Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system

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The *mtr* (multiple transferable resistance) system of *Neisseria gonorrhoeae* determines levels of gonococcal resistance to hydrophobic agents (HAs), including detergent-like fatty acids and bile salts that bathe certain mucosal surfaces. The genetic organization of the *mtr* system was determined and found to consist of the *mtrR* gene, which encodes a transcriptional regulator (MtrR), and three tandemly linked genes termed *mtrCDE*. The *mtrCDE* genes were organized in the same apparent transcriptional unit, upstream and divergent from the *mtrR* gene. The *mtrCDE*-encoded proteins of *N. gonorrhoeae* were analogous to a family of bacterial efflux/transport proteins, notably the MexABOprK proteins of *Pseudomonas aeruginosa* and the AcrAE and EnvCD proteins of *Escherichia coli*, that mediate resistance to drugs, dyes, and detergents. Inactivation of the *mtrC* gene resulted in loss of the MtrC lipoprotein and rendered gonococci hypersusceptible to structurally diverse HAs; this revealed the importance of the *mtr* system in determining HA^R in gonococci. Further support for a role of the *mtrCDE* gene complex in determining levels of HA^R in gonococci was evident when transformants bearing mutations in the *mtrR* gene were analysed. In this respect, missense and null mutations in the *mtrR* gene were found to result in increased levels of MtrC and HA^R. However, high levels of MtrC and HA^R, similar to those observed for clinical isolates, were associated with a single bp deletion in a 13 bp inverted repeat sequence that intervened the divergent *mtrR* and *mtrC* genes. We propose that the 13 bp inverted-repeat sequence represents a transcriptional control element that regulates expression of the *mtrRCDE* gene complex, thereby modulating levels of gonococcal susceptibility to HAs.

Keywords: antibiotic resistance, efflux, gonococci, membrane proteins, regulation

INTRODUCTION

The capacity of *Neisseria gonorrhoeae* to resist the toxic action of faecal lipids and bile salts is likely required for gonococci to survive at certain mucosal surfaces (Shafer *et al.*, 1984). Strains resistant to multiple, structurally diverse antimicrobial hydrophobic agents (HAs) are frequently isolated from homosexual males with rectal

infections (Morse *et al.*, 1982), presumably because toxic faecal lipids provide strong selective pressure for mutations that serve to enhance gonococcal HA^R. The molecular mechanism(s) responsible for gonococcal HA^R is unclear, although the *mtr* system has been implicated (Sparling *et al.*, 1978). In this respect, a report from over a decade ago (Morse *et al.*, 1982) estimated that 12.5% of patients with gonococcal disease are infected with strains that are HA^R due to the *mtr* system.

Although the gonococcal *mtr* system has been known for over 20 years (Maness & Sparling, 1973), it was not until recently (Pan & Spratt, 1994) that a mutation having the capacity to confer HA^R upon a highly sensitive strain (FA19) was identified. This mutation was shown to occur

Abbreviations: CBB, Coomassie Brilliant Blue; HA, hydrophobic agents; IEF, isoelectric focusing; mAb, monoclonal antibody; MFP, membrane fusion protein.

The *mtrC* nucleotide sequence has been assigned the GenBank accession number U14993.

in the *mtrR* gene, which encodes a 210 amino acid protein bearing significant similarity to numerous repressors of transcription (summarized by Pan & Spratt, 1994), particularly those of the tetracycline repressor family (Brow *et al.*, 1985), and the LuxR transcriptional-activator protein (Schwartzman *et al.*, 1992). The MtrR protein from strain FA19 differed from that possessed by two HA^R clinical isolates by only a single amino acid, which was outside the proposed DNA-binding domain of the MtrR protein. Accordingly, HA^R in gonococci was postulated to be due to a reduction of MtrR repressor activity, which resulted in enhanced expression of other gene(s) that mediate resistance. This hypothesis was consistent with earlier studies (Guymon *et al.*, 1978) that showed elevated levels of a membrane protein, particularly a lipoprotein termed the Mtr-associated protein (Chen *et al.*, 1985), in an HA^R transformant of strain FA19.

Subsequent cloning of the gene for the Mtr-associated protein, *mtrC*, which encodes a 44 kDa membrane lipoprotein described herein, revealed its close proximity to the *mtrR* gene. Divergently transcribed from *mtrR*, *mtrC* mapped 250 bp upstream of the regulatory gene and was considered a candidate for regulation by MtrR (Pan & Spratt, 1994). The predicted amino acid sequence for MtrC indicated that it was highly similar to two *Escherichia coli* membrane lipoproteins termed AcrA (Ma *et al.*, 1993) and EnvC (Klein *et al.*, 1991), and MexA of *Pseudomonas aeruginosa* (Poole *et al.*, 1993b). These proteins, together with AcrE, EnvD and MexB (along with OprK), are known to be important in determining levels of bacterial resistance to multiple antibiotics (Poole *et al.*, 1993b) and acridine dyes (Ma *et al.*, 1993) by an efflux-based mechanism (Nikaido, 1994). The levels of AcrAE and EnvCD in *E. coli* may be controlled by their respective regulatory proteins, AcrR and EnvR (Pan & Spratt, 1994), which are analogous to the MtrR protein of the gonococcus.

Our goal in this study was to determine the relationship between the gonococcal *mtr* system and other bacterial systems (Nikaido, 1994) that mediate resistance to structurally diverse antimicrobial agents. We now report the complete amino acid sequence for the MtrC lipoprotein and show that it is encoded within a four-gene complex, the *mtrRCDE* system, that mediates HA^R in gonococci. The *mtrRCDE* region of the gonococcal chromosome encodes four distinct proteins sharing homology to the products of three bacterial operons, *acrRAE* and *envRCD* of *E. coli* and *mexABoprK* of *P. aeruginosa*, involved in mediating resistance to structurally diverse antimicrobial agents by an efflux-based mechanism. Based on these similarities, we propose that the capacity of gonococci to resist the antimicrobial action of structurally diverse hydrophobic compounds, including fatty acids and bile salts that bathe mucosal surfaces, is due to an efflux-based system mediated by the MtrCDE proteins.

