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Differential Regulation of *ponA* and *pilMNOPQ* Expression by the MtrR Transcriptional Regulatory Protein in *Neisseria gonorrhoeae*▽

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***Neisseria gonorrhoeae* utilizes the *mtrCDE*-encoded efflux pump system to resist not only host-derived, hydrophobic antimicrobials that bathe mucosal surfaces, which likely aids in its ability to colonize and infect numerous sites within the human host, but also antibiotics that have been used clinically to treat infections. Recently, overexpression of the MtrC-MtrD-MtrE efflux pump was shown to be critically involved in the capacity of gonococci to develop chromosomally mediated resistance to penicillin G, which for over 40 years was used to treat gonococcal infections. Mutations in either the promoter or the coding sequence of the *mtrR* gene, which encodes a repressor of the efflux pump operon, decrease gonococcal susceptibility to penicillin. We now describe the capacity of MtrR to directly or indirectly influence the expression of two other loci that are involved in gonococcal susceptibility to penicillin: *ponA*, which encodes penicillin-binding protein 1 (PBP 1), and the *pilMNOPQ* operon, which encodes components of the type IV pilus secretion system, with PilQ acting as a channel for entry for penicillin. We determined that MtrR increases the expression of *ponA* directly or indirectly, resulting in increased levels of PBP 1, while repressing the expression of the divergently transcribed *pilM* gene, the first gene in the *pilMNOPQ* operon. Taken together with other studies, the results presented herein indicate that transcriptional regulation of gonococcal genes by MtrR is centrally involved in determining levels of gonococcal susceptibility to penicillin and provides a framework for understanding how resistance developed over the years.**

The strict human pathogen *Neisseria gonorrhoeae* continues to be a public health problem due to its frequency of infection (estimated at over 60 million cases per year), its ability to evade the immune system, and its increasing resistance to antibiotics (2). Antibiotic resistance is especially worrisome in underdeveloped countries, where effective treatment often requires the use of more expensive drugs that are not always available. The relatively inexpensive antibiotic penicillin G was used clinically for over 40 years until 1985, when it became evident that there was an unacceptably high prevalence of clinically resistant strains. These strains often did not produce β-lactamase (9) but instead had a number of chromosomal mutations that affected penicillin entry, target recognition, or efflux (5, 11, 24, 27, 36, 38). The report (10) of one such resistant strain in the United States prompted the removal of penicillin from the CDC-recommended treatment regimen.

Mutations in at least five chromosomal genes are required for high-level chromosomally mediated penicillin resistance in gonococci. These genes encode mutated forms of penicillin-binding protein 1 (PBP 1) (27) and PBP 2 (36), the major outer membrane protein $PorB_{IB}$ (24), the type IV pilus secretin protein PilQ (44), and the transcriptional repressor MtrR (14, 25), which negatively regulates the expression of the *mtrCDE*-encoded efflux pump. Accumulating evidence (25, 38) has revealed the importance of overexpression of the MtrC-MtrD-MtrE efflux pump, which has the capacity to recognize and export structurally diverse antimicrobials (14), in gonococcal penicillin resistance due to *mtrR* mutations. The 23-kDa MtrR protein, a member of the TetR family of transcriptional repressors with a helix-turn-helix motif near its N terminus (25), binds in a specific manner to the *mtrCDE* promoter (20) through two homodimers that recognize two pseudo-direct repeats within the *mtrCDE* promoter (16). MtrR may have a more global regulatory property in that it has been shown to negatively regulate the expression of two additional genes: *mtrF*, which encodes an inner membrane accessory protein required for maximal efflux of antimicrobials by the MtrC-MtrD-MtrE efflux pump in *mtrR* mutants (39, 40), and *farR*, which encodes the transcriptional repressor of the *farAB*-encoded efflux pump (18, 19). Moreover, earlier work by Dougherty et al. (5) suggested that introduction of a then-undefined *mtrR* mutation into a penicillin-susceptible recipient strain resulted in a decreased level of penicillin binding to PBP 1 (encoded by *ponA*) as determined by [^{3}H]penicillin G binding. Because changes in the expression of the *mtrCDE* efflux pump would not be expected to have an effect on [^{3}H]penicillin G binding to isolated membranes used in the experiments described by Dougherty et al. (5), we hypothesized that the *mtrR* mutation might modulate the expression of *ponA*, in addition to its primary role of regulating the expression of the *mtrCDE* efflux pump. Interestingly, *ponA* is adjacent to, but transcriptionally divergent from, the *pilMNOPQ* operon. This operon encodes components of the type IV pilus secretion system (6, 7), and recent studies (3, 44) have implicated the multimeric PilQ secretin in antibiotic (including penicillin) permeation in gonococci.

Since mutations in *ponA* and *pilQ* are necessary for gono-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or relevant genotype	Source or reference
Strains		
Neisseria gonorrhoeae		
FA19	Wild type	13
FA19 Δ*mtrR*	98% deletion of the coding sequence of *mtrR*	14
FA19 Δ*mtrR* GC3*mtrR*	Δ*mtrR* complemented with a wild-type copy of *mtrR* inserted into the gonococcal chromosome (Em^r)	This study
FA19 *ponA-lacZ*	Translational fusion of the promoter region of *ponA* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 Δ*mtrR ponA-lacZ*	Δ*mtrR*; translational fusion of the promoter region of *ponA* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 Δ*mtrR* GC3*mtrR ponA-lacZ*	Complemented Δ*mtrR*; translational fusion of the promoter region of *ponA* to the *lacZ* gene and inserted into the chromosome (Em^r) (Cm^r)	This study
FA19 *pilM-lacZ*	Translational fusion of the promoter region of *pilM* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 Δ*mtrR pilM-lacZ*	Δ*mtrR*; translational fusion of the promoter region of *pilM* to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 Δ*mtrR* GC3*mtrR pilM-lacZ*	Complemented Δ*mtrR*; translational fusion of the promoter region of *pilM* to the *lacZ* gene and inserted into the chromosome (Em^r) (Cm^r)	This study
FA19 *ponA-lacZ*¥	Translational fusion of the promoter region of *ponA*, containing the 8-bp mutation, to the *lacZ* gene and inserted into the chromosome (Cm^r)	This study
FA19 Δ*mtrR ponA-lacZ*¥	Δ*mtrR*; translational fusion of the *ponA* promoter region, containing the 8-bp mutation, to the *lacZ* gene and inserted into the chromosome (Cm^r)	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*	29
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)	33
p*PponA*	pLES94 containing the 300-bp upstream sequence of *ponA* at the BamHI site	This study
p*PpilM*	pLES94 containing the 300-bp upstream sequence of *pilM* at the BamHI site	This study
p*PponA*¥	pLES94 containing the 300-bp upstream sequence of *ponA*, containing the 8-bp mutation, at the BamH1 site	This study
pGCC3	pHSS6-derived plasmid for genetic complementation at a secondary site within the *N. gonorrhoeae* chromosome (Km^r)(Em^r)	22
pGCC3*mtrR*	pGCC3 containing a wild-type copy of *mtrR* and upstream promoter sequences	This study

