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A Novel Mechanism of High-Level, Broad-Spectrum Antibiotic Resistance Caused by a Single Base Pair Change in Neisseria gonorrhoeae

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ABSTRACT The MtrC-MtrD-MtrE multidrug efflux pump of Neisseria gonorrhoeae confers resistance to a diverse array of antimicrobial agents by transporting these toxic compounds out of the gonococcus. Frequently in gonococcal strains, the expression of the mtrCDE operon is differentially regulated by both a repressor, MtrR, and an activator, MtrA. The mtrR gene lies 250 bp upstream of and is transcribed divergently from the mtrCDE operon. Previous research has shown that mutations in the mtrR coding region and in the mtrR-mtrCDE intergenic region increase levels of gonococcal antibiotic resistance and in vivo fitness. Recently, a C-to-T transition mutation 120 bp upstream of the mtrC start codon, termed mtrC120, was identified in strain MS11 and shown to be sufficient to confer high levels of antimicrobial resistance when introduced into strain FA19. Here we report that this mutation results in a consensus −10 element and that its presence generates a novel promoter for mtrCDE transcription. This newly generated promoter was found to be stronger than the wild-type promoter and does not appear to be subject to MtrR repression or MtrA activation. Although rare, the mtrC120 mutation was identified in an additional clinical isolate during sequence analysis of antibiotic-resistant strains cultured from patients with gonococcal infections. We propose that cis-acting mutations can develop in gonococci that significantly alter the regulation of the mtrCDE operon and result in increased resistance to antimicrobials.

IMPORTANCE Gonorrhea is the second most prevalent sexually transmitted bacterial infection and a worldwide public health concern. As there is currently no vaccine against Neisseria gonorrhoeae, appropriate diagnostics and subsequent antibiotic therapy remain the primary means of infection control. However, the effectiveness of antibiotic treatment is constantly challenged by the emergence of resistant strains, mandating a thorough understanding of resistance mechanisms to aid in the development of new antimicrobial therapies and genetic methods for antimicrobial resistance testing. This study was undertaken to characterize a novel mechanism of antibiotic resistance regulation in N. gonorrhoeae. Here we show that a single base pair mutation generates a second, stronger promoter for mtrCDE transcription that acts independently of the known efflux system regulators and results in high-level antimicrobial resistance.

Neisseria gonorrhoeae, the causative agent of the sexually transmitted infection gonorrhea, is a Gram-negative diplococcus and is strictly a human pathogen. Clinical isolates of N. gonorrhoeae frequently exhibit high levels of antimicrobial resistance mediated by multiple mechanisms, including active efflux of antimicrobials by four known efflux pumps (1–5). The MtrC-MtrD-MtrE efflux pump is a well-characterized system that recognizes and exports a wide variety of antimicrobial agents, including macrolide and β-lactam antibiotics, detergents, and host antimicrobial factors (1, 6). Transcription of the mtrCDE operon is differentially regulated by a repressor, MtrR, and an activator, MtrA (7–9). The mtrR gene is located 250 bp upstream of and is transcribed divergently from mtrCDE (3). MtrR represses the expression of mtrCDE via binding of two homodimers to pseudodirect repeats within the mtrCDE promoter (10); the MtrR helix-turn-helix (HTH) DNA-binding motif resides between residues 32 and 53 (7, 11).

A variety of mutations in mtrR and in the mtrR-mtrCDE intergenic region have been identified in antibiotic-resistant gonococcal strains recovered from outbreak investigations (12). Strain MS11, originally isolated in the 1960s from a patient with an uncomplicated cervical infection (13) and since used extensively by many researchers, exhibits higher levels of intrinsic in vitro resistance to MtrC-MtrD-MtrE substrates than do other laboratory
Additionally, we report that while the mtrCDE promoter appears to function independently of MtrR and MtrA regulation, the consensus sequence generated by the mtrCDE promoter elements, the consensus −10 sequence generated by the mtrCDE promoter, and the putative −35 element for the mtrCDE promoter are indicated in the expanded sequence. The transcription start site from the previously characterized mtrCDE promoter is marked by a single cross; the transcription start point from the mtrCDE promoter is marked by a double cross.

