The MtrD protein of *Neisseria gonorrhoeae* is a member of the resistance/nodulation/division protein family constituting part of an efflux system

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The *mtr* (multiple transferable resistance) system of *Neisseria gonorrhoeae* mediates resistance of gonococci to structurally diverse hydrophobic agents (HAs) through an energy-dependent efflux process. Recently, complete or partial ORFs that encode membrane proteins (MtrC, MtrD, MtrE) forming an efflux pump responsible for removal of HAs from gonococci were identified and appeared to constitute a single transcriptional unit. In this study, the complete nucleotide sequence of the *mtrD* gene was determined, permitting the characterization of the MtrD protein. The full-length MtrD protein has a predicted molecular mass of nearly 114 kDa, putatively containing a 56 amino acid signal peptide. MtrD displays significant amino acid sequence similarity to a family of cytoplasmic membrane proteins, termed resistance/nodulation/division (RND) proteins, which function as energy-dependent transporters of antibacterial agents and secrete bacterial products to the extracellular fluid. The predicted topology of the MtrD transporter protein revealed 12 potential membrane-spanning domains, which were clustered within the central and C-terminal regions of the primary sequence. Loss of MtrD due to insertional inactivation of the *mtrD* gene rendered gonococci hypersusceptible to several structurally diverse HAs, including two fatty acids (capric acid and palmitic acid) and a bile salt (cholic acid), but not hydrophilic antibiotics such as ciprofloxacin and streptomycin. Since gonococci often infect mucosal sites rich in toxic fatty acids and bile salts, the expression of the *mtr* efflux system may promote growth of gonococci under hostile conditions encountered *in vivo*.

Keywords: *Neisseria gonorrhoeae*, antimicrobial resistance, efflux, transporter, MtrD

INTRODUCTION

Neisseria gonorrhoeae infects mucosal surfaces that are often bathed in antibacterial hydrophobic agents (HAs) such as fatty acids and bile salts (McFarland *et al.*, 1983; Morse *et al.*, 1982). The capacity of gonococci to infect sites rich in HAs has been associated with the *mtr*

(multiple transferable resistance) system (Morse *et al.*, 1982; Shafer *et al.*, 1984) since *mtr* confers broad-spectrum resistance to hydrophobic drugs, dyes and detergents (Guymon & Sparling, 1975; Maness & Sparling, 1973; Shafer *et al.*, 1984). Strains expressing HA-resistance (HA^R) due to *mtr* are frequently isolated from patients with rectal infections (Morse *et al.*, 1982) as well as uncomplicated urogenital infections (Urdez & Shafer, unpublished observations).

The *mtr* system was originally proposed to regulate the permeability barrier of the gonococcal cell envelope to HAs (Guymon & Sparling, 1975), but more recent genetic (Hagman *et al.*, 1995; Pan & Spratt, 1994) and physiological (Lucas *et al.*, 1995) studies demonstrated

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Abbreviations: HA, hydrophobic agent; RND, resistance/nodulation/division; for other abbreviations see footnote to Table 1.

The GenBank accession number for the *mtrD* nucleotide sequence reported in this paper is U60099.

Fig. 1. Genetic organization of the *mtr* efflux operon in *N. gonorrhoeae*. The divergent *mtrR* and *mtrCDE* genes (Hagman *et al.*, 1995) are shown, together with the intervening region containing their divergent promoters (P_{mtrR} and P_{mtrCDE} as described by Hagman & Shafer, 1995) and the approximate sites of annealing of the major oligonucleotide primers (*mtrD1*, *mtrD7*, *mtrD10* and *mtrE1*) described in the text.

that *mtr* functions as an energy-dependent efflux pump that removes HAs by a mechanism requiring the proton motive force. Thus, the *mtr* efflux system resembles other bacterial efflux pumps, notably the *mexABoprM*-encoded system of *Pseudomonas aeruginosa* (Li *et al.*, 1995; Poole *et al.*, 1993a,b) and the *acrAB*- and *acrEF*-encoded systems of *Escherichia coli* (Ma *et al.*, 1994); *acrAB* was previously termed *acrEF* (Ma *et al.*, 1993), while *acrEF* was previously termed *envCD* (Klein *et al.*, 1991). Nucleotide sequencing studies (Hagman *et al.*, 1995; Pan & Spratt, 1994) revealed that the *mtr* gene complex is composed of a regulatory gene (*mtrR*) encoding a transcriptional repressor (MtrR), and three genes encoding membrane proteins (MtrC, MtrD and MtrE) that form an efflux pump (Lucas *et al.*, 1995).

