Loss-of-function mutations in the *mtr* efflux system of *Neisseria gonorrhoeae*

Wendy L. Veal,¹ Ansley Yellen,¹ Jacqueline T. Balthazar,^{1,3} Wubin Pan,^{2†} Brian G. Spratt^{2‡} and William M. Shafer^{1,3}

Author for correspondence: William M. Shafer. Tel: +1 404 728 7688. Fax: +1 404 329 2210.
e-mail: wshafer@emory.edu

¹ Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA

² Microbial Genetics Group, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK

³ Laboratories of Microbial Pathogenesis, Medical Research Service, VA Medical Center (Atlanta), Decatur, GA 30033, USA

Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents (HAs) has been ascribed to the *mtr* (multiple transferable resistance) operon. This operon is composed of the *mtrR* gene, which encodes a transcriptional repressor (MtrR), and a three-gene complex (*mtrCDE*), which encodes cell envelope proteins (MtrC–MtrD–MtrE) that form an energy-dependent efflux pump. HA-hypersusceptible strains are often isolated from patients, but the genetic basis for such hypersusceptibility was heretofore unknown. The genetic basis of HA hypersusceptibility in laboratory-derived strains BR54 and BR87 was studied to learn if this trait could be linked to mutations in the *mtr* operon. Mutations in the *mtrR* gene of these strains that could be phenotypically suppressed by mutations in their *mtrC* or *mtrD* genes were identified. Thus, small deletions (4–10 bp) in the *mtrC* or *mtrD* genes of strains BR87 and BR54 that would result in the production of truncated efflux pump proteins that serve as a membrane fusion protein (MtrC) or transporter of HAs (MtrD) were found to be responsible for their HA-hypersusceptible property.

Keywords: gonococci, efflux pump, antibiotic hypersusceptibility

INTRODUCTION

Following the introduction of penicillin to treat gonorrhoea, strains of *Neisseria gonorrhoeae* expressing clinically significant levels of penicillin resistance emerged. Gonococcal clinical isolates displaying hypersusceptibility to penicillin and structurally diverse hydrophobic agents (HAs) such as drugs, dyes, detergents and host-derived compounds (fatty acids and bile salts) also arose at nearly the same time as penicillin-resistant strains appeared. In one particular study, conducted in North Carolina in 1978, antibiotic-hypersusceptible strains represented upwards of 15% of all gonococcal clinical isolates (Eisenstein & Sparling, 1978). The antibiotic-hypersusceptibility profiles of

these clinical isolates were remarkably similar to a set of laboratory-derived mutants and transformants that contained mutations in different loci termed *env* (envelope) (Sarubbi *et al.*, 1975). On the basis of recombination studies (Eisenstein & Sparling, 1978; Shafer *et al.*, 1984), these antibiotic-hypersusceptible clinical isolates appeared to contain mutations in one of three different *env* loci, but most *env* mutations appeared to map at or near the *env-2* locus of the laboratory-derived HA-hypersusceptible strain BR87. Although *Env*[−] mutants and transformant strains were found to be hypersusceptible to structurally diverse HAs, many also contained a phenotypically suppressed mutation, now known to be in the *mtrR* gene (Hagman *et al.*, 1995), which could confer HA resistance when introduced into wild-type strain FA19 (Maness & Sparling, 1973; Sarubbi *et al.*, 1975; Shafer *et al.*, 1984). The biochemical basis for the *Env*[−] property of such mutants has not been resolved, although earlier studies suggested that such strains had decreased amounts of a 52 kDa outer-membrane protein and decreased cross-linking of their peptidoglycan (Guymon *et al.*, 1978). These biochemical changes in the cell envelope chemistry of *Env*[−] strains were postulated to be responsible for their enhanced permeability to HAs.

The *mtr* locus in *N. gonorrhoeae* (Maness & Sparling,

† **Present address:** TerraGen Diversity Inc., University of British Columbia, Vancouver, Canada V6T 1Z3.

