Analysis of the clonal relationships between strains of *Neisseria meningitidis* by pulsed field gel electrophoresis

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Fingerprint patterns were generated from strains of *Neisseria meningitidis* by digestion of chromosomal DNA samples with 'rare-site' restriction endonucleases and resolution of the resultant fragments by pulsed field gel electrophoresis (PFGE). The potential of this technique for the rapid establishment of the clonal relationships between different isolates of the meningococcus was investigated. The fingerprint patterns from various serogroup A strains, previously assigned to clonal subgroups on the basis of their electrophoretic types (ETs), were compared. Fingerprints generated with the endonucleases *Sfi*I, *Spe*I and *Nhe*I each gave distinctive patterns for the clonal subgroups I–IV of serogroup A. Further, the endonucleases *Spe*I and, particularly, *Nhe*I were capable of resolving differences between various subgroup III strains isolated at different times and geographical locations. Strains isolated during the 'new wave' pandemic, which was associated with the Haj, from Europe, America, and Africa, had a characteristic fingerprint pattern and appeared to be distinct from 'old wave' pandemic strains. The PFGE technique is a relatively rapid and sensitive method for establishing clonal relationships among epidemic strains of *N. meningitidis*.

Introduction

Neisseria meningitidis causes bacterial meningitis and septicaemia worldwide (Schwartz *et al.*, 1989). The mechanisms of pathogenicity of this organism are still poorly understood (DeVoe, 1982). In Western Europe and North America the disease occurs endemically as a sporadic disease primarily of infants, young children and adolescents. These cases are most frequently caused by serogroup B or C organisms (Peltola, 1983). In other parts of the world, notably China, the Middle East, and Africa, epidemic outbreaks occur, caused by serogroup A organisms. Although polysaccharide vaccines are available against organisms of the A, C, W-135 and Y serogroups, these are poorly effective in young children (Reingold *et al.*, 1985) and there is no effective vaccine against the group B capsule (Frasch *et al.*, 1988). In recent years there has been considerable interest in the development of new vaccines, particularly against serogroup B organisms (Frasch, 1989). A thorough understanding of the epidemiology and a rapid, precise means of identifying strains are central to the design and implementation of vaccine trials.

The population genetics of a limited number of bacterial species, mainly pathogens, have been studied, principally by the techniques of multilocus enzyme electrophoresis (MLEE; Selander *et al.*, 1986, 1987). Populations of *N. meningitidis*, in common with those of the other bacteria investigated, have a clonal structure (Caugant *et al.*, 1986, 1987; Olyhoek *et al.*, 1987); that is, isolates of meningococci can be resolved into a limited number of genetically related types, or clones, each thought to contain strains with a common ancestry. The pandemics of serogroup A meningococci are caused by individual clones spreading across continents (Achtman, 1990), sometimes in conjunction with large population movements, for example the Haj (Moore *et al.*, 1989; Achtman, 1990; Jones & Sutcliffe, 1990; Achtman *et al.*, 1992). These clones are genetically and serologically stable over time, and during intercontinental spread (Crowe *et al.*, 1988, 1989; Achtman, 1990; Salih *et al.*, 1990). The group B and C organisms that cause sporadic, endemic disease are genetically more diverse, particularly the group B meningococci (Caugant *et al.*, 1990); however, single clones of group B organisms are known to have caused epidemic outbreaks, for example the European outbreaks caused by the serogroup B serotype 15 subtype P1.16 strains belonging to the ET-5 cluster (Caugant *et al.*, 1986).

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A number of alternative techniques for the determination of the clonal types present in bacterial populations have been investigated. These have included the analysis of endonuclease digests by conventional agarose gel electrophoresis (Bjorvatn *et al.*, 1984; Olyhoek *et al.*, 1988); restriction fragment length polymorphism (RFLP) types of specific parts of the chromosome (Fox *et al.*, 1991), particularly of ribosomal RNA operons (Stull *et al.*, 1988); and the analysis of bacterial chromosomes by pulsed field gel electrophoresis (PFGE; Schwartz & Cantor, 1984; Carle *et al.*, 1986) for the analysis of large DNA molecules (Arbeit *et al.*, 1990). In the present work, chromosomal DNA isolated from strains of *N. meningitidis* was digested with restriction endonucleases that cut the chromosome into 10–40 fragments, which were resolved into fingerprint patterns by PFGE. These patterns were produced and interpreted relatively simply and quickly, and were stable for at least eight passages of the strain *in vitro*. Comparison of these fingerprints was capable of distinguishing and identifying clonal subgroups of meningococcal isolates and of assessing the genetical relationships between strains belonging to the same clonal subgroup.

