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# Genetic Diversity of Penicillin-Binding Protein 2 Genes of Penicillin-Resistant Strains of *Neisseria meningitidis* Revealed by Fingerprinting of Amplified DNA

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**A 2-kilobase fragment containing the penicillin-binding protein 2 gene (*penA*) was amplified by using the polymerase chain reaction with DNA prepared from 35 penicillin-resistant strains of *Neisseria meningitidis* isolated in England, Ireland, and Spain (MICs, 0.16 to 1.28 μg of benzylpenicillin per ml) and from 10 penicillin-susceptible strains (MICs, ≤ 0.04 μg of benzylpenicillin per ml). The *penA* genes were digested with *Hpa*II or *Taq*YI; and the resulting fragments were end-labeled, fractionated on a polyacrylamide gel, and autoradiographed, to produce gene fingerprints. The fingerprints of the *penA* genes of the 10 penicillin-susceptible strains were identical. In contrast, the fingerprints of the *penA* genes of all but two of the penicillin-resistant strains differed markedly from those of the penicillin-susceptible strains. The altered *penA* genes of group B penicillin-resistant strains were very diverse, and 14 different fingerprints were found among the 15 strains that we examined. The *penA* genes of the 19 resistant group C strains were less diverse, and two major fingerprint patterns predominated.**

In recent years clinical isolates of *Neisseria meningitidis* that have increased levels of resistance to penicillin have been reported from the United Kingdom (10), Spain (7), and South Africa (1). Whereas for typical meningococci MICs are ≤0.04 μg of benzylpenicillin per ml, for penicillin-resistant strains, the MICs are 0.16 to 1.28 μg/ml. Although infections with these strains seem to respond to penicillin therapy, any further increase in their level of resistance may lead to treatment failure.

The decreased susceptibility of *N. meningitidis* to penicillin is at least in part due to the production of altered forms of penicillin-binding protein 2 (PBP 2) that have decreased affinity for the antibiotic (5, 6, 9). Previously (5, 6), the altered PBP in penicillin-resistant meningococci was called PBP 3. However, as it is the homolog of PBP 2 of *Neisseria gonorrhoeae* (9, 11), for consistency, we refer to it here as PBP 2. Comparison of the nucleotide sequence of the PBP 2 gene (*penA*) of a penicillin-resistant meningococcus (strain S738) with those of penicillin-susceptible meningococci has shown that the *penA* gene of the resistant strain has a mosaic structure consisting of regions that are identical to those in susceptible strains alternating with regions that show 22% sequence divergence (9). The two diverged blocks in the *penA* gene of the penicillin-resistant strain appear to have been introduced by genetic transformation from the *penA* gene of *Neisseria flavescens* to result in a hybrid form of PBP 2 that has a decreased affinity for penicillin (9).

Increased resistance to penicillin has so far been reported only in serogroup B and C meningococci and appears to be mainly restricted to Europe, although there are reports of such strains from South Africa (1, 7, 10). Apart from a single isolate (S738) from the United Kingdom in 1978, the emergence of penicillin-resistant strains appears to have occurred in the last few years. Multilocus enzyme electrophoresis has, however, demonstrated that the penicillin-resistant strains of *N. meningitidis* found in Spain are genetically diverse, and the appearance of these strains in recent years is not due to the emergence and spread of a single resistant clone (6). Since meningococci are naturally transformable, it remains possible that an altered *penA* gene arose only once, but has spread horizontally through the meningococcal population to result in the observed genetic diversity of resistant strains. We used DNA fingerprinting to show that the *penA* genes of almost all penicillin-resistant meningococci are considerably altered compared with those of penicillin-susceptible strains, which have very uniform fingerprints, and demonstrate that the altered *penA* genes of serogroup B, and to a lesser extent, serogroup C, penicillin-resistant meningococci, are very diverse.

