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Transcriptional Control of the *mtr* Efflux System of *Neisseria gonorrhoeae*

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The capacity of *Neisseria gonorrhoeae* to resist structurally diverse hydrophobic agents (HAs) because of the *mtr* (multiple transferrable resistance) efflux system was found to be regulated at the level of transcription by two distinct mechanisms. This was surmised because a deletion that removed >90% of the coding sequence of the *mtrR* (multiple transferrable resistance regulator) gene or a single-base-pair deletion within a 13-bp inverted repeat sequence located in its promoter resulted in altered expression of the *mtrC* gene; *mtrC* encodes a 44-kDa membrane lipoprotein essential for the efflux of HAs. However, the single-base-pair deletion had the more significant impact on gene expression since it resulted in the loss of expression of *mtrR* and a threefold increase in the expression of *mtrC*. Hence, the *mtr* efflux system in gonococci is subject to both MtrR-dependent and MtrR-independent regulation, and the levels of *mtrC* mRNA correlate well with HA resistance levels in gonococci.

The genetic organization of the *mtr* system in gonococci (17) was recently determined (4, 12) and was found to be remarkably similar to that of the *mexAB-oprK* efflux system of *Pseudomonas aeruginosa* (13) as well as those of the *acrAE* (7) and *envCD* (5) efflux pumps possessed by *Escherichia coli*; the *E. coli* efflux operons have been renamed *acrAB* and *acrEF*, respectively (8). Recent studies confirmed (6) the energy-dependent efflux action capacity of the *mtr* system. As was emphasized in a recent review by Nikaido (11), efflux systems have importance for bacterial resistance to structurally diverse antimicrobial agents, including antibiotics, dyes, and detergents. Efflux systems may be of importance for gonococcal survival in hostile environments rich in hydrophobic agents (HAs) since clinical isolates expressing resistance to multiple HAs because of *mtr* are frequently recovered from patients (10), particularly those with rectal infections in which toxic fecal lipids and bile salts are likely to select for resistant variants in vivo (9).

The *mtrR*-encoded protein resembles numerous transcriptional repressors (12), such as the tetracycline repressor of pSC101 (2), and at least one activator of transcription, LuxR of *Vibrio harveyi* (18). The *mtrCDE* gene complex probably constitutes a single transcriptional unit since promoter sequences have not been found between these genes (4). The *mtrCDE* operon is positioned 250 bp upstream of and is divergently transcribed from the *mtrR* gene. Mutations within the *mtrR* coding or promoter region were found (4) to result in both enhanced levels of HA resistance and elevated levels of MtrC, a 44-kDa lipoprotein that resembles members of the membrane fusion protein family (14) essential for the efflux of antimicrobial agents (11). Missense mutations in *mtrR* that result in radical amino acid replacements within the helix-turn-helix motif or a downstream domain of the MtrR protein were recently found to enhance the resistance of HA-sensitive strain FA19 (4, 12, 15, 16). However, this resistance was substantially less (4, 15) than that due to a single-base-pair deletion within a 13-bp inverted repeat positioned between the −10 and the −35 sequences of the *mtrR* promoter and overlapping the proposed −35 region of the *mtrC* promoter (Fig. 1). In order to test the hypothesis that the *mtr* efflux system is regulated by both MtrR-dependent and MtrR-independent mechanisms, the levels of *mtrR*- and *mtrC*-specific transcripts in *Neisseria gonorrhoeae* FA19 and transformants that contained a major deletion (>90%) of the *mtrR* coding sequence or a single-base-pair deletion within the *mtrR* promoter region were determined.

(A preliminary account of these studies was presented at the Ninth International Pathogenic *Neisseria* Conference held in Winchester, United Kingdom, 26 to 30 September 1994.)