FIG 1  The mtr locus in N. gonorrhoeae. (A) Organization of the mtr locus. Bent arrows mark the mtrR and mtrCDE promoters (P). mtrR and mtrCDE are divergently transcribed on opposite strands. The locations of the mtrCDE promoter and the mtrC-lacZ fusion start sites are indicated. The hatched box represents the location of the expanded sequence. (B) Sequence of the mtrR-mtrCDE intergenic region. The previously characterized mtrR and mtrCDE promoter elements, the consensus −10 sequence generated by the mtrCDE promoter, and the putative −35 element for the mtrCDE promoter are indicated in the expanded sequence. The transcription start site from the previously characterized mtrCDE promoter is marked by a single cross; the transcription start point from the mtrCDE promoter is marked by a double cross.

strains (12). Sequence analysis of the MS11 mtr locus revealed that MS11 is a natural mtr mutant, containing an alanine-to-threonine substitution at position 39 in the MtrR DNA-binding domain, as well as a novel C-to-T transition mutation located 120 bp upstream of the mtrC promoter start codon (mtrC) (12). Introduction of the mtrC mutation into laboratory strain FA19 yielded one of the highest reported levels of MtrC-MtrD-MtrE-based antimicrobial resistance (12). Additionally, this mutation increased resistance to the host-derived antimicrobial compounds progesterone and CRAMP-38, the murine homologue of the human cathelicidin LL-37, suggesting that the mtrC mutation facilitates resistance to host defense mechanisms (12). In agreement with this hypothesis, the mtrC mutation increased in vivo fitness in a female mouse model of lower genital tract infection by nearly 3 logs compared to the wild-type strain FA19 during competitive infection in the absence of antibiotic treatment (12).

Here we demonstrate that the mechanism of mtrC-based antimicrobial resistance is the generation of a consensus −10 element (14) that acts as a second, stronger promoter for mtrCDE transcription, resulting in substantially increased pump expression and enhanced resistance to antimicrobials. This promoter appears to function independently of MtrR and MtrA regulation. Additionally, we report that while the mtrCDE mutation is rarer than other mutations that enhance mtrCDE expression, it was found in an additional multidrug-resistant strain that has been included in the 2008 WHO N. gonorrhoeae reference strain panel (15).

RESULTS
Analysis of the mtrCDE locus in MS11 revealed that this C-to-T transition creates the consensus sequence for a −10 element (TA TAAT) (Fig. 1) (14). A near-consensus −35 element sequence (TTGAGA) was located upstream of the potential −10 element; however, this putative −35 hexamer was separated from the potential −10 element by 24 bp rather than the optimal 17 bp (14). To determine if this −10 element could act to promote transcription, primer extension of mtrC was performed using total RNA from FA19 and DW120 cultures at mid-log phase (Fig. 2). As expected, primer extension of RNA from FA19 yielded a single mtrC transcript, which mapped to the previously identified transcription start site (Fig. 1) (1). The presence of the mtrC mutation, however, resulted in a second, shorter mtrC transcript that was more intense than the wild-type mtrC transcript, suggesting a higher concentration of the shorter transcript. Importantly, the start site for this second transcript mapped to a site 7 bp downstream of the −10 consensus sequence generated by mtrC, a reasonable distance to suggest that this −10 sequence could act to promote the expression of this transcript.

To rule out the possibility that the mutant transcript was a result of differential mRNA processing due to the mtrC mutation, β-galactosidase assays were carried out with FA19 containing a promoterless lacZ gene translationally fused to either the entire mtrC promoter region (mtrC-lacZ) or a truncated region lacking the wild-type mtrC promoter (mtrC-lacZ) (Fig. 3A) (16). The

FIG 2  Primer extension analysis of mtrC from FA19 and DW120. Primer extension products were generated using an mtrC-specific oligonucleotide (Table 4) hybridized to 50 μg of total RNA harvested from each strain. The DNA sequence was produced using the same oligonucleotide and is complementary to the mRNA. The wild-type and mutant sequences at the mtrC locus are expanded, with the mutated nucleotide in bold.

