

MtrR Modulates *rpoH* Expression and Levels of Antimicrobial Resistance in *Neisseria gonorrhoeae*[∇]

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The MtrR transcriptional-regulatory protein is known to repress transcription of the *mtrCDE* operon, which encodes a multidrug efflux pump possessed by *Neisseria gonorrhoeae* that is important in the ability of gonococci to resist certain hydrophobic antibiotics, detergents, dyes, and host-derived antimicrobials. In order to determine whether MtrR can exert regulatory action on other gonococcal genes, we performed a whole-genome microarray analysis using total RNA extracted from actively growing broth cultures of isogenic MtrR-positive and MtrR-negative gonococci. We determined that, at a minimum, 69 genes are directly or indirectly subject to MtrR control, with 47 being MtrR repressed and 22 being MtrR activated. *rpoH*, which encodes the general stress response sigma factor RpoH (sigma 32), was found by DNA-binding studies to be directly repressed by MtrR, as it was found to bind to a DNA sequence upstream of *rpoH* that included sites within the *rpoH* promoter. MtrR also repressed the expression of certain RpoH-regulated genes, but this regulation was likely indirect and a reflection of MtrR control of *rpoH* expression. Inducible expression of MtrR was found to repress *rpoH* expression and to increase gonococcal susceptibility to hydrogen peroxide (H₂O₂) and an antibiotic (erythromycin) recognized by the MtrC-MtrD-MtrE efflux pump system. We propose that, apart from its ability to control the expression of the *mtrCDE*-encoded efflux pump operon and, as a consequence, levels of gonococcal resistance to host antimicrobials (e.g., antimicrobial peptides) recognized by the efflux pump, the ability of MtrR to regulate the expression levels of *rpoH* and RpoH-regulated genes also modulates levels of gonococcal susceptibility to H₂O₂.

Neisseria gonorrhoeae is a gram-negative strict human pathogen and the causative agent of the sexually transmitted disease gonorrhea. Worldwide, over 60 million cases of gonorrhea occur each year (7, 33). Although the incidence of disease in the United States has decreased since the 1970s, there has since been an increase in antibiotic-resistant strains reported in the United States and elsewhere (25, 33). Indeed, the prevalence of gonococci resistant to penicillin, tetracycline, and/or fluoroquinolones became so significant over the past 25 years that in 2007 the Centers for Disease Control and Prevention added *N. gonorrhoeae* to the list of “superbugs”.

The capacity of gonococci and other bacteria to employ drug efflux pumps to recognize and export antibiotics has attracted considerable attention, as these pumps can impact the efficacy of antibiotic therapy of infections (18, 19, 25, 30, 34, 35). Moreover, since certain efflux pumps recognize host-derived antimicrobial agents, such as antimicrobial peptides (24), there is increasing suspicion that they provide bacteria with a mechanism to escape innate host defenses (13, 21). The gonococcal MtrC-MtrD-MtrE efflux pump can export a variety of antibi-

otics (17, 28), including penicillin (30) and macrolides (35), and antimicrobial peptides, such as human LL-37 (24).

The *mtrCDE* operon is negatively regulated by the MtrR repressor (10, 16, 23), which is encoded by a gene (*mtrR*) immediately upstream of, but transcriptionally divergent from, *mtrCDE* (19). Two homodimers of MtrR bind to a DNA sequence within the *mtrCDE* promoter (12). Point mutations in the MtrR-binding site (16), a single-base-pair deletion within a 13-bp inverted-repeat sequence in the *mtrR* promoter (10), or missense mutations that cause radical amino acid replacements within the helix-turn-helix motif of MtrR all can result in derepression of *mtrCDE* expression (23). Derepression of *mtrCDE* results in decreased antimicrobial susceptibility of gonococci (10, 11, 23) and increased fitness of gonococci in an experimental murine lower genital tract infection model (31).

Consequently, it is important to define the regulatory properties of MtrR. DNA-binding proteins such as MtrR that negatively regulate bacterial efflux pump genes have been considered to be “local” gene regulators, although there is increasing evidence that they can directly or indirectly influence the expression of other genes (6, 15). In order to define the genes in gonococci regulated by MtrR, we employed a microarray analysis of total RNA extracted from mid-logarithmic-phase broth cultures of isogenic MtrR-positive and MtrR-negative gonococci. Through this analysis, we found that MtrR can directly or indirectly regulate 69 genes, which are distributed throughout the genome. We identified 47 MtrR-repressed genes (including the previously described *mtrCDE* operon) and 22

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TABLE 1. Gonococcal strains and plasmids used

Strain or plasmid	Relevant genotype or remarks	Source or reference
Strains		
FA19	Wild type	P. F. Sparling 5
JF1	Like FA19 but $\Delta mtrR$	This study
JF6	Like JF1 but $mtrR^{+a}$	This study
JF7	Like JF1 but $mtrR^{+b}$ $rpoH-lacZ$	This study
JF8	Like FA19 but $rpoH-lacZ$	This study
JF9	Like JF1 but $rpoH-lacZ$	This study
JF10	Like JF6 but $rpoH-lacZ$	This study
JF11	Like FA19 but $grpE-lacZ$	This study
JF12	Like JF1 but $grpE-lacZ$	This study
JF13	Like JF6 but $grpE-lacZ$	This study
JF14	Like FA19 but $rpoH1-lacZ^c$	This study
JF15	Like JF1 but $rpoH1-lacZ^c$	This study
JF16	Like FA19 but $mtrR-lacZ$	This study
Plasmids		
pLES94	Cloning vector containing promoterless <i>lacZ</i> for insertion of gonococcal genes between <i>proA</i> and <i>proB</i> (27)	V. Clark
pGCC3	NICS vector (25) used for insertion of <i>mtrR</i> between <i>lctP</i> and <i>aspC</i>	H. Seifert
pGCC4	Same as pGCC3 with IPTG-inducible <i>lacZ</i> promoter	H. Seifert
pJF1	Like pGCC3 but $mtrR^{+}$	This study
pJF2	Like pGCC4 but $mtrR^{+}$	This study

^a *mtrR* from pJF1 is under the control of its own promoter in this strain.

^b *mtrR* from pJF2 is under the control of the *lacZ* promoter in this strain.

^c This fusion lacks the 172-bp *rpoH* leader sequence.

MtrR-activated genes. An MtrR-repressed gene of particular interest, which was the subject of this investigation, was *rpoH*. *rpoH* encodes the alternative stress response sigma factor RpoH (sigma 32), which appears to be essential for gonococcal viability even under normal growth conditions (3, 8). Earlier studies showed that *rpoH* expression is increased during exposure of gonococci to certain stress conditions, such as growth at elevated temperatures (8), and during bacterial contact with cultured epithelial cells (2, 3). Moreover, certain RpoH-regulated genes are differentially expressed during exposure of gonococci to H₂O₂ (29). Thus, apart from its regulation of *mtrCDE* and levels of gonococcal resistance to hydrophobic antimicrobial agents (20, 21, 24, 30), MtrR regulation of *rpoH* expression may have significance for gonococcal survival in vivo, particularly at sites rich in neutrophils producing large quantities of H₂O₂.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strain TOP10 (Invitrogen, Carlsbad, CA) or strain DH5 α mcr was 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 (22). *N. gonorrhoeae* strains were grown on gonococcal medium base (GCB) agar (Difco Laboratories, Detroit, MI) containing glucose and iron supplements (22) at 37°C under 3.8% (vol/vol) CO₂ or in GCB broth with supplements and sodium bicarbonate as described previously (22). The plasmids and oligonucleotide primers used in this investigation are listed in Tables 1 and 2. All chemicals were purchased from Sigma Biochemical (St. Louis, MO).