cocci to express high-level chromosomally mediated resistance to penicillin (27, 32) and such strains also bear mutations in *mtrR*, we investigated whether MtrR also regulates these genes. We now report that MtrR can positively regulate the expression of *ponA* while repressing *pilMNOPQ* transcription and propose that this DNA-binding protein functions in modulating gonococcal susceptibility to penicillin by controlling the expression of multiple genes. (A preliminary account of these findings was presented at the 15th International Pathogenic Neisseria Conference [10 to 15 September 2006] in Cairns, Australia.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strain TOP10 (Invitrogen, Carlsbad, CA) and *E. coli* DH5α mcr (29) were used in all cloning experiments. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates at 37°C. *N. gonorrhoeae* strain FA19 was used as the primary gonococcal strain (21, 35). *N. gonorrhoeae* 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 as described previously (30). All chemicals were purchased from Sigma Biochemical (St. Louis, MO).

Construction of strain FA19 Δ*mtrR GC3mtrR*. In order to complement the *mtrR* deletion in FA19 Δ*mtrR*, the *mtrR* gene and promoter region were amplified by PCR (pGC35′*mtrR*, 5′-GGTTAATTAACGCCTTAGAAGCATAAAAAGC-3′; 3′*mtrR*, 5′-GGGTTTAAACTTATTTCCGGCGCAGGCAG-3′) from wild-type strain FA19, which produces a functional MtrR repressor (20). The resulting DNA fragment was inserted into the PmeI and PacI sites of pGCC3 (22) (kindly provided by A. Jerse and H. Seifert) to produce pGC3*mtrR*, and the correct orientation and nucleotide sequence were confirmed by DNA sequencing. pGC3*mtrR* was digested with ClaI, and the fragment containing the gonococcal *lctP* gene, *mtrR*, *ermC* (an erythromycin resistance cassette), and *aspC* was purified and used to transform gonococcal strain FA19 Δ*mtrR*. Transformations were performed as previously described (12). Transformants were selected on GCB containing 1 μg/ml of erythromycin.

Construction of the *ponA-lacZ* and *pilM-lacZ* fusions in gonococci. Translational *lacZ* fusions were constructed as previously described (34). In brief, the promoter sequence of *ponA* was amplified by PCR from strain FA19 using primers 5′P*ponA* (5′-GGGGATCCTTCCAATTGAATTTGGTTTAAACT-3′) and 3′P*ponA* (5′-GGGGATCCCGAATCATAGCTGAATAATAATTTAC-3′). The promoter sequence of *pilM* was amplified by PCR from strain FA19 using primers 5′P*pilM* (5′-ATGGATCCAACGGCATTTTAGGCTGGTAA-3′) and 3′P*pilM* (5′-ATGGATCCCGGCGCATGATGAAAGTTCCTG-3′). The resulting DNA fragments were inserted into the BamHI site of pLES94 (33), and the recombinant plasmids were introduced into *E. coli* DH5α mcr by transformation. Correct insertion and orientation were confirmed by PCR analysis and DNA sequencing. The plasmids were used to transform strains FA19, FA19 Δ*mtrR*,

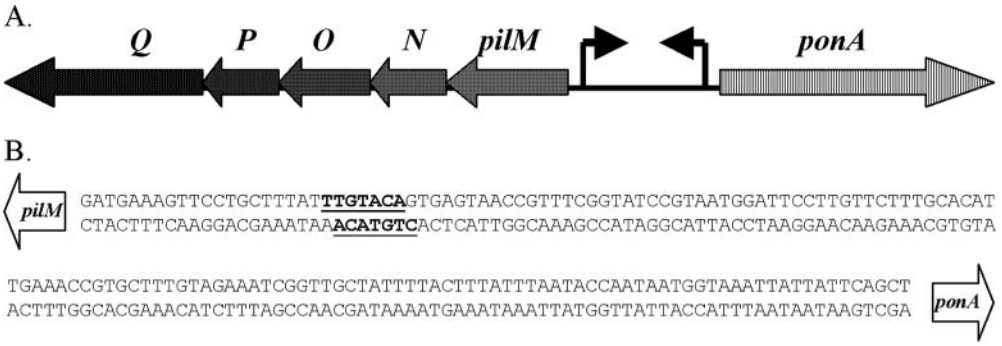

FIG. 1. (A) Genetic organization of the divergently transcribed *ponA* and *pilMNOPQ* genes. (B) DNA sequence of the 150-bp intergenic region. Repeat I (upper strand) and repeat II (lower strand, opposite direction) are shown underlined and bolded, indicating the location of the MtrR-binding site.

and FA19 Δ*mtrR* GC3*mtrR* to allow insertion into the chromosomal *proAB* locus. Transformants were selected on GCB agar containing 1 μg/ml of chloramphenicol.

Preparation of cell extracts and β-Gal assays. The strains containing *lacZ* translational fusions were grown overnight on GCB agar plates containing 1 μg/ml of chloramphenicol. 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 (β-Gal) assays were performed as previously described (34).