## MATERIALS AND METHODS

**Growth of bacteria and preparation of chromosomal DNA.** Meningococci were grown on gonococcal (GC) agar base plus supplements at 37°C in a 5% $CO_2$ atmosphere. The bacterial growth from a single agar plate was suspended in 6 ml of 50 mM Tris hydrochloride–20 mM EDTA (pH 7.5), and the bacteria were lysed by the addition, at room temperature, of 20 μl of 10 mg of lysozyme per ml for 10 min followed by the addition of 6 ml of 2% Triton X-100–50 mM Tris hydrochloride (pH 7.5). The crude viscous lysate was used directly in the polymerase chain reaction (8). Meningococci were serogrouped by coagglutination by using polyclonal sera and were serotyped and subtyped by a dot blot technique with monoclonal antibodies (4). MICs of penicillin were determined by agar dilution.

**Amplification of the *penA* gene.** The coding region for the entire *penA* gene was amplified by the polymerase chain reaction (8) by using the oligonucleotide primers GC11

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(dGCCTGTGTGCCGGAATCG), which primes from a position 55 base pairs upstream of the initiation codon, and GCdown3 (dTCGTGAATTCGGGGATATAACTGCGGCC GTC), which primes from a position 187 base pairs downstream of the termination codon (9). The 100-μl reaction mixture contained 2 μl of crude cell lysate, 1 μM each of GC11 and GCdown3, 50 mM KCl, 10 mM Tris hydrochloride (pH 8.4), 2.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, and 200 μg of gelatin per ml. The mixture was heated at 95°C for 5 min and was then cooled over 30 min to 52°C. *Taq* polymerase (2 U; Cetus) was added; and amplification was carried out for 20 cycles by using a cycle of 3 min at 72°C, 1 min at 95°C, and 2 min at 52°C. The final extension step at 72°C was extended for an additional 5 min, and the sample was cooled to 4°C over 10 min. The samples were applied to a 0.6% agarose gel; and the amplified 2-kilobase *penA* fragment was excised from the gel, purified by Geneclean (Bio 101 Inc., La Jolla, Calif.), and suspended at about 10 ng/μl in 10 mM Tris hydrochloride–1 mM EDTA (pH 7.4).

**Gene fingerprinting.** A total of 1 μl of the purified *penA* gene fragment (approximately 10 ng) was added to 4 μl of an enzyme mixture (10 mM Tris hydrochloride [pH 7.4], 10 mM $MgCl_2$, 50 mM NaCl, 0.25 U of *Hpa*I or *Taq*YI per μl). After 1 h at 37°C (or 65°C for *Taq*YI), 2 μl of a labeling mixture (10 mM Tris hydrochloride [pH 7.4], 10 mM $MgCl_2$, 6 mM dithiothreitol, 0.1 mM dATP, 0.1 mM dTTP, 0.1 mM dGTP, 10 μCi of [α-$^{32}$P]dCTP per μl, 0.4 U of Klenow fragment per μl) was added, and after 10 min at room temperature, 2 μl of loading buffer was added and 4 μl of the sample was applied to a 6% nondenaturing polyacrylamide gel (31 by 39 cm). pBR322 DNA, which was digested with *Hpa*II and end-labeled with [α-$^{32}$P]dCTP (as described above), was used as a size marker. The samples were electrophoresed at 1,000 V until the bromophenol blue reached 10 cm from the bottom of the gel; and the gel was fixed, transferred to Whatman no. 1 filter paper, and dried under vacuum. Autoradiography was done for 6 to 24 h with X-ray film (RX; Fuji).

## RESULTS

**Fingerprints of the *penA* gene from penicillin-susceptible meningococci.** Figure 1 shows the fingerprints obtained by digesting the amplified *penA* genes of the 10 penicillin-susceptible meningococci (MICs, ≤0.04 μg of benzylpenicillin per ml) with *Hpa*II. All of these strains had an identical pattern of 23 major *Hpa*II fragments and also showed the same pattern of minor bands, which are presumably partial *Hpa*II digestion products. All of the penicillin-susceptible strains also gave an identical pattern of seven fragments when they were digested with *Taq*YI (data not shown). The *penA* genes of the 10 susceptible meningococci, which included one group A strain that was isolated in the 1930s and several recently isolated group B and C strains, were therefore very uniform.

**Fingerprints of the *penA* genes from penicillin-resistant group B meningococci.** Figure 1 shows the *Hpa*II fingerprints of the *penA* genes of group B penicillin-resistant strains. Of the 15 strains, 2 strains (H481 and H782) had *penA* genes that could not be distinguished from those of the penicillin-susceptible strains. The *penA* genes of these two strains also could not be distinguished from those of the penicillin-susceptible strains by *Taq*YI fingerprinting (data not shown).