RNA isolation. RNA was isolated from 50-ml GCB broth cultures of strains FA19 and JF1 (the same as FA19 but with *mtrR* deleted [$\Delta mtrR$] [5]) grown to mid-log phase using a hot-phenol method as previously described (4). Samples were then DNase treated on column (Qiagen DNase kit), and the RNA was recovered using the Qiagen RNeasy Mini kit. RNA was quantified by NanoDrop1000 (NanoDrop Technologies), and the RNA integrity was analyzed on an

Agilent bioanalyzer (Agilent Technologies). The RNA samples were stored at -80°C until further use.

Microarray design. A custom multipathogen Affymetrix GeneChip, MPAUT1a520274F, was utilized for the microarray experiments in the present study. Probe pairs were designed for each gene, consisting of 11 probe pairs (22 features) with a perfect match and a single-nucleotide mismatch. The *N. gonorrhoeae* genome probe set (1,925 genes) was represented on the array.

TABLE 2. Oligonucleotides used

Oligonucleotide primer name	Sequence (5'→3')
5'PrpoH	GGGATCCGGCGAAACGCCCTAT ATGAA
3'PrpoHB	GGGATCCCCGATTCATTTGGGCA TTTCCTTT
5'ΔPrpoH	GGACAGGATGAGTTGTTTGG
3'ΔPrpoH	TATCGGCGGTTGTAAACCTGAT AGCTCAATTC
3'PrpoHB	GGGATCCCCGATTCATTTGGGCA TTTCCTTT
5'ΔPrpoH	GGACAGGATGAGTTGTTTGG
3'ΔPrpoH	TATCGGCGGTTGTAAACCTGAT AGCTCAATTC
5'mtrR	GGTTAATTAACCGCCCTCGTCAA ACCGA
3'mtrR	GGGTTTAAACTTATTTCGGGCGC AGGCAG
mtrC1	CGGGATCCCCGAGCCATTATTTAT CCTATCTGTC
pmtrR	GGATCCTCTCATAATGGCGTTTT CGTTTCG
5'PgrpE	GGGGATCCACACGGTTTGGTGC AAAAAAC
3'PgrpE	GGGGATCCGGCTCATATCGTAT CCCTCAA

cDNA labeling and hybridization. Sample preparation and hybridization were done at the University of Iowa DNA Facility (<http://dna-9.int.med.uiowa.edu/>) according to the Affymetrix Genechip Expression Analysis technical manual prokaryotic protocol. Briefly, biotinylated cDNA was generated from 10 μ g of total RNA and fragmented prior to hybridization to the custom chip at 45°C for 16 h. The arrays were stained using streptavidin-phycoerythrin (ProkGE-WS2v3_450 protocol; Affymetrix, Inc.), and the scanned images (Affymetrix GeneChip Scanner 3000) were processed using Genechip Operating Software version 1.4 (Affymetrix, Inc.). Hybridizations were done on three separate biological replicates of each isolate.

Array analysis. Data files generated by Genechip Operating Software (Affymetrix, Inc.) were imported into GeneSpring GX 7.3.1 (Agilent Technologies) and normalized per chip by dividing each measurement by the 50th percentile of all the measurements in that sample and per gene by dividing each gene by the median of its measurements across all samples. The cross-gene error model was operating based on replicates. The normalized data from all samples were filtered on genes flagged as present or marginal, with the resulting gene list used for further gene expression analysis and clustering. Change was expressed as the ratio of gene expression of the wild-type FA19 over the same gene expression for the mutant. Genes with a differential expression of ≥ 1.5 -fold and a *P* value of 0.05 were selected.

Construction of *mtrR*-complemented strains, inducible expression of *mtrR*, detection of MtrR, and antimicrobial susceptibility in induced cultures. The construction of pJF1, which contains the wild-type *mtrR* coding sequence and promoter sequence from strain FA19 cloned into pGCC3 (27), and strain JF6 (like JF1 but with this *mtrR* sequence, including its promoter, located between *aspC* and *lctP*) has been described by Folster and Shafer (5). In order to engineer an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible copy of *mtrR*, the *mtrR* coding sequence with its ribosome binding site was PCR amplified from chromosomal DNA prepared from wild-type strain FA19 using primers 5'*mtrR* and 3'*mtrR* (Table 2). The resulting DNA fragment was inserted into the PmeI and PacI sites of pGCC4, which contains the *lacZ* promoter (27) (kindly provided by A. Jerse and H. Seifert) to produce pJF2, and the correct orientation and nucleotide sequence were confirmed by DNA sequencing. pJF2 was digested with ClaI, and the fragment containing the gonococcal *lctP* gene, *mtrR*, *ermC* (an erythromycin [Ery] resistance cassette), and *aspC* was purified and used to transform gonococcal strain FA19 Δ *mtrR*. Transformations were performed as previously described (9). Transformants were selected on GCB agar containing 1 μ g/ml of Ery, and the resulting strain was named JF7 (like JF1, but *mtrR*⁺).

For inducible expression of *mtrR*, strain JF7 was grown on GCB agar as described above. The overnight growth was then used to inoculate 50 ml of GCB broth, and the culture was grown at 37°C with shaking (200 rpm) until the optical density at 600 nm reached 0.2, at which time it was split into two equal samples. One sample received IPTG (1 mM), while the other received an equal volume of sterile distilled water. These cultures were grown for 1 h, and samples were then harvested by centrifugation. MtrR was detected in solubilized cell extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting as described previously (6). MtrR was detected using a rabbit anti-MtrR antiserum (1:5,000 dilution) prepared by Protein Tech Group, Inc. (Chicago, IL), with purified MtrR fused to maltose-binding protein (MBP) (15) as the antigen and was visualized on the blot using goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase (1:5,000 dilution). IPTG-induced and control samples were also evaluated for their susceptibility to Ery (0.5 μ g/ml) by an efficiency-of-plating assay described previously (22), using agar plates with or without 1 mM IPTG. H₂O₂ susceptibility was determined using a disk diffusion assay containing filter disks (1 cm) presoaked in 3% (vol/vol) H₂O₂ that were placed on freshly inoculated GCB agar plates that contained or lacked 1 mM IPTG.