Methods

Propagation and maintenance of meningococcal strains. Meningococcal strains were obtained from the United Kingdom Public Health Laboratory Service, the Max-Planck Institut für Molekulare Genetik, Berlin, and the China Committee for the Culture Collection of Micro-organisms, as detailed in Table 1. The strains were maintained at -70°C in a mixture of 50% Mueller-Hinton broth and 50% 'freezing mix' (glycerol, 10%, v/v; sodium citrate, 3 mM; ammonium sulphate, 1 mM; potassium phosphate, 100 mM, pH 6.6). Strains were grown at 37°C in an atmosphere of 5% CO_2 on heated-blood (chocolate) agar plates for 8–16 h.

Preparation of chromosomal DNA samples. Cells were grown as described above and the growth from five plates was scraped into TE (Tris/HCl, 10 mM; EDTA, 1 mM; pH 8.0; 5 ml). The cell suspension was mixed with an equal volume of melted Sea-Plaque agarose [1.0% (w/v) in TE; FMC Bioproducts] at 37°C . The mixture was directly dispensed into plastic moulds (0.1 ml capacity; Pharmacia) at ice temperature, and allowed to set. The plugs were removed from the moulds and incubated overnight at 55°C in SaET [Sarkosyl, 1% (w/v); EDTA, 0.5 mM; Tris/HCl, 10 mM pH 9.5] with pronase E (1 mg ml^{-1} ; Sigma). They were then washed twice at room temperature in SaET without pronase E. The plugs could be stored in this buffer at 4°C for at least one year. Prior to use, the plugs were washed three times in TE buffer, and then incubated in a restriction buffer appropriate to the endonuclease to be used (New England Biolabs) with preboiled ribonuclease A (1 $\mu\text{g ml}^{-1}$; Sigma) at 37°C for 30 min and rewashed in the same buffer twice.

Restriction endonuclease digestion and storage of digested DNA. As required, plugs containing chromosomal DNA were equilibrated with appropriate restriction enzyme buffer, as described above, melted at 65°C and digested with restriction endonucleases (New England Biolabs) overnight at 37°C . At the end of the incubation, the reaction was stopped by the addition of stop buffer [Tris-acetate, 20 mM;

EDTA, 0.5 mM; Orange-G marker dye (Sigma) 1 $\mu\text{g ml}^{-1}$; 1% (w/v) sea plaque agarose] and heating to 65°C . Samples were either stored at -20°C or loaded on to the gels immediately. Melting the plugs did not cause non-specific degradation of the chromosomal DNA, and facilitated the subsequent manipulation of the samples.

Pulsed field gel electrophoresis. Pulsed field gel electrophoresis was carried out with a Pharmacia 'Pulsaphor' apparatus. The standard conditions were a constant voltage of 160 V and starting current of 80 mA: the electrophoresis buffer was maintained at 14°C throughout the experiment. An interpolated program was used: 5 s pulses to 10 s pulses in 10 h, followed by 10 s pulses to 30 s pulses in 8 h, and 30 s pulses to 40 s pulses in 4 h. The gel and running buffer was $0.5 \times \text{TBE}$ (Tris-borate, 5 mM; EDTA, 0.5 mM; pH 8.0). After electrophoresis, the gels were stained with ethidium bromide, visualized on an ultraviolet light transilluminator and photographed.

Analysis of data. Restriction fragment sizes were calculated by reference to bacteriophage λ DNA of known molecular size. The migration distances were transformed by the inverse of their square root. This gave the closest approximation to a linear relationship between migration distance and molecular size for DNA molecules in the range 50–400 kbp separated by the conditions described above: for sizes smaller than 50 kbp, a logarithmic transformation of the data was used. The molecular sizes of the chromosomal fragments were estimated by interpolation within the standard curve, assisted by a BASIC computer program. The reproducibility of band sizes was generally good, with band sizes from different gels usually within 5%. The resolution of the technique was limited mainly by the size of the photograph. The sizes and presence of the smaller restriction endonuclease fragments was confirmed by conventional submerged agarose gel electrophoresis as described by Maniatis *et al.* (1982).