‡ **Present address:** Wellcome Trust Centre for the Epidemiology of Infectious Diseases, Department of Zoology, Oxford University, South Parks Road, Oxford OX1 3PS, UK.

Abbreviations: HA, hydrophobic agent; MFP, membrane fusion protein; TX-100, Triton X-100.

The GenBank accession number for the sequence of the *mtrC* gene of strain BR87 reported in this paper is AF037041 and that of the *mtrD* gene of strain BR54 is AF037040.

Table 1. Properties of HA resistant and susceptible isogenic strains

Strain	Genotype	Source	MIC ($\mu\text{g ml}^{-1}$)*		
			Ery	PenG	TX-100
FA19	Wild-type	P. F. Sparling	0.25	0.007	100
FA140	<i>penA penB mtrR-140</i>	P. F. Sparling	2.00	1.000	>16000
BR54	<i>penA penB mtrR-140 mtrD-54 (env-3)†</i>	P. F. Sparling	0.06	0.060	25
BR87	<i>penA penB mtrR-87 mtrC-87 (env-2)†</i>	P. F. Sparling	0.06	0.060	25
WV1	As BR54 but <i>mtrD</i> ⁺	FA19‡ × BR54	2.00	1.000	>16000
WV2	As BR54 but <i>mtrD</i> ⁺	Spontaneous mutant	1.00	0.500	>16000
WV3	As BR54 but <i>mtrD</i> ⁺	WV2‡ × BR54	1.00	0.500	>16000
AY1	As BR87 but <i>mtrC</i> ⁺	FA19‡ × BR87	1.00	0.125	>16000

* Ery, erythromycin; PenG, penicillin G.

† Former designations (Sarubbi *et al.*, 1975) for the *mtrD-54 (env-3)* and *mtrC-87 (env-2)* genes of strains BR54 and BR87, respectively, are in parentheses.

‡ Transformant strain WV1 was obtained using the 964 bp PCR product from the *mtrD* gene of strain FA19 (Fig. 1), transformant WV3 was obtained using the same 964 bp PCR product from WV2 and transformant AY1 was obtained using the PCR-amplified *mtrC* gene from strain FA19.

1973; Guymon & Sparling, 1975) has been recently characterized (Pan & Spratt, 1994; Hagman *et al.*, 1995) and consists of the *mtrR* gene, positioned 250 bp upstream and divergently transcribed from a three-gene (*mtrCDE*) complex (Fig. 1) encoding cell envelope proteins that form an energy-dependent efflux pump (Lucas *et al.*, 1995). This efflux pump, not altered cell envelope permeability (Guymon & Sparling, 1975), appears to be responsible for the HA-resistant property exhibited by certain strains of gonococci (Pan & Spratt, 1994; Shafer *et al.*, 1995). Moreover, the *mtr* efflux system is crucial for expression of maximal levels of chromosomally mediated penicillin resistance that results from mutations at unlinked genes (Sparling *et al.*, 1975).

The MtrR protein appears to be a transcriptional repressor and performs a critical role in regulating transcription of the *mtrCDE* gene complex (Hagman *et al.*, 1995). Thus, mutations within the *mtrR*-coding region or a single base pair deletion in a 13 bp inverted repeat in its promoter enhance *mtrCDE* gene expression leading to elevated HA resistance (Hagman *et al.*, 1995). However, overexpression of the *mtrCDE* gene complex has a physiological consequence since it is known (Eisenstein & Sparling, 1978) that transformant strains bearing *mtrR* mutations display a slower growth rate than that of the wild-type parental strain. A possible selective advantage *in vivo*, in the absence of HAs (or penicillin), for strains bearing *env* mutations is that the reduced growth rate imparted by mutations in *mtrR* can be reversed to wild-type levels by a co-resident mutation in *env-2* or *env-3* (Eisenstein & Sparling, 1978).