The other 13 group B strains each had fingerprints that differed both from each other and from those of the penicillin-susceptible strains. The *penA* genes of group B penicillin-resistant meningococci are therefore very diverse.

**Fingerprints of the *penA* genes from penicillin-resistant group C meningococci.** Figure 2 shows the *Hpa*II fingerprints of the *penA* genes of penicillin-resistant group C meningococci. Only six different *Hpa*II fingerprints were found among the 19 group C strains. No further subdivision of the six fingerprint patterns was obtained by using *Taq*YI for fingerprinting.

Two fingerprint patterns predominated. One pattern (the $C^1$ fingerprint; Table 1) was found in seven serotype 2b strains and the polyagglutinable strain 7203. For these strains, MIC was 0.64 or 1.28 μg of benzylpenicillin per ml. The second predominant pattern (the $C^2$ fingerprint) was found in five serotype 2b strains and two nontypeable strains, for which the MIC was 0.32 or 0.64 μg/ml. Two other serotype 2b strains (J1027 and K589) had identical *penA* fingerprints (the $C^3$ fingerprint), whereas the other three strains each had distinct fingerprints, although that of the serotype 2a strain, K740, was very similar (but not identical) to the $C^2$ fingerprint (Table 1).

## DISCUSSION

The method of gene fingerprinting of amplified DNA that we described provides a convenient high-resolution method for the examination of the genetic diversity of particular loci. In the case of the *penA* gene of *N. meningitidis*, high resolution was obtained by the use of a single restriction enzyme, *Hpa*II, which provided approximately 23 DNA fragments from the gene. The use of a second enzyme, *Taq*YI, which produced only about seven fragments, provided no additional resolution, as we found no examples of *penA* genes that appeared to be identical by *Hpa*II fingerprinting but which could be distinguished by *Taq*YI fingerprinting, although the converse was not true. In other cases, enzymes that produce sufficient numbers of fragments may not be available, and high-resolution fingerprinting may require the use of several restriction enzymes.

The identical patterns of both the *Hpa*II and *Taq*YI fingerprints from the 10 penicillin-susceptible meningococci, which included group A, B, and C strains, suggest that the *penA* gene of meningococci is very uniform. Nucleotide sequencing of the *penA* genes of the group A strain NCTC 10025, the group B strain C311, the group C strain J1657, and one additional group C penicillin-susceptible strain (that was not included in the fingerprinting study) has directly confirmed the low level of diversity in this gene (<0.3% divergence for the two most dissimilar strains; Q.-Y. Zhang and B. G. Spratt, unpublished data).

The low level of sequence diversity in the *penA* genes of this small sample of penicillin-susceptible strains contrasts with the extensive genetic diversity that has been found among meningococci by multilocus enzyme electrophoresis (2, 3). At present, it is unclear whether multilocus enzyme electrophoresis exaggerates the extent of nucleotide sequence diversity in meningococci, or whether the lack of diversity in the *penA* gene is not typical of most meningococcal genes. The fingerprinting, or nucleotide sequencing, of other meningococcal genes that are not subject to obvious selection should resolve this issue. As far as we are aware, there are no other examples of meningococcal genes (except those for antigenically variable cell surface proteins) that have been sequenced from several different natural isolates.

The *penA* genes of group B penicillin-resistant meningococci were more diverse than those of the group C strains. Thirteen different fingerprints were found among the 15 resistant group B strains, whereas only 6 distinct fingerprints

