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

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2011. A Novel Mechanism of High-Level, Broad-Spectrum Antibiotic Resistance Caused by a Single Base Pair Change in *Neisseria gonorrhoeae*. mBio 2(5): . doi:10.1128/mBio.00187-11.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
AUG 2011		**00-00-2011 to 00-00-2011**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
A Novel Mechanism of High-Level, Broad-Spectrum Antibiotic Resistance Caused by a Single Base Pair Change in Neisseria gonorrhoeae	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Emory University School of Medicine,Department of Microbiology and Immunology,1648 Pierce Drive, N.E.,Atlanta,GA,30322**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
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15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**9**	

RESEARCH ARTICLE

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 mtr_{120}, 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 mtr_{120} 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.**

Received 17 August 2011 **Accepted** 23 August 2011 **Published** 20 September 2011

Citation Ohneck EA, et al. 2011. A novel mechanism of high-level, broad-spectrum antibiotic resistance caused by a single base pair change in *Neisseria gonorrhoeae*. mBio 2(5):e00187-11. doi:10.1128/mBio.00187-11.

Editor E. Peter Greenberg, University of Washington

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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

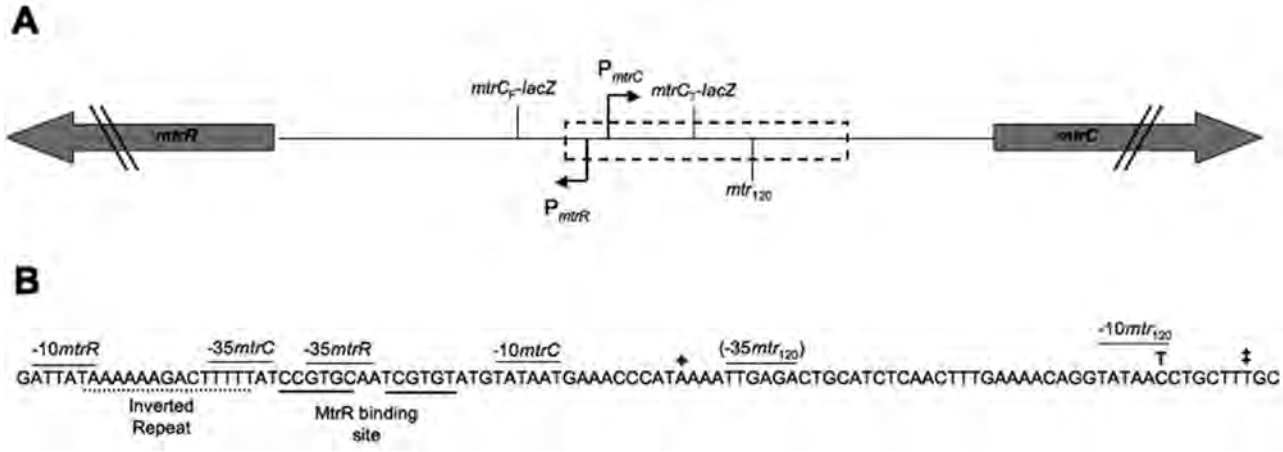

FIG 1 The *mtr* locus in *N. gonorrhoeae*. (A) Organization of the *mtr* locus. Bent arrows mark the *mtrR* and *mtrCDE* promoters (P). *mtrR* and *mtrCDE* are divergently transcribed on opposite strands. The locations of the mtr_{120} mutation and the *mtrC-lacZ* fusion start sites are indicated. The 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 mtr_{120} mutation, and the putative −35 element for the mtr_{120} 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 mtr_{120} 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* start codon (mtr_{120}) (12). Introduction of the mtr_{120} 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 mtr_{120} mutation facilitates resistance to host defense mechanisms (12). In agreement with this hypothesis, the mtr_{120} mutation increased *in vivo* fitness in a female mouse model of lower genital tract infection by nearly 3 logs compared to wild-type strain FA19 during competitive infection in the absence of antibiotic treatment (12).

