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Molecular Characterization of Rifampin-Resistant *Neisseria meningitidis*

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Primers were designed to amplify the *rpoB* gene of *Neisseria meningitidis*. The region of the gene amplified covered clusters I and II of the rifampin resistance (Rif^r) mutation sites identified in *Escherichia coli*. DNAs from six Rif^r isolates and 21 rifampin-susceptible isolates from the United Kingdom representing a number of serogroups were amplified and sequenced. All six Rif^r isolates had identical DNA sequences and the same amino acid change, a His to an Asn change at position 35 (H35N). This His residue is equivalent to the His residue at position 526 in *E. coli*, one of the known Rif^r mutation sites. DNAs from an additional six Rif^r mutants generated in vitro were amplified and sequenced. Three had H35Y changes, one had an H35R change, one had an H35N change and one had an S40F change. The predominance of mutations at the His residue at position 35 in Rif^r *N. meningitidis* isolates suggests that it plays a critical role in the selection of antibiotic-resistant variants. All six Rif^r isolates belonged to the same clonal group when analyzed by restriction enzyme analysis and pulsed-field gel electrophoresis. These data suggest that a single clone of Rif^r *N. meningitidis* is present and widespread throughout the United Kingdom.

Infection with *Neisseria meningitidis* remains an important health problem among children and young adults. Mortality rates of 10% have been reported. The ease with which *N. meningitidis* can spread among families and people in close-knit communities increases the risk of developing the disease by 500- to 800-fold compared with the risk of spread from the general population (18). Treatment of close contacts with antibiotics is routinely performed during outbreaks of meningococcal infection to help prevent the spread of the disease. Two drugs that have been used for chemoprophylaxis are rifampin and minocycline. These are effective in eradicating nasopharyngeal colonization by *N. meningitidis*, although the use of minocycline is limited by the high rate of adverse reactions that it causes (6). Nasopharyngeal carriage of *N. meningitidis* can be reduced by ~90% by using rifampin (5); however, strains of rifampin-resistant (Rif^r) meningococci have been recovered from recipients of the drug (9, 28). Furthermore, Rif^r meningococci are known to cause systemic disease, and rifampin prophylaxis may fail to prevent secondary cases of infection (28). The spread of Rif^r meningococcal strains may pose serious problems in the management of *N. meningitidis* infections.

Rifampin is effective against a wide range of bacteria and mycobacteria. It acts by binding to the β subunit of the RNA polymerase enzyme, preventing transcription of DNA to RNA (10). Mutations that confer resistance to rifampin have been characterized in isolates of *Escherichia coli* (20), *Mycobacterium leprae* (11), and *Mycobacterium tuberculosis* (27). The mutations occur in the *rpoB* gene, which encodes the β subunit of the polymerase. To date, 15 amino acid positions which are altered in Rif^r mutants have been identified. The majority of these mutation sites (14 of 15) occur in a short conserved region (amino acids 507 to 687 in *E. coli*) of the β subunit. Within this region, the mutation sites are located in three clusters, with a total of 13 sites occurring in clusters I and II (13). Each of the clusters is thought to occur in a part of the *rpoB* gene that codes for the rifampin-binding site in the subunit. It has also been observed that some of these mutations are associated with a number of conditional defects such as temperature sensitivity of growth as well as rifampin resistance (14).

The spread of antibiotic-resistant strains of *N. meningitidis* has been examined by a number of molecular techniques (2, 17, 23). Like many other bacteria, populations of *N. meningitidis* appear to have a clonal structure, being made up of numbers of different groups of organisms that are closely related and that are derived from a common ancestry (3). In the study described here, we used direct sequencing techniques to establish the molecular basis of rifampin resistance in *N. meningitidis* and also determined the clonal relationship of resistant isolates by using restriction enzyme analysis (REA) and pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Bacterial strains. Strains of *N. meningitidis* were obtained from the Aberdeen Royal Infirmary, R. Fallon (Glasgow, United Kingdom), and D. Jones (Manchester, United Kingdom); details about the strains have been described previously (17). Serogroup, serotype, and antibiotic resistance data for the 6 Rif^r, 21 rifampin-susceptible (Rif^s), and 6 in vitro-generated Rif^r mutant isolates used in the study are presented in Table 1. Isolates were stored on beads at −70°C (Protect Vials; Technical Services Consultants, Bury, United Kingdom).

