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Regulation of *mtrF* Expression in *Neisseria gonorrhoeae* and Its Role in High-Level Antimicrobial Resistance

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The obligate human pathogen *Neisseria gonorrhoeae* uses the MtrC-MtrD-MtrE efflux pump to resist structurally diverse hydrophobic antimicrobial agents (HAs), some of which bathe mucosal surfaces that become infected during transmission of gonococci. Constitutive high-level HA resistance occurs by the loss of a repressor (MtrR) that negatively controls transcription of the *mtrCDE* operon. This high-level HA resistance also requires the product of the *mtrF* gene, which is located downstream and transcriptionally divergent from *mtrCDE*. MtrF is a putative inner membrane protein, but its role in HA resistance mediated by the MtrC-MtrD-MtrE efflux pump remains to be determined. High-level HA resistance can also be mediated through an induction process that requires enhanced transcription of *mtrCDE* when gonococci are grown in the presence of a sublethal concentration of Triton X-100. We now report that inactivation of *mtrF* results in a significant reduction in the induction of HA resistance and that the expression of *mtrF* is enhanced when gonococci are grown under inducing conditions. However, no effect was observed on the induction of *mtrCDE* expression in an MtrF-negative strain. The expression of *mtrF* was repressed by MtrR, the major repressor of *mtrCDE* expression. In addition to MtrR, another repressor (MpeR) can downregulate the expression of *mtrF*. Repression of *mtrF* by MtrR and MpeR was additive, demonstrating that the repressive effects mediated by these regulators are independent processes.

Neisseria gonorrhoeae is an obligate human pathogen and the causative agent of the sexually transmitted disease gonorrhea. In the United States alone, over 350,000 cases of gonorrhea were reported in 2002 (3). Recent studies have shown that untreated cases exceed treated cases, suggesting that the number of gonococcal infections is much greater than reported (36). Although the number of gonorrhea cases in the United States has declined since the 1970s, the trend of increasing antimicrobial resistance has become a significant problem.

In the last two decades, antibiotic resistance has dramatically increased for several pathogenic bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *N. gonorrhoeae* (1, 12, 14, 26). The dramatic rise in microbial antibiotic resistance has become a major public health concern worldwide. The active pumping of these antibacterial agents out of the cell by efflux pump systems has been recognized as a major contributor to bacterial resistance to antibiotics. Although high-level expression of efflux pumps may permit clinically significant levels of resistance of bacteria to antimicrobial agents, the expression of these pumps and the active removal of these agents is an energy-expensive process. Therefore, the expression of efflux systems is usually tightly regulated (7).

Resistance of *N. gonorrhoeae* to structurally diverse hydrophobic agents (HAs) can be mediated by the *mtr* (multiple transferable resistance) locus (39, 40). HAs include antibiotics (including penicillin and erythromycin), nonionic detergents (including the spermacide, nonoxynol-9), and certain antimicrobial peptides that are produced at host mucosal surfaces. The *mtr* locus encodes an energy-dependent efflux system composed of three membrane proteins (MtrC, MtrD, and MtrE) that form the core components of the efflux pump, a transcriptional repressor (MtrR), and a gene (*mtrF*) that encodes an inner membrane protein (9, 37). MtrD is located in the inner membrane and functions as the transporter component of the pump. MtrE is an outer membrane protein whose function is similar to that of the *Escherichia coli* TolC protein and forms the channel for export of agents to the extracellular milieu. MtrC is a periplasmic protein with significant homology to a class of proteins termed membrane fusion proteins. MtrC functions as a bridge contacting the inner membrane component, MtrD, and outer membrane component, MtrE, of the efflux apparatus.

The *mtrCDE* operon is regulated by both positive and negative control mechanisms. These systems serve to tightly regulate the expression of the pump apparatus and to allow induction during exposure to HAs (10). The *mtrR* gene encodes a transcriptional repressor of *mtrCDE* expression (27), whereas the *mtrA* gene encodes a transcriptional activator similar to members of the AraC/XylS family and is required for inducible HA resistance. Mutations in *mtrR* or its promoter can enhance constitutive levels of HA resistance in gonococci due to increased expression of *mtrCDE*. In contrast, mutations in *mtrA* inhibit the ability of gonococci to express inducible levels of HA resistance.

The *mtrF* gene, which is required for high-level constitutive HA resistance mediated by the MtrC-MtrD-MtrE efflux pump, is located just downstream and transcribed divergent from *mtrR*. MtrF is a putative 58-kDa inner membrane protein composed of 12 transmembrane domains. Homologs of *mtrF* were identified in a number of diverse bacteria. With the exception