EMSAs. Electrophoretic mobility shift assays (EMSAs) using purified MtrR fused to maltose-binding protein (MBP) were performed as previously described (20). All probes were amplified by PCR from FA19 chromosomal DNA. In brief, the intergenic region of *ponA-pilM* was amplified by PCR using 3′P*ponA* and 3′P*pilM*, and the promoter sequence of *mtrC* was PCR amplified using 5′P*mtrC* (5′-CGTTTCGGGTCGGTTTGACG-3′) and 3′P*mtrC* (5′-GCTTTGATACCC GAATGTTCG-3′). The overlapping probes used in the MtrR-binding site study were amplified by PCR using the following primer pairs: 5′*ponA*225 (5′-GCAA CCAGACCCACTCCA-3′) and 3′*ponA*75 (5′-TTGAAACCGTGCTTTGTAG-3′), 5′*ponA*75 (5′-TGTGCAAAGAACAAGGAATCC-3′) and 3′*pilM*-75 (5′-ATTGAGTCCCGAAGATTTTTTA-3′), 5′*ponA*50 (5′-CGGATACCGAAAC GGTTAC-3′) and 3′*pilM*-100 (5′-TATCGATGCCGATTGCCGC-3′), and 5′*ponA*25 (5′-TACAAATAAAGCAGGAACTTTCA-3′) and 3′*pilM*-125 (5′-A CCATTTTGATGGAATGCTGG-3′). The resulting PCR products were end labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled DNA fragments (10 ng) were incubated with purified MBP-MtrR in 30 μl of reaction buffer [10 nM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.5 EDTA, 4% (vol/vol) glycerol, 1 mM $MgCl_2$, 50 mM NaCl, poly(dI-dC) (0.5 μg/ml), salmon sperm (200 ng/ml)] at 4°C for 20 min. Samples were subjected to electrophoresis on a 6% (wt/vol) polyacrylamide gel at 4°C, followed by autoradiography. Densitometry was performed using Scion Image (v. alpha 4.0.3.2; Frederick, MD).

Mutagenesis of the MtrR-binding site. PCR mutagenesis was performed using the overlapping primers 5′mutMBS (5′-CGGTTACTCAAGTGCACCATAAA GCAGG-3′) and 3′mutMBS (5′-CCTGCTTTATGGTGCACTTGAGTAACC G-3′), each containing the 8-bp transversion mutation of the MtrR-binding site. First, two fragments were amplified by PCR from FA19 chromosomal DNA using the primer sets 3′P*ponA*/3′mutMBS and 3′P*pilM*/5′mutMBS. The resultant fragments were gel purified using a QIAquick purification kit (QIAGEN Inc., Valencia, CA), and these fragments then served as both primers and templates for a second PCR. After 8 reaction cycles, primers 3′P*ponA* and 3′P*ponA* were added to the PCR and amplification continued for an additional 25 cycles. The resulting DNA fragment containing the mutation was purified and served as the template for the last PCR, using primers 5′P*ponA* and 3′P*ponA*. The resulting DNA fragment was inserted into the BamHI site of pLES94, resulting in the *ponA-lacZ*$^{\psi}$ construct. The recombinant plasmid was introduced into DH5α mcr by transformation. Correct insertion and orientation were confirmed by PCR analysis, and DNA sequencing analysis confirmed the mutation of the MtrR-binding site. The plasmid was used to transform strains FA19 and FA19 Δ*mtrR* to allow insertion into the chromosomal *proAB* gene. Transformants were selected on GCB agar containing 1 μg/ml of chloramphenicol.

Western blotting of PBP 1. Western blotting of PBP 1 was done essentially as described previously (28). Briefly, cultures of FA19, FA19 Δ*mtrR*, and FA19 Δ*mtrR* GC3*mtrR* were grown overnight on GC plates, and cells were swabbed into GC broth containing 10 mM $MgCl_2$ and diluted to an 0.18 optical density at 600 nm. One-milliliter aliquots of each sample were pelleted, the cell pellets were dissolved in 100 μl of 1× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis buffer and boiled, and aliquots were separated on an 8% polyacrylamide-SDS gel. Alternatively, the cells were lysed, the particulate fractions were isolated, and equal levels of protein were electrophoresed as described above. The proteins were transferred to polyvinylidene difluoride membranes in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], pH 11, 5% methanol, 0.005% SDS for 4 h at 200 mA on a semidry blotting apparatus (Owl Scientific, Portsmouth, NH). The blot was incubated with a 1/5,000 dilution of rabbit PBP 1 antisera (28) followed by horseradish peroxidase-conjugated goat anti-rabbit antibody, and the protein bands were visualized with Pierce Super-Signal West Pico chemiluminescence reagent (Rockford, IL). Films were imaged on a Bio-Rad Fluor-S system and quantified with Bio-Rad QuantityOne software (Bio-Rad, Hercules, CA).

RESULTS

We examined the capacity of MtrR to regulate the expression of the *ponA-pilMNOPQ* locus (Fig. 1) in the gonococcal chromosome to determine if this DNA-binding protein can control the expression of proteins involved in penicillin resistance in addition to the *mtrCDE*-encoded efflux pump. We chose to analyze this region of the gonococcal chromosome because levels of PBP 1 may be affected by *mtrR* mutations (5), the multimeric PilQ has been implicated in penicillin permeation, and a mutation in *pilQ* (*pilQ2*, previously termed *penC*) is necessary for high-level chromosomally mediated penicillin resistance in stepwise transformants of FA19 (27).