An assessment of the degree of divergence between related fingerprint patterns was obtained by comparing each fingerprint pattern with each related pattern and scoring the number of shared bands. This figure was divided by the mean of the bands present in the two fingerprint patterns to give a number less than or equal to one (for exact pattern matching), to account for the differences in the number of bands between some pairs of fingerprints. The divergence between all possible pairs of fingerprint pattern was calculated and a phenogram constructed, using the average linkage (UPGMA) method of Sokal & Michener (1958). This gave a graphical representation of the relationships between strains of the same clonal subgroup.

Results

Pulsed field electrophoresis of meningococcal DNA

Appropriate parameters for the analysis of meningococcal DNA were established with chromosomal DNA plugs prepared from strain 2053 (Table 1), a B:15:P1.7,16 organism isolated during an outbreak of meningococcal disease in Stroud, Gloucestershire, UK (Cartwright *et al.*, 1986). Seven restriction endonucleases with 'rare' recognition sequences (*NheI*; *NotI*; *PacI*; *SfiI*; *SpeI*; *XbaI*; and *XhoI*) were used in preliminary studies. These endonucleases gave patterns of varying complexity. The simplest pattern was obtained with the endonuclease *SfiI*: this fingerprint comprised seven bands of between 20 and 600 kbp in size. The most complex fingerprints were those obtained with endo-

Table 1. *Neisseria meningitidis* strains used in this work

NIBSC number (used in this work), original strain number, serological and clonal information (if known) are given, together with the geographical source of the isolate. Details of the clonal subgroup type strains (2151–2190) are given in Olyhoek *et al.* (1987). Strains 2033 and 2225 were the same isolate from different sources. Strains 2183 and 2222 were also from the same original isolate.

NIBSC number	Strain number	Serogroup	Serotype /subtype	Clone	Geographical location of isolation	Year of isolation	Source
2009	10025	A		IV-3	USA	1937	NCTC
2002	29001	A					CCCCM
2053	F126	B	15:P1.16	ET-5	UK	1986	PHLS
2033	H2182	A			UK	1987	PHLS
2034	H2224	A			UK	1987	PHLS
2035	H2283	A			UK	1987	PHLS
2036	H2360	A			UK	1987	PHLS
2037	H2525	A			UK	1987	PHLS
2038	H2576	A			UK	1987	PHLS
2039	H2591	A			UK	1987	PHLS
2040	J78	A			UK	1988	PHLS
2041	J117	A			UK	1988	PHLS
2042	J126	A			UK	1988	PHLS
2151	B40	A	P1.10	I-1 (ET-1)	Morocco	1967	MPIMG
2177	B534	A		II-1 (ET-27)	UK	1941	MPIMG
2183	B54	A	P1.9,X	III-1 (ET-34)	Finland	1975	MPIMG
2184	B227	A	P1.9,5	III-1 (ET-36)	Denmark	1974	MPIMG
2185	B554	A		III-1 (ET-37)	Finland	1977	MPIMG
2186	B515	A		III-2 (ET-38)	Finland	1975	MPIMG
2187	B306	A		III-3 (ET-39)	Nepal	1983	MPIMG
2188	B503	A		III-4 (ET-40)	China	1984	MPIMG
2189	B506	A		III-4 (ET-41)	Brazil	1976	MPIMG
2190	B48	A	P1.7	IV-1 (ET-42)	Gambia	1983	MPIMG
2221	Z4088	A		III-1	China	1987	MPIMG
2222	Z1054	A		III-1	Finland	1975	MPIMG
2223	Z3905	A		III-1	China	1966	MPIMG
2224	Z3703	A		III-1	Sweden	1973	MPIMG
2225	Z3771	A		III-1	UK	1987	MPIMG
2226	Z3768	A		III-1	Norway	1969	MPIMG
2227	Z3524	A		III-1	Chad	1988	MPIMG
2228	Z3529	A		III-1	Chad	1988	MPIMG
2230	Z3747	A		III-1	Sudan	1988	MPIMG
2233	Z3726	A		III-1	Sweden	1983	MPIMG
2236	Z3696	A		III-1	Sudan	1988	MPIMG
2237	Z3506	A		III-1	USA	1987	MPIMG

NCTC, National Collection of Type Cultures.