In vitro generation of rifampin-resistant mutants. Rifampin-susceptible strains of *N. meningitidis* were plated onto chocolate agar supplemented with 50 μg of rifampin (Rifactane; Ciba Laboratories, Horsham, United Kingdom) per ml. Plates were incubated at 37°C for 48 h. Single colonies were replated on chocolate agar containing 50 μg of rifampin per ml, and the MICs for the isolates were determined by the E test (1).

DNA isolation. DNA was extracted from strains of *N. meningitidis* by a modification of the guanidinium thiocyanate

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TABLE 1. Isolates analyzed in the study

Isolate (location, year of isolation)[a]	Serogroup	Serotype	MIC (μg/ml)	REA clonal group	Mutation[b]
A5	C	NS[c]	<0.064	7	
A10	C	2a	<0.064	1	
A16	C	2	<0.064	2	
F4	C	2a	<0.064	1	
F5	C	2a	<0.064	1	
F6	C	2a	<0.064	1	
F7	C	2b	<0.064	2	
F10	C	2b	<0.064	3	
F38	NG[d]	NS	<0.064		
G6	W135	NS	<0.064		
J9	C	2b	<0.064	2	
J23	C	NS	<0.064	1	
A11 (Aberdeen, 1987)	C	2a	24	1	H35N
A14 (Aberdeen, 1986)	C	2b	24	1	H35N
F1 (Falkirk, NK[e])	C	2a	24	1	H35N
F2 (Falkirk, NK)	C	2a	24	1	H35N
F3 (Aberdeen, 1987)	C	2a	24	1	H35N
J37 (Lancaster, 1987)	C	2a	24	1	H35N
G5	Y		<0.064		
J25	C	NS	<0.064	11	
J35	C	NS	<0.064	5	
J39	C	NS	<0.064	11	
J28	B	15	<0.064		
J34	C	1	<0.064	6	
A6	A		<0.064		
F18	C	15	<0.064	9	
F19	B	4	<0.064		
A6M	A		>256		H35Y
A10M	C	2a	>256	1	H35R
F5M	C	2a	>256	1	H35N
F19M	B	4	>256		S40F
F38M	NG	NS	>256		H35Y
G6M	W135	NS	>256		H35Y

[a] All isolates except the six Rif[r] mutants A6M, A10M, F5M, F19M, F38M, and G6M are grouped according to the silent mutations of the *rpoB* gene (see text). All locations are in the United Kingdom.
[b] Numbering refers to the *N. meningitidis* rpoB gene sequence shown in Fig. 1.
[c] NS, nonserotypeable.
[d] NG, nonserogroupable.
[e] NK, not known.

method of Pitcher et al. (21) as described by Jordens and Pennington (17).

PCR. The primers used for the amplification of the *N. meningitidis rpoB* gene were based on sequence data from the *E. coli* (19) and *Salmonella typhimurium* (26) *rpoB* genes. Primer RPO-1 (5′-TGA TGC CNC AAG AYA TGA T, where Y = T or C) corresponds to nucleic acid residues 1541 to 1559 (*E. coli* numbering) (19), and RPO-2 (5′-TCR AAG TTR TAR CCG TTC CA, where R = A or G) corresponds to residues 2500 to 2519. Additional primers were designed from sequence data obtained from the *N. meningitidis rpoB* gene. All primers were synthesized on an Applied Biosystems 891 DNA synthesizer.

