. Based on this result, we next monitored *mpeR-lacZ* expression during growth of strain AD2 under iron-replete and iron-depleted (with deferoxamine mesylate) conditions and harvested cells at the late log phase of growth. As expected, the culture growing under iron-depleted conditions had a severe growth defect compared to the control culture, yet expressed *mpeR* at higher levels (Fig. 3). This result was consistent with the conclusions reached by Ducey et al. (8) and Jackson et al. (19) that *mpeR* is an iron-repressed gene in gonococci.

Antimicrobial susceptibility of gonococci can be modulated by MpeR and iron availability. In order to determine whether production of MpeR, which negatively regulates the expression of *mtrF* (12), can influence levels of gonococcal resistance to antimicrobials recognized by the Mtr efflux pump system in an iron-dependent manner, we assessed the susceptibility of strain FA140 to Triton X-100, a known substrate of the pump when grown under iron-replete and iron-depleted conditions. We used FA140 for this purpose because loss of *mtrF* in this strain, but not in wild-type strain FA19, has a phenotype (e.g., hypersusceptibility to Triton X-100 [34]). Interestingly, Triton X-100 is structurally similar to the over-the-counter spermicide nonoxynol-9, which also displays antigonococcal action (12, 34). FA140 expresses a high level of resistance to Triton X-100 mediated by the Mtr efflux

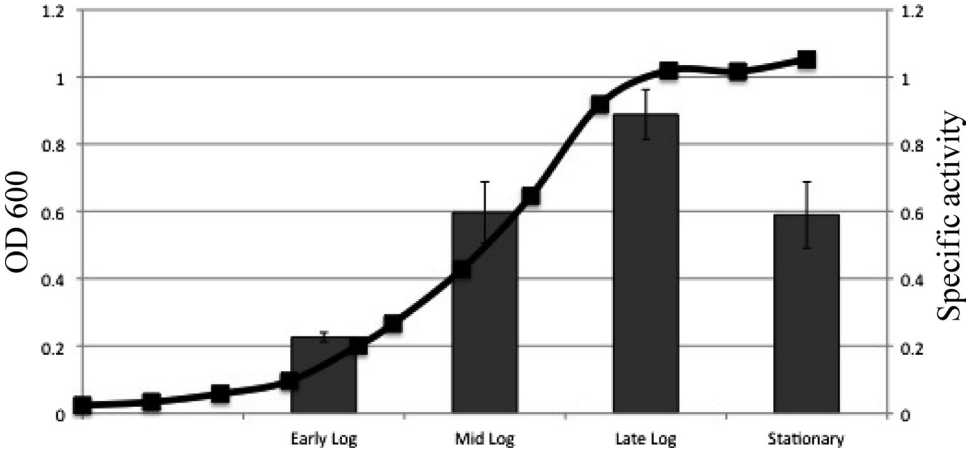

FIG 2 Maximal expression of *mpeR*. The specific activities (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per mg of protein) for measuring *mpeR-lacZ* expression in strain AD2 (FA19 *mpeR-lacZ* [Table 1]) at different phases of growth under iron-replete conditions are shown. The above-described experiment was done in triplicate and is a representative example of three independent experiments. Error bars represent one standard deviation, and differences in expression of *mpeR-lacZ* at the early log, mid-log, late log, and stationary phases of growth were significant ($P < 0.05$).