Construction and analysis of *rpoH-lacZ*, *grpE-lacZ*, and *mtrR-lacZ* fusions. Translational *lacZ* fusions were constructed as previously described (5, 6). In brief, the promoter sequence of *rpoH* was amplified by PCR from strain FA19 using primers 5'PrpoH and 3'PrpoHB (Table 2). The resulting DNA fragment was inserted into the BamHI site of pLES94 (26), and the recombinant plasmid was introduced into *E. coli* DH5 α mcr by transformation. The correct orientation of the insertion was confirmed by PCR analysis and DNA sequencing. The plasmid was used to transform strains FA19, JF1, and JF6 to allow insertion into the chromosomal *proAB* gene, and transformants were selected on GCB agar containing 1 μ g/ml of chloramphenicol. Representative transformants obtained with the three recipient strains were termed JF8, JF9, and JF10, respectively (Table 1). Strains bearing a *grpE-lacZ* fusion were prepared essentially as described above, but the oligonucleotide primers 5'PgrpE and 3'PgrpE (Table 2) were used to PCR amplify a 300-bp fragment encompassing the upstream sequence of *grpE* and the first two codons. The strains bearing the *grpE-lacZ* fusion

were termed JF11 (FA19 *grpE-lacZ*), JF12 (FA19 Δ *mtrR* *grpE-lacZ*), and JF13 (FA19 Δ *mtrR* *mtrR*⁺ *grpE-lacZ*), respectively (Table 1). For construction of a *mtrR-lacZ* fusion in strain FA19, oligonucleotide primers mtrC1 and pmtrR (Table 2) were used to PCR amplify *mtrR*; the strain bearing the *mtrR-lacZ* fusion was termed JF16 (FA19 *mtrR-lacZ*). β -Galactosidase (β -Gal) assays were conducted as previously described (5, 6).

Deletion of the *rpoH* extended leader sequence. PCR mutagenesis was used to delete the 172-bp *rpoH* leader sequence and was performed using the overlapping primers 5' Δ PrpoH and 3' Δ PrpoH (Table 2). First, two fragments were amplified by PCR from FA19 chromosomal DNA using the primer set pairs 5'PrpoH/3' Δ PrpoH and 3'PrpoHB/5' Δ PrpoH. The resultant fragments were purified after agarose gel electrophoresis using the Qiaquick purification kit (Qiagen Inc., Valencia, CA), and they then served as both primers and templates for a second PCR. After eight reaction cycles, primers 5'PrpoH and 3'PrpoHB were added to the PCR mixture, and amplification continued for an additional 25 cycles. The resulting DNA fragment containing the deletion was purified and served as the template for the last PCR using primers 5'PrpoH and 3'PrpoHB. The resulting DNA fragment was inserted into the BamHI site of pLES94, resulting in the *rpoH* Δ -*lacZ* construct. The recombinant plasmid was introduced into DH5 α mcr by transformation. Correct insertion and orientation were confirmed by PCR analysis, and DNA-sequencing analysis confirmed the mutation of the MtrR-binding site (data not presented). The plasmid was used to transform strains FA19 and JF1 to allow insertion into the chromosomal *proAB* gene. Transformants were selected on GCB agar containing 1 μ g/ml of chloramphenicol; representative transformants obtained with recipient strains FA19 and JF1 were termed JF14 and JF15, respectively (Table 1).

EMSA and DNase I protection. Electrophoretic mobility shift assays (EMSAs) using purified MBP fused to MtrR were performed as previously described (5, 6, 15). In brief, the 184-bp promoter region of *rpoH* lacking the leader sequence was amplified by PCR using 5'PrpoH and 3'PrpoHB from strain JF14 (Table 1). The 184-bp nonspecific probe was PCR amplified from FA19 chromosomal DNA using primers 5' Δ PrpoH and 3'PrpoHB (Table 2). The resulting PCR products were end labeled with [γ -³²P]dATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The labeled DNA fragments (10 ng) were incubated with purified MtrR in 30 μ l of reaction buffer [10 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.5 mM EDTA, 4% (vol/vol) glycerol, 1 mM MgCl₂, 50 mM NaCl, poly(dI-dC) (0.05 μ g/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.

DNase I protection assays were performed as previously described (16). Target DNA sequences for DNase I footprints were generated by PCR using the oligonucleotide primers 5'PrpoH and 3'PrpoHB (Table 2) to generate a 184-bp promoter region of *rpoH* lacking the leader sequence from strain JF14. Target DNA was labeled at the 5' end of one strand as described for EMSA. Purified MBP-MtrR protein was allowed to bind in the binding buffer as in the EMSA before CaCl₂ and MgCl₂ were added to final concentrations of 2.5 mM and 5 mM, respectively. Five units of DNase I (Promega, Madison, WI) were then added to the reaction mixture, which was incubated at room temperature for 1 min. Digestion was stopped by the addition of NaCl to 250 mM, and the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) before precipitation with 100% ethanol for 30 min at -20°C. The pellet was washed four times with 70% (vol/vol) ethanol, vacuum dried, and resuspended in gel loading buffer, which consisted of 0.1 M NaOH-formamide (1:2 [vol/vol]), 0.1% (vol/vol) xylene cyanol, and 0.1% (vol/vol) bromophenol blue. Regions protected from DNase I digestion were resolved on a 6% denaturing polyacrylamide gel, which was dried and exposed to X-ray film for autoradiography.

Microarray data expression number. Gene expression data for all microarray experiments can be retrieved from the Gene Expression Omnibus (GEO) database at NCBI (<http://www.ncbi.nih.gov/geo/>) (accession number GSE12686).

RESULTS AND DISCUSSION

Identification of MtrR-regulated genes in gonococci. To identify MtrR-regulated genes in *N. gonorrhoeae*, a microarray analysis was performed that permitted a comparison of gonococcal transcript levels in total RNA prepared from mid-logarithmic-phase cultures of wild-type strain FA19 and its isogenic *mtrR* deletion mutant, strain JF1 (Table 1). Using an expression difference of 1.5-fold (*P* \leq 0.05), analysis of the microarray results allowed us to establish an MtrR regulon that at a

