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Off-Target Gene Regulation Mediated by Transcriptional Repressors of Antimicrobial Efflux Pump Genes in *Neisseria gonorrhoeae*▽

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DNA-binding proteins that control expression of drug efflux pump genes have been termed "local regulators" as their encoding gene is often located adjacent to the gene(s) that they regulate. However, results from recent studies indicate that they can control genes outside efflux pump-encoding loci, which we term as being "off target." For example, the MtrR repressor was initially recognized for its ability to repress transcription of the *mtrCDE*-encoded efflux pump operon in the strict human pathogen *Neisseria gonorrhoeae*, but recent results from genetic and microarray studies have shown that it can control expression of nearly 70 genes scattered throughout the chromosome. One of the off-target MtrR-repressed genes is *glnA*, which encodes glutamine synthetase. Herein, we confirm the capacity of MtrR to repress *glnA* expression and provide evidence that such repression is due to its ability to negatively influence the binding of a second DNA-binding protein (FarR), which activates *glnA*. FarR was previously recognized as a transcriptional repressor of the *farAB*-encoded efflux pump operon. Thus, two DNA-binding proteins previously characterized as repressors of genes encoding efflux pumps that contribute to gonococcal resistance to antimicrobials can act in an opposing manner to modulate expression of a gene involved in basic metabolism.

Over the past 2 decades, considerable progress has been made in characterizing the biochemistry, genetics, regulation, and function of efflux pump systems that export antimicrobials and contribute to bacterial resistance to antibiotics (1, 2, 8, 18, 20–22). Prior to the availability of genome sequence information, many of these efflux pumps were identified by the isolation of laboratory-derived mutants that expressed decreased susceptibility to antibiotics or other antimicrobials (18). These mutations frequently mapped to genes encoding DNA-binding proteins that normally function to dampen transcription of efflux pump protein-encoding genes. In this respect, the identification of the *mtr* (*m*ultiple *t*ransferable *r*esistance) system of *Neisseria gonorrhoeae* by Pan and Spratt (19) was made possible by their cloning and sequencing of DNA fragments harboring mutant alleles of the *mtrR* gene, which encodes a repressor (MtrR) of the transcriptionally divergent *mtrCDE* efflux pump operon (10, 11, 19). When they are introduced into a wild-type strain of gonococci, mutant *mtrR* alleles can confer resistance to structurally diverse hydrophobic antimicrobial agents (9, 10, 19, 26, 28).

MtrR is a member of the TetR/QacR family of repressors (reviewed in reference 8), which are known to perform important roles in controlling expression of a number of bacterial efflux pump-encoding genes (21). With respect to repression of *mtrCDE*, two homodimers of MtrR bind the DNA sequence that overlaps the promoter used for *mtrCDE* transcription (12, 16), and a pseudo-direct repeat element (CCGTGCA and TC GTGTA), separated by a single nucleotide, within this binding site is important for MtrR recognition (12). Due to its ability to modulate levels of antimicrobial resistance and the frequent occurrence of *mtrR* mutants in clinical isolates (4, 25, 30, 31), we have been interested in how MtrR controls gene expression in gonococci (5–7, 15). Although repressors like MtrR have been thought of as local regulators (17) that control adjacent efflux pump genes, it is likely that they have the capacity to regulate additional genes outside those involved in drug efflux; we term these genes as being "off target." Since it is unclear as to whether the model (12, 16) developed for MtrR repression of target genes (e.g., *mtrCDE*) would hold true for off-target genes, we examined its ability to control expression of *glnA*, which was identified in a recent microarray study (7) as being MtrR repressed. We were especially interested in MtrR regulation of *glnA* because *glnA* in *Corynebacterium glutamicum* has been shown (3) to be negatively controlled by another member of the TetR/QacR family (AmtR), and we thought that studies with MtrR would, in general, provide insights regarding the mechanism by which members of the TetR/QacR family regulate genes outside those encoding drug efflux proteins. We now report evidence that MtrR represses *glnA* in gonococci by negatively influencing the binding of a second DNA-binding protein (FarR), also involved in repressing expression of an antimicrobial efflux pump (15), which activates *glnA* expression. Taken together, our results indicate that DNA-binding proteins recognized for their ability to control expression of drug efflux pump genes and modulate levels of bacterial susceptibility to antimicrobials can have alternative targets and modes of regulation.