METHODS

Bacterial strains. The *N. gonorrhoeae* strains used in this study are described in Table 1. Strains FA19, FA171 and BR87 were kindly provided by P. F. Sparling (University of North

Carolina, Chapel Hill, NC, USA). Strains FA171 and BR87 were constructed in the early 1970s (Sarubbi *et al.*, 1975; Sparling *et al.*, 1975) by transformation and require special comment since they are now known to contain different mutations that can transform strain FA19 for different levels of HA^R (Table 1). In constructing strain FA171, donor DNA from strain FA48 (as FA19 but *mtrR-2* and *penB2*) was used to transform strain FA19 for HA^R. The construction of strain BR87 involved the introduction of DNA from strain FA47 (as FA19 but *env-2 str-7 penA2*) into strain FA140 (as FA19 but *penA2 mtrR-171 penB2*). Strain BR87, however, contains a phenotypically suppressed mutation in its *mtrR* gene, due to the presence of an unlinked mutation in the *env-2* locus (Sarubbi *et al.*, 1975), and it displays increased susceptibility to HAs (Table 1); phenotypic suppression of the *mtrR* gene in strain BR87 was inferred because its DNA could transform strain FA19 for HA^R (Sarubbi *et al.*, 1975). All other strains listed in Table 1 were constructed by transformation as described below.

Bacterial growth conditions. Gonococci were grown as P⁺ Op⁻ on GCB agar (Difco) containing defined supplements I and II (Shafer *et al.*, 1984), at 37 °C under 3·8% (v/v) CO₂. Liquid cultures of gonococci were grown in GCB broth containing supplements I and II and 0·043% (w/v) sodium bicarbonate at 37 °C with shaking. The MICs of various antimicrobial agents (listed in Table 1) were determined using twofold serial dilutions of the agent, as described previously (Sarubbi *et al.*, 1975). *E. coli* XL1-Blue was grown on either LB agar (Difco) plates or in LB broth at 37 °C in the presence or absence of 100 µg ampicillin ml⁻¹ (Sigma).

Analysis of MtrC. Whole cell lysates or membrane fractions (Clark *et al.*, 1987) of *N. gonorrhoeae* or *E. coli* were solubilized in Laemmli solubilization buffer (Laemmli, 1970), and subjected to SDS-PAGE and Western blotting as described previously (Judd *et al.*, 1991). Amounts of total protein in membranes were determined by the method of Bradford (1976). MtrC was detected using an anti-MtrC monoclonal antibody (mAb) (Judd *et al.*, 1991), or a polyclonal rabbit antiserum [kindly provided by S. Morse, Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA]. Both the rabbit antiserum and the goat anti-rabbit IgG (Cappel Laboratories) were absorbed three times with heat-killed *E. coli* XL1-Blue containing pBluescript when Western blots of *E. coli* whole cell lysates were analysed for the production of MtrC. Additional mAbs were used to ensure that equal amounts of protein from various whole cell lysates or membrane fractions were applied to SDS-polyacrylamide gels. These mAbs included 2E6 (kindly provided by M. Tam, Genetic Systems, Seattle, WA, USA), which recognizes the reduction-modifiable membrane-protein (Rmp) (Swanson *et al.*, 1982) and ML-30 (kindly provided by T. Shinnick, CDC) that recognizes a 43 kDa gonococcal protein bearing antigenic similarity to the 66 kDa stress protein of *Mycobacterium tuberculosis* (DeMarco de Hormaeche *et al.*, 1991). Proteins reactive with the mAbs or the polyclonal rabbit antiserum were detected with goat anti-mouse or goat anti-rabbit IgG coupled to alkaline phosphatase as described by Blake *et al.* (1984).

In certain experiments, proteins in whole cell lysates were separated by a two-dimensional (2-D) isoelectric focusing (IEF)-SDS-PAGE system (O'Farrell *et al.*, 1977; Dunbar *et al.*, 1990), using the Bio-Rad Mini-PROTEAN II apparatus. IEF was performed at 700 V for 4 h. The tube gels were then equilibrated with SDS-PAGE sample buffer for 20 min at room temperature and loaded onto 15% (w/v) acrylamide slab gels for electrophoresis in the second dimension. Proteins were visualized by staining with Coomassie Brilliant Blue (CBB), or were transferred to nitrocellulose membranes for subsequent probing with the anti-MtrC mAb.

Fig. 1. Organization of the *mtrRCDE* region in *N. gonorrhoeae*. The order and transcriptional orientation of the *mtrRCDE* genes are shown. The restriction fragment in pKH9 is highlighted by the bar labelled 2.57 kb. The sites of the Km^R cassette in the *mtrR* gene in strain KH9, and in the *mtrC* gene in strains KH12 and KH13 are shown. S, *Sma*I; D, *Dra*I; M, *Mlu*I; N, *Not*I; Bg, *Bgl*II; NI, *Nla*IV; H, *Hinc*II; B, *Bsp*106; P, promoter region. The shaded area in *mtrC* signifies the *Not*I-*Nla*IV fragment used to detect *mtrC* in Southern hybridization experiments. The size of the *mtrD* gene was estimated based on results obtained from agarose gel electrophoresis of a PCR-amplified product.

Lipoproteins, including MtrC, were labelled during growth of gonococcal strains in 5 ml GCB broth containing $10 \mu\text{Ci ml}^{-1}$ (370 kBq ml^{-1}) [^3H]palmitate (Chen *et al.*, 1985). Whole cell lysates of labelled gonococci (strains KH8 and KH13) were subjected to SDS-PAGE and autoradiography. The dried SDS-polyacrylamide gel was exposed to Kodak X-OMAT X-ray film for two weeks at -70°C prior to development.

