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Rifampin Resistance in *Neisseria meningitidis* Due to Alterations in Membrane Permeability

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Rifampin-resistant (Rifr) *Neisseria meningitidis* strains are known to have single point mutations in the central conserved regions of the *rpoB* gene. We have demonstrated two distinct resistance phenotypes in strains with identical mutations in this region, an intermediate level of resistance in Rifr clinical isolates and a high level of resistance in mutants selected in vitro. The possible role of membrane permeability in the latter was investigated by measuring MICs in the presence of Tween 80; values for high-level-resistance mutants were reduced to intermediate levels, whereas those for intermediate-level-resistance strains were unaffected. The highly resistant mutants were also found to have increased resistance to Triton X-100 and gentian violet. Sequencing of the meningococcal *mtrR* gene and its promoter region (which determine resistance to hydrophobic agents in *Neisseria gonorrhoeae*) from susceptible or intermediate strains and highly resistant mutants generated from them showed no mutation within this region. Two-dimensional gel electrophoresis of two parent and Rifr mutant strains showed identical shifts in the pI of one protein, indicating that differences between the parent and the highly Rifr mutant are not confined to the *rpoB* gene. These results indicate that both permeability and *rpoB* mutations play a role in determining the resistance of *N. meningitidis* to rifampin.

*Neisseria meningitidis* is a major cause of bacterial meningitis among children and young adults. Prophylactic treatment for close contacts of an index case usually involves administration of rifampin. This can reduce carriage of *N. meningitidis* and help prevent transmission (7). However, the development of resistance to rifampin has been observed during the course of a meningococcal outbreak following prophylactic treatment (22). Such resistant strains may prove problematic for the management of meningococcal meningitis.

A wide variety of pathogenic bacteria are known to develop resistance to rifampin after exposure to the antibiotic. Molecular characterization of rifampin-resistant (Rifr) isolates has identified a short central region of the *rpoB* gene (which codes for the β subunit of RNA polymerase) in which mutations giving rise to the resistance phenotype can occur (12, 14, 20). These alterations prevent rifampin from binding to and inhibiting RNA polymerase (11). Mutations within this region of the *rpoB* gene of *N. meningitidis* are also known to occur and are responsible for rifampin resistance (4).

Bacterial resistance to rifampin may also be due to alterations in membrane permeability. Previous studies of mycobacteria suggested that the natural resistance of some mycobacterial species to rifampin is due to a decrease in membrane permeability relative to that of rifampin-susceptible (Rifs) organisms (13). Purification of the RNA polymerase from these resistant species showed that it was sensitive to the effects of rifampin. The natural resistance of *Pseudomonas aeruginosa* to a variety of antibiotics is also thought to be due to the low-level permeability of the outer membrane of the organism (2). Thus, the ability of bacteria to resist the action of rifampin may in part be due to their ability to prevent it from entering the cell.

It has been known for over 20 years that the ability of *Neisseria gonorrhoeae* to resist the action of hydrophobic agents is due to mutations in the so-called multiple transferable resistance, or *mtr*, gene (19). More recently, it has been shown that alterations in the membrane permeability of *N. gonorrhoeae* are due to mutations in the *mtr* gene (18), a transcriptional repressor of the *mtr* gene complex. This complex consists of three tandemly linked genes (*mtrCDE*) constituting a single transcriptional unit. The complex is situated 250 bp upstream of, and transcribed divergently from, the *mtrR* gene. It is homologous to a number of bacterial efflux systems, including *mexAB*-opr*K* of *P. aeruginosa*. These bacterial efflux systems are thought to be responsible for resistance to a number of antibiotics and reagents and act by removing the antibiotic from the cytoplasm. Different resistance phenotypes in *N. gonorrhoeae* are associated with different mutations within the *mtrR* gene (18) and its promoter (10). These mutations alter the expression of the efflux system and thus provide a mechanism whereby the organism can adapt to the presence of hydrophobic reagents.