CCCCM, China Committee for the Culture Collection of Micro-organisms.

PHLS, Public Health Laboratory Service, Withington Hospital, Manchester, UK.

MPIMG, Max-Planck Institut für Molekulare Genetik, Berlin, FRG.

nucleases *Xho*I and *Xba*I: each of these endonucleases produced more than 30 fragments, the majority between 15 and 50 kbp in size. The restriction endonuclease *Not*I gave 34 fragments ranging between 4 and 130 kbp; *Nhe*I, 16 fragments between 5 and 400 kbp; *Pac*I, 14 fragments of 4–400 kbp; and *Spe*I, 19 fragments of 6–300 kbp. To test the stability of the restriction endonuclease fingerprints obtained, the strain was passaged eight times on heated-blood agar plates and restriction patterns were resolved from DNA isolated from cells of each passage. No changes were observed in any endonuclease fingerprint. The fingerprints obtained with the endonuclease *Sfi*I for 16 meningococcal reference strains were com-

pared. Each strain included in this analysis had a different fingerprint pattern. The pattern diversity seen in this relatively small sample of strains indicated that the sequence divergence between meningococcal strains was measurable by this technique. Calculations based on these and other data suggested that the meningococcal chromosome was 1.8–2.0 Mbp in size.

Analysis of serogroup A meningococcal reference strains

Representative serogroup A strains of each of the known clonal subgroups of this serogroup (I–IV; Table 1;

Fig. 1. Restriction endonuclease digestions of chromosomal DNA isolated from serogroup A strains of known clonal type with *Sfi*I resolved into fingerprint patterns by PFGE. Lanes: S, standards, i.e. concatamerized phage λ DNA mixed with *Hind*III-digested phage λ DNA; lane 1, strain 2151 (clone I-1); lane 2, strain 2177 (clone II-1); lane 3, strain 2183 (clone III-1); lane 4, strain 2190 (clone IV-1). Sizes (kbp) of standard DNA molecules are indicated.

Olyhoek *et al.*, 1988) were analysed with the three restriction endonucleases *Sfi*I (Fig. 1), *Spe*I (data not shown), and *Nhe*I (Fig. 2a). Each of these endonucleases gave fingerprint patterns unique to the clonal subgroups studied, although there was some similarity between the *Sfi*I fingerprints of some of the clones.

The diversity of *Sfi*I, *Spe*I, and *Nhe*I restriction fingerprints within a clonal subgroup was assessed by the analysis of seven electrophoretic types of subgroup III (strains 2183–2189; Table 1). These analyses showed that whilst all seven variants had the same *Sfi*I fingerprint pattern (lane 3, Fig. 1), there were minor variations in the *Spe*I and *Nhe*I fingerprints (Fig. 2b). Each of the *Nhe*I fingerprint patterns gave a total genome size of between 1.94 and 1.96 Mbp in size, with the exception of strain 2189, which gave a size of 2.12 Mbp.

Assessment of genetical relationships within subgroup III by PFGE analysis

An analysis of the *Sfi*I and *Nhe*I fingerprints of 9 group A meningococcal clinical isolates, a NCTC serogroup A reference strain, and a serogroup A strain obtained from the China Committee for the Culture Collection of Micro-organisms, was carried out to determine their clonal relationships and genetical diversity. Three of these strains (2022, 2035, 2037) had *Sfi*I, *Spe*I, and *Nhe*I fingerprints similar to that of the subgroup I reference strain (Fig. 1, Fig. 2a, and data not shown). The remainder of the strains had *Sfi*I fingerprint patterns similar to the subgroup III strains examined. Further analyses with *Nhe*I were consistent with these results, but showed greater pattern diversity, particularly within the 9 strains belonging to subgroup III (Table 3). Similar results were observed with fingerprints generated by *Spe*I.