Based on the deduced amino acid sequences of the MtrC–MtrD–MtrE proteins, it is clear that the *mtr* efflux pump in gonococci is similar to the MexA–MexB–OprM pump of *Pseudomonas aeruginosa* and

the AcrA/E–AcrB/F–TolC systems of *Escherichia coli* (reviewed by Nikaido, 1994, 1996; Paulsen *et al.*, 1996). The gonococcal MtrC protein belongs to the membrane fusion protein (MFP) family (Saier *et al.*, 1994) that links the cytoplasmic-membrane MtrD transporter protein, which belongs (Hagman *et al.*, 1997) to the resistance/modulation/division protein family (Saier *et al.*, 1994), to the MtrE outer-membrane protein. The MtrE protein probably forms an outer-membrane channel that facilitates export of HAs (Delahay *et al.*, 1997), in a manner proposed for the TolC and OprM outer-membrane proteins of *E. coli* and *P. aeruginosa*, respectively (Nikaido, 1996). As the *mtrCDE*-encoded efflux pump performs a predominant role in determining levels of gonococcal resistance to HAs, including toxic fatty acids and bile salts that bathe certain mucosal surfaces (Morse *et al.*, 1982), we analysed whether the *Env*[−] strains studied previously by Sarubbi *et al.* (1975) contain mutations in their *mtrCDE* gene complex. We now report that these *Env*[−] strains contain small deletions in their *mtrC* or *mtrD* genes and that these mutations are responsible for their HA-hypersusceptible trait.

METHODS

Strains of *N. gonorrhoeae* employed and growth conditions.

Strain FA19 and a panel of isogenic transformant strains (Table 1) were kindly provided by P. F. Sparling (University of North Carolina, Chapel Hill, NC, USA). HA hypersusceptibility was defined when the MIC of erythromycin was <0.12 $\mu\text{g ml}^{-1}$ and that of Triton X-100 (TX-100) was <62 $\mu\text{g ml}^{-1}$. All strains were propagated on GCB agar (Difco) containing glucose and iron supplements (Shafer *et al.*, 1984) with incubation at 37 °C under 3.8% (v/v) CO₂.

PCR amplification and DNA sequencing. Chromosomal DNA was prepared by the method of McAllister & Stephens (1993) and used in PCR amplification reactions as described pre-

Table 2. Oligonucleotides used to amplify *mtrRCDE* genes

Gene amplified	Oligonucleotide	Sequence	Site*
<i>mtrR</i>	KH9#3	5'-GACGACAGTGCCAATGCAACG-3'	47 nt downstream of <i>mtrC</i> start; C strand of <i>mtrR</i>
	CEL#1	5'-GACAATGTTTCATGCGATGATAGG-3'	114 nt downstream of <i>mtrR</i> stop; NC strand of <i>mtrR</i>
<i>mtrC</i>	KH9#1	5'-GTCGCAGATACGTTGGAACAACG-3'	174 nt downstream of <i>mtrR</i> start; C strand of <i>mtrC</i>
	<i>mtrD</i> #2	5'-GCCGTACCCAACTGCCCTTGC-3'	702 nt downstream of <i>mtrD</i> start; NC strand of <i>mtrC</i>
<i>mtrD</i>	KH9#4	5'-CATCACTTGTGCGGACGCACC-3'	151 nt downstream of <i>mtrD</i> start; NC strand of <i>mtrC</i>
	<i>mtrD</i> #1	5'-CGGCATCTGAAGCCAAACCTGC-3'	10 nt upstream of <i>mtrC</i> stop; C strand of <i>mtrD</i>
	<i>mtrE</i> #1	5'-GATGGAAGAAACCGATGTGTCG-3'	120 nt downstream of <i>mtrE</i> start; NC strand of <i>mtrD</i>
	<i>mtrD</i> #10	5'-AGCATCAACCTGCAAGACCGC-3'	2028 nt downstream of <i>mtrD</i> start; C strand of <i>mtrD</i>
	<i>mtrD</i> #7	5'-ATATACAGGGGAACCACGCCC-3'	208 nt upstream of <i>mtrD</i> stop; NC strand of <i>mtrD</i>
<i>mtrE</i>	<i>mtrE</i> #2	5'-CCTTTGCATTGTCTGCCTGCAC-3'	43 nt downstream of <i>mtrE</i> start; C strand of <i>mtrE</i>
	<i>mtrE</i> #3	5'-CGGTTTGGGTATCCGTTTCAATC-3'	5 nt upstream of <i>mtrE</i> stop; NC strand of <i>mtrE</i>