In previous work on rifampin resistance in *N. meningitidis*, we observed two different resistance phenotypes (4). One in vitro-generated mutant had a much higher level of rifampin resistance than the resistant isolates from patients but the same mutation in the *rpoB* gene, suggesting that other factors may be involved in resistance. The hydrophobic nature of rifampin suggested that resistance may be due to changes in membrane permeability or in the ability of the pathogen to remove the antibiotic from the cell. To determine if this was the case, we examined the effect of Tween 80 on MICs and identified and sequenced an *mtrR* homolog in Rifr and Rifr isolates of *N. meningitidis*.

**MATERIALS AND METHODS**

**Bacterial strains.** Details of the bacterial strains used have been described elsewhere (4). In vitro generation of the Rifr mutants F1M and A1M from parental strains F1 and A1, respectively, was carried out by plating on chocolate agar supplemented with 50 μg of rifampin per ml as described previously (4). The serogroups, serotypes, and rifampin MICs for the strains used in this study are detailed in Table 1. Isolates were stored on beads at −70°C (Protec Vials; Technical Services Consultants, Bury, United Kingdom).
MIC determination. MICs of rifampin were determined by the E-test (AB-Biodisk, Solna, Sweden) (1, 3), which consists of commercially available antibiotic-impregnated strips which allow direct evaluation of MICs, and by broth microdilution tests carried out by the procedure recommended by the National Committee for Clinical Laboratory Standards (16). MICs of Triton X-100, gentian violet, and sodium dodecyl sulfate (SDS) were determined by agar dilution tests as described by the National Committee for Clinical Laboratory Standards (16). MICs of rifampin in the presence and absence of Tween 80 (0.05%, wt/vol) were determined by the broth microdilution test. All tests were carried out in duplicate on separate occasions. Culture purity was examined before and after each test by subculturing on chocolate agar.

PCR detection and amplification of mtr genes. PCR was carried out as described previously (4). An annealing temperature of 50°C was used for all mtr gene amplifications. The primers used and their relative orientations with respect to the mtrR and mtrC genes are given in Table 2 and Fig. 1. Primers used for amplification of the N. meningitidis mtrR gene and its promoter were based on the published sequence (10, 18) of this gene from N. gonorrhoeae and subsequent sequence data obtained in this study. All primers were synthesized on an ABI 373A automated DNA synthesizer. Amplified products from the PCR were characterized by electrophoresis on 2% agarose gels followed by staining in ethidium bromide (0.5 μg/ml).

Sequencing. Sequencing of PCR products was carried out as described previously (4). All products were purified on Centricon C-100 columns (Amicon, Stonehouse, United Kingdom) and sequenced on an ABI 373A automated DNA sequencer fitted with a stretch upgrade. Sequence analysis was carried out with the Seqed sequence analysis program (Applied Biosystems, Warrington, United Kingdom).

2D gel electrophoresis. Strains of meningococci and the corresponding RifR mutants were grown overnight on chocolate agar, and the soluble bacterial proteins were prepared as described by Cash et al. (5). One-half plate of bacterial growth was harvested with a sterile swab and resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 8% [vol/vol] urea, 50 mM dithiothreitol, 2 mM phenylmethylsulfonylfluoride, 10% [vol/vol] glycerol, 5% [vol/vol] Nonidet P-40 [Fluka, Gillingham, United Kingdom], 200 μg of RNase per ml, 6% [vol/vol] carrier ampholytes [pH 3.5 to 10] [Resolyte; Merck, Stonehouse, United Kingdom]). Insoluble cellular debris was removed by centrifugation (11,000 × g, 5 min). The supernatant was stored at −20°C until required. Soluble bacterial proteins were analyzed by two-dimensional (2D) gel electrophoresis as described by Cash et al. (5). Isoelectric focusing was performed in the first dimension followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. The protein gels were stained in 0.2% Coomassie blue. The marker used for the estimation of pI values was carbamylated creatine phosphokinase (Merck).