The strains used in this investigation and their relevant phenotypes are described in Table 1. The sequence of the *mtrR* coding and promoter regions in HA-sensitive strain FA19 was recently described (4, 12). HA-resistant transformant strains KH11 and KH15 were constructed in a previous study (4). Strain KH11, which expresses an intermediate level of HA resistance (Table 1), contains a deletion in the *mtrR* gene that leaves only 60 nucleotides at the 5′ end of the coding sequence, but this does not include the DNA sequence that encodes the helix-turn-helix domain of the protein (12). Strain KH11 contains the 13-bp inverted repeat sequence that is positioned between the −10 and −35 sequences of the *mtrR* promoter. Highly HA-resistant strain KH15 has an *mtrR* coding sequence identical to that of strain FA19 but contains a single-base-pair deletion in the 13-bp inverted repeat sequence described above (Fig. 1).

Analysis of *mtrR* and *mtrC* gene expression. Nonpiliated, transparent colony types were routinely grown on GCB agar or in GCB broth (Difco Laboratories, Detroit, Mich.) as previously described (3, 4). Total RNA was prepared from gonococci by the method of Baker and Yanofsky (1), and RNA concentrations were determined by UV spectrophotometry at A_{260} in which 1.0 absorbance unit equals 40 μg of RNA. Primer extension analysis of *mtrR* was performed with the Promega primer extension kit (Promega Co., Madison, Wis.) according to the instructions provided by the manufacturer. Briefly, 20 μg of total RNA was reverse transcribed by annealing a ^{32}P-,

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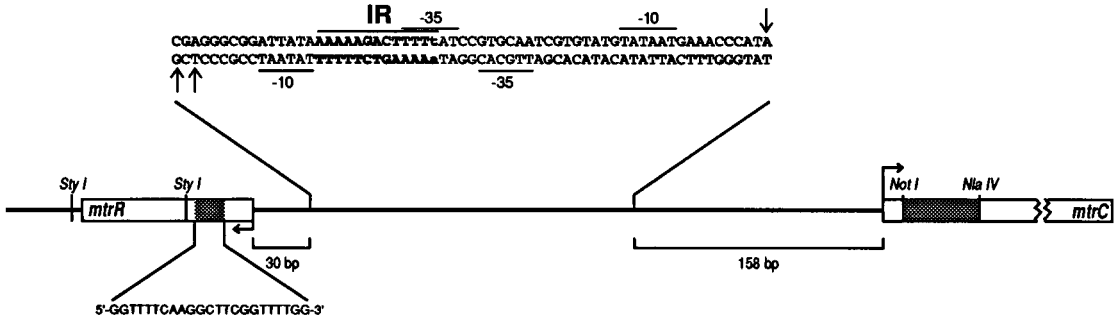

FIG. 1. Schematic representation of the *mtrR* and *mtrC* genes. The double-stranded nucleotide sequence between the start points of transcription (arrows) for the *mtrR* and *mtrC* genes is shown. The 13-bp inverted repeat (IR) is denoted in bold. Lowercase letters represent the single-base-pair deletion within the 13-bp inverted repeat. The −10 and −35 promoter sequences for both genes are shown in overlines or underlines. The location and sequence of the oligonucleotide used in the primer extension analysis of *mtrR* are shown shaded within the *mtrR* gene. The *Not*I-*Nla*IV fragment used for the *mtrC*-specific probe is shaded within the *mtrC* gene.