FIG 1  The mtr locus in N. gonorrhoeae. (A) Organization of the mtr locus. Bent arrows mark the mtrR and mtrCDE promoters (P). mtrR and mtrCDE are divergently transcribed on opposite strands. The locations of the mtrCDE promoter and the mtrC-lacZ fusion start sites are indicated. The hatched box represents the location of the expanded sequence. (B) Sequence of the mtrR-mtrCDE intergenic region. The previously characterized mtrR and mtrCDE promoter elements, the consensus −10 sequence generated by the mtrCDE promoter, and the putative −35 element for the mtrCDE promoter are indicated in the expanded sequence. The transcription start site from the previously characterized mtrCDE promoter is marked by a single cross; the transcription start point from the mtrCDE promoter is marked by a double cross.
mtrC-lacZ expression remained high when the wild-type (wt) promoter was disrupted and the sequence at position 120 was wild type (Fig. 3B). Expression of mtrC-lacZ expression was abrogated when the wild-type promoter was disrupted. (B) The mtrC mutant showed levels of mtrC-lacZ expression significantly higher than the wild-type promoter, in agreement with the more intense band seen in the primer extension from the wild-type promoter (17), the mtrC wild-type sequence at this locus (Fig. 4). Additionally, the absence of mtrR and mtrA did not affect levels of resistance to pump substrates in strains with mtr120 suggesting that, unlike the wild-type promoter (17), the mtr120 promoter is not subject to MtrR or MtrA regulation.

To verify that the observed increased antimicrobial resistance was due to increased levels of MtrC-MtrD-MtrE pump production, Western blot analysis was conducted to determine the effect of the mtr120 mutation on MtrE production. In agreement with the results of the MIC assays, strains bearing the mtr120 mutation produced much greater amounts of MtrE than strains with a wild-type sequence at this locus (Fig. 4). Additionally, the absence of MtrR and MtrA did not affect levels of resistance to pump substrates in strains with mtr120, further suggesting that these regulators do not act on the promoter generated by this mutation.

The mtr120 mutation was originally identified in strain MS11 (12), which is a commonly utilized laboratory strain that has been used in gonococcal research for many years. Thus, to determine if the mtr120 mutation is present in strains isolated during recent clinical infection, as well as to compare its frequency to other mtr locus mutations in a clinical setting, the mtrR gene and the mtrR-

mtrCDE wild-type promoter, affect the resistance levels conferred by this mutation, we determined the MICs of antimicrobials against strains FA19 and DW120 and mtrR and mtrA knockout derivatives of each strain (genotypes described in Table 1, results shown in Table 2). In agreement with the increased mRNA levels detected in the primer extension experiment and high levels of β-galactosidase activity from the mtrC-lacZ fusions carrying the mtr120 mutation, the presence of mtr120 resulted in increased resistance to the MtrC-MtrD-MtrE pump substrates erythromycin (Erm), rifampin (Rif), crystal violet (CV), and Triton X-100 (TX-100). In contrast, the presence of mtr120 did not affect resistance to the nonpump substrate kanamycin (Km) in strains isogenic for mtrR or mtrA; increased Km resistance in mtrR and mtrA knockout strains is due to the presence of the aphA-3 cassette within these genes, and differences in resistance between strains with mtrR disruption and mtrA disruption may be attributed to differences in promoter strength for these genes. Importantly, the absence of MtrR and MtrA did not affect levels of resistance to pump substrates in strains with mtr120 suggesting that, unlike the wild-type promoter (17), the mtr120 promoter is not subject to MtrR or MtrA regulation.