Expression of *ponA* is activated by MtrR. To monitor the regulation of *ponA* expression by MtrR, a translational reporter fusion system was employed. For this purpose, a *ponA* promoter-*lacZ* fusion was constructed and transformed into the isogenic strains FA19 and FA19 Δ*mtrR*, which resulted in a single copy of the *ponA* promoter fused translationally to *lacZ* within the *proAB* chromosomal locus. β-Gal activity in cell lysates from these strains indicated that the expression of *ponA* decreased about twofold in strain FA19 Δ*mtrR* strain compared to that in its otherwise isogenic wild-type parental strain, FA19 (Fig. 2A). To confirm that these results were due to deletion of *mtrR* and not to a polar effect, FA19 Δ*mtrR* *ponA-lacZ* was complemented with the wild-type *mtrR* gene from strain FA19, which was inserted at a secondary site within the gonococcal chromosome (between the *lctP* and *aspC* genes) to create FA19 Δ*mtrR* GC3*mtrR* *ponA-lacZ* (22). Using

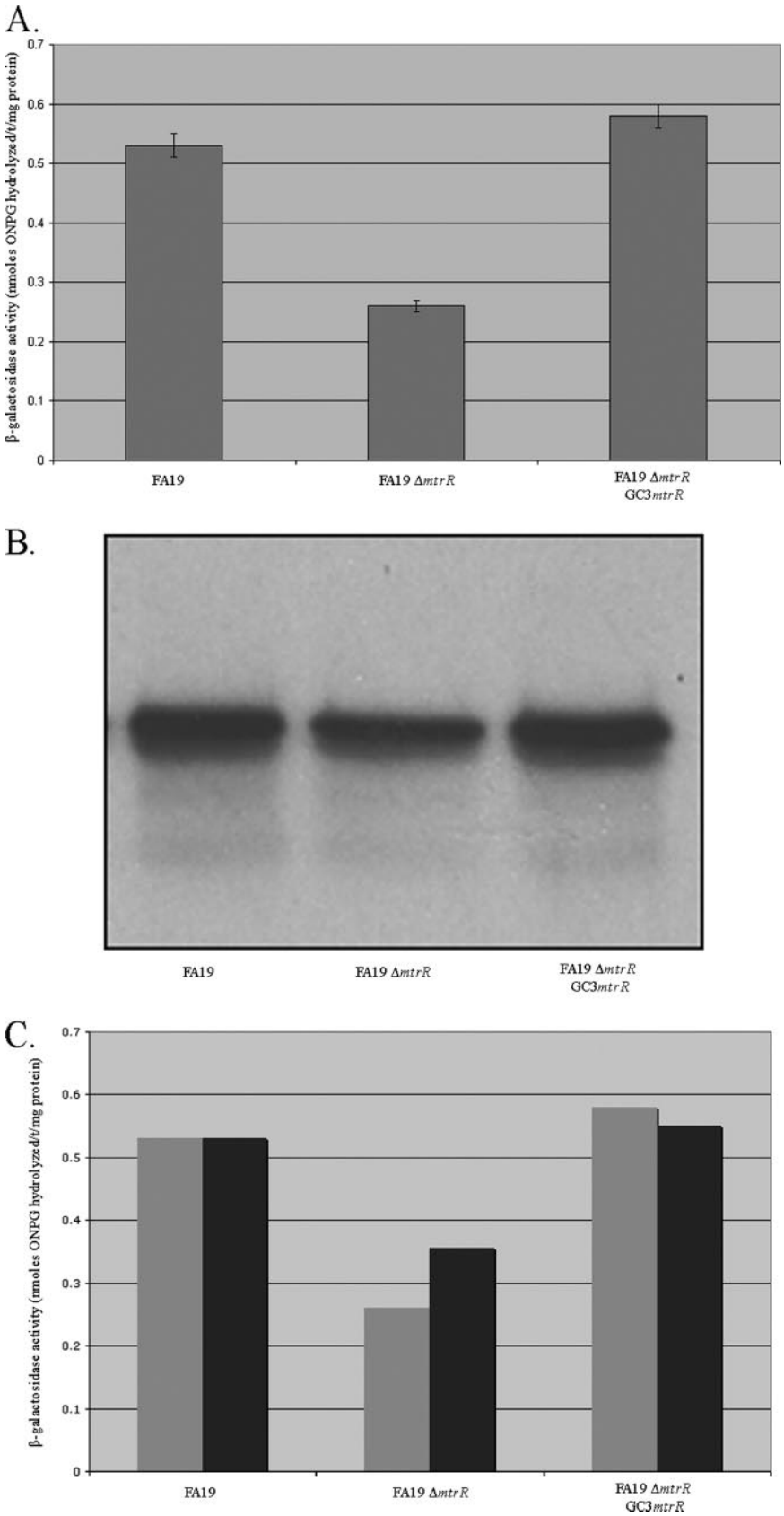

FIG. 2. (A) Regulation of *ponA* expression by MtrR. Shown are the β-Gal activities per mg of total protein in cell extracts of reporter strains FA19 *ponA-lacZ*, FA19 Δ*mtrR ponA-lacZ*, and FA19 Δ*mtrR* GC3*mtrR ponA-lacZ*. The figure represents one experiment of three replicates; each