* Sites of annealing of oligonucleotides at nucleotide positions are shown upstream or downstream from translational start or stop codons for *mtrRCDE* genes that are on coding (C) or non-coding (NC) strands.

viously (Hagman *et al.*, 1995). The oligonucleotide primers used in this investigation are described in Table 2. DNA sequencing was performed on PCR products using the cycle sequencing protocol (Hagman *et al.*, 1995) or by automatic DNA sequencing; automatic sequencing was performed by the Nucleic Acid Core Facility of Emory University. Nucleotide and amino acid sequence analysis was performed using DNASTar.

Transformation and isolation of spontaneous mutants. Piliated gonococci were transformed with bulk chromosomal DNA or PCR products prepared from strain FA19 essentially as described by Sarubbi *et al.* (1975). Transformation experiments initially employed PCR products immediately after the amplification reaction. In subsequent experiments, products with transforming activity were purified by agarose gel electrophoresis to exclude transformation due to the input template DNA. Erythromycin-resistant transformants of strains BR54 and BR87 were selected using erythromycin at 0.12 or 0.25 µg ml⁻¹. Transformants were scored for levels of resistance to erythromycin, penicillin G and TX-100 as described previously (Shafer *et al.*, 1984). Spontaneous HA-resistant mutants were selected by plating suspensions onto GCB agar containing erythromycin (0.12 or 0.25 µg ml⁻¹) or TX-100 (500 µg ml⁻¹).

SDS-PAGE and Western blotting. Whole-cell lysates of gonococcal strains were prepared as described previously (Hagman *et al.*, 1995), while total cell envelope proteins were prepared as described by Clark *et al.* (1987). Proteins were separated by SDS-PAGE (Laemmli, 1970) and electroblotted to nylon membranes (Hagman *et al.*, 1995). MtrE was detected using an anti-MtrE mouse antiserum (kindly provided by C. Ison, St

Mary's Hospital Medical School, London, UK) and goat anti-mouse IgG conjugated to alkaline phosphatase.

RESULTS AND DISCUSSION

Presence of phenotypically suppressed *mtrR* mutations in HA-hypersusceptible transformant strains

HA-hypersusceptible strains BR54 (*env-3*) and BR87 (*env-2*) were generated in P. F. Sparling's laboratory in the mid-1970s (Sarubbi *et al.*, 1975) using donor DNA from HA-hypersusceptible strains FA32 (for BR54) or FA47 (for BR87) to transform HA-resistant strain FA140. The *env-3* and *env-2* mutations in strains BR54 and BR87 were postulated to occur at different sites because they recombine frequently, yielding HA-resistant transformants (Sarubbi *et al.*, 1975; Eisenstein & Sparling, 1978; Shafer *et al.*, 1984). Sarubbi *et al.* (1975) found that strains BR54 and BR87 could also donate high-level HA resistance to wild-type strain FA19 in these transformation experiments, suggesting that they also contain a phenotypically suppressed mutation in their *mtrR* gene (Pan & Spratt, 1994). In support of this hypothesis, we previously found (Hagman *et al.*, 1995) that the *mtrR* gene of strain BR87 differed from that of strain FA19 by a missense mutation at codon 45 (GGC to GAC), which resulted in the replacement of the glycine residue with an aspartic acid at position 45, which is within the helix-turn-helix region of the MtrR