The *mtrCDE* gene complex in gonococci represents a single transcriptional unit that is located 250 bp upstream and transcribed divergently from the *mtrR* gene (Fig. 1). The entire *mtrC* gene from strain FA19 was previously identified (Hagman *et al.*, 1995) in a cloned 2.57 kb *DraI* fragment that also contained partial ORFs for *mtrR* and *mtrD*; the translational start codon for *mtrD* was located 11 nucleotides downstream from the *mtrC* translational stop codon. A second plasmid construct obtained with DNA from a clinical isolate contained the remainder of the *mtrD* gene and the start of a third ORF. This third ORF was termed *mtrE* and the limited amino acid sequence that could be deduced suggested that its product would be similar to the OprM outer-membrane lipoprotein of *P. aeruginosa* (Li *et al.*, 1995) that is required for efflux of antibiotics (Poole *et al.*, 1993a,b) from this pathogen.

Homology studies (Hagman *et al.*, 1995) of the deduced amino acid sequence for MtrC revealed a consensus fatty acid acylation site at the N-terminus of the mature protein and a high degree of similarity to a growing list of bacterial proteins termed membrane fusion proteins (Saier *et al.*, 1994). The partially characterized (Hagman *et al.*, 1995) MtrD protein consists of 243 amino acids that have homology with the N-terminal amino acid sequences of members of the resistance/nodulation/division (RND) family of cytoplasmic membrane proteins (Saier *et al.*, 1994). These RND proteins have been proposed to serve as transporters that interact with a companion membrane fusion protein for energy-dependent efflux of antibacterial compounds (Ma *et al.*, 1994; Saier *et al.*, 1994). To gain a better understanding of the structural components of the *mtr* efflux pump, we

determined the complete nucleotide sequence of the entire *mtrD* gene and constructed transformants of *N. gonorrhoeae* strain FA19 bearing an insertionally inactivated *mtrD* gene.

METHODS

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

The gonococcal strains employed in this investigation are given in Table 1. Strain FA19 (Sparling *et al.*, 1975) is the parental strain of transformant strains KH8, KH12, KH14, KH15 and LD1. Strains KH8, KH12 and KH15 were constructed previously (Hagman *et al.*, 1995), while strain LD1 was constructed by transformation using donor DNA from strain KH14 to transform strain KH15 (see below). These strains were grown on GCB agar with defined supplements at 37 °C under 3.8% (v/v) CO₂ or in GC broth as previously described (Shafer *et al.*, 1984). For transformation experiments, piliated transparent colony types were used (Sparling *et al.*, 1975), but nonpiliated transparent variants were used to determine susceptibility to antimicrobial agents.

Antimicrobial agent susceptibility testing. The susceptibility of gonococcal strains to capric acid (CA), cholic acid (CHA), ciprofloxacin (CP), crystal violet (CV), erythromycin (ERY), palmitic acid (PA), streptomycin (STR) and Triton X-100 (TX-100) was determined by the agar plating method described by Shafer *et al.* (1984). All of these compounds were purchased from Sigma except for CP, which was obtained from the Pharmacy Service of the Atlanta VA Medical Center. CA, CHA, ERY and PA were dissolved in 100% (v/v) ethanol.

PCR amplification and nucleotide sequencing of the *mtrD* gene.