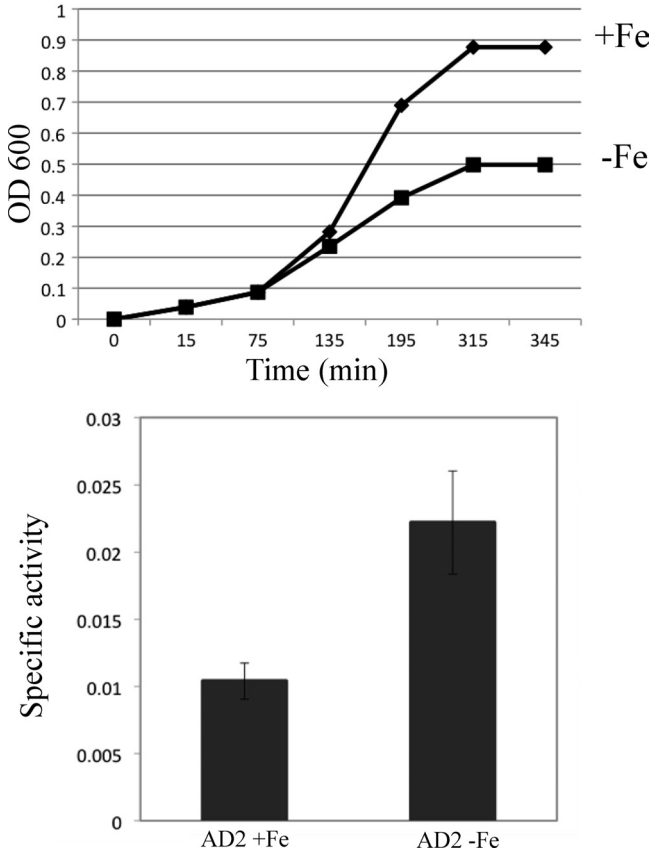

FIG 3 Expression of *mpeR* under iron-replete and -depleted conditions. The growth of strain AD2 (FA19 *mpeR-lacZ* [Table 1]) under either iron-replete (+Fe) or iron-depleted (−Fe) conditions (top panel) and the expression of *mpeR-lacZ* (bottom panel) are shown. Samples from the growth curve were harvested from gonococci at the late log phase of growth. The experiment was done in triplicate and is a representative example of three independent experiments. Error bars represent one standard deviation. The difference in expression of *mpeR-lacZ* between cultures grown under iron-replete and iron-depleted conditions was significant ($P = 0.007$).

pump due to a single base pair deletion in the *mtrR* promoter that abrogates *mtrR* expression, resulting in elevated expression of *mtrCDE* (33). With FA140, we found that Triton X-100 resistance was substantially higher (>64-fold) in the iron-replete culture than in the iron-depleted culture (Table 3). In order to determine whether this iron-dependent Triton X-100 resistance required *mpeR* and/or *mtrF*, we next examined genetic derivatives of FA140 that contained inactivated copies of these genes. Using strain WV16 (as FA140 but *mtrF*::Km), we confirmed that the loss of *mtrF* significantly enhances gonococcal susceptibility to Triton X-100 (Table 3). Importantly, there was no difference between the Triton X-100 MIC values for the cultures grown under iron-replete and iron-depleted conditions. In contrast to parent strain FA140, strain AD9 (as FA140 *mpeR*::Km) expressed a high level of Triton X-100 resistance independent of iron availability (Table 3), indicating that the iron-dependent phenotype of antimicrobial susceptibility requires MpeR.

Identification of MpeR-regulated genes associated with antimicrobial resistance. In order to identify MpeR-regulated genes besides *mtrF* that are involved in antimicrobial resistance, we used RNA from strains FA19 and JF5 to identify the MpeR regulon in wild-type strain FA19. As is shown in Tables 4 and 5, microarray analysis of RNA pairs prepared from mid-logarithmic and late logarithmic phase cultures of these strains revealed a total of 67 MpeR-regulated genes, with 46 being MpeR repressed (30 at mid-log and 16 at late log) and 21 activated in the presence of MpeR (16 at mid-log and 5 at late log). The finding that *mtrF* expression was

TABLE 3 Effect of iron and *mpeR* on high-level Triton X-100 resistance due to *mtrR* mutations

Strain	MIC (μg of Triton X-100/ml)[a]
FA140 +Fe	>16,000
FA140 −Fe	375
WV16 +Fe	125
WV16 −Fe	125
AD9 +Fe	>16,000
AD9 −Fe	>16,000

[a] All values represent average results from three independent experiments.