Cloning of *mtrCDE*. A λ ZAPII library of *Dra*I-digested FA19 chromosomal DNA fragments with attached *Eco*RI linkers (Cornelissen *et al.*, 1993) was kindly provided by C. N. Cornelissen and P. F. Sparling (University of North Carolina, Chapel Hill). This phage bank was propagated on *E. coli* XL1-Blue in the presence of IPTG (Sigma) (Sambrook *et al.*, 1989). Plaque lifts, representing over 30000 individual plaques, were screened with the rabbit anti-MtrC antiserum for production of MtrC. Six immunoreactive plaques were recovered and then rescreened in the absence of IPTG. Phagemid DNA was excised (Sambrook *et al.*, 1989) from two positive plaques obtained after this second screening, and the gonococcal insert was liberated by digestion with *Eco*RI. A limited restriction map of the gonococcal insert from one of the phagemid constructs (pKH9) was prepared after digestion with a variety of restriction endonucleases (see Fig. 1). Defined deletions of the insertion in pKH9 were prepared by digesting pKH9 with appropriate restriction endonucleases followed by ligation (Sambrook *et al.*, 1989). These constructs were introduced into *E. coli* XL1-Blue by electroporation and were analysed for production of MtrC by SDS-PAGE and Western blotting. The Western blots were probed with the rabbit antiserum described above.

A DNA fragment containing the remainder of the *mtrD* gene and the 5'-end of the *mtrE* gene was identified from a plasmid library of partial *Sau*III-digested chromosomal DNA prepared from strain CH95 (Pan & Spratt, 1994), using a *Hind*III-*Dra*I fragment of the *mtrD* gene from pKH9 (Fig. 1) as a probe in colony hybridizations.

Molecular analyses. Several oligonucleotide primers specific for the *mtr* region and two primers that anneal to the pBluescript vector (CM6, 5'-TCACACAGGAAACAGCTATGACC-3' and CM7, 5'-CCAGTCACGCGTTGTAAACG-3') were prepared by L. Jones (Microchemical Facility, Emory University) and used in sequencing the gonococcal DNA insert in pKH9 (or its subclones); additional primers were prepared as necessary to complete the sequencing. Primers were labelled with [γ - ^{32}P]ATP (NEN DuPont) and both strands of the original insert were sequenced in their entirety using the AmpliTaq cycle sequencing kit (Perkin Elmer) as recommended by the manufacturer. The DNA sequence of the ends of the insert containing the partial *mtrD* and *mtrE* genes was determined using both universal and reverse M13/pUC primers. PCR products were sequenced by the cycle sequencing procedure. Typically, two different PCR

reactions were analysed to account for possible errors due to *Taq* polymerase. Dried gels were exposed to Kodak XAR film at room temperature and developed after 24–96 h. Nucleotide and amino acid sequence analysis was performed using DNASTar. Predicted amino acid homologies were determined after searching the GBTRANS/Swiss-Prot/EMBL databases using the DNASTar Proscan program. The nucleotide sequence for the *mtrC* gene has been deposited in GenBank under accession number U14993.

Oligonucleotide primers CEL-1 (5'-GACAATGTTTCATGCG-ATGATAGG-3'), which anneals 120 nucleotides downstream from the translational stop codon for *mtrR*, and KH9#3 (5'-GACGACAGTGCCCAATGCAACG-3'), which anneals 24 nucleotides downstream of the *mtrC* translational start codon, were used to amplify a 1087 bp product containing the *mtrR* region from chromosomal DNA prepared from strains FA19, FA171, BR87 and KH8 and their respective HA^R transformants. Conditions of PCR reactions were essentially as described previously (McAllister & Stephens, 1993). Amplification was carried out for 30 cycles using a two-step programme consisting of a 1 min denaturation at 95°C followed by a 1 min anneal and extension at 60°C using a Perkin Elmer 480 thermal cyclor.

Primer extensions were performed with $40 \mu\text{g}$ total RNA, prepared by the method of Baker & Yanofsky (1968), employing oligonucleotides that annealed within 100 bases of the 5'-end of the transcripts and the AMV Reverse Transcriptase Primer Extension System from Promega. To determine transcriptional start points, the extension products were analysed on a 6% sequencing gel beside reference sequencing reactions derived from the same oligonucleotides used for primer extension analysis.

Transformation experiments. Gonococcal chromosomal DNA was extracted from 16 h agar-grown cultures as described by McAllister & Stephens (1993). For transformation, PCR-amplified *mtrR* genes were purified by agarose gel electrophoresis, electroeluted into TBE buffer, ethanol precipitated and dissolved in TE buffer. Recipient FA19 P^+ organisms were scraped from GCB agar plates after 16–18 h growth and resuspended in GC broth containing defined supplements I and II, 2 mM MgCl_2 and 0.043% (w/v) sodium bicarbonate to a density of approximately $1 \times 10^8 \text{ c.f.u. ml}^{-1}$. Gonococci were incubated with $0.5 \mu\text{g DNA ml}^{-1}$ for 30 min at 37°C prior to plating onto GCB agar plates. After 4–6 h incubation at 37°C , agar overlays containing appropriate antibiotics were added to the plates with continued incubation at 37°C , under 3.8% (v/v) CO_2 for 48 h.

To introduce cloned *mtrR* fragments into strain FA19, replicative-form DNA from M13mp18 clones containing either the 750 bp *mtrR*-95 gene fragment from strain CH95 or

mtrR : : Km^R (Pan & Spratt, 1994) was used in transformation as described above to construct strain KH8 (as FA19 but *mtrR*-95) and strain KH9 (as FA19 but *mtrR* : : Km^R). Strain KH11 was constructed using a plasmid that contains a deletion of the *mtrR* gene from the *StyI* site located 21 codons into the start of the *mtrR*-coding region to the *StyI* site located six nucleotides past the translational stop codon (Fig. 1). Strains KH12 and KH13 are transformants of strains FA19 and KH8 respectively that contain a 1.3 kb Km^R cassette (Stoker *et al.*, 1982) in the 5'-proximal *NotI* site of the *mtrC* gene. Strains KH15, KH16, and KH17 were constructed by transformation using PCR-amplified products of 1087 bp, corresponding to the *mtrR* region from strains FA171, BR87, and KH8, respectively. Km^R transformants were selected using 50 µg kanamycin ml⁻¹, while Ery^R transformants were selected using 0.5 µg erythromycin ml⁻¹.

