JOURNAL OF BACTERIOLOGY, July 1997, p. 4123–4128 Vol. 179, No. 13
0021-9193/97/$04.00+0

The MtrR Repressor Binds the DNA Sequence between the *mtrR* and *mtrC* Genes of *Neisseria gonorrhoeae*

CLARESSA E. LUCAS,[1,2] JACQUELINE T. BALTHAZAR,[1] KAYLA E. HAGMAN,[3] AND WILLIAM M. SHAFER[1,2]*

Laboratories of Microbial Pathogenesis, VA Medical Research Service, VA Medical Center (Atlanta), Decatur, Georgia 30033[1]; Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322[2]; and Department of Microbiology and Immunology, University of Texas Southwestern School of Medicine, Dallas, Texas 75235[3]

Received 16 August 1996/Accepted 29 April 1997

Gonococcal resistance to antimicrobial hydrophobic agents (HAs) is due to energy-dependent removal of HAs from the bacterial cell by the MtrCDE membrane-associated efflux pump. The *mtrR* (multiple transferrable resistance Regulator) gene encodes a putative transcriptional repressor protein (MtrR) believed to be responsible for regulation of *mtrCDE* gene expression. Gel mobility shift and DNase I footprint assays that used a maltose-binding protein (MBP)–MtrR fusion protein demonstrated that the MtrR repressor is capable of specifically binding the DNA sequence between the *mtrR* and *mtrC* genes. This binding site was localized to a 26-nucleotide stretch that includes the promoter utilized for *mtrCDE* transcription and, on the complementary strand, a 22-nucleotide stretch that contains the −35 region of the *mtrR* promoter. A single transition mutation (A→G) within the MtrR-binding site decreased the affinity of the target DNA for MtrR and enhanced gonococcal resistance to HAs when introduced into HA-susceptible strain FA19 by transformation. Since this mutation enhanced expression of the *mtrCDE* gene complex but decreased expression of the *mtrR* gene, the data are consistent with the notion that MtrR acts as a transcriptional repressor of the *mtrCDE* efflux pump protein genes.

Resistance of *Neisseria gonorrhoeae* to structurally diverse hydrophobic agents (HAs) has been the subject of several recent investigations (5, 6, 10, 20, 23). Although earlier studies indicated that the *mtr* (multiple transferrable resistance) locus modifies the permeability barrier of the gonococcal cell envelope (3, 4, 13, 16, 22, 24), more recent studies revealed that *mtr* encodes an energy-dependent efflux system (5, 6, 10, 20, 23). The membrane proteins (MtrC, MtrD, and MtrE) forming the efflux pump share considerable amino acid sequence similarity with other efflux proteins in *Escherichia coli* (12, 17) and *Pseudomonas aeruginosa* (21). Active removal of toxic compounds by bacteria is an important mechanism of multiple antibiotic resistance (8, 17) and may confer a selective advantage on organisms, such as gonococci, when they colonize mucosal sites bathed in fluids containing antibacterial fatty acids and bile salts or encounter other environmental stresses (8, 11, 12, 16–18, 23).

Production of the MtrCDE efflux proteins is controlled at the level of transcription by both *cis*- and *trans*-acting factors involving the *mtrR* gene (5, 6, 20, 23). The *mtrR* gene is located 250 bp upstream of the *mtrCDE* gene complex and transcribed divergently (5, 6, 20). It encodes a 210-amino-acid protein with a molecular mass of approximately 23 kDa that contains a putative helix-turn-helix motif near its N terminus (5, 20, 23). MtrR has considerable amino acid sequence similarities to several transcriptional repressors, most notably, the tetracycline repressor of pSC101 (1, 7, 20). Recent investigations have revealed that missense or deletion mutations within the *mtrR* coding region result in enhanced *mtrCDE* transcription and gonococcal resistance to HAs (6, 23). Thus, the available genetic data strongly suggest that MtrR is a repressor whose function is to regulate transcription of the *mtrCDE* genes. However, prior to the present study, there was no direct evidence demonstrating the ability of the putative MtrR repressor to bind a target DNA sequence. In the present study, we examined the DNA-binding capacity of the MtrR repressor and found that it specifically binds to the intergenic sequence between the *mtrR* and *mtrCDE* genes.

(A preliminary account of this study was presented at the Tenth International Pathogenic *Neisseria* Conference in Baltimore, Md., 8 to 13 September 1996.)

MATERIALS AND METHODS

Bacterial strains and growth conditions used in this study. Strain FA19 and its isogenic transformant, strain KH16, have been described previously (5) and were the sources of DNAs for construction of maltose-binding protein (MBP)-MtrR fusion proteins. Briefly, strain KH16 contains a single missense mutation in its *mtrR* gene that results in replacement of the wild-type glycine residue with aspartic acid at position 45 in the MtrR amino acid sequence. Strain CL1 is a transformant of FA19 constructed in this study (see below) and contains a single point (transition) mutation that lies within the *mtrCDE* promoter. Strain KH15 contains a single-base-pair deletion in a 13-bp inverted repeat within the *mtrR* promoter (5, 6) and was used in experiments that measured levels of *mtrR* and *mtrC* mRNAs (see below). Gonococcal strains were grown on GC agar plates (Difco Laboratories, Detroit, Mich.) with defined supplements I and II at 37°C under 3.8% (vol/vol) CO_2 as previously described (5). *E. coli* TB-1 harboring fusion protein vectors was grown in Luria broth with 100-μg/ml ampicillin (Difco Laboratories) at 37°C with aeration.

