JOURNAL OF BACTERIOLOGY, Dec. 2003, p. 7145–7152 Vol. 185, No. 24
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.24.7145–7152.2003

FarR Regulates the *farAB*-Encoded Efflux Pump of *Neisseria gonorrhoeae* via an MtrR Regulatory Mechanism

E.-H. Lee,[1] C. Rouquette-Loughlin,[1] J. P. Folster,[1] and W. M. Shafer[1,2]*

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322,[1] and Laboratories of Microbial Pathogenesis, Veterans Affairs Medical Center, Decatur, Georgia 30033[2]

Received 16 July 2003/Accepted 8 September 2003

The *farAB* operon of *Neisseria gonorrhoeae* encodes an efflux pump which mediates gonococcal resistance to antibacterial fatty acids. It was previously observed that expression of the *farAB* operon was positively regulated by MtrR, which is a repressor of the *mtrCDE*-encoded efflux pump system (E.-H. Lee and W. M. Shafer, Mol. Microbiol. 33:839–845, 1999). This regulation was believed to be indirect since MtrR did not bind to the *farAB* promoter. In this study, computer analysis of the gonococcal genome sequence database, *lacZ* reporter fusions, and gel mobility shift assays were used to elucidate the regulatory mechanism by which expression of the *farAB* operon is modulated by MtrR in gonococci. We identified a regulatory protein belonging to the MarR family of transcriptional repressors and found that it negatively controls expression of *farAB* by directly binding to the *farAB* promoter. We designated this regulator FarR to signify its role in regulating the *farAB* operon. We found that MtrR binds to the *farR* promoter, thereby repressing *farR* expression. Hence, MtrR regulates *farAB* in a positive fashion by modulating *farR* expression. This MtrR regulatory cascade seems to play an important role in adjusting levels of the FarAB and MtrCDE efflux pumps to prevent their excess expression in gonococci.

Neisseria gonorrhoeae is a strictly human pathogen that causes the sexually transmitted disease gonorrhea. Gonococci often infect mucosal sites bathed in fluids containing host-derived antimicrobial hydrophobic agents (HAs), such as free fatty acids (FAs) and bile salts. Gonococcal resistance to certain antimicrobial HAs has been attributed to the *mtrCDE* (for "multiple transferable resistance")-encoded efflux pump, which exports a number of host-derived antimicrobial HAs (e.g., bile salts, antibacterial peptides, and FAs) (11, 28, 35). The *mtr* locus consists of three tandemly linked genes (*mtrCDE*) encoding cell envelope proteins that are divergently transcribed from the *mtrR* gene, positioned 250 bp upstream (11). The MtrR protein is a transcriptional repressor belonging to the TetR family of proteins and plays a critical role in modulating transcription of the *mtrCDE* operon. Thus, mutations within the *mtrR* coding region or the intergenic region between *mtrR* and *mtrCDE* enhanced *mtrCDE* expression, leading to elevated resistance to HAs (11, 12).

A recent study by Lee and Shafer (15) revealed a second efflux pump system that can modulate levels of gonococcal resistance to a subset of HAs. This system was termed *far* (for "FA resistance") because it confers resistance to long-chain FAs. The *far* system was found to be responsible for the *mtr*-independent mechanism of FA resistance, which was previously observed in a number of clinical isolates obtained from homosexual men with rectal infections (22). The *far* system is composed of the FarA membrane fusion protein and the FarB cytoplasmic membrane transporter protein. This efflux pump requires the MtrE protein (5) as the outer membrane channel to export antibacterial FAs from inside the cell (15). Although the *mtr*- and the *far*-encoded systems independently mediate gonococcal resistance to host-derived antimicrobial HAs, their expression is controlled by MtrR. In contrast to that of the *mtr* system, expression of *farAB* was positively associated with the presence of a functional MtrR protein. However, the results indicated that this regulation was indirect because MtrR did not bind to the *farAB* operon (15).

At the amino acid sequence level, the *farAB* system is similar to the *emrAB* efflux pump system of *Escherichia coli*, which provides resistance to uncoupling agents and certain antibiotics (17). The *emrAB* operon is negatively controlled by the product of the *emrR* gene, which is located upstream of *emrA* (18). EmrR belongs to the MarR family of transcriptional regulatory proteins, which control a variety of biological functions, including resistance to antimicrobial agents (e.g., antibiotics, organic solvents, and oxidative stress agents) (1, 24). In addition to EmrR, at least two MarR family proteins are involved in the resistance of *E. coli* (MarR) and *Pseudomonas aeruginosa* (MexR) to antimicrobial agents by modulating the expression of efflux pump systems. MarR is a negative regulator of the *marRAB* operon in *E. coli* (21, 32). Mutations in the *marR* gene or certain inducing conditions cause the overexpression of the MarA activator, resulting in activation of a number of genetic loci, including the *acrAB* efflux pump system, and enhance bacterial resistance to antimicrobial agents (10, 27). In *P. aeruginosa*, inactivation of the MexR repressor results in the overexpression of the *mexA-mexB-oprM* efflux pump system, which is a major determinant for resistance to a broad range of antimicrobials (29, 31, 37, 40). The analysis of the crystal structures for MarR and MexR suggested that MarR family proteins bind to DNA as dimers through a con-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322. Phone: (404) 728-7688. Fax: (404) 329-2210. E-mail: wshafer@emory.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference(s)
N. gonorrhoeae		
FA19	Wild type	8, 9, 11, 33
EL12	FA19 (pLFAB1)	This study
EL24	FA19 *farR*::Km^r	This study
EL26	FA19 *farR*::Km^r (pLFAB1)	This study
EL27	FA19 *marR2*::Km^r	This study
EL29	FA19 *marR2*::Km^r (pLFAB1)	This study
EL33	FA19 (pLFAR1)	This study
EL35	FA19 *farR*::Km^r (pLFAR1)	This study
EL37	FA19 Δ*mtrR* (pLFAR1)	This study
KH11	FA19 Δ*mtrR*	11
E. coli TOP10	(F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*)ϕ80 *lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r *endA1 nupG*)	Invitrogen
Plasmids		
pLES94	pUC18 derivative allowing chromosomal *lacZ* fusion at *proAB* site in *N. gonorrhoeae*; Ap^r Cm^r	36
pLFAB1	pLES94 derivative carrying 300-bp upstream sequence of *farA* at *Bam*HI site	This study
pLFAR1	pLES94 derivative carrying 305-bp upstream sequence of *farA* at *Bam*HI site	This study
pBAD-TOPO	pUC-derived protein expression vector carrying C-terminal six-His tag; Ap^r	Invitrogen
pBFAR1	pBAD-TOPO derivative carrying 441-bp coding sequence of *farR*	This study

served helix-turn-helix motif (2, 3). This binding often occurs through recognition of inverted or direct repeat sequences (6, 21, 39).