RESULTS

Characterization of the *mtrC*-encoded lipoprotein in gonococci

In a previous report the complete amino acid sequence for the MtrR regulatory protein of *N. gonorrhoeae* was presented (Pan & Spratt, 1994). Upstream and divergently transcribed from the *mtrR* gene, an incomplete open reading frame (ORF) was identified and termed *mtrC*. The partial amino acid sequence of *mtrC* appeared to encode a membrane lipoprotein similar to the AcrA and EnvC lipoproteins of *E. coli*. Analysis of the sequence upstream of the *acrA* and *envC* genes of *E. coli* revealed the presence of the *acrR* and *envR* genes, respectively.

We have cloned a complete *mtrC* gene on a 2578 bp DNA fragment, derived from strain FA19, in pKH9 (Fig. 1). pKH9 directed the synthesis in *E. coli* XL1-Blue of a 40 kDa protein (Fig. 2) that reacted in Western blots with the rabbit antiserum against the previously termed Mtr-associated lipoprotein (Chen *et al.*, 1985). Deletions at the 3'-end of the cloned fragment up to the *BspI*106 site (Fig. 1) had no effect on synthesis of this protein when such subclones of pKH9 were introduced into *E. coli* (data not presented). However, a deletion variant of pKH9 that lacked the 5'-end of the fragment up to the *NotI* site (Fig. 1) permitted the synthesis of this protein only when IPTG was added to the culture (data not presented), suggesting that a functioning promoter region for *mtrC* in *E. coli* was upstream of the *NotI* site. Hence, these studies permitted the localization of the *mtrC* gene to the region shown in Fig. 1. When a 980 bp *NotI*-*NlaIV* fragment (Fig. 1, shaded area) was used to probe Southern blots of *DraI*-digested FA19 chromosomal DNA, it was determined that gonococci possess a single copy of the *mtrC* gene (data not presented).

The nucleotide sequence of the insert in pKH9 confirmed the presence of a complete *mtrC* gene. Translation of the *mtrC* gene revealed a 412 amino acid lipoprotein having a molecular mass of 42777 Da (Fig. 3) with a bacterial signal peptidase II consensus site (LSSC) (von Heijne, 1989) located at positions 22-25. Acylation of the Cys-25 residue by a fatty acid moiety occurs prior to cleavage of the signal peptide from the mature lipoprotein. Indeed, insertional-inactivation of the *mtrC* gene in gonococci,

Fig. 2. Production of MtrC from *N. gonorrhoeae* in *E. coli*. Western blot analysis demonstrating expression of MtrC in *E. coli* XL1-Blue(pKH9) and *N. gonorrhoeae* strain FA19. Solubilized, whole-cell lysates from FA19 (lane 1), XL1-Blue(pKH9) (lane 2) and the control XL1-Blue(pBluescript) (lane 3) were separated by SDS-PAGE and the blot was probed with a 1:10000 dilution of the polyclonal rabbit antiserum. The faint band above the predominant band is likely to be MtrC with an uncleaved leader peptide. Approximate molecular masses, which were determined using Bio-Rad low range standards, are indicated on the left in kDa.

due to the presence of a Km^R cassette in the *NotI* site located at the 5'-end of the gene (Fig. 1), resulted in the loss of a comparatively minor lipoprotein that in the parental strain migrated with an apparent molecular mass of 47 kDa (data not presented). Removal of the leader sequence by signal peptidase II would result in the unmodified MtrC protein having a molecular mass of 40437 Da and a pI of 8.01; the calculated pI for MtrC was consistent with its electrophoretic mobility in IEF gels (see below). Hydropathy analysis of MtrC failed to reveal, outside of the signal sequence, extended regions of significant hydrophobicity expected for a typical membrane protein (data not presented). This characteristic is not unusual since MtrC-homologues (see below) often display a similar property (Saier *et al.*, 1994).

At the amino acid level, the MtrC lipoprotein from strain FA19 was 99.24% identical to the partial (263 amino acids) sequence reported for the MtrC protein of strain CH95 (Pan & Spratt, 1994). Using the DNASTar Proscan program to search the GBTRANS/Swiss-Prot/EMBL databases, the similarity of MtrC with AcrA, EnvC and MexA was confirmed (Fig. 3). MexA (Poole *et al.*, 1993b) has been reported to display similarity with certain bacterial efflux and transporter proteins, which belong to the membrane fusion protein (MFP) family described by Saier *et al.* (1994). These proteins include CzcB (Nies *et al.*, 1989), EmrA (Lomovskaya & Lewis, 1992), HlyD (Hess *et al.*, 1986), and LktD (Guthmiller *et al.*, 1990). These MFPs apparently have the same general topology and structure (Saier *et al.*, 1994), but display considerable sequence differences. Alignment of the *mtrC* sequence

Fig. 3. Complete amino acid sequence for the *N. gonorrhoeae* MtrC lipoprotein deduced from the nucleotide sequence of the *mtrC* gene from strain FA19, and its alignment with the AcrA, EnvC and MexA lipoproteins. The amino acids are denoted by the single-letter amino acid code. Conserved amino acid substitutions are shown in lightly shaded boxes. A proposed consensus sequence in which at least three out of four amino acids are identical is shown.

with CzcB, HlyD, LktD and EmrA revealed (data not presented) regions of identity, which consisted of overlaps of 49 amino acids (EmrA) to 324 amino acids (CzcB). Over the regions of overlap, MtrC was 27.8 % identical to CzcB, 22.3 % identical to HlyD, 17.9 % identical to LktD, and 24.5 % identical to EmrA.