Chromosomal DNA from strain FA19 was prepared by the method of McAllister & Stephens (1993). Oligonucleotide primers *mtrD1* (5'-CGGCATCTGAAGCCAAACCTGC-3') and *mtrE1* (5'-GATGGAAGAAACCGATGTGTCG-3') were used in PCR amplification of the *mtrD* sequence under conditions described previously (Hagman *et al.*, 1995). Oligonucleotides *mtrD1* and *mtrE1* anneal 24 nucleotides upstream of the *mtrD* translational start codon and 171 nucleotides downstream from the *mtrD* translational stop codon, respectively (Fig. 1). DNA sequencing was conducted on both strands using *mtrD1*, *mtrE1* and additional oligonucleotide primers, which were prepared based on determined sequences by the cycle sequencing method (Hagman *et al.*, 1995) that employs AmpliTaq DNA polymerase; primers were labelled with [γ -³²P]ATP (NEN DuPont).

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 signal peptide of MtrD was identified using the PSORT program which is available on the World Wide Web

Table 1. Susceptibility of gonococci to antimicrobial agents

Strain	Genotype	MIC ($\mu\text{g ml}^{-1}$) of:*							
		CA	PA	CHA	CV	TX-100	ERY	STR	CP
FA19	Wild-type	300	200	300	2.5	100	0.25	25	0.002
KH8	As FA19 but <i>mtrR</i> -95	ND	ND	ND	10	> 16000	2.0	25	0.002
KH12	As FA19 but <i>mtrC</i> ::Km ^R	25	25	150	< 1.25	25	0.06	25	0.002
KH14	As FA19 but <i>mtrD</i> ::Km ^R	12.5	25	100	< 1.25	25	0.06	25	0.002
KH15	As FA19 but <i>mtrR</i> -171	> 500	800	> 500	10	> 16000	2.0	25	0.002
LD1	As KH15 but <i>mtrD</i> ::Km ^R	25	25	100	ND	25	0.06	ND	0.002

ND, Not determined.

*CA, capric acid; PA, palmitic acid; CHA, cholic acid; CV, crystal violet; TX-100, Triton X-100; ERY, erythromycin; STR, streptomycin; CP, ciprofloxacin.

(<http://www.PSORT.nibb.oc.jp>). A predicted topology model was developed for MtrD using the algorithm of von Heijne (1989, 1992) as implemented by the TopPred II program (Claros & von Heijne, 1994). A hydrophobicity profile was created using the GcH1 scale (von Heijne, 1992) with the window size set to 21 amino acids.

Insertional inactivation of the *mtrD* gene. A 758 bp region representing the 5'-end of the *mtrD* gene was prepared by PCR amplification of FA19 chromosomal DNA. The PCR reaction employed oligonucleotide primers *mtrD1* (see above) and *mtrD2*. Primer *mtrD2* (5'-GCCGT ACCCAACTGCCCTT GC-3') anneals 712 nucleotides downstream from the *mtrD* translational start codon. The 758 bp product contained a unique *HindIII* site 328 nucleotides downstream of the translational start codon that was used for insertional inactivation of the *mtrD* gene. This *mtrD* sequence was cloned into pCR-Script (Stratagene) following the manufacturer's directions. The cloned *mtrD* fragment was removed from the pCR-Script vector by digesting with *EcoRI* and *NotI*, subjected to agarose gel electrophoresis and subsequently purified using Qiaex beads according to manufacturer's directions (Qiagen). The purified *mtrD* fragment was cloned into *EcoRI/NotI*-digested pBSΔH3, which is pBluescript lacking the unique *HindIII* site; pBSΔH3 was created by digesting pBluescript with *HindIII*, filling in the overhangs with Klenow fragment (Boehringer Mannheim) and ligating the blunt ends. Once the *mtrD* fragment had been cloned into pBSΔH3, the construct (pBS*mtrD1*) was digested with *HindIII* and the kanamycin resistance (Km^R) cassette from pH45ΩKm^R (Prentki & Krisch, 1984) was inserted. *E. coli* strain XL1-Blue was transformed with the construct and transformants resistant to ampicillin (100 $\mu\text{g ml}^{-1}$) and kanamycin (50 $\mu\text{g ml}^{-1}$) were selected. Oligonucleotide primers *mtrD1* and *mtrD2* were used in PCR reactions to verify insertion of the Km^R cassette in the *mtrD* gene (Fig. 1) of selected transformants and a representative PCR product was used to transform FA19 to Km^R (50 $\mu\text{g ml}^{-1}$). DNA from a representative transformant (strain KH14) was subjected to PCR-amplification using oligonucleotide primers *mtrD1* and *mtrD2* to verify insertional inactivation of *mtrD* and for transformation experiments using strain KH15 (FA19 *mtrR*-171) as the recipient.