MATERIALS AND METHODS

Strains used and growth conditions. All of the strains of *N. gonorrhoeae* employed (Table 1) are genetic derivatives of strain FA19 and were constructed in previous investigations (5–7, 15) or in this study. Gonococci were routinely cultured as nonpiliated, opacity-negative (P^- Opa^-) variants on GCB agar with defined supplements I and II (23) at 37°C under 3.8% (vol/vol) CO_2; transfor-

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TABLE 1. Gonococcal strains and plasmids used in this study

Strain or plasmid	Relevant genotype or remarks	Source or reference
Strains		
FA19	Wild type	P. F. Sparling
EL24	As FA19 but *farR*::*kan*	15
EL28	As EL24 but *farR*$^+$	15
JF1	As FA19 but Δ*mtrR*	5
JF6	As JF1 but *mtrR*$^+$	7
PJ9	As FA19 but *glnA*::*lacZ*	This study
PJ10	As JF1 but *glnA*::*lacZ*	This study
PJ11	As EL24 but *glnA*::*lacZ*	This study
PJ12	As JF6 but *glnA*::*lacZ*	This study
PJ13	As PJ10 but *farR*::*kan*	This study
PJ14	As EL28 but *glnA*::*lacZ*	This study
PJ15	As PJ14 but *farR*$^+$	This study
PJ16	As PJ12 but *farR*::*kan*	This study
PJ22	As PJ13 but *mtrR*$^+$	This study
Plasmids		
pLES94	Cloning vector containing promoterless *lacZ* for insertion of gonococcal genes between *proA* and *proB*	V. Clark
pJF3	As pLES94 but *glnA*::*lacZ*	This study
pGCC3	NICS vector used for insertion of gonococcal genes between *lctP* and *aspC*	H. Seifert

TABLE 2. Oligonucleotides used in this study

Oligonucleotide used[a]	Sequence (5′ → 3′)
5′pglnA	GGGGATCCCATAAAGGCGGGGCGGTC
3′pglnA	GGGGATCCCGGGACATCTTCAGCTCC TGAA
glnA1	GCAACCGCCTGTTTCAAAAAATG
glnA2	GGACATCTTCAGCTCCTGAAAAAG
glnAsec1F	TAACGTTTGCCCCGCAACC
glnAsec1R	CCCCCGCTACGCCGTTTTC
glnAsec2F	GGGCGTGCATAGTCATATTC
glnAsec2R	GCGTCAAATTCCAAGCCGG
glnAsec3F	AAACCGGTTTCAGACGGCAT
glnAsec3R	GGACATCTTCAGCTCCTGAA
glnAtrunckf	CATGGATCCGATGAATCTGCGGCGA TTTG
rmpF	CATGTTTCTACAGCGGCCTG
rmpR	CGGCAAGATATTACCTAGCCT
16SRTR	CATCGGTATTCCTCCACATCTC
16SRTF	GTAGGGTGCGAGCGTTAATC
glnARTR	GGCTTTGGCGTGTTTGATG
glnARTF	TCCGATACCGCGCTCTACTAC

[a] Primers with ending letters RTR or RTF were used in qRT-PCR for the 16S rRNA and *glnA* transcripts.

mation experiments used piliated (P^+) Opa^- variants. Gonococci were also cultured in GCB broth (Difco Laboratories, Detroit, MI) with supplements I and II and 0.048% (wt/vol) sodium bicarbonate with shaking at 37°C. *Escherichia coli* strain DH5α *mcr* was routinely cultured in Luria-Bertani (LB) broth or on LB agar (Difco Laboratories) with antibiotics (100 μg/ml of ampicillin or kanamycin); antibiotics were purchased from Sigma Chemical Corporation (St. Louis, MO).

qRT-PCR. The essentials of the quantitative real-time reverse transcription (RT)-PCR (qRT-PCR) protocol employed have been described previously (13). In order to confirm the microarray data reported by Folster et al. (7), a portion of each of the three RNA samples from parent strain FA19 and strain JF1 (as FA19 but Δ*mtrR*) used in that study was employed in qRT-PCR; these RNA samples were kindly provided by L. Jackson and D. Dyer (University of Oklahoma Health Sciences Center, Oklahoma City, OK). cDNA from each sample was synthesized in three independent RT reactions with random hexamer primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The specific primers for *glnA* and 16S RNA used in qRT-PCR are listed in Table 2. qRT-PCR was performed on an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). iQSYBR Green Supermix (Bio-Rad Laboratories) was employed in a reaction volume of 25 μl with 200 nM 5′ and 3′ primers and 5-fold dilutions of RT reaction mixtures. 16S RNA cDNA was employed as the internal control. The results for changes in *glnA* expression due to loss of *mtrR* are reported (see below) as an average value (± standard deviation).