Here we demonstrate that the mechanism of mtr_{120}-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 mtr_{120} 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 mtr_{120} locus in MS11 revealed that this C-to-T transition creates the consensus sequence for a −10 element (TA TAA**T**) (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 mtr_{120} 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 mtr_{120}, 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 mtr_{120} mutation, β-galactosidase assays were carried out with FA19 containing a promoterless *lacZ* gene translationally fused to either the entire *mtrC* promoter region ($mtrC_F$-*lacZ*) or a truncated region lacking the wild-type *mtrC* promoter ($mtrC_T$-*lacZ*) (Fig. 3A) (16). The

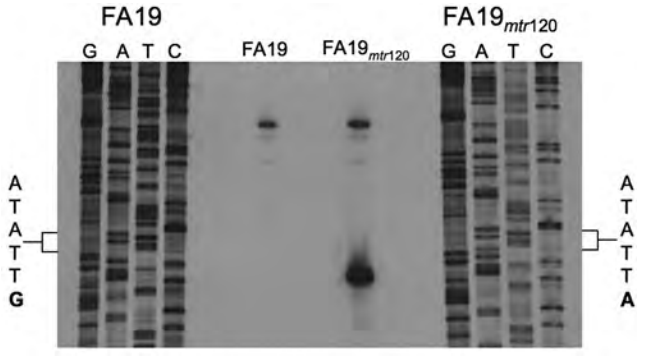

FIG 2 Primer extension analysis of *mtrC* from FA19 and DW120. Primer extension products were generated using an *mtrC*-specific oligonucleotide (Table 4) hybridized to 50 μg of total RNA harvested from each strain. The DNA sequence was produced using the same oligonucleotide and is complementary to the mRNA. The wild-type and mutant sequences at the mtr_{120} locus are expanded, with the mutated nucleotide in bold.

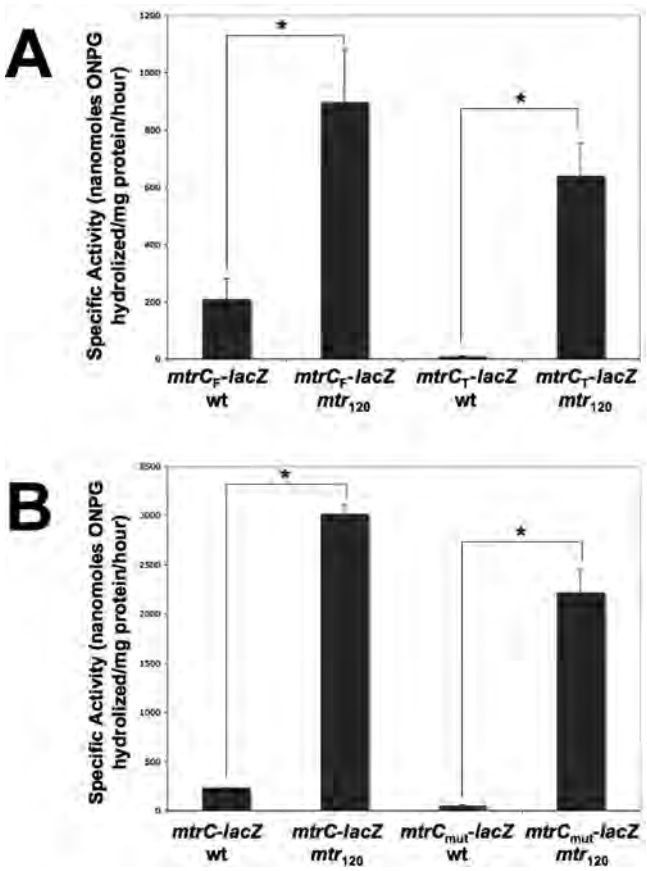

FIG 3 Expression of β-galactosidase from the mtr_{120} locus. The β-galactosidase activities per milligram of total protein in cell extracts of FA19 containing translational *mtrC-lacZ* fusions are shown. Assays were performed in triplicate. Error bars represent 1 standard deviation. Asterisks correspond to a *P* value of <0.01 (Student's *t* test). (A) The mtr_{120} locus is sufficient for *mtrC-lacZ* expression in the absence of the wild-type (wt) promoter. (B) The mtr_{120} locus promotes *mtrC-lacZ* expression when the wild-type *mtrC* promoter is inactivated.

