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DNA Fingerprinting of *Streptococcus pneumoniae* Strains by Pulsed-Field Gel Electrophoresis

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Pulsed-field gel electrophoresis of genomic DNA was carried out on *Streptococcus pneumoniae* strains to determine its value in the epidemiological survey of pneumococcal infections. Twenty-one clinical strains were chosen to cover a broad range of diversity according to geographic location, penicillin susceptibility, serotype, and multilocus enzyme electrophoresis (MLEE) pattern. The restriction endonucleases *Apa*I and *Sma*I were used to digest intact chromosomes, and the fragments were resolved by field inversion gel electrophoresis (FIGE). Each digest produced 10 to 19 fragments for comparison between strains. All the strains, including strains of the same serotype and strains with the same MLEE profile, had different FIGE patterns. In some cases, the restriction patterns differed by only a few fragment bands, and two isolates differed only in the location of a single DNA fragment. The polymorphism obtained with FIGE was greater than those obtained with serotyping and MLEE analysis. The stability of the FIGE profiles was established by testing of two independent clones derived from pneumococcus strain R36A. These results indicated that pulsed-field gel electrophoresis should be an effective tool for the typing of *S. pneumoniae* strains, capable of subdividing serotypes or MLEE types and of tracing the origin of pneumococcal strains.

Streptococcus pneumoniae is a common microorganism of the normal human respiratory flora and a major cause of human morbidity and mortality worldwide. Pneumococcal infection is one of the most frequent causes of pneumonia, meningitis, otitis media, and bacteremia in children and adults worldwide (3, 4, 21). Moreover, the emergence of strains of *S. pneumoniae* resistant to penicillin and other antibiotics and the spread of that resistance over the world have become a major concern for antimicrobial therapy of such infections and increase the need for epidemiological surveillance (7, 12). Therefore, specific epidemiological markers are required to survey *S. pneumoniae* infections. Phenotypic and genotypic schemes have been developed to assist in epidemiological investigations. These include serotyping, penicillin-binding protein (PBP) patterns, multilocus enzyme electrophoresis (MLEE) patterns, DNA fingerprinting, and ribotyping (8, 15, 17, 18, 26, 30). However, difficulties are associated with variable expression of the polysaccharide capsule in serotyping, and some isolates are not typeable. PBP pattern analysis is limited to changes in genes encoding penicillin resistance proteins. DNA endonuclease analysis using conventional electrophoresis is difficult to interpret because of the large number of bands. MLEE analysis detects mutations in a variety of genes for metabolic enzymes throughout the entire chromosome, and DNA restriction fragment length polymorphisms of rRNA genes (ribotyping) is simpler to interpret than chromosomal DNA endonuclease analysis. Moreover, these two methods appeared to be more discriminating than were serotyping and PBP patterns in discerning *S. pneumoniae* strain differences (18, 26).

We have been using a variation of pulsed-field gel electrophoresis, field inversion gel electrophoresis (FIGE), to analyze *S. pneumoniae* genomic DNA with low-frequency-cleavage restriction endonucleases; a physical map of the *S. pneumoniae* chromosome that should provide a useful framework for further molecular and genetic investigations has been constructed (6). In this study, we utilized FIGE following *Apa*I and *Sma*I restriction digestion of chromosomal DNA to conduct a molecular analysis of *S. pneumoniae* strains from different geographic locations, with the goal of determining whether this technique can differentiate epidemiologically independent strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Twenty-one clinical isolates of penicillin-susceptible and -resistant *S. pneumoniae* were kindly donated by R. Hakenbeck, Max-Planck Institute, Berlin, Germany. They were a sample drawn from a larger collection of strains previously characterized (26). These strains and their relevant properties are listed in Table 1. They were chosen to cover a broad range of diversity according to geographic location, penicillin susceptibility, serotype, and MLEE type (MLEET). MLEE analysis with 17 enzymes was previously used to investigate the degree of genetic variation between strains (26).

Strains Cl3 and R6 are two independent clones derived from pneumococcus strain R36A (27). A subculture of *S. pneumoniae* 801, derived from the R6 strain, was used in this study (14) along with a multiply marked strain (strain 119) (29).

The culture medium has been described elsewhere (5). Cultures were incubated at 37°C without aeration and were maintained at pH 7.8 during growth by the addition of 1 N NaOH.