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype[a]	Source or reference
Strains		
Neisseria gonorrhoeae		
FA19	Wild type	8
JF1	Δ*mtrR*	9
FA19 *mtrF*::Km^r	Inactivation of *mtrF* with insertion of *aphA*-3	This study
FA19 *mtrC-lacZ*	Translational fusion of the promoter region of *mtrC* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 *mtrF*::Km^r *mtrC-lacZ*	Inactivation of *mtrF* with insertion of *aphA*-3, translational fusion of the promoter region of *mtrC* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 *mtrF-lacZ*	Translational fusion of the promoter region of *mtrF* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
JF1 *mtrF-lacZ*	Δ*mtrR*, translational fusion of the promoter region of *mtrF* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 *mpeR*::Km^r *mtrF-lacZ*	Inactivation of *mpeR* with insertion of *aphA*-3, translational fusion of the promoter region of *mtrF* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 *mpeR*::Km^r *mtrC-lacZ*	Inactivation of *mpeR* with insertion of *aphA*-3, translational fusion of the promoter region of *mtrC* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
JF1 *mpeR*::Km^r *mtrF-lacZ*	Δ*mtrR*, translational fusion of the promoter region of *mtrF* to the *lacZ* gene and inserted into the chromosome (Cm^r), inactivation of *mpeR* with the insertion of *aphA*-3	This study
Escherichia coli		
DH5α mcr	[F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*]	31
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		
pBAD-TOPO-T/A	pUC-derived protein expression vector; Ap^r	Invitrogen
pLES94	pUC18-derivative allowing a chromosomal *lacZ* fusion at the *proAB* site in *Ng*; Ap^r Cm^r	34
p*PmtrC*	pLES94 containing the 248-bp upstream sequence of *mtrC* at the BamHI site	This study
p*PmtrF*	pLES94 containing the 138-bp upstream sequence of *mtrF* at the BamHI site	This study

[a] Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance.

of the AbgT transporter of *E. coli*, a transporter of *p*-aminobenzoyl-glutamate (13), all identified homologs were hypothetical proteins with unknown functions. Inactivation of *mtrF* had no discernible effect on the HA susceptibility property of gonococcal strain FA19 to hydrophobic agents, including erythromycin, Triton X-100 (TX-100), and crystal violet (37). However, inactivation of *mtrF* in an *mtrR* mutant resulted in a significant decrease in resistance to HAs. These results suggested that *mtrF* is necessary for high-level resistance in gonococcal strains lacking MtrR. The capacity of bacteria to express inducible levels of resistance to antimicrobial agents through efflux-dependent processes requires certain transcriptional regulatory proteins (6, 9) and membrane proteins other than those that are thought to be core components of the pump (24, 25). In order to gain insight regarding the involvement of MtrF in efflux of HAs, we examined whether it is required for inducible HA resistance and studied the regulation of *mtrF* expression. We also report that the expression of *mtrF* is negatively regulated by MtrR and a newly described transcriptional regulator, MpeR.

(A preliminary report of these findings was presented at the 14th International Pathogenic *Neisseria* Conference held in Milwaukee, Wis., September 5 to 10, 2004.)

MATERIALS AND METHODS

Bacterial strains, culture conditions, and HA susceptibility testing. The bacterial strains used in the present study are listed in Table 1. *E. coli* strain TOP10 (Invitrogen, Carlsbad, CA) and *E. coli* DH5α mcr (31) were used in all cloning experiments. *E. coli* strains were grown in Luria-Bertani broth or on Luria-Bertani agar plates at 37°C. *N. gonorrhoeae* strain FA19 was used as the primary gonococcal strain. Gonococcal strains were grown on gonococcal medium base (GCB) agar (Difco Laboratories, Detroit, MI) containing glucose and iron supplements at 37°C under 3.8% (vol/vol) CO_2. All chemicals were purchased from Sigma Biochemical (St. Louis, MO). The MICs of selected antimicrobial agents against all strains were determined as previously described (8).

Construction of the insertional inactivation of *mtrF* and *mpeR*. The *mtrF* gene was previously inactivated in FA19 by the insertion of a kanamycin resistance (Km^r) cassette (*aphA*-3) to generate strain WV9 (22). Chromosomal DNA from strain WV9 was used to transform FA19, and transformants were selected on GCB agar plates containing 50 μg of kanamycin/ml. A single colony was selected and sequencing analysis of the *mtrF* gene confirmed the genotype of FA19 *mtrF*::Km^r. DNA sequencing was performed by using the Nucleic Acid Sequencing Core Facility of Emory University. The nucleotide sequence for *mpeR* is available through GenBank accession number AY941321. The *mpeR* gene was inactivated by the insertion of a kanamycin resistance cassette (*aphA-3*). Primers 5′*mpeR* (5′-ATGAACACCGCCGCCATCT-3′) and 3′*mpeR* (5′-GCACTTTTT CACATCCGAAGG-3′) were used to PCR amplify *mpeR* from FA19 chromosomal DNA. The gene was inserted into pBAD-TOPO-T/A and transformed into *E. coli* TOP10 as described in the manufacturer's protocol (Invitrogen). The nonpolar *aphA-3* cassette (22) was removed from pUC18K after digestion with SmaI and cloned into a NaeI restriction site of *mpeR*. This recombinant plasmid was introduced into DH5α TOP10 by transformation. The plasmid was purified, and the inactivated *mpeR*::Km^r sequence was PCR amplified by using the primers 5′*mpeR* and 3′*mpeR*. The amplified product was used to transform FA19 and JF1 and selected for by growth on GCB containing 50 μg of kanamycin/ml. PCR and sequencing analysis confirmed the insertion of the kanamycin cassette into the chromosomal *mpeR* gene.

