Missense mutations that alter the DNA-binding domain of the MtrR protein occur frequently in rectal isolates of *Neisseria gonorrhoeae* that are resistant to faecal lipids

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Resistance of *Neisseria gonorrhoeae* to structurally diverse hydrophobic agents (HAs) has been associated with missense or deletion mutations in the *mtrR* (multiple transferable resistance Regulator) gene of laboratory-derived strains but their prevalence in clinical isolates was heretofore unknown. Since faecal lipids provide strong selective pressure for the emergence of variants resistant to HAs (HA^R), the nucleotide sequence of the *mtrR* gene from rectal isolates of *N. gonorrhoeae*, which displayed different levels of HA^R, was determined. Compared to the *mtrR* gene possessed by the HA-sensitive strain FA19, each clinical isolate contained mutations in the coding and/or promoter regions of their *mtrR* gene. A missense mutation in codon 45 (Gly-45 to Asp) was the most common mutation found in the strains studied and impacted the structure of the helix–turn–helix domain of the MtrR protein thought to be important in DNA-binding activity. Two clinical isolates bearing a missense mutation in codon 45 also contained a single basepair deletion in a 13 bp inverted sequence positioned within the *mtrR* promoter region. Introduction of *mtrR* sequences amplified from the clinical isolates into strain FA19 revealed that acquisition of the single basepair deletion was correlated with high level HA^R while mutations in the *mtrR*-coding region provided for an intermediate level of HA^R.

Keywords: gonococci, transcriptional regulator, missense mutations, antimicrobial agents, *Neisseria gonorrhoeae*

INTRODUCTION

The genetic control of multi-antibiotic resistance expressed by *Neisseria gonorrhoeae*, as exemplified by the *mtr* (multiple transferable resistance) system, is of considerable interest because a single mutation can lead to resistance to not only antibiotics but also antibacterial compounds (e.g. fatty acids and bile salts) that bathe mucosal surfaces. Maness & Sparling (1973) initially described the *mtr* system and showed that it mediated resistance to a panel of hydrophobic agents (HAs) including drugs, dyes and detergents. Subsequent reports indicated that such resistant strains accounted for 12.5% of all clinical isolates (Morse *et al.*, 1982). Moreover, strains displaying hyper-

sensitivity to penicillin and HAs, due to mutations in *env* loci, often contained a phenotypically suppressed mutation in their *mtrR* gene (Eisenstein & Sparling, 1978); these strains could serve as genetic donors in the horizontal spread of HA^R (Shafer *et al.*, 1984).

The *mtr* system is a multi-gene complex consisting of the *mtrR* gene, encoding the MtrR transcriptional repressor protein (Pan & Spratt, 1994), and three tandemly linked genes (*mtrCDE*) that are positioned 250 bp upstream and transcribed divergently from the *mtrR* gene (Hagman *et al.*, 1995). The *mtrCDE* genes constitute a single transcriptional unit and encode proteins (MtrC, MtrD and MtrE) similar to the MexABOprK membrane proteins of *Pseudomonas aeruginosa* (Poole *et al.*, 1993a), which mediate bacterial resistance to antibiotics by an efflux-based mechanism (Poole *et al.*, 1993b).

Missense mutations in the *mtrR*-coding region (Pan &

Abbreviations: CV, crystal violet; Ery, erythromycin; HA, hydrophobic agent; HTH, helix–turn–helix; TX-100, Triton X-100.

Spratt, 1994; Hagman *et al.*, 1995), as well as a single basepair deletion in the *mtrR* promoter region (Hagman *et al.*, 1995), were able to confer HA^R when introduced by transformation into the HA^S strain FA19. Since the frequency and importance of these mutations among clinical isolates was unknown, we determined the nucleotide sequence of *mtrR* genes from six strains isolated from patients with rectal gonorrhoea. These isolates were chosen for analysis because antibacterial faecal lipids have been proposed to select for HA^R variants (Morse *et al.*, 1982). The results revealed that missense mutations that cause radical amino acid substitutions within the proposed DNA-binding domain of the MtrR protein of laboratory-derived strains also occur frequently in HA^R clinical isolates.