Strain construction and β-Gal assays. Translational *lacZ* fusions were constructed using pLES94 (27) by transformation into strains FA19, JF1, JF6, and EL24 or their derivatives. The strains bearing translational fusions are described in Table 1. β-Galactosidase (β-Gal) assays were performed as described by Folster and Shafer (5). Plasmid pJF3, which contained a *glnA-lacZ* fusion, was prepared essentially as described by Folster et al. (7). Briefly, the promoter sequence of *glnA* (summarized in Fig. 1) was amplified by PCR from strain FA19 using primers 5′pglnA and 3′pglnA (Table 2). Following amplification by PCR, the DNA sequence was cloned into the BamHI cloning site of pLES94 (27). The resulting plasmid construct was then transformed into *E. coli* DH5α *mcr* and transformants were recovered by ampicillin selection (100 μg/ml). The cloned fragment in the resulting transformant was identified by PCR analysis and DNA sequencing. This plasmid (pJF3) was then transformed (5) into strains FA19, JF1, JF6, and EL24, with insertion occurring at the nonessential *proAB* locus (27) of the recipient strains. Transformants were selected on GCB agar containing 1 μg/ml of chloramphenicol. The resulting transformants (Table 1) were then used in β-Gal assays as previously described (7).

Protein purification and DNA-binding studies. The production and purification of the maltose-binding protein (MBP)-MtrR and the histidine (His)-tagged FarR fusion proteins used in this study have been described previously (6, 15). These proteins were used in electrophoretic mobility shift assays (EMSAs) and DNase I protection studies using previously described methods (7, 15). The DNA probes for EMSAs consisted of PCR products that were obtained using sets of oligonucleotide primers (Table 2); the generation of specific PCR products is described in the relevant figure legend. These PCR products were end labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (New England BioLabs, Beverly, MA) as described previously (15). The labeled PCR-generated products were incubated with purified MtrR-MBP or FarR-His, or both, in a final reaction volume of 30 μl consisting of the 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) (0.05 μg/ml)] and distilled H_2O at 25°C for 30 min. Following incubation, the reaction mixtures were subjected to gel electrophoresis utilizing a 6% (wt/vol) polyacrylamide gel at 4°C and dried, and autoradiography was performed for visualization. Competitive EMSAs were performed in the same manner, but with the addition of unlabeled specific competitor, generated from the same sequence as the target, or nonspecific competitor, generated from the *rmp* gene using primers rmpF and rmpR (Table 2). DNase I protection assays were performed essentially as described by Folster et al. (7). PCR-generated target DNA sequences were synthesized using primers glnAsec1F, glnAsec1R, glnAsec2F, or glnAsec2R and were labeled at the 5′ end of one strand as described for EMSA. Purified MtrR-MBP or FarR-His was then incubated with the target DNA sequence under the same binding conditions used in the EMSA for 15 min at 37°C. Following the addition of $MgCl_2$ (5 mM) and $CaCl_2$ (2.5 mM), DNase I (Promega, Madison, WI) was added and the reaction mixtures were incubated at 37°C for 1 min. The reactions were stopped with DNase I stop buffer (95% ethanol, 7.5 mM ammonium acetate, and nuclease-free H_2O), snap-frozen on dry ice for 15 min, and then precipitated at −80°C overnight. The pellet was then washed in 70% (vol/vol) ethanol, dried, and resuspended in gel loading buffer (Epicentre, Madison, WI). Reaction mixtures were then loaded on 8% denaturing polyacrylamide gel, subjected to gel electrophoresis, and dried, and autoradiography was performed for visualization as described previously (6, 7).

RESULTS AND DISCUSSION

MtrR repression of off-target gene, *glnA*. We first sought to confirm the earlier microarray data of Folster et al. (7), which revealed the capacity of MtrR to negatively control *glnA* expression by 1.6-fold ($P < 0.05$). Results from qRT-PCR experiments, which used total RNA isolated from isogenic strains