RESULTS

rpoB gene. Sequencing of the 950-bp region of the rpoB gene known to contain the mutation sites conferring rifampin resistance showed that the high-level-resistance mutants F1M and A11M had sequences identical to those of their intermediate-level-resistance parent strains, F1 and A11. All had a His-to-Asn mutation at position 35 (H35N mutation) (4) (Table 1).

Rifampin sensitivity. To investigate the possible role of membrane permeability in the high-level resistance mechanism, the MICs of rifampin were determined in the presence and absence of Tween 80 (0.05%) (Table 3), a reagent thought to increase the permeability of the membrane (13). The very high MICs observed for mutant strains A11M, F5M, and G6M were reduced more than 10-fold in the presence of Tween 80. Addition of Tween 80 had no effect on the MICs for intermediate isolates (A11 and F1) or the susceptible isolate (G6). The Tween 80 had no observable effect on the growth of parent or mutant strains. Resistances to other hydrophobic reagents and dyes were also measured (Table 3). Mutant strains were more resistant to the Triton X-100 and gentian violet than their parental strains, though differences in resistance to SDS and acriflavine were not apparent.

2D gel electrophoresis. 2D gel electrophoresis was used to detect other differences between the phenotypes of parents and RifR mutants (5). Comparison of the soluble-protein profiles showed a shift in mobility of one spot in two resistant isolates (F1M and F5M) in comparison with their parent strains (Fig. 2). This spot corresponded to a protein of approximately 18.9 kDa in size, and the shift was due to an alteration in the pl from 6 to <5.9 (outwith the range of the markers). Other parent and mutant strains showed no alteration in the electrophoretic mobility of this protein (A11 and A11M, G6 and G6M).

The mtr system. Initial attempts to use the gonococcal primers MTR-Up and MTR-Down (18) to amplify the mtrR gene in meningococci were unsuccessful. More than one band was consistently observed when these two primers were used together. Additional primers specific for the mtrR gene were synthesized. MTR4 is based on a region of homology between mtrR and other repressor-activator proteins. MTR4 is based on the gonococcal sequence. PCR using both of these primers amplified a central region of the mtrR gene from all isolates, giving a band of 470 bp. To ensure that the gene was present in N. meningitidis and to rule out the possibility of sample contamination, the published sequence (10, 18) of this gene from N. gonorrhoeae was amplified by using the gonococcal primers MTR-Up and MTR-Down (18) to amplify the mtrR gene in meningococci, showing the locations and orientations of the primers used in this study. MTR-D, MTR-Down (Table 2).

![Image of 2D gel electrophoresis](image)

**FIG. 1.** The mtrR gene, part of the mtrC gene, and the intervening promoter region of N. gonorrhoeae, showing the locations and orientations of the primers used in this study. MTR-D, MTR-Down (Table 2).

| Isolate | Serogroup | Serotype | MIC (μg/ml) | Mutation
|---------|-----------|----------|-------------|-----------
| A11M    | C         | 2a       | >256        | H35N
| F1M     | C         | 2a       | >256        | H35N
| F1      | C         | 2a       | 24          | H35N
| F5      | C         | 2a       | <0.064      | H35N
| F5M     | C         | 2a       | >256        | H35N
| G6      | W135      | NS       | <0.047      | H35Y
| G6M     | W135      | NS       | >256        | H35Y

* Rifampin MIC measured by E-test.

b Numbering as in reference 4.

* NS, nonserotypeable.