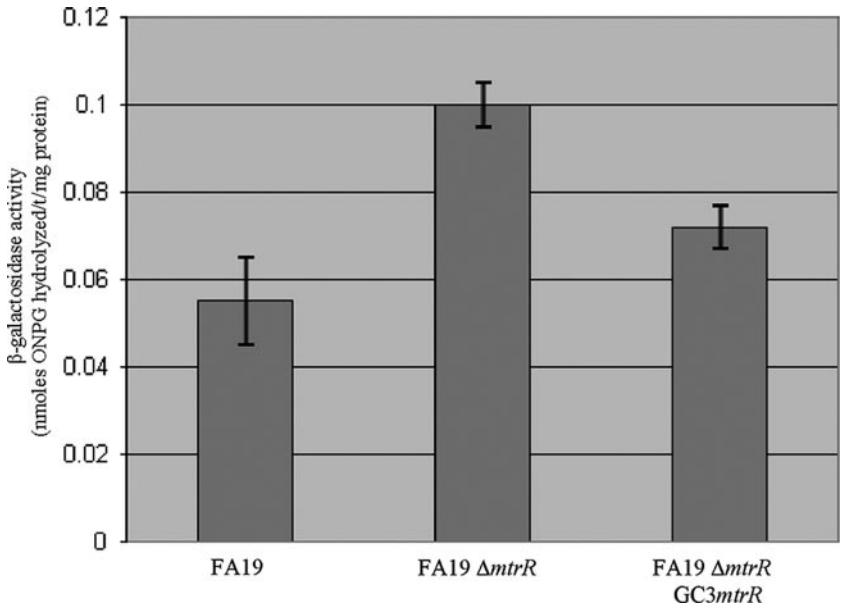

FIG. 3. Regulation of *pilM* expression by MtrR. β-Gal activities per mg of total protein in cell extracts of reporter strains FA19 *pilM-lacZ*, FA19 Δ*mtrR pilM-lacZ*, and FA19 Δ*mtrR* GC3*mtrR pilM-lacZ* are shown. The figure represents one experiment of three replicates; each replicate was performed in triplicate. Error bars represent 1 standard deviation. The *P* value (Student's *t* test) between strains FA19 *pilM-lacZ* and FA19 Δ*mtrR pilM-lacZ* was 0.0024, and that between strains FA19 Δ*mtrR pilM-lacZ* and FA19 Δ*mtrR* GC3*mtrR pilM-lacZ* was 0.0017.

this strain, we found that complementation of FA19 Δ*mtrR ponA-lacZ* with the *mtrR* gene restored *ponA* expression to a level similar but slightly higher than that observed in FA19 *ponA-lacZ* (Fig. 2A).

Based on these results, we next examined whether protein levels of PBP 1 were decreased due to the *mtrR* deletion mutation. Accordingly, total membrane fractions were prepared from strains FA19, FA19 Δ*mtrR*, and FA19 Δ*mtrR* GC3*mtrR*, and PBP 1 levels were determined by Western blotting using rabbit anti-PBP 1 antisera (28) (Fig. 2B). Densitometry was performed to determine the ratio of PBP 1 levels in FA19 relative to those in FA19 Δ*mtrR* and FA19 Δ*mtrR* GC3*mtrR*. These values were then plotted on the same graph as the gene expression (β-Gal activity) data, with the densitometry levels adjusted such that the value observed in FA19 was equal to that determined for gene expression in strain FA19 (Fig. 2C). These data show that PBP 1 levels were decreased in strain FA19 Δ*mtrR* to nearly the same extent as that determined in the gene expression assay and recovered to wild-type levels in the complemented strain FA19 Δ*mtrR* GC3*mtrR*. This experiment was repeated two other times with whole-cell lysates of the three strains, and results similar to those with membranes were obtained (data not shown). Taken together, these results suggested that MtrR directly or indirectly stimulates *ponA* expression, resulting in increased levels of PBP 1.

Expression of *pilM* is repressed by MtrR. *pilM* is the first gene in the gonococcal *pilMNOPQ* operon (Fig. 1), and transcription of this operon, including *pilQ*, appears to be driven by a promoter upstream of *pilM*. The *ponA-pilM* intergenic region is only 150 bp, and the predicted −10 and −35 consensus sites for these loci appear to overlap. Based on this gene organization, we tested whether MtrR regulates *pilM* expression as was observed for the divergently transcribed *ponA* gene. For this purpose, a *pilM* promoter-*lacZ* fusion was constructed and transformed into FA19, FA19 Δ*mtrR*, and FA19 Δ*mtrR* GC3*mtrR*, which resulted in a single copy of the promoter of *pilM* fused translationally to *lacZ* within the *proAB* locus of the gonococcal chromosome. Expression of *pilM* increased greater than twofold in strain FA19 Δ*mtrR* versus that in the parental strain, FA19, indicating that MtrR represses the expression of *pilM* (Fig. 3). Complementation of *mtrR* in strain FA19 Δ*mtrR* GC3*mtrR* restored the repression of *pilM* to wild-type levels.

MtrR directly binds to the *ponA-pilM* promoter region. Because MtrR appeared to increase *ponA* expression (Fig. 2A) while repressing *pilM* expression (Fig. 3), we examined whether

replicate was performed in triplicate. Error bars represent 1 standard deviation. The differences between strains FA19 *ponA-lacZ* and FA19 Δ*mtrR ponA-lacZ* as well as between strains FA19 Δ*mtrR ponA-lacZ* and FA19 Δ*mtrR* GC3*mtrR ponA-lacZ* were significant ($P < 0.0001$). (B) Total membranes from strains FA19, FA19 Δ*mtrR*, and FA19 Δ*mtrR* GC3*mtrR* were prepared, and PBP 1 levels were determined by Western blotting using rabbit anti-PBP 1 antisera. Equal amounts of protein (3 μg) were loaded in each lane. Western blotting of whole-cell lysates from these same strains gave similar results (data not presented). (C) Comparison of the MtrR-dependent regulation of *ponA* gene expression and PBP 1 protein levels determined by densitometry. Densitometry was performed to determine the relative ratios of PBP 1 in strains FA19, FA19 Δ*mtrR*, and FA19 Δ*mtrR* GC3*mtrR*. These values were plotted on the same graph as that shown in panel A, with the densitometry levels for FA19 PBP 1 set to the same values as the β-Gal activity levels for FA19 *ponA-lacZ* for purposes of comparison. Light gray bars represent gene expression, while dark gray bars represent protein levels.

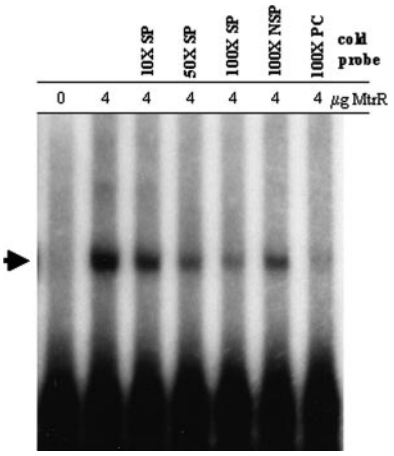

FIG. 4. Competitive EMSA using purified MBP-MtrR fusion protein. The radiolabeled probe migrating at the bottom of the gel is the 150-bp intergenic region of *ponA-pilM*. SP, specific cold probe (*ponA-pilM* intergenic region); NSP, nonspecific cold probe (internal region of *mtrC*); PC, positive-control cold probe (*mtrR-mtrC* intergenic region). The lane without MtrR is designated by 0 and shows the electrophoretic migration of the labeled probe alone. The arrow indicates the protein/DNA complex. At a 100-fold level of unlabeled probe, SP was nearly 5-fold more effective than the NSP in competing with the labeled probe for MtrR-binding.