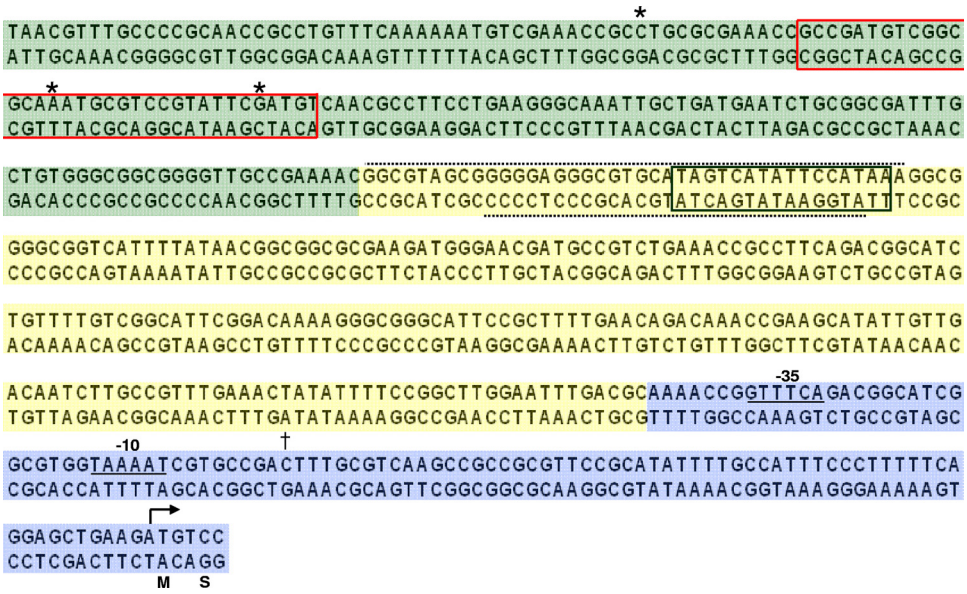

FIG. 1. Nucleotide sequence upstream of *glnA*. The nucleotide sequence of the DNA upstream of *glnA* is shown, including the first two codons encoding methionine (M) and serine (S); the translational start codon is identified by the bent arrow. This 506-bp sequence is divided into three sections that were used in EMSA experiments, with section I shown in green, section II in yellow, and section III in blue. The −10 and −35 hexamer sequences of the *glnA* promoter (section III) are identified with the −10 and −35 notation as well as a line under the sequences. The start point of transcription is shown by the † symbol and was determined by primer extension analysis as described previously (10). The MtrR-binding site in section I that is boxed in red was predicted on the basis of similarity to the binding site upstream of *mtrCDE* (12, 16). DNase I-hypersensitive sites observed in protection assays with MtrR are shown by asterisks. The FarR-binding site predicted by sequence similarity with that upstream of *farAB* (15) is shown in the black boxed area in section II, and the binding sites identified by DNase I protection (Fig. 4) are noted by the dotted line above the sense strand and below the antisense strand.

FA19 and JF1 (as FA19 but Δ*mtrR*; Table 1), confirmed that MtrR could dampen (1.9- ± 0.23-fold; $P < 0.05$) *glnA* expression. As additional confirmation, we employed a translational promoterless *lacZ* expression system that measures gene expression and regulation from an ectopic site in gonococci (27). The *glnA-lacZ* translational fusion employed in this study consisted of 500 bp of DNA upstream and the first two codons of *glnA* (Fig. 1). Using this system, we found (Fig. 2) that *glnA-lacZ* expression in isogenic MtrR-positive and -negative genetic derivatives differed and was significantly ($P = 0.005$) elevated in MtrR-negative strain PJ10 compared to MtrR-positive strain PJ9. Importantly, this increase in *glnA-lacZ* expression in the MtrR-negative strain was reversed ($P < 0.005$) to wild-type levels when the mutation was complemented (see strain PJ12) with an ectopically expressed wild-type *mtrR* gene (Fig. 2).

Since the qRT-PCR and *lacZ* translational fusion data confirmed the microarray data of Folster et al. (7) that *glnA* is subject to MtrR repression in gonococci, we tested if MtrR could bind the DNA sequence upstream of *glnA*. In preliminary EMSA experiments, we found that an MBP-MtrR fusion protein could bind in a specific manner (data not presented) to a labeled PCR probe that consisted of the DNA upstream of *glnA* shown in Fig. 1. In order to localize the site of specific DNA binding by MtrR, we prepared three probes (termed sections I, II, and III in Fig. 1) that consisted of truncated sequences upstream of *glnA*. Using EMSA, we found that MtrR bound only section I in a specific manner (data not presented and Fig. 3). On the basis of the MtrR-binding site sequence upstream of *mtrCDE* (12, 16), we identified a potential MtrR-binding site upstream of *glnA* within section I, which is shown in the red box in Fig. 1. However, when we used DNase I footprinting to identify potential sites for MtrR binding within section I, we could not reproducibly detect defined regions of protection. Interestingly, on the coding strand, at least three DNase I-hypersusceptible sites were evident (summarized in Fig. 1), suggesting the occurrence of MtrR-DNA interactions. Two of these hypersensitive sites were within the predicted MtrR-binding site (12, 16), while the third was 12 nucleotides upstream of this region.

FarR activates *glnA* expression. Although MtrR can directly regulate certain genes in gonococci, it can also indirectly regulate others (7, 15). For instance, expression of the *farAB* efflux pump-encoding operon (15) is increased in an *mtrR*-null mutant because MtrR can repress *farR*, which encodes a direct repressor of the *farAB*-encoded efflux pump operon (15). Accordingly, we tested if MtrR regulation of *glnA* also involves FarR. For this purpose we used the translational *glnA* fusion system described above and monitored its expression in strains containing a *farR*-null mutation with and without a coresident *mtrR*-null mutation. Evidence for FarR regulation of *glnA* was obtained (Fig. 2) when the *glnA-lacZ* translational fusion was expressed in strain FA19 bearing a wild-type (strain PJ9) or an insertionally inactivated (strain PJ11) *farR* gene. With these strains we found (Fig. 2) that expression of *glnA* was significantly ($P = 0.007$) higher in FarR-positive strain PJ9.