Organization and importance of the *mtrRCDE* region in gonococci

The DNA sequence upstream from the *mtrC* gene in pKH9 contained the incomplete *mtrR* ORF which is transcribed divergently from *mtrC* (Pan & Spratt, 1994). It also revealed the presence of a 13 bp inverted repeat sequence (Fig. 4) positioned between the -10 and -35 regions of the *mtrR* promoter. A consensus -10 DNA sequence for the *mtrC* promoter was identified (Fig. 4), but only a weak -35 DNA sequence (5'-TTATCC-3'), beginning within the 13 bp inverted repeat, could be identified. A comparison of this sequence with the corresponding DNA sequence from strain CH95 revealed that strain CH95 had a single bp (T/A) deletion in the 13 bp inverted repeat. In subsequent experiments (see below), it was determined that the single bp deletion in the 13 bp inverted repeat was important in determining high level HA^R in gonococci.

The DNA sequence 12 bp downstream from the translational stop codon of the *mtrC* gene in pKH9 revealed the start of a third ORF, *mtrD* (Fig. 1). This incomplete ORF encoded 243 amino acids of a protein, MtrD, with similarity to the AcrE, EnvD and MexB proteins (Fig. 5) that have reported molecular masses of 110 kDa. MFPs, such as MtrC, are thought to act with certain transporter proteins (e.g. EnvC with EnvD), which belong to the resistance/nodulation/cell division (RND) family, to promote energy-dependent efflux of their substrates across bacterial membranes. Accordingly, MtrD is predicted to belong to the RND family of proteins. In order to obtain the remainder of the *mtrD* gene, a DNA fragment from the end of the incomplete *mtrD* gene in pKH9 was used as a probe to identify plasmids with overlapping fragments from a library of partial *Sau*III-digested DNA of strain CH95 (Pan & Spratt, 1994). A plasmid was isolated that contained the remainder of an *mtrD* gene including the putative translational stop codon (data not presented). The size of the complete *mtrD* gene in strains FA19 and CH95 was estimated by PCR analysis and oligonucleotide primers that annealed to the 5'-ends of the coding and non-coding strands. The complete *mtrD* gene was estimated to be 3 kb, a value consistent with an MtrD protein having a molecular mass of nearly 110 kDa (data not presented).

Fig. 4. Presence of a 13 bp inverted repeat between the *mtrR* and *mtrC* genes and identification of their promoter regions. The 13 bp inverted repeat nucleotide sequence between the divergent translational start codons for the *mtrR* and *mtrC* genes in *N. gonorrhoeae* strain FA19 is shown in the boxed area. The site of the single bp deletion in strains CH95 and FA171 and transformants (KH8, KH15 and KH17) of strain FA19 obtained with DNA from strains CH95 or FA171 is underlined. The transcriptional start points (*) of the *mtrR* and *mtrC* genes were mapped by primer extension and the -10 and -35 sequences were deduced as shown; two start points of transcription for the *mtrR* gene were reproducibly observed.

Fig. 5. Alignment of partial MtrD and MtrE sequences with similar bacterial proteins. (a) N-terminal amino acid sequence for MtrD and its alignment with the MexB, AcrE and EnvD proteins, with a proposed consensus sequence where at least 3 out of 4 amino acids are identical. (b) N-terminal amino acid sequence for the putative MtrE lipoprotein, alignment with the OprK lipoprotein from *P. aeruginosa*, and a proposed consensus sequence.

The plasmid containing the partial *mtrD* gene also contained the start of the ORF that would encode a putative lipoprotein that was homologous (36.2% identical over 69 amino acids) to OprK of *P. aeruginosa* (Fig. 5). The incomplete *mtrE* gene was located 36 bp downstream from the translational stop codon of the *mtrD* gene. Since consensus -10 and -35 regions could not be detected

between the *mtrC* and *mtrD* genes or the *mtrD* and *mtrE* genes, it is likely that these three genes represent a single transcriptional unit, a property similar to the *acrAE* (Ma *et al.*, 1993) and *emwCD* (Klein *et al.*, 1991) operons in *E. coli* and *mexABoprK* in *P. aeruginosa* (Poole *et al.*, 1993a). Further support for the hypothesis that *mtrCDE* represents a single transcriptional unit is the lack of the DNA

Table 1. Susceptibility of gonococcal strains to antimicrobial agents, and levels of MtrC

Strain	Minimum inhibitory concentrations ($\mu\text{g ml}^{-1}$) of*:					MtrC†
	Ery	Rif	Pen	TX	CV	
FA19 (wild-type)	0.25	0.06	0.008	125	2.5	+
FA171 (<i>mtrR-171</i>)	2.0	0.25	0.032	> 16 000	10	+++++
KH8 (<i>mtrR-95</i>)	2.0	0.25	0.032	> 16 000	10	+++++
KH9 (<i>mtrR::Km^R</i>)	1.0	0.25	0.016	1000	5	+++
KH11 ($\Delta mtrR$)	1.0	0.25	0.016	1000	5	+++
KH12 (<i>mtrC::Km^R</i>)	0.06	0.015	0.008	31	1.25	—
KH13 (<i>mtrC::Km^R mtrR-95</i>)	0.06	0.015	0.008	31	1.25	—
KH15 (<i>mtrR-171</i>)‡	2.0	ND	ND	> 16 000	10	+++++
KH16 (<i>mtrR-87</i>)§	1.0	ND	ND	1000	5	+++
KH17 (<i>mtrR-95</i>)	2.0	ND	ND	> 16 000	10	+++++
KH20 (<i>mtrR-171::Km^R</i>)	2.0	ND	ND	> 16 000	10	ND
BR87 (<i>mtrR-87, env-2</i>)	0.06	0.03	0.06¶	62.5	1.25	+

ND, Not done.

* Ery, erythromycin; Rif, rifampin; Pen, penicillin; TX, Triton X-100; CV, crystal violet.