mtr_{120} $mtrC_F$-*lacZ* fusion showed significantly higher β-galactosidase activity than the wild-type $mtrC_F$-*lacZ* fusion. As expected, the wild-type $mtrC_T$-*lacZ* fusion showed no β-galactosidase activity. However, the mtr_{120} $mtrC_T$-*lacZ* fusion showed levels of β-galactosidase activity significantly higher than those of the wild-type $mtrC_F$-*lacZ* fusion, demonstrating that the −10 element generated by the mtr_{120} mutation is sufficient for transcription in the absence of the wild-type promoter and is stronger than the wild-type promoter, in agreement with the more intense band seen in the primer extension from mtr_{120}. As further verification, we prepared *mtrC-lacZ* translational fusions containing a mutated −10 sequence (TATAAT to TGTCAC) of the wild-type *mtrCDE* promoter ($mtrC_{mut}$-*lacZ*). We observed that *mtrC-lacZ* expression was abrogated when the wild-type promoter was mutated and the sequence at position 120 was wild type (Fig. 3B). However, *mtrC-lacZ* expression remained high when the mtr_{120} mutation was present. On the basis of these findings, we propose that the mtr_{120} mutation defines a new and highly active promoter for *mtrCDE* transcription.

To characterize the resistance phenotype of the mtr_{120} mutation and determine if MtrR and MtrA, the known regulators of the *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 mtr_{120} mutation, the presence of mtr_{120} 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 mtr_{120} 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 mtr_{120}, suggesting that, unlike the wild-type promoter (17), the mtr_{120} 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 mtr_{120} mutation on MtrE production. In agreement with the results of the MIC assays, strains bearing the mtr_{120} 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 appear to affect MtrE levels in the presence of mtr_{120}, further suggesting that these regulators do not act on the promoter generated by this mutation.

The mtr_{120} 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 mtr_{120} 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*-

TABLE 1 Strains used in this study

Strain	Relevant genotype	Reference
FA19	Wild type	45
DW120	FA19 mtr_{120}	12
KH9	FA19 *mtrR*::Km^r	7
CR1	FA19 *mtrA*::Km^r	8
EO1	FA19 mtr_{120} *mtrR*::Km^r	This study
EO2	FA19 mtr_{120} *mtrA*::Km^r	This study
RD1	*mtrE*::Km^r	44

TABLE 2 Sensitivity to substrates of the MtrC-MtrD-MtrE efflux system

		MIC (μg/ml)				
Strain	Genotype	Erm	Rif	CV	TX-100	Km
FA19	Wild type	0.25	0.06	0.6	125	30
KH9	FA19 *mtrR*::Km^r	1	0.12	1.25	250	480
CR1	FA19 *mtrA*::Km^r	0.25	0.06	0.6	125	240
DW120	FA19 mtr_{120}	2	0.25	2.5	>16,000	30
EO1	FA19 mtr_{120} *mtrR*::Km^r	2	0.25	2.5	>16,000	480
EO2	FA19 mtr_{120} *mtrA*::Km^r	2	0.25	2.5	>16,000	240

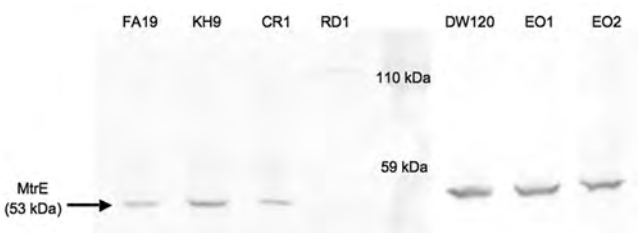

FIG 4 Expression of MtrE by wild-type and mtr_{120} mutant strains. Western blot analysis of whole-cell lysates from late-log-phase cultures was conducted using polyclonal rabbit MtrE-specific antibodies, followed by goat anti-rabbit IgG-alkaline phosphatase. Strain RD1 (44) contains a Km^r insertion in *mtrE* and was used as a negative control. The total protein from all strains was equally loaded, as assessed by Coomassie blue staining of a separate SDS-PAGE gel (data not shown).

mtrC intergenic region of 113 clinical isolates and 8 WHO reference strains were selected on the basis of 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 mtr_{120} 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 mtr_{120} 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 mtr_{120} 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 mtr_{120} 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 mtr_{120} 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 mtr_{120} mutation acts as a second, independent promoter for *mtrCDE* transcription.