TABLE 4 MpeR-regulated genes at mid-log phase in *Neisseria gonorrhoeae*

Gene[a]	Common name	Fold change	Functional classification
MpeR repressed			
NGO0018	NGO0018	1.53	Unknown
NGO0205	*lolA*	1.60	Putative outer membrane lipoprotein carrier protein
NGO0373	NGO0373	1.75	Putative ABC transporter permease
NGO0393	NGO0393	1.52	Putative TetR family transcriptional regulator
NGO0678	NGO0678	1.59	Unknown
NGO0679	*leuC*	1.51	Isopropylmalate isomerase large subunit
NGO0754	*mobA*	1.56	Putative molybdoprotein guanine dinucleotide biosynthesis protein
NGO0795	*bfrB*	2.24	Bacterioferritin B
NGO0863	NGO0863	1.69	Putative oxidoreductase
NGO0891	NGO0891	1.95	Unknown
NGO0916	*sucB*	1.65	Dihydrolipoamide acetyltransferase
NGO1046	*clpB*	1.66	Putative ClpB protein
NGO1273	NGO1273	1.51	Unknown
NGO1368	***mtrF***	**2.22**	**Mtr efflux pump protein component**
NGO1416	*nqrD*	1.80	NADH-ubiquinone reductase
NGO1418	*nqrF*	1.70	Na^+-translocating NADH-quinone reductase subunit F
NGO1422	*grpE*	1.71	Putative heat shock protein
NGO1428	NGO1428	1.53	Unknown
NGO1494	*potF*	1.71	Spermidine/putrescine ABC transporter
NGO1600	*glnA*	1.95	Glutamine synthetase
NGO1665	*ilvE*	1.62	Branched-chain amino acid aminotransferase
NGO1685	NGO1685	1.68	Unknown
NGO1749	*nuoC*	1.52	NADH dehydrogenase subunit C
NGO1765	pglA	1.51	Putative glycosyltransferase
NGO1770	*prlC*	1.54	Oligopeptidase A
NGO1780	NGO1780	1.67	Unknown
NGO1809	*valS*	1.50	Valyl-tRNA synthetase
NGO2013	*glnQ*	1.82	Putative ABC transporter, ATP-binding protein
NGO2014	*cjaA*	2.15	Putative ABC transporter, periplasmic binding protein
NGO2094	*groES*	1.84	Cochaperonin GroES
MpeR activated			
NGO0221	NGO0221	1.57	Putative deoxyribonucleotide triphosphate pyrophosphatase
NG00527	NGO0527	1.58	Unknown
NGO0567	NGO0567	1.69	Putative hydrolase
NGO0606	NGO0606	1.58	Putative sodium-dependent transport Protein
NGO0694	NGO0694	1.62	Unknown
NGO0820	*mesJ*	1.75	Putative cell cycle protein
NGO0876	NGO0876	1.90	Unknown
NGO0958	*rph*	1.62	Putative RNase PH
NGO1058	*surE*	1.64	Stationary-phase survival protein
NGO1059	**NGO1059**	**2.17**	**Putative tellurium resistance gene**
NGO1079	NGO1079	1.69	Putative oxidoreductase
NGO1406	*gcvT*	1.55	Aminomethyltransferase
NGO1488	NGO1488	1.53	Unknown
NGO1810	NGO1810	1.62	Unknown
NGO2132	NGO2132	1.58	Unknown
NGO2167	NGO2167	1.51	Unknown

[a] FA1090 genome annotation. Boldface indicates genes selected for study.

elevated in strain JF5 is consistent with earlier work (12) (Fig. 1B) that *mtrF* is an MpeR-repressed gene (Table 4), and it served as an internal control that validated our use of the microarray system to detect MpeR-regulated genes. Interestingly, for both MpeR-repressed and -activated genes, there was no overlap of regulated genes in the mid-log and late log RNA samples.

