Introduction

Neisseria meningitidis causes serious disease leading frequently to death in children and elderly people, though less commonly in all other age groups. Despite the high virulence of some strains, Maiden & Feavers estimated that only one in 10,000 infections results in a case of disease under endemic circumstances, while in an epidemic, one in 100 infections could be the cause of serious illness. The remaining infections result in carrier status.

In general, two different types of population structure can be distinguished. Bacteria exhibiting a population structure that is characterized by strong vertical inheritance are called ‘clonal’, while those showing vertical and horizontal inheritance are called ‘panmictic’; in the latter type the genetic material is shuffled through horizontal transfer of DNA. For a long time, scientists believed meningococci to be clonal, but a series of studies and publications led to the conclusion that within the genus Neisseria, and in particular within N. meningitidis, there are population structures ranging from clonal to varying degrees of panmictic.\(^{4,5}\) The position on the scale of population structures, ranging from clonal to panmictic, depends on the frequency of horizontal genetic exchange.\(^{5}\) This mechanism has been demonstrated for a variety of gene loci, including penicillin binding proteins\(^{6}\) or the \(\text{dhps}\) locus.\(^{7,8}\)

Horizontal genetic transfer between species could occur more frequently in strains from a carrier, since these have the opportunity to coexist with clinically unimportant Neisseria spp. living in the upper respiratory tract of humans.\(^{3}\) This assumption is of particular interest in the study of resistance to antibiotics because, via horizontal transfer, even a chromosomally encoded resistance mechanism can, at least in part, be transferred. One example of such a mechanism has been demonstrated for the gene coding for a penicillin binding protein in Neisseriaeae.\(^{6}\)

For close contacts of index cases of neisserial disease, chemoprophylactic treatment with the antibiotic rifampicin is recommended. Rifampicin acts on the \(\beta\) subunit (encoded by \(\text{rpoB}\)) of DNA-directed RNA polymerase (EC 2.7.7.6) by inhibiting the elongation of the initiated RNA chain.\(^{9}\) Frequently, cases have been described of meningococci with clinically significant rifampicin resistance, which...
has even caused death in secondary cases. The reports describing the mechanism of resistance against rifampicin include the species Mycobacterium tuberculosis and N. meningitidis. In most cases, a single point mutation in a specific region of the rpoB gene has been identified, leading to an amino acid substitution, although more recently A. Badi et al. have demonstrated that membrane permeability effects may play an important role in resistant meningococci, generated in vitro, which show extremely high levels of rifampicin resistance. In addition, some strains of M. tuberculosis are resistant to rifampicin without any changes in the nucleotide sequence within the subgenic fragment of rpoB being considered responsible.

However, in most studies published, the evidence for the point mutation as the cause of resistance has been provided by sequencing unrelated strains and/or in-vitro generated strains. These sequence data were compared with those from wild-type strains assuming strong conservation of the gene. In the present study, direct evidence is given for the mechanism by which meningococci acquire rifampicin resistance. In addition, the genetic variability of the subgenic region encompassing the site of rifampicin resistance is analysed. The results are discussed with respect to the population structure of meningococci.

### Materials and methods

#### Bacterial strains

The N. meningitidis strains studied were obtained from the National Reference Laboratory for Meningococci (NRLM) at the Hygiene Institute, University of Heidelberg, Germany, except for strains 9409B and 9419B, which were kindly provided by J. A. Vazquez, Majadahonda, Spain. For all strains, serogroup, serotype and serosubtype were determined according to standard serological techniques, namely latex agglutination for serogroup and whole-cell enzyme-linked immunosorbent assay (ELISA) for serotype and subtype. A nitrobiotic resistance data were obtained using either the Etest procedure (strains above the line in Table I) or the agar dilution method (strains below the line in Table I), by testing the antibiotics routinely used in the NRLM. All data available for the strains studied are summarized in Table I.