Fig. 1. Organization of the *mtr* efflux operon of *N. gonorrhoeae* strain FA19. On the line marked FA19, the gene products and their function, the size (not drawn to scale) in bp of each gene and the approximate sites of annealing and direction of oligonucleotide primers used to amplify the *mtrCDE* genes from strain FA19 for transformation experiments are described. The arrows with dashed lines underneath the promoter region show the direction of transcription of the *mtrR* and *mtrCDE* genes (Hagman & Shafer, 1995). The glycine residue at position 45 of the MtrR protein produced by strain FA19 is shown (G). The glycine-45 to aspartic acid (D) substitution within the MtrR protein produced by strains BR87 and BR54 is shown. In addition to this missense mutation, strain BR54 also contains a single base pair deletion within the 13 bp inverted repeat localized within the *mtrR* promoter (data not presented). The regions of the *mtr* operon of strain FA19 capable of transforming strains BR87 and BR54 for HA resistance are shown in the boxed regions on their respective lines. The darkened box within the *mtrD* gene of strain BR54 shows the region (nucleotide positions 2029–2993) mapped by transformation that contained the 10 bp deletion responsible for HA hypersusceptibility in this strain.

repressor (Pan & Spratt, 1994). This radical amino acid substitution abrogates binding of the MtrR repressor to its target DNA sequence, the overlapping, divergent promoters used for *mtrR* and *mtrCDE* expression (Lucas *et al.*, 1997). Importantly, when introduced into strain FA19 by transformation, this missense mutation provided for increased HA resistance (Hagman *et al.*, 1995).

To determine the nature of the phenotypically suppressed *mtrR* gene in strain BR54, we sequenced its *mtrR* gene and that of HA-resistant parental strain FA140. Using oligonucleotide primers previously employed (Table 2; Shafer *et al.*, 1995) to amplify the *mtrR* gene, we determined that the *mtrR* gene from strains BR54 and FA140 also contained the missense mutation in codon 45 present in BR87 (Fig. 1) but, unlike BR87, they also contained a single base pair deletion in the 13 bp inverted repeat within the promoter utilized for *mtrR* gene transcription (Hagman & Shafer, 1995). This base pair deletion in the *mtrR* promoter abrogates transcription of *mtrR* but enhances transcription from the overlapping, divergent promoter used for *mtrCDE* gene transcription (Hagman & Shafer, 1995) and is sufficient to provide high-level HA resistance (Hagman *et al.*, 1995; Shafer *et al.*, 1995).

Strains BR87 and BR54 contain small deletions in their *mtrC* and *mtrD* genes, respectively

Since the HA-susceptibility profiles of strains BR54 and BR87 (Sarubbi *et al.*, 1975 and Table 1) resembled those of mutants of strain FA19 that contained insertionally

inactivated *mtrCDE* genes (Hagman *et al.*, 1995, 1997; Delahay *et al.*, 1997), we hypothesized that the *env-2* and *env-3* mutations might reside within the *mtrCDE* gene complex. To test this hypothesis, the *mtrC*, *mtrD* and *mtrE* genes from wild-type strain FA19 were amplified by PCR and used to transform strains BR54 and BR87 for HA resistance.

With strain BR87 (*env-2*) as the recipient, only the *mtrC*-encoding sequence (obtained with oligonucleotide primers KH9#1 and *mtrD*#2; Table 2) from strain FA19 exhibited transforming activity (Fig. 1). A representative transformant (strain AY1) displayed enhanced resistance to TX-100 and a twofold increase in penicillin resistance. DNA sequencing of the *mtrC* gene from strains BR87, FA140 and AY1 was undertaken to identify the mutation(s) that might be responsible for HA hypersusceptibility in BR87. The results showed that compared to the *mtrC* gene from strain FA19 (GenBank accession no. U14993; Hagman *et al.*, 1995), the *mtrC* gene of strain BR87 contained a 4 bp deletion between nucleotide positions 349 and 352 and single nucleotide changes at codons 152 (GCG to GTG), 161 (GAA to GAG), 162 (GCA to GCG), 163 (AGC to GGC) and 390 (AAC to AAT) (Fig. 2). All of the single nucleotide changes, but not the 4 bp deletion, were also present in parental strain FA140 (data not presented). The 4 bp deletion represents, on the *mtrC*-coding strand, the last four nucleotides of a six dinucleotide (GC) repeat (341-GCGCGCGCGCGC-352; underline shows deletion in BR87) and generates a new stop codon (Fig. 2). Due to this frameshift, the MtrC protein produced by