Amplification of the *N. meningitidis rpoB* gene was performed by following the method of Saiki et al. (24). Each amplification reaction mixture contained DNA (1 ng/μl), primers (250 nM each), *Taq* polymerase (0.025 U/μl; CAMBIO, Cambridge, United Kingdom), and the four deoxynucleoside triphosphates (0.2 mM each), all in the buffer supplied with the *Taq* polymerase. PCR was performed by initially heating the samples at 94°C for 4 min; this was followed by 30 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 2 min). Prior to the addition to the reaction mixture, meningococcal DNA was heated at 95°C for 5 min. All reactions were carried out on a Perkin-Elmer Cetus 480 thermal cycler. The reaction products were characterized by electrophoresis on 2% agarose gels and then by staining in 0.5 μg of ethidium bromide per ml.

Sequencing. The PCR products were sequenced directly on an Applied Biosystems 373A automated DNA sequencer. Samples which gave a single band on agarose gels were purified for sequencing by using Centricon-100 columns (Amicon, Stonehouse, United Kingdom) to remove excess primers and nucleotides. Sequencing was carried out by using a *Taq* dye deoxy terminator cycle sequencing kit (Applied Biosystems, Warrington, United Kingdom) by following the protocol described by the manufacturer.

REA. Five to 8 μg of DNA was digested with 8 to 10 U of

*Stu*I (Pharmacia/LKB, Milton Keynes, United Kingdom) according to the manufacturer's instructions. Fragments were separated on a 0.8% agarose gel run at a constant voltage of 100 V for 13 h. Bands were visualized and photographed under UV light after staining with ethidium bromide (0.5 μg/ml).

PFGE. Inserts for PFGE were prepared as described by Poh and Lau (22). Approximately one-sixth of the insert was equilibrated in restriction enzyme buffer, 1 mM dithiothreitol, and 0.1% [wt/vol] Oxoid skimmed milk powder overnight at 4°C and was then digested by adding 20 U of restriction endonuclease and incubating the mixture for 24 h at 37°C. The digested inserts were loaded onto a 1% (wt/vol) agarose gel, and the fragments were separated on a CHEF DR II electrophoresis apparatus (Bio-Rad) run in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at 200 V for 22 h with a pulse time of 10 s. The gels were stained with ethidium bromide (0.5 μg/ml) and were visualized under UV light.

Statistical analysis. The percent similarity of banding patterns from REA or PFGE was estimated by the method of Dice (6): percent similarity = [(number of matching bands between two isolates × 2)/sum of the number of bands in each isolate] × 100. All bands visible on PFGE gels were used in the Dice analysis. For REA, only bands between 0.15 and 1.7 kb were included.

RESULTS

PCR amplification and sequencing of the *rpoB* gene of *N. meningitidis*. Amplification of *N. meningitidis* genomic DNA with the primers RPO-1 and RPO-2 gave a single band on agarose gels of approximately 950 bp, the size expected on the basis of the *E. coli* (19) and *S. typhimurium* (26) *rpoB* sequence data. This fragment covers the region of the *E. coli rpoB* gene containing 14 of the 15 known mutation sites responsible for rifampin resistance (13). The 950-bp PCR fragment was purified from two strains of *N. meningitidis*, strains G5 (serogroup Y) and J39 (serogroup C) (both Rif^s), and the sequence data were obtained with primers RPO-1 and RPO-2. These data allowed internal primers to be designed, and the 950-bp PCR fragment was sequenced completely. Comparison of the sequence data for strains G5 and J39 identified 23 base differences in the DNA sequence but only one amino acid difference (data not shown). Alignment of the protein sequence associated with the 950-bp fragment showed that the *N. meningitidis* β subunit of the RNA polymerase is 77% identical to the RNA polymerase β subunit of *E. coli* and *S. typhimurium*.

A fragment of the *rpoB* gene from the 6 Rif^r and 21 Rif^s *N. meningitidis* strains was amplified by using RPO-1 and RPO-5 (5′-GCG GTA AGG CGT TTC CAA GA). A single band of approximately 300 bp was observed. This fragment covers clusters I and II of the *E. coli* Rif^r mutation sites and could be sequenced completely by using the two amplification primers. The Rif^s isolates represented a number of different serogroups, which are described in detail in Table 1.