<table>
<thead>
<tr>
<th>TABLE 1 Strains used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>FA19</td>
</tr>
<tr>
<td>DW120</td>
</tr>
<tr>
<td>KH9</td>
</tr>
<tr>
<td>CR1</td>
</tr>
<tr>
<td>EO1</td>
</tr>
<tr>
<td>EO2</td>
</tr>
<tr>
<td>RD1</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>TABLE 2 Sensitivity to substrates of the MtrC-MtrD-MtrE efflux system</th>
</tr>
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<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>--------</td>
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<tr>
<td></td>
</tr>
<tr>
<td>FA19</td>
</tr>
<tr>
<td>KH9</td>
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<tr>
<td>CR1</td>
</tr>
<tr>
<td>DW120</td>
</tr>
<tr>
<td>EO1</td>
</tr>
<tr>
<td>EO2</td>
</tr>
</tbody>
</table>
mtrC intergenic region of 113 clinical isolates and 8 WHO reference strains were selected based on their azithromycin MICs and sequenced. The azithromycin MIC range for the N. gonorrhoeae isolates sequenced, including the eight 2008 WHO reference strains (15), was 0.125 to 8 μg/ml, with 76% of the strains found to be resistant to azithromycin by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org) standard (MIC, >0.5 μg/ml). Among these strains, only one, the 2008 WHO reference strain WHO L (15), was found to contain the mtrR120 mutation. This strain was also found to contain the previously described G45D mutation in the HTH DNA-binding domain of MtrR (11). In contrast to the low frequency of the mtrR120 mutation, the previously defined single nucleotide (A) deletion in the 13-bp inverted repeat located between the −10 and −35 sequences of the mtrR promoter was found in 86 isolates (71%), of which 8 (7%) also had the G45D amino acid alteration in the coding region of mtrR (7). Moreover, five additional mutations in the promoter region of mtrR were found in a total of 12 isolates. Alteration of G45 [G45D (n = 11) and G45S (n = 3)] in the mtrR coding region alone was present in 14 isolates (12%). Other frequently occurring amino acid alterations found in the coding region of mtrR were A86T, which was found in 109 isolates (90%); Y105H, which was found in 25 isolates (21%); D79N, which was found in 9 isolates (7%); A39T, which was found in 7 isolates (6%); and L99G/H, which was found in 3 isolates (2%) (Table 3).

### DISCUSSION

The mtrR120 mutation is novel in its mechanism of providing antimicrobial resistance in that it creates an entirely new promoter for mtrCDE transcription and acts independently of the MtrR and MtrA transcription regulatory proteins. To our knowledge, this is the first report of such a mechanism of efflux pump regulation. Two precedents exist in N. gonorrhoeae, however, for the upregulation of efflux pumps through cis-acting point mutations at existing promoters. First, the expression of norM, a gene encoding a multidrug and toxic compound extrusion family exporter that contributes to quaternary ammonium compound, norfloxacin, and ciprofloxacin resistance, is upregulated by point mutations in the −35 hexamer (C to T) of the norM promoter or in the ribosome-binding site (A to G) (5). Second, a point mutation in the −10 hexamer (G to T) of the promoter for macAB, which encodes an ABC transporter family efflux system that contributes to macrolide resistance, results in the increased expression of this efflux pump (4). Like the mtrR120 mutation, these point mutations bring their respective promoter elements closer to the consensus sequences (TTGACA for the −35 element; AGGAGG for the ribosome-binding site), thereby enhancing recognition by RNA polymerase or, in the case of norM, the ribosome (14, 18). The mtrR120 mutation is novel, however, in that there is no expression from the wild-type sequence at this locus, and the consensus −10 element generated by the mtrR120 mutation acts as a second, independent promoter for mtrCDE transcription.

The mtrR120 mutation appears to offer the gonococcus a relatively simple and convenient mechanism of antibiotic resistance. The change of this single base pair significantly increases mtrCDE expression.

### TABLE 3

The MIC of azithromycin and frequency of recovery of mutations in the promoter region of mtrR and the coding region of mtrR and the mtrR120 mutation in 113 N. gonorrhoeae clinical isolates from 2002 to 2009 and 8 WHO N. gonorrhoeae reference strains from 2008

<table>
<thead>
<tr>
<th>Azithromycin MIC (μg/ml)</th>
<th>mtrR Promoter Mutation</th>
<th>Nonsynonymous Mutation in mtrR Coding Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azithromycin MIC (μg/ml) (no. of isolates)</td>
<td>ΔAΔ</td>
<td>C→A</td>
</tr>
<tr>
<td>0.125 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 (4)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>0.38 (14)</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>0.5 (9)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>0.75 (43)</td>
<td>34</td>
<td>1</td>
</tr>
<tr>
<td>1 (30)</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>1.5 (8)</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>2 (4)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>4 (2)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>6 (4)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>8 (1)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total (121)</td>
<td>86</td>
<td>6</td>
</tr>
</tbody>
</table>

\[a\] Deletion of A in 13-bp inverted repeat in the mtrR promoter.