PAGE and Western blotting of proteins. Whole-cell lysates were prepared and analysed by SDS-PAGE as described by Laemmli (1970). Gels were stained sequentially with silver and Coomassie Brilliant Blue or electrophoretically blotted to PVDF membranes. Membranes were blocked with Dulbecco's PBS/0.05% (v/v) Tween-20. Western blots were probed with a monoclonal antibody to MtrC as described previously (Judd *et al.*, 1991; Hagman *et al.*, 1995) or a rabbit antiserum against a synthetic peptide of MtrD (FNKKFDSWTHGYEGRVA), which corresponds to amino acids 513–529 of the full-length protein. This peptide was synthesized by the University of Montana Molecular Biology Facility and was used to immunize female New Zealand White rabbits using standard protocols. Briefly, 0.25 ml of a 1 mg ml⁻¹ stock peptide solution in complete Freund's adjuvant was injected subcutaneously at two inguinal and two axillary sites, followed by one intramuscular injection of 0.25 ml of the peptide stock solution 1 week later. Intravenous injections followed at 2 week intervals. Prior to the initial injection, pre-bleeds of the rabbits were obtained and sera was subsequently collected after peptide injections at weekly intervals. The anti-MtrD peptide antiserum was pre-absorbed three times with a whole-cell Triton X-100 lysate of *E. coli* XL-1 Blue (Hagman *et al.*, 1995) and clarified by centrifugation prior to use. The anti-MtrD peptide antiserum was used at a 1:5 dilution. Antigen-antibody reactions were detected using horseradish peroxidase-conjugated antibodies against mouse IgG or rabbit IgG prepared in goats.

Analysis of *mtrCD* gene expression. Total RNA was prepared from gonococcal strains by the method of Baker & Yanofsky (1968). Transcripts corresponding to products of the *mtrC*, *mtrD* and *rmp* genes were detected using a slot-blot hybridization procedure (Hagman & Shafer, 1995) that employed gene-specific probes. The *rmp* gene in gonococci is not under MtrR control (Hagman & Shafer, 1995) and its mRNA serves as an internal control in slot-blot hybridizations. The *mtrC* and *rmp* gene probes were described previously (Hagman & Shafer, 1995). An *mtrD*-specific gene probe was prepared by PCR amplification of chromosomal DNA prepared from strain FA19. This probe was prepared using oligonucleotide primers *mtrD7* (5'-ATATACAGGGGAACACGCC-3')

and *mtrD10* (5'-AGCATCAACCTGCAAGACCGC-3'). *mtrD7* anneals 202 nucleotides from the translational stop codon of *mtrD*, while *mtrD10* anneals 1171 nucleotides from the *mtrD* stop codon (Fig. 1). The 969 bp product that resulted from PCR amplification corresponds to a sequence 1699 bp downstream of the Km^R cassette insertion in strain KH14 (Fig. 1). DNA probes were labelled with [α - ^{32}P]dGTP using the Boehringer Mannheim random primer labelling kit as directed by the manufacturer. All conditions used in slot-blot hybridizations were as described previously (Hagman & Shafer, 1995).