With respect to genes known or possibly involved in antimicrobial resistance, we identified, in addition to *mtrF*, two genes of interest: *mtrR* and *terC*. The expression of *mtrR*, which encodes the major transcriptional repressor (MtrR) of the *mtrCDE* operon (15) and can also negatively control *mtrF* independently of MpeR (12), was identified as an MpeR-repressed gene (1.74-fold change) in the late log culture of strain FA19. In contrast, *terC* (NGO1059), which has been provisionally annotated (http://www.genome.ou.edu) as encoding a putative membrane protein (TerC) involved in tellurium resistance (3), was found to be MpeR activated (2.17-fold change) but only in the mid-log phase of growth. While much is known about *mtrR* (10, 11, 15, 21, 24, 27, 36), little information is available about *terC* in gonococci. The gene *terC* is of interest, however, because it contains a polynucleotide repeat (A-8) in its

TABLE 5 MpeR-regulated genes at late log phase in *Neisseria gonorrhoeae*

Gene[a]	Common name	Fold change	Functional classification
MpeR repressed			
NGO0365	*dcmG*	1.94	Site-specific DNA methyltransferase
NGO0672	NGO0672	1.98	Unknown
NGO0924	NGO0924	1.51	Unknown
NGO0952	NGO0952	1.51	Putative TonB-dependent receptor protein
NGO1107	NGO1107	1.62	Unknown
NGO1159	NGO1159	1.59	Unknown
NGO1176	NGO1176	1.88	Unknown
NGO1313	NGO1313	1.76	Unknown
NGO1342	*dhpS*	1.67	Dihydropteroate synthase
NGO1366	***mtrR***	**1.74**	***mtrCDE* transcriptional regulator, repressor**
NGO1481	*bioC*	1.52	Biotin synthesis protein
NGO1771	NGO1771	1.63	Unknown
NGO1847	NGO1847	1.65	Unknown
NGO1915	*kdtA*	1.81	3-Deoxy-D-manno-octulosonic-acid transferase
NGO1951	*prfB*	2.40	Peptide chain release factor 2
NGO2042	*pilS*	1.87	Pilin silent gene cassette
MpeR activated			
NGO1151	NGO1151	1.63	Unknown
NGO1170	NGO1170	1.95	Unknown
NGO1179	NGO1179	1.97	Unknown
NGO1270	NGO1270	1.95	Unknown
NGO1498	NGO1498	1.58	Unknown

[a] FA1090 genome annotation. Boldface indicates gene selected for further study.

coding sequence, making it a candidate for being a phase-variable gene. Since analysis of the FA1090 genome sequence (http://www.genome.ou.edu) and our own sequencing of the *terC* gene in FA19 (data not shown) showed that *terC* would be in the "phase-on" sequence in these strains, we asked whether it was involved in resistance to tellurium as well as antimicrobials recognized by the Mtr efflux pump. For this purpose, a null mutation in *terC* was constructed in strain FA19 (see Materials and Methods); this mutant strain was named AD6. Strain AD6 was then complemented with the wild-type *terC* gene from FA19, which was cloned into pGCC3 and expressed from its own promoter between *lctP* and *aspC* genes in the chromosome; this complemented strain was termed AD7. Using these strains, we found that the loss of *terC* increased gonococcal susceptibility to potassium tellurite (Table 6) but not other tested antimicrobials (e.g., silver nitrate and Triton X-100), including one (Triton X-100) recognized by the Mtr efflux pump; this change in tellurium susceptibility endowed by the null mutation could be reversed by complementation. Consistent with *terC* being an MpeR-activated gene, the loss of *mpeR* in strain JF5 also resulted in hypersusceptibility of gonococci to potassium tellurite, but this was reversed by complementation when a wild-type copy of *mpeR* was expressed ectopically (see strain AD5 in Table 6). Since the *terC* mutant did not show hypersusceptibility to a substrate of the Mtr efflux pump (e.g., Triton X-100), we concentrated on MpeR control of *mtrR* and a target of MtrR regulation (*mtrCDE*).

MpeR regulation of *mtrR* and *mtrCDE*. The observation that

TABLE 6 Effect of *terC* and *mpeR* on the susceptibility of gonococci to potassium tellurite

Strain	MIC (μg/ml)[a]		
	K_2TeO_3	$AgNO_3$	Triton X-100
FA19	1.0	20	100
AD6	0.05	20	100
AD7	0.5	20	100
JF5	0.05	20	100
AD5	1	20	100

[a] K_2TeO_3, potassium tellurite; $AgNO_3$, silver nitrate. All values represent average results from three independent experiments.