\[b\] Transversion from C to A 19 nucleotides upstream of where the A deletion occurs.

\[c\] Insertion of one T 10 nucleotides downstream of where the A deletion occurs.

\[d\] Deletion of one G 34 nucleotides upstream of where the A deletion occurs.

\[e\] Deletion of one T 21 nucleotides upstream of where the A deletion occurs.

\[f\] Transversion from A to G in the inverted repeat 3 nucleotides upstream of where the A deletion occurs.
expression and confers high-level antimicrobial resistance with‐
out disrupting other components of the efflux system, including
the regulators MtrR and MtrA. Both MtrR and MtrA are global
regulators in \textit{N. gonorrhoeae}, controlling a multitude of genes out‐
side the mtrCDE operon, many of which are important for viru‐
ulence and \textit{in vivo} fitness (19, 20). Thus, the ability to upregulate
mtrCDE without affecting the regulation of other genes needed for
infection and survival would be a highly efficient and minimally
disruptive mechanism of developing antimicrobial resistance. It is
therefore somewhat surprising that this mutation is so rare, espe‐
cially compared to the frequency of mtrR promoter and coding
region mutations in the clinical isolates examined in this study.

The reason for the rarity of the mtr120 mutation is thus a matter
of speculation. The production of efflux pumps is an energy‐
expensive process, and it is therefore possible that the high levels
of MtrC-MtrD-MtrE production stimulated by this mutation stress
the gonococcus, resulting in slower or defective growth. In
this respect, Eisenstein and Sparling noted that a single base pair
deletion in the inverted repeat of the mtrR promoter, a mutation
which also confers high-level antibiotic resistance through in‐
creased transcription of mtrCDE, results in a lower growth rate \textit{in
vitro} (7, 21). However, unlike mtr120, this mutation was recovered
with high frequency in the strains sequenced in this study, and
with the more recent finding that MtrR acts as a global regulator in
the gonococcus, it is possible that the observed growth defect was
at least in part due to lack of mtrR expression. Additionally, we
have noticed no difference in the \textit{in vitro} growth kinetics of strains
carrying mtr120 and strains wild type at this site (data not shown),
and Warner et al. found that strain DW120 has a fitness advantage
\textit{in vivo} over FA19 in a female mouse model of lower genital tract
infection (12).

Another possibility to explain the rarity of the mtr120 mutation
is that the mutational event required for this change occurs less
frequently than those required for other mutations, particularly
the deletion in the inverted repeat. A specific nucleotide change at
a single base pair locus is required to generate the mtr120 pheno‐
type. However, one of any five base pairs may be deleted in the
inverted repeat to cause high-level resistance. Thus, it may be that
the mtr120 mutation is simply less likely to occur, which would
account for its scarcity in the isolates sequenced. Further analysis
of the mtr120 mutation and its overall effects is required to elucid‐
ate the reason for this mutation’s relative infrequency.

It is important to note that the mtr120 mutation was originally
identified in strain MS11 (12). This strain has been used exten‐
sively in the laboratory for studies on neisserial pili (22–25), Opa
proteins (26, 27), \textit{in vitro} cell infection (28, 29), antimicrobial
resistance (30), and \textit{in vivo} pathogenesis in male volunteers (31–
34). The possession of this rare mutation, however, makes MS11
uncommon compared to other gonococcal strains, enhancing its
resistance not only to antibiotics but also to host antimicrobial
compounds that are MtrC-MtrD-MtrE substrates, such as the an‐
imicrobial peptide LL-37 (6). MS11 has been found to be more
infectious than another commonly studied \textit{N. gonorrhoeae} strain,
FA1090, in experimental infection of male volunteers (35), and it
is likely that increased resistance to host antimicrobial com‐
ounds due to the mtr120 mutation plays an important role in this
increased infectivity. It is therefore important to consider this mu‐
tation when interpreting findings from previous studies using
strain MS11, particularly those involving antimicrobial resistance
and pathogenesis and human volunteer studies in which virulence

factors important in the evasion of innate defenses were assessed
(35).