TABLE 3. MtrR-regulated genes^a in *N. gonorrhoeae*

Gene	Common name	Change (fold)	Functional classification
MtrR repressed			
NGO0006	<i>leuS</i>	1.59	Leucyl-tRNA synthetase
NGO0018	NGO0018	1.65	Unknown
NGO0195	<i>potH</i>	1.56	Putative ABC-type polyamine transporter
NGO0288	<i>rpoH</i>	1.58	Alternative sigma factor
NGO0302	NGO0302	1.98	Unknown
NGO0308	<i>bioA</i>	1.59	Putative aminotransferase
NGO0309	<i>bioD</i>	1.53	Dithiobiotin synthetase
NGO0658	<i>pdxH</i>	1.51	Pyrodoxamine 5'-phosphate oxidase
NGO0756	NGO0756	1.53	Unknown
NGO0795	<i>brfB</i>	1.98	Bacterioferritin B
NGO0846	<i>umpA</i>	1.54	Putative prolipoprotein diacylglycerol transferase
NGO0863	NGO0863	1.71	Unknown
NGO0891	NGO0891	1.92	Unknown
NGO0916	<i>accB</i>	1.58	Dihydrolipoamide acetyltransferase
NGO0927	NGO0927	1.51	Unknown
NGO1002	NGO1002	1.54	Unknown (putative phage associated)
NGO1029	<i>fumC</i>	1.57	Fumarate hydratase
NGO1046	<i>clpB</i>	2.10	Heat shock chaperone
NGO1069	NGO1069	1.63	Unknown
NGO1084	NGO1084	1.75	Unknown
NGO1189	<i>hsp33</i>	2.24	Heat shock chaperonin
NGO1244	NGO1244	1.52	MarR family regulator
NGO1293	NGO1293	2.11	Unknown
NGO1294	<i>ltp</i>	1.77	AsnC family transcriptional regulator
NGO1314	NGO1314	1.80	Putative protease
NGO1325	<i>gcvP</i>	2.35	Glycine dehydrogenase
NGO1363	<i>mtrE</i>	2.20	Mtr efflux pump protein component
NGO1364	<i>mtrD</i>	2.40	Mtr efflux pump protein component
NGO1365	<i>mtrC</i>	1.78	Mtr efflux pump protein component
NGO1368	<i>mtrF</i>	2.21	Mtr efflux pump protein component
NGO1418	<i>nqrF</i>	1.74	Subunit F, NADH-quinone reductase
NGO1422	<i>grpE</i>	1.83	Heat shock protein
NGO1494	<i>potF</i>	1.58	Putative periplasmic polyamine binding protein
NGO1520	NGO1520	1.55	Unknown
NGO1600	<i>glnA</i>	1.63	Glutamine synthetase
NGO1699	NGO1699	1.56	Unknown
NGO1738	<i>nuoM</i>	1.52	NADH dehydrogenase subunit M
NGO1741	<i>nuoK</i>	1.80	NADH dehydrogenase kappa subunit
NGO1742	<i>nuoJ</i>	1.56	NADH dehydrogenase I chain J
NGO1749	<i>nuoC</i>	1.57	NADH dehydrogenase subunit C
NGO1751	<i>nuoA</i>	1.78	Putative NADH dehydrogenase I chain A
NGO1765	<i>pglA</i>	1.63	Glycosyltransferase
NGO1890	<i>gltS</i>	1.66	Putative glutamate permease
NGO2011	<i>glnM</i>	1.55	Putative ABC-type polyamine transporter
NGO2013	<i>glnQ</i>	1.75	Putative ATP-binding protein
NGO2014	<i>cjaA</i>	2.00	Amino acid periplasmic binding protein
MtrR activated			
NGO0113	<i>mg</i>	1.57	Putative RNase G/cytoplasmic axial filament protein
NGO0150	NGO0150	1.61	Unknown
NGO0290	NGO0290	1.57	Unknown
NGO0481	NGO0481	1.85	Unknown (putative phage associated)
NGO0739	NGO0739	1.62	Putative DNA helicase
NGO0876	NGO0876	1.83	Unknown
NGO1024	NGO1024	1.57	Unknown
NGO1059	NGO1059	1.77	Unknown
NGO1062	NGO1062	1.71	Unknown
NGO1097	NGO1097	1.70	Unknown (phage associated)
NGO1420	<i>abpE</i>	1.65	Thiamine biosynthesis
NGO1460	NGO1460	1.72	Unknown
NGO1488	NGO1488	1.53	Unknown
NGO1511	NGO1511	1.67	Unknown
NGO1722	<i>recQ</i>	1.67	Recombination
NGO1728	NGO1728	1.60	Unknown
NGO1758	<i>glnE</i>	7.04	Glutamine synthetase adenylyltransferase
NGO1759	NGO1759	148.70	Unknown
NGO1760	NGO1760	1.73	Unknown
NGO1874	NGO1874	1.58	Unknown
NGO1897	<i>rfbB</i>	1.73	dDTP-D-glucose-4,6 dehydratase
NGO2135	NGO2135	1.63	Putative transglycosylase

^a Genes of interest to this investigation are highlighted in boldface.

minimum consists of 47 MtrR-repressed and 22 MtrR-activated genes (Table 3); these MtrR-regulated genes were distributed throughout the genome (Fig. 1). Four previously identified MtrR-repressed genes (*mtrC*, *mtrD*, *mtrE*, and *mtrF*) were confirmed as being MtrR repressed by the array, which

validated the array as a means to identify MtrR-regulated genes. Based on the annotation of the FA1090 genome sequence (GenBank accession no. AE004969), the majority of MtrR-regulated genes were predicted to encode hypothetical proteins, but several genes encoding proteins with known or

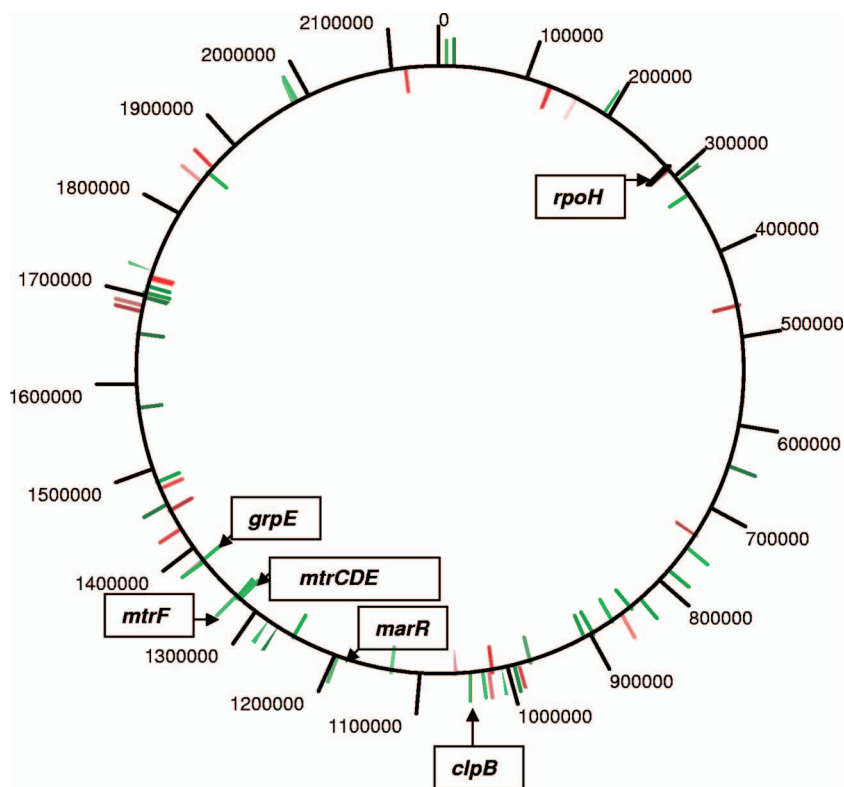


FIG. 1. Chromosomal-map positions of MtrR-regulated genes. The positions of MtrR-regulated genes in strain FA19 that were identified by the microarray analysis (Table 3) are shown on the circular map of the gonococcal chromosome (strain FA1090). MtrR-activated genes are shown in red, while MtrR-repressed genes are shown in green.

putative functions were identified (Table 3). In particular, we noted that expression of *rpoH*, which encodes the stress-related sigma factor RpoH, and some RpoH-regulated genes (*grpE*, *clpB*, and *marR* [8, 29]) were increased in strain JF1 relative to the MtrR-positive parental strain FA19, suggesting that they are MtrR repressed. Since RpoH expression in gonococci is increased during exposure to certain environmental stresses (8) when gonococci bind to cervical epithelial cells (2, 3) and is essential for viability (3, 8), we studied the ability of MtrR to regulate *rpoH* expression in *N. gonorrhoeae*.