mtrR expression was repressed by MpeR in the late log phase culture of strain FA19 (Table 5) suggested that MpeR could indirectly regulate the *mtrCDE* efflux pump operon through modulation of MtrR levels and that such regulation could be iron dependent. To test this hypothesis, we examined *mtrR* and *mtrC* expression in MpeR-positive and MpeR-negative strains grown under iron-replete and iron-depleted conditions. Using the FA19 *mtrR-lacZ* and JF5 *mtrR-lacZ* strains and the *mpeR*-complemented strain with the *mtrR-lacZ* fusion (AD3), we found that *mtrR* expression was decreased in the MpeR-positive strains compared to that in the MpeR-negative strain JF5, confirming that it is an MpeR-repressible gene (Fig. 4). Based on this result, we next tested whether levels of MtrR would differ in wild-type strain FA19 grown under iron-replete and iron-depleted conditions. Using SDS-PAGE and Western blot analysis of whole-cell lysates that employed anti-MtrR antiserum, we found that the level of MtrR in strain FA19 was increased by 42% ($P = 0.03$) when grown under iron-replete conditions compared to that under iron-depleted conditions (Fig. 5).

Our finding that levels of MtrR in gonococci can change due to the presence of MpeR and levels of iron suggested that MtrR control of the *mtrCDE* efflux pump operon is regulated by MpeR and iron availability. As a further test of this model, we monitored the expression of an *mtrC-lacZ* fusion in FA19, JF5, and the *mpeR*-

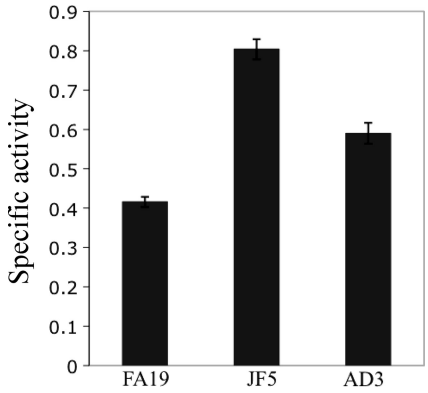

FIG 4 Regulation of *mtrR* by MpeR. *mtrR* expression in the FA19 *mtrR-lacZ*, JF5 *mtrR-lacZ*, and AD3 (JF5 *mtrR-lacZ mpeR*$^+$ [Table 1]) strains as determined by translational *lacZ* fusions. Samples were harvested from gonococci at the late log phase of growth. The above-described experiment was done in triplicate and is a representative example of three independent experiments. Error bars represent one standard deviation. The difference in expression of *mtrR-lacZ* between FA19 and JF5 was significant ($P \leq 0.001$), as was the difference in *mtrR-lacZ* expression between JF5 and AD3 ($P \leq 0.001$).

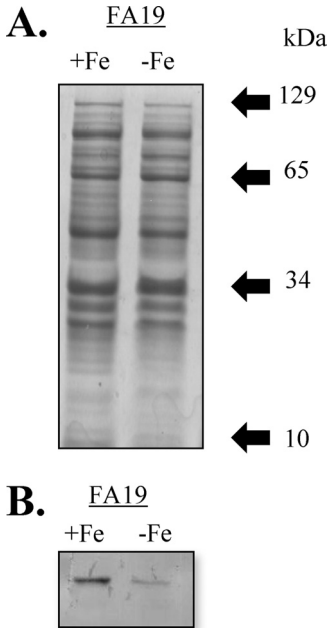

FIG 5 Iron modulates levels of MtrR. Wild-type strain FA19 was grown under iron-replete and -depleted conditions. (A) Samples for each growth condition were harvested at the late log phase of growth, solubilized, and separated by SDS-PAGE; the electrophoretic mobility of each molecular mass marker is shown by the arrow. (B) Following transfer to nitrocellulose, the blot was probed with anti-MtrR antiserum and the difference in MtrR levels was determined as described in Materials and Methods.