Since we previously observed that the *farAB* operon was regulated indirectly by the MtrR repressor, we sought to elucidate the molecular mechanism by which *farAB* is regulated via a MtrR-dependent mechanism. Accordingly, we asked if there is a regulatory protein in gonococci that directly controls expression of the *farAB* operon. We now report on the identification of a regulatory protein (FarR) in gonococci that belongs to the MarR family and show that it directly controls expression of *farAB*. Furthermore, we found that the MtrR repressor modulates expression of *farR* and is consequently implicated in positive regulation of the *farAB* operon. Thus, MtrR appears to be of importance in differentially controlling two efflux pumps, those encoded by *mtrCDE* and *farAB*, in gonococci.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* strain TOP10 (Invitrogen, Carlsbad, Calif.) was used in all cloning experiments. *N. gonorrhoeae* strain FA19 was used as the primary gonococcal strain. *E. coli* strains were grown in Luria-Bertani broth at 37°C. Gonococcal strains were grown on gonococcal medium base (GCB) agar (Difco Laboratories, Detroit, Mich.) containing glucose and iron supplements at 37°C under 3.8% (vol/vol) CO_2 (33).

Efficiency-of-plating (EOP) analysis. After overnight growth on GCB agar plates with or without supplementation of palmitic acid (150 μg/ml; Sigma, St. Louis, Mo.), the CFU of gonococci were determined from GCB-palmitic acid plates and compared with the CFU on GCB plates as previously described (30).

Construction of an insertional mutation in *marR1* (*farR*) and *marR2*. A 956-bp region encompassing the *marR1* (*farR*) gene was prepared by PCR using oligo-

TABLE 2. Oligonucleotide sequences used in this study

Primer	Sequence (5′–3′)	Location[a]
farA26	CCCAAAAGAGAGATGCCTTGC	−213; C strand of *farA*
farA26B	CCCAAAAGAGGGATTCCTTGC	−213; C strand of *farA*
farA52B	CCATAAGATTGGGATCCGAATTTCC	+34; NC strand of *farA*
farB1	TTAGATACGACCATCGCCAACG	+79; C strand of *farB*
farB2	ACAACGACGGTCATTGCC	+426; NC strand of *farB*
farRB1	GCCGATGGGATCCCTTGTTG	+214; C strand of *farR*
farRB2	GGTCATCAGGGGATCCCTTG	−69; NC strand of *farR*
KH9#2	CGTTTCGGGTCGGTTTGACG	−12; NC strand of *mtrR*
KH9#3	GACGACAGTGCCAATGCAACG	+71; NC strand of *mtrC*
marR2	GTTTATGCACAGCATCACGG	+727; NC strand of *farR*
marR4	CCGGAAGCCAAGTTTCGAGA	−924; C strand of *farR*
marR7	ATGCCTACCCA ATCAAAACATGCG	+1; C strand of *farR*
marR8	CGAGTTCAACGCATCCTCG	−438; NC strand of *farR*
R2#1	CACACTGCTGATTCTGTTCGGC	−709; C strand of *marR2*
R2#2	CATCCACAGATAAGTGCCGAC	+1198; NC strand of *marR2*
R2#5	CAAAACCCTTGCCCGGGAAGGGTTGATTG	+191; C strand of *marR2*
R2#6	CAATCAACCCTTCCCGGGCAAGGGTTTTG	+220; NC strand of *marR*
RT1B	AAAATGCAGTTTGGATTCGAATGG	+11; C strand of *degR*

[a] Relative to translation start site of *farA* in the *lacZ* fusion. C, coding strand; NC, noncoding strand 45.

nucleotide primers *marR2* and *marR4* (Table 2). This PCR product was cloned into the pBAD-TOPO vector (Invitrogen) according to the manufacturer's instructions. The resulting construct was digested with *Stu*I, which cleaved the plasmid at a unique restriction site located at the 5′-end sequence of the insert. The nonpolar *aphA-3* cassette (23) was digested with *Sma*I from pUC18K and cloned into the *Stu*I site of the construct so that it would be in the same transcriptional orientation as *farR*. This recombinant plasmid was introduced into *E. coli* TOP10 by transformation. Transformants were selected with kanamycin (50 μg/ml; Sigma) after gene expression was induced with 0.002% (vol/vol) arabinose. The plasmid was then purified from the host *E. coli* and used to transform *N. gonorrhoeae* strain FA19 as described previously (11). The transformants were selected on GCB agar plates containing kanamycin (50 μg/ml). An insertional mutation in the *marR2* gene was also created with the kanamycin cassette as described above, with modifications. Briefly, two steps of PCR were carried out to create a *Sma*I restriction site in the middle of *marR2*. In the first step, two PCRs amplified each half of *marR2*. One reaction encompassed the upstream sequence and the 5′-end region of *marR2*, and the other one included the 3′ end of *marR2* and its downstream sequence. *Sma*I restriction sites were introduced at the 3′ end of the 5′ region with primer *R2#5* and at the 5′ end of the 3′ section of the *marR2* gene with primer *R2#6* (Table 2). An 1,800-bp fragment encompassing the entire *marR2* gene in which the *Sma*I site was created was then amplified with primers *R2#1* and *R2#2* (Table 2). The resulting DNA was cloned into the pUC18 vector, and the *aphA-3* cassette was introduced into the created *Sma*I site of the *marR2* gene.