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TABLE 1. Isolates used in this study

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FIG. 2. Analysis of cell lysates from F5 and F5M by 2D PAGE. Soluble proteins from F5 and F5M were prepared and analyzed by 2D PAGE as described in the text. The bacterial protein found to alter its mobility in the F5M strain (arrows) is indicated. The gels are oriented with high-molecular-weight proteins at the top and acidic proteins to the left.

contamination, this fragment was labelled with digoxigenin during the amplification process and a Southern blot of restricted meningococcal genomic DNA was probed. A single band was observed among all isolates analyzed (results not shown). Two more primers (MTR5 and MTR6) based on sequence data obtained from the MTR3 and MTR4 amplification product were synthesized. Amplification using MTR-Up plus MTR6 or MTR5 plus MTR-Down gave products which encompassed the complete mtrR gene from N. meningitidis (Fig. 1). For strain G6 and its RifR mutant G6M, amplification with MTR5 and MTR-Down failed to give any product. Another primer, MTR7, which is based on the 3′ noncoding gonococcal sequence (18), was synthesized, and amplification with this primer and MTR5 was successful. Comparison of the mtrR sequence data for isolates A11, A11M, F1, F1M, F5, F5M, G6, and G6M showed that all had identical protein sequences (Fig. 3), although a number of silent mutations were observed in the gene sequence (results not shown). Comparison of the protein sequence with the gonococcal sequence showed them to be highly homologous (97% identity), with only a few amino acid changes (positions 78, 86, 105, 181, 183, and 206) (Fig. 3). One of the amino acid differences at position 105 (Tyr in N. meningitidis and His in N. gonorrhoeae) (Fig. 3) was identical to a mutation found in isolates of gonococci resistant to hydrophobic agents (10, 18).

Primers for the mtrC gene were synthesized, on the basis of the mtrC sequence of N. meningitidis (results not shown) to allow specific primers to be designed. To obtain the promoter region of the mtrR gene, meningococcal DNA was amplified with MTR6 and MTR1rev. A band of approximately 980 bp was consistently observed. Sequencing of the promoter region contained within this amplification product using primers MTR3rev and MTR1rev showed that an additional 158 bp was present in comparison with the gonococcal sequence (Fig. 4). Comparison of the promoter regions from RifR parents and their resistant mutants showed them to be identical, although a small number of differences between strains were observed, including a 4-bp insertion in G6 and G6M (position 196; Fig. 4). A 13-bp inverted repeat present within the gonococcal promoter (10) was found to contain one mismatch within the meningococcal promoter.

DISCUSSION

Resistance to rifampin is classically associated with mutations in the rpoB gene coding for the β subunit of the RNA polymerase. These mutations prevent rifampin from binding to the enzyme and inhibiting its activity. The vast majority of mutations that have been described, including those in N. meningitidis, occur as single point mutations in a short central region of the rpoB gene (4, 12, 14, 20). Identical single point mutations might be expected to cause similar or identical MICs when found in strains of the same organism. The observation of significantly different MICs for independently isolated strains with the same point mutation suggests that additional mutations in other genes and different mechanisms may be involved in the resistance phenotype.

During the course of our previous work, two RifR phenotypes were observed: an intermediate resistance phenotype (MIC, 24 μg/ml) in a group of closely related clinical isolates (represented by A11 and F1 in this study) and a high-level resistance phenotype (MIC, >256 μg/ml) among in vitro-isolated mutants (4). Rifampin is a derivative of rifamycin and is characteristically hydrophobic in nature. Possible mechanisms of resistance to rifampin may be a reduction of the ability of the antibiotic to enter bacteria due to alterations in the structure of the outer membrane or an enhancement of the rate of removal from the cytoplasm following changes in efflux mechanisms (17). Because of the hydrophobic nature of rifampin, its entry into bacteria is thought to be via the lipid bilayer of the outer membrane rather than through outer membrane pores. Thus, alteration of the outer membrane structure will influence the entry of rifampin. Gas chromatographic analysis of the outer membranes of one mutant and its parent has shown an alteration in lipid composition with a significant decrease in the level of C16 lipid material of the mutant (results not shown), suggesting that the increased resistance may be due to differences in the membrane composition.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTR-Up</td>
<td>5′-ATA CAT ACA CGA TTG CAC GG</td>
</tr>
<tr>
<td>MTR-Down</td>
<td>5′-TTT GTG TCA TTT TGA TGC CG</td>
</tr>
<tr>
<td>MTR3</td>
<td>5′-GGG CTC TAY TGG CAY TTY AA</td>
</tr>
<tr>
<td>MTR3rev</td>
<td>5′-TTG AAA TGC CAA TAG AGC GC</td>
</tr>
<tr>
<td>MTR4</td>
<td>5′-TTY TCN AAR TTR TCC ATC AT</td>
</tr>
<tr>
<td>MTR5</td>
<td>5′-TGG TCC AAC GTA TCT GCC</td>
</tr>
<tr>
<td>MTR6</td>
<td>5′-TCG CAG GAA GAG AAC CAC</td>
</tr>
<tr>
<td>MTR7</td>
<td>5′-TTC AGA CGG CAT TGT TAC</td>
</tr>
<tr>
<td>MTR1rev</td>
<td>5′-CAT CGC CTG AGA AGC ATA</td>
</tr>
</tbody>
</table>

Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTR-Up</td>
<td>5′-ATA CAT ACA CGA TTG CAC GG</td>
</tr>
<tr>
<td>MTR-Down</td>
<td>5′-TTT GTG TCA TTT TGA TGC CG</td>
</tr>
<tr>
<td>MTR3</td>
<td>5′-GGG CTC TAY TGG CAY TTY AA</td>
</tr>
<tr>
<td>MTR3rev</td>
<td>5′-TTG AAA TGC CAA TAG AGC GC</td>
</tr>
<tr>
<td>MTR4</td>
<td>5′-TTY TCN AAR TTR TCC ATC AT</td>
</tr>
<tr>
<td>MTR5</td>
<td>5′-TGG TCC AAC GTA TCT GCC</td>
</tr>
<tr>
<td>MTR6</td>
<td>5′-TCG CAG GAA GAG AAC CAC</td>
</tr>
<tr>
<td>MTR7</td>
<td>5′-TTC AGA CGG CAT TGT TAC</td>
</tr>
<tr>
<td>MTR1rev</td>
<td>5′-CAT CGC CTG AGA AGC ATA</td>
</tr>
</tbody>
</table>

Table 3. MICs of rifampin, Triton X-100, gentian violet, SDS, and acriflavine for parent and mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rifampin</th>
<th>Reagents and dyes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>E-test</td>
<td>Microdilution</td>
</tr>
<tr>
<td></td>
<td>With Tween</td>
<td>Without Tween</td>
</tr>
<tr>
<td>A11</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>A11M</td>
<td>&gt;256</td>
<td>50</td>
</tr>
<tr>
<td>F5</td>
<td>&lt;0.064</td>
<td>0.08</td>
</tr>
<tr>
<td>F5M</td>
<td>&gt;256</td>
<td>50</td>
</tr>
<tr>
<td>G6</td>
<td>&lt;0.047</td>
<td>0.08</td>
</tr>
<tr>
<td>G6M</td>
<td>&gt;256</td>
<td>100</td>
</tr>
</tbody>
</table>

a. MICs for reagents and dyes were determined by agar dilution tests (16), and rifampin MICs were determined by E-test with strips graduated to 256 μg/ml and by broth microdilution (16) (see Materials and Methods).

b. TX100, Triton X-100; GV, gentian violet; AF, acriflavine.

c. Tween 80 at 0.05%.

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All the resistance phenotypes in this study were found to have a single point mutation in the central conserved region of the rpoB gene (Table 1). The possibility of a permeability barrier to rifampin led us to investigate the role of the membrane in determining resistance. The addition of 0.05% Tween 80 was found to greatly reduce the observed MIC in the highly resistant isolates while having no effect on the intermediate or susceptible isolates. Tween 80 is a detergent which is used in liquid medium preparation to increase the uptake of nutrients. It has also been used to investigate membrane permeability in mycobacteria, in which it also acts as a growth supplement (13). No difference in growth rates in the presence and absence of Tween 80 was observed for the susceptible, intermediate, or highly resistant isolates. These results suggest that the basal level of resistance to rifampin imposed by a single point mutation in the rpoB gene gives an MIC of approximately 25 to 100 μg/ml. The fall in MIC for the highly resistant mutants in the presence of Tween 80 implies that additional mechanisms are involved in determining the levels of resistance to rifampin.