The mtr_{120} 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*

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 mtr_{120} mutation in 113 *N. gonorrhoeae* clinical isolates from 2002 to 2009 and 8 WHO *N. gonorrhoeae* reference strains from 2008

	No. of isolates with:											
	mtrR promoter mutation						Nonsynonymous mutation in *mtrR* coding region					
Azithromycin MIC (μg/ml) (no. of isolates)	ΔA[a]	C→A[b]	T[c]	G[d]	T[e]	A→C[f]	G45D/G45S	A86T	Y105H	D79N	A39T	L99G/L99H	mtr_{120}
0.125 (2)								1					
0.25 (4)	4						2	4					
0.38 (14)	7	3					2	9	2	5	2	2	
0.5 (9)	4	2					1	7	2	2	1	1	1
0.75 (43)	34		1	1	2		10	41	12	1	2		
1 (30)	26	1					3	29	3	1	2		
1.5 (8)	7						1	8	1				
2 (4)	2					1	1	4	1				
4 (2)	2							2					
6 (4)			1				3	3	3				
8 (1)							1	1	1				
Total (121)	86	6	2	1	2	1	24	109	25	9	7	3	1

[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 C in the inverted repeat 3 nucleotides upstream of where the A deletion occurs.

expression and confers high-level antimicrobial resistance without disrupting other components of the efflux system, including the regulators MtrR and MtrA. Both MtrR and MtrA are global regulators in *N. gonorrhoeae*, controlling a multitude of genes outside the *mtrCDE* operon, many of which are important for virulence and *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, especially 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 mtr_{120} 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 in the *mtrR* promoter, a mutation which also confers high-level antibiotic resistance through increased transcription of *mtrCDE*, results in a lower growth rate *in vitro* (7, 21). However, unlike mtr_{120}, 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 *in vitro* growth kinetics of strains carrying mtr_{120} and strains wild type at this site (data not shown), and Warner et al. found that strain DW120 has a fitness advantage *in vivo* over FA19 in a female mouse model of lower genital tract infection (12).

Another possibility to explain the rarity of the mtr_{120} 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 mtr_{120} phenotype. 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 mtr_{120} mutation is simply less likely to occur, which would account for its scarcity in the isolates sequenced. Further analysis of the mtr_{120} mutation and its overall effects is required to elucidate the reason for this mutation's relative infrequency.

It is important to note that the mtr_{120} mutation was originally identified in strain MS11 (12). This strain has been used extensively in the laboratory for studies on neisserial pili (22–25), Opa proteins (26, 27), *in vitro* cell infection (28, 29), antimicrobial resistance (30), and *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 antimicrobial peptide LL-37 (6). MS11 has been found to be more infectious than another commonly studied *N. gonorrhoeae* strain, FA1090, in experimental infection of male volunteers (35), and it is likely that increased resistance to host antimicrobial compounds due to the mtr_{120} mutation plays an important role in this increased infectivity. It is therefore important to consider this mutation 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 mtr_{120} 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 attributable 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 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 *cis*-regulatory mechanism for efflux pump expression. Sequence analysis of the promoter regions of other efflux pumps, both in *Neisseria* and in other pathogenic organisms, will determine if this mechanism is widely used among pathogens or is unique to *N. gonorrhoeae* and *mtrCDE*. Additionally, the mtr_{120} mutation provides a unique opportunity for study of the physiological consequences of efflux pump overexpression on bacterial cells. This single point mutation 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 examined without the introduction of confounding effects on cell physiology due to altered regulation of other genes, a challenge which has been difficult to overcome. Further study of the mtr_{120} mutation will help advance our understanding of antimicrobial resistance mechanisms, as well as elucidate the physiological consequences of efflux pump overexpression on bacterial cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Gonococci were routinely grown on GCB agar (Difco Laboratories, Detroit, MI) containing defined supplements I and II (36) at 37°C under 4% CO_2 or in GCB broth (Difco Laboratories, Detroit, MI) containing defined supplements I and II and 0.048% (vol/vol) sodium bicarbonate with shaking at 37°C. *E. coli* DH5α was routinely grown on LB agar or in LB broth (Difco Laboratories, Detroit, MI).