Construction and purification of MBP-MtrR fusion proteins. MtrR was fused in frame to MBP by using the pMal-c2 fusion vector (New England Biolabs, Beverly, Mass.). Briefly, the *mtrR* gene was PCR amplified from chromosomal DNA prepared (14) from strain FA19 and KH16 DNAs (14) by using primers, R1 (5′-GTCAGGTCTCTATGAGAAAAACCAAAACCGAAGC-3′) and R2 (5′-ACTGAAGCTTATTTCCGGCGCAGGCAGGG-3′), that contained engineered restriction sites, i.e., *Bsa*I at the start of translation in R1 and *Hin*dIII at the translational stop codon in R2. After digestion with *Bsa*I, the PCR products were blunt ended with Klenow and digested with *Hin*dIII to allow directional ligation into pMal-c2 digested with *Xmn*I and *Hin*dIII. Plasmids pMBP-R1 or pMBP-R16 (generated from the *mtrR* coding sequence of strain FA19 or KH16, respectively) were transformed into *E. coli* TB-1, and the entire insert of each plasmid was sequenced to ensure fidelity of PCR amplification and in-frame

* Corresponding author. Phone: (404) 728-7688. Fax: (404) 329-2210. E-mail: wshafer@emory.edu.

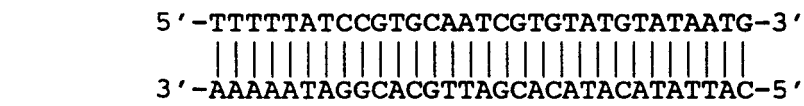

FIG. 1. Double-stranded 31-bp oligonucleotide encompassing the mtr-binding site on the *mtrC* coding strand and the complementary sequence.

ligation with *malE*. After 4 h of induction of MBP-MtrR overexpression with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG), cell lysates were purified by amylose affinity column chromatography. The eluted fusion protein resolved as a single band of approximately 65.7 kDa on sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis, and factor Xa digestion of the fusion protein released the 42.7-kDa MBP and 23-kDa MtrR proteins (data not shown). The N-terminal sequence of the MtrR band was determined, and the first 10 amino acids of the recovered protein were MRKTKTEALK, which are identical to those predicted by DNA sequencing (20, 23). Similar results were obtained in the purification of MBP-MtrR16. Repeated attempts to recover the MtrR protein from the MBP-MtrR factor Xa digests were unsuccessful. The MtrR protein was found to be highly susceptible to proteolytic activity that could not be eliminated by the addition of protease inhibitors (data not shown). Therefore, we elected to use intact fusion proteins in subsequent experiments, since MBP fusion proteins have been successfully used in other investigations of DNA-binding proteins (9, 15).

DNA-binding assays. The optimal binding buffer for gel mobility shift assays contained 10 mM Tris/Cl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DL-dithiothreitol, 1 mM EDTA, 4% (vol/vol) glycerol, and 1-mg/ml salmon sperm DNA. Target DNA fragments were PCR amplified from FA19 chromosomal DNA by using oligonucleotide primers KH9#2 (5′-CGTTTCGGGTCGGTTTGACG-3′) and CEL2A (5′-GCTTTGATACCCGAATGTTCG-3′). Primer KH9#2 anneals 11 nucleotides upstream of the translational start of *mtrR* (5, 6, 20, 23); primer CEL2A anneals 168 nucleotides upstream. One primer in the target DNA amplification reaction was radiolabeled with [λ-^{32}P]ATP (NEN du Pont, Atlanta, Ga.) and T4 polynucleotide kinase (New England Biolabs) to obtain a PCR product labeled at the 5′ end of one strand. The concentration of the labeled FA19A target was determined by spectrophotometry, and the specific activity was found to be 5,000 cpm/pmol; 1 pmol of the target was used per binding reaction. After the addition of MBP-MtrR fusion proteins, binding reactions were incubated at room temperature for 15 min. Protein-DNA complexes were resolved on a 6% (80:1 acrylamide to bisacrylamide) nondenaturing polyacrylamide gel. Results were analyzed by ImageQuant 3.2 for use with the Molecular Dynamics PhosphorImager Series 400.

A double-stranded, 31-bp oligonucleotide encompassing the MtrR-binding site on the *mtrC* coding strand (Fig. 1) and the complementary sequence were prepared and purified by J. Pohl (Microchemical Facility, Emory University). Four variant double-stranded oligonucleotides containing single-nucleotide replacements at positions 14, 15, 17, and 21 of the 31-bp oligonucleotide were also prepared. These oligonucleotides were employed in competitive gel shift mobility experiments that measured their capacity to inhibit binding of MBP-MtrR to a ^{32}P-labeled double-stranded oligonucleotide having the wild-type MtrR-binding site. Protein-DNA complexes were resolved by polyacrylamide gel electrophoresis as described above.