Construction of the *mtrC-lacZ* and *mtrF-lacZ* fusions in gonococci. The translational *lacZ* fusions were constructed as previously described (34). In brief, the promoter sequence of *mtrF* was PCR amplified from strain FA19 by using the primers 5′P*mtrF* (5′-TTGGATCCGAATAACGATGTGGGCATTTTC-3′) and 3′P*mtrF* (5′-TTGGATCCCGACTCATCTGCTTCTCCTTAA-3′). The promoter sequence of *mtrC* was PCR amplified from strain FA19 by using the primers 5′P*mtrC* (5′-TTGGATCCCGTCTCATAATGGCGTTTTCGT-3′) and 3′P*mtrC* (5′-CGGGATCCCGAGCCATTATTTATCCTATCTG-3′). The resulting DNA fragments were inserted into the BamHI site of pLES94. These recombinant plasmids were introduced into DH5α TOP10 by transformation. Correct insertion and orientation was confirmed by PCR analysis and DNA sequencing analysis. The plasmids were used to transform strains FA19, JF1, FA19 *mtrF*::Km^r,

TABLE 2. MtrF is required for maximal levels of inducible resistance to TX-100

Strain	MIC (μg of TX-100/ml)[a]	
	Without TX-100 induction	With TX-100 induction
FA19	125	> 10,000
FA19 *mtrF*::Km^r	125	500
FA19 *mpeR*::Km^r	125	> 10,000

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

FA19 *mpeR*::Km^r, and JF1 *mpeR*::Km^r to allow insertion into the chromosomal *proAB* gene. Transformants were selected on GCB agar containing 1 μg of chloramphenicol/ml.

Preparation of cell extracts and β-galactosidase assays. The strains containing *lacZ* translational fusions were grown overnight on GCB agar plates containing 1 μg of chloramphenicol/ml and 50 μg of TX-100/ml where indicated. Cells were scraped, washed once with phosphate-buffered saline (pH 7.4), and resuspended in lysis buffer (0.25 mM Tris [pH 8.0]). Cells were broken by repeated freeze-thaw cycles. The cell debris was removed by centrifugation at 15,000 × *g* for 8 min at 4°C. β-Galactosidase assays were performed as previously described (35).

RESULTS

***mtrF* is required for inducible high-level resistance to TX-100 but does not influence expression of *mtrCDE*.** Previous studies in our laboratory demonstrated that *mtrF* was required for constitutive high-level HA resistance in gonococcal strains that lacked a functional MtrR repressor (37). High-level resistance can arise by either mutations in the expression or activity of MtrR or by induction of the efflux pump operon during growth of gonococci in sublethal concentrations of TX-100; both mechanisms result in increased expression of *mtrCDE* (9, 29). To determine whether *mtrF* was required for inducible high-level resistance to TX-100, the *mtrF* gene was inactivated by insertion of a nonpolar Km^r cassette in strain FA19. Strains FA19 and FA19 *mtrF*::Km^r were grown overnight on GC agar plates with or without the addition of a sublethal concentration (50 μg/ml) of TX-100. Cells were collected and spotted onto GC agar plates containing increasing concentrations of TX-100 to determine the MIC of this HA against each strain (Table 2). We noted no difference in the MICs of TX-100 for FA19 and FA19 *mtrF*::Km^r under noninducing conditions, confirming our earlier result that *mtrF* is not required for basal levels of TX-100 resistance (37). However, under inducing conditions, the TX-100 MIC against FA19 *mtrF*::Km^r was significantly (>20-fold) decreased from those observed for FA19, demonstrating that *mtrF* is required for high-level, inducible resistance of gonococci to TX-100.

The high-level constitutive HA resistance of gonococci that results due to mutations in *mtrR* or inducible resistance that can occur in the presence of sublethal concentrations of TX-100 requires increased expression of the *mtrCDE* operon (29, 38). In order to determine whether *mtrF* expression impacts expression of the *mtrCDE* operon under constitutive or inducible conditions, a translational reporter fusion system was used. For this purpose, the upstream region of *mtrCDE*, containing the promoter sequences, ribosome-binding site, and sequence encoding the first two codons of *mtrC*, were PCR amplified and cloned into pLES94 (34), which contains a promoter-less *lacZ* gene located between the *proAB* genes of *N. gonorrhoeae*. The *mtrC-lacZ* fusion was transformed into FA19 and FA19 *mtrF*::Km^r, which resulted in a single copy of the promoter of *mtrC* fused translationally to *lacZ* within the *proAB* chromosomal site. Strains FA19 *mtrC-lacZ* and FA19 *mtrF*::Km^r *mtrC-lacZ* were grown overnight on GC agar alone or GC agar with the addition of 50 μg of TX-100/ml. Cell extracts were harvested, and the expression of *mtrC-lacZ* was determined by measuring the β-galactosidase activity (Fig. 1). The expression of *mtrC* significantly increased in both FA19 and FA19 *mtrF*::Km^r under inducing versus noninducing conditions. No significant difference was observed for the expression of *mtrC* between FA19 and FA19 *mtrF*::Km^r under both noninducing and inducing conditions. These results demonstrate that *mtrF* is not required for the increased expression of *mtrCDE* under inducing conditions.