As this result suggested that FarR is a positive regulator of *glnA* expression, we tested if complementation of the *farR*-null mutant with the wild-type *farR* gene expressed ectopically (strain PJ14) would restore levels of *glnA* expression and found

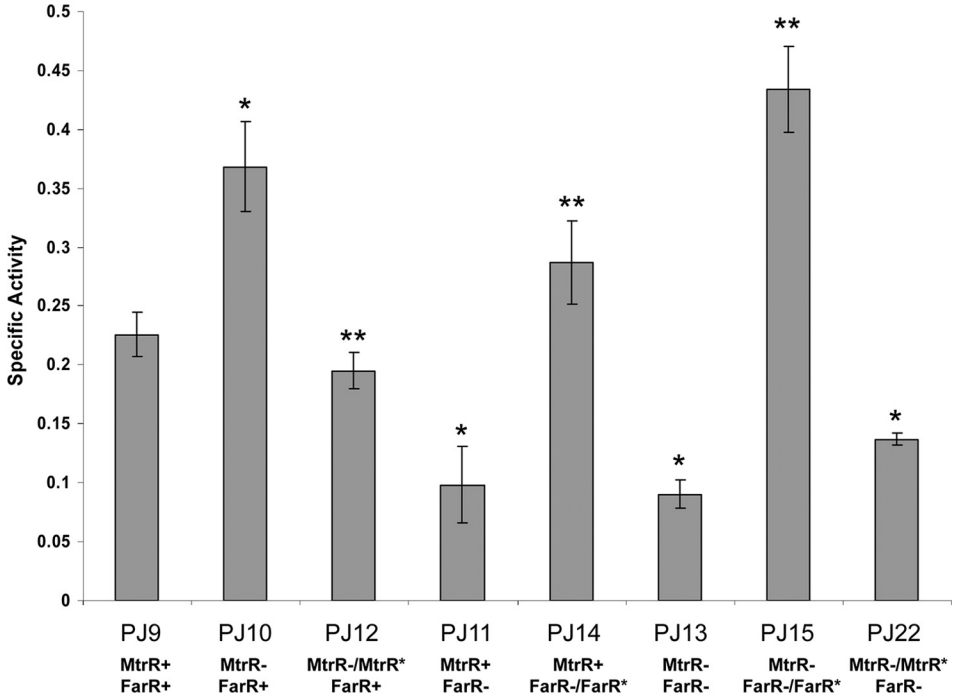

FIG. 2. MtrR and FarR regulation of *glnA* expression. The specific activity of β-Gal (expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per mg of protein) in strains PJ9 (FA19 *glnA*::*lacZ*), PJ10 (JF1 *glnA*::*lacZ*), PJ12 (JF6 *glnA*::*lacZ*), PJ11 (EL24 *glnA*::*lacZ*), PJ14 (EL28 *glnA*::*lacZ*), PJ13 (PJ10 *farR*::*kn glnA lacZ*), PJ15 (PJ14 *farR*$^+$), and PJ22 (PJ13 *mtrR*$^+$). The results are shown as an average value (± standard deviation) from three independent experiments. The single asterisk above a bar denotes a significant ($P < 0.01$) difference between the indicated strain and wild-type strain PJ9, while the double asterisk denotes a significant difference ($P < 0.01$) between a complemented strain and its respective mutant (comparisons of PJ12 versus PJ10, PJ14 versus PJ11, and PJ15 versus PJ13); the specific *P* values are provided in the text.