Target DNA sequences for DNase I footprints were generated by PCR using oligonucleotide primers KH9#1 (5′-GTCGCAGATACGTTGGAACAACG-3′) and KH9#3 (5′-GACGACAGTGCCAATGCAACG-3′) to amplify a 518-bp product from chromosomal DNAs of strains FA19 and CL1. Target DNA was labeled at the 5′ end of one strand as described for gel mobility shift assays. Fusion proteins were allowed to bind in the binding buffer as in gel mobility shift assays before $CaCl_2$ and $MgCl_2$ were added to final concentrations of 2.5 and 5 mM, respectively. Five units of DNase I (Promega, Madison, Wis.) was then added to the reaction mixture, which was incubated at room temperature for 1 min. Digestion was stopped by 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% 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 for autoradiography.

Transformation experiments. PCR products were generated by successive rounds of PCR amplification for use in transformation experiments. The first round of PCR amplification utilized two separate reactions with the primers CEL1 (5′-GACAATGTTCATGCGATGATAGG-3′) and A14R (5′-TTTTTATCCGTGCGATCGTGTATGTATAATG-3′) or KH9#6 (5′-GATACTGATGCCTTCCACAACC-3′) and A14C (5′-CATTATACATACACGATCTGCACGGATAAAAA-3′). Wild-type FA19 chromosomal DNA was used as the template in these reactions. After recovery of the PCR products from a 1% agarose gel by electroelution, they were used in a second round of PCR amplification using primers CEL1 and KH9#3 (5′-GACGACAGTGCCAATGCAACG-3′) to amplify across the 31-nucleotide overlap of the two template PCR products. This reaction yielded a 1,094-bp PCR product which incorporated the transitional mutation at position 14 and was used to transform strain FA19 to increased erythromycin resistance, as described previously (23). A 1,094-bp PCR product containing the wild-type sequence was also produced as a negative control for transformation experiments. It was generated in a manner similar to that described above, except that the first round of PCR amplifications paired primers CEL1 and WtR (5′-TTTTTATCCGTGCAATCGTGTATGTATAATG-3′) or KH9#6 and WtC (5′-CATTATA CATACACCATTGCACGGATAAAAA-3′).

Analysis of *mtrR* and *mtrC* transcription. Total RNA was prepared from strains FA19, KH15, and CL1 and examined for *mtrR*, *mtrC*, and *rmp* (reduction modifiable protein) mRNA transcripts by using the RNA slot blot hybridization procedure described previously (6); the *rmp* transcript was used as a control since expression of *rmp* is not controlled by MtrR (6). Gene-specific probes were prepared by PCR (6) and labeled with [α-^{32}P]dCTP with the Boehringer Mannheim random primer labeling kit as described by the manufacturer. For hybridization, 20 μg of RNA was immobilized on a Zeta Probe GT nylon membrane (Bio-Rad Laboratories, Richmond, Calif.) and processed as described previously (6). Transcript levels were compared by analysis of the densitometric scans of the PhosphorImager profiles.

RESULTS AND DISCUSSION

MtrR binds to the DNA region between the *mtrR* and *mtrC* genes. Results from genetic experiments (5, 6, 20, 23) suggested that the gonococcal MtrR protein is a transcriptional repressor of the *mtrCDE* gene complex. To test this hypothesis, gel mobility shift assays were performed to determine whether the wild-type MtrR protein from strain FA19 is capable of binding a 157-bp target DNA sequence (FA19A) that intervenes between the translational start codons of the divergent *mtrR* and *mtrC* genes. Increasing amounts (25 to 250 ng) of MBP-MtrR incubated with FA19A target DNA resulted in two distinct shifted bands (Fig. 2, lanes 3 to 10). An additional, diffuse band near the wells was also present at high protein concentrations. We determined the molecular weights of the protein-DNA complexes by the method of Orchard and May (19) and found target-to-protein ratios of 1:1 in shift 1, 1:2 in shift 2, and at least 1:4 in the diffuse band at the top. In contrast, the MBP–β-Galα fusion protein expressed from the pMal-c2 vector was incapable of retarding the target band (Fig. 2, lane 2), demonstrating that the DNA-binding activity of the MBP-MtrR fusion protein was due to the MtrR moiety. DNA targets of sequences nearer to the translational start of *mtrC* were not shifted by MBP-MtrR, nor were they or salmon sperm DNA able to compete with the FA19A target (data not shown), indicating the specificity of MtrR-DNA binding.

Previous studies (5, 23) demonstrated that a missense mutation at codon 45 in the *mtrR* coding sequence of strain KH16, which would result in a radical amino acid substitution (Gly-45 to Asp) within the helix-turn-helix motif of the MtrR repressor, could enhance gonococcal resistance to HAs four- to eightfold. To determine whether this radical amino acid substitution alters the DNA-binding activity of MtrR, we employed an MBP-MtrR fusion protein that contained the MtrR amino acid sequence from strain KH16. No retardation of target DNA was observed when MBP-MtrR16 (Gly-45 to Asp) was used in a gel mobility shift assay (Fig. 2, lanes 11 to 18), indicating that this mutant protein was deficient in DNA binding.