RESULTS AND DISCUSSION

Sequence analysis and characteristics of MtrD

The N-terminal 243 amino acids of MtrD were deduced previously by sequencing a cloned DNA fragment from strain FA19 that contained a partial *mtrR* gene, a complete *mtrC* gene and a partial *mtrD* gene (Hagman *et al.*, 1995). The limited amino acid sequence that could be deduced for MtrD suggested that this protein was similar to other bacterial transporter proteins that participate in efflux of antimicrobial agents. To confirm this hypothesis, it was necessary to obtain the complete *mtrD* gene. Thus, oligonucleotide primers *mtrD1* and *mtrE1* (Fig. 1) were used to amplify the entire *mtrD* region from chromosomal DNA of strain FA19. DNA sequencing of the resulting 3.3 kb PCR product revealed a large ORF (3204 nucleotides) that encodes a 1067 amino acid protein, MtrD (Fig. 2). The first 243 amino acids of the full-length MtrD protein were identical to those predicted earlier by DNA sequencing of the partial *mtrD* gene in pKH9 (Hagman *et al.*, 1995). The full-length MtrD protein was predicted to have a molecular mass of 113.9 kDa and a pI of 6.86. Using the PSORT program, a potential signal I peptidase cleavage site for

MtrD was located between Val-56 and Met-57; removal of the 56 amino acid signal peptide would result in the mature MtrD protein having a molecular mass of 107.9 kDa and a pI of 6.1; the relatively long signal peptide in MtrD is similar in length to that proposed (Poole *et al.*, 1993a) for the MexB protein possessed by *P. aeruginosa*. Alignment of MtrD with MexB of *P. aeruginosa* and AcrB and AcrF of *E. coli* showed that it is highly similar to these transporter proteins, confirming that it is a member of the RND protein family. MtrD is 49% identical to MexB (GenBank accession number GBL11616) over 1057 amino acids, 43% identical to AcrB (GenBank accession number U00734) over 978 amino acids and 43% identical to AcrF (GenBank accession number X57948) over 990 amino acids.

Membrane topology of MtrD

The RND protein family is thought to represent integral cytoplasmic membrane proteins with numerous transmembrane domains that are presumed to facilitate interactions with their respective partner membrane fusion proteins (Saier *et al.*, 1994). The predicted topology of full-length MtrD revealed that it has 12 α -helical transmembrane segments (Fig. 3). The clustering of these potential membrane-spanning segments within the central and C-terminal regions of MtrD is remarkably similar to that proposed for MexB (Poole *et al.*, 1993a), as well as AcrB (Ma *et al.*, 1993), AcrF (Ma *et al.*, 1994) and AcrD (Nilles & Bertrand, unpublished observations). The model shown in Fig. 3 can be viewed as consisting of two nearly symmetrical halves, with each having a similar predicted structure. Thus, each half has an initial transmembrane segment (numbered 1-7 in Fig. 3), followed by two large loops (labelled P1

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-----+-----+-----+-----+-----+-----+-----+
1 MAKFFIDRPIFAWVISIFIIAAGIFGIKSLPVSQYPSVAAPTITLHAIYPGASAQVMEGS 60
61 VLSVIERNMNGVEGLDYMSTADSSSGSGSVSLTFTPDTDENLAQVEVQNKLEVLRLTPA 120
121 TVQQYGVTVSKARSNFLMIVMLSSDVQSTEEMNDYAQRNVVPELQRIEGVGQVRLFGAQR 180
181 AMRIWVDPKKLQNYNLSFADVGSALSQAQNIQISAGSIGSLPAVRGQTVTATVTAQGQLGT 240
241 AEEFGNVILRANTDGSNIYKLDVAKVGLGMEDYSSSTRLNGVNTTGMVMSLSNSGNAMAT 300
-----+-----+-----+-----+-----+-----+-----+
301 AKAVKERLAVLEKYFPQGMWKTPTDTSKFVEISIEKVIHTLIEAMVLVVFVVMYLFQNI 360
361 RYTLIPTIVVPIISLLGGFAFISYMGMSINVLTMFAMILVIGIVVDDAIVVVENVERIMAG 420
421 EGLPPKEATKKAMGQISGAVIGITAVLISVFVPLAMFSGAAGNIYKQFALTMASSIAFSA 480
481 FLALTLTPALCATMLKTIPKGHHEEKKGFFGWFNKKFDSWTHGYEGRVAKVLRKTFRMMV 540
541 VYIGLAVVGVLFLMRLPTSFLPTEDQGFMVSVQLPAGATKERTDATLAQVTQLAKSIPE 600
-----+-----+-----+-----+-----+-----+-----+
601 IENIITVSGFSFSGSQNMAMGFAIFKDNERTASGSDAVAVAGKLTGMMMGTLDGFGI 660
661 AVVPPPIELGNGSGLSINLQDRNNTGHTGIAGEGNELIQMRASGLFDPSTVRAGGLED 720
721 SPQLKIDINRAAAAQGISFADIRTAASALSSSYVSDFPNQGRQLQRMVQADEDARMQP 780
781 ADILNLTVPNKSGVAVPLSTIATVSWENGTEQSVRFNGYPSMKLSASPATGVSTGQAMEA 840
841 VQKMVDELGGGYSFEWGGQSSEEAKGGSQTLLYGLAVAAVFLVLAALYESWSIPLAVIL 900
-----+-----+-----+-----+-----+-----+-----+
901 VIPLGLIGAAAGVTGRNLFEGLLGSVPSFANDIYFQVGFTVMGLSAKNAILIIEFAKDL 960
961 QAQKSAVEAALEAARLRFRPIIMTSFAFILGVVPLYIAAGASSASQRAIGTTVFWGMLV 1020
1021 GTLLSVFLVPLFYVVVRKFFKETAHEHEMAVRHASKAGITGSDDKQY. 1068