† Intensity of anti-MtrC mAb reactivity in Western blots with all values compared to that of the level of MtrC in strain FA19.

‡ Obtained with the *mtrR-171* sequence derived from PCR amplification of chromosomal DNA from strain FA171, and contains the single bp deletion in the 13 bp inverted repeat in the *mtrR/mtrC* promoter region.

§ Obtained with the *mtrR* sequence derived from PCR amplification of chromosomal DNA from strain BR87.

|| Obtained with the *mtrR-95* sequence derived from PCR amplification of chromosomal DNA from strain KH8.

¶ BR87 is derived from strain FA47 (as FA19 but *env-2 str-7 penA2*) and FA140 (as FA19 but *penA2 mtrR-171 penB2*); see Methods.

uptake sequence (5'-GCCGTCTGAA-3') between these genes, which is often part of putative transcriptional terminators (Goodman & Scocca, 1988).

To confirm the importance of the *mtrCDE* region in determining levels of HA^R in gonococci, the effect of *mtrC*-inactivation in strain FA19 was determined. For this purpose a 4.3 kb *PvuII* fragment from pKH10 containing *mtrC::Km^R* was used to transform strain FA19 for Km^R. Southern hybridization experiments (data not presented) demonstrated that the single copy of the *mtrC* gene possessed by transformant strain KH12 had been inactivated by the 1.3 kb Km^R cassette. Compared to parental strain FA19, strain KH12 was found to be hypersusceptible to a panel of HAs (Table 1). To determine whether inactivation of the *mtrC* gene possessed by a highly HA-resistant strain would also result in hypersusceptibility to HAs, the *mtrC* gene of strain KH8 (as FA19 but *mtrR-95*; see Table 1) was inactivated by insertion of the 1.3 kb Km^R cassette at the *NotI* site as described in Methods. Compared to parental strain KH8, a representative Km^R transformant, strain KH13, was found to be hypersusceptible to HAs and penicillin (Table 1), but was equally sensitive to the hydrophilic antibiotic streptomycin (MIC equal to 40 μg streptomycin ml⁻¹). These results confirmed the importance of the *mtrCDE* region in determining HA^R in gonococci.

Taken together, the organization of the *mtrRCDE* region and its contribution to HA^R in gonococci represents a

combination of features displayed by the *acrRAE* and *envRCD* operons in *E. coli* and the *mexABoprK* system in *P. aeruginosa*. *N. gonorrhoeae* appears to have combined features of both systems, a regulatory gene, *mtrR*, similar to *acrR* and *envR* of *E. coli* and three genes, *mtrCDE*, encoding proteins similar to the *mexABoprK*-encoded membrane proteins possessed by *P. aeruginosa*.

Mutations in the *mtrR* gene enhance levels of MtrC and HA^R

Both missense and null mutations in the *mtrR* gene are known to enhance levels of gonococcal HA^R (Pan & Spratt, 1994). Earlier, an undefined mutation in the *mtrR* gene possessed by HA^R transformants of strain FA19 was correlated with increased production of the membrane lipoprotein (Chen *et al.*, 1985), now known to be MtrC. In order to directly test the effects of mutations in the *mtrR* gene on levels of MtrC, the level of MtrC in transformant strains KH8 (*mtrR-95*), KH9 (*mtrR::Km^R*) and KH11 ($\Delta mtrR$), was examined and compared to that in parental strain FA19. Strain KH8 exhibits high level HA^R (Table 1) due to the *mtrR-95* gene from strain CH95 (Pan & Spratt, 1994). Both strains KH9 and KH11 exhibited increased HA^R but the level of resistance was not as great as that seen with strain KH8 (Table 1). A Western blot of total membrane protein from strains FA19, KH8, KH9, and KH11 probed with the anti-MtrC mAb showed that the level of MtrC was enhanced in all three of the

Fig. 6. 2-D IEF-SDS-PAGE and Western blot analysis of gonococcal transformant strains. Whole-cell lysates (WC) from strains FA19, KH8, KH12 and KH13 were subjected to IEF and then SDS-PAGE. Proteins were either visualized by CBB-staining (left panel) or transferred to nylon membranes and probed with the anti-MtrC mAb (labelled as 44 kDa-specific mAb in the right panel). WC of FA19 were also run, without prior IEF, in the SDS-polyacrylamide gel to determine electrophoretic mobility of proteins, and reactivity in Western blots with the anti-MtrC mAb. Molecular mass markers in kDa are shown between the CBB-stained gels and Western blots. The electrophoretic mobilities of the gonococcal porin protein (Por) and the 44 kDa protein are identified. The relative migration of the MtrC protein in the 2-D gels is marked with an arrowhead (<). The arrow in the CBB-stained gels indicates the acidic 44 kDa protein that is present in FA19 and KH8, but absent in KH12 and KH13. The electrophoretic position of the 44 kDa proteins that react with the anti-MtrC mAb is shown (<).

transformants, above that observed in strain FA19, but the effect was greatest in strain KH8 (Table 1).

Since the MtrC-homologues in *E. coli* (AcrA and EnvC) and *P. aeruginosa* (MexA) are thought to be minor proteins we used 2-D IEF-SDS-PAGE coupled with Western blotting or CBB-staining to more precisely compare levels of MtrC in strains FA19 and KH8, and to ascertain whether the level of other proteins was changed due to acquisition of the *mtrR-95* allele. MtrC was localized in the Western blots of these 2-D electropherograms with the anti-MtrC mAb. The anti-MtrC mAb weakly reacted with two relatively basic proteins at 44 kDa in the FA19 whole cell lysate (Fig. 6). The migration of MtrC in the IEF gel, well toward the basic region of the IEF separation, was consistent with a sequence-based predicted pI of 8.01. Importantly, the mAb reaction was intensified and more diffuse in the whole cell lysate of strain KH8 (*mtrR-95*), ranging in pI from 7.0 to 8.0. This elongation was probably due to both overproduction of

MtrC and secondary components, such as fatty acid acylation of the mature MtrC protein. Analysis of the CBB-stained 2-D electropherograms revealed that a basic protein, which migrated similarly to the mAb-reactive region, was more intensely stained in the KH8 protein sample (see < in the CBB-stained gels) than that seen with the FA19 protein sample, further indicating that the level of MtrC was increased in strain KH8 due to the presence of *mtrR-95*. These results also indicated that like EnvC (Klein *et al.*, 1991) and AcrA (Ma *et al.*, 1993) in *E. coli*, MtrC is a relatively minor protein.