Fig. 2. Identification of the 4 bp deletion (dashed line) and point mutations (double-underlined nucleotides) in the *mtrC* gene of HA-hypersusceptible strain BR87 compared to the sequences possessed by strains FA19 and AY1. The four solid lines flanked by numbers at the top of the figure show the nucleotide positions of the relevant sequences in the *mtrC* gene. The new stop codon (TAA) in the BR87 sequence is shown with a single underline.

	340	360	450	463	480	490	1167	1180
FA19	AGCGCGCGCGCAACTGGCA	CGATGCTGCGGTAA	CGAAGCAAGCG	AAACCAAGCTGCCG				
	GC Repeat							
BR87	AGCGCGCGC - - - AACTGGCA	CGATGCTG <u>TGGTAA</u>	CGA <u>GCGCGCGC</u>	AAATCAAGCTGCCG				
	4 bp Deletion		Stop					
AY1	AGCGCGCGCGCAACTGGCA	CGATGCTGCGGTAA	CGAAGCAAGCG	AAACCAAGCTGCCG				

FA19	2721	GGT GCG GCT GCG GGC <u>GTA ACC GGG</u> CGC AAC CTG TTT GAA GGA CTG TTG GGC	2772
		G A A A G V T G R N L F E G L L G	
		912	
BR54		GGT GCG GCT GCG GGC GCA ACC TGT TTG AAG GAG TGT TGG GCA GCG TTC CCT	
		G A A A G A T C L K D C W A A F P	
		**	
WV1		GGT GCG GCT GCG GGC <u>GTA ACC GGG</u> CGC AAC CTG TTT GAA GGA CTG TTG GGC	
		G A A A G V T G R N L F E G L L G	
WV2		GGT GCG GCT GCG GGC - - - AAC CTG TTT GAA GGA CTG TTG GGC	
		G A A A G N L F E G L L G	

Fig. 3. Nucleotide sequence of a 51 nucleotide region (nucleotides 2721–2772) of the *mtrD* gene of strains FA19, BR54, WV1 and WV2. The 10 bp region that is deleted in strain BR54 is shown in the underlined stretch in the sequence of strains FA19 and HA-resistant transformant strain WV1. The encoded amino acids are shown in the single-letter code. The additional 2 bp that are deleted in spontaneous mutant WV2 are shown with asterisks in the BR54 sequence. The 12 bp deleted in WV2 are shown by dashed lines.

strain BR87 would be only 152 amino acids in length, diverging from the MtrC protein of strain FA19 (412 amino acids; molecular mass of 42777 Da) after amino acid position 116. The nucleotide sequence of the *mtrC* gene from transformant strain AY1 (Table 1) was determined and the results showed that the 4 bp deletion (Fig. 2) and each of the six point mutations described above had been repaired so as to resemble the wild-type sequence. Since the MtrC lipoprotein is a member of the MFP family (Saier *et al.*, 1994), it is likely that the severely truncated MtrC protein produced by BR87 is unable to interact with MtrD or MtrE or both, resulting in the loss of overall structural integrity of the *mtr* efflux pump.

In contrast with the results obtained with BR87, only the *mtrD* sequence (obtained with oligonucleotide primers *mtrD*#1 and *mtrE*#1; Table 2) from strain FA19 was able to transform strain BR54 for HA resistance (Fig. 1). To localize the region of the 3.2 kb *mtrD* gene in strain BR54 responsible for its HA-hypersusceptible property, a panel of oligonucleotide primers that would amplify overlapping regions of the *mtrD* gene from strain FA19 (GenBank accession no. U60099) was used to generate PCR products for transformation. With these different PCR products, the transforming activity of the *mtrD*

sequence could be narrowed to a 964 bp fragment (obtained with oligonucleotide primers *mtrD*#10 and *mtrD*#7; Table 2) located near the 3'-end of the gene (shown as nucleotide positions 2029–2993 in Fig. 1). A representative transformant (WV1) was as HA resistant as the immediate parental strain of BR54 (strain FA140; Table 1).