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Fig. 2. Amino acid sequence of MtrD. The full-length MtrD protein from *N. gonorrhoeae* strain FA19 is shown in single amino acid letter code.

Fig. 3. Topology and potential membrane-spanning domains of MtrD. From the hydrophobicity profile of MtrD, a model was generated by applying the positive-inside rule (von Heijne, 1992) to determine the preferred topology model, which is shown. The 12 transmembrane domains of full-length MtrD are numbered sequentially, 1–12. Periplasmic domains (P1–P6) and cytoplasmic domains (C1–C7) are shown. Pluses indicate the positions of basic amino acids (arginine and lysine), while minuses indicate the positions of acidic amino acids (aspartate and glutamate). Amino acids in MtrD that are identical to two of three similar transporters (MexB, AcrB or AcrF) are shown as filled circles.

and P4 in Fig. 3) that may extend into the periplasm, facilitating interactions with its companion membrane fusion protein, MtrC. Not surprisingly, the topology model for MtrD is similar to models constructed for other homologues in the RND family of transporters, including MexB, AcrB, AcrF and AcrD (Nilles & Bertrand, unpublished observations). Importantly, the signature sequence of R(LIV)RP(LIV)₂MT(ATS)L, identified by Saier *et al.* (1994) as being highly conserved even among distantly related members of the RND family, was found within a likely membrane-spanning region between residues 978 and 987 of full-length MtrD. The near consensus signature sequence in MtrD (RFRPILMTSF) differs from that of AcrF and MexB by two conservative phenylalanine substitutions (positions 979 and 987) for leucines at positions 901 and 909 in AcrF, and 970 and 978 in MexB.

Insertional inactivation of *mtrD* renders gonococci hypersusceptible to HAs

The wild-type copy of the *mtrD* gene in strain FA19 (Fig. 1) was replaced with an insertional inactivated *mtrD* sequence that contained the Km^R cassette from

pH45ΩKm^R (Prentki & Krisch, 1984). Agarose gel electrophoresis of PCR-amplified *mtrD* products derived from chromosomal DNA preparations of strains FA19 and a representative transformant (strain KH14) confirmed the expected shift-up of the *mtrD* band in the KH14 PCR product (data not presented). Transformant strain KH14 did not differ significantly from parental strain FA19 in growth rate in GCB broth (data not presented). However, compared to parental strain FA19, strain KH14 was hypersusceptible to a panel of structurally diverse HAs (CA, CHA, CV, ERY, PA and TX-100; Table 1), but not hydrophilic antibiotics such as CP and STR (Table 1). The HA-susceptibility phenotype displayed by strain KH14 was similar to that of strain KH12, which bears an insertionally inactivated *mtrC* gene (Table 1). Moreover, insertional inactivation of the *mtrD* gene in strain KH15, which bears the *mtrR*-171 mutation that affords high-level HA^R due to a single bp deletion in the *mtrR* promoter (Hagman & Shafer, 1995; Hagman *et al.*, 1995), also resulted in hypersusceptibility of gonococci to HAs (see strain LD1 in Table 1). We recognized that insertional inactivation of *mtrD* by the Km^R cassette from pH45ΩKm^R would also result in loss of expression of the tandemly linked *mtrE*