It is also important to note that WHO reference strain WHO L
carries the mtr120 mutation. Although its levels of resistance to
Erm and azithromycin were found to be slightly higher than those
of reference strains with the single nucleotide deletion in the 13-bp
inverted repeat of the mtrR promoter, WHO L does not contain
this deletion, and its resistance was thus considered to be attribu‐
table to the G45D mutation in the HTH domain of MtrR (15).
However, as mutations in the HTH domain of MtrR generally
confer only low levels of antimicrobial resistance, it is far more
likely that the higher level of azithromycin resistance of WHO L is
due to the presence of \textit{mtr}120, which is an important factor to
consider in its use as a reference strain.

This study demonstrates the significant impact single base pair
mutations may have on gene expression and the development of
antimicrobial resistance and characterizes a novel \textit{cis}-regulatory
mechanism for efflux pump expression. Sequence analysis of
the promoter regions of other efflux pumps, both in \textit{Neisseria} and
in other pathogenic organisms, will determine if this mechanism is
widely used among pathogens or is unique to \textit{N. gonorrhoeae} and
\textit{mtrCDE}. Additionally, the mtr120 mutation provides a unique op‐
portunity for study of the physiological consequences of efflux
pump overexpression on bacterial cells. This single point muta‐
tion in a noncoding region allows overproduction of the MtrC-
MtrD-MtrE efflux pump without disruption or altered expression
of local or global regulatory proteins. Thus, the direct phenotypic
consequences of high-level efflux pump production can be exam‐
ined without the introduction of confounding effects on cell physi‐
ology due to altered regulation of other genes, a challenge which
has been difficult to overcome. Further study of the mtr120 muta‐
tion will help advance our understanding of antimicrobial resis‐
tance mechanisms, as well as elucidate the physiological conse‐
quences of efflux pump overexpression on bacterial cells.

\section*{Materials and Methods}

\subsection*{Bacterial strains and culture conditions.}

\textit{Gonococci} were routinely grown on GCB agar (\textit{Difco Laboratories}, Detroit, MI) containing defined supple‐
ments I and II (36) at 37°C under 4% CO$_2$ or in GCB broth (\textit{Difco Labora‐
tories}, Detroit, MI) containing defined supplements I and II and 0.048% (vol/vol) sodium bicarbonate with shaking at 37°C. \textit{E. coli} DH5$\alpha$ was rou‐
tinely grown on LB agar or in LB broth (\textit{Difco Laboratories}, Detroit, MI).

\textit{N. gonorrhoeae} strains used in this study are described in Table 1. The oligonucleotide primers used are listed in Table 4. Strains DW120,
KH9, and CR1 were previously described (1, 8, 12). Strain EO1 was con‐
structed by transformation of DW120 with the \textit{mtrA}\texttt{-}Km$^+$ gene from KH9
chromosomal DNA by PCR amplification using primers KH9\#10B, which
anneals 10 bp downstream of the \textit{mtrR} translational start site, and CEL1,
which anneals 120 bp downstream of the \textit{mtrR} translational stop site (1). Strain
EO2 was constructed by transformation of DW120 with the \textit{mtrA}: Km$^+$
gene from CR1 PCR amplified using primers C6, which anneals 255 bp
upstream of the \textit{mtrA} translational stop site, and C7, which anneals
232 bp downstream of the \textit{mtrA} translational start site. \textit{PCR} products were
purified using the Qiagen Quick PCR purification kit (Qiagen Inc., Valencia,
CA). Purified products were transformed into FA19, and transformants
were selected on GCB agar supplemented with 50 \textmu g/ml Km. Transforma‐
tions were performed as previously described (37). All transformants
were confirmed by \textit{PCR}.