Table 2. Restriction endonuclease fragments generated by *Nhe*I from *N. meningitidis* strains of clone III

Twenty-seven strains were analysed on three pulsed gels, and the resultant bands measured and assigned. The size of the smallest band (no. 1) was estimated from the same samples analysed on conventional gels. The mean band sizes, standard deviation, and standard error of means of all the bands considered to be equivalent are shown.

Band number	Band size (kbp)	SD	SEM	Number of bands measured (number of gels)
1	5.1	0.1	0.02	27 (3)
2	12.3	0.67	0.13	26 (3)
3	16.5			1 (1)
4	19.1	1.0	0.9	4 (3)
5	22.7	1.7	0.3	25 (3)
6	37	4.6	1.4	11 (3)
7	44.8	3	2.15	2 (2)
8	49.9			1 (1)
9	57.8	3.2	0.6	26 (3)
10	69.5	3	0.6	26 (3)
11	76.2	3.9	1.2	9 (3)
12	78.8	4.6	0.89	27 (3)
13	84.9			1 (1)
14	89.8	3.3	0.64	26 (3)
15	92.6			1 (1)
16	104.3			1 (1)
17	109	0.6	0.3	3 (3)
18	110	3.5	0.67	27 (3)
19	131			1 (1)
20	132	3.5	0.7	27 (3)
21	134	4.3	1.2	12 (3)
22	147	3.8	0.72	27 (3)
23	159			1 (1)
24	175	3.2	0.82	15 (3)
25	193	5.2	1.0	27 (3)
26	225	5.7	1.1	27 (3)
27	242			1 (1)
28	251			1 (1)
29	267	4.8	0.96	25 (3)
30	293	2.8	2	2 (1)
31	376	10.9	2.2	24 (3)

Fig. 3. K^+ content (a) and viability (b) of cells treated with streptomycin at different K^+ concentrations. Cells of *E. coli* TK1110 were grown at the following K^+ concentrations: 0.5 mM ($\circ$); 10 mM ($\bullet$); 30 mM ($\square$) and 115 mM ($\blacksquare$). Streptomycin ($50 \mu g \text{ ml}^{-1}$) was added at zero time. The K^+ content of the cells and number of viable cells of the suspension were determined as a function of time.

Permeability changes for other ions

Under the conditions where the cells lost K^+ , they took up 20% more Na^+ than they lost K^+ (Fig. 5a). Thus, streptomycin not only affects the transmembrane concentration gradient of K^+ and Rb^+ , but also that of another inorganic cation. In the experiment of Fig. 5(a), part of the Na^+ taken up in excess of K^+ was electrically compensated for by the efflux of H^+ , since the internal pH of the cells increased by 0.15 units (Fig. 5b). Sodium was not required for K^+ loss, since streptomycin-treated cells grown in the absence of Na^+ lost K^+ with a time course and to an extent similar to those of cells grown in the presence of Na^+ (Fig. 5c, e). Cells grown in the absence of Na^+ did not exchange K^+ for H^+ after streptomycin treatment, since the internal pH of these cells increased rather than decreased (Fig. 5d). This indicates that under these conditions the uptake of unidentified cations (possibly choline) or the exit of unidentified anions accompanies K^+ loss. Omission of choline and Na^+ from the medium led to a slightly different behaviour: K^+ loss became transient (Fig. 5e) and the internal pH of the treated cells was only initially

Fig. 4. The addition of an excess of K^+ to cells treated with streptomycin reverses K^+ loss (a) but does not affect the rate with which cells lose their viability (b). Two cultures of *E. coli* TK1110 were grown in K0.5 medium. At zero time $50 \mu g \text{ streptomycin } ml^{-1}$ was added to the two cell suspensions. At $t = 9 \text{ min}$ one culture received 10 mM-KCl ($\bullet$). The second culture served as the control ($\circ$). The K^+ content of the cells and the number of viable cells were determined as a function of time.

higher than that of control cells (Fig. 5f). We will return to the phenomenon of transient K^+ loss below.