Fig. 4. SDS-PAGE profile of whole-cell lysates of strains FA19, KH14, KH8 and KH12. Whole-cell lysates of gonococcal strains were prepared, separated on 15% SDS-PAGE gels and stained sequentially with silver and Coomassie Brilliant Blue as described in Methods. The right panel shows the expanded top region of the gel in the left panel so as to enhance viewing of higher molecular mass proteins. Arrows signify putative migration of the 47 kDa MtrC lipoprotein (Hagman *et al.*, 1995) and MtrD. A diamond identifies the putative MtrD protein in strain KH8. Molecular mass markers (M) were from the Bio-Rad low molecular mass kit.

gene (Hagman *et al.*, 1995) that would likely be co-transcribed with *mtrCD*. To circumvent this problem, we attempted to insertionally inactivate *mtrD* with the nonpolar *aphA-3* cassette (Ménard *et al.*, 1993) as was done for generating an insertionally inactivated *mtrE* gene (Delahay *et al.*, 1997). Unfortunately, repeated attempts to do so failed, perhaps because production of the MtrE outer-membrane protein without the MtrD transporter protein is lethal.

Electrophoretic analysis of gonococcal proteins

To determine the impact of insertional inactivation of *mtrD* on gonococcal proteins and to visualize the MtrD protein, total cellular proteins in whole-cell lysates of isogenic strains were analysed by SDS-PAGE and Western immunoblotting. When whole-cell lysates from strains FA19, KH8 [which, like strain KH15 overproduces MtrC (Hagman *et al.*, 1995) due to a single bp deletion (*mtrR-171*) in a 13 bp inverted repeat in the *mtrR* promoter (Hagman & Shafer, 1995)], KH12 (*mtrC::Km^R*) and KH14 (*mtrD::Km^R*) were examined by SDS-PAGE a protein of nearly 97 kDa appeared to be correlated with MtrD. Thus, a ~ 97 kDa stained band was present in strain FA19 and was present in elevated amounts in strain KH8 but in decreased amounts in strains KH12 and KH14 (Fig. 4). Due to the nature of the mutations in strains KH8, KH12 and KH14, and the fact that the *mtrCDE* gene complex represents a single transcriptional unit (Hagman *et al.*, 1995), these results suggested that the ~ 97 kDa staining band might contain mature MtrD among other co-migrating proteins. To determine whether MtrD migrated as a ~ 97 kDa

protein in SDS-PAGE, an anti-MtrD peptide antiserum (see Methods) was used to probe a Western blot containing whole-cell lysates from these isogenic strains. This polyclonal antiserum and a pre-bleed serum (data not presented) reacted with at least five gonococcal proteins in the 50–80 kDa region of the SDS-PAGE. However, only the anti-MtrD peptide antiserum specifically recognized a protein of nearly 97 kDa in whole-cell lysates prepared from strains KH8 and FA19 but was absent in strains KH12 and KH14 (Fig. 5). The lower apparent molecular mass of MtrD in whole-cell lysates of strains FA19 and KH8, as judged by SDS-PAGE and Western blotting, compared to that predicted by DNA sequence analysis may reflect proteolytic processing events other than removal of the signal peptide or its electrophoretic mobility in SDS-PAGE may not accurately reflect its molecular mass.

To determine whether loss of MtrD affected the level of its companion membrane fusion protein, the level of MtrC in strains FA19, KH8, KH12 and KH14 was determined by Western blotting using an anti-MtrC monoclonal antibody (Fig. 5). In both the whole-cell lysate (Fig. 5) and total membrane preparations (data not presented), the MtrC protein was present in strain FA19, present in elevated levels in strain KH8, and absent in strain KH12. Unexpectedly, however, the level of the MtrC protein in strain KH14 was reduced. This was not due to reduced expression of the *mtrC* gene, which lies immediately upstream of *mtrD* in the same transcriptional unit (Fig. 1), because a slot-blot RNA hybridization study revealed that strains FA19 and KH14 contained very similar levels of *mtrC* mRNA (Fig.