DNA preparation and restriction enzyme digestion. *S. pneumoniae* DNA embedded in agarose blocks was prepared by a method derived from that described previously for *Haemophilus influenzae* (11). Cell cultures were grown to an optical density at 560 nm of 0.3 to 0.5, centrifuged at 5,000 × *g* for 10 min at 0°C, and washed with 1 M NaCl–10 mM Tris hydrochloride (pH 8.0). Washed cells were resuspended

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TABLE 1. Characteristics of the 23 isolates of *S. pneumoniae*

Strain	Origin[a]	Yr of isolation	Serogroup or type	MLEET	MIC (μg/ml)	FIGE pattern	
						*Apa*I	*Sma*I
R6	USA	1947	NT[b]	ND[c]	S[d]	1	1a
Cl3	USA	1947	NT	ND	S	1	1b
635	Sp	1987	18	1	S	2	2
629	Sp	1986	19F	2	0.25	3	3
2039	SA	1978–1980	6A	5	2	4	4
63509	SA	1987	19A	5	1	5	5
644	Sp	1988	14	6	2	6	6
665	Sp	1988	9V	13	1	7a	7
676	Sp	1988	9	13	1–2	7b	7
8250	SA	1978–1980	14	14	0.25	8	8
662	Sp	1986	6B	16	1	9	9
668	Sp	1986	6B	16	1–2	10	10
681	Sp	1986	6B	16	1	11	11
43352	Fi	1987	6	17	1	12	12
456	Sp	1984	23F	18a	1–2	13	13
673	Sp	1988	23	18a	1	14	14
496	Sp	1985	19F	18a	1	15	15
56741	SA	1987	23F	18b	4	16	16
56828	SA	1987	19F	22	0.5	17	17
242	G	1986	23F	25	0.25–0.5	18	18
43356	Fi	1987	19A	26	0.25–0.5	19	19
45607	SA	1987	29	27	0.12	20	20
SpR	G	1986	6A	28	0.25	21	21

[a] SA, South Africa; Sp, Spain (Barcelona); Fi, Finland; G, Germany; USA, United States of America.
[b] NT, nontypeable.
[c] ND, not determined.
[d] S, sensitive (0.007 to 0.03 μg/ml for penicillin).

in the same buffer and adjusted to an optical density at 560 nm of 0.6. The bacterial suspension was warmed to 42 to 45°C and mixed with an equal volume of 1% low-melting-point agarose at the same temperature. The molten mixture was poured into Plexiglas molds (100 μl). After solidification, the cells embedded in agarose were lysed by addition of 0.1 M EDTA–10 mM Tris hydrochloride–0.5% Brij–0.2% deoxycholate–0.5% sarcosyl and incubated for 2 h at 37°C. The agarose blocks were transferred in an equal volume of 0.25 M EDTA–1% sarcosyl–100 μg of proteinase K per ml and incubated overnight at 50°C. They were suspended in TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride, incubated at room temperature for 1 to 2 h with gentle mixing, and washed three times with TE buffer.

For digestion of DNA, the agarose blocks were equilibrated with 1× restriction buffer before the addition of 250 U of restriction enzyme (New England Biolabs or Bethesda Research Laboratories) per ml. The blocks were mixed with *Sma*I or *Apa*I restriction enzyme for 2 h, transferred to 100 mM EDTA, and placed in ice for 15 min. Before being loaded onto the gel, the blocks were melted at 62°C for 10 to 15 min.

FIGE of macrorestriction fragments. FIGE was performed in 1% agarose for 20 h at 8°C at 5 V/cm in a 0.5× solution of Tris-borate-EDTA (16). Field inversion was supplied by a ramp (programmable power inverter; model PPI 200, MJ Research) that controls the time for the forward and reverse cycles. Two programs were used. Program A was used for 0.15 to 12.03 s in the forward direction and 0.05 to 4.01 s in the reverse direction. Program B was used for 0.3 to 30 s in forward direction and 0.1 to 10 s in the reverse direction. Ab program means 16 h of program A followed by 4 h of program B. Ba program means 16 h of program B followed by 4 h of program A.

Following electrophoresis, the gels were stained with ethidium bromide and the DNA bands were visualized with a UV transilluminator.

Coefficients of similarity [CS = (number of shared bands × 2 × 100)/total number of bands in the two samples] were determined for some isolates by visual comparison of macrorestriction patterns as a means of quantitating the relatedness or lack thereof among these isolates.