Construction and use of *farAB-lacZ* and *farR-lacZ* fusions in gonococci. The *farAB-lacZ* and *farR-lacZ* fusions used in this study were prepared in pLES94 as previously described (36). Briefly, an approximately 300-bp sequence upstream of *farA* and *farR* was amplified by PCR using primers *farA26B* and *farA52B* and *farRB1* and *farRB2* (Table 2), respectively, and ligated into the *Bam*HI site of pLES94. The ligation mixtures were introduced into *E. coli* TOP10 by transformation, and transformants were selected on LB agar plates supplemented with ampicillin (100 μg/ml; Sigma). After the orientation and sequence of the insert were checked and found correct, the resulting plasmids were used to transform strain FA19 to allow insertion of the *farAB-lacZ* or *farR-lacZ* fusion at the *proAB* site on the gonococcal chromosome (36). Transformants were selected on GCB plates containing chloramphenicol (1 μg/ml; Sigma). To create a *farR* mutation in the *farR-lacZ* fusion strain (EL33), a 2.5-kb DNA sequence encompassing the *farR* coding region, interrupted by a kanamycin resistance cassette, was amplified by PCR using oligonucleotide primers *marR2* and *marR4* from strain EL24 (*farR*::Km^r) and this product was introduced into strain EL33 by transformation. For the *mtrR* mutation in strain EL33, a 720-bp region containing the deleted *mtrR* gene (11) was amplified from DNA of strain KH11 (same as FA19 except Δ*mtrR*) with primers *RT1B* and *KH9#3* and transformed into EL33 as described above. Transformants were selected on GCB agar containing kanamycin (50 μg/ml) for the *farR* mutation and erythromycin (0.5 μg/ml) for the *mtrR* mutation.

β-Gal assay. Nonpiliated, transparent colony types were routinely grown on GCB agar or in GCB broth (Difco) as previously described (11). GCB-grown cells were harvested when the optical density of culture at 600 nm reached 0.3, and plate culture cells were taken after 20 h of growth. The cells were washed once in phosphate-buffered saline (pH 7.4) and frozen at −70°C overnight. The cell pellets were suspended in lysate buffer (1 mM dithiothreitol, 100 mM potassium phosphate, pH 7.8) and broken by repeated freeze-thaw cycles. The cell debris was removed by centrifugation at 15,000 × *g* for 10 min at 4°C. The amount of β-galactosidase (β-Gal) in the cell extracts was measured with a chemiluminescent β-Gal assay kit (Clontech, Palo Alto, Calif.) by following the manufacturer's instructions.

Purification of FarR and MtrR. To overexpress and purify FarR, a 438-bp region containing the *farR* structural gene was amplified by PCR using oligonucleotides primers *marR7* and *marR8*. This PCR product was cloned into pBAD (Invitrogen), producing a C-terminal fusion with a histidine tag. The recombinant plasmid was introduced by transformation into *E. coli* TOP10, and the resulting transformants were selected on LB plates supplemented with ampicillin (100 μg/ml). The *E. coli* strain containing pBFAR1 was grown in 200 ml of LB broth at 37°C with vigorous aeration. When the optical density of the culture at 600 nm reached 0.6, FarR expression was induced by the addition of 0.002% (wt/vol) arabinose for 3 h. Cells were harvested by centrifugation at 6,000 × *g* for 10 min and resuspended in 20 ml of binding buffer (1 mM phenylmethylsulfonyl fluoride, 5 mM imidazole, 20 mM phosphate, 500 mM NaCl, pH 7.8). After addition of lysozyme (100 μg/ml), cells were broken by repeated freeze-thaw and sonication cycles. Insoluble debris was removed by centrifugation at 3,000 × *g* for 15 min, and the supernatant was passed through a 0.8-μm-pore-size syringe filter (Millipore). All further purification was carried out by following the manufacturer's instruction (Invitrogen). The supernatant was loaded on a mini-Ni^{2+} affinity column that was equilibrated with native binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8). After the column was washed with washing buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0) several times, the FarR-His protein was eluted with 5 ml of each of five imidazole elution buffers that had increasing imidazole concentrations (50, 100, 200. 350, and 500 mM). All fractions were collected and subjected to electrophoresis on a sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) gel (14). A major peak of FarR was eluted with a minor peak of non-FarR material at about 350 to 450 mM imidazole. To remove this minor contamination, the FarR fraction was further purified by high-pressure liquid chromatography (HPLC; Jupiter 5μ C4, 300 A, 250 by 4.6 mm; Phenomenex). A major peak of FarR was collected in a solution of 70% acetonitrile–10% water–0.1% (vol/vol) trifluoroacetic acid and lyophilized. The lyophilized FarR protein was dissolved in water, dialyzed overnight against a buffer composed of 5 mM Tris (pH 8.0), 5 mM EDTA, 2 mM dithiothreitol, and 0.01% Triton X-100, and concentrated with a Centricon YM-3 centrifugal filter (Amicon; Millipore). The N-terminal amino acid sequence of FarR was analyzed by the automated Edman degradation method using cLC-Procise sequenator (Applied Biosystems, Foster City, Calif.). The MtrR-maltose binding protein (MBP) was purified as described previously (19).

Electrophoretic mobility shift assay (EMSA). The *farAB* and *farR* promoter fragments were amplified by PCR from FA19 chromosomal DNA with oligonucleotides pairs *farA26* and *farA52B* for the *farAB* promoter, *farRB1*and *farRB2* for the *farR* promoter, *KH9#2* and *KH9#3* for the *mtrR-CDE* intervening region, and *farB1* and *farB2* for the *farB* coding region, (Table 2). The PCR products were end labeled with [γ-^{32}P]dATP by using T4 polynucleotide kinase (New England Biolabs). Approximately 5 ng of the labeled DNA fragment was incubated with FarR or MtrR in 30 μl of reaction buffer (10 mM Tris-HCl [pH 7.5], 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% [vol/vol] glycerol, 1 mM $MgCl_2$, 50 mM NaCl, poly[dI-dC] [0.5 μg/ml], salmon sperm [200 μg/ml]) at room temperature for 25 min. For the competition assay, a nonlabeled target or irrelevant DNA was added in the binding reaction buffer. Samples were subjected to electrophoresis in a 4.5% native polyacrylamide gel at 4°C, followed by autoradiography (19).

RESULTS AND DISCUSSION

Identification of a regulatory protein (FarR) involved in negative regulation of the *farAB* operon. Members of the MarR family of repressors (EmrR [*E. coli*], MarR [*E. coli*], and MexR [*P. aeruginosa*]) are involved in the regulation of efflux pump operons (3, 4, 6, 16, 18, 21, 29, 32, 39). In an attempt to identify a transcriptional regulatory protein that directly modulates *farAB*, we sought to identify a MarR-like protein(s) in gonococci. Since *Neisseria meningitidis* and *N. gonorrhoeae* are genetically closely related and since the complete annotation of the meningococcal genome sequencing database was available, we searched for a meningococcal *marR*-like gene(s) in the online database of *N. meningitidis* strain MC58 (www.tigr.org). This search identified two meningococcal open reading frames that encode transcriptional regulators of the MarR family (TIGR locus names: NMB1843 and NMB1585). Furthermore, sequences homologous to these genes, *marR1* for NMB1843 and *marR2* for NMB1585, were subsequently identified in the *N. gonorrhoeae* FA1090 genome sequence database (www.genome.ou.edu). When the putative products of *marR1* and *marR2* were compared with other MarR family regulatory proteins that modulate efflux pump operons, they were found to have 20 to 25% amino acid identity (data not presented).