5′-end-labeled oligonucleotide primer complementary to nucleotides 11 to 33 of the *mtrR* coding sequence (Fig. 1). The primer extension products were electrophoresed on a DNA sequencing gel (4) along with the sequencing reaction products of a cloned partial *mtrR* gene from plasmid pKH9 (4) from strain FA19; the details of this plasmid construct were recently described (4). Previously, we used primer extension to map the transcriptional start point of the *mtrC* gene in strain KH15 (4). However, primer extension analysis failed to detect *mtrC* mRNA in strains FA19 and KH11 (data not presented), presumably because of the lower expression of *mtrC* in these strains (Table 1). Accordingly, for the detection of *mtrC* mRNA among the test strains, a slot blot hybridization procedure was employed. A 980-bp *Not*I-*Nla*IV fragment internal to the *mtrC* gene was removed from pKH9 (4) and served as the *mtrC*-specific gene probe (Fig. 1). The probe was labeled with [α-^{32}P]dCTP with the Boehringer Mannheim random primer labeling kit as described by the manufacturer. To assure that equal amounts of RNA were applied per slot, a duplicate blot was hybridized with a gene probe specific for the *rmp* (reduction modifiable protein) gene, which is not controlled by *mtr* (3); pTp.III containing the full-length *rmp* gene was kindly provided by M. Blake (Rockefeller University, New York, N.Y.). For hybridization, RNA was immobilized on a Zeta Probe GT nylon membrane (Bio-Rad Laboratories, Richmond, Calif.) and prehybridized for 10 min in prehybridization buffer (0.5 M $NaHPO_4$ [pH 7.2], 1 mM EDTA, 7% [wt/vol] sodium dodecyl sulfate [SDS]) at 65°C. Hybridization was performed overnight at 65°C in this buffer, which also contained 10^6 cpm of the labeled DNA probe ml^{-1}. Following hybridization, the membrane was washed at 65°C (30 min per wash), twice with 40 mM $NaHPO_4$ (pH 7.2)–1 mM EDTA–5% SDS and then twice with the same buffer except that it contained only 1% SDS. The blot was then exposed to a Kodak phosphoimaging screen for 18 h. Densitometric analysis of the blot was done with a Molecular Dynamics phosphoimager, and peak areas were calculated. The blots were also exposed to Kodak X-Omat X-ray film for photodocumentation.

Impact of defined mutations in *mtrR* on *mtrR* and *mtrC* gene expression. A comparison of the *mtrR* primer extension products obtained with total RNA prepared from strains FA19, KH11, and KH15 was performed to measure *mtrR* transcript levels. The results showed (Fig. 2) that deletion of the *mtrR* gene in strain KH11 still permitted transcription from the *mtrR* promoter, as was evidenced by the presence of the two extension products seen in parental strain FA19. However, the single-base-pair deletion in the 13-bp inverted repeat in the overlapping promoter regions of the *mtrR* and *mtrC* genes significantly reduced, to an undetectable level, transcription of the *mtrR* gene in strain KH15. Since the *mtrR* coding regions of strains FA19 and KH15 are identical (4), the undetectable expression of *mtrR* in strain KH15 is apparently due to the base pair deletion in the 13-bp inverted sequence. This base pair deletion would result in a change in the spacing between the −10 and −35 regions of the *mtrR* promoter from 17 bp to 16 bp. Thus, the 13-bp inverted repeat sequence in parental strain FA19 is proposed to be a *cis*-acting control element that enhances expression of *mtrR*.

The levels of the 44-kDa MtrC membrane lipoprotein in strains FA19, KH15, and KH11 were recently determined (4)

TABLE 1. Strains of *Neisseria gonorrhoeae* employed

Strain	Genotype	Level of HA resistance[a]	Level of MtrC[b]
FA19	Wild type	Sensitive	+
KH11	Δ*mtrR*	Intermediate	+++
KH15	*mtrR-171*	High	+++++

[a] As defined in reference 4 with respect to resistance to erythromycin. The MICs of erythromycin for strains FA19, KH11, and KH15 are 0.25, 1.0 and 2.0 μg ml^{-1}, respectively, and those for Triton X-100 are 125, 1,000, and ≥16,000 μg ml^{-1}, respectively.

[b] As defined in reference 4. The levels of MtrC were determined by Western blotting (immunoblotting) analysis with an anti-MtrC monoclonal antibody.