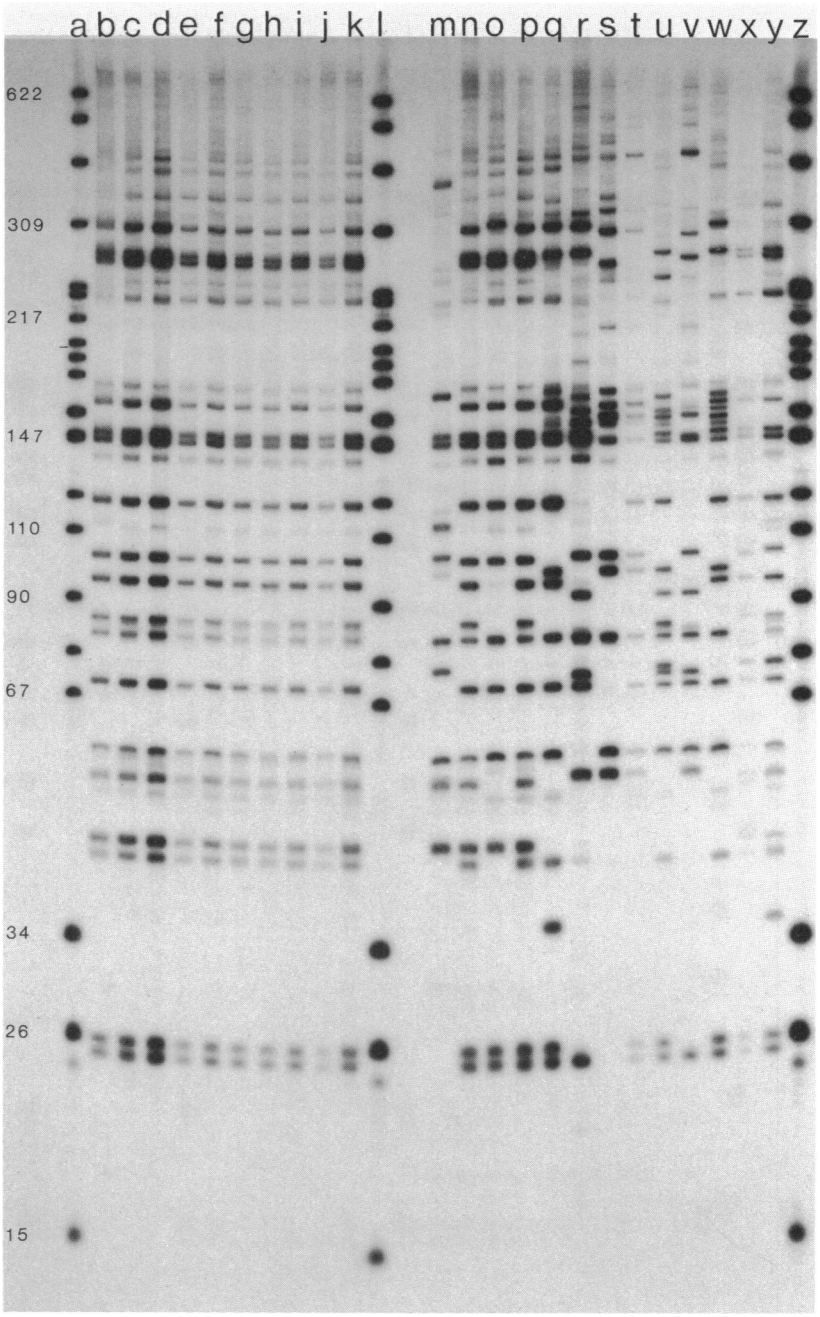



FIG. 1. *Hpa*II fingerprints of the *penA* genes of penicillin-susceptible and penicillin-resistant group B meningococci. The $^{32}$P-labeled *Hpa*II fragments from the *penA* gene of meningococci were fractionated on a 6% polyacrylamide gel and autoradiographed as described in the text. Lanes b through k, Fingerprints of the *penA* genes of penicillin-susceptible strains; lanes m through y, fingerprints of penicillin-resistant group B strains. Fingerprints of the following strains are shown in the indicated lanes: NCTC10025 (b), J1657 (c), C311 (d), K1707 (e), K1921 (f), K1934 (g), K1894 (h), K1924 (i), K1938 (j), 4476 (k), S738 (m), H481 (n), H557 (o), H782 (p), J2312 (q), J2411 (r), K196 (s), K197 (t), K270 (u), K662 (v), K825 (w), K1206 (x), and 7230 (y). Lanes a, l, and z are size markers (pBR322 digested with *Hpa*II); and the sizes of some of the fragments (in base pairs) are shown to the left of lane a. The sizes of the *Hpa*II fragments of pBR322 are 622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34, 26, 15, and (not visible) 9 base pairs.