\subsection*{Primer extension of \textit{mtrC}.}

Total RNA was prepared from gonococci by the method of Baker and Yanofsky (38). Primer extension analysis
of \textit{mtrC} was performed using SuperScript II reverse transcriptase (Invitro‐
gen Co., Carlsbad, CA) according to the manufacturer’s instructions.
Table 4: Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH9#10B</td>
<td>5’CCAAAAACGAAGCCCTTGAGAAAACCAA3’</td>
<td>mtrR::Km' amplification</td>
</tr>
<tr>
<td>CEL1</td>
<td>5’GACATGTCATGCGAGTATGAG3’</td>
<td>mtrR::Km' amplification</td>
</tr>
<tr>
<td>C6</td>
<td>5’CGACATTCCATTGCCTTCCGG3’</td>
<td>mtrA::Km' amplification</td>
</tr>
<tr>
<td>C7</td>
<td>5’GCCACGACGAAATGCGGAG3’</td>
<td>mtrA::Km' amplification</td>
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<tr>
<td>PEmtrC181</td>
<td>5’CCCTAGAAGCATAAAAAAGCCAT3’</td>
<td>Primer extension of mtrC</td>
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<tr>
<td>mtrC_3</td>
<td>5’AGTCGGATCCGGTTGACGAGGGCGGAT3’</td>
<td>Full mtrC-lacZ fusion</td>
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<tr>
<td>mtrC_4</td>
<td>5’AGTCGGATCCCAATTGAGACTGCATCTCAACT3’</td>
<td>Truncated mtrC-lacZ fusion</td>
</tr>
<tr>
<td>PmtrCmut</td>
<td>5’AGTCGGATCCGGTTGACGAGGGCGGATTATTTTTTATTCCGTGCAA TGCTATATGCAGCAAGAACCCA3’</td>
<td>Wild-type mtrC promoter 10 mutation for lacZ fusion</td>
</tr>
<tr>
<td>mtrC_7</td>
<td>5’AGTCGGATCCGAAAGCATAAAAAAGCC3’</td>
<td>Reverse mtrC promoter primer</td>
</tr>
<tr>
<td>mtrC_F</td>
<td>5’CGTTGCGGGTGCGTTTACGG3’</td>
<td>mtrR-mtrC intergenic region amplification</td>
</tr>
<tr>
<td>mtrC_R</td>
<td>5’CATCGCCTTAGAAGCATAAAAAAGCC3’</td>
<td>mtrR-mtrC intergenic region amplification</td>
</tr>
<tr>
<td>MTR1</td>
<td>5’AACAGGCCATCTTTATTCAG3’</td>
<td>mtrR amplification</td>
</tr>
<tr>
<td>MTR2</td>
<td>5’TTGAAGAATGCTTTTGGTC3’</td>
<td>mtrR amplification</td>
</tr>
</tbody>
</table>

Briefly, 50 μg of total RNA was reverse transcribed using the 5’-end 32P-labeled oligonucleotide primer PEmtrC181, which anneals to the first seven codons of mtrC (Table 4). To determine transcription start sites, primer extension products were electrophoresed on a 6% sequencing gel alongside reference sequencing reaction products derived from the PEmtrC181 primer using an mtrCDE promoter region PCR product amplified using primers mtrC_F and mtrC_R as templates. Sequencing was performed using the SequiTherm EXCEL II DNA sequencing kit (Epicenter Biotechnologies, Madison, WI) by following the manufacturer’s instructions. The dried gel was exposed to Kodak XAR film overnight at −70°C and developed using a Kodak X-Omat 1000A film processor.

Construction of mtrC-lacZ fusions. Translational lacZ fusions were constructed as previously described (16). Briefly, the mtrC promoter region from FA19 or DW120 was amplified using primers that introduce a BamHI restriction site at the end of the PCR products; primer sequences are listed in Table 4. For all four primers, mtrC_7, which anneals to the first six codons of mtrC, was used as the reverse primer. Forward primer mtrC_3 was used to amplify the mtrC promoter sequence beginning 239 bp upstream of the mtrC start codon, encompassing all mtrC promoter elements, to make fusions wild-type mtrC_lacZ and mtrC_120 mtrC_lacZ. Forward primer mtrC_4 was used to PCR amplify a region beginning 157 bp upstream of the mtrC start codon from FA19 or DW120, excluding the previously identified mtrC promoter and transcription start site (1), to make the wild-type mtrC_lacZ and mtrC_120 mtrC_lacZ fusions. Forward primer PmtrCmut was used to PCR amplify a region beginning 254 bp upstream of the mtrC start codon from FA19 or DW120 and mutate the −10 region of the wild-type mtrC promoter from TATAAT to TGTCAC. PCR products were digested with BamHI, and the resulting DNA fragments were inserted into the BamHI site of pLES94 (16). Recombinant plasmids were transformed into E. coli DH5α. Transformants were selected on LB agar containing 100 μg/ml ampicillin. Correct insertion and orientation were confirmed by PCR analysis and DNA sequencing. The plasmids were transformed into FA19 to allow insertion into the chromosomal proAB locus. Transformants were selected on GCB agar containing 1 μg/ml chloramphenicol.