The so-called sibling strains are numbered 3981B and 3982B. Strain 3981B was isolated in March 1995 from a 10-year-old child who was admitted to the university hospital in Bonn, Germany, with petechiae and a Waterhouse-Friderichsen-like syndrome. Six days later, strain 3982B was isolated from the child’s 11-year-old sibling who had

#### Table I. Characterization of all strains studied

<table>
<thead>
<tr>
<th>Strain</th>
<th>Subtype</th>
<th>Ci (mg/L)</th>
<th>Ct (mg/L)</th>
<th>Tc (mg/L)</th>
<th>PG (mg/L)</th>
<th>Ri (mg/L)</th>
<th>Ca (mg/L)</th>
<th>Am (mg/L)</th>
<th>Isolated from</th>
</tr>
</thead>
<tbody>
<tr>
<td>3981B</td>
<td>B:15:P1.7,16</td>
<td>0.006</td>
<td>0.006</td>
<td>0.75</td>
<td>0.064</td>
<td>0.064</td>
<td>NT</td>
<td>NT</td>
<td>patient</td>
</tr>
<tr>
<td>3982B</td>
<td>B:15:P1.7,16</td>
<td>0.004</td>
<td>0.004</td>
<td>0.50</td>
<td>0.064</td>
<td>&gt;32</td>
<td>NT</td>
<td>NT</td>
<td>patient</td>
</tr>
<tr>
<td>9409B</td>
<td>B:4:P1.4</td>
<td>0.006</td>
<td>0.008</td>
<td>0.75</td>
<td>0.094</td>
<td>&gt;32</td>
<td>NT</td>
<td>NT</td>
<td>patient</td>
</tr>
<tr>
<td>9419B</td>
<td>B:4:P1.4</td>
<td>0.006</td>
<td>0.012</td>
<td>0.75</td>
<td>0.125</td>
<td>&gt;32</td>
<td>NT</td>
<td>NT</td>
<td>patient</td>
</tr>
<tr>
<td>3377B</td>
<td>B:NT:P1.1</td>
<td>0.008</td>
<td>0.008</td>
<td>0.75</td>
<td>0.016</td>
<td>0.008</td>
<td>NT</td>
<td>NT</td>
<td>carrier</td>
</tr>
<tr>
<td>3654A</td>
<td>A:4:NT</td>
<td>NT</td>
<td>NT</td>
<td>0.5</td>
<td>0.06</td>
<td>0.5</td>
<td>1</td>
<td>0.125</td>
<td>patient</td>
</tr>
<tr>
<td>3704W</td>
<td>W135:NT:P1.10</td>
<td>NT</td>
<td>NT</td>
<td>0.5</td>
<td>0.03</td>
<td>0.015</td>
<td>1</td>
<td>0.06</td>
<td>patient</td>
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<tr>
<td>3708Y</td>
<td>Y:14:NT</td>
<td>NT</td>
<td>NT</td>
<td>2</td>
<td>0.06</td>
<td>0.015</td>
<td>1</td>
<td>0.06</td>
<td>patient</td>
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<tr>
<td>3722B</td>
<td>B:NT:P1.2,5</td>
<td>0.004</td>
<td>NT</td>
<td>0.5</td>
<td>0.03</td>
<td>0.03</td>
<td>2</td>
<td>0.015</td>
<td>patient</td>
</tr>
<tr>
<td>3724E</td>
<td>29E:4:P1.5</td>
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<td>NT</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>1</td>
<td>0.06</td>
<td>carrier</td>
</tr>
<tr>
<td>3730C</td>
<td>C:4:NT</td>
<td>0.004</td>
<td>NT</td>
<td>0.5</td>
<td>0.125</td>
<td>0.008</td>
<td>1</td>
<td>0.06</td>
<td>patient</td>
</tr>
<tr>
<td>3735P</td>
<td>poly:NT:P1.7</td>
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<td>NT</td>
<td>0.5</td>
<td>0.125</td>
<td>0.06</td>
<td>1</td>
<td>0.06</td>
<td>carrier</td>
</tr>
<tr>
<td>N. gonorrhoeae</td>
<td>0.008</td>
<td>0.008</td>
<td>NT</td>
<td>0.64</td>
<td>0.023</td>
<td>NT</td>
<td>NT</td>
<td>patient</td>
<td></td>
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<tr>
<td>N. lactamica</td>
<td>0.008</td>
<td>0.016</td>
<td>NT</td>
<td>0.38</td>
<td>1.0</td>
<td>NT</td>
<td>NT</td>
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</tr>
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<td>N. flava</td>
<td>0.008</td>
<td>0.016</td>
<td>NT</td>
<td>0.75</td>
<td>0.38</td>
<td>NT</td>
<td>NT</td>
<td>carrier</td>
<td></td>
</tr>
<tr>
<td>N. subflava</td>
<td>0.016</td>
<td>0.032</td>
<td>NT</td>
<td>1.0</td>
<td>1.0</td>
<td>NT</td>
<td>NT</td>
<td>carrier</td>
<td></td>
</tr>
</tbody>
</table>

a poly, polyagglutinable; NT, not typable; NST, not subtypable.