Comparison of the DNA sequence data for the 21 Rif^s isolates showed a number of differences. There was no apparent correlation between sequence and serogroup (Table 1). Members of the same serogroup had different *rpoB* gene sequences, and members of different serogroups had identical sequences. All the differences observed among the *N. meningitidis rpoB* gene sequences were silent mutations, and the deduced amino acid sequences of the 21 Rif^s isolates were identical. The DNA sequences of the six Rif^r strains were identical. Comparison of the Rif^r and Rif^s protein sequences showed only one difference at position 35 (Fig. 1), a His to an Asn mutation resulting from a single base change in the His codon (CAT to AAT) (referred to hereafter as H35N). Specifically, the silent mutations observed among the *N. meningitidis rpoB* gene sequences were as follows (the numbers refer to the sequence of the *N. meningitidis rpoB* gene shown in Fig. 1). Isolate A6 had mutations at C84T, T120C, C156A, C159A, C162T, A168G, and T250C. Isolate F18 had mutations at C84T, T120C, C156A, C159A, C162T, A168G, and C195T. Isolate F19 had mutations at C114T, T120C, A150G, and A168G. Isolates G5, J25, J35, and J39 had mutations at C114T, C156A, C159A, C162T, A168G, and C195T. Isolates J28 and J34 had mutations at T120C.

To establish that the mutation seen in the Rif^r isolates is responsible for the resistance phenotype, Rif^s strains were plated out on rifampin-containing agar, and the Rif^r mutants were isolated. Rifampin-resistant mutants A6M, A10M, F5M, F19M, F38M, and G6M (Table 1) were obtained. The DNAs of the mutants were purified, and the *rpoB* gene was amplified and sequenced by using RPO-1 and RPO-5. A number of different mutations were obtained. Five of the six mutants had an alteration at the same amino acid position (position 35; Fig. 1) that was observed previously for the six Rif^r isolates. Three of the five mutants (A6M, F38M, and G6M) had an H35Y mutation (CAT to TAT), one mutant (A10M) had an H35R mutation (CAT to CGT), and one mutant (F5M) had an H35N mutation that was seen previously. One of the six mutants (F19M) had an alteration at position 40 (Fig. 1) which changed a Ser residue to a Phe residue (TCC to TTC). Apart from these missense mutations, the DNA sequences of the fragments from the mutants were identical to the sequences of their Rif^s progenitors. The identities of the mutant and the parent were confirmed by REA.

Clonal relationships of Rif^r isolates. The different geographical distributions and the fact that the *rpoB* gene sequences of the six Rif^r isolates were identical led us to investigate the clonal relationships of these isolates. Digestion of the genomic DNAs from 34 group C *N. meningitidis* isolates with *Stu*I (REA) gave banding patterns with a range of Dice coefficients (35 to 100% similarity; data not shown). Isolates with Dice coefficients of greater than 95% were considered to be clonally related. A total of 11 clonal groups were observed. The six Rif^r isolates belonged to the same clonal group (Table 1), which also included a number of Rif^s isolates (Fig. 2). Digestion with the rarely cutting enzyme *Nhe*I (PFGE) gave banding patterns made up of 10 to 18 fragments. Analysis of these patterns also showed that the six Rif^r strains were clonally related (Fig. 3). Members of the same clonal group had identical *rpoB* gene sequences (Table 1).

DISCUSSION

The treatment of *N. meningitidis* infections with rifampin is considered the most appropriate prophylaxis, despite reports of the frequent development of antibiotic resistance (8, 9). No information is available on the types of mutations in *N. meningitidis* that are responsible for Rif^r strains, although studies of *E. coli* and *M. leprae* identified specific regions in the *rpoB* genes which are altered in resistant strains (11, 13). Using this information, we designed primers which can amplify part of the *rpoB* gene of *N. meningitidis* and used these primers to study Rif^r isolates. Primers RPO-1 and RPO-2 amplified a 900-bp fragment of the *rpoB* gene, and two serogroups of meningococci were chosen to determine their similarities. Complete sequencing of the fragment revealed only one amino acid difference between the two serogroups, although there were a number of silent mutations. The degree of similarity between the amino acid sequences of *N. meningitidis*, *E. coli*,