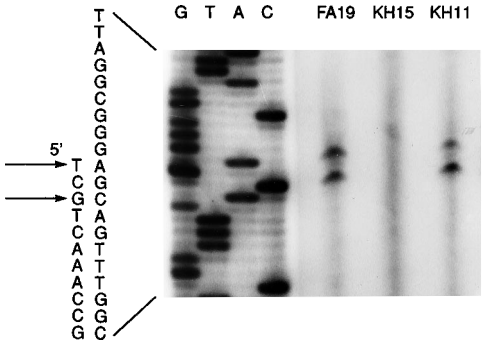

FIG. 2. Primer extension analysis of *mtrR* from strains FA19, KH15, and KH11. Primer extension products were generated from the *mtrR*-specific oligonucleotide (defined in Fig. 1) hybridized to 20 μg of total RNA isolated from each strain. The DNA sequence was produced by the same oligonucleotide and is therefore complementary to that of the mRNA. The actual RNA sequence is listed beside the DNA sequence, and the start points are marked with arrows.

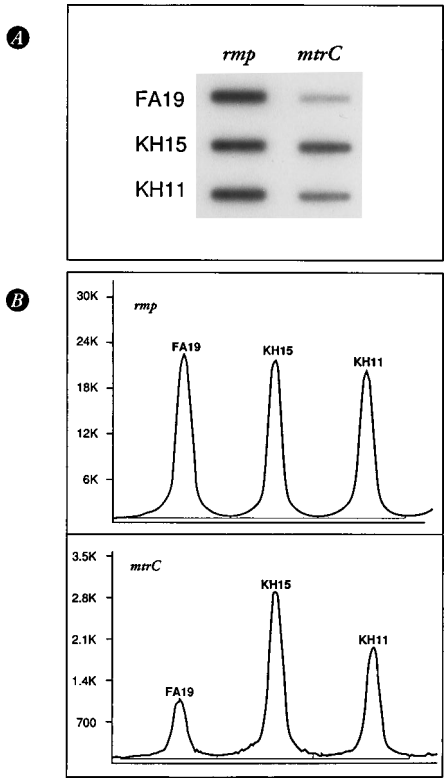

FIG. 3. Slot blot analysis of *mtrC* mRNA levels. Equal amounts of total RNA from each strain were applied per slot and hybridized with ^{32}P-labeled probe specific for either *rmp* mRNA or *mtrC* mRNA. (A) Autoradiograph of the slot blot hybridization. (B) Densitometric analysis of signal intensities from the same blot. The analysis was carried out with a Molecular Dynamics phosphoimager. The *y* axes are marked in phosphoimager units as designated by Molecular Dynamics.

and are summarized in Table 1. Levels of MtrC in these strains also correlate with levels of resistance to structurally diverse HAs (Table 1). In order to ascertain whether the mutations described above enhance expression of the *mtrC* gene, a comparison of the levels of *mtrC* mRNA in total RNA samples prepared from strains FA19, KH11, and KH15 was made. The results (Fig. 3A) confirmed that strain KH15 produced the highest level of *mtrC*-specific mRNA, strain KH11 produced the next highest, and HA-sensitive strain FA19 produced the lowest level. The qualitative assessment of the intensity of the hybridizing signals (Fig. 3A) was confirmed by the densitometric tracing (Fig. 3B) of the phosphoimager-generated profiles. Hence, the quantitative differences in *mtrC* mRNA among these strains paralleled those found in earlier studies that measured levels of MtrC in whole-cell lysates or membrane preparations.

The results presented here suggest two mechanisms by which the *mtr* system in gonococci can be regulated. Both mechanisms have importance for the expression of the divergently transcribed *mtrR* and *mtrC* genes. First, the MtrR protein, through its proposed transcriptional repressor action (12), can down-regulate expression of *mtrC* and presumably of *mtrD* and *mtrE* as well, as they are in the same transcriptional unit as *mtrC* (4). Our results demonstrate that the loss of MtrR in strain KH11 resulted in enhanced expression of *mtrC* (Fig. 2), but not to the extent as that caused by the single-base-pair deletion in the *mtrR* promoter region. This observation is entirely consistent with earlier studies that showed differences in MtrC levels among strains FA19, KH11, and KH15.