To determine whether the putative *marR1*- or *marR2*-encoded protein or both regulate *farAB* gene expression, we created an insertional mutation within the *marR1* and *marR2* genes in *N. gonorrhoeae* strain FA19 (see Materials and Methods for details). Transformants of strain FA19 containing the kanamycin resistance (Km^r) cassette in *marR1* or *marR2* were

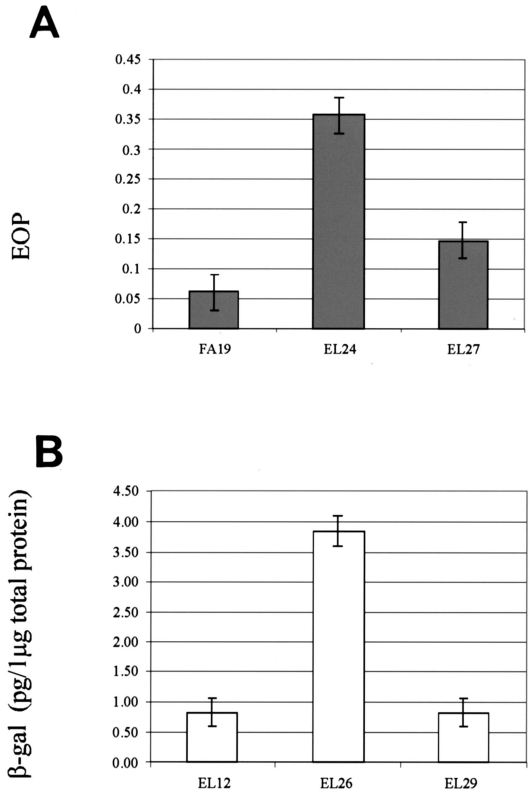

FIG. 1. Effect of the *marR1* mutation on FA resistance and *farAB* expression in *N. gonorrhoeae* FA19. (A) An EOP experiment was performed with strains FA19, EL24 (same as FA19 except *marR1*::Km^r) and EL27 (same as FA19 except *marR2*::Km^r) on GCB agar plates containing palmitic acid (150 μg/ml). EOPs are average values (± standard deviations [SD]) from at least three independent experiments. (B) Expression of *farAB* in EL12 (FA19[pLFAB1]) and its isogenic mutant strains EL26 (EL24[pLFAB1]) and EL29 (EL27[pLFAB1]). Shown are the amounts of β-Gal in cell extracts prepared as described in Materials and Methods from reporter strains EL12 and EL26, which contained the *farAB*::*lacZ* fusion. The results are averages of at least four independent experiments; error bars represent 1 SD.

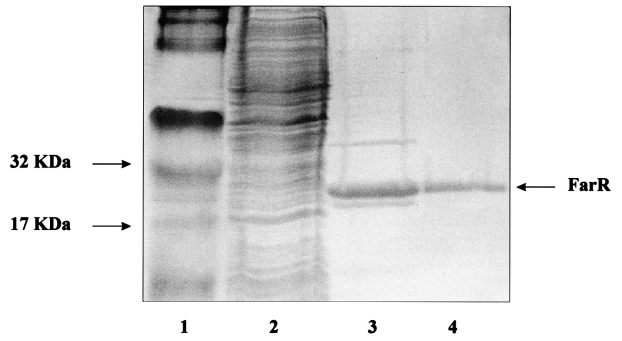

FIG. 2. Expression and purification of FarR-His. Protein samples collected during the purification were analyzed on SDS–15% PAGE gels stained with Coomassie brilliant blue. Lane 1, molecular weight standard markers (arrows [left], 17- and 32-kDa markers); lane 2, cell lysate after induction; lane 3, pooled fraction after Ni^{2+} affinity chromatography; lane 4, purified FarR after HPLC purification. Arrow (right), location of the FarR-His monomer.

examined for their susceptibility to long-chain FAs. Since these types of FAs are very hydrophobic, with limited solubility, it was impossible to obtain a FA concentration higher than the MIC for the wild-type strain FA19. We therefore performed an EOP experiment using palmitic acid as described previously (30). CFU of transformants EL24 (*marR1*::Km^r) and EL27 (*marR2*::Km^r) were calculated from bacterial growth on GCB agar plates supplemented or not supplemented with palmitic acid (150 μg/ml). The results demonstrated that the *marR1*::Km^r mutation had a more significant ($P = 0.01$) impact on gonococcal susceptibility to palmitic acid than the *marR2*::Km^r mutation ($P = 0.07$). In this respect, the EOP of strain EL24 was sixfold higher than that of the parental strain, FA19 (Fig. 1A).

To ascertain if MarR1 or MarR2 regulates the *farAB* operon, a *farAB-lacZ* reporter fusion was constructed in strain FA19. For this purpose, 300 bp of the *farAB* upstream region, including the ATG start codon and the codons for the first seven amino acids, was amplified by PCR and cloned into pLES94, resulting in a translational *lacZ* fusion. The resulting recombinant plasmid was introduced into the *proAB* site on the gonococcal chromosome by homologous recombination (36). We performed a chemiluminescence β-Gal assay using stationary cells grown overnight on GCB agar to permit maximal expression of *farAB*. The results showed that the *marR1* mutation (strain EL26) caused a fourfold increase ($P = 0.02$) in the amount of β-Gal production (Fig. 1B), while the *marR2* mutation (strain EL29) did not ($P = 0.5$) impact *farAB* expression (Fig. 1B). On the basis of these results and the FA susceptibility data (Fig. 1A), we hypothesized that MarR1 negatively regulates *farAB* expression. Because MarR1 had the most significant impact on gonococcal susceptibility to FAs and *farAB* expression, we studied it in more detail. MarR1 was renamed FarR to signify its role in regulation of *farAB* expression.