RESULTS

Typeability, reproducibility, and stability of FIGE fingerprintings. Digestion of *S. pneumoniae* DNA with *Apa*I or *Sma*I produced 10 to 19 fragments for comparison between strains (Fig. 1 and 2). FIGE analysis of 23 *S. pneumoniae* isolates with *Apa*I or *Sma*I revealed 22 different pattern types (Table 1). Two isolates which appeared to be identical after FIGE of *Apa*I restriction fragments (FIGE type 1) and two isolates which were identical after FIGE of *Sma*I restriction fragments (FIGE type 7) differed by one fragment band after FIGE analysis with the other endonuclease.

Reproducibility of the FIGE profiles was established by repeated testing of the same isolate on separate occasions; when multiple gels of any single pneumococcal DNA preparation were obtained, the FIGE patterns were identical. For example, more than 50 assays with the DNA of strain R6 have given the same profile.

Original FIGE patterns were also maintained after multiple in vitro passages. *Apa*I FIGE fingerprints of strains R6 and Cl3, derived from pneumococcus strain R36A, appeared to be identical (Fig. 1, lanes 2 and 3). The two strains exhibited identical *Sma*I FIGE fingerprints, except that strain Cl3 had an additional DNA fragment band of 220 kb (Fig. 2, lanes 2 and 3). However, more than 15,000 genera-

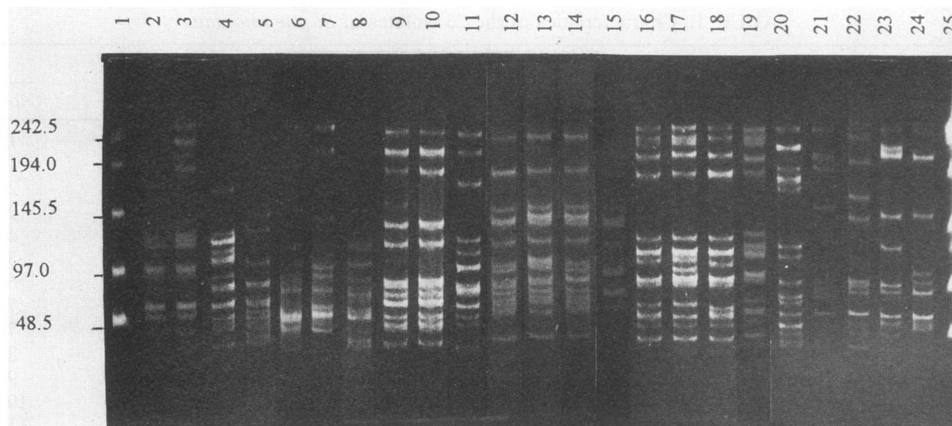

FIG. 1. FIGE separation of *Apa*I restriction fragments of *S. pneumoniae* strains of different MLEETs. Lanes 1 and 25, lambda DNA ladder (sizes [in kilobases] are indicated on the left); lanes 2 and 3, strains R6 and Cl3; lanes 4 to 24, the *S. pneumoniae* clinical strains described in Table 1: lane 4, strain 635, MLEET 1; lane 5, strain 629, MLEET 2; lanes 6 and 7, strains 2039 and 63509, MLEET 5; lane 8, strain 644, MLEET 6; lanes 9 and 10, strains 665 and 676, MLEET 13; lane 11, strain 8250, MLEET 14; lanes 12 to 14, strains 662, 668, and 681, MLEET 16; lane 15, strain 43352, MLEET 17; lanes 16 to 18, strains 456, 673, and 496, MLEET 18a; lane 19, strain 56741, MLEET 18b; lane 20, strain 56828, MLEET 22; lane 21, strain 242, MLEET 25; lane 22, strain 43356, MLEET 26; lane 23, strain 45607, MLEET 27; lane 24, strain SpR, MLEET 28.

tions separated these two strains, which have been cultured independently in two laboratories.

We have looked for other differences with another program (Ba) designed for better discrimination between large fragments. Indeed, more *Sma*I and *Apa*I bands were observed. The total number of bands resulting from the two enzymes and the two programs was 30 for strains Cl3 and R6 (data not shown). However, the Ba program was not more efficient in discriminating between closely related strains than was the Ab program used routinely in this study.