METHODS

Gonococcal strains and growth conditions. The strains employed in this investigation and their levels of resistance to HAs are listed in Table 1. Strains FA171 and BR87 are genetic derivatives of strain FA19 that were constructed in the 1970s (Sparling *et al.*, 1975; Sarubbi *et al.*, 1975). Strain BR87, hypersusceptible to HAs, contains a missense mutation in codon 45 of its *mtrR* gene (Hagman *et al.*, 1995) that is phenotypically suppressed by an unlinked mutation, *env-2* (Sarubbi *et al.*, 1975). The presence of this missense mutation in strain BR87 prompted this investigation. Strain FA171 is a transformant of strain FA19. It was constructed (Sparling *et al.*, 1975) using donor DNA from an HA^R mutant (FA48) derived from an HA hypersusceptible mutant (FA47) of strain FA19 (Sarubbi *et al.*, 1975). Strains AP776, AP1058, AP1169, AP1192, NRL31115 and NRL31376 were rectal isolates obtained from non-partner homosexual men with gonorrhoea. These isolates were obtained in Seattle, Washington during the period of 1978–1980 and were previously found to be resistant to the antibacterial action of faecal lipids (Morse *et al.*, 1982). They exhibited varying levels of resistance to HAs (Table 1). All strains were grown as P⁺Op⁻ variants on GCB agar containing glucose and iron supplements (Shafer *et al.*, 1984) under 3.8% (v/v) CO₂ at 37 °C.

Antimicrobial susceptibility testing. The minimal inhibitory concentrations (MIC) of the HAs crystal violet (CV), erythromycin (Ery) and Triton X-100 (TX-100) for the strains studied were determined as described previously (Shafer *et al.*, 1984) and are presented in Table 1.

PCR amplification and DNA sequencing. A chromosomal DNA sequence encompassing the 633 bp *mtrR* coding region, 120 bp of sequence information past the translational stop codon, and 272 bp of sequence information upstream of the translational start codon was obtained by PCR amplification of chromosomal DNA preparations (McAllister & Stephens, 1993) using the oligonucleotide primers CEL-1 (5' GACAATGTTC-ATGCGATGATAGG 3') and KH9#3 (5' GACGACAGTG-CCAATGCAACG 3'). PCR amplification was for 30 cycles using a two-step programme consisting of a 1 min denaturation at 95 °C followed by extension at 60 °C. PCR products for use in sequencing and transformation were purified by agarose gel electrophoresis and electroeluted into TBE buffer (9 mM Tris, 9 mM boric acid, 2 mM EDTA, pH 8.0); at least two independent lots of PCR-amplified DNA were obtained from each strain. DNA sequencing was performed using the above described oligonucleotide primers, labeled with [γ -³²P]ATP (NEN DuPont). The AmpliTaq cycle sequencing kit (Perkin Elmer) was used as recommended by the manufacturer.

Transformation. A 0.5 ml sample of a culture of strain FA19 (P⁺) was incubated with 0.05 µg of agarose-gel-purified, PCR-amplified DNA in transformation broth (Shafer *et al.*, 1984) for 0.5 h prior to plating onto GCB agar. After incubation for 5 h at 37 °C under 3.8% (v/v) CO₂, transformants resistant to Ery (0.5 µg ml⁻¹) were selected using an agar overlay procedure (Sparling *et al.*, 1975). Transformants were screened for levels of resistance to CV, Ery and TX-100.

RESULTS

Deduced amino acid sequences of MtrR proteins

The nucleotide sequences of the *mtrR*-coding regions from the isogenic gonococcal strains FA19, FA171 and BR87, and the clinical isolates CH95 and 3035, were

Table 1. Strains of *N. gonorrhoeae* and susceptibility to HAs

Strain	Source	MIC* of:		
		CV	Ery	TX-100
FA19	P. F. Sparling, Dept of Medicine, University of North Carolina	2.5	0.25	0.125
BR87†		1.25	0.06	0.031
FA171		10	2.0	> 16
KH15	Transformant‡	10	2.0	> 16
AP776	Clinical isolates	10	2.0	2
AP1058		5	1.0	> 16
NRL31115		5	1.0	2
AP1169		10	2.0	> 16
AP1192		20	2.0	> 16
NRL31376		10	1.0	0.5

* Expressed as µg ml⁻¹ for CV and Ery and mg ml⁻¹ for TX-100.