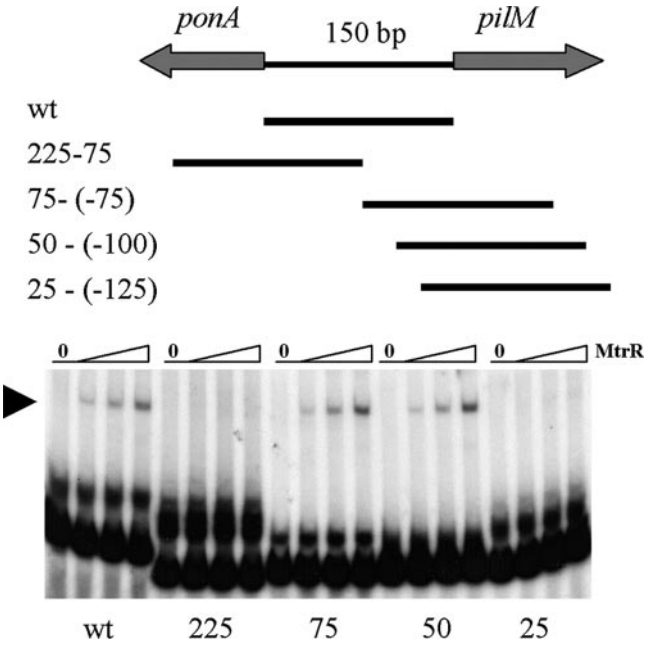

FIG. 5. EMSAs performed on the 150-bp overlapping radiolabeled probes within the *ponA-pilM* intergenic region using purified MBP-MtrR protein. The diagram at top indicates the location of each probe. Increasing amounts (0, 2, 4, and 8 μg) of MBP-MtrR protein were added to each binding reaction. The lane without MtrR is designated by 0 and shows the electrophoretic migration of the labeled probes alone. The arrow indicates the protein/DNA complex.

MtrR could bind in a specific manner to the *ponA-pilM* promoter region. MtrR has been shown previously to bind specifically to the *mtrR-mtrC* intervening region (20), and this interaction served as a positive control in the DNA-binding experiments described below. Initial DNA-binding experiments using EMSA showed that MtrR bound to both the *mtrR-mtrC* and the *ponA-pilM* promoter regions in a concentration-dependent manner (data not shown). To verify the specificity of this interaction, a competitive EMSA was performed using specific unlabeled PCR-derived probes from the *ponA-pilM* and *mtrR-mtrC* promoter regions and a nonspecific probe derived from an internal region of the *mtrC* gene. We observed that increasing amounts of the unlabeled *ponA-pilM* probe competed with MtrR binding to the labeled *ponA-pilM* region (Fig. 4), whereas the unlabeled, nonspecific probe was nearly fivefold less inhibitory, as determined by densitometric analysis. It is important to note that whereas 100× unlabeled *ponA-pilM* promoter probe decreased MtrR binding to the labeled *ponA-pilM* probe, 100× unlabeled *mtrR-mtrC* promoter probe completely abolished MtrR binding, suggesting that MtrR binds to the *mtrC-mtrR* promoter region with much higher affinity than to the *ponA-pilM* promoter region.

Identification of the MtrR-binding site. The results from the EMSA experiments suggested that MtrR has the capacity to bind within the *ponA-pilM* intergenic region. Therefore, we sought to identify the location of the MtrR-binding site within this 150-bp region. Initial attempts to do this by DNase I footprinting gave inconsistent results (data not presented), perhaps due to the lower affinity of MtrR for this site than for the *mtrCDE* promoter region. Accordingly, a series of 150-bp overlapping probes (Fig. 5) were used in EMSAs to delimit the region required for MtrR binding. In the absence of MtrR, each labeled probe migrated as two bands, likely due to differences in the secondary structure of the DNA; the migration position of the MtrR-DNA complexes is designated by the arrow in Fig. 5. MtrR failed to bind to the probe (from bp 225 to bp 75) encompassing part of the *ponA* coding sequence and the first 75 bp of the intergenic region (Fig. 5). However, MtrR did bind to the probe (from bp 75 to bp −75) encompassing the last 75 bp of the intergenic region and part of *pilM*, suggesting that the MtrR-binding site is located within the last 75 bp of the *ponA-pilM* intergenic region. Within this 75-bp fragment, MtrR bound to the probe from bp 50 to bp −100 but was unable to bind to the probe from bp 25 to bp −125. Taken together, these results suggested that a 25-bp region located between 50 and 25 bp upstream of the *pilM* start codon contains either part of or the entire MtrR-binding site.

MtrR binds to the *mtrR-mtrC* intergenic region (20), and a 15-bp binding site containing two pseudo-direct repeats (underlined; 5′-CCGTGCAATCGTGTA-3′) was recently shown to be necessary and sufficient for MtrR binding within the *mtrCDE* promoter (16). Based on homology to these repeats, a possible MtrR-binding site containing two pseudorepeats within the required 25-bp sequence of the *ponA-pilM* region was identified (5′-TTGTACA-3′ and 5′-CTGTACA-3′ [bold, underlined sequences in Fig. 1]). One of these repeats overlapped the putative −10 promoter hexamer sequence used for transcription of *ponA* that was previously predicted by Ropp and Nicholas (28). To determine if these repeats were required for MtrR binding, pseudorepeat I was mutated from 5′-TTGTAC A-3′ to 5′-GGTGCAC-3′ and pseudorepeat II was mutated from 5′-CTGTACA-3′ to 5′-AGTGCAC-3′. The impact of these mutations on MtrR binding to the intergenic region was then deter-