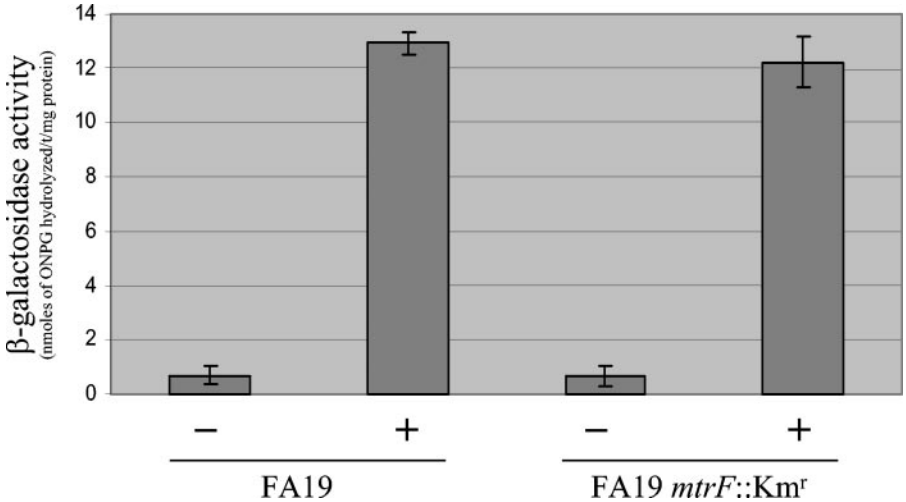

FIG. 1. Regulatory affect of the *mtrF* mutation on the expression of *mtrC*. Shown is the specific β-galactosidase activity per mg of total protein in cell extracts of reporter strains, FA19 *mtrC-lacZ* and FA19 *mtrF*::Km^r *mtrC-lacZ*, containing the *mtrC-lacZ* fusion. −, Growth on GCB agar plates; +, growth on GCB agar plates containing 50 μg of TX-100/ml. The results shown are the average of three independent experiments. Error bars represent one standard deviation. The *P* value (Student *t* test) between the "−" and "+" TX-100 for both strains was <0.0001.

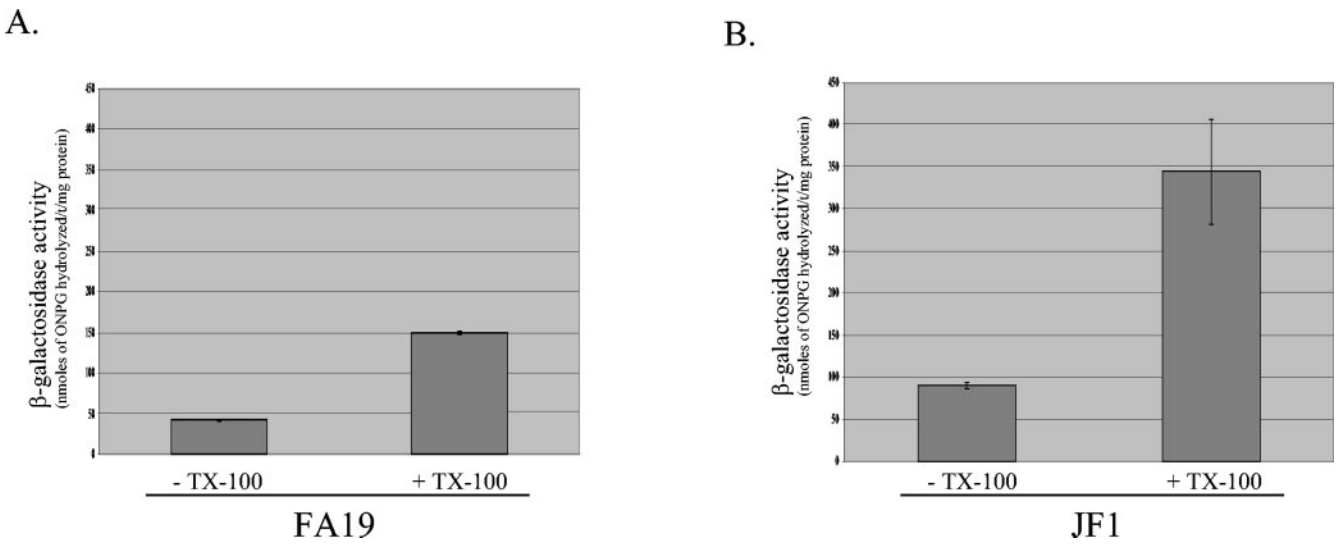

FIG. 2. Regulatory affects of TX-100 induction and the *mtrR* mutation on *mtrF* expression. Shown is the specific β-galactosidase activity per mg of total protein in cell extracts of reporter strains, FA19 *mtrF-lacZ* and JF1 *mtrF-lacZ*, containing the *mtrF-lacZ* fusion. (A) Effect of TX-100 induction on *mtrF* expression. FA19 *mtrF-lacZ* was grown overnight on GCB agar plates alone or containing 50 μg of TX-100/ml. (B) Effect of the *mtrR* mutation and TX-100 induction on *mtrF* expression. JF1 *mtrF-lacZ* was grown overnight on GCB agar plates alone or containing 50 μg/ml of TX-100. Strain JF1 has a deletion of *mtrR*. The results shown are the average of three independent experiments. Error bars represent one standard deviation. The *P* value (Student *t* test) between FA19 and JF1 under TX-100 induction was <0.015. The *P* value (Student *t* test) between the rest of the strains and conditions was <0.0001.