DNA-binding properties of FarR. To determine whether FarR regulates the expression of *farAB* by directly binding to the *farAB* operon, FarR was purified. The *farR* coding sequence was cloned into the pBAD-TOPO vector to form a C-terminal fusion with a histidine tag with expression under the control of the arabinose-inducible promoter in *E. coli* TOP10. Crude cell extracts were prepared from a 200-ml culture and passed through a mini-Ni^{2+} affinity column. Analysis of fractions eluted from this matrix by SDS-PAGE revealed that the FarR-His fusion protein was slightly contaminated with a protein of about 40 kDa (Fig. 2, lane 3). This contaminating 40-kDa protein was removed from the FarR-His fusion protein by HPLC (Fig. 2, lane 4). The recovered FarR-His fusion protein was shown to have a molecular mass of 20 kDa when analyzed by HPLC, which is in good agreement with the predicted mass of 20.68 kDa (data not shown). N-terminal amino acid sequencing confirmed that the first nine amino acids of the recovered protein (MPTQSKHAS) were identical to the corresponding amino acid sequence predicted by DNA sequence analysis of *farR* (data not presented). The DNA-binding capacity of the FarR-His protein was studied by EMSA. The target DNA consisted of approximately 300 bp of the *farA* upstream region that included the *farAB* promoter (P*farAB*). Using EMSA, we detected two potential FarR-DNA

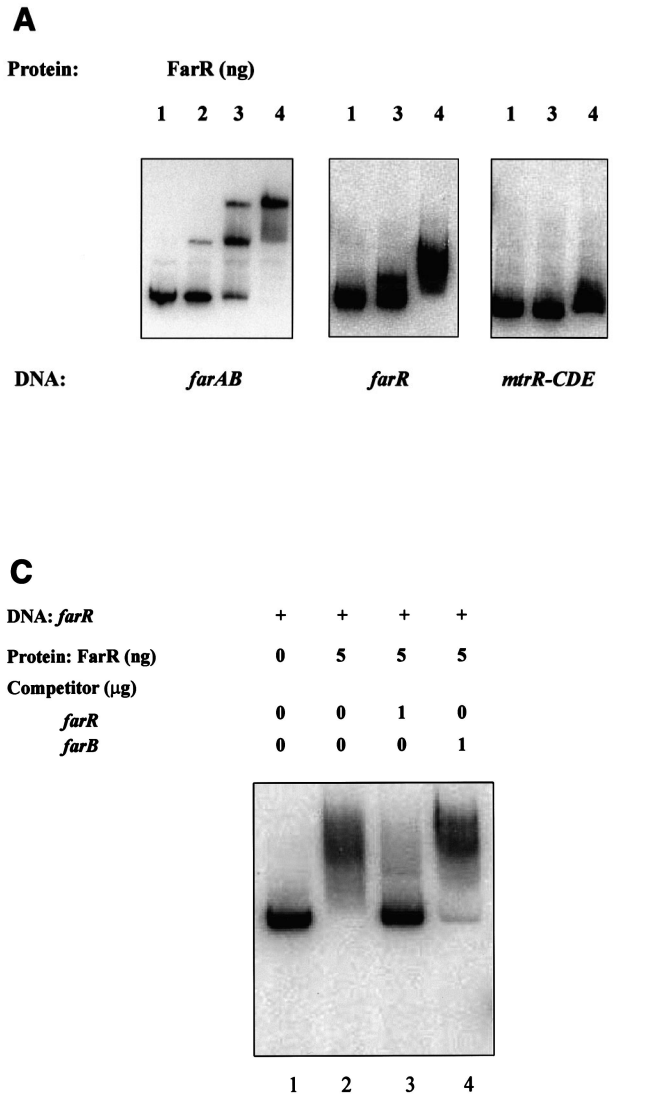

FIG. 3. DNA-binding properties of FarR. Shown is the binding of the purified His-tagged FarR protein to target DNA sequences. (A) FarR binding to *farAB*, *farR*, and *mtrR-CDE* promoter regions. Lanes 1, free labeled probe; lanes 2, probe with 0.2 ng of FarR; lanes 3, probe with 1 ng of FarR; lanes 4, probe with 5 ng of FarR. The probe used is indicated at the bottom of each panel. (B and C) Competition assays. (B) ^{32}P-labeled 300-bp DNA encompassing the *farAB* promoter region was incubated with 5 ng of FarR. This binding was competed with unlabeled *farAB* (300 bp) or the *mtrR-CDE* intergenic region (310 bp). Lane 1, no protein added; lane 2, FarR; lane 3, FarR with 0.1 μg of *farAB* DNA; lane 4, FarR with 1 μg of *farAB* DNA; lane 5, FarR with 0.1 μg of *mtrR-CDE* DNA; lane 6, FarR with 1 μg of *mtrR-CDE* DNA. (C) The ^{32}P-labeled 305-bp *farR* promoter region was incubated with 5 ng of FarR. This binding was competed with the unlabeled *farR* promoter (305 bp) or a DNA sequence containing the *farB* coding region (365 bp). Lane 1, no protein added; lane 2, FarR; lane 3, FarR with 1 μg of *farR* DNA; lane 4, FarR with 1 μg of *farB* DNA.

complexes. The first (form I) was observed at a level of 0.2 ng of FarR, and a slower-migrating complex (form II) was observed with increasing levels of FarR (1 to 5 ng) (Fig. 3A), suggesting that FarR binds to at least two sites in the upstream sequence of *farA*. To show the binding specificity of FarR, we performed competition assays. Addition of a 200-fold molar excess of a heterologous unlabeled DNA fragment containing 310 bp of the *mtrR-CDE* intervening region had no effect on binding (Fig. 3B). However, addition of unlabeled P*farAB* inhibited the binding of FarR to the labeled P*farAB* fragment. These results indicated that FarR binds to the *farAB* promoter in a specific manner.

Autoregulation of *farR* expression. Regulatory proteins that control expression of efflux pump operons are often subject to autoregulation (20, 29). To explore this possibility, we conducted an EMSA using FarR and a DNA fragment containing the *farR* promoter. A retarded complex was observed at the concentration of 5 ng, indicating that FarR might regulate its own expression (Fig. 3A). The specificity of this binding was confirmed by performing a competition assay, the results of which are shown in Fig. 3C. This experiment revealed that an unlabeled *farR* promoter sequence but not a DNA sequence within the *farB* coding region could compete with *farR* binding to the labeled *farR* promoter sequence. To determine if FarR regulates its own expression, the *farR* gene was inactivated by a nonpolar kanamycin resistance cassette in the *farR-lacZ* fusion strain EL33, giving rise to strain EL35, and β-Gal activity was assessed. The results (Table 3) revealed that inactivation of *farR* in strain EL35 resulted in a greater-than-twofold increase in β-Gal activity, indicating that FarR represses its own expression.