Membrane potential

The effects of streptomycin on cell membrane permeability are not large since streptomycin-treated cells still maintained substantial concentration gradients of K^+ (Rb^+) and H^+ across their cytoplasmic membranes (Figs 1–5). To obtain more information on this point we measured the membrane potential of cells of strain AS-1, in which this potential can be measured without pretreatment with EDTA (Hirota *et al.*, 1981). Remarkably, streptomycin caused hyperpolarization during the period that the cells lost K^+ (Fig. 6). This effect may be partially due to the inhibition of protein synthesis by streptomycin since chloramphenicol, added at a concentration at which it blocks protein synthesis completely, caused an even larger hyperpolarization (Fig. 6a). This

Table 3. *NheI* restriction fragment fingerprint patterns for strains of serogroup A, clonal group III

NheI-generated fingerprint patterns of 26 strains with the *SfiI* fingerprint pattern characteristic of clonal group III were analysed. Each band was measured and assigned a band number on the basis of the data presented in Table 2. The presence of a band in a given strain is indicated with a 1, its absence with a 0.

Strain	Band number																															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
2009	1	1	0	1	1	0	0	0	1	1	1	1	0	0	0	0	1	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2033	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2034	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2038	1	1	0	0	1	1	0	0	1	1	1	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2039	1	1	0	0	1	0	0	0	1	0	1	1	0	1	0	0	1	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2040	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2041	1	0	0	0	1	1	0	0	1	1	1	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2042	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2183	1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2184	1	1	1	0	1	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2185	1	1	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2186	1	1	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2187	1	1	0	1	1	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	1	0	0	1	
2188	1	1	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2189	1	1	0	0	1	0	1	0	1	1	0	1	0	1	0	0	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	
2221	1	1	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2222	1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2223	1	1	0	1	0	0	0	0	1	1	1	1	1	1	0	1	0	1	0	1	0	1	1	1	1	1	0	0	0	1	0	
2224	1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2225	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2226	1	1	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	
2227	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2228	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2230	1	1	0	0	1	0	1	1	0	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2233	1	1	0	0	1	0	0	0	1	1	1	1	0	1	1	0	0	1	0	1	0	1	0	1	1	1	0	0	1	1	0	
2236	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	
2237	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	0	1	0	1	1	1	0	0	1	1	0	0	1	0	1	

chromosome. Thus, the relationships between strains with diverse fingerprints is difficult to assess solely from the number of shared bands. A phenogram such as that illustrated in Fig. 4 is therefore a guide to the interpretation of the fingerprint data rather than a true representation of phylogenetic relationships.

Relationships between serogroup A strains with identical SfiI fingerprints revealed by comparative analysis of NheI fingerprints

The strains included in the cluster analysis (Fig. 4) were isolated between 1937 and 1988 and were from various geographical locations, including Asia, North and South America, Europe and Africa. With the exception of strains 2223 (isolated in China in 1966) and 2189 (isolated in Brazil in 1976) these strains divided into two branches of the tree. One of these comprises only strains isolated in 1987/8 in Africa, Europe and America ('new wave pandemic'; Moore *et al.*, 1989; Salih *et al.*, 1990; Achtman *et al.*, 1992), many of these strains have fingerprints that are identical, which is consistent with the rapid spread of this disease-causing clone. The other

branch is more diverse, perhaps reflecting a more gradual spread of the disease caused by subgroup III meningococci during the 'old wave' pandemic. Some strains isolated in 1987 and 1988 cluster with the 'old wave' strains. It is possible that these strains were 'old wave' strains still present during the 'new wave' pandemic. The *NheI* fingerprint patterns appear to be stable over at least several decades and during geographical spread of the strains.

Comparison of PFGE fingerprint data with clonal subgroups determined by MLEE

There are a number of discrepancies between the PFGE fingerprint patterns and the subgroups determined by MLEE. These discrepancies presumably reflect differences in the nature of the data used by each technique. In the absence of a physical and genetical map of the meningococcal chromosome, it is not possible to compare these data directly. Discrepancies could be caused by differences between the chromosomal locations of the genes encoding the proteins used in MLEE and the *NheI* sites, or by changes in chromosome structure which did

Fig. 3. *NheI* fingerprints of thirteen serogroup A strains belonging to clone III from various dates and geographical locations. Standards (S) as Fig. 1. Lane 1, strain 2223 (China, 1966); lane 2, strain 2226 (Norway, 1969); lane 3, strain 2224 (Sweden, 1973); lane 4, strain 2222 (Finland, 1975); lane 5, strain 2221 (China, 1987); lane 6, strain 2225 (UK, 1987); lane 7, strain 2237 (USA, 1987); lane 8, strain 2233 (Sweden, 1983); lane 9, strain 2227 (Chad, 1988); lane 10, strain 2228 (Chad, 1988); lane 11, strain 2230 (Sudan, 1987); lane 12, strain 2236 (Sudan, 1988).

not affect the phenotypic characters used in MLEE studies.