The number refers in each case to the designated strain number in the collection of the National Reference Laboratory for Meningococci (NRLM) at the Hygiene Institute, University of Heidelberg, Germany. For each strain, the results of serological typing, the antibiotic resistance data and the source of isolation (patient or carrier) are given. Strains above the line were tested using the E test procedure, but strains below that line (isolated 1994 or earlier) were examined by an agar dilution method (which reflects the change in routine drug susceptibility testing at the NRLM, Heidelberg). NT not tested. A nitrobiotics tested were: Ci, ciprofloxacin; Ct, cephotaxim; Tc, tetracycline; PG, penicillin G; Ri, rifampicin; Ca, chloramphenicol; Am, ampicillin.
received prophylactic rifampicin treatment for 4 days, but also developed petechiae and was admitted to the same hospital. The siblings recovered after treatment with penicillin. Isolated strains were sent to the N R L M in Heidelberg for further typing and antibiotic susceptibility testing. In addition to these strains, a variety of rifampicin-resistant strains belonging to different serogroups as well as four other species of the genus Neisseria (Neisseria lac-tamica, Neisseria flava, Neisseria subflava and Neisseria gonorrhoeae) were studied (Table I). For each of the most common meningococcal serogroups, one strain was chosen randomly from the collection of strains at the N R L M in Heidelberg. N. gonorrhoeae and the clinically unimportant species were also taken from the N R L M. For these species, detailed typing other than for antimicrobial susceptibility testing was not performed. The strains below the line in Table I (except strains of the clinically unimportant species) were used in previous studies to characterize a gene probe. 15

DNA isolation
Chromosomal DNA was extracted according to a modified procedure from E. C. G otschlich (personal communication) and is described here briefly. Meningococci were scraped into 10 mM Tris-1mM EDTA (TE) buffer (pH 8.0) from an overnight culture on chocolate agar plates and washed twice. After brief centrifugation, the bacterial pellet was resuspended in 250 µL TE buffer containing sodium dodecyl sulphate (SDS) to a final concentration of 1%. Following incubation for about 30 min at 65°C, the solution turned clear (cell lysis). Proteinase K (Merck, Darmstadt, Germany) was added to a final concentration of 0.5 mg/mL and incubated for an additional 4 h at 37°C. The solution was extracted twice with TE-saturated phenol, twice with chloroform/isoamylalcohol (24:1 v/v), and finally with phenol/chloroform/isoamylalcohol (25:24:1 by volume). Chromosomal DNA in the aqueous solution was pelleted with 0.1 volume of 3 M sodium acetate and 2 volumes of absolute ethanol, followed by a washing step with 80% ethanol. After vacuum drying, DNA was resuspended in 1 mL of TE buffer containing RNase (D Nase-free, Boehringer Mannheim, Mannheim, Germany) and incubated for an additional hour at 37°C. Following an extrac-tion step, the DNA was analysed on a 0.7% agarose gel, the concentration was measured and the DNA diluted to give a working solution of 100 pg/µL.

PCR
To determine the molecular mechanism of rifampicin resistance, a 790 bp fragment from the rpoB gene, covering the region associated with rifampicin resistance, was amplified by PCR. The region in which the mutation conferring resistance can occur was known from previous studies in other organisms. 12, 13 Sequence analysis of the whole rpoB gene (E M B L/G enB ank/D D B J database accession number Z54353) showed two sites, 471 bp apart, for the restriction enzyme ClaI within the 790 bp fragment. Digestion with the isoschizomeric enzyme Bsu15I (Fermentas, Vilnius, Lithuania) yielded a 471 bp central fragment which was cloned into the vector pBluescript SK – (Stratagene, La Jolla, CA, U SA) with high efficacy. Primers R P O R R 1 and R P O R R 5 (Table II), which were to amplify the 790 bp fragment, were chosen from the analysis of the whole rpoB gene. This reference sequence is referred to later as the BNCV (serogroup B, noncapsulated variant) sequence or strain.