were found among the 19 resistant group C strains. Only two group B resistant strains had the same fingerprints, and this fingerprint was identical to that of the penicillin-susceptible strains. The increased resistance to penicillin in these two group B strains (MICs, 0.32 μg/ml), which were both isolated from patients with infections in 1987 in eastern England, may not be due to the production of altered forms of PBP 2 that have a decreased affinity for penicillin, and could, for example, be due to decreased permeability of the meningococcal outer membrane. However, the latter strains may have alterations of *penA* that result in the production of a low-affinity form of PBP 2 but which do not alter the distribution of *Hpa*II or *Taq*YI restriction sites.

The eight penicillin-resistant strains that had the C$^1$ *penA* fingerprint were, with the exception of the polyagglutinable strain 7203, all serotype 2b strains that had relatively high

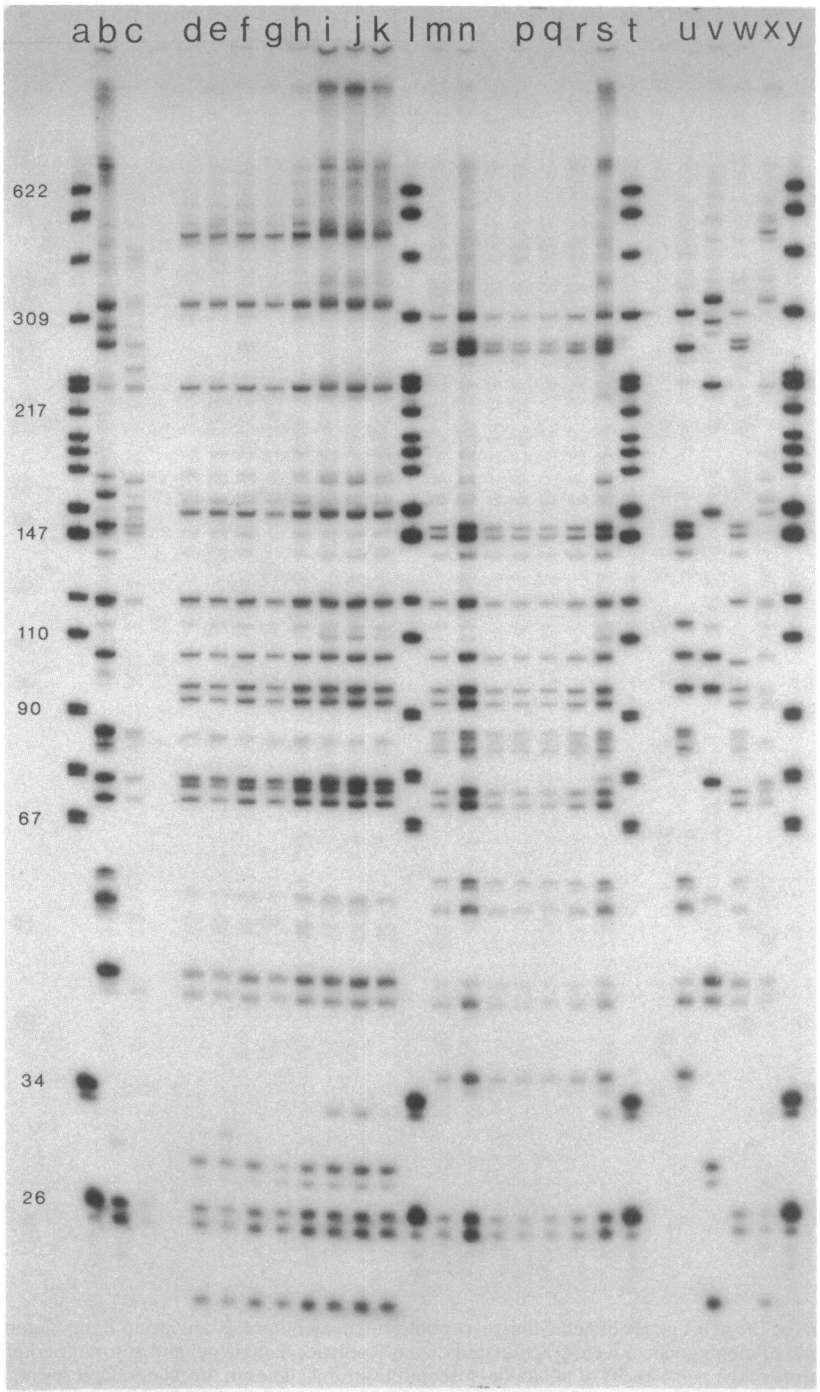



FIG. 2. *Hpa*II fingerprints of the *penA* genes of penicillin-resistant group B and C meningococci. Lanes b and c, Fingerprints of penicillin-resistant group B strains; lane e, a polyagglutinable strain, lanes d, f through k, m through s, and u through x, fingerprints of penicillin-resistant group C strains. Lanes a, l, t, and y are pBR322 digested with *Hpa*II size markers. Fingerprints of the following strains are shown in the indicated lanes: 32-IC (b), 5959 (c), H717 (d), 7203 (e), J588 (f), J724 (g), K1375 (h), 1-DA (i), 39-CS (j), 6044 (k), H675 (m), H1189 (n), J1106 (o), J1162 (p), K1380 (q), K1393 (r), 74-JC (s), J1027 (u), H2437 (v), K740 (w), and 6037 (x). Slight differences in a few of the very minor bands (partial digestion products) were occasionally found in *penA* genes that were considered to have the same fingerprints (e.g., compare the minor bands around 30 base pairs in lanes d through k). These minor differences were not reproducible and probably arose from incomplete *Hpa*II digestion in some of the samples.

levels of resistance to benzylpenicillin (MIC, 0.64 or 1.28 μg/ml). These strains were obtained from several locations within the United Kingdom and Spain and may be members of the same penicillin-resistant clone. The $C^2$ fingerprint was found in five serotype 2b strains and two nontypeable strains obtained from several locations in the United Kingdom, Spain, and Ireland. The latter strains had slightly lower levels of penicillin resistance (MIC, 0.32 or 0.64 μg/ml) than

TABLE 1. Fingerprint patterns and characteristics of strains examined in this study[a]