† The *env-2* mutation renders strain BR87 hypersusceptible to HAs (Sarubbi *et al.*, 1975).

‡ Transformant of strain FA19 constructed using a PCR-amplified product from strain FA171 (Hagman *et al.*, 1995).

Compared to strain FA19, all six HA^R rectal isolates possessed nucleotide differences in their *mtrR*-coding sequences. Strain NRL31115 was of particular interest because it contained an 11 bp insert (5' TCTGCGACG AC 3') at nucleotide position 204 that represented an exact duplication of nucleotides 193–203 of the *mtrR*-coding region. This duplication would result in the production of a protein of 117 amino acids with a C-terminal domain that diverges from the FA19 MtrR sequence at amino acid position 69 (Fig. 1) and is truncated by 93 amino acids (data not presented). The other five rectal isolates lacked this duplication but each contained single missense mutations that resulted in radical amino acid replacements in their respective MtrR proteins. A missense mutation in codon 45 (GGC to GAC) occurred most frequently (strains AP1058, AP1169, AP1192 and NRL31376); this mutation was identical to that observed in the *mtrR* gene possessed by strain BR87. Strain AP776 contained a missense mutation in codon 40 (GCC to ACC), which resulted in the replacement of a hydrophobic amino acid

Representative transformants were scored for levels of cross-resistance to CV and TX-100 and the results (Table 2) showed that the transformants expressed either intermediate or high levels of HA^R. Differences in levels of HA^R expressed by the transformant strains were most apparent with respect to TX-100 resistance (TX-100^R). Thus, intermediate levels of TX-100^R (0.5–2.0 mg TX-100 ml⁻¹) were obtained when the donor DNA possessed only a missense mutation in codon 40 (strain AP776) or codon 45 (strain AP1058) or the nonsense mutation (strain NRL31115). However, donor DNA samples containing the single bp deletion in the *mtrR* promoter

Table 2. Transforming activity of PCR-amplified *mtrR* genes

Donor PCR-DNA	Nonsense or missense mutation	Single bp deletion	Transformation frequency†	MIC* towards transformants of:		
				CV	Ery	TX-100
FA19‡	—	—	< 10 ⁻⁹	NA	NA	NA
FA171	—	+	4 × 10 ⁻⁴	10	2.0	> 16
NRL31115	+	—	8 × 10 ⁻⁵	5	1.0	2
AP776	+	—	4 × 10 ⁻⁵	5	1.0	2
AP1058	+	—	5 × 10 ⁻⁶	5	1.0	0.5
AP1169	+	+	6 × 10 ⁻⁵	10	2.0	> 16

* Expressed as µg ml⁻¹ for CV and Ery, and mg ml⁻¹ for TX-100. High-level resistance is defined as TX-100 MIC > 16 mg ml⁻¹ and intermediate resistance is defined as TX-100 MIC 0.5–2.0 mg ml⁻¹. NA, Not applicable.

† Transformation frequencies were calculated as transformants per mg DNA per 10⁸ c.f.u. ml⁻¹.

‡ The MIC value for donor strain FA19 is shown in Table 1.

region with (strain AP1169) or without (strain AP171) an accompanying missense mutation in codon 45 were capable of transforming strain FA19 to high level TX-100^R (> 16 mg TX-100 ml⁻¹). The level of HA^R expressed by transformants obtained with the FA171, NRL31115, AP776 and AP1169 PCR products resembled that of their respective donor strains (Table 1). However, the level of HA^R expressed by transformants obtained with the PCR product from strain AP1058 was less than that of the donor strain (Table 2). This difference was more obvious with TX-100^R, suggesting that strain AP1058 contained a second but *mtr*-independent mechanism for resistance to the membrane-damaging action of this detergent. An *mtr*-independent mechanism for determining levels of gonococcal resistance to faecal lipids, which exert detergent-like action in membranes, has been previously proposed (McFarland *et al.*, 1983) and is supported by this data.