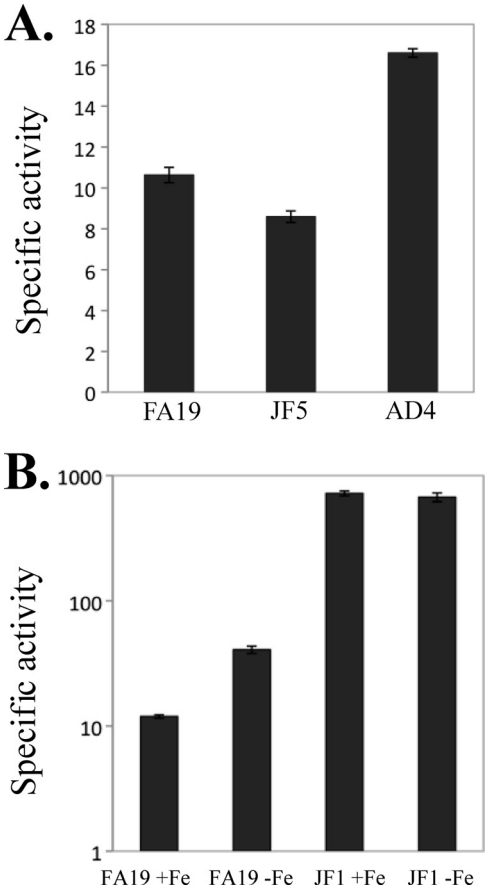

FIG 6 Regulation of *mtrCDE* expression is modulated by *mpeR* expression and levels of free iron but not *mtrR* expression. (A) *mtrCDE* expression levels in the FA19 *mtrC-lacZ*, JF5 *mtrC-lacZ*, and AD4 (JF5 *mtrC-lacZ mpeR*$^+$ [Table 1]) strains were measured. The difference in *mtrCDE* expression between strains FA19 and JF5 was significant ($P = 0.002$), as was that between strains JF5 and AD4 ($P = 0.0001$). (B) *mtrCDE* expression levels in the FA19 *mtrC-lacZ* and JF1 *mtrC-lacZ* (Table 1) strains grown under either iron-replete or iron-depleted conditions were determined. The difference in *mtrCDE* expression between FA19 +Fe and FA19 −Fe was significant ($P = 0.0023$), while that between JF1 +Fe and JF1 −Fe was not significant ($P = 0.3132$). The above-described experiments were done in triplicate and are representative examples of three independent experiments. Error bars represent one standard deviation.

complemented strain termed AD4 (Table 1). We found that the loss of *mpeR* in strain JF5 decreased *mtrC-lacZ* expression by nearly 20% ($P = 0.002$), which was reversed by complementation when the wild-type *mpeR* gene was expressed ectopically in strain AD4 (Fig. 6A); this small but significant difference in *mtrC-lacZ* expression may explain why the *mtrCDE* genes were not identified in the microarray studies as being MpeR regulated. Additionally, we observed that iron-depleted conditions (with deferoxamine mesylate) enhanced *mtrC-lacZ* expression in strain FA19 (Fig. 6B). Since MtrR is a direct repressor of *mtrCDE* (15, 24) and its expression can be controlled by levels of iron and MpeR, we hypothesized that the expression of *mtrC-lacZ* would not be affected by the availability of iron in an MtrR-negative strain. In order to test this possibility, we used strains FA19 and JF1 (as FA19 but Δ*mtrR*) containing an *mtrC-lacZ* translational fusion. We found that although the level of *mtrC-lacZ* expression was higher in the JF1 background, unlike that of strain FA19, it was not influenced by iron limitation (Fig. 6B).

Binding of MpeR upstream of *mtrR*. In order to determine whether MpeR-mediated repression of *mtrR* was direct, we used EMSA to detect specific binding of an MBP-MpeR fusion protein to a target 360-bp DNA sequence that contained 186 bp of the upstream region containing the *mtrR* promoter (14, 15) and part (174 bp) of the *mtrR* coding sequence. Using a competitive EMSA, we found that incubation of the target DNA with 15 μg of the MBP-MpeR fusion protein resulted in the appearance of two shifted complexes. Of these two complexes, only the slower-migrating species (shown by the arrow in Fig. 7) gave evidence of being a specific complex, as it was reduced in intensity by the presence of a specific, unlabeled probe of similar length but not a nonspecific probe (*misR*).