A second mechanism by which the *mtr* efflux system in gonococci is regulated involves the 13-bp inverted repeat sequence that is positioned within the *mtrR* promoter. That this inverted repeat serves as a *cis*-acting control sequence is supported by the observation that a single-base-pair deletion within it has opposite effects on *mtrR* and *mtrC* gene expression. With respect to *mtrR* expression, the primer extension analysis revealed that the deletion represses transcription of *mtrR* (Fig. 2). Most likely, this repressive activity is due to a shortening of the distance between the −10 and −35 regions of the *mtrR* promoter, which thereby reduces the binding of RNA polymerase. An alternative hypothesis to explain the data is that the 13-bp inverted repeat is a binding site for an activator of *mtrR* and that the single-base-pair deletion abrogates activator binding, thus reducing *mtrR* transcription.

The single-base-pair deletion in the inverted repeat also serves to enhance expression of *mtrC* (Fig. 3). However, this effect cannot be explained simply by the loss of MtrR, since strain KH11 (Δ*mtrR*) has a lower level of MtrC than strain KH15 (*mtrR-171*). Moreover, in other *mtrR* null mutants, high-level expression of MtrC and HA resistance required that the null mutant also contain the single-base-pair deletion in the inverted repeat (4). Thus, it may be that the single-base-pair deletion within the 13-bp inverted repeat permits enhanced binding to the *mtrC* promoter of either RNA polymerase or a transcriptional activator that increases expression of *mtrCDE*. The enhanced binding of either RNA polymerase or an activator to the *mtrC* promoter may be due to decreased competition for binding on the same region on the DNA. If the *mtrR* promoter, which overlaps the −35 promoter sequence of *mtrC*, is unoccupied by RNA polymerase or a transcriptional activator for *mtrR* because of the single-base-pair deletion, then perhaps the *mtrC* promoter would be unobstructed and more available for transcription factors to bind to it. The base pair deletion may also simply reduce the binding of an unidentified repressor for *mtrC*. These possibilities are now being examined.

When we compared the nucleotide sequence between the *mtrR* and *mtrC* genes to that which separates *acrR* and *acrA* of *E. coli*, it was noted that the *acrRAB* sequence (GenBank accession number U00734) (7) contains a 13-bp symmetrical repeat, 5′-TTTACATACATTT-3′, positioned 26 nucleotides upstream from *acrR* and 102 nucleotides upstream from *acrA*. Whether this represents a regulatory element similar to the 13-bp inverted repeat of the *mtr* system is not known. AcrR appears to be a repressor of *acrAB* expression (8) just as MtrR is a repressor of *mtrCDE* expression. Information concerning additional regulatory factors for the *acrRAB* genes has not been presented, to our knowledge.

Recent studies with highly HA-resistant gonococcal clinical isolates obtained from males with rectal infections (15) or urogenital infections (16) showed that all contained the base pair deletion present in strain KH15. Although the run of five T/A base pairs in the inverted repeat (Fig. 1) precludes an identification of the deleted base pair, it is important to note that a deletion occurring in the other eight base pairs of the inverted repeat has not been observed among HA-resistant isolates. Accordingly, the 5-bp T/A sequence may be an important sequence in transcriptional control of the *mtr* efflux system. It is important to note, however, that other clinical isolates that were obtained from male patients with rectal or urogenital infections but that express lower levels of HA resistance display the intact 13-bp inverted repeat but typically have missense mutations that alter the DNA-binding domain

of MtrR (15, 16). Our examination of these clinical isolates has yet to identify a clinically isolated strain containing only the base pair deletion. Accordingly, it may be that intermediately HA-resistant variants containing missense mutations in the *mtrR* gene are first selected and that subsequent mutations in the promoter region (such as the base pair deletion in the inverted repeat) are required for survival at sites with higher concentrations of HAs.

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