FIGE fingerprints with respect to MLEETs. Digestion of *S. pneumoniae* genomic DNA with *Apa*I produced 12 to 19 well-resolved fragments of 20 to 250 kb (Fig. 1). All the 21 clinical isolates, including strains of the same serotype and strains with the same MLEET, had different FIGE patterns. In some cases, the restriction patterns of strains with the same MLEET differed by only a few fragment bands. For example, strains 665 and 676 (MLEET 13) appeared to be identical but the former strain showed one more band of 160 kb and a similarity value of 97% (Fig. 1, lanes 9 and 10); strains 662, 668, and 681 (MLEET 16) differed by four fragments only (Fig. 1, lanes 12 to 14); strains 456, 673, and 496 (MLEET 18a) differed in the location of two bands at 78 and 105 kb; strains 456 and 673 had an additional DNA fragment of 225 kb; and strain 673 had an additional fragment of 100 kb (Fig. 1, lanes 16 to 18). On the other hand, the FIGE profiles of strains with the same MLEET could show greater differences: the MLEET 5 strains isolated from South Africa in 1980 and in 1987 differed from each other markedly. The CS was 36% (Fig. 1, lanes 6 and 7).

As shown in Fig. 2, each *Sma*I fingerprint contained from 10 to 16 fragments of 20 to 300 kb. All the clinical strains but

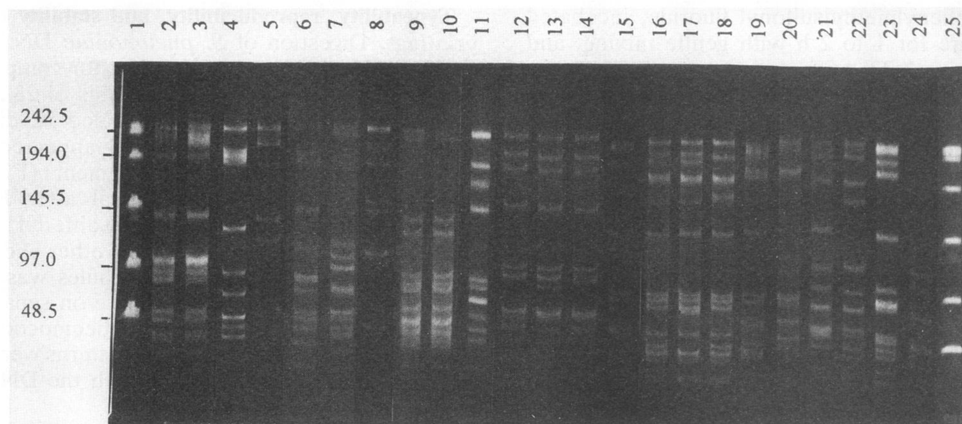

FIG. 2. FIGE separation of *Sma*I restriction fragments of *S. pneumoniae* strains of different MLEETs. Lanes 1 and 25, lambda DNA ladder (sizes [in kilobases] are indicated on the left); lanes 2 and 3, strains R6 and Cl3; lanes 4 to 24, the *S. pneumoniae* clinical strains described in Table 1: lane 4, strain 635, MLEET 1; lane 5, strain 629, MLEET 2; lanes 6 and 7, strains 2039 and 63509, MLEET 5; lane 8, strain 644, MLEET 6; lanes 9 and 10, strains 665 and 676, MLEET 13; lane 11, strain 8250, MLEET 14; lanes 12 to 14, strains 662, 668, and 681, MLEET 16; lane 15, strain 43352, MLEET 17; lanes 16 to 18, strains 456, 673, and 496, MLEET 18a; lane 19, strain 56741, MLEET 18b; lane 20, strain 56828, MLEET 22; lane 21, strain 242, MLEET 25; lane 22, strain 43356, MLEET 26; lane 23, strain 45607, MLEET 27; lane 24, strain SpR, MLEET 28.

two had different genome patterns; strains 665 and 676 (MLEET 13) were identical (Fig. 2, lanes 9 and 10). There was no strain polymorphism by *Sma*I testing which was greater than that by *Apa*I testing. For example, strains 662, 668, and 681 (MLEET 16) differed by two fragments only (Fig. 2, lanes 12 to 14); strains 456, 673, and 496 (MLEET 18a) differed in the location of one band at 175 kb; strains 456 and 673 had an additional DNA fragment of 230 kb; strains 456 and 496 had an additional fragment of 50 kb; and strain 673 had an additional fragment of 80 kb (Fig. 2, lanes 16 to 18). The FIGE profiles of the MLEET 5 strains did not show greater differences by *Sma*I testing than those observed with *Apa*I (CS = 38%) (Fig. 2, lanes 6 and 7). However, FIGE of *Sma*I restriction fragments, as for *Apa*I restriction fragments, could differentiate epidemiologically independent *S. pneumoniae* strains.