DISCUSSION

Based on the data presented herein, we propose the existence of a regulatory pathway in gonococci that controls the expression of the *mtr* efflux pump locus. In this model (Fig. 8), the expression of *mtrF* is repressed by MpeR in an iron-responsive process shown by others to involve Fur- plus iron-mediated repression of *mpeR* (8, 19). We propose that MpeR also indirectly enhances *mtrCDE* expression due to its ability to directly repress *mtrR*, which encodes the main repressor of this operon (27), especially when free iron levels are decreased. Taken together, we suggest that since MtrR is the direct repressor of *mtrCDE* (15), levels of free iron would modulate the expression of this operon by controlling levels of MpeR and in turn MtrR. We cannot presently rule out, however, that MpeR binding to the *mtrR-mtrCDE* intervening sequence might additionally activate *mtrCDE* expression directly, and additional DNA-

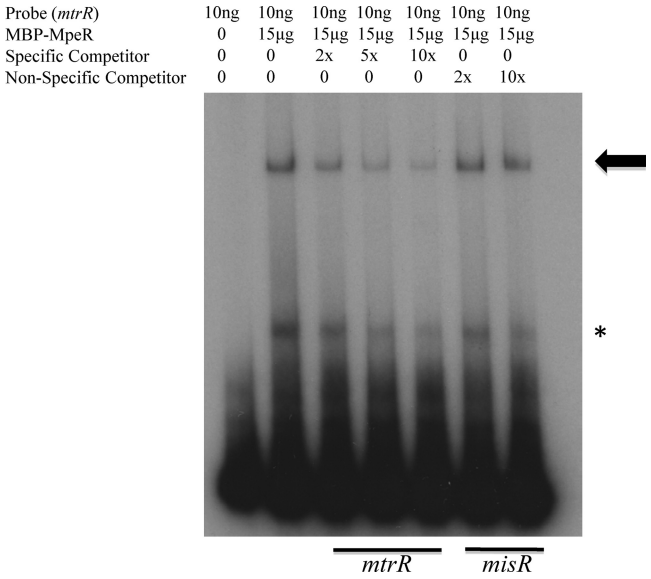

FIG 7 MpeR binds specifically to the upstream region of *mtrR*. The 363-bp *mtrR* upstream region was radiolabeled, and 10 ng of this DNA was incubated with 15 μg of MBP-MpeR alone. In order to demonstrate that binding of MBP-MpeR to *mtrR* was specific, increasing concentrations (2×, 5×, and 10×) of cold specific competitor (*mtrR*) or increasing concentrations (2× and 10×) of cold nonspecific competitor (*misR*) were added. The arrow indicates the specific complex, while the asterisk indicates the nonspecific complex.

binding studies are required to resolve this issue. The regulation of *mtrF* is also complex in that it is negatively controlled by both MpeR and MtrR. The mechanism(s) by which these two transcriptional regulators function together or in opposition under iron-replete or -depleted conditions is not yet clear and is the subject of ongoing investigation.

How might our observations help in understanding the biology and antimicrobial resistance potential of the gonococcus during infection on the genital mucosal surface, especially when this human pathogen is confronted with antimicrobials recognized by the Mtr efflux pump? For instance, in an infection where the gonococcus is confronted with an iron-restricted environment, the negative influence of Fur on *mpeR* expression would be diminished. We hypothesize that iron restriction would increase *mpeR* expression and that the increased level of MpeR would then dampen transcription of *mtrR*. As a consequence, levels of the Mtr efflux pump would increase, and this would enhance gonococcal infectivity as well as decrease bacterial susceptibility to antimicrobials recognized by the pump. In contrast to the iron-restricted situation, women infected with gonococci can develop serious complications and often present with pelvic inflammatory disease within days of menses onset when iron levels would be elevated due to blood flow. In this scenario, *mpeR* expression would be reduced because the repressive action of Fur would be favored and levels of MtrR would be higher than in an iron-restricted environment. Consequently, *mtrCDE* expression and antimicrobial resistance mediated by the pump would be lowered. We do not yet know whether conditions of iron restriction are responsible for the remarkable differences in the number and types of genes that were observed by microarray analysis to be regulated by MpeR during the mid- and late logarithmic phases of growth (Tables 4 and 5). Despite this uncertainty, our data emphasize that transcriptional regulators such as MpeR can exhibit very different regulons depending on growth phase.