Expression of *rpoH* and certain RpoH-regulated genes is repressed by MtrR. To study the capacity of MtrR to regulate the expression of *rpoH*, a translational *lacZ* reporter fusion system was engineered. This translational fusion consisted of 365 bp of DNA sequence upstream of *rpoH*, which included the promoter region (14), the first two codons of *rpoH* (Fig. 2), and a promoterless *lacZ* gene. This construct was transformed into isogenic strains FA19 and JF1 to create the transformant strains JF8 (FA19 *rpoH-lacZ*) and JF9 (FA19 Δ *mtrR rpoH-lacZ*), respectively, resulting in a single copy of the *rpoH* promoter fused translationally to *lacZ* within the *proAB* chromosomal locus. β -Gal activity in cell lysates from these strains indicated that expression of *rpoH* increased more than eightfold in the MtrR-negative strain JF9 compared to the MtrR-positive strain JF8 (FA19 *rpoH-lacZ*) (Fig. 3A). To confirm that these results were due to deletion of *mtrR* and not a polar effect on other genes, strain JF9 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 strain JF10 (FA19 Δ *mtrR mtrR*⁺ *rpoH-lacZ*) (Table 1). Using this strain, we found that complementation of the *mtrR* deletion in JF9 (FA19 Δ *mtrR rpoH-lacZ*) with the wild-type *mtrR* gene restored *rpoH* repression to a level similar to that of JF8 (like FA19, but *rpoH-lacZ*) (Fig. 3A).

The sequence of the gonococcal *rpoH* promoter, as well as the transcriptional start point and ribosome binding site, were defined previously (8) (Fig. 2). This region contains an extended 172-bp RNA leader sequence, which was previously observed to be involved in *rpoH* repression during growth under normal conditions (14). To determine if the leader sequence was involved in MtrR repression of *rpoH*, an internal deletion was engineered within the *rpoH-lacZ* construct and transformed into strains FA19 and JF1 (FA19 Δ *mtrR*) to create strains JF14 (FA19 *rpoH-lacZ*) and JF15 (FA19 Δ *mtrR rpoH-lacZ*) (Table 1), respectively, and expression was measured. We found that *rpoH-lacZ* expression from the wild-type promoter and the promoter with the leader deleted did not differ in either the MtrR-positive (JF14) or MtrR-negative (JF15) background (Fig. 3B), indicating that MtrR-mediated repression of *rpoH* is independent of the leader sequence.

Gunsekere et al. (8) identified a panel of RpoH-regulated genes in gonococci, and Stohl et al. (29) showed that some of these were upregulated during bacterial exposure to H₂O₂. Since our microarray analysis (Table 3) suggested that a subset of these RpoH-regulated genes (*clpB*, *grpE*, and *marR*) is also

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GGCGAAACGCCCTATATGAAAACCGGCAGTTCATGGTGGTTGATGGGCATATTGACCCTAGCCGCACTGAT
CCGCTTTGCGGGATATACTTTTGGCCGTCAGTACCACCAACTACCCGTATAAAGTGGGATCGGCGTGACTA

TCTTTTCATCTTCCGAAACAAAGAACACTGATACCTGCACAGTCCGTCGGGACGGCTGTCTTTTTTCAA
AGAAAAGTAGAAGGCTTTGTTTCTGTGACTATGGACGTGTCAGGCAGGCTGCCCGACAGAAAAAGTTT

          -35          -10
CCGGCCGTTTACATACAACCACGTATGTAATATAATTCTAAAATCGAATTGAGCTATCAGGTTTACAAAAT
GGCCGGCAAATGTATGTTGGTGCATACATTATATTAAGATTTTAGCTTAACTCGATAGTCCAAATGTTTAA

CAATCAAACCTTGCCTTACAAAATTTCTGTCTTTCAAACCTTAGCGTATATCTCCTGCCAACATACAAA
GTTAGTTTGAACGGAATGTTTTAAAGGACAGAAAAGTTTGGAAATCGCATATAGAGGACGGTTTGTATGTTT
-----
RBS
ATACAAGCCAAGCAGTGATATACTGCATCCCAACACACCAAACCGCCGATACGCAGTTTCAGCCGAAAGGA
TATGTTTCGGTTCGTCACTATATGACGTAGGGTTGTGTGGTTTGGCGGCTATGCGTCAAAGTCGGCTTTCCT
-----