Localization of MtrR-binding sites. DNase I footprint analyses were performed to identify the MtrR-binding site(s) on the target DNA sequence from strain FA19. Binding of MBP-MtrR to the target DNA was evaluated by using 0, 5, and 10 μg of MBP-MtrR. As shown in Fig. 3, 26 nucleotides on the *mtrC* coding sequence that included the promoter utilized for *mtrCDE* transcription (Fig. 3A) and the complementary sequence of the −35 region of the *mtrR* promoter could be protected from DNase I digestion. It is important to note that we were unable to detect sites protected from DNase I digestion when 10 μg of the MBP-MtrR16 fusion protein, which does not bind the target DNA (Fig. 2), was used in similar

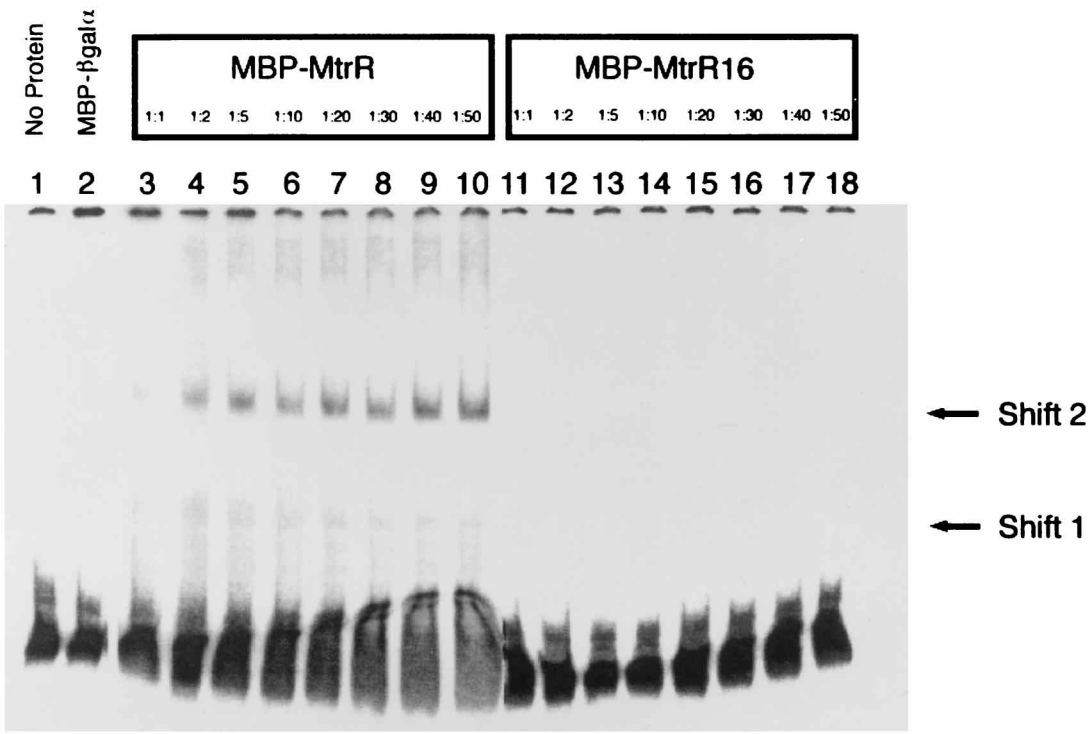

FIG. 2. Gel mobility shift assays of MBP-MtrR fusion proteins. Shown is the autoradiographic profile that resulted from incubation of the radiolabeled 157-bp FA19A target sequence with the MBP-MtrR (lanes 3 to 10) and MBP-MtrR16 (lanes 11 to 18) fusion proteins. The target-to-protein molar ratios are shown above the lanes. Lanes 1 and 2 show the electrophoretic mobilities of probe DNA alone and probe DNA incubated with a 50-fold molar excess of MBP–β-Galα, respectively.

experiments (data not shown). We also evaluated protection of the *mtrR* coding strand by MBP-MtrR and found that most of the 22 nucleotides that could be protected were complementary to the region identified on the *mtrC* coding strand (Fig. 3B). Additionally, we observed the presence of a band hypersensitive to DNase I cleavage only in the presence of MBP-MtrR (Fig. 3B). This DNase I-hypersensitive band is in the A-T-rich region adjacent to the target sequence and may indicate a change in DNA conformation upon MtrR binding. The protected region on the *mtrR* coding strand included the −35 region of the *mtrR* promoter. The nucleotide sequences on the *mtrR* and *mtrC* coding strands that bind MBP-MtrR are summarized in Fig. 4.