FIGE fingerprints with respect to serotype and penicillin susceptibility. Strains 456, 673, and 496 (MLEET 18a) were isolates of different serotypes (23F, 23, and 19F, respectively). They appeared to be genetically closely related after FIGE of *Apa*I or *Sma*I restriction fragments, with similarity values above 85% (Fig. 1 and 2, lanes 16 to 18). The same observation could be done for strains 665 and 676 (MLEET 13; serotypes 9V and 9, respectively). Conversely, the serotype of strains 56741 and 242 was the same (23F) and these strains showed quite different FIGE profiles, with similarity values below 30% (Fig. 1 and 2, lanes 19 and 21).

Very similar FIGE patterns were found for multiresistant strains 456 and 496 from Spain and for the multiresistant strain 56741 isolated from South Africa (CS above 80%). Their *Apa*I FIGE profiles differed by two bands at 120 and 220 kb, and their *Sma*I FIGE profiles differed by three bands at 70, 85, and 220 kb (Fig. 1 and 2, lanes 16, 18, and 19). This suggests a common origin for these three strains.

DISCUSSION

The stability of the FIGE pattern could be tested since the original Avery R36A strain has been subcultured independently in two laboratories. In 1947, H. Ephrussi-Taylor moved from the Rockefeller Institute to Paris, where almost every day the strain, now named C13, was subcultured by 1/20 dilution to keep it competent until 1962, when frozen cultures started being used. This strain was grown for more than 15,000 generations. The Rockefeller strain, R6, a sister strain of C13, was also continuously, but less frequently, subcultured by a passage every 3 months (10). In any case, more than 15,000 generations separated these two strains. Nevertheless, their profiles were nearly identical except for one band. This shows that FIGE patterns are quite stable and can be a good typing method to trace the origins of pneumococcal strains. In our work, 30 bands resulting from *Sma*I and *Apa*I restriction digests with 180 bases were investigated. As only one fragment was changed, 1 base out of 180 had mutated. Thus, the minimal rate of divergence would be 4×10^{-7} per base per generation.

The results obtained by FIGE separations of restriction fragments of *S. pneumoniae* DNA indicate that there is no correlation demonstrated between FIGE profiles and serotyping. In a previous study, DNA fingerprint differences were found among serotypes, with significantly greater similarity among strains of a given serotype (30). Our data clearly show that different capsular types can be genetically closely related whereas strains with the same serotype may show quite different FIGE fingerprints. They confirm that capsular type is not a good criterion for genetic relatedness as reported recently (26).

It has been proposed that the Spanish and South African serotype 23F multiresistant strains are representative of the same serotype 23F clone that has recently been described as occurring in Spain, Great Britain, and the United States (18, 19). The FIGE profiles observed in this study confirm that these strains are genetically closely related (CS = 88%) and might have originated from the same clone. The Spanish serotype 23F clone is shared by a Spanish serotype 19F multiresistant strain (CS = 88%); a relationship between these two strains had already been suggested by their identical PBP properties and MLEETs (26). Our results also indicate that there is considerable DNA polymorphism within the species *S. pneumoniae* and even among strains from the same geographic location, with the same MLEET. The polymorphism obtained with FIGE fingerprints is greater than that obtained with the MLEE analysis, which has been reported to be as discriminating as ribotyping (18) and more discriminating than was the PBP pattern analysis (26). Thus, FIGE appears to be a powerful tool for performing molecular epidemiological studies of pneumococcal infections, capable of subdividing MLEETs, serotypes, or ribotypes. The results of this study, coupled with others recently reported with *S. pneumoniae* isolates (18, 26), confirm the observations made for other bacterial species: DNA fingerprinting by pulsed-field gel electrophoresis is more effective than MLEE analysis or ribotyping in the differentiation of epidemiologically unrelated strains (22, 23, 25). Restriction endonuclease analysis of chromosomal DNA using conventional electrophoresis has been used for fingerprinting pneumococcal strains (30). However, because of the large number of bands, comparison of different fingerprints is tedious and does not allow for quantitative or objective appraisal. Conversely, pulsed-field gel electrophoresis with low-frequency-cleavage restriction endonucleases reduces the number of bands but still provides discriminating data. Pulsed-field gel electrophoresis has been used to compare chromosome restriction patterns in *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa* (1), *Pseudomonas cepacia* (2), *Legionella pneumophila* (25), *Enterococcus faecalis* (20), *Mycoplasma hominis* (13), *Leptospira* sp. (9), *Campylobacter hyointestinalis* (24), and *Staphylococcus aureus* (23, 28). The technique is relatively simple, is applicable to a wide range of bacteria with little modification, and appears to have sufficient discriminatory power to be useful for epidemiological tracking.

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