AATGCCCAAATGAAT
TACGGGGTTTACTTA
      M N

```

FIG. 2. Nucleotide sequence upstream of *rpoH* and identification of the MtrR-binding site. The nucleotide sequence of the DNA upstream of *rpoH* is shown with the 172-bp leader sequence indicated by the dashed lines; the first two codons encoding methionine (M) and asparagine (N) are also shown in boldface. The position of the transcriptional start point (8) is shown by the boldface A nucleotide downstream of the -10 sequence. The -10 and -35 sequences of the *rpoH* promoter identified previously (8) are shown with the -10 and -35 hexamers and are highlighted by a single line under the sequence. The 4-bp ribosome binding site (RBS) sequence is shown by an underline. The MtrR-binding site identified by DNase I protection (Fig. 6) is shown by the wide bars above the coding strand and below the noncoding strand. The boxed region shows a 15-nucleotide sequence on the coding strand (5'-GGC[-]CGTTTACATACA-3') that is 73.3% identical (the identical nucleotides are boldface in above sequence) to the MtrR-binding sequence (5'-GGCACGTTAGCACATA-3') upstream of *mtrCDE* on the noncoding strand (12); the hyphen in the sequence above represents a single-nucleotide gap in the *rpoH* sequence compared to the *mtrCDE* bound by MtrR.

subject to MtrR regulation, we examined the ability of MtrR to control the expression of RpoH-regulated genes in gonococci and used *grpE* for this purpose. We examined its expression in isogenic MtrR-positive and MtrR-negative gonococci utilizing a *grpE-lacZ* construct introduced into strains FA19, JF1 (FA19 $\Delta mtrR$), and JF6 (FA19 $\Delta mtrR mtrR^+$); the transformed strains were termed JF11, JF12, and JF13, respectively (Table 1). The results indicated that expression of *grpE* (Fig. 4) was elevated

in the absence of MtrR (see strain JF12) and restored to near-wild-type levels (strain JF11) by complementation with the intact *mtrR* gene (strain JF13).

As expression of RpoH-regulated *grpE* is induced during exposure of gonococci to low levels of H₂O₂ (29), we next evaluated whether expression of *mtrR*, *rpoH*, and *grpE* was altered when gonococci were exposed to the oxidizing agent. Using the *lacZ* fusion strains JF8 (FA19 *rpoH-lacZ*), JF11

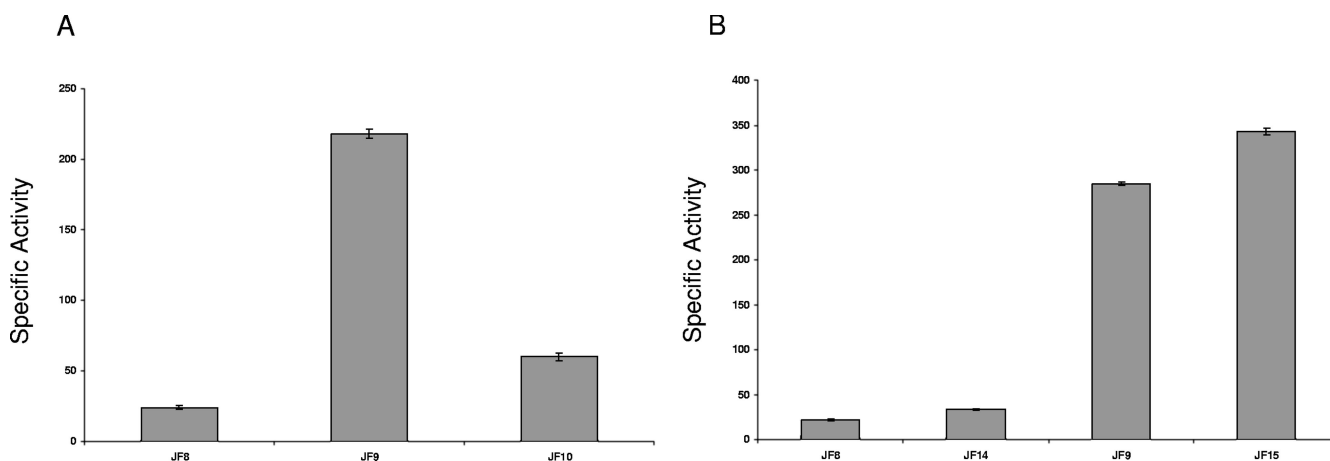


FIG. 3. MtrR regulation of *rpoH* expression. (A) Levels of β -Gal activity (specific activity expressed as nanomoles of *o*-nitrophenyl- β -D-galactopyranoside hydrolyzed per mg of protein) in strains JF8 (like FA19, but *rpoH-lacZ*), JF9 (like JF1, but *rpoH-lacZ*), and JF10 (like JF6 [Table 1], but *rpoH-lacZ*). Complementation of the *mtrR* deletion mutation resulted in reduced *rpoH* expression. The results are shown as average values (\pm standard deviations) from three independent assays. The difference in β -Gal activities between strains JF8 and JF9 was significant ($P < 0.001$), as was that between JF9 and JF10 ($P < 0.001$). (B) MtrR repression of *rpoH* was independent of the 172-bp leader sequence upstream of *rpoH*, as shown by similar levels of β -Gal production between isogenic pairs (MtrR-positive strains JF8 and JF14 and MtrR-negative strains JF9 and JF15) with (strains JF8 and JF9) or without (JF14 and JF15) the 172-bp leader sequence. The differences in β -Gal activities between strains JF8 and JF14 and between JF9 and JF15 were not statistically significant.

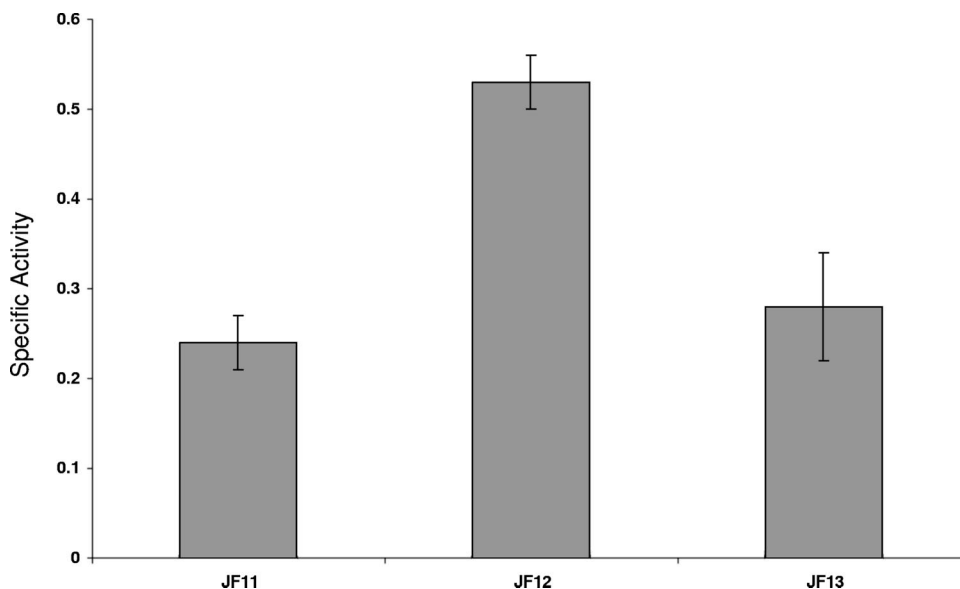


FIG. 4. MtrR regulation of the RpoH-regulated *grpE* gene. Shown are the β -Gal-specific activities expressed in *grpE-lacZ* fusion strains JF11 (MtrR positive) and JF12 (MtrR negative) and the *mtrR*⁺ complemented strain JF13. The results are shown as average values (\pm standard deviations) from three independent assays. The differences between strains JF11 and JF12 and between JF12 and JF13 were significant ($P < 0.001$ and 0.003, respectively).

(FA19 *grpE-lacZ*), and JF16 (FA19 *mtrR-lacZ*) (Table 1), we monitored the expression of *rpoH*, *grpE*, and *mtrR* during a brief (15-min) exposure of gonococci to 1.5 mM H₂O₂; this concentration of H₂O₂ did not reduce viability (data not presented). As is shown in Fig. 5, we found that under these conditions, expression of both *rpoH* and *grpE* was increased significantly ($P < 0.0001$ and 0.002, respectively), while *mtrR* expression was slightly but significantly ($P = 0.049$) dampened. In order to determine if H₂O₂ induction of *rpoH* expression would be enhanced in the absence of MtrR, we next compared its expression when strains JF8 (FA19 *rpoH-lacZ*) and JF9 (like JF8, but Δ *mtrR*) were exposed to 1.0 mM H₂O₂ for 15 min.

Although *rpoH* expression was nearly 15-fold greater in strain JF9 than in JF8, the changes in expression after H₂O₂ exposure were nearly identical (data not presented). We propose that although MtrR can control constitutive levels of *rpoH* and *grpE* expression, it is not necessarily involved in their H₂O₂-inducible expression. Alternatively, under the experimental conditions employed, *rpoH* expression in JF9 reached maximal levels, and induction above the observed level was not possible or detectable.

MtrR directly binds to the *rpoH* promoter region. Initial EMSA experiments showed that MtrR bound to the DNA sequence upstream of *rpoH* encompassing its promoter region in a specific, concentration-dependent manner and did not

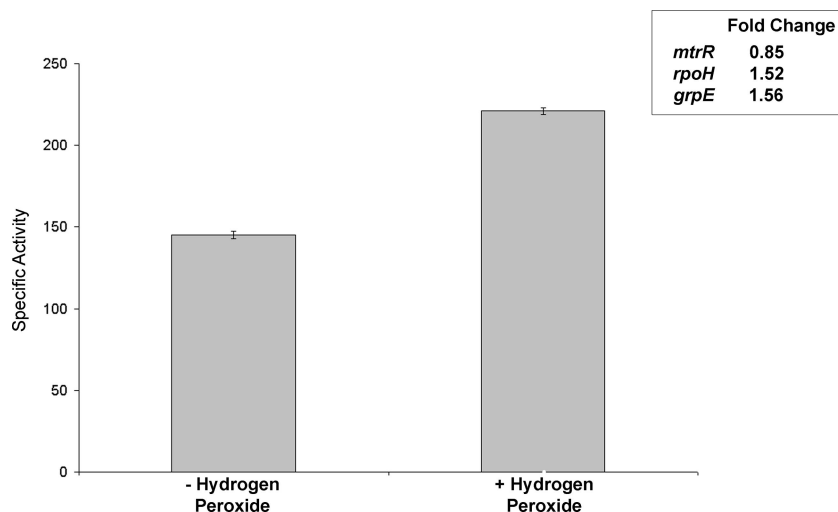