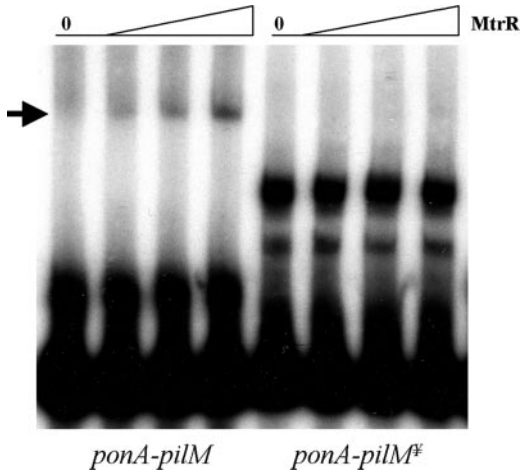

FIG. 6. EMSA performed on a wild-type or mutated *ponA-pilM* promoter region using purified MBP-MtrR protein. Probe *ponA-pilM* contains the wild-type MtrR-binding site, while the *ponA-pilM*¥ probe contains the mutated MtrR-binding site. Increasing amounts (0, 2, 4, and 8 μg) of MBP-MtrR protein were added to each binding reaction. The lane without MtrR is designated by 0 and shows the electrophoretic migration of the labeled probes alone. The arrow indicates the protein/DNA complex.

mined by EMSA. As opposed to its binding to the wild-type target DNA (Fig. 6), MtrR failed to bind the mutated target DNA, indicating that at least one (and likely both) of these two sites is required for its binding to the transcriptional regulator.

An MtrR-binding site mutation deregulates *ponA* expression. Since mutation of the MtrR-binding site resulted in the inability of MtrR to bind to the *ponA-pilM* intergenic region, we next tested whether these mutations would prevent MtrR-dependent regulation of these genes. The above-mentioned mutations were introduced into the pP*ponA-lacZ* plasmid, resulting in plasmid construct pP*ponA-lacZ*¥. This construct was then transformed into strains FA19 and FA19 Δ*mtrR*, and *ponA* expression was assessed by quantifying β-Gal activity. As previously observed, the expression of *ponA* from the wild-type promoter was decreased in FA19 Δ*mtrR* compared to that in its parental strain, FA19 (Fig. 7). However, there was no significant difference in *ponA* expression observed in the FA19 *ponA-lacZ*¥ and FA19 Δ*mtrR ponA-lacZ*¥ strains, with the *ponA* promoter containing the mutation (Fig. 7). Therefore, mutation of the MtrR-binding site within the *ponA* promoter results in the loss of MtrR-dependent regulation of *ponA*. Interestingly, the mutation of the MtrR-binding site should have also disrupted the predicted −10 region of the *ponA* promoter. However, our data clearly demonstrate that *ponA* expression was not abolished by this mutation, as expression in both FA19 *ponA-lacZ*¥ and FA19 Δ*mtrR ponA-lacZ*¥ was at least as great as that observed for FA19 Δ*mtrR ponA-lacZ* (Fig. 7), suggesting that this was not the location of the −10 region. Taken together, these results are consistent with MtrR serving as an enhancer of *ponA* expression.

DISCUSSION

The loss of penicillin as an effective antibiotic in the treatment of gonorrhea resulted not from the acquisition of the β-lactamase plasmid (still rarely found in gonococci) that was reported in the 1970s (9) but rather from the sequential accumulation over a 40-year time period of mutations in the *mtrR*, $porB_{1B}$, and *ponA* genes as well as the creation and horizontal transfer of mosaic *penA* genes (24, 27, 36, 44). Each of these

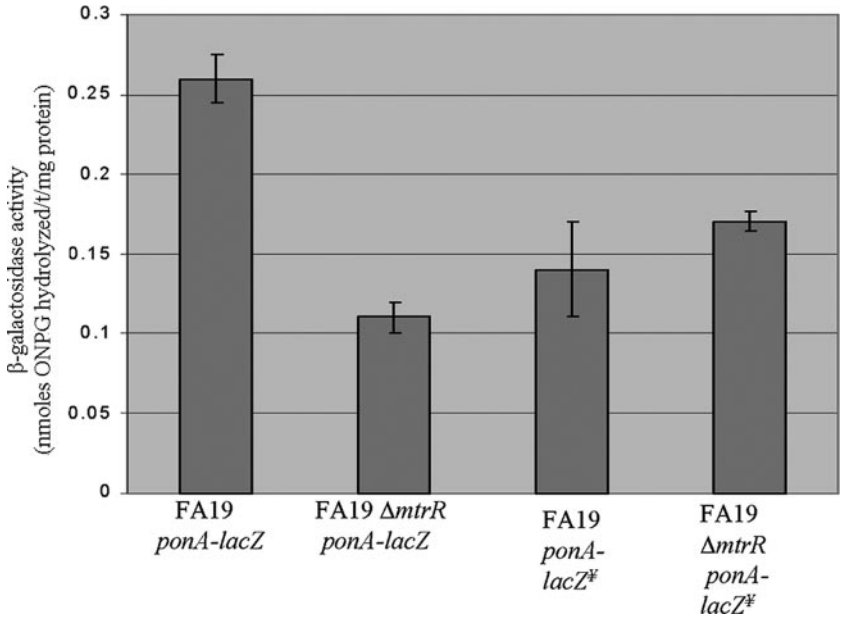

FIG. 7. Mutation of the MtrR-binding sites within the *ponA-pilMNOPQ* intergenic region disrupts MtrR-dependent regulation of *ponA* expression. Shown are the β-Gal activities per mg of total protein in cell extracts of reporter strains FA19 *ponA-lacZ*, FA19 Δ*mtrR ponA-lacZ*, FA19 *ponA-lacZ*¥, and FA19 Δ*mtrR ponA-lacZ*¥. The figure represents one experiment of three replicates; each replicate was performed in triplicate. Error bars represent 1 standard deviation. The *P* value (Student's *t* test) between strains FA19 *ponA-lacZ* and FA19 Δ*mtrR ponA-lacZ* was <0.0001. The difference in activity determined for FA19 *ponA-lacZ*¥ and FA19 Δ*mtrR ponA-lacZ*¥ was not significant.