Mutations in the MtrR-binding site. To verify that the MtrR-binding site deduced by the DNase I footprint analysis is indeed important in mediating MtrR binding, double-stranded oligonucleotides containing the wild-type MtrR-binding site or four variants with single-base-pair substitutions were constructed (see Materials and Methods). In preliminary experiments, it was found that a radiolabeled 31-mer double-stranded oligonucleotide (see Materials and Methods) containing the MtrR-binding site yielded two shifted bands after incubation with MBP-MtrR (data not shown). Four double-stranded mutant oligonucleotides that contained single-nucleotide replacements at positions 14 (A→G), 15 (A→T), 17 (C→T), and 21 (T→C) in the 31-mer target DNA and the wild-type sequence were studied for possible differences in affinity for MBP-MtrR. In this competition experiment, the target DNA was radiolabeled 31-mer double-stranded oligonucleotide described in Materials and Methods, and the average results from three competition experiments are shown in Fig. 5A. The T21→C transition did not significantly alter the affinity of the labeled target DNA for the MtrR repressor, but the C17→T transition decreased MtrR binding about twofold. In contrast, the A14→G transition reduced binding approximately 5-fold, while the A15→T transversion increased target binding nearly 20-fold.

We reasoned that since MtrR appears to be a transcriptional repressor that regulates transcription of the *mtrCDE* efflux genes (5, 6, 20), a reduction in MtrR binding to a target DNA sequence with the A14→G transition would lead to enhanced resistance of HA-susceptible strain FA19. To test the importance of this transition mutation in regulating *mtrR* and *mtrCDE* gene expression, two 1,094-bp products containing the A14→G mutation or the wild-type sequence were constructed as described in Materials and Methods and used to transform strain FA19 for increased resistance to erythromycin. Transformants were recovered with the PCR product containing the A14→G transition at a frequency of 10^{-6}, but none ($\leq 5 \times 10^{-8}$) were recovered with the control PCR product from parental strain FA19. Compared to parental strain FA19, a selected transformant (CL1) had enhanced resistance to both erythromycin (MICs, 0.25 and 2.0 μg/ml, respectively) and Triton X-100 (MICs, 100 and >5,000 μg/ml, respectively). The *mtrR* gene and the region between the *mtrR* and *mtrC* genes were sequenced from CL1. The single difference between it and the parental FA19 sequence was the A14→G change present in the donor DNA sequence (data not shown). The affinity of MBP-MtrR for the DNA sequence between the *mtrR* and *mtrC* genes in strains FA19 and CL1 was next examined in a competitive gel mobility shift assay that employed the 157-bp target sequence described above (Fig. 2). As shown in Fig. 5B, the A14→G transition resulted in decreased affinity of the target DNA for MBP-MtrR in vitro. This result is in good agreement with the finding that the A14→G transition in the synthetic 31-mer double-stranded oligonucleotide reduced its

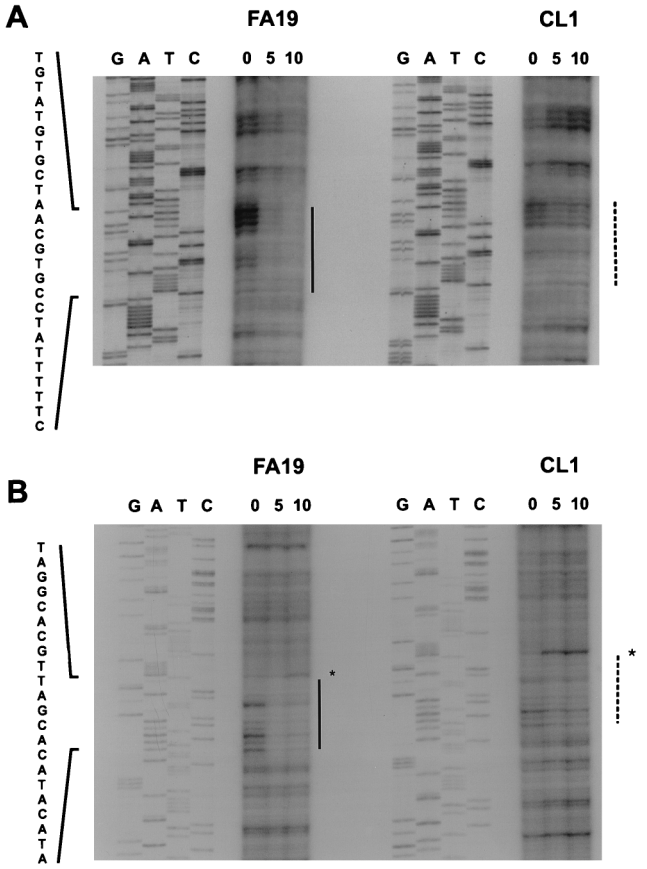

FIG. 3. DNase I footprint of FA19 and CL1 target DNA samples by MBP-MtrR. The 518-bp FA19 and CL1 target DNA samples (1 pmol) labeled on the *mtrC* (A) or *mtrR* (B) coding strand were incubated with 0, 5, and 10 μg (equivalent to a 500 to 1,000-fold molar excess) of MBP-MtrR and subjected to DNase I digestion. The general areas of protection in the FA19 sequence are indicated by the shaded bars, while the corresponding but unprotected regions in the CL1 sequence are indicated by the dashed lines. The DNase I-hypersensitive site on the *mtrR* coding strand is shown by an asterisk.