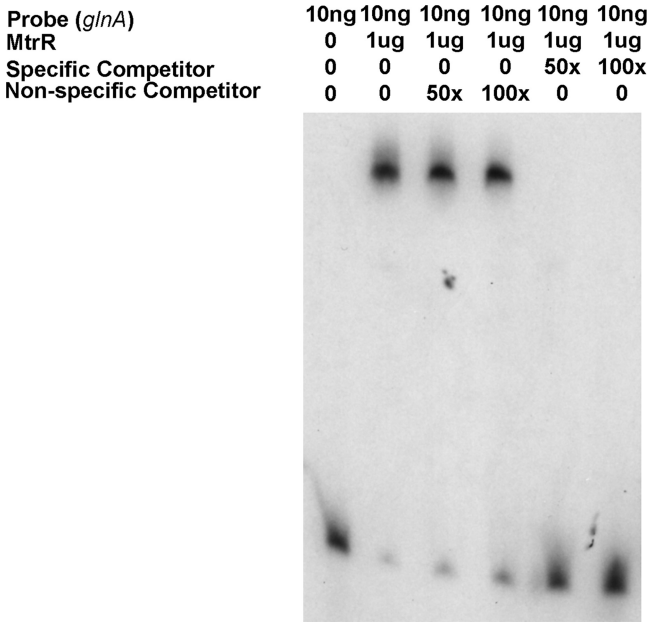

FIG. 3. Specific binding of MtrR upstream of *glnA*. The area of MtrR-specific binding was identified using the three sections of the full-length DNA sequence described in Fig. 1. The full-length probe was PCR amplified from DNA of strain FA19 using primers 5′pglnA and 3′pglnA (Table 2), while binding site I was amplified with primers glnAsec1F and glnAsec1R. The nonspecific probe was prepared using primers rmpF and rmpR. MtrR was found to specifically bind only (data not presented) to section I since specific competitor DNA, but not nonspecific competitor (up to 100 times), was able to abrogate the shifting of section I *glnA* DNA in EMSA; this result is shown in the figure.

this to be the case (Fig. 2); the difference in *glnA* expression between strains PJ11 and PJ14 was significant ($P = 0.002$). Due to these results, we next examined *glnA* expression in an MtrR$^-$ FarR$^-$ double mutant (strain PJ13). In double mutant strain PJ13, *glnA* expression mimicked (Fig. 2) that of the single mutant that only lacked FarR (strain PJ11). Moreover, complementation of the double mutant with a wild-type *farR* gene (strain PJ15) but not *mtrR* (strain PJ22) expressed ectopically enhanced *glnA* expression to a level that resembled that in MtrR$^-$ FarR$^+$ strain PJ10 (Fig. 2); the difference in *glnA* expression between strains PJ13 and PJ15 was significant ($P < 0.0001$).

Evidence that FarR can bind upstream of *glnA* was obtained by both EMSA and DNase I protection. In EMSA experiments that monitored specific binding of FarR to sections I, II, and III (Fig. 1), we found that it bound only section II in a specific manner (data not presented and Fig. 4A). Using DNase I protection, we identified (Fig. 4B) three sites protected by FarR on the sense strand, all three of which had adjacent DNase I-hypersensitive nucleotides. Importantly, only one of the FarR-protected sites was within section II (shown by the # sign next to the vertical bar in Fig. 4B), which bound FarR in a specific manner (Fig. 4A). On the antisense strand, only a single predominant region gave evidence of protection. This protected region was complementary to a 28-nucleotide stretch on the sense strand in section II that was also protected by FarR (summarized in Fig. 1). Overall, this area of protection is located nearly 200 bp upstream of the previously annotated