Strain 2009, which is subgroup IV-3 (strain M1027 in Olyhoek *et al.*, 1987; M. Achtman, personal communication) on the basis of MLEE data, appears to be more closely related to the 'old wave' subgroup III-1 than to strain 2190 (subgroup IV-1) (Figs 1 and 2). It is difficult to explain this result, although it is possible that the chromosome structures of strains assigned to clone IV-3 are more closely related to those of strains of subgroup III-1 than to those of subgroup IV-1. Data from more strains assigned to subgroup IV is required to resolve this discrepancy.

Strain 2230 (isolated from Sudan in 1987, from the 'new wave' pandemic) differed in four bands (loss of bands 6 and 9 and gain of bands 7 and 8) from the other 'new wave' pandemic strains, and hence appeared to be quite distinct from, although still related to, these strains. These band differences could be explained by a single inversion that changed the relative position of only one nucleotide site if bands 6 and 9 are contiguous. Chromosome rearrangement events could change the

fingerprint pattern with relatively little effect on the MLEE type; this type of event could explain the relatively large difference between the fingerprint of strain 2223 and other III-1 strains.

Strains 2188 and 2189 are both subgroup III-4, but have different fingerprint patterns. The chromosome of strain 2189 is apparently larger (2.1 Mbp) than that of strains 2183–2188 (1.94–1.96 Mbp). It is possible that this difference in fingerprint pattern is caused by the presence of a large episome or a genetical element such as a prophage integrated into the chromosome introducing two extra *NheI* sites into the largest *NheI* fragment. In the case of strains 2185 (ET-38, subgroup III-1) and 2186 (ET-39, subgroup III-2), which had the same fingerprint pattern, the fingerprint data appeared to be less discriminatory than the MLEE data, although on the basis of their epidemiology (both strains were isolated in Finland in the 1970s) the strains could be closely related.

The present work demonstrates that PFGE fingerprints can be used to identify relationships among clinical isolates of meningococci with two restriction endonucleases (*SfiI* and *NheI*). The *NheI* fingerprints

Fig. 4. Phenogram illustrating differences between the *NheI* fingerprints of 27 serogroup A, clone III meningococcal strains. The percentage similarity between fingerprints was calculated on the basis of the number of bands shared between each possible pair of fingerprints. All the strains represented in this figure had identical *SfiI* fingerprint patterns.

were capable of greater resolution and distinguished 'old wave' and 'new wave' strains belonging to subgroup III-1. Clonal analysis with PFGE fingerprint data has a number of advantages. The fingerprint is a characteristic of a particular strain which enables the analysis of the whole chromosome of the target organism. The patterns depend on many independent chromosomal loci, the number of which can be altered by the appropriate choice of restriction endonuclease. Inclusion of data from several different endonucleases also improves the coverage of the chromosome, and examines the possibility that the data are adversely affected by factors such as site-specific methylation in the chromosome. The patterns, which are stable for a number of passages *in vitro* and probably over many years in culture collections, are readily recognized and the fragment sizes may be compared with those obtained for other isolates to establish the relationships within clonal subgroups. The fingerprint patterns are independent of variable antigens, the genes of which may change at a faster rate than the rest of the chromosome. The development of DNA probes to particular genes will enable the relation of fingerprint data directly to chromosome maps, giving a more precise estimation of the chromosome structures being analysed. Finally, the PFGE technique is relatively simple and rapid. A more detailed analysis is being undertaken to compare the results obtained by PFGE

fingerprint data and MLEE techniques. The PFGE approach is particularly useful for the analysis of closely related strains, such as those isolated from a single outbreak of the disease.

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