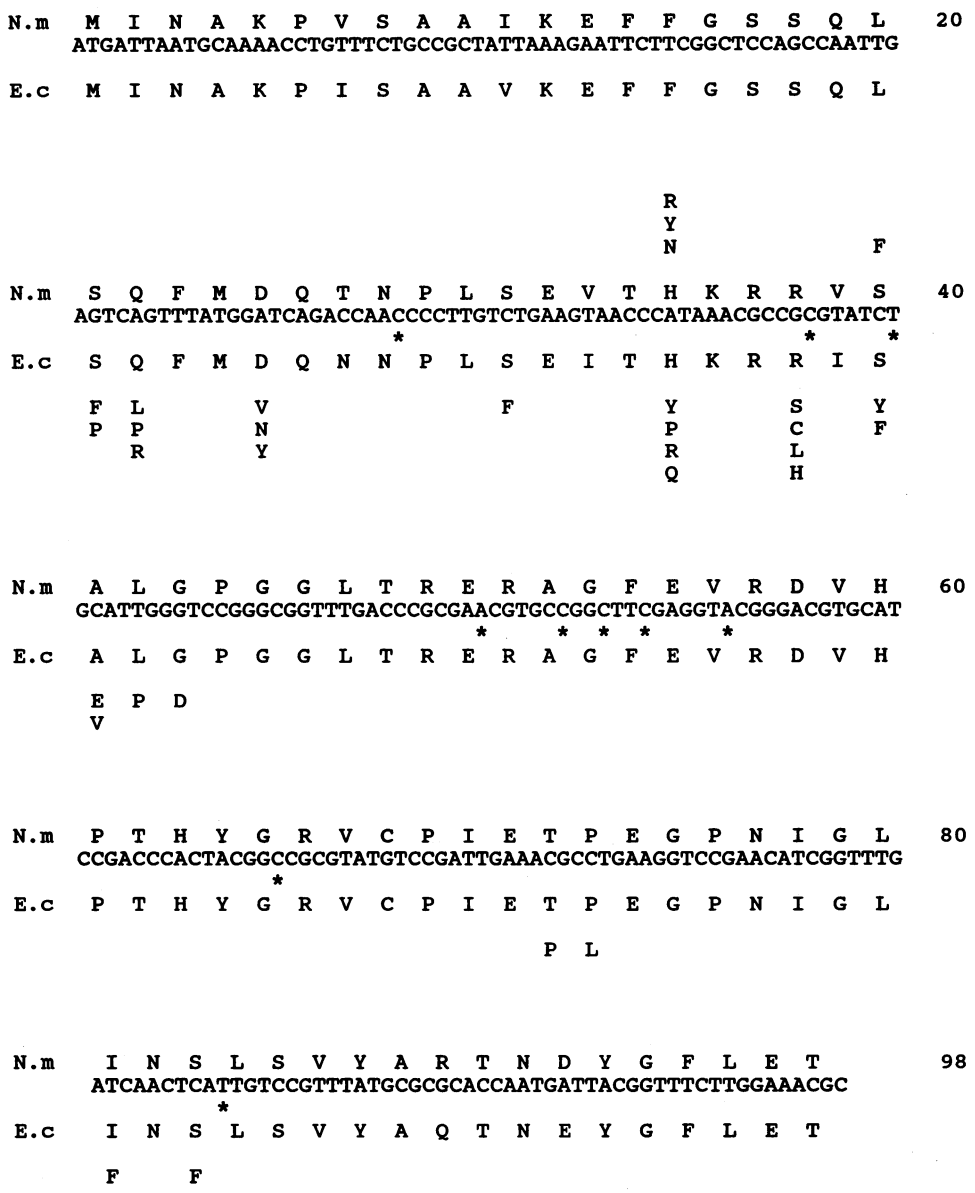

FIG. 1. The *rpoB* DNA sequence of Rif^s *N. meningitidis*. Positions of the silent mutations are marked with asterisks. The protein sequence deduced from the DNA fragment and the mutations found in Rif^r *N. meningitidis* isolates are shown above the sequence. The protein sequence of the *E. coli* β subunit covering clusters I and II and the associated Rif^r mutations are also shown (25). N.m, *N. meningitidis*; E.c, *E. coli*.

and *S. typhimurium* reflects the conservation of sequence of the β subunit of the RNA polymerase between bacterial species.