Fig. 5. Western immunoblot analysis for detection of MtrD and MtrC. Samples were treated as described in Fig. 4 legend (a), and proteins were then electrophoretically transferred to PVDF membranes and probed with an anti-MtrC monoclonal antibody (Hagman *et al.*, 1995) (b) or anti-MtrD peptide antiserum as described in Methods (c). Arrows identify the migration of the immunoreactive MtrC and MtrD proteins, and the diamond identifies the MtrD band in the lane containing whole-cell lysates from strain KH8. Molecular mass markers were as described in Fig. 4 legend.

Fig. 6. Expression of the *mtrCD* genes in gonococcal strains. A phosphorimager-derived profile is shown of a slot-blot hybridization of total RNA from strains FA19, KH14 and KH15. Twenty micrograms of total RNA was hybridized with gene-specific probes to detect *mtrC*, *mtrD* and *rmp* transcripts. The *mtrCD* transcripts are elevated in strain KH15 due to the *mtrR*-171 mutation. Note that while the control *rmp*-specific transcript was detected in all three strains, the *mtrD* transcript was only detected in strains FA19 and KH15.

6). Thus, we propose that without the anchoring effect supplied by the cytoplasmic-membrane-bound MtrD, the MtrC membrane fusion protein is rapidly turned over.

Determination of the complete nucleotide sequence for the *mtrD* gene allowed us to unambiguously place the MtrD protein in the RND family of bacterial cytoplasmic membrane proteins that are critical for export of antibacterial agents (Ma *et al.*, 1994; Saier *et al.*, 1994). Our earlier characterization of the MtrC membrane fusion protein and evidence (Hagman *et al.*, 1995) for the existence of an OprM homologue in gonococci

(MtrE), which would form a channel in the outer membrane, indicates that the membrane organization of the *mtr* efflux pump is similar to that proposed by Ma *et al.* (1994) for the MexA-MexB-OprM efflux pump possessed by *P. aeruginosa*. The description of the gonococcal MtrE protein and its importance in determining HA^R in gonococci are described by Delahay *et al.* (1997). Despite the apparent structural similarities between the MtrC-MtrD-MtrE and MexA-MexB-OprM efflux pumps it is important to stress that these systems likely display functional differences. Thus, the efflux pump possessed by *P. aeruginosa* can apparently enhance resistance to ciprofloxacin (Li *et al.*, 1994), while the highly similar gonococcal *mtr* pump appears unable to modulate susceptibility to this hydrophilic antibiotic (Table 1) and other quinolone antibiotics (Shafer *et al.*, unpublished observations). Moreover, the transcriptional repressors [MtrR for *N. gonorrhoeae* (Pan & Spratt, 1994) and MexR for *P. aeruginosa* (Poole *et al.*, 1996)] do not show structural similarities.

Efflux pumps presumably evolved long before the introduction of antibiotics in the clinical setting, perhaps arising from some other transport system, permitting bacteria to resist antimicrobial agents in their natural environment (Levy, 1992; Ma *et al.*, 1994). From an ecological viewpoint, the capacity of efflux pumps, such as the *mtrCDE*-encoded system, to remove structurally diverse antimicrobial agents would provide bacteria with a growth advantage in environments containing

multiple and structurally diverse antimicrobial agents. Since the broad substrate specificity displayed by many efflux pumps (reviewed by Ma *et al.*, 1994) endows pathogens with a mechanism to resist diverse, multiple antimicrobial compounds, including those encountered at mucosal surfaces, the defensive activity of efflux pumps should be considered a virulence factor. During gonococcal infections, *mtr*-mediated resistance to HAs might enhance survival at mucosal surfaces, such as the rectum, where toxic fatty acids and bile salts could have anti-gonococcal activity (Morse *et al.*, 1982). The increased susceptibility to CA, CHA and PA of strains bearing inactivated *mtrCDE* genes supports this hypothesis.

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