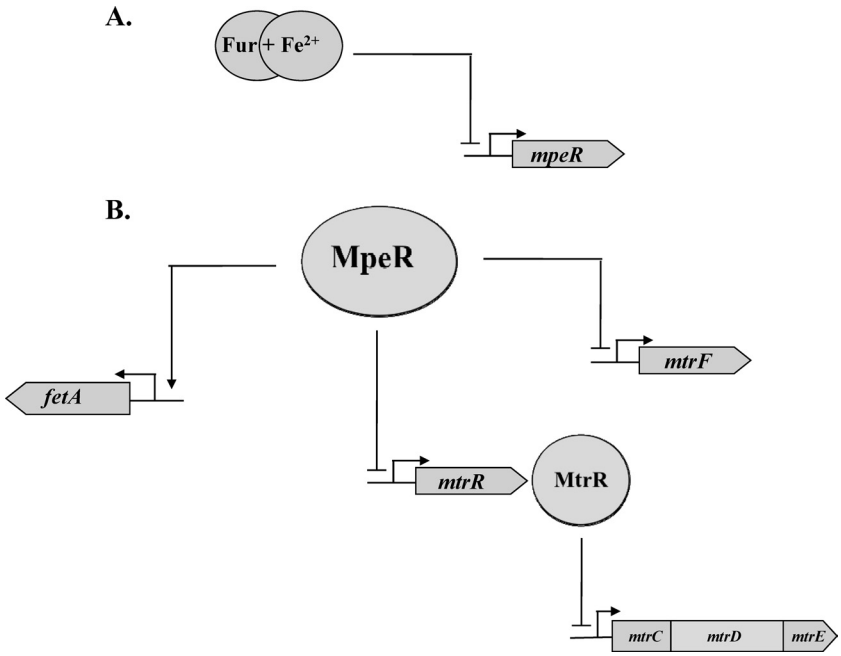

FIG 8 Model demonstrating the regulatory properties of MpeR that affect the high-level resistance mediated by the MtrCDE efflux system in an iron-dependent manner. (A) Under iron-replete conditions, Fur complexed with iron represses the expression of *mpeR*. (B) Under iron-depleted conditions, *mpeR* is derepressed and acts to repress the expression levels of both *mtrF* and *mtrR* in strain FA19. In strain FA1090, MpeR activates the expression of *fetA* (16). For all genes, the bent arrows identify promoter elements, while straight arrows indicate MpeR-activated genes and blocked lines designate MpeR-repressed genes.

Therefore, transcriptional profiling studies, particularly those involving investigations on antimicrobial resistance, should take this into account.

In addition to its importance in regulating *mtr*-associated efflux genes in gonococci, MpeR has recently been found by Hollander et al. (16) to directly activate transcription of *fetA* in strain FA1090 under iron-replete conditions. It is important to note that strain FA1090 employed by Hollander et al. is unrelated to strain FA19 employed in this investigation, and differences in how *mpeR* expression is regulated in these two strains may account for why we did not detect *fetA* as an MpeR-regulated gene in our array studies. Despite this uncertainty, FetA is of interest as it is the outer membrane transporter of a TonB-dependent receptor complex employed by gonococci to obtain iron bound to enterobactin-like siderophores produced by other bacteria (2, 16). FetA is also produced in *Neisseria meningitidis* (2) and is immunogenic, as evidenced by the presence of anti-FetA antibodies in convalescent-phase serum from patients recovering from meningococcal disease; these antibodies also cross-react with gonococci (1).

In conclusion, although MpeR was first identified based on its ability to control levels of gonococcal resistance to antimicrobials (12), it may have even greater significance in controlling genes of pathogenic *Neisseria* that encode proteins involved in iron utilization and perhaps other systems that are important in virulence expressed by gonococci and meningococci.

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