Following the observation that mutant isolates were also more resistant than their parents to other hydrophobic agents (Triton X-100 and gentian violet), we investigated the possible role of the mtr locus in the resistance phenotype. Previous studies had shown that this locus was responsible for the resistance of N. gonorrhoeae to a range of hydrophobic agents. Mutations in the mtrR gene and its promoter were shown to cause high-level resistance, including increased resistance to rifampin (10, 18). Mutations in mtrR lead to increased levels of the lipoprotein MtrC, which is homologous to the efflux protein from P. aeruginosa. A homolog of the gonococcal mtr locus was found in meningococci, and sequences of the mtrR gene and its promoter were obtained. Parent and mutant strains were found to have identical sequences of both the mtrR gene and its promoter (Fig. 3 and 4). This suggests that the mechanism of increased resistance to rifampin in these isolates is...
not related to the hydrophobic resistance seen in gonococci. The sequence of the mtrR protein was almost identical to the gonococcal sequence (18). This would be expected for two closely related species which differ by only 2% in their housekeeping genes. At one position (amino acid 105) (Fig. 3), the meningococcal sequence was identical to the sequence of a resistant mutant gonococcus (10). The precise role this may play in the function of mtrR in meningococci is difficult to assess, although we found that the MICs of Triton X-100 and gentian violet were higher for RifS meningococci than gonococci. The promoter region of the N. meningitidis mtr locus homolog was 97.2% identical to the gonococcal sequence but contained a 158-bp insertion (Fig. 4). An inverted repeat present in the gonococcal sequence was also present in meningococci in an imperfect form. This repeat is considered to be important in determining the resistance of gonococci to hydrophobic agents. A single-base-pair deletion in the repeat is responsible for the high levels of resistance seen in gonococcal mutants. The precise role, if any, of the mtr region in meningococci is not clear. Control of the levels of MtrC in gonococci is important, as it enables these organisms to survive two different environments, the genital tract and the rectum. Meningococci are carried in the upper respiratory tract and do not appear to require resistance to hydrophobic agents. However, there are a number of reports of N. meningitidis being isolated from genital and anorectal sites (8, 21), and it may be that these isolates have acquired the mtr locus, which performs the same function as in gonococci.

Evidence that other mutations within the meningococcal genome (apart from the change in the rpoB gene) were associated with the increased resistance to rifampin came from the studies using 2D gel electrophoresis, which showed an alteration in the position of one protein spot in two mutants, F1M and F5M. This change resulted from the decrease in pl of a protein of approximately 19 kDa (Fig. 2). This difference was not observed in A11M or G6M. This could possibly result from there being a different mutation in A11M or G6M which does not alter the mobility of the protein. Further studies are under way to identify this protein and determine the mutation giving rise to the altered mobility.

Most studies have suggested that the acquisition of resistance to rifampin is due to a single event, namely, the rpoB mutation. However, there are some reports which suggest that resistance can be due to mutations in other genes. Resistance to a number of antibiotics, including rifampin, can be induced in Escherichia coli by growth on tetracycline or chloramphenicol (9). The resistance phenotype is controlled by the mar locus, and mutations within this locus give rise to resistant isolates (6). Alterations in the membrane permeability of N. gonorrhoeae not associated with the mtr locus have also been described (15). Such experiments suggest that different levels of resistance to rifampin can be imparted by mechanisms other than mutations in the rpoB or mtr gene. It is likely that increased resistance to rifampin in the isolates studied here is due in part to a decrease in the ability of the drug to enter the bacterium, mediated by a hitherto-undescribed mechanism, in combination with mutations in the rpoB gene.

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