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Genome Fingerprinting of *Pseudomonas aeruginosa* Indicates Colonization of Cystic Fibrosis Siblings with Closely Related Strains

DIETMAR GROTHUES,[1] UTA KOOPMANN,[1,2] HORST VON DER HARDT,[2] AND BURKHARD TÜMMLER[1,2]*

Cystic Fibrosis Research Group, Zentrum Biochemie II, OE 4350,[1] and Zentrum Kinderheilkunde, OE 6710,[2] Medizinische Hochschule Hannover, D-3000 Hannover 61, Federal Republic of Germany

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The epidemiology of *Pseudomonas aeruginosa* infection at a cystic fibrosis (CF) center was monitored over a 3-year period. A total of 835 isolates from 72 unrelated patients and 22 siblings with CF were analyzed by genome fingerprinting and serotyping, bacteriophage typing, and pyocin typing. For genome fingerprinting, bacterial chromosomes were digested with one of the restriction endonucleases *Spe*I, *Dra*I, *Xba*I, *Ssp*I, and *Nhe*I, which cut only rarely, and subsequently separated by field inversion gel electrophoresis. The physical genome analysis allowed us to classify *P. aeruginosa* strains in terms of DNA relatedness. Related strains differed by fewer than six *Dra*I bands in the fingerprint, whereas unrelated strains differed by more than 20 *Dra*I bands. All unrelated CF patients were colonized with different strains. The absence of a nosocomial spread of organisms at the CF center was attributed to the strict hygiene measures observed at the hospital. CF siblings were harboring either identical or closely related strains; transmission within the family is thought to be the most likely cause.

In cystic fibrosis (CF), chronic lung infection with *Pseudomonas aeruginosa*, which is found in the majority of patients, is thought to be primarily responsible for pulmonary deterioration and reduced life expectation (15). Cross infection among CF patients may increase the prevalence of infections caused by these bacteria. We tried to determine the extent of *P. aeruginosa* cross infection by typing respiratory isolates. Besides the established methods of serotyping, bacteriophage typing, and pyocin typing, the novel technique of genome fingerprinting was applied to this problem as follows (8). Chromosomal DNA was digested with restriction endonucleases that cut only rarely, and the large fragments thus obtained were subsequently separated by field inversion gel electrophoresis (FIGE) (3). The pattern of restriction fragments is characteristic for each strain and provides an estimate of the degree of genomic relationship among the strains.

MATERIALS AND METHODS

Isolation of strains. The *P. aeruginosa* strains were isolated from deep-throat swabs taken from CF patients over a period of 3 years at 6-month intervals. The specimens were plated onto MacConkey agar (Oxoid Ltd., Basingstoke, England), tryptic soy agar (Difco Laboratories, Detroit, Mich.), Mueller-Hinton agar (Difco), and chocolate agar (Difco) containing 8% erythrocytes and 2% horse serum. Colonies dissimilar in macroscopic appearance were processed separately (17, 20). For samples taken from CF siblings, at least five colony representatives were analyzed from each throat swab. Second subcultures of the *P. aeruginosa* strains were stored in soy tryptone broth supplemented with 15% (vol/vol) glycerol at −70°C until used.

Serotyping, phage typing, and pyocin typing. Strains were serotyped by agglutination tests with commercial antisera (Pasteur Diagnostika, Munich, Federal Republic of Germany) (16). The phage typing pattern was assessed with the routine set of 20 bacteriophages described by Asheshov (2). Pyocin typing (7) was carried out by the spotting method (5).