MtrR binds to the FarR promoter, resulting in regulation of *farAB*. Previously, we observed that MtrR, a repressor of the *mtr* efflux system (19, 28), was involved in positive regulation of the *farAB* operon (15). The presence of both MtrR and its DNA-binding activity was shown to be required for the basal level of *farAB* expression and FA resistance in gonococci. However, because MtrR did not bind to the *farAB* promoter in a specific manner (Fig. 4), we hypothesized that MtrR may indirectly regulate the *farAB* operon through its capacity to

TABLE 3. Effect of *farR* or *mtrR* mutation on *farR* expression in *N. gonorrhoeae*[a]

Strain	Vector	β-Gal sp act[b] (ng/10^7 CFU)
EL33 (MtrR$^+$ FarR$^+$)	pLFAR1[c]	248 ± 71.8
EL35 (MtrR$^+$ FarR$^-$)	pLFAR1	718 ± 190.2
EL37 (MtrR$^-$ FarR$^+$)	pLFAR1	381 ± 125.6

[a] *N. gonorrhoeae* (Opa$^-$ Pil$^-$) fusion strains were grown to late log phase in GCB broth and assayed for β-Gal activities.

[b] Data are averages of four experiments, each in duplicate, ± standard deviations. The differences between strains were significant (*P* values: EL33 versus EL35, 0.002; EL33 versus EL37, 0.009) as determined by Student's *t* test.

[c] pLFAR1 containing a translational *farAB-lacZ* fusion was inserted into the chromosomal *proAB* site of *N. gonorrhoeae.*

regulate another gene, perhaps a transcriptional repressor of *farAB* (15). Accordingly, we asked whether MtrR modulates *farR* expression. We noted that the putative promoter region for the *farR* gene contained a sequence (5′-GATTAAAATA TAACTATTAA-3′) resembling the *mtrR-CDE* intervening region. This sequence encompassed the −10 region of the *mtrR* promoter and the 13-bp inverted repeat sequence (homologous nucleotides are underlined), which was previously shown to be important for MtrR regulation of *mtrCDE* (12). However, this site only partially overlaps the MtrR-binding site previously identified by Lucas et al. (19), suggesting that the precise nucleotides for MtrR regulation remain unidentified. Therefore, to determine if MtrR binds to the *farR* promoter, we performed an EMSA using a purified MBP-MtrR fusion protein (19). MBP-MtrR-dependent gel shifts were observed for the *mtrR-mtrCDE* intergenic and the *farR* promoter regions (Fig. 4). The specificity of this binding was indicated by the finding that an unlabeled *farR* promoter DNA sequence could compete with the labeled complexes but that the *farAB* upstream sequence was unable to compete with this binding (data not shown).

To ascertain the in vivo effects of MtrR binding to the *farR* promoter, we constructed an *mtrR* deletion strain from a derivative of strain FA19 that contained a translational *farR-lacZ* fusion (strain EL37). The level of β-Gal activity, directly correlating with *farR* expression, in cells taken at late log phase of growth was assessed. The results demonstrated that deletion of

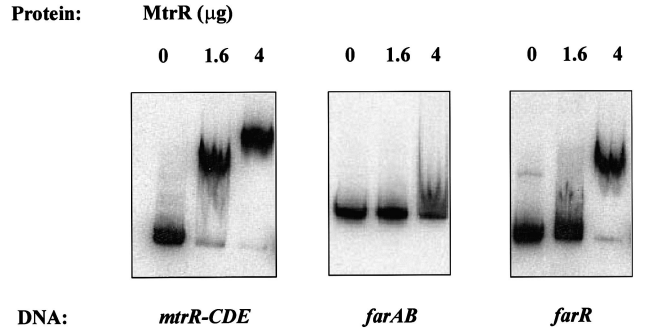

FIG. 4. MtrR binds to the DNA sequence upstream of *farR.* Shown is the binding of MBP-MtrR to target DNA sequences, the *mtrR-CDE*, *farAB*, and *farR* promoter regions. Lanes 1 (from left), free labeled probe; lanes 2, probe with 1.6 μg of MBP-MtrR; lanes 3, probe with 4 μg of MBP-MtrR. The probe used is indicated at the bottom.

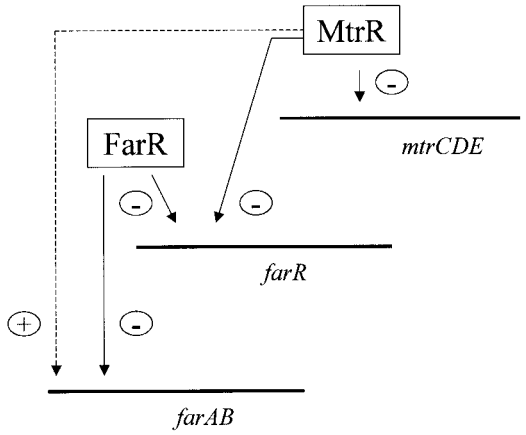

FIG. 5. A model for MtrR regulation of *farAB* and *mtrCDE* efflux *pump* operons in *N. gonorrhoeae*. This model describes the ability of MtrR to positively regulate (+) *farAB* expression by repressing (−) *farR* and *mtrCDE* expression. This MtrR regulatory circuit is most likely to be important in preventing the excess expression of these efflux pumps in gonococci.

mtrR resulted in a 54% increase in *farR-lacZ* expression compared with that for the parent strain (Table 3). These results indicate that MtrR negatively regulates *farR* expression. It is important to emphasize that the modest increase in *farR* expression due to the loss of MtrR is not an unusual phenomenon for efflux pump operons since inactivation of *acrR* and *mexR* was also shown to cause only a small increase in expression of the *acrAB* and *mexAB-oprM* efflux pump operons, respectively (20, 29). Taken together, these results suggest that MtrR is involved in positive regulation of *farAB* expression by downregulating expression of *farR*.