A m plification was carried out in an automated thermal cycler (Grant Autogene II, Grant Instruments, B arrington, U K) using DNA from all strains listed in Table I. The reaction mixture was as follows: D N A (100 pg per reaction), 50 pmol of each primer, and the four deoxynucleotides (Phar-macia, B romma, Sweden) each at a final concentration of 0.2 mM and T a q polymerase (5 U/µL, 1 U per reaction; Fermentas, Vilnius, Lithuania) were mixed. The reaction buffer supplied with the polymerase was used, and MgCl2 was added to a final concentration of 4 mM. The following temperature profile was used: denaturation at 94°C for 0.7 min, annealing at 52°C for 1.5 min and extension at 72°C for 1 min. A m plification products were analysed on 1.5% agarose gel, and restriction enzyme analysis performed using the enzyme Bsu15I.

Sequence analysis of the PCR products
PCR products from strains 3981B (rifampicin-sensitive strain), 3982B, 9409B and 9419B (all of which are rifampicin-resistant) were cloned into pBluescript SK – at the ClaI site according to the manufacturer’s instructions. At least three clones from each isolate were sequenced. Sequencing reactions were performed manually by the chain termination method 16, 17 using the Sequenase 2.0 kit (A mershaw B uchler, B raunschweig, G ermany) according to the manufacturer’s instructions. In addition, undigested amplification products were sequenced using the Ampli Cycle sequencing kit (Perkin E Imr, R oche Molecular Systems, B ranchburg, N J, U SA). A m plification products were concentrated using G ene Clean II K it (B io101, V ista, C A, U SA), following the supplier’s instructions. Ninety microlitres of each reaction mixture were washed, and the DNA resuspended from the silica matrix in 25 µL TE buffer. Three microlitres of this DNA preparation were usually sufficient to obtain readable sequences. Sequencing reactions were performed in an automated thermal cycler (G rant A utogene II, G rant I nstruments, B arrington, U K).

Three microlitres of DNA, 3 µL [α-32P]dATP (>1000 Ci/mmol; A mershaw B uchler, B raunschweig, G ermany), 4 µL reaction buffer including T aq polymerase, 2 µL sequencing primer (10 pmol/µL) and 20 µL water were mixed; 8 µL of this mixture was added to 2 µL of each termination mix in a 0.5 mL reaction tube. The mixture was
Table II. Primers used in the present study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5'–3')</th>
<th>Position</th>
<th>Tm (°C)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPORR1</td>
<td>sense</td>
<td>AAA AAC TGT CCG AAG CCC AAC AAA ACT CT</td>
<td>1247–1275</td>
<td>82</td>
<td>790 bp amplicon</td>
</tr>
<tr>
<td>RPORR5</td>
<td>antisense</td>
<td>ATA TAT TGG ACG CGG TCG GGC GCT T</td>
<td>2036–2013</td>
<td>80</td>
<td>790 bp amplicon</td>
</tr>
<tr>
<td>NmB9F</td>
<td>sense</td>
<td>ATG ATT AAT GCA AAA CCT GT</td>
<td>1552–1571</td>
<td>56</td>
<td>sequencing primer</td>
</tr>
<tr>
<td>NmB24R</td>
<td>antisense</td>
<td>TTC GGA CCT TCA GGC GTT T</td>
<td>1780–1762</td>
<td>58</td>
<td>sequencing primer</td>
</tr>
<tr>
<td>NSF25R</td>
<td>antisense</td>
<td>CCA ATG TTT GGC CCT TCA G</td>
<td>1786–1786</td>
<td>58</td>
<td>sequencing primer</td>
</tr>
</tbody>
</table>

Besides the name of each primer the orientation, sequence and position with respect to the BNCV reference sequence (EMBL/GenBank/DDBJ accession number Z45535) are given. The melting temperature (Tm) as supplied by the manufacturer (TIB MolBiol, Berlin, Germany) is given. For all primers, the function (sequencing or amplification) is indicated. Primer NSF25R was used instead of NmB24R for sequencing the PCR fragment derived by amplifying DNA from N. subflava.
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flava and N. gonorrhoeae yielded a single band of expected size on a 1.5% agarose gel. However, PCR using DNA from Neisseria sicca failed to amplify the desired fragment. DNA fragments of the meningococcal strains (and that of N. gonorrhoeae) have two internal sites for the restriction enzyme Clal, approximately 471 bp apart. After digestion with the isoschizomeric enzyme Bsu15I, the central 471 bp, which includes the 295 bp segment previously analysed for rifampicin resistance by Carter et al. (which will be referred to as s74030) was cloned into pBluescript SK−.