Within *E. coli* and *M. leprae* Rif^r strains, the majority of mutations are confined to a short central fragment of the *rpoB* gene. Sequencing of this fragment from 6 Rif^r and 21 Rif^s isolates revealed only one amino acid difference. In all six Rif^r isolates, a C-to-A transversion at position 103 (Fig. 1) alters the His codon to that of Asn (H35N). This His is homologous to the His residue at position 526 (His-526) in the *E. coli* sequence (Fig. 1), one of the known mutation sites for Rif^r strains (13). Mutations in the *E. coli rpoB* gene sequence alter His-526 to Tyr, Gln, Arg, or Pro (25), all of which are Rif^r. H526Y mutations are known to be particularly defective at terminating RNA synthesis (15) and are also incompatible with a number of conditional alleles responsible for *rho*, *nus*, and *dnaA* mutations (12). Such pleiotropic effects of a change at His-526 may play an additional role in the selection of particular forms of Rif^r mutations.

The protein sequences from the RPO-1 and RPO-5 fragments of *N. meningitidis* are 93% identical to the sequence from *E. coli*. All of the amino acids which are mutated in this region in Rif^r strains of *E. coli* are present unchanged in the *N. meningitidis* sequence. This, together with the fact that the H35N mutation is the only change in this region, suggests that this mutation confers resistance to rifampin in *N. meningitidis*.

Further evidence for the role of the H35N mutation in Rif^r *N. meningitidis* came from in vitro-generated Rif^r mutants. The six mutants all had a single nucleic acid and amino acid change compared with the sequence of the parent strain. Five of the six mutants had mutations at the His residue at position 35, indicating a role for this mutation in rifampin resistance. The H35N mutation has not previously been observed in either *E.*

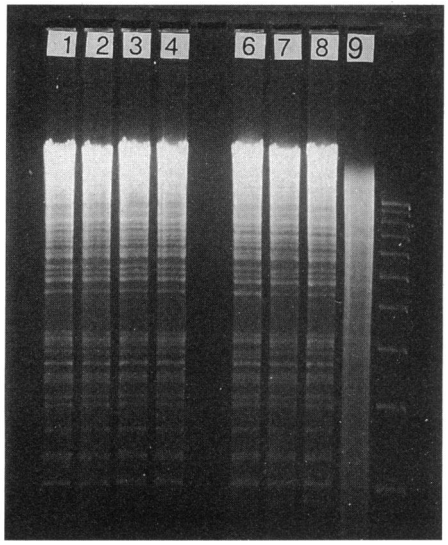

FIG. 2. Separation of meningococcal genomic DNA digested with *Stu*I. Lane 1, A11 (Rif^r); lane 2, A14 (Rif^r); lane 3, F1 (Rif^r); lane 4, F2 (Rif^r); lane 6, J37 (Rif^r); lane 7, F5 (Rif^s); lane 8, F6 (Rif^s); lane 9, F10 (Rif^s); unnumbered lane on the right, 1-kb ladder (Bio-Rad). Lanes 1 to 4 and 6 to 8 contain members of the same clonal group.

coli or *M. leprae*. The other mutation, S40F, has been found in *E. coli* Rif^r isolates. The MICs for the six in vitro-generated mutants indicated a higher level of resistance to rifampin than was observed for the resistant isolates obtained initially (Table 1). One of the six mutants, F5M, had the same mutation seen in the Rif^r isolates (H35N) and was a member of the same clonal group, but the level of resistance to rifampin was at least 10 times higher. This suggests that other factors may be involved in resistance. It was observed that although the H35N mutation was found in all of the Rif^r isolates, the commonest