Preparation of cell extracts and β-galactosidase assays. Strains containing translational mtrC-lacZ fusions were grown overnight on GCB agar plates containing 1 μg/ml chloramphenicol. Cells were scraped, washed once with phosphate-buffered saline (pH 7.4), and resuspended in lysis buffer (24 mM Na2HPO4, 16 mM NaH2PO4, 4 mM KCl, 0.4 mM MgSO4 ·7 H2O). Cells were lysed by repeated freeze-thaw cycles, and cell debris was removed by centrifugation at 9,300 x g for 10 min at 4°C. β-Galactosidase assays were performed as previously described (39).

MIC assays. The MICs of Erm, Rif, CV, TX-100, and Km were determined by 2-fold agar dilution assay (36). Strains were grown on GCB agar and resuspended in GCB broth to an optical density of 600 nm of 0.1, and 5-μl samples of these suspensions were inoculated onto GCB agar plates containing 2-fold serial dilutions of antibiotics. Plates were incubated
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overnight at 37°C under 4% CO₂. Differences in MIC values greater than 2-fold were considered significant.

**Western blot analysis of MtrE expression.** Whole-cell lysates from late-log-phase cultures (approximately 10⁶ cells per sample) were subjected to 10% SDS-PAGE and transferred to nitrocellulose. The membrane was probed with a 1:10,000 dilution of rabbit polyclonal antibodies against amino acids 110 to 120 of MtrE (RQGSLSGGVSV) (20). Detection was performed with a 1:10,000 dilution of goat-antirabbit IgG secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories, Hercules, CA) exposed to 5-bromo-4-chloro-3-indolylphosphate (BCIP) and Nitro Blue Tetrazolium (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions.

**Detection of the mtrR mutation in clinical isolates.** All examined clinical *N. gonorrhoeae* isolates (n = 113) were obtained at the National Reference Laboratory for Pathogenic Neisseria, Örebro University Hospital, Örebro, Sweden, from 2002 through 2009. Isolates were cultured from patients exposed to infection in many countries worldwide and were included based on having an azithromycin MIC of ≥0.25 µg/ml. Furthermore, the 2008 WHO *N. gonorrhoeae* reference strains (n = 8) were included for examination and quality control in all assays (15). The identities of all isolates were confirmed to the species level by the sugar utilization test and/or the Phadebat GC Monoclonal Test (Boule Diagnostics AB, Huddinge, Sweden), and they were preserved as previously described (40). The MIC (µg/ml) of azithromycin was determined using the Etest method (AB bioMérieux, Solna, Sweden) as previously described (41). The breakpoints used for susceptibility, intermediate susceptibility, and resistance were according to EUCAST; for azithromycin, susceptibility was ≤0.25 µg/ml and resistance was >0.5 µg/ml.

*N. gonorrhoeae* DNA was isolated in the NorDiag Bullet instrument (Nordlaj ASA Company, Oslo, Norway) using the BUGS’n BEADS STL-fast kit (Nordlaj ASA Company), according to the manufacturer’s instructions. To identify putative mutations that cause enhanced expression of the MtrC-MtrD-MtrE efflux pump, the mtrR-mtrC intergenic region was amplified in a LightCycler 1.2 real-time PCR system (Roche Molecular Biochemicals, Mannheim, Germany) using primers mtrC_F, which anneals 11 bp upstream of the mtrR start codon and 249 bp upstream of the mtrC start codon (17), and mtrC_R, which anneals 24 nucleotides downstream of the mtrC translational start (1). Additionally, the promoter and coding region of mtrR was amplified using primers MTR1 and MTR2 (42) as previously described (43). All PCR amplification products were purified prior to sequencing using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). Both DNA strands of amplicons were sequenced using the same primers as in the PCR amplification described above using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI 3120 Genetic Analyzer (Applied Biosystems) according to the manufacturer’s instructions. Nucleotide and amino acid multiple-sequence alignments were performed using the BioEdit (version 5.0.9) software.

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