Cloning was performed using digested amplification products from four strains, namely, 3981B, 3982B, 9409B and 9419B. Sequence data for the remaining strains listed in Table I were obtained with the cycle sequencing technique. The unambiguous sequences are presented in Figure 1.

Sequence data from the PCR products

Comparison between the already published sequence of the 295 bp rpoB fragment (accession number s74030) and the corresponding fragment on the reference sequence of the whole rpoB gene (BNCV sequence) showed only two nucleotide substitutions, the one responsible for rifampicin resistance (aa 552, nt 1654) and another one, resulting in an amino acid substitution: a methionine codon (ATG) is substituted by a lysine codon (TTG; aa 518, nt 1552) in the BNCV strain; the mutations are numbered according to the whole rpoB gene.

Sequence analysis of strains 3981B and 3982B isolated from the siblings gave identical sequences, except for one base substitution at aa 548 (nt 1643). This single difference between the two fragments led to an amino acid substitution. A serine residue in the rifampicin-resistant strain 3981B is replaced by phenylalanine in the rifampicin-resistant strain 3982B. Compared with s74030 and the sequence from the BNCV strain a further eight altered nucleotide positions could be detected in strains from the siblings. With two exceptions, these substitutions were neutral. One at aa 572 (nt 1714) led to glutamine in strains 3981B and 3982B compared with glutamic acid in all other strains.

Figure 1. Matrix showing the point mutations within all sequenced PCR products. Only the altered sites are presented, as indicated by the numbers above (numbers are written vertically). Dots indicate the same base as in the reference sequence, which was derived from the BNCV strain; minus signs indicate data not available; letters printed in bold indicate the altered sites conferring rifampicin resistance (also indicated by an asterisk above the sequence); plus signs show the sites where amino acid substitutions occurred. Numbering is according to the BNCV reference sequence (EMBL/GenBank/DDBJ accession number Z54353). s74030 is the accession number of the sequence published previously by Carter et al.13 which starts in the original paper with number 1. All other abbreviations are the same as in Table I. The lines demonstrate the division into sequence groups.
studied, the other one at residue 590 (nt 1768) led to a substitution of proline by serine. As both substitutions occurred in both the rifampicin-sensitive strain 3981B and the resistant strain 3982B, they can be seen as normal genetic variations. Furthermore, the mutations are genetic markers for strains 3981B and 3982B, demonstrating the strong clonal relatedness of these two strains. The substitution at aa 548 which conferred rifampicin resistance has not been described previously for meningococci, but has been noted in M. tuberculosis.  

The two strains supplied by V azquez (94098/9419B) were identical according to sequence analysis. The deduced amino acid sequence was identical to that from the BNCV strain with the exception of one substitution at aa 552 (nt 1654), where a histidine residue was substituted by a tyrosine residue as described previously for three strains, selected in vitro for rifampicin resistance. A t aa 623 (nt 1879), which is downstream from the known rifampicin resistance region, both strains from Spain had a lysine residue, whereas in the BNCV strain glutamic acid was found. The same kind of mutation was observed in three more strains from Germany, indicating that this mutation is not linked to the degree of rifampicin susceptibility.  

Besides the point mutations that led to substitutions of amino acids, further changes have been observed in the sequences of amplification products of all strains which did not affect the deduced amino acid translation and are therefore neutral. Within the meningococcal strains and that of N. gonorrhoeae, between one and ten neutral mutations in comparison with the BNCV sequence could be detected (Figure 1). The clinically unimportant Neisseria species differed in nucleotide sequence between 7% and nearly 10%; most of the mutations were neutral. In Figure 1, the changes in the nucleotide sequences of all strains sequenced are shown in comparison with the BNCV sequence which is taken as a reference.  