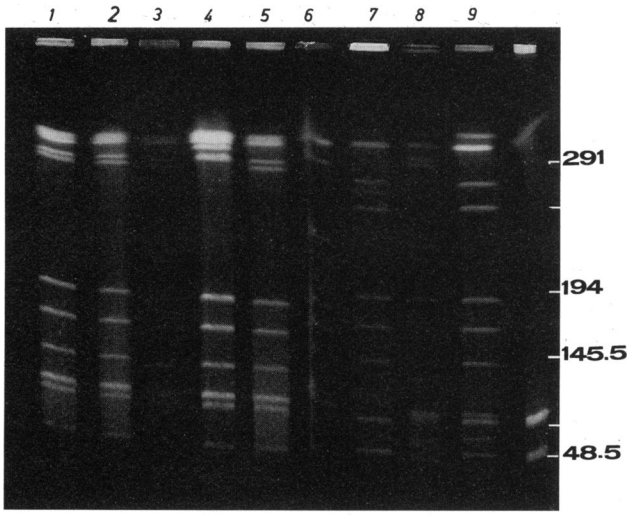

FIG. 3. Separation of *Nhe*I-digested meningococcal DNA by PFGE. Lane 1, A11 (Rif^r); lane 2, A14 (Rif^r); lane 3, F1 (Rif^r); lane 4, J37 (Rif^r); lane 5, F6 (Rif^s); lane 6, J35 (Rif^s); lane 7, J25 (Rif^s); lane 8, F7 (Rif^s); lane 9, J39 (Rif^s). Lanes 1 to 5 contain isolates that are considered to be members of the same clonal group. Lanes 6 to 9 contain members of other clonal groups. DNA size standards (marked on the right) are given in kilobase pairs.

mutation among the in vitro-generated mutants was H35Y. Current work is aimed at identifying other possible mutations within the *rpoB* gene of *N. meningitidis* and determining the effects of the different mutations on the mutant phenotypes.

We applied REA and PFGE, techniques which directly index the genotypes of strains and which provide information on the clonal relationships among bacteria (2, 17), to determine the genetic relationship between the six Rif^r isolates. All six isolates were found to belong to the same clonal group (Table 1). The large number of clonal groups observed among the small number of isolates may reflect the high degree of genetic diversity previously observed among serogroup C isolates (4). Sequence data for the 21 Rif^s isolates showed that this group included members of the same clonal group as the Rif^r isolates as well as members of other clonal groups plus isolates of serogroups Y, B, W135, and NG (Table 1). Although there was no correlation between the *rpoB* gene sequence and serogroup, members of the same clonal group had identical sequences. The sequence information confirmed the relationships obtained by macromolecular techniques, although it was not as discriminatory. The 23 differences noted between isolates G5 and J39 may provide further information on the clonal distribution of the *rpoB* alleles.

There are few reports of the isolation of Rif^r *N. meningitidis* isolates from patients with secondary cases of infection or from patients with disease, despite the frequent development of resistance and the widespread use of rifampin in prophylaxis. Serogroup C strains are responsible for 25% of the cases of disease caused by meningococci in the United Kingdom (16); cases are sporadic rather than epidemic. Two of our Rif^r isolates (F1 and F2) came from "kissing contacts"; the rest were obtained either at different times or from different geographical locations. The clonal group containing the Rif^r isolates was the largest among the serogroup C strains examined and contained a number of Rif^s isolates. The close genetic relationship between all of the Rif^r isolates supports the hypothesis that this phenotype arose once as a mutational event and then spread through the United Kingdom. Alternatively, the particular Rif^r mutation that we detected is preferentially selected (with other Rif^r mutations having pleiotropic effects [14] which, for example, diminish their ability to grow in human hosts) and that its restriction to one serogroup C clone is due to chance. Further work is in progress to distinguish between these alternative hypotheses. Because the possibility that rifampin-resistant strains of *N. meningitidis* have become established and widespread remains strong, surveillance for this character should be conducted in a systematic way.

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