In order to confirm that the mAb-reactive protein correlated with the CBB-staining proteins in strains FA19 and KH8, whole cell lysates from strains KH12 and KH13 (as KH8 but *mtrC::Km^R*) were separated by 2-D IEF-SDS-PAGE. These mAb reactions were not observed with either the KH12 or KH13 samples, confirming that insertional-inactivation of *mtrC* resulted in loss of MtrC. However, the CBB-stained 2-D electro-

Fig. 7. Mutations affecting HA^R in *N. gonorrhoeae*: single bp deletion in the *mtrR*/*mtrC* promoter regions or missense mutations in *mtrR* genes. The figure is a schematic representation of the *mtrR* region of the strains employed. The presence of glycine (G) or aspartic acid (D) at position 45, and histidine (H) or tyrosine (Y) at position 105 in the MtrR proteins of respective strains is shown. The number of thymidines (T) in the 13 bp inverted repeat in the 5'-flanking region of each *mtrR* gene is shown. A 15 bp sequence containing the 13 bp inverted repeat with a possible site of the single bp deletion is shown at the bottom. Levels of HA^R are from Table 1. Levels of MtrC were determined by evaluating the relative intensity of reaction with the anti-MtrC mAb in Western blots.

phorograms also revealed a major difference in the 44 kDa range between strains FA19 and KH8 compared to their respective *mtrC*::Km^R transformant strains, KH12 and KH13. Strains KH12 and KH13 lost expression of a relatively acidic 44 kDa protein present in the CBB-stained gels of their respective parental strains, FA19 and KH8 (see arrows in the CBB-stained gels). The amount of this 44 kDa protein, as judged by CBB-staining, appeared to be similar in strains FA19 and KH8, suggesting that, unlike MtrC, its level was not regulated by *mtrR*; the characteristics of this protein are under investigation.

Importance of distinct mutations in the *mtrR* gene in determining levels of HA^R in gonococci

Results from DNA sequencing of the *mtrR* genes from strains FA171 and BR87 suggested that levels of HA^R and MtrC can be modulated by mutations either in the *mtrR*-coding sequence or in a 13 bp inverted repeat sequence that intervenes the promoters utilized by the divergent *mtrR* and *mtrC* genes (Fig. 4). The *mtrR*-coding sequence from strains FA19 and FA171 were identical (Fig. 7), even though strain FA171 (as FA19 but *mtrR*-171) displays high-level HA^R (Table 1). Strain FA171 differed from strain FA19 by a single bp (T/A) deletion in the 13 bp inverted repeat sequence (Fig. 4). In contrast, strain BR87 contained a single mutation in the *mtrR*-coding sequence located at codon 45 (GGC to GAC) resulting in a glycine → aspartic acid change. This substitution occurred in the second helical domain of the proposed helix-turn-helix motif of the MtrR protein (Pan & Spratt, 1994).

To confirm that the missense mutation at codon 45 could result in HA^R in gonococci, the 1087 bp PCR product from strain BR87 that included the *mtrR* locus and the 13 bp inverted repeat region was used to transform strain FA19 for resistance to 0.5 µg erythromycin ml⁻¹; transformants were recovered at a frequency of 4×10^{-4} per 10^8 c.f.u. per µg DNA. Representative transformants were

scored for levels of resistance to the HAs crystal violet, erythromycin and Triton X-100. The transformants (e.g. strain KH16) displayed an intermediate level of resistance to HAs, which was most clearly evident with respect to Triton X-100 susceptibility (Table 1). Moreover, MtrC expression in strain KH16 did not equal the level seen with strains FA171 and KH8 although it was enhanced above that of parental strain FA19 (Fig. 7). In this respect, strain KH16 resembled strains bearing null mutations in the *mtrR* gene [e.g. strains KH9 and KH11 (Table 1)]. The nucleotide sequence of the *mtrR* region from strain KH16 was determined, and the results confirmed the presence of the missense mutation at codon 45 (Fig. 7) and the 13 bp inverted repeat DNA sequence in the promoter region.

The importance of the single bp deletion in the 13 bp inverted repeat sequence in mediating high-level HA^R was confirmed by transformation. The *mtrR* region from an HA^R transformant (strain KH15) of strain FA19, which was obtained with the PCR product from strain FA171 used in the sequencing study, also displayed this single bp deletion but was otherwise identical to the *mtrR* sequences of strains FA19 and FA171 (Fig. 7). Transformant strain KH15, like strains FA171 and KH8, displayed both high levels of HA^R (Table 1) and MtrC (Fig. 7). Hence, the single bp deletion in the 13 bp inverted repeat in the promoter regions of the *mtrR* and *mtrC* genes was responsible for these phenotypes in strain FA171. The capacity of the single bp deletion to provide high-level HA^R was found to be independent of the MtrR protein because transformants of strain KH15 (e.g. strain KH20; Table 1), obtained with DNA from strain KH9 (*mtrR*::Km^R), containing an insertionally inactivated *mtrR* gene, but maintaining the single bp deletion, continued to express high levels of HA^R. However, from this transformation a second class of Km^R transformants were recovered that displayed the intermediate HA^R property of donor strain KH9. DNA sequence analysis of

PCR products encompassing the *mtrR* promoter region from two of these transformants revealed that these transformants now contained the 13 bp inverted sequence, indicating repair of the single bp deletion was sufficient to reduce HA^R.