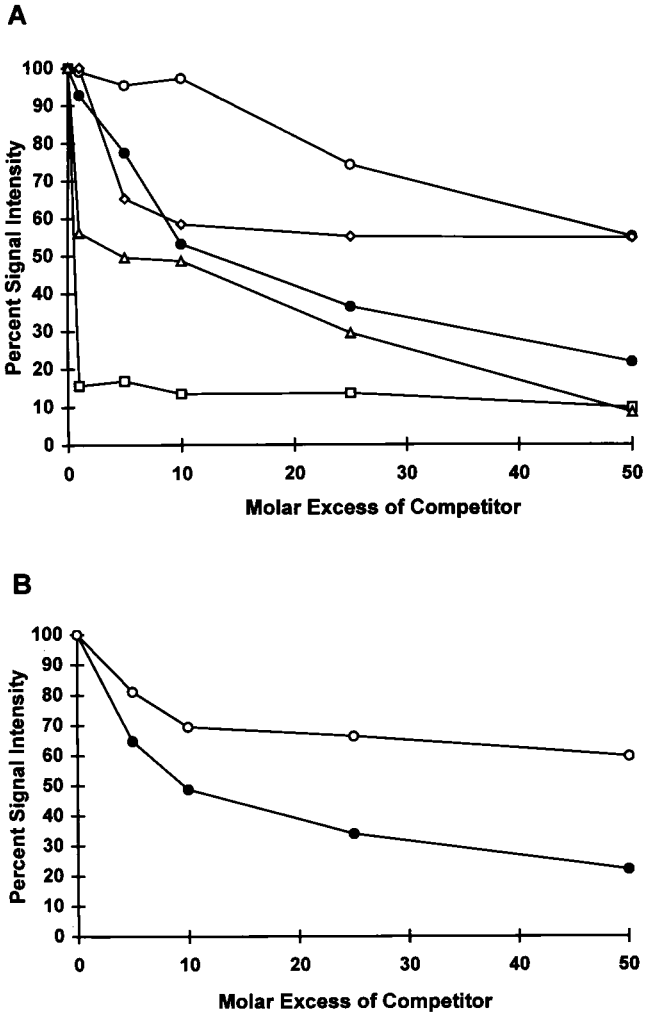

FIG. 5. Binding of MBP-MtrR to mutant target sequences. (A) Double-stranded 31-mer oligonucleotides were used to compete for the binding of MBP-MtrR to the ^{32}P-labeled wild-type 31-mer oligonucleotide. Symbols: ●, wild-type 31-mer oligomer; ○, A14→G; □, A15→T; ◇, C17→T; △, T21→C. (B) Capacity of the 157-bp PCR products from strains FA19 (●) and CL1 (○) to compete for binding of MBP-MtrR to the labeled 157-bp fragment from strain FA19. Unlabeled competing DNAs in both A and B were used at 0-, 1-, 5-, 10-, 25-, and 50-fold molar excesses over the labeled target DNA.

binding affinity for MBP-MtrR (Fig. 5A). In the experiment shown in Fig. 3, which employed 0, 5, and 10 μg of MBP-MtrR and FA19 target DNA, we also utilized target DNA from CL1 in the DNase I footprinting assay. Compared to the results obtained with the FA19 target DNA, neither the *mtrR* nor the *mtrRCDE* promoter regions from CL1 showed evidence of being protected. However, we cannot exclude the possibility of some binding of MBP-MtrR to the CL1 target sequence because the hypersensitive band noted with the FA19 target was also present in the CL1 sequence (Fig. 3). Nevertheless, on the basis of both competitive gel mobility shift and DNase I footprinting assays, we concluded that the A14→G transition in strain CL1 significantly reduced recognition of MtrR. This provides strong evidence that the *mtrR* and *mtrRCDE* promoter regions are important targets for MtrR binding.

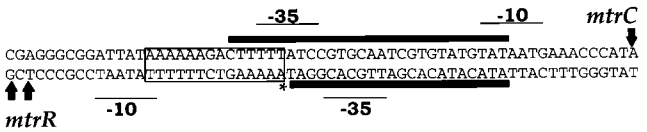

FIG. 4. Nucleotide sequence of sites recognized by the MtrR repressor. The intergenic nucleotide sequence between the transcriptional start points of the *mtrR* and *mtrC* genes in strain FA19 is given. The promoter regions are defined by black bars, and the transcriptional start points are indicated by arrows (5, 6). The regions protected in DNase I footprinting analyses are indicated by shaded bars, and the DNase I-hypersensitive site on the *mtrR* coding strand is shown by an asterisk. The 13-bp inverted repeat is encompassed within the boxed area.