N. gonorrhoeae possesses *far*- and *mtr*-encoded efflux pumps to independently meditate gonococcal resistance to host-derived HAs. FarAB belongs to the major facilitator superfamily, while MtrCDE belongs to the resistance/nodulation/division family, of drug efflux pumps. Despite the structural dissimilarities between FarAB and MtrCDE, their expression is related in that both of the efflux operons are regulated by the same transcriptional regulatory protein (MtrR). Previously, Lee and Shafer (15) observed that MtrR was indirectly involved in the positive regulation of *farAB* whereas MtrR repressed *mtrCDE*. The results presented herein strongly suggest that MtrR modulates *farAB* indirectly via regulation of a second gene, *farR*, which encodes a repressor of *farAB* (Fig. 5). It is important that, because *farAB* expression is less than that of *mtrCDE*, the decreased amount of MtrE, which is shared by both efflux pumps in an MtrR-positive strain (e.g., FA19), is likely to be sufficient for maximal FarAB activity (15).

The mechanism by which MtrR regulates expression of the *far* and *mtr* systems emphasizes two important features. First, the *farAB* operon can be directly regulated in a negative manner by FarR. Our data also support the notion that this operon can be upregulated by MtrR and by FarR repression of *farR* (Fig. 5). This is different from many other efflux pump operons, which are directly regulated by an activator (e.g., the MexT activator of *mexEF-oprN* in *P. aeruginosa* [13]) or a repressor (e.g., the EmrR repressor of the *emrAB* operon in *E. coli* [18]).

The ability of MtrR to regulate *mtr* and *far* efflux operons in opposite ways highlights an important feature of gene control in gonococci (Fig. 5) because loss of MtrR repressor activity resulted in increased expression of *mtrCDE* but decreased expression of *farAB.* A similar regulatory scheme has been reported for the production of two major porins, OmpF and OmpC, which determine the permeability of the outer membrane in *E. coli*. The production of OmpF and OmpC is under the control of EnvZ and OmpR, a two-component signal transduction system encoded by the *ompB* locus. The level of OmpF, which forms a larger pore, relative to that of OmpC was modulated by the status of OmpF phosphorylation in response to environmental conditions (7, 25). Switching between OmpF and OmpC seems to be an important part of bacterial adaptation and survival under stress conditions (26).

N. gonorrhoeae uses the *mtr* and *far* efflux pump systems to resist the antimicrobial agents that bathe certain mucosal sites which this organism infects (15, 22, 34). However, overproduction of an efflux pump seems to be detrimental, as gonococcal growth was slowed when the *mtr* system was overproduced (9). In dealing with this problem, gonococci seem to use MtrR to adjust the total activity of efflux pumps. Our results may also explain why McFarland et al. (22) observed an Mtr-independent mechanism by which gonococci resist fecal lipids since their test strains did not express resistance to HAs such as erythromycin or Triton X-100, which would have required *mtrR* mutations to cause overexpression of *mtrCDE*. During rectal infections where gonococci would be confronted with toxic fecal lipids, those strains producing an active MtrR repressor would increase *farAB* expression due to the ability of MtrR to reduce *farR* expression. This hypothesis is in keeping with the model described in Fig. 5.

We observed that a DNA sequence upstream of *farR* resembles the *mtrR-CDE* intervening region encompassing an inverted repeat sequence. Conventional and competitive EMSA experiments that used a PCR product encompassing the sequence upstream of *farR* revealed that MtrR could bind to this region. This observation, coupled with the results from β-Gal fusion assays (Table 3), demonstrates that MtrR is a multigene regulator in gonococci. We are now addressing this hypothesis and are attempting to identify other MtrR-regulated genes through a combination of proteomic and genomic approaches. Because an MtrR-like protein was identified as a potential virulence factor in *P. aeruginosa* (38), it may be that MtrR in gonococci and similar proteins in other bacteria regulate genes involved in virulence.

ACKNOWLEDGMENTS

We are grateful to J. Pohl and O. Stuchlik of the Emory Microchemical Facility for their help in FarR purification and N-terminal sequencing of FarR. We are thankful to the Gonococcal Genome Sequencing Project (supported by NIH grant AI-38399) of the University of Oklahoma (B. A. Roe, S. P. Lin, L. Song, X. Yuan, S. Clifton, T. Dulcey, L. Lewis, and D. W. Dyer) for providing the sequence of the FA1090 genome online. We are also thankful to L. Pucko, S. Katzif, and S. Satola for helping with manuscript preparation and careful reading.

The protein purification and sequencing work performed at the Microchemical Facility was supported by NIH-NCRR grants 02878, 12878, and 13948. Work in our laboratories was supported by NIH grants. AI-21150-17 (W.M.S.) and AI-37945 (R. Lehrer, UCLA Health Sciences Center). J.P.F. was supported by NIH training grant 5T32 AI-07470. W.M.S. is the recipient of a Senior Research Career Scientist Award from the VA Medical Research Service.