DISCUSSION

A recent review (Nikaido, 1994) on the mechanisms by which bacteria resist multiple antimicrobial agents, including the HAs studied herein, stressed the importance of permeability and efflux systems in allowing for resistance to structurally diverse compounds. Indeed, changes in cell envelope permeability due to the *mtr* system have been proposed to account for resistance of gonococci to structurally diverse HAs (Guymon *et al.*, 1975). The striking amino acid homology of the MtrCDE proteins with other proteins involved in transport of toxic compounds across membranes requires that this model be re-evaluated. In this respect, expression of the *mexABoprK* operon in *P. aeruginosa* has been shown to impart nonspecific resistance to a variety of antibiotics by an efflux mechanism (Poole *et al.*, 1993b). A complex of the MexA-MexB-OprK proteins is thought to form a continuous channel from the cytoplasm through the outer membrane that facilitates transport of agents (including membrane-damaging detergents) into the extracellular fluid. For agents that damage the membrane, it is postulated that they are captured in the membrane bilayer and transported through the channel (Nikaido, 1994). Like its homologues in *E. coli* and *P. aeruginosa*, MtrC is clearly located in the gonococcal cell envelope, where it would be needed to participate in efflux, but appears to be bound to peptidoglycan (Hill & Judd, 1989). This interaction with peptidoglycan perhaps explains why MtrC fails to separate in sucrose-density gradients with either the outer or the cytoplasmic membranes, but instead fractionates in the mixed-membrane region of the gradient (J. Smith & R. C. Judd, unpublished observations). This cellular location for MtrC is consistent with it belonging to the MFP family of proteins (Saier *et al.*, 1994), which are thought to cause localized fusion of the inner and outer membranes. Hence, a continuous channel, resembling the MexA-MexB-OprK complex of *P. aeruginosa*, consisting of MtrCDE may account for the capacity of gonococci to resist HAs that act in the cytoplasm or by damaging the membrane.

Our observations that mutations in the *mtrR* gene of *N. gonorrhoeae* resulted in HA^R with a concomitant increase in the level of MtrC, while loss of MtrC resulted in hypersusceptibility of gonococci to structurally diverse HAs, confirms a role for MtrC in resistance. Since *mtrD* and *mtrE* are in the same apparent transcriptional unit as *mtrC*, mutations affecting *mtrC* expression are likely to affect *mtrDE* expression also. This is not unexpected since a mutation in *envC* is known to have pleiotropic effects (Klein *et al.*, 1991; Starkova *et al.*, 1978) that include hypersusceptibility of *E. coli* to a variety of antimicrobial agents and chain formation at high temperature, altered phospholipid composition, and loss of EnvD. Com-

plementation of these phenotypes requires both *envC* and *envD* gene products (Klein *et al.*, 1991).

Our studies confirm that the *mtr* system of HA^R in gonococci can be controlled by an MtrR-dependent mechanism of regulation that can be bypassed by missense or null mutations in the *mtrR*-coding sequence; such mutations provide for an intermediate level of resistance. The MtrR protein resembles a DNA-binding protein similar to the tetracycline repressor of pSC101 (Brow *et al.*, 1985). Interestingly, the *mtrR* gene was found (Pan & Spratt, 1994) to be homologous to previously unidentified ORFs (*acrR*, *envR*) that are upstream of the *E. coli* *acrAE* and *envCD* operons. Our studies with gonococci suggest that mutations in the coding sequence of these *mtrR* homologues would enhance the resistance of *E. coli* to antimicrobial agents by increasing the level of the AcrAE and EnvCD proteins. Pan & Spratt (1994) found that a single amino acid change at position 105 (histidine → tyrosine) in MtrR was sufficient for HA^R in a transformant of strain FA19 that was constructed by transformation using the cloned *mtrR*-95 allele; recent studies, however, indicate that this missense mutation confers an intermediate level of HA^R similar to that of strains KH9, KH11 and KH16 (data not presented). The mechanism by which this single amino acid substitution would change the putative regulatory property of MtrR is not clear since the substitution is downstream of the helix–turn–helix motif, which is presumed to be important in DNA-binding and/or regulatory functions exhibited by MtrR. More easily understood is the phenotypically suppressed *mtrR* mutation (*mtrR*-87) in strain BR87 because it results in a single, but radical, amino acid change (glycine → aspartic acid) at position 45, which is within the second helical domain of proposed helix–turn–helix motif of MtrR. This radical substitution in the second helical domain of helix–turn–helix motif may have a direct effect on DNA-binding activity because it is predicted to increase the helical characteristic of the adjacent turn region (data not presented). Thus, it is likely that mutations in the *mtrR*-coding sequence serve to reduce or otherwise inhibit repressor activity.

By themselves, mutations in the *mtrR*-coding sequence afford only an intermediate level of HA^R when introduced into strain FA19. It is clear from our studies that high-level HA^R and MtrC production involves a single bp deletion in the 13 bp inverted repeat sequence that was located between the divergent *mtrR* and *mtrC* genes (Fig. 4). The influence of the single bp deletion was independent of the MtrR protein, since HA^R was observed in strains possessing an inactivated *mtrR* gene. Moreover, since repair of this deletion, in the absence of an intact *mtrR* gene, resulted in intermediate HA^R, we propose that this 13 bp inverted repeat is a transcriptional regulatory element. It is likely that the single bp deletion in this sequence serves to up-regulate expression of the *mtrCDE* gene complex. The deletion would also shorten the distance between the –10 and –35 regions of the *mtrR* promoter, thereby reducing expression of the *mtrR* gene. Whether this single bp deletion enhances *mtrCDE* promoter utilization by RNA polymerase or modifies

binding of a transcriptional regulatory protein is unclear. To our knowledge this is the first description of this type of mutation affecting resistance of bacteria to structurally diverse agents, such as the HAs studied herein, and suggests a novel genetic mechanism by which bacteria modulate levels of resistance to structurally diverse agents.

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