The sequences can obviously be divided into two groups. The first group is characterized by having alterations at two nucleotide sites relative to the BNCV sequence; these changes are shared by all strains of this group (the strains above the line in Figure 1). The second group (strains below the line in Figure 1) is characterized by six point mutations, compared with the BNCV sequence, which are also shared by all strains of this group. Within each of these two groups, additional point mutations are observed in some strains. Using all sequences obtained by sequencing the 471 bp fragment and s74030, a tree was constructed using the option CLUSTREE (program CLUSTAL W), which uses the neighbour-joining method. In the resulting tree, the clinically unimportant Neisseria species N. flava, N. subflava and N. lactamica clustered separately from the pathogenic species N. gonorrhoeae and N. meningitidis. The relationships are shown in the dendrogram in Figure 2. Bootstrapping of the dendrogram revealed that most knots were not of statistical significance. However, computer analysis using various options of the PHYLIP package, e.g. constructing trees based on a distance matrix according to Kimura with the method of Fitch or the UPGMA method, always resulted in comparable trees. Only strain 3735P was positioned differently but always within the second group of sequences. In each tree, the first group of sequences were positioned in identical clusters (except sequence s74030), and all trees revealed a comparable clustering of the second group of strains. However, using the UPGMA method, which requires an outgroup, the clustering of strains belonging to the second group is more obvious than with the neighbour-joining method (Figure 2). This clustering indicates that the strains of the first group are closely related to each other, whereas although the strains constituting the second group seem to be related, this is only to a lesser extent. Most strains of the second cluster differ by serogroup. Interestingly, the sequence of N. gonorrhoeae is positioned within the meningococcal sequences due to its high degree of similarity. The gonococcal sequence is absolutely identical to the sequence determined for strain 3924E.  

A nalysis of the sequence data revealed a cluster of four neutral mutations within five consecutive codons (numbers 1707, 1710, 1713 and 1719 in Figure 1) in the rpoB gene fragment of some meningococcal strains. Interestingly, Carter et al. detected identical neutral point mutations in some of the strains they studied.  

Discussion  

Rifampicin resistance  

Mutations leading to rifampicin resistance have been studied for a variety of bacteria including E. coli, M ycobacterium leprae, M. tuberculosis and N. meningitidis. In most cases a single mutation in the rpoB gene, which codes for the β subunit of DNA-directed RNA polymerase (EC 2.7.7.6), could be detected, resulting in an amino acid substitution. For M. tuberculosis, a multitude of such single mutations are possible. Recently, membrane permeability effects were demonstrated to be responsible for very high levels of rifampicin resistance in meningococci. However, M. tuberculosis strains exist where the reason for resistance could not be detected. In most studies, the sensitive reference strain was clonally unrelated to the resistant strain analysed. Only in the study by Williams et al. were two M. tuberculosis strains from the same patient, i.e. clonally related, examined before and after acquiring resistance to rifampicin.  

In the present study, meningococcal strains from very closely related patients were studied. Strains 3981B and 3982B were isolated from siblings. Both strains were subjected to typing by standard methods. No differences between the two strains could be detected except alteration of the MIC of rifampicin. Sequencing of the 471 bp DNA fragment generated by PCR and restriction enzyme digestion yielded identical sequences for both strains except for
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Due to the altered nucleotide, a phenylalanine replaced a serine. This amino acid substitution has been described for *M. tuberculosis* as one possible cause of rifampicin resistance. Together with the anamnestic data, the mutation in the sibling strain 3982B is considered to be responsible for resistance against rifampicin. By comparing all the data available for the two strains (including the nucleotide sequence data which give the finest resolution of genetic diversity), it is likely that both siblings were infected by meningococci from one clone. The point mutation conferring resistance to rifampicin must have occurred either during or before prophylactic treatment of the second sibling. However, the MICs of the antibiotics tested differ slightly, especially for tetracycline (Table I). These differences are only in the range of one step on the E test strip, though, they may be interpreted as variations due to the test procedure. Thus, in this case the point mutation in the resistant strain 3982B is direct evidence of the mechanism of development of rifampicin resistance.