The enhanced HA resistance expressed by transformant strain CL1 and the reduced capacity of its MtrR-binding site to recognize MBP-MtrR suggested that expression of the *mtrR* and/or *mtrCDE* genes in this strain might be altered due to the A14→G mutation. To test this hypothesis, total RNA was prepared from FA19, CL1, and KH15 (same as FA19 but *mtrR171*) and used in slot blot hybridization assays with gene-specific probes for the *mtrR*, *mtrC*, and *rmp* transcripts. (Strain KH15 is a transformant of FA19 [5] that contains a single-base-pair deletion in the 13-bp inverted repeat sequence [Fig. 4] within the *mtrR* promoter. This deletion abrogates transcription of *mtrR* and enhances transcription of *mtrCDE* [6]. We found that strain CL1 had enhanced (twofold) expression of *mtrC* compared to that in parental strain FA19 (Fig. 6), but the

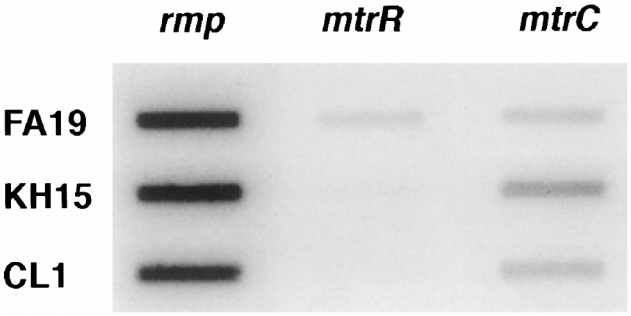

FIG. 6. Levels of *mtrR* and *mtrC* transcripts in isogenic strains FA19, CL1, and KH15. Twenty micrograms of total RNA from each strain was hybridized with ^{32}P-labeled *mtrR*, *mtrC*, and *rmp* gene probes (see Materials and Methods). Densitometric analyses of signal intensities were carried out with a Molecular Dynamics PhosphorImager. The *mtrC* transcript levels in strains CL1 and KH15 were found to be elevated over that in strain FA19 by factors of 2 and 3.9, respectively, when the ratios of the *mtrC*/*rmp* transcript levels of these strains were compared.

mtrC transcript level was not as elevated as in strain KH15, which was increased nearly fourfold, similar to that found earlier (6). Interestingly, in three different experiments with different lots of RNA, we also found that the *mtrR* transcript level was reduced significantly in CL1 compared with that in FA19 and was similar to that in strain KH15 (Fig. 6). We do not believe that the reduced capacity of MtrR to bind to its target sequence obtained from CL1 explains the reduced expression of *mtrR* (e.g., MtrR does not serve as a positive regulator of *mtrR* expression) because transformants of strain FA19 that have an insertionally inactivated or deleted *mtrR* coding sequence exhibit wild-type *mtrR* transcript levels (data not shown). Thus, the enhanced HA resistance phenotype of CL1 is likely due to the combined effects of decreased transcription of *mtrR*, which may be due to reduced binding of RNA polymerase to the −35 region of the *mtrR* promoter impacted by the A14→G mutation (Fig. 4), and the reduced capacity of any synthesized MtrR to bind its target sequence on the *mtrCDE* coding strand.

The position of the MtrR-binding site between the −10 and −35 hexamers of the *mtrC* promoter was deduced from the results of the DNase I protection assays. However, because large amounts of MBP-MtrR (5 and 10 μg) were required for protection, we constructed mutant double-stranded oligonucleotides containing the deduced MtrR-binding site but with single-nucleotide changes within the spacer region between the −10 and −35 regions of the *mtrCDE* promoter and examined their capacity to bind MBP-MtrR. Because two specific base pair changes resulted in target DNA sites with decreased (the A14→G transition) or increased (A15→T transversion) affinity for MBP-MtrR, the in vitro data (Fig. 2 and 3) corroborate our conclusion that MtrR acts as a transcriptional repressor by binding within the *mtrCDE* promoter. We propose that transcriptional inhibition of the *mtrCDE* gene complex by MtrR is due to steric hindrance of RNA polymerase binding to the −10 and −35 hexamers of the *mtrCDE* promoter. Additional studies are required, however, to better define the nucleotides within this region that are important for MtrR and RNA polymerase binding.

The inability of the MBP-MtrR16 mutant to either shift target DNA (Fig. 2) or protect the target from DNase I digestion (data not shown) lends credence to the theory advanced by Pan and Spratt (20) that the proposed helix-turn-helix motif of the MtrR repressor is important for DNA binding. This missense mutation, which substitutes an aspartic acid for glycine at codon 45, was predicted (23) to perturb the second helix of the helix-turn-helix motif, such that it better resembles an extended turn. DNA-binding proteins of the helix-turn-helix family utilize the second helix as a bridge across the major groove of the target DNA (2, 25). This contact is required to stabilize the protein-DNA interaction and may be important in mediating conformational changes of the DNA itself.

Transcriptional control of the *mtr* efflux system may allow gonococci to respond to the antibacterial effects of HAs (e.g., bile salts and fatty acids) that bathe certain mucosal surfaces which this organism colonizes. Recent work with the *E. coli* *acrAB*-encoded efflux pump (11) has demonstrated that the AcrR repressor, which is similar to MtrR (20), modulates induction of *acrAB* gene expression in response to global stresses (12). Thus, our studies and those reported by others highlight the complexity inherent in the regulation of efflux pump gene expression.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI-21150 and funds from the VA Medical Research Service, both awarded to W.M.S. W.M.S. is the recipient of an Associate Career Scientist Award from the VA Medical Research Service. C.E.L. was supported by a predoctoral fellowship provided by the American Society for Microbiology.