DISCUSSION

These results confirm and extend previous studies (Pan & Spratt, 1994; Hagman *et al.*, 1995) that implicated the MtrR protein as a regulator of HA^R in gonococci. The similarity between MtrR and certain transcriptional repressors, notably those belonging to the tetracycline repressor family (Brow *et al.*, 1985), have led to the suggestion that MtrR may function to control expression of genes that are responsible for the efflux of HAs. Indeed, our recent studies (Hagman *et al.*, 1995) indicate that MtrR regulates expression of the tandemly linked *mtrCDE* genes because mutations in the *mtrR*-coding or upstream sequences results in enhanced levels of the MtrC lipoprotein. MtrC (Hagman *et al.*, 1995) shares more than 40% identity at the amino acid level to the MexA (Poole *et al.*, 1993), AcrA (Ma *et al.*, 1993) and EnvC (Klein *et al.*, 1991) lipoproteins that participate in the efflux of antimicrobial agents from *P. aeruginosa* (MexA) and *E. coli* (AcrA and EnvC).

The missense mutation in codon 45 that we identified in four of the six clinical isolates was identical to that observed previously (Hagman *et al.*, 1995) in a laboratory-derived strain (BR87). Since this mutation is phenotypically suppressed in strain BR87 by an unlinked mutation in the *emv-2* locus (Sarubbi *et al.*, 1975) its significance was unknown. Hence, the presence of this mutation among the clinical isolates studied herein indicates that it is important in HA^R *in vivo*. Moreover, this and a second missense mutation in codon 40, which also results in a radical amino acid substitution within the HTH motif of MtrR, suggests that this domain is important in the ability of MtrR to regulate levels of HA^R in gonococci. It is possible that amino acid replacements in the first (Ala-40 to Thr) or second (Gly-45 to Asp) helical domains reduces the ability of MtrR to bind to its DNA target site. We hypothesize that this reduction in MtrR binding allows for enhanced expression of the *mtrCDE* genes. The mechanism by which the missense mutation in codon 105 (His-105 to Tyr) in the *mtrR* gene of strains CH95 and 3035 results in HA^R is more difficult to understand since the amino acid replacement is outside of the proposed DNA-binding domain of MtrR. However, changes in amino acid sequence downstream of the HTH region could affect tertiary structure or subunit interactions that are important in DNA-binding. The impact of this mutation emphasizes the complexity of MtrR-mediated regulation of HA^R in gonococci.

High level HA^R in gonococci due to the *mtr* system is dependent on a single basepair deletion located within the *mtrR* promoter region. This deletion is located within a novel 13 bp inverted repeat sequence (Fig. 2); however, the significance of this repeat sequence and the impact of the deletion are not yet clear. Nevertheless, two hypotheses can be advanced, which are not mutually exclusive, that could account for their roles in determining HA^R in gonococci. First, the inverted repeat sequence could serve as a recognition site for MtrR and loss of a single basepair might reduce binding. Second, by de-

creasing the spacing between the -10 and -35 regions of the *mtrR* promoter by a single bp the fidelity of transcription could be affected by this deletion, resulting in loss of *mtrR* expression. It is important to note that transformants of strain FA19 bearing a null mutation in the *mtrR* gene exhibited different levels of HA^R depending on whether they contained an intact 13 bp inverted repeat sequence (Hagman *et al.*, 1995). In this respect, strains bearing both a null mutation in *mtrR* and the single basepair deletion displayed higher levels of HA^R than isogenic strains containing the null mutation but having an intact 13 bp inverted repeat. Recent transcriptional studies indicate that this single basepair deletion inhibits expression of *mtrR* but enhances expression of the *mtrCDE* gene complex (K. E. Hagman & W. M. Shafer, unpublished results), a result that is consistent with the notion that the 13 bp inverted repeat sequence is a *cis*-acting transcriptional control element.

Mutations in the *mtrR* gene capable of transforming strain FA19 for enhanced resistance to HAs have been detected in rectal isolates and in strains isolated from patients with urogenital infections (Pan & Spratt, 1994; W. M. Shafer and others, unpublished observations), an observation that is consistent with the relatively high frequency at which HA^R strains are isolated (Morse *et al.*, 1982). The capacity of gonococci to increase HA^R through mutations in the *mtrR*-coding region or the *mtrR* promoter region, or by an *mtr*-independent mechanism, provides this pathogen with several mechanisms to resist toxic agents that bathe certain mucosal surfaces. Whether the different levels of HA^R afforded by these different genetic mechanisms are important in the ability of gonococci to survive at mucosal sites rich in HAs has yet to be determined.

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