resistance determinants, which are transferred from a resistant strain to a susceptible strain in a specific order (27, 36, 40, 44), incrementally increases in resistance until treatment failure occurs (MIC $\geq$ 2 μg/ml). Mutations in *ponA* and *penA* decrease penicillin susceptibility in gonococci by decreasing the rate of penicillin acylation of PBPs 1 and 2, respectively, while *mtrR* mutations result in overexpression of the MtrC-MtrD-MtrE efflux pump. Although overexpression of the efflux pump on its own results in only a small increase in resistance to penicillin, *mtrR* mutations are required for gonococci to exhibit high-level chromosomally mediated resistance to penicillin (25, 38). The *penB* resistance determinant, which encodes $PorB_{1B}$ variants with multiple amino acid changes in the putative loop 3 constriction loop, was originally thought to reduce antibiotic entry by altering permeation of antibiotics through the porin channel, but this has recently been brought into question (17). Finally, recent evidence indicates that mutations in *pilQ* also affect antibiotic entry into gonococci (3).

Because of the ability of MtrR to control the expression of *mtrCDE* (14) and the impact of *mtrR* mutations on pump levels and penicillin resistance (38), we asked if this DNA-binding protein might also regulate other genes important for penicillin resistance. The evidence presented herein strongly suggests that the transcriptionally divergent *ponA-pilMNOPQ* gene cluster is subject to MtrR transcriptional control. Specifically, *ponA* expression appears to be increased in the presence of MtrR while *pilMNOPQ* expression is subject to MtrR repression at a level similar to that observed for *mtrCDE* (14, 15). EMSA and mutagenesis studies strongly suggest that the pseudorepeats TTGTACA and CTGTACA, located within the *ponA-pilM* intergenic region, form the MtrR-binding site. Interestingly, these sites are on opposite strands of the DNA, a feature not previously observed for the TetR family of transcriptional regulators but perhaps necessary for control of the divergently transcribed *ponA-pilMNOPQ* region.

Increasing evidence indicates that MtrR plays a central role in modulating levels of gonococcal susceptibility to antimicrobial agents (15), and there is strong evidence to suggest that it performs this function primarily by modulating levels of the MtrC-MtrD-MtrE efflux pump. We have also determined that MtrR regulates the expression of *mtrF* (11), which encodes a cytoplasmic membrane protein needed for high-level hemagglutinin resistance mediated by the MtrC-MtrD-MtrE pump, as well as *farR*, which encodes the transcriptional repressor of the *farAB*-encoded efflux pump that exports long-chain fatty acids (18). The results presented herein implicate MtrR as a regulator of *ponA* and *pilMNOPQ* expression and lend further support to the notion that MtrR has more global regulatory properties than previously appreciated (15, 37). A recurring theme of the MtrR-regulated genes identified thus far is that all appear to be involved in resistance to host-derived antimicrobials or classical antibiotics. With respect to PilQ, a point mutation in *pilQ* (*pilQ1*) was previously shown to result in the decreased resistance of gonococci to a number of antimicrobials through a change in the structure of the PilQ outer membrane pore that enhanced the entry of antimicrobials (3). A second *pilQ* point mutation, termed *penC* (*pilQ2*) (44), appeared to cause a defect in PilQ multimerization and a subsequent loss of antibiotic entry. Moreover, this mutation was found to be essential for the phenotypic expression of high-level penicillin resistance in laboratory transformation experiments. Interestingly, the increase in resistance due to *pilQ2* was observed only in strains containing the *penA*, *mtrR*, and *penB* resistance determinants, and together with *ponA*, these five determinants were capable of conferring high-level penicillin resistance to the same level as that found in clinical isolates.

In contrast to that of other MtrR-regulated genes, *ponA* gene expression appears to be transcriptionally enhanced by MtrR, a finding that was somewhat surprising as members of the TetR family are usually repressors. However, one other TetR member, DhaS from *Lactococcus lacti*, has been shown to be an activator of gene expression (4). While the physiological relevance of *mtrCDE* regulation by MtrR and the impact of *mtrR* mutations in gonococci have been documented (38), the benefit of *ponA* and *pilMNOPQ* regulation by this transcriptional regulator is less clear. At first glance, it would seem to be disadvantageous in vivo for gonococci to differentially regulate *ponA* and *pilMNOPQ*, as such changes in gene expression that occur in *mtrR* mutants would be expected to increase antibiotic uptake due to increased expression of *pilQ* and decrease peptidoglycan structure or bacterial growth rate due to decreased expression of *ponA*. However, since the type IV pilus has been shown to be required for motility, DNA uptake, virulence, and biofilm formation (26, 37, 41, 42), increased expression of the pilus apparatus genes may give gonococci with *mtrR* mutations a survival advantage, along with increased MtrC-MtrD-MtrE pump levels, during host infections. We propose that MtrR activation of *ponA* either directly or indirectly provides gonococci a mechanism to resist the lethal action of the host environment, perhaps due to changes in peptidoglycan structure, which makes the gonococci less susceptible to damaging agents. In *Neisseria meningitidis*, alterations in PBP 2 have been shown to result in compositional modifications in peptidoglycan structure (1). Thus, host antimicrobials that damage the cell envelope integrity may have reduced activities against *mtrR* mutants, which were shown previously (13) to have altered peptidoglycan cross-linking and lytic behavior. More-in-depth interpretations of the biological significance of the data presented herein will require additional experimentation. However, in support of our hypothesis that these changes in gene expression are not disadvantageous in vivo are reports that gonococci with *mtrR* mutations are often isolated from patients with rectal or urogenital infections (8, 23, 31, 43). Moreover, recent experiments showed that mutations in *mtrR* can enhance gonococcal fitness in a murine vaginal infection model (D. M. Warner, J. P. Folster, W. M. Shafer, and A. Jerse, unpublished results). Hence, we propose that under certain circumstances and infections, MtrR regulation of gene expression is an important determinant of gonococcal survival in vivo. A thorough understanding of the MtrR regulon in gonococci should therefore provide important insights regarding antibiotic resistance and pathogenesis as well as the link between these two phenotypes.

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