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

Kayla E. Hagman,1,2† Claressa E. Lucas,1,2 Jacqueline T. Balthazar,1,3 Lori Snyder,1,2 Matthew Nilles,4 Ralph C. Judd5 and William M. Shafer1,2,3

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

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 (HA8) 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

1,2 Program in Microbiology and Molecular Genetics of the Graduate Division of Biological and Biomedical Sciences1 and Dept of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA
3 Laboratories of Microbial Pathogenesis, VA Medical Research Service, VA Medical Center (Atlanta), Decatur, GA 30033, USA
4 Department of Microbiology and Immunology, University of Kentucky, Lexington, KY 40536-0084, USA
5 Department of Biological and Molecular Genetics, VA Medical Center (Atlanta), Decatur, GA 30033, USA

1Present address: Department of Microbiology and Immunology, University of Texas Southwestern Medical School, Dallas, TX 75235, USA.

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.
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 Dral 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 (Ma et al., 1995) of the deduced 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/godulation/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'-CCGCATCTGAAAGCCAAAACCTGC-3') and mtrE1 (5'-GATGGAAAGAAAACCGATGTGTCG-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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>MIC (µg ml⁻¹) of:*</th>
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<tr>
<td></td>
<td></td>
<td>CA</td>
</tr>
<tr>
<td>FA19</td>
<td>Wild-type</td>
<td>300</td>
</tr>
<tr>
<td>KH8</td>
<td>As FA19 but</td>
<td>ND</td>
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<tr>
<td></td>
<td>mtrR-95</td>
<td></td>
</tr>
<tr>
<td>KH12</td>
<td>As FA19 but</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>mtrC::KmR</td>
<td></td>
</tr>
<tr>
<td>KH14</td>
<td>As FA19 but</td>
<td>12-5</td>
</tr>
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<td></td>
<td>mtrD::KmR</td>
<td></td>
</tr>
<tr>
<td>KH15</td>
<td>As FA19 but</td>
<td>&gt;500</td>
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<tr>
<td></td>
<td>mtrR-171</td>
<td></td>
</tr>
<tr>
<td>LD1</td>
<td>As KH15 but</td>
<td>25</td>
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<td>mtrD::KmR</td>
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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.or.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 S' 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 ACCCAACTGCCCTTGCC-3') anneals 712 nucleotides downstream from the mtrD translational start codon. The 758 bp product contained a unique HindIII site; pBSAH3 was created by digesting was transformed with the construct and transformants resistant to ampicillin (100 pg ml⁻¹) and kanamycin (50 pg ml⁻¹) were selected. Oligonucleotide primers mtrD1 and mtrD2 were used in PCR reactions to verify insertion of the KmR cassette in the mtrD gene (Fig. 1) of selected transformants and a representative PCR product was used to transform FA19 to KmR (50 µg 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 MtrCAs 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. 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 prepared 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'-ATATACAGGGGAACCACGCCC-3')
and mtrD10 (5’-AGCATCAACCTGAAAGCCG-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 KmR cassette insertion in strain KH14 (Fig. 1). DNA probes were labelled with [α-32P]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 mtrD1 (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 amino acid protein, MtrD (Fig. 2). The first 243 amino acids of the full-length MtrD protein are identical to amino acid protein, MtrD (Fig. 2). The first 243 amino acids of the full-length MtrD protein were identical to amino acid protein, MtrD (Fig. 2).

Alignment of MtrD with MexB of P. aeruginosa 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 UU00734) 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., 1993), 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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**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.

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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)MT(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 insertionally inactivated mtrD sequence that contained the KmR cassette from pH45ΩKmR (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 HASs (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 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 KmR cassette from pH45ΩKmR would also result in loss of expression of the tandemly linked mtrE
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 over-produces 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::KmR) and KH14 (mtrD::KmR) 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. 4). 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. 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.

Fig. 5. Western blot analysis of whole-cell lysates of strains FA19, KH14, KH8 and KH12. Western blots were probed with an anti-MtrC monoclonal antibody to detect the MtrC protein. The MtrC protein is 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 HAR 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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REFERENCES


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