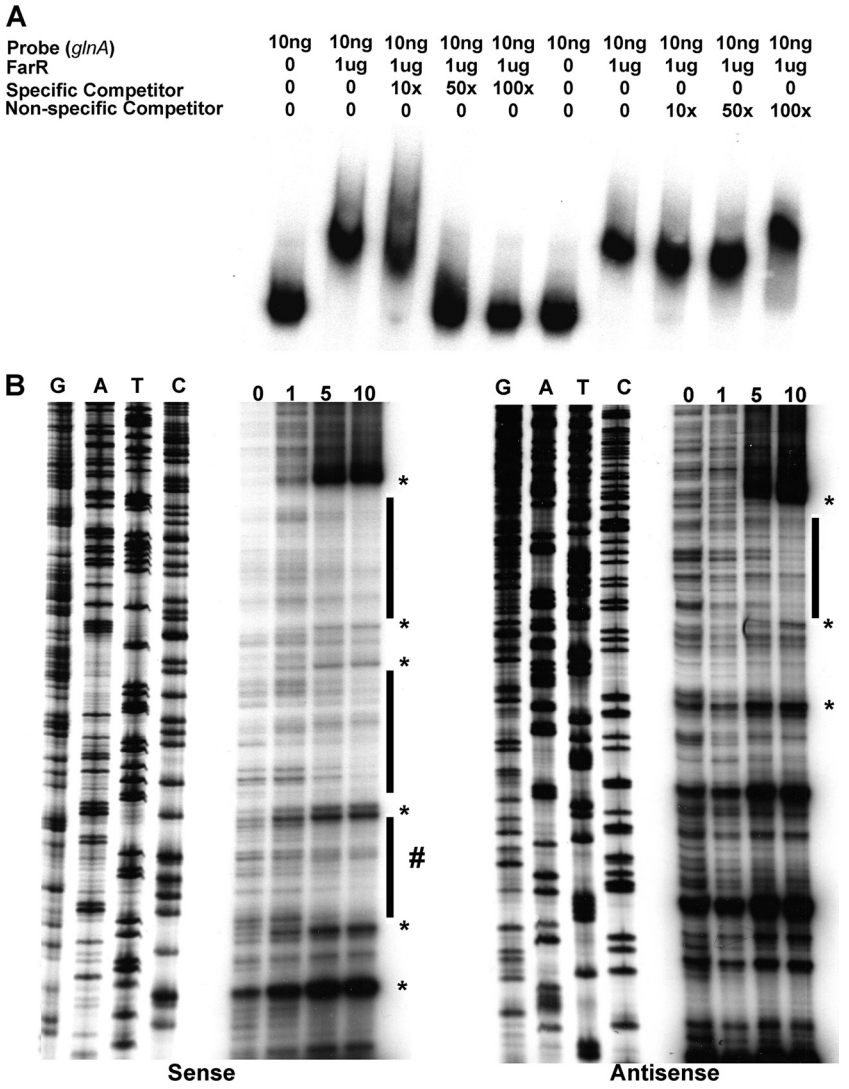

FIG. 4. Identification of the FarR-binding site upstream of the *glnA* promoter. (A) The area of FarR-specific binding was first identified using the subsections of the full-length *glnA* sequence shown in Fig. 1. The full-length probe was PCR amplified using primers 5′pglnA and 3′pglnA, while binding site II was amplified with primers glnAsec2F and glnAsec2R. The nonspecific probe was prepared using primers rmpF and rmpR. FarR was found (data not presented) to bind only section II in a specific manner, as determined by competitive EMSA (shown in the figure). (B) The FarR-binding sites on the sense and antisense strands were identified by DNase I protection assays that employed increasing amounts of purified FarR-His (0, 1, 5, and 10 μg). The protected regions on each probe are identified by the black bars, with the protected region on the sense strand within section II being denoted by a # sign next to the bar to distinguish it from the other two protected regions that lie within section I. DNase I-hypersensitive sites are shown by an asterisk. The sequencing reactions for each probe are adjacent to the DNase I protection reactions and oriented G, A, T, C.

glnA promoter, which we verified (data not presented) by primer extension analysis.

MtrR binding to *glnA* negatively influences FarR binding. We hypothesized that the ability of MtrR to repress *glnA* expression could be due to its ability to impact FarR binding to a target DNA sequence upstream of *glnA*. In order to test if FarR::DNA complexes can be influenced by the presence of MtrR, the target DNA was preincubated with a fixed concentration of one protein and then incubated with increasing concentrations of the second protein. The results (Fig. 5) showed that in the absence of competing protein, MtrR and FarR gave distinct shifts of the probe. However, as the amount of FarR was increased after the DNA had been preincubated with a fixed amount of MtrR (0.1 μg), only the lower of the two FarR::DNA complexes appeared at ≥0.25 μg of competing FarR. Importantly, the electrophoretic mobility of the preformed MtrR::DNA complex showed only minor changes in mobility. In sharp contrast to these results, the pattern of the FarR-specific shifts was significantly changed by the addition of increasing amounts of MtrR. Specifically, the slower-migrating FarR-specific shift was lost and higher-molecular-weight complexes became evident and seemed to predominate, especially at amounts of MtrR of ≥0.25 μg; one of these complexes comigrated with the MtrR-specific shift (see the lane with 0.25 μg of competing MtrR in Fig. 5). The complexes that had a slower electrophoretic mobility than that of the MtrR-specific

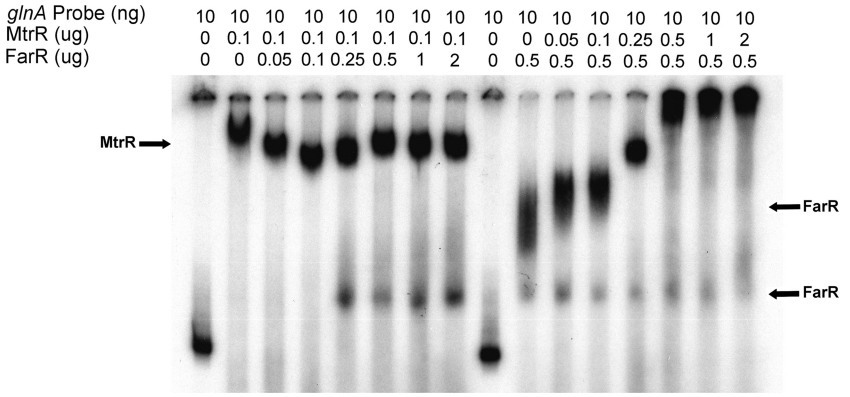

FIG. 5. MtrR influences FarR::DNA complexes. Shown are the results from an EMSA experiment that evaluated the binding of MtrR and FarR to the ^{32}P-labeled full-length *glnA* probe (Fig. 1) that was prepared as described in the legend to Fig. 3. The lane assignments are as follows, from left to right: probe alone; probe plus 0.1 μg of MtrR-MBP; probe plus 0.1 μg of MtrR-MBP and increasing amounts of FarR-His (0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 μg); probe alone; and probe plus 0.5 μg of FarR-His with increasing amounts of MtrR-MBP (0.05, 0.1, 0.25, 0.5, 1.0, and 2.0 μg). The positions of MtrR and FarR shifted bands in the absence of competing protein are shown.