The specific point mutation causing rifampicin resistance, i.e. the substitution of serine in the susceptible strain by phenylalanine in the resistant strain at aa 548 of the
rpoB gene fragment, was not found in the collection of strains studied by Carter et al., who always found the same substitution, namely, a histidine to arginine substitution at aa 552 in all resistant strains from patients. In some in-vitro generated rifampicin-resistant strains, the authors observed a histidine to tyrosine substitution. The same mutation was found to be responsible for rifampicin resistance of the two strains from Spain examined in the present study. A substitution in the case of rifampicin resistance in M. tuberculosis, more than one probable point mutation can confer resistance. All point mutations in the rpoB gene known so far seem to induce comparable levels of resistance, as determined by the E test. The data give clear evidence that rifampicin resistance can develop rapidly under treatment with this antibiotic. Such resistant strains may subsequently be spread through the population by carriers. There is a lack of information on the possibility of horizontal transfer of rifampicin resistance from one Neisseria species to another. However, studies in Nigeria and Southern Europe have demonstrated that the prevalence of carriage of rifampicin-resistant meningococci could be extremely high after initial rifampicin treatment. This demonstrates the possible existence of a horizontal spread of rifampicin resistance besides a simple point mutation induced by chemophylaxis. The nucleotide sequence data for three clinically unimportant Neisseria spp. presented provide the information for recognizing horizontal transfer at this specific locus if it occurred.

Carter et al., found identical sequences in the six resistant isolates sampled from patients in the UK, all of them belonging to serogroup C, subtype 2a, providing proof of a clonal spread of a resistant strain throughout the country. In a collection of more than 20 strains, these authors observed a set of additional point mutations, all of which were silent, whereas in the data presented here, seven amino acid substitutions within the meningococcal strains were detected. Two of these amino acid substitutions are responsible for rifampicin resistance, the remaining five being natural variation. These amino acid changes cannot be responsible for resistance, as the mutations occur either in rifampicin-sensitive strains or in sensitive and resistant strains.

In the strains studied here, a comparable number of silent mutations to those described by Carter et al., could be detected. A nalysis of the clonal relationships by means of constructing dendrograms from the sequenced strains resulted in a tree with two more or less focused clusters of meningococcal strains. The first cluster (sequence group 1) seems to be highly clonally related, whereas the second cluster is somewhat weaker. This is interesting because the strains belonging to the first cluster have diverse geographical origins (US, Spain, Germany and s74030 from the UK), whereas most strains from the second cluster were collected in Germany over the period of one year. Some strains studied by Carter et al., are of similar sequence to the strains from the second sequence group in this study, a fact that supports the hypothesis of clonal spread of virulent strains.

The genetic relationships observed for the N. meningitidis strains could thus imply clonality. This contrasts with the findings of Spratt et al., and a number of other researchers who claim that N. meningitidis and in particular subpopulations of serogroup B and C tend to be panmictic. This conflict is probably a problem of sampling. Except for three meningococcal strains, all those used in the present study were isolated from patients suffering from disease. On the assumption that under endemic circumstances only one in 1000 or more strains will cause disease the collection of strains do not reflect the epidemiological situation. Most studies on the genetic variability of meningococcal gene loci are biased because a large proportion of strains are isolated from patients, while only a few of those studied are isolated from carriers; thus this situation is the opposite of the normal epidemiological one. The assumption of a more or less panmictic population structure is based on the study of carrier strains. It remains unclear whether the conserved nature of the studied DNA fragment has any impact on the observed genetic relationships. A multiple alignment using the meningococcal rpoB gene and some homologous genes from other organisms showed high conservation in the amino acid sequence within the amplified and studied fragment (not shown).

The results presented here demonstrate the importance in all species of studying a sample of strains that is in accordance with the observed or known epidemiology of that particular species, as otherwise clonal analysis will not be reliable in the context of population genetics. However, there is no direct evidence in the sequence data presented here, that horizontal transfer could have occurred. The sequence data from the clinically unimportant species provide the opportunity to search in further studies for intragenic horizontal exchange at this specific locus. Data from strains isolated from siblings provided direct evidence that rifampicin resistance can occur rapidly in vivo following exposure to this antibiotic. The study confirmed that a single point mutation is responsible for resistance in vivo and demonstrated that in vivo more than one amino acid can be the subject of a resistance-conferring point mutation.

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References


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