A mutation in the *mtrD* gene from strain BR54 was identified by sequencing the aforementioned PCR product obtained from strains FA19, BR54 and transformant strain WV1. From this analysis it was determined that compared to strains FA19, FA140 and WV1, strain BR54 contained a 10 bp deletion (5'-CGTAACCGGG-3'; nucleotide positions 2736–2745 in the *mtrD* gene of strain FA19), while WV1 had this deletion repaired (Fig. 3). The 10 bp deletion would result in the production of an MtrD protein that would diverge from the wild-type sequence at amino acid position 912. Moreover, due to the introduction of a new stop codon (TGA; nucleotides 2825–2827 in the *mtrD* gene of strain FA19), the MtrD protein produced by strain BR54 would contain 938 amino acids (molecular mass of 99935 Da) compared to the 1067 amino acid MtrD protein produced by strain FA19 (molecular mass of 113928 Da). Importantly, a topology model for the MtrD protein of strain FA19 has been deduced (Hagman *et al.*, 1997) and it predicts the existence of 12 transmembrane domains, seven cytoplasmic loops and six periplasmic loops. From this model, the MtrD protein produced by BR54 would lack the last three C-terminal transmembrane domains as well as two periplasmic and cytoplasmic loops with the last 26 amino acids being located in the cytoplasm (M. Nilles, personal communication).

Isolation of spontaneous HA-resistant mutants

To identify mutations that might reverse the effects of the small deletions in the *mtrC* and *mtrD* genes in BR87 and BR54, respectively, spontaneous HA-resistant mutants of strains BR54 and BR87 were sought. Erythromycin-resistant mutants of strain BR54 arose at a low frequency (4×10^{-9}) but such mutants of BR87 could not be recovered (frequency $< 10^{-10}$) despite repeated efforts. A spontaneous erythromycin-resistant mutant (WV2) of BR54 displayed high-level resistance to TX-100 (MIC $> 16000 \mu\text{g ml}^{-1}$) but resistance to erythromycin and penicillin was twofold lower than that of FA140 or WV1 (Table 1). The DNA sequence of the

mtrD gene from strain WV2 that could be amplified by PCR with primers *mtrD*#10 and *mtrD*#7 was determined. The results showed that an additional 2 bp had been deleted in the *mtrD* sequence of strain WV2 (shown in Fig. 3 with asterisks above the BR54 sequence). This 2 bp deletion in strain WV2 would restore the correct reading frame for the *mtrD* gene but would also result in a loss of four amino acids (Val-Thr-Gly-Arg) prior to returning to the wild-type amino acid sequence (Fig. 3). The Thr-Gly-Arg sequence represents the first three amino acids of the fifth periplasmic loop predicted to exist for MtrD (Hagman *et al.*, 1997), but the deletion of the Val-Thr-Gly-Arg peptide is not predicted to significantly alter the topology map of MtrD (M. Nilles, personal communication). We confirmed the importance of the 2 bp deletion in the *mtrD* gene sequence in WV2 by transformation. Using a PCR product containing this region, we were able to obtain transformants of strain BR54 expressing enhanced HA resistance (see WV3 in Table 1) similar to that observed with donor strain WV2.

Heretofore, all mutations that altered HA resistance in gonococci have mapped to the *mtrR* regulatory gene (mutations that enhance resistance) or were laboratory-derived insertional mutations in the *mtrCDE* gene complex (mutations that decrease HA resistance). Moreover, a survey of the literature suggests that all other mutations in the resistance/nodulation/division protein class of efflux pumps (Saier *et al.*, 1994), of which the *mtr* system is a member, that decrease efflux pump activity represent regulatory mutants or strains with purposeful, laboratory-derived insertional mutations. Our finding that HA-hypersusceptible transformant strains BR54 and BR87 contain small deletions in their *mtrCD* genes is the first report, to our knowledge, of loss-of-function-type mutations in bacterial efflux pump genes.