shift likely represent multimers of MtrR bound to the target DNA, as they were observed (data not presented) in other EMSAs that used large amounts of MtrR (≥0.5 μg) but not FarR.

DNA-binding proteins that control efflux gene expression in bacteria have been termed "local regulators" (17), as their encoding gene is frequently located adjacent to their respective target genes that encode efflux pump proteins. While the ability of such regulators to control expression of efflux pump genes is certainly important in modulating levels of bacterial susceptibility to antimicrobials, they may have, on the basis of our work with MtrR described herein and elsewhere (6, 7, 15), as well as work with *Salmonella enterica* serovar Typhimurium (2), a more global action and control expression of genes, which we term off-target, that contribute to important physiological processes. The question addressed herein was whether the model developed for MtrR repression of the *mtrCDE* efflux pump locus, which involves DNA binding to the promoter region (12, 16), would be similar for a model off-target and repressed gene. In sharp contrast to MtrR repression of *mtrCDE* (12, 16), the results presented herein suggest that MtrR represses *glnA* both by its ability to reduce expression of *farR* (15) and by negatively influencing binding of FarR to its target site upstream of *glnA* or decreasing stability of such complexes (Fig. 5), which would normally result in activation of *glnA*. Thus, as summarized in Fig. 6, MtrR can directly repress its target sequence (the *mtrCDE* efflux pump operon [12, 16]) as well as off-target sequences upstream of *farR* (21) and *glnA* (Fig. 2). Conversely, transcriptional activation of *glnA* is mediated by FarR, which directly represses the *farAB* efflux operon (21), by its binding upstream of *glnA* (section II shown in Fig. 6). However, the binding of FarR to this target can be negatively influenced by binding of MtrR upstream of the FarR-binding site (Fig. 5).

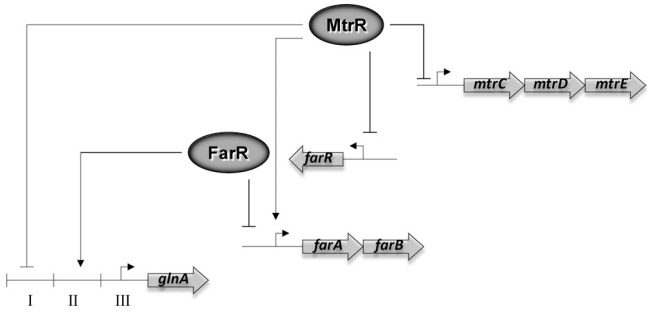

FIG. 6. Model of *glnA* regulation by MtrR and FarR. Direct repression and activation of genes is shown by the solid-barred and arrowed lines, respectively, while indirect activation (i.e., MtrR activation of *farAB*) is show by the dashed, arrowed line. The binding of proteins is shown adjacent to promoter regions (bent arrow). With respect to *glnA*, the binding of MtrR is shown within section I and the binding of FarR is shown within section II of the DNA sequence shown in Fig. 1. The ability of MtrR to repress *glnA* is proposed to be due to its capacity to repress *farR* expression (15) and its ability to diminish FarR binding within section II (Fig. 5).

The ability of a transcriptional regulator in the TetR/QacR family to repress *glnA* is not without precedent, as AmtR of *Corynebacterium glutamicum* was recently shown to negatively control *glnA* expression (3) by a yet-to-be-defined mechanism. Although measurements of glutamine synthetase activity in isogenic variants of strain FA19 bearing wild-type or mutant alleles of *mtrR* or *farR* did not reveal significant differences when such strains were grown in GCB broth lacking glutamine (P. J. T. Johnson and W. M. Shafer, unpublished observations), levels of glutamine at mucosal surfaces and within phagolysosomes (14), two important sites for harboring gonococci during infection (24), are scarce. We hypothesize that regulatory systems involving MtrR which modulate expression of *glnA* and other genes may be important for optimal growth and fitness of gonococci during infection. Accordingly, we are now using a murine model of vaginal infection, which previously revealed the importance of MtrR in determining levels of *in vivo* fitness of gonococci (29), to test the importance of off-target genes regulated by MtrR during infection.

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