Expression of *mtrF* is induced by growth on sublethal concentrations of TX-100. We next examined the regulation of *mtrF* expression during growth of gonococci in the presence of a sublethal concentration of TX-100. Expression of the *mtrCDE* operon is regulated by at least two independent mechanisms: induction by growth in sublethal concentrations of TX-100 and repression under constitutive conditions by MtrR. To monitor the regulation of *mtrF* expression, the translational *lacZ* fusion system described above was utilized (34). For this purpose, the upstream region of *mtrF*, containing the putative promoter sequences, ribosome-binding site, and sequence encoding the first two codons of *mtrF*, were PCR amplified and cloned into pLES94. The *mtrF-lacZ* fusion was transformed into strain FA19. To determine whether expression of *mtrF* was inducible by TX-100, strain FA19 *mtrF-lacZ* was grown overnight on GC agar alone or GC agar with the addition of 50 μg of TX-100/ml. Cell extracts were harvested, and the expression of *mtrF-lacZ* was determined by measuring the β-galactosidase activity in whole-cell extracts. Expression of *mtrF* increased more than threefold in the presence of TX-100, compared to GC agar alone, in FA19 (Fig. 2A). These results demonstrate that the expression of *mtrF* is inducible by growth of gonococci in the presence of TX-100.

Expression of *mtrF* is repressed by MtrR. Since the *mtrF* gene is located adjacent to *mtrR* and the *mtrCDE* operon (37), we tested whether MtrR can regulate *mtrF* expression during growth of gonococci under normal or inducing conditions. The *mtrF-lacZ* fusion described above was transformed into strain JF1, which is derived from FA19 but contains a deletion of 98% of the *mtrR* coding sequence (see Table 1). To determine whether expression of *mtrF* was repressed by MtrR, strains FA19 *mtrF-lacZ* and JF1 *mtrF-lacZ* were grown overnight on GC agar alone or GC agar with the addition of 50 μg of TX-100/ml. Cell extracts were prepared, and the expression of *mtrF-lacZ* was determined by measuring the β-galactosidase activity. In both the presence or the absence of TX-100, expression of *mtrF* increased ~2-fold in strain JF1 over strain FA19 (Fig. 2A and B). These data demonstrate that *mtrF*, like *mtrCDE*, is subjected to repression by MtrR. However, in both strains FA19 and JF1, expression of *mtrF* increased ~3-fold in the presence of TX-100 over that observed for the absence of TX-100 (Fig. 2A and B). Since no significant difference was observed for the induction of *mtrF* expression in strain FA19 versus JF1, these data also demonstrate that inducible expression of *mtrF* is an MtrR-independent process.

Previous studies have demonstrated that the negative regulation of *mtrCDE*, mediated by MtrR, is due to specific binding of MtrR to the promoter of the *mtrCDE* operon (19). Electrophoretic mobility shift analysis (EMSA) coupled with DNase footprinting analysis has identified the MtrR binding region within the *mtrCDE* promoter (19). Since MtrR repressed *mtrF* expression (Fig. 2), we next sought to determine whether MtrR could bind in a specific manner to the *mtrF* promoter region. As previously described (16), MtrR was expressed and purified as a fusion protein to MalE and used in EMSA. In competitive EMSA, purified MalE-MtrR demonstrated specific binding of MtrR to the *mtrCDE* promoter (data not shown). However, competitive EMSA analysis failed to demonstrate specific binding of MtrR to the promoter region of *mtrF*, suggesting that the MtrR-specific regulation of *mtrF* was indirect (data not shown). These data indicated that other transcriptional regulatory proteins could regulate *mtrF* expression.

Identification of *mpeR*, an AraC-like transcriptional regulator that represses *mtrF*. The lack of specific binding of MtrR to the *mtrF* promoter prompted us to search for other regulatory proteins that control *mtrF* expression. A search of the *N. gonorrhoeae* genomic database revealed an open reading frame that would encode a member of the AraC family of transcrip-