FIG. 5. H₂O₂ induction of *rpoH* expression. Shown in the graph are the β -Gal-specific activities in *rpoH-lacZ* fusion strain JF8 after a 15-min exposure to 1 mM H₂O₂. The results are shown as average values (\pm standard deviations) from three independent assays; the difference was significant ($P < 0.00001$). The inset shows the changes in expression for *mtrR*, *rpoH*, and *grpE* when fusion strains were exposed to H₂O₂.

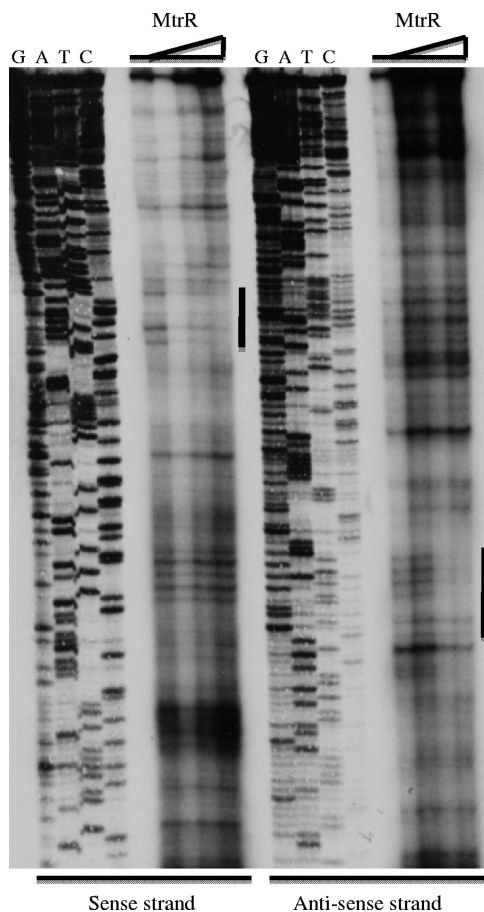


FIG. 6. Identification of the MtrR-binding site within the *rpoH* promoter. The MtrR-binding site (see the nucleotide sequences in Fig. 2 and its legend) was identified by DNase I protection using increasing amounts of MtrR (0, 5, 10, and 20 μg) in assays that employed sense and antisense probes. The site of protection on each strand is shown by the vertical bar adjacent to the protected region. The nucleotide-sequencing reaction for each strand is shown (G, A, T, C) adjacent to the lanes with the protection reactions.

require the 172-bp leader sequence (data not presented). In order to determine if MtrR directly or indirectly regulates the RpoH-regulated *grpE* gene, an EMSA was performed using MtrR and a 305-bp probe containing the upstream DNA (including a putative promoter element) of the *grpE* coding sequence. Unlike its binding to the DNA sequence upstream of *mtrC* and *rpoH*, MtrR failed to bind the *grpE* target DNA sequence (data not presented). Accordingly, we believe that the ability of MtrR to regulate *grpE* is indirect and due to its capacity to repress *rpoH*.

In order to identify the MtrR-binding site within the *rpoH* promoter region, a DNase I protection assay was performed. The *rpoH* promoter probe described above was radiolabeled separately at its 5' and 3' ends and utilized in DNase I protection assays that employed increasing amounts (0 to 20 μg) of MtrR (Fig. 6). With increasing amounts of MtrR, an area of protection of seventeen nucleotides on the sense strand (Fig. 2) could be identified as being involved in binding MtrR. A second area of protection of sixteen nucleotides was observed on the antisense strand (Fig. 2), which overlapped the area

observed on the sense strand. The region of protection indicates that MtrR binds to the *rpoH* promoter at an area that overlaps the -35 hexamer sequence of the *rpoH* promoter. Using the nucleotide sequence of the MtrR-binding site on the noncoding strand within the *mtrCDE* promoter (5'-GGCACGTTAGCACATA-3') defined by Hoffman et al. (12), we were able to identify a region of similarity (73.3% identity) (Fig. 2) within the *rpoH* promoter region, which was also found by DNase I protection to bind MtrR.

Modulation of MtrR expression and levels of antimicrobial resistance. In order to further connect MtrR expression with levels of *rpoH* expression and to assess the biologic consequence of MtrR gene control in gonococci, we next inserted an IPTG-inducible copy of the wild-type *mtrR* gene from strain FA19 into its transformant strain JF9 (FA19 $\Delta mtrR$ *rpoH-lacZ*) to create strain JF7 (FA19 $\Delta mtrR$ *mtrR*⁺ *rpoH-lacZ*). Strain JF7 was grown in GCB broth to mid-log phase and split into two samples, one receiving 1 mM IPTG and one left untreated. After 1 h of induction, MtrR was clearly produced by the IPTG-treated culture (Fig. 7B), and *rpoH* expression was decreased by nearly twofold (Fig. 7C) compared to the untreated sample. In order to determine whether induction of *mtrR* expression resulted in biological changes of gonococci, we next examined the susceptibility of the IPTG-treated and control cultures to Ery and H₂O₂. Ery susceptibility analysis was performed because MtrR repression of the *mtrCDE*-encoded efflux pump increases gonococcal susceptibility to this antibiotic and other hydrophobic antimicrobial agents (10, 11, 19). In support of this, we found that inducible expression of *mtrR* resulted in increased susceptibility of gonococci to Ery (Fig. 7D). H₂O₂ assays were performed because loss of RpoH production was found to enhance the susceptibility of *Brucella melitensis* (1) to the oxidizing agent. Since we (unpublished observations) and others (3, 8, 14) have been unsuccessful in obtaining an *rpoH* null mutant, we hypothesized that under conditions where *rpoH* expression is dampened due to the repressive action of MtrR, gonococcal susceptibility to H₂O₂ would increase. Indeed, we found that inducible expression of *mtrR* increased gonococcal susceptibility to H₂O₂ (Fig. 7E).

We believe that the MtrR regulon defined in this study represents the minimum number of gonococcal genes under MtrR control, as other genes (*farR*, *ponA*, and *pilMNO PQ*) known (6, 15) to be controlled by MtrR were not identified as being regulated by ≥ 1.5 -fold in the microarray data set. In this respect, the MtrR-repressed *farR* (15) and *pilM* (6) genes were measured as being repressed by 1.44- and 1.40-fold, respectively, in the microarray data set, while the MtrA-activated *ponA* gene was measured to be 1.24-fold activated. Other MtrR-controlled genes might be uncovered under different growth conditions or by exposure of gonococci to stress conditions or antimicrobial agents.

Although the microarray results indicated that expression of *rpoH* was modestly repressed by MtrR, the level of repression (1.58-fold) was only slightly less than those of the *mtrCDE* genes (1.7- to 2.2-fold). Since loss of MtrR repression of *mtrCDE* in strain JF1 (FA19 $\Delta mtrR$) can have important consequences for certain gonococcal properties (levels of antimicrobial susceptibility and in vivo fitness [31]), we elected to use *rpoH* as a model gene outside of the *mtrCDE* locus to study the

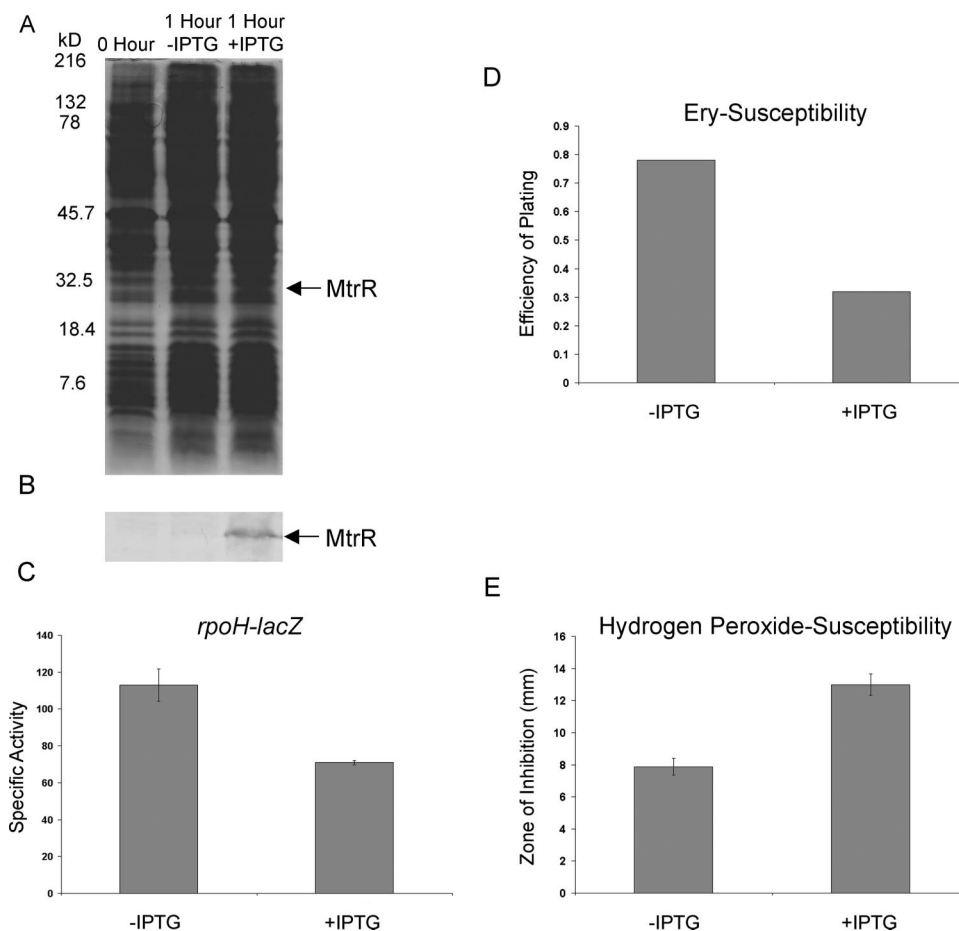