The *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 constructed by transformation of DW120 with the *mtrR*::Km^r gene from KH9 chromosomal DNA by PCR amplification using primers KH9#10B, which anneals 10 bp downstream of the *mtrR* translational start site, and CEL1, which anneals 120 bp downstream of the *mtrR* translational stop site (1). Strain EO2 was constructed by transformation of DW120 with the *mtrA*::Km^r gene from CR1 PCR amplified using primers C6, which anneals 255 bp upstream of the *mtrA* translational stop site, and C7, which anneals 232 bp downstream of the *mtrA* translational start site. PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). Purified products were transformed into FA19, and transformants were selected on GCB agar supplemented with 50 μg/ml Km. Transformations were performed as previously described (37). All transformants were confirmed by PCR.

Primer extension of *mtrC*. Total RNA was prepared from gonococci by the method of Baker and Yanofsky (38). Primer extension analysis of *mtrC* was performed using SuperScript II reverse transcriptase (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions.

TABLE 4 Oligonucleotide primers used in this study

Name	Sequence	Purpose
KH9#10B	5′ CCAAAACCGAAGCCTTGAAAACCAA 3′	*mtrR*::Km^r amplification
CEL1	5′ GACAATGTTCATGCGATGATAGG 3′	*mtrR*::Km^r amplification
C6	5′ CGACATTCCATTCGTCTTCCGG 3′	*mtrA*::Km^r amplification
C7	5′ GCCACGACGGAAAATGCGGAG 3′	*mtrA*::Km^r amplification
PEmtrC181	5′ CCTTAGAAGCATAAAAAGCCAT 3′	Primer extension of *mtrC*
mtrC_3	5′ AGTCGGATCCGGTTTGACGAGGGCGGAT 3′	Full *mtrC-lacZ* fusion
mtrC_4	5′ AGTCGGATCCAATTGAGACTGCATCT CAACT 3′	Truncated *mtrC-lacZ* fusion
PmtrCmut	5′ AGTGGATCCGTTTCGGGTCGGTTTGACGAGGG CGGATTATAAAAAAGACTTTTTATCCGTGCAA TCGTGTATGTAGCACGAAACCCA 3′	Wild-type *mtrC* promoter −10 mutation for *lacZ* fusion
mtrC_7	5′ AGTCGGATCCGAAGCATAAAAAGCC 3′	Reverse *mtrC* promoter primer
mtrC_F	5′ CGTTTCGGGTCGGTTTGACG 3′	*mtrR-mtrC* intergenic region amplification
mtrC_R	5′ CATCGCCTTAGAAGCATAAAAAGCC 3′	*mtrR-mtrC* intergenic region amplification
MTR1	5′ AACAGGCATTCTTATTTCAG 3′	*mtrR* amplification
MTR2	5′ TTAGAAGAATGCTTTGTGTC 3′	*mtrR* amplification

Briefly, 50 μg of total RNA was reverse transcribed using the 5′-end ^{32}P-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 fusions, 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 $mtrC_F$-*lacZ* and mtr_{120} $mtrC_F$-*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 $mtrC_T$-*lacZ* and mtr_{120} $mtrC_T$-*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 Na_2HPO_4, 16 mM NaH_2PO_4, 4 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$). Cells were lysed by repeated freeze-thaw cycles, and cell debris was removed by centrifugation at 9,300 × *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 at 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

overnight at 37°C under 4% CO_2. 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 10^8 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 (RQGSLSGGNVS) (20). Detection was performed with a 1:10,000 dilution of goat anti-rabbit 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 mtr_{120} 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 ≥0.38 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 Phadebact 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 (Nordiag ASA Company, Oslo, Norway) using the BUGS'n BEADS STI-*fast* kit (Nordiag 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.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI021150-26 (W.M.S.) and AI031496-21 (P. F. Sparling, University of North Carolina), by a VA Merit Review Grant to W.M.S., and by grants from the Örebro County Council Research Committee and the Foundation for Medical Research at Örebro University Hospital, Örebro, Sweden (M.U.). W.M.S. is the recipient of a Senior Research Career Scientist Award from the VA Medical Research Service. E.A.O. was supported in part by NIH training grant 2T32AI007470.

We thank L. Pucko for help in manuscript preparation.

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