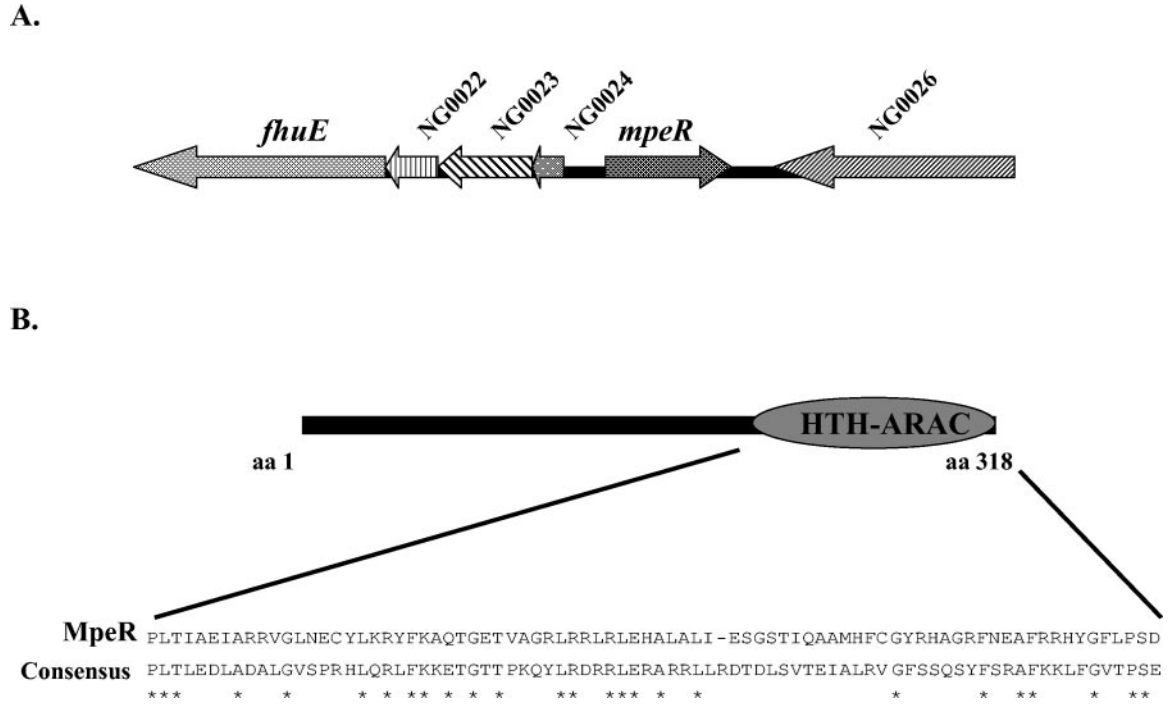

FIG. 3. (A) Genetic organization of *mpeR* and adjacent genes. (B) Predicted domain structure of MpeR and amino acid alignment of the C-terminal region of MpeR to the consensus helix-turn-helix (HTH-ARAC) motif of AraC transcriptional regulators (17, 33). The top sequence represents MpeR, and the bottom sequence represents the consensus HTH motif. The asterisks the indicate the locations of identical amino acids.

tional regulators, which we termed MpeR (NG0025 [http://www.stdgen.lanl.gov/stdgen/bacteria/ngon/]), and it was identified by its high homology to the conserved helix-turn-helix motif located at its C terminus (HTH-ARAC, SMART database) (Fig. 3) (17, 33). The chromosomal location of *mpeR* is shown in Fig. 3. Several members of the AraC family of transcriptional regulators are involved in the regulation of homologous efflux-pump systems, including MarA, SoxS, and Rob, which are involved in the regulation of the *acrAB*-encoded efflux pump of *E. coli* (5, 23). The *mpeR* gene encodes a 318-amino-acid protein with the predicted mass of 35.7 kDa. The predicted helix-turn-helix motif of MpeR has significant amino acid sequence identity to helix-turn-helix motifs of other AraC-like proteins including YbtA (27% identity) in *E. coli* (4), PchR (32%) in *Pseudomonas aeruginosa* (11), and to AlcR (29%), an AraC-like activator of iron transport in *Bordetella pertussis* (2, 28). The *mpeR* gene could be PCR amplified from eight additional gonococcal strains (FA1090, FA62, FA889, DG1 1918, EU75, RD5, and UU1), suggesting that it is a conserved gonococcal gene (data not shown). A search of meningococcal genomic databases (www.tigr.organd www.sanger.ac.uk) identified *mpeR* homologs in serogroups A (Z2491), B (MC58), and C (FAM18). However, the homologous sequence identified in MC58 is predicted to encode two polypeptides (NMB1878 and NMB1879), which may or may not encode functional proteins.

To determine whether MpeR is needed for *mtrF* expression, *mpeR* was inactivated by the insertion of the nonpolar *aphA*-3 cassette, and the resulting plasmid construct was used to transform strain FA19. To measure the expression of *mtrF*, the previously described *mtrF-lacZ* fusion was inserted into the chromosome of strains FA19 and FA19 *mpeR*::Km^r. Strains FA19 *mtrF-lacZ* and FA19 *mpeR*::Km^r *mtrF-lacZ* were grown overnight on GC agar alone or GC agar with the addition of 50 μg of TX-100/ml. Cell extracts were harvested and the expression of *mtrF-lacZ* was determined by measuring the β-galactosidase activity (Fig. 4). Expression of *mtrF* increased approximately twofold (<0.0001) under both inducing (+TX-100) and noninducing (−TX-100) conditions in the *mpeR*-deficient strain, suggesting that *mpeR* encodes a repressor of *mtrF* expression.

Evidence that repression of *mtrF* expression by MtrR and MpeR are independent regulatory processes. Our studies have identified two repressors of *mtrF* expression, MtrR and MpeR. Since we were unable to demonstrate specific binding of MtrR to the promoter region of *mtrF*, we suspected that MtrR regulation of *mtrF* is indirect. To determine whether the repressive regulation observed for MtrR was dependent on MpeR, a double mutant FA19 strain was engineered. The *mpeR* gene of the *mtrR* deletion strain, JF1, was inactivated by insertion of a nonpolar kanamycin cassette (Km^r), resulting in strain JF1 *mpeR*::Km^r. To measure the expression of *mtrF* in these mutant strains, the previously described *mtrF-lacZ* fusion was inserted into the chromosome of strains FA19, JF1, FA19 *mpeR*::Km^r, and JF1 *mpeR*::Km^r. All four strains were grown overnight on GC agar. Cell extracts were harvested, and the expression of *mtrF-lacZ* was determined by measuring the β-galactosidase activity. As previously observed, the expression of *mtrF* increased in an *mtrR*-deficient strain (JF1) and increased in an *mpeR*-deficient strain (FA19 *mpeR*::Km^r) (Fig. 5). In the strain lacking both MtrR and MpeR, expression increased greater than when either single mutation was present. Repression of *mtrF* by MtrR and MpeR was additive,