FIG. 7. Inducible production of MtrR represses *rpoH* expression and modulates antimicrobial susceptibility levels in gonococci. (A and B) Strain JF7 was grown in the presence or absence of 1 mM IPTG as described in Materials and Methods, and cell lysates before induction (0 Hour) or after 1 h of incubation in the absence (–) or presence (+) of IPTG were solubilized and subjected to SDS-PAGE using a 12.5% (wt/vol) SDS-PAGE gel with the separated proteins stained by Coomassie brilliant blue (A); the positions of the molecular mass markers are shown on the left of the gel, and the approximate positions of MtrR as determined by immunoblotting with detection using anti-MtrR antiserum (B) are shown. (C) Specific activity values for β -Gal levels in the control (–IPTG) and induced (+IPTG) cultures. The results are shown as average values (\pm standard deviations) from three independent assays. The difference was significant ($P = 0.0012$). (D) The efficiencies of plating of the control and IPTG-induced cultures on GCB agar with or without 0.5 μ g/ml of Ery are shown. (E) The H_2O_2 susceptibilities of the control and IPTG-induced cultures were assessed by a disk diffusion assay, and the difference in growth inhibition between the cultures was significant ($P < 0.001$).

global regulatory properties of MtrR. The combined translational fusion data that examined *rpoH* expression in the presence or absence of MtrR, especially those involving the inducible production of MtrR (Fig. 7), the increased expression of three RpoH-activated genes (*clpB*, *marR*, and *grpE*) in the MtrR-deficient strain JF1, and the ability of MtrR to bind within the *rpoH* promoter (Fig. 6), led us to conclude that MtrR acts as a direct repressor of *rpoH*. As EMSA experiments failed to show MtrR binding to *grpE*, we also conclude that the ability of MtrR to control the expression of this RpoH-regulated gene is likely indirect and is most easily explained by the ability of MtrR to dampen *rpoH* expression.

What is the biological significance of MtrR regulation of *rpoH*? We considered the possibility that because *rpoH* expression can enhance bacterial resistance to killing by H_2O_2 (1) and because certain genes under RpoH control are upregulated in gonococci during bacterial exposure to H_2O_2 (29), MtrR ex-

pression in gonococci would impact the levels of such resistance. The results presented here support this hypothesis. Moreover, based on these results, we suggest that MtrR is a general regulator of levels of gonococcal susceptibility to antimicrobial agents of the innate host defense, particularly the nonoxidative and oxidative killing systems of neutrophils. Thus, by repressing *mtrCDE* expression, MtrR can enhance gonococcal susceptibility to LL-37, which is produced by neutrophils, epithelial cells, and certain organs (e.g., testis) (24) and is a mediator of nonoxidative killing of bacteria. As MtrR production also increases bacterial susceptibility to H_2O_2 , its control of gene expression, along with other resistance systems, may be important in the ability of gonococci to survive oxidative killing by neutrophils. We do not yet know if the observed decrease in H_2O_2 susceptibility in *mtrR* mutants directly or indirectly involves RpoH, but the ability of MtrR to control *rpoH* expression suggests that this regulatory scheme is important in the general stress response. We also noted that *hsp33*

expression is also subject to MtrR repression (2.24-fold) (Table 3). This is of interest because Hsp33 has been implicated in bacterial responses to heat and peroxide (32). Unlike, *grpE*, MtrR regulation of *hsp33* may be direct, as a potential MtrR-binding site consisting of 31 bp is positioned from nucleotides -110 to -141 (data not presented); this putative binding site is 61.3% identical to the 31-bp region upstream of *mtrCDE* shown by Lucas et al. (16) to bind MtrR. Taking these data together, we propose that naturally occurring *mtrR* mutants (22, 23, 34, 35) may have an advantage during infection, based on their increased resistance to LL-37 (24) and H₂O₂ (Fig. 7). This linkage of bacterial resistance to distinct mediators of innate host defense through MtrR may help to explain the recent observation of Warner et al. (31) that *mtrR* mutants have a competitive advantage over otherwise wild-type gonococci in a female mouse lower genital tract infection model. The potential roles of other transcriptional regulators similar to MtrR in modulating bacterial resistance to innate host defense mechanisms should be considered.

Against this background, it should be asked why *mtrR* mutants are not the dominant strain in the community. At first glance, it seems to be a disadvantage for gonococci to have maintained *mtrR*, as its presence would downregulate the expression of genes needed for resistance to antimicrobials. We propose that because MtrR can also transcriptionally activate certain genes (Table 3) involved in metabolism (e.g., *abpE*, *gluE*, and *rfbB*), the need to resist host-derived antimicrobials through constitutive MtrR-repressible systems may be essential only at those sites rich in these agents. For most strains and infections, the need to have expression of MtrR-activated genes is more critical and overrides any advantage afforded by *mtrR* mutations, particularly since the *mtrCDE* efflux operon can be transcriptionally induced by MtrA in the presence of sublethal levels of antimicrobials (20), even in the presence of a functional MtrR.

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