We thank Charles P. Moran for critical reading of the manuscript. We also thank him for suggesting the use of mutant oligonucleotides for studying MtrR binding and the PCR strategy to construct mutant DNA sequences for transformation.

REFERENCES

1. **Bertrand, K. P., K. Postle, L. V. J. Wray, and W. S. Reznikoff.** 1983. Overlapping divergent promoters control expression of Tn*10* tetracycline resistance. Gene **23:**149–156.
2. **Brennan, R. G., and B. W. I. Matthews.** 1989. Structural basis of DNA-protein recognition. Trends Biochem. Sci. **14:**286–290.
3. **Guymon, L. F., and P. F. Sparling.** 1975. Altered crystal violet permeability and lytic behavior in antibiotic-resistant and -sensitive strains of *Neisseria gonorrhoeae*. J. Bacteriol. **124:**757–763.
4. **Guymon, L. F., D. L. Walstad, and P. F. Sparling.** 1978. Cell envelope alterations in antibiotic-sensitive and -resistant strains of *Neisseria gonorrhoeae*. J. Bacteriol. **136:**391–401.
5. **Hagman, K. E., W. Pan, B. G. Spratt, J. T. Balthazar, R. C. Judd, and W. M. Shafer.** 1995. Resistance of *Neisseria gonorrhoeae* to hydrophobic antimicrobial agents is modulated by the *mtrRCDE* efflux system. Microbiology **141:** 611–622.
6. **Hagman, K. E., and W. M. Shafer.** 1995. Transcriptional control of the *mtr* efflux system of *Neisseria gonorrhoeae*. J. Bacteriol. **177:**4162–4165.
7. **Hillin, W., and C. Berens.** 1994. Mechanisms underlying expression of Tn*10* encoded tetracycline resistance. Annu. Rev. Microbiol. **48:**345–369.
8. **Levy, S. B.** 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36:**695–703.
9. **Lu, F., and G. Churchward.** 1994. Conjugative transposition: Tn*916* integrase contains two independent DNA binding domains that recognize different DNA sequences. EMBO J. **13:**1541–1548.
10. **Lucas, C. E., K. E. Hagman, D. C. Stein, and W. M. Shafer.** 1995. Importance of lipooligosaccharide structure in determining gonococcal resistance to hydrophobic antimicrobial agents resulting from the *mtr* efflux system. Mol. Microbiol. **16:**1001–1009.
11. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19:**101–112.
12. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
13. **Maness, M. J., and P. F. Sparling.** 1973. Multiple antibiotic resistance due to a single mutation in *Neisseria gonorrhoeae*. J. Infect. Dis. **128:**321–330.
14. **McAllister, C. F., and D. S. Stephens.** 1973. Analysis in *Neisseria meningitidis* and other *Neisseria* species of genes homologous to the FKBP immunophilin family. Mol. Microbiol. **10:**13–24.
15. **McIver, K. S., A. S. Heath, B. D. Green, and J. R. Scott.** 1995. Specific binding of the activator Mga to promoter sequences of the *emm* and *scpA* genes in the group A streptococcus. J. Bacteriol. **177:**6619–6624.
16. **Morse, S. A., P. G. Lysko, L. McFarland, J. S. Knapp, E. Sandstrom, C. Critchlow, and K. K. Holmes.** 1982. Gonococcal strains from homosexual men have outer membranes with reduced permeability to hydrophobic molecules. Infect. Immun. **37:**432–438.

17. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
18. **Okuso, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
19. **Orchard, K., and G. E. May.** 1993. An EMSA-based method for determining the molecular weight of a protein-DNA complex. Nucleic Acids Res. **21:** 3335–3336.
20. **Pan, W., and B. G. Spratt.** 1994. Regulation of the permeability of the gonococcal cell envelope by the *mtr* system. Mol. Microbiol. **11:**769–775.
21. **Poole, K., D. E. Heinrichs, and S. Neshat.** 1993. Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of the siderophore pyoverdine. Mol. Microbiol. **10:**529–544.
22. **Scudamore, R. A., T. J. Beveridge, and M. Goldner.** 1979. Penetrability of the outer membrane of *Neisseria gonorrhoeae* in relation to acquired resistance to penicillin and other antibiotics. Antimicrob. Agents Chemother. **15:**820–827.
23. **Shafer, W. M., J. T. Balthazar, K. E. Hagman, and S. A. Morse.** 1995. Missense mutations that alter the DNA-binding domain of the MtrR protein occur frequently in rectal isolates of *Neisseria gonorrhoeae* that are resistant to faecal lipids. Microbiology **141:**907–911.
24. **Sparling, P. F., F. A. Sarubbi, and E. Blackman.** 1975. Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in *Neisseria gonorrhoeae*. J. Bacteriol. **124:**740–749.
25. **Suzuki, M., N. Yagi, and M. Gerstein.** 1995. DNA recognition and superstructure formation by helix-turn-helix proteins. Protein Eng. **8:**329–338.