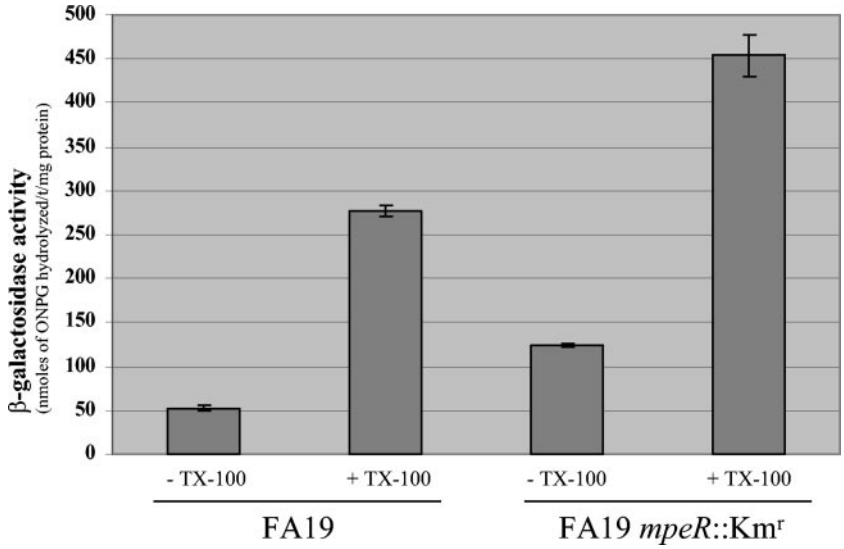

FIG. 4. Regulatory affects of TX-100 induction and the *mpeR* mutation on *mtrF* expression. Shown is the specific β-galactosidase activity per mg of total protein in cell extracts of reporter strains, FA19 *mtrF-lacZ* and FA19 *mpeR*::Km^r *mtrF-lacZ*, containing the *mtrF-lacZ* fusion. (A) Effect of TX-100 induction on *mtrF* expression. FA19 *mtrF-lacZ* was grown overnight on GCB agar plates alone or containing 50 μg of TX-100/ml. (B) Effect of the *mtrR* mutation and TX-100 induction on *mtrF* expression. FA19 *mpeR*::Km^r *mtrF-lacZ* was grown overnight on GCB agar plates alone or containing 50 μg of TX-100/ml. The results shown are the average of three independent experiments. Error bars represent one standard deviation. The *P* value (Student *t* test) between all of the strains and conditions tested was <0.0001.

suggesting that MtrR- and MpeR-mediated repression of *mtrF* expression are independent processes.

DISCUSSION

Production of efflux pumps can result in elevated resistance of bacteria to antimicrobials recognized by the given pump (18, 20). Such pump expression can be constitutive, usually the result of loss of production of a functional transcriptional repressor that controls efflux gene expression (7). Elevated resistance of bacteria to antimicrobials can also occur transiently in the presence of sublethal levels of antimicrobials recognized by certain efflux pumps or environmental signals. This inducible resistance seems to require transcriptional activators and certain cell envelope proteins (20, 29). This inducible resistance of bacteria to antimicrobials would allow for a quick response to potentially lethal levels of an antimicrobial that is

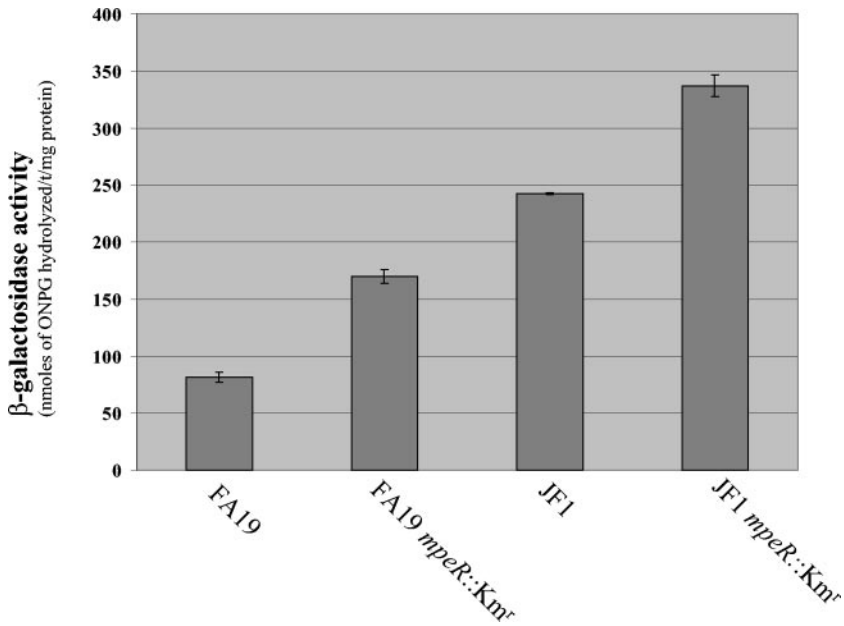

FIG. 5. Regulatory affect of the *mpeR*/*mtrR* double mutation on *mtrF* expression. The specific β-galactosidase activity per milligram of total protein in cell extracts of reporter strains, FA19 *mtrF-lacZ*, FA19 *mpeR*::Km^r *mtrF-lacZ*, JF1 *mtrF-lacZ*, and JF1 *mpeR*::Km^r *mtrF-lacZ*, containing the *mtrF-lacZ* fusion, is shown. Strain JF1 has a deletion of *mtrR*. The results shown are the average of three independent experiments. Error bars represent one standard deviation. The *P* value (Student *t* test) between all of the strains tested was <0.0001.

given therapeutically or via a natural host product. In the latter situation, inducible resistance of gonococci to HAs through the *mtr* efflux system would enhance its growth capability at mucosal surfaces that contain antimicrobial peptides and other antimicrobial agents. Indeed, recent studies by Jerse et al. (15) showed that a functional *mtr* efflux system was required for gonococci to cause a sustained vaginal infection in mice.