REFERENCES

1. **Alekshun, M. N., and S. B. Levy.** 1999. The *mar* regulon: multiple resistance to antibiotics and other toxic chemicals. Trends Microbiol. **7:**410–413.
2. **Alekshun, M. N., Y. S. Kim, and S. B. Levy.** 2000. Mutational analysis of MarR, the negative regulator of *marRAB* expression in *Escherichia coli*, suggests the presence of two regions required for DNA binding. Mol. Microbiol. **35:**1394–1404.
3. **Alekshun, M. N., S. B. Levy, T. R. Mealy, B. A. Seaton, and J. F. Head.** 2001. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 Å resolution. Nat. Struct. Biol. **8:**710–714.
4. **Cohen, S. P., H. Hachler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
5. **Delahay, R. M., B. D. Robertson, J. T. Balthazar, W. M. Shafer, and C. A. Ison.** 1997. Involvement of the gonococcal MtrE protein in the resistance of *Neisseria gonorrhoeae* to toxic hydrophobic compounds. Microbiology **143:** 2127–2133.
6. **Evans, K., L. Adewoye, and K. Poole.** 2001. MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic binding sites in the *mexA-mexR* intergenic region. J. Bacteriol. **183:**807–812.
7. **Forst, S., and M. Inouye.** 1988. Environmentally regulated gene expression for membrane proteins in *Escherichia coli*. Annu. Rev. Cell Biol. **4:**21–42.
8. **Guymon, L. F., and P. F. Sparling.** 1975. Altered crystal violet permeability and lytic behavior in antibiotic-resistant and -sensitive strains of *Neisseria gonorrhoeae*. J. Bacteriol. **124:**757–763.
9. **Guymon, L. F., D. L. Walstad, and P. F. Sparling.** 1978. Cell envelope alterations in antibiotic-sensitive and -resistant strains of *Neisseria gonorrhoeae*. J. Bacteriol. **136:**391–401.
10. **Hächler, H., S. P. Cohen, and S. B. Levy.** 1991. *marA*, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **173:**5532–5538.
11. **Hagman, K. E., W. Pan, B. G. Spratt, J. T. Balthazar, R. C. Judd, and W. M. Shafer.** 1995. Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrCDE* efflux system. Microbiology **141:**611–622.
12. **Hagman, K. E., and W. M. Shafer.** 1995. Transcriptional control of the *mtr* efflux system of *Neisseria gonorrhoeae*. J. Bacteriol. **177:**4162–4165.
13. **Köhler, T., S. F. Epp, L. K. Curty, and J.-C. Pechère.** 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol. **181:**6300–6305.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
15. **Lee, E.-H., and W. M. Shafer.** 1999. The *farAB*-encoded efflux pump mediates resistance of gonococci to long-chained antibacterial fatty acids. Mol. Microbiol. **33:**839–845.
16. **Lim, D., K. Poole, and N. Strynadka.** 2002. Crystal structure of the MexR repressor of the *mexAB-oprM* multi-drug efflux operon of *Pseudomonas aeruginosa*. J. Biol. Chem. **277:**29253–29259.
17. **Lomovskaya, O., and K. Lewis.** 1992. *emr*, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
18. **Lomovskaya, O., K. Lewis, and A. Martin.** 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. J. Bacteriol. **177:**2328–2334.
19. **Lucas, C. E., J. T. Balthazar, K. E. Hagman, and W. M. Shafer.** 1997. The MtrR repressor binds the DNA sequence between the *mtrR* and *mtrC* genes of *Neisseria gonorrhoeae*. J. Bacteriol. **179:**4123–4128.
20. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19:**101–112.
21. **Martin, R. G., and J. L. Rosner.** 1995. Binding of purified multiple antibiotic-resistance repressor protein (MarR) to *mar* operator sequences. Proc. Natl. Acad. Sci. USA **92:**5456–5460.
22. **McFarland, L., T. A. Mietzner, J. S. Knapp, E. Sandstrom, K. K. Homes, and S. A. Morse.** 1983. Gonococcal susceptibility to fecal lipids can be mediated by an *mtr*-independent mechanism. J. Clin. Microbiol. **18:**121–127.
23. **Ménard, R., P. J. Sansonetti, and C. Parsot.** 1993. Nonpolar mutagenesis of *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. J. Bacteriol. **175:**5899–5906.
24. **Miller, P. F., and M. C. Sulavik.** 1996. Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in *Escherichia coli*. Mol. Microbiol. **21:**441–448.
25. **Mizuno, T., and S. Mizushima.** 1990. Signal transduction and gene regulation through the phosphorylation of two regulatory components: the molecular basis for the osmotic regulation of the porin genes. Mol. Microbiol. **4:**1077–1082.
26. **Nikaido, H., and M. Vaara.** 1987. Outer membrane, p. 7–22. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.). *Escherichia coli* and *Salmonella typhimurium*: cellular

and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
27. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
28. **Pan, W., and B. G. Spratt.** 1994. Regulation of the permeability of the gonococcal cell envelope by the *mtr* system. Mol. Microbiol. **11:**769–775.
29. **Poole, K., K. Tetro, Q. Zhao, S. Neshat, D. Heinrichs, and N. Bianco.** 1996. Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40:**2021–2028.
30. **Rouquette-Loughlin, C., I. Stojiljkovic, T. Hrobowski, J. T. Balthazar, and W. M. Shafer.** 2002. Inducible, but not constitutive, resistance of gonococci to hydrophobic agents due to the MtrC-MtrD-MtrE efflux pump requires TonB-ExbB-ExbD proteins. Antimicrob. Agents Chemother. **46:**561–565.
31. **Saito, K., H. Yoneyama, and T. Nakae.** 1999. *nalB*-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the *mexR* gene of the *Pseudomonas aeruginosa* chromosome. FEMS Microbiol. Lett. **179:**67–72.
32. **Seoane, A. S., and S. B. Levy.** 1995. Characterization of MarR, the repressor of the multiple antibiotic resistance (*mar*) operon of *Escherichia coli*. J. Bacteriol. **177:**3414–3419.
33. **Shafer, W. M., L. F. Guymon, I. Lind, and P. F. Sparling.** 1984. Identification of an envelope mutation (*env-10*) resulting in increased antibiotic susceptibility and pyocin resistance in a clinical isolate of *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **25:**767–769.
34. **Shafer, W. M., J. T. Balthazar, K. E. Hagman, and S. A. Morse.** 1995. Missense mutations that alter the DNA-binding domain of the MtrR protein occur frequently in rectal isolates of *Neisseria gonorrhoeae* that are resistant to faecal lipids. Microbiology **141:**904–911.
35. **Shafer, W. M., X.-D. Qu, A. J. Waring, and R. I. Lehrer.** 1998. Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. Proc. Natl. Acad. Sci. USA **95:**1829–1833.
36. **Silver, L. E., and V. L. Clark.** 1995. Construction of a translational *lacZ* fusion system to study gene regulation in *Neisseria gonorrhoeae*. Gene **166:** 101–104.
37. **Srikumar, R., C. J. Paul, and K. Poole.** 2000. Influence of mutations in the *mexR* repressor gene on expression of the MexA-MexB-oprM multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol. **182:**1410–1414.
38. **Tan, M. W., L. G. Rahme, J. A. Sternberg, R. G. Tompkins, and F. M. Ausubel.** 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. Proc. Natl. Acad. Sci. USA **96:**2408–2413.
39. **Xiong, A., A. Gottman, C. Park, M. Baetens, S. Pandza, and A. Martin.** 2000. The EmrR protein represses the *Escherichia coli emrRAB* multidrug resistance operon by directly binding to its promoter region. Antimicrob. Agents Chemother. **44:**2905–2907.
40. **Ziha-Zarifi, I., C. Llanes, T. Kohler, J. C. Pechere, and P. Plesiat.** 1999. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrob. Agents Chemother. **43:**287–291.