The findings presented in this report clarify the mechanism by which strains BR54 and BR87, the first laboratory-derived HA-hypersusceptible strains isolated in the gonococcus (Sarubbi *et al.*, 1975), exhibit HA hypersusceptibility. While it was originally thought (Guymon & Sparling, 1975) that these strains had increased permeability for HAs, it is now clear that their respective *mtr* efflux pumps are abnormal. Thus, the small deletions in the *mtrC* or *mtrD* genes in strains BR87 and BR54 would result in truncated, partially nonsensical MtrC or MtrD proteins. Since MtrC is in the family of MFPs (Saier *et al.*, 1994) that are presumed to link the cytoplasmic-membrane efflux transporter protein to the outer-membrane protein that serves as a channel for exported antimicrobials (Nikaido, 1996), it is likely that this function is abolished in strain BR87. Truncation of the MtrD protein in strain BR54 could result in HA hypersusceptibility due to loss of its transporter capacity or an inability to interact with its cognate MFP protein (MtrC). We considered the possibility that the 10 bp deletion in the *mtrD* gene would impact expression of the *mtrE* gene, which is immediately downstream and in the same transcriptional unit,

but an anti-MtrE antiserum (Delahay *et al.*, 1997) recognized this 50 kDa MtrE protein in strains FA19, BR54, WV1 and WV2 (data not presented). Thus, the HA-hypersusceptible phenotype in strain BR54 seems to be due to an altered MtrD transporter protein and not loss or diminished expression of *mtrE*.

We also confirmed that HA-hypersusceptible strains BR54 and BR87 contain phenotypically suppressed mutations in their *mtrR* gene, which is consistent with the earlier prediction of Sarubbi *et al.* (1975). The mutations occur within the *mtrR*-coding sequence that determines the amino acid composition of the helix-turn-helix domain of MtrR or in a 13 bp inverted repeat sequence that lies between the -10 and -35 regions of the *mtrR* promoter. When transferred to wild-type strain FA19, any of these mutations are sufficient to increase HA resistance (Hagman *et al.*, 1995; Shafer *et al.*, 1995). We have evaluated two recent HA-hypersusceptible clinical isolates because the original report of Eisenstein & Sparling (1978) suggested that such strains would have both phenotypically suppressed mutations in *mtrR* and mutations similar to the *mtrC* mutation in strain BR87. We found that these two recent clinical isolates contained a single missense mutation in their *mtrR*-coding region that mapped to codon 40 (GCC to ACC), causing a replacement of alanine with threonine in the helix-turn-helix region of the MtrR repressor; this replacement is sufficient to mediate increased HA resistance in gonococci (Shafer *et al.*, 1995). Surprisingly, however, the genetic lesion for HA hypersusceptibility in these strains appears not to map within the *mtrCDE* complex (data not presented). Accordingly, these recent clinical isolates, as opposed to those studied in the 1970s (Eisenstein & Sparling, 1978), could have mutations that affect alternative efflux pumps or permeability systems or unlinked control systems that modulate *mtr* efflux activity. Nevertheless, the presence of phenotypically suppressed *mtrR* mutations in these strains and the presence of an unlinked mutation(s) that results in hypersusceptibility of gonococci to HAs is consistent with the earlier hypothesis (Shafer *et al.*, 1984) that *mtrR* mutations are first selected when gonococci are introduced into an environment bathed in HAs (such as the rectum). Subsequent secondary mutations that reverse the effect of *mtrR* mutations (HA resistance and slower growth rate) could arise when gonococci enter other sites that may be hostile for those slower growing gonococci harbouring *mtrR* mutations. The genetic basis for HA hypersusceptibility in these clinical isolates is now under investigation.

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