Although constitutive HA resistance in gonococci due to overproduction of the MtrC-MtrD-MtrE has been extensively studied (21, 38), considerably less is known about the molecular mechanisms that mediate inducible resistance. However, some progress in understanding inducible HA resistance has been made through studies that revealed the necessity for the transcriptional activator, MtrA (29), and energy supplied by the TonB-ExbB-ExbD system (30). In the present study we document a role for MtrF, a putative cytoplasmic membrane protein, in such resistance. Previous studies by Veal and Shafer (37) showed that MtrF is also required for high-level HA resistance that occurs in MtrR-deficient strains. In the work presented here, we set out to further define the role of *mtrF* in high-level HA resistance in gonococci and began to examine the regulation of *mtrF* expression.

MtrF was discovered by the observation that mutations in *mtrF* could phenotypically suppress mutations in *mtrR* that normally result in constitutive HA resistance (37). The mechanism of MtrF and how it modulates levels of constitutive or inducible HA resistance is not yet known. MtrF shares homology with AbgT of *E. coli*, a transporter of *p*-aminobenzoylglutamate (13). However, plasmid expressed recombinant MtrF failed to complement an AbgT-inactivated strain of *E. coli*, and therefore we do not believe that is the function of MtrF (37). Previous studies demonstrated that disruption of *mtrF* does not affect the transport of proteins across the inner membrane, change the lipooligosaccharide profile, or change the membrane phospholipid profile (37). One possible role for MtrF is that it directly interacts with one or more of the efflux pump components, and these interactions are necessary for high-level activity of the pump. It is interesting that putative homologs of *mtrF* have been identified in many diverse species of bacteria (37). The majority of these bacteria have at least one putative RND-transporter similar to MtrD, suggesting the possibility that MtrF may be a conserved member of RND-transporters.

The MIC results presented here demonstrate that *mtrF* is required for high-level HA resistance of gonococci due to TX-100 induction (Fig. 1). However, inactivation of *mtrF* had no effect on basal levels of HA resistance under noninducing conditions. Therefore, *mtrF* is required for high-level HA resistance that results from inactivation of MtrR or TX-100 induction. To our surprise, inactivation of *mtrF* had no effect on the expression of *mtrC* when induced by growth on sublethal concentrations of TX-100 (Fig. 2). Similar results were observed for inactivation of *mtrF* in an MtrR mutant gonococcal strain (data not shown). Taken together, these data suggest that *mtrF* does not participate in the regulation of expression of the *mtrCDE*-encoded efflux pump. Therefore, we believe that *mtrF* is required for the proper mechanism of the pump, during conditions which would normally result in high-level activity of the pump.

To gain a better understanding of the function of *mtrF*, we have begun to examine the regulation of *mtrF* expression. The major repressor of *mtrCDE*, MtrR, was shown to repress the expression of *mtrF* (Fig. 2). The regulation of *mtrF* by MtrR seems to be indirect because the repressor does not bind in a specific manner to a DNA sequence upstream of *mtrF*, as was previously seen for MtrR repression of *mtrCDE*. Expression of the *mtrF* gene was also shown to be inducible upon growth of gonococci on sublethal concentrations of TX-100 (Fig. 2).

Due to our lack of evidence for MtrR binding to the *mtrF* promoter, we sought to identify other transcriptional regulators that might be participating in controlling *mtrF* expression. We described previously an AraC-like protein (MtrA) in gonococci (29). However, results from *lacZ* expression experiments revealed that loss of MtrA did not impact *mtrF* expression (data not presented). We subsequently identified a second putative AraC regulator, MpeR (for Mtr protein efflux regulator). β-Galactosidase assays suggested that *mpeR* encodes a repressor of *mtrF* expression (Fig. 4). Although AraC-like regulators are commonly activators of transcription, several AraC-like regulators have been identified that function as both activators and repressors, including MarA in *E. coli* (32), YbtA (4), and PchR (11). Also similar to the repression of *mtrF* by MtrR, MpeR-dependent repression had no effect on the TX-100 induction of *mtrF* expression (Fig. 4). The level of repression of *mtrF* mediated by MpeR was very similar to that observed for MtrR (Fig. 2 versus Fig. 4). That result, coupled with the lack of binding of MtrR to the *mtrF* upstream region, suggested that these repressors may function via a single regulatory process. The repression of *mtrF* by MtrR and MpeR was additive, and therefore, independent processes (Fig. 5). β-Galactosidase assays showed no difference in the expression of *mtrC* in wild-type FA19 versus FA19 *mpeR*::Km^r, demonstrating that *mpeR* is not involved in the regulation of *mtrCDE* (data not shown). Moreover, MICs for TX-100 against strains FA19 and FA19 *mpeR*::Km^r showed no difference in resistance, confirming that MpeR is not involved in the regulation of *mtrCDE* expression (Table 2). Taken together, these results demonstrate that besides the regulatory mechanisms previously observed for *mtrCDE* (9), *mtrF* has at least one additional level of regulation and that this regulatory process involves MpeR. The mechanism by which MpeR modulates *mtrF* expression is now under investigation.

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