| Strain | Serogroup or serotype | Site of isolation | MIC (μg/ml) | Yr of isolation | Origin | Fingerprint class |
|---|---|---|---|---|---|---|
| NCTC 10025 | A | CSF | 0.02 | 1937 | Chicago, Ill. | S |
| C311 | B NT | CSF | 0.02 | 1986 | Liverpool, UK | S |
| K1707 | B 2a | CSF | 0.04 | 1989 | Hartlepool, UK | S |
| K1921 | B 15 P1.16 P1.7 | CSF | 0.02 | 1989 | Manchester, UK | S |
| K1934 | B NT P1.16 | Blood | 0.02 | 1989 | Hartlepool, UK | S |
| 4476 | B 15 P1.16 | Not known | 0.04 | 1986 | Norway | S |
| S738 | B | Blood | 1.28 | 1978 | Southampton, UK | $B^1$ |
| H481 | B NT | Blood and CSF | 0.32 | 1987 | Bury, UK | S |
| H557 | B NT | Blood | 0.32 | 1987 | Dublin, Ireland | $B^2$ |
| H782 | B 15 P1.16 | CSF | 0.32 | 1987 | Lincoln, UK | S |
| J2312 | B 15 P1.16 | Blood and CSF | 0.32 | 1988 | Dublin, Ireland | $B^3$ |
| J2411 | B 2b | Blood | 0.32 | 1988 | Hull, UK | $B^4$ |
| K196 | B 2a | CSF | 0.64 | 1989 | Dublin, Ireland | $B^5$ |
| K197 | B 2a | Blood and CSF | 0.64 | 1989 | Dublin, Ireland | $B^6$ |
| K270 | B NT P1.15 | CSF | 0.32 | 1989 | Nottingham, UK | $B^7$ |
| K662 | B 2a P1.2 | CSF | 0.32 | 1989 | London, UK | $B^8$ |
| K825 | B 2b P1.6 | CSF | 0.32 | 1989 | Ashford, UK | $B^9$ |
| K1206 | B 15 P1.16 | Blood | 0.32 | 1989 | Grimsby, UK | $B^{10}$ |
| 32-IC | B 4 P1.15 | Pharynx | 0.64 | 1988 | San Sebastian, Spain | $B^{11}$ |
| 7230 | B 15 P1.15 | Blood | 0.16 | 1988 | Barcelona, Spain | $B^{12}$ |
| 5959 | B 15 P1.7,16 | CSF | 0.64 | 1986 | Barcelona, Spain | $B^{13}$ |
| J1657 | C 14 | CSF | 0.02 | 1988 | Kettering, UK | S |
| K1894 | C NT | CSF | 0.02 | 1989 | Manchester, UK | S |
| K1924 | C 1 P1.15 | CSF | 0.02 | 1989 | Sidcup, UK | S |
| K1938 | C 2a | CSF | 0.04 | 1989 | London, UK | S |
| H675 | C 2b | Blood and CSF | 0.32 | 1987 | Uxbridge, UK | $C^2$ |
| H717 | C 2b | Blood and CSF | 0.64 | 1987 | Taunton, UK | $C^1$ |
| H1189 | C NT | CSF | 0.32 | 1987 | London, UK | $C^2$ |
| H2437 | C 2b | CSF | 1.28 | 1987 | London, UK | $C^4$ |
| J588 | C 2b | Blood | 1.28 | 1988 | Carshalton, UK | $C^1$ |
| J724 | C 2b | CSF | 1.28 | 1988 | Worksop, UK | $C^1$ |
| J1027 | C 2b | Blood and CSF | 0.64 | 1988 | London, UK | $C^3$ |
| J1106 | C 2b | Blood | 0.64 | 1988 | Chatham, UK | $C^2$ |
| J1162 | C NT | Blood and CSF | 0.32 | 1988 | Ashford, UK | $C^2$ |
| K589 | C 2b | Pharynx | 0.64 | 1989 | Hull, UK | $C^3$ |
| K740 | C 2a | CSF | 0.32 | 1989 | Northampton, UK | $C^5$ |
| K1375 | C 2b | CSF | 1.28 | 1989 | Orpington, UK | $C^1$ |
| K1380 | C 2b | Blood | 0.32 | 1989 | Cambridge, UK | $C^2$ |
| K1393 | C 2b | CSF | 0.64 | 1989 | Sligo, Ireland | $C^2$ |
| 1-DA | C 2b | Blood | 1.28 | 1987 | San Sebastian, Spain | $C^1$ |
| 39-CS | C 2b | Pharynx | 1.28 | 1988 | San Sebastian, Spain | $C^1$ |
| 74-JC | C 2b | CSF | 0.64 | 1988 | San Sebastian, Spain | $C^2$ |
| 6044 | C 2b | Blood | 0.64 | 1986 | Barcelona, Spain | $C^1$ |
| 6037 | C 2b | Blood | 1.28 | 1986 | Barcelona, Spain | $C^6$ |
| 7203 | PA 1 P1.2 | CSF | 0.64 | 1988 | Castellon, Spain | $C^1$ |

[a] Abbreviations: CSF, cerebrospinal fluid; NT, nontypeable; UK, United Kingdom; PA, polyagglutinable.

those of the strains that possessed the $C^1$ fingerprint and may be members of a second penicillin-resistant clone.

The diversity of *penA* genes in penicillin-resistant meningococci parallels the genetic diversity found among these strains by multilocus enzyme electrophoresis (8). It is therefore unlikely that an altered *penA* gene arose on a single occasion and has subsequently spread horizontally to produce the observed diversity of penicillin-resistant clones. Comparisons of the nucleotide sequences of the *penA* genes of several penicillin-resistant meningococci (unpublished data) confirm that altered *penA* genes have arisen on several occasions and support our earlier report (9) that they have emerged by the replacement, presumably by genetic transformation, of parts of the meningococcal *penA* gene by the corresponding regions from commensal *Neisseria* species. The extensive diversity found in the *penA* genes from penicillin-resistant meningococci is thus a consequence of the creation, on multiple occasions, of mosaic genes. Further diversity is probably generated by truncation of the ends of the regions introduced from commensal species, and perhaps by mismatch repair processes, as the original mosaic *penA* genes have spread horizontally through the meningococcal population.

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