Genome fingerprinting by FIGE. *P. aeruginosa* strains were grown in tryptone-water to the exponential phase. The cells were pelleted, washed once with 75mM NaCl–25 mM EDTA (pH 7.4; SE buffer), and adjusted to 10^{10} CFU/ml in SE buffer. For the preparation of unsheared genomic DNA, the bacterial suspension was mixed at 45°C with the same volume of 2% (wt/vol) low-gelling agarose (type 7; Sigma Chemical Co., St. Louis, Mo.) in SE buffer. The mixture was dispensed into slots (10 by 6 by 1 mm) and allowed to solidify for 30 min at 4°C. The agarose blocks were incubated in an Eppendorf tube containing 0.5 mg of proteinase K (Boehringer Mannheim Biochemicals, Tutzing, Federal Republic of Germany) per ml, 1% (wt/vol) *N*-lauroylsarcosine, and 0.5 M EDTA (pH 9.5) for 15 h at 56°C. The blocks were then equilibrated with 10 mM Tris–10 mM EDTA (pH 7.4; TE), cut in half, and stored at 4°C until used. Restriction digestions of genomic DNA were regularly performed with 10 U of *Dra*I (recognition sequence, AAATTT) (New England BioLabs, Inc., Beverly, Mass.). For 30 strains, the chromosomes were also cleaved with 10 U of *Nhe*I (GCTAGC), *Spe*I (ACTAGT), *Ssp*I (AATATT), or *Xba*I (TCTAGA) (New England BioLabs). After equilibration of agarose blocks with the appropriate buffer, two half-blocks were incubated for 15 h at 37°C in 155 μl of buffer containing 1.5 mM dithiothreitol, 0.15 mg of bovine serum albumin per ml, and 10 U of the respective restriction endonuclease. The reaction was stopped by the addition of 1 ml of TE buffer at 4°C. Restriction fragments were separated by FIGE with a home-built apparatus on 1% agarose gels (20 by 20 by 0.7 cm, with 23 lanes) in 45 mM Tris–10 mM phosphate–1 mM EDTA (pH 8.6). FIGE was run at 14°C with a field strength of 5.6 V/cm. The forward-to-reverse ratio was chosen to be 3:1. Subsequently, the gels were stained with ethidium bromide. λ DNA oligomers (monomer, 43.3 kilobase pairs [kbp]) prepared from intact λgt10 phages (8) were used as size markers. To evaluate the relatedness of *P. aeruginosa* strains, we analyzed the 20 restriction digests on one gel by side-to-side visual comparison. Fingerprints from different FIGE gels were compared with the aid of λ oligomers and

* Corresponding author.

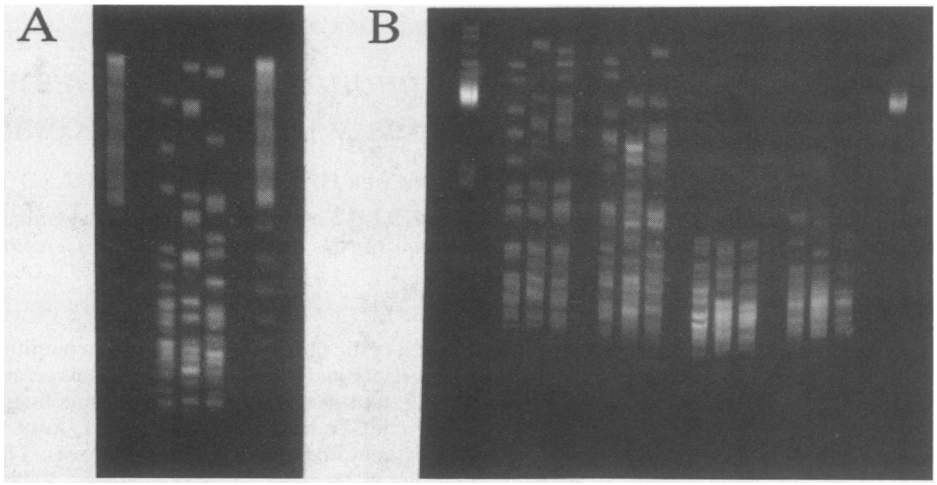

FIG. 1. FIGE analysis of three *P. aeruginosa* strains isolated from a single throat swab of a CF patient. Chromosomes were digested (from left to right) with (A) *Spe*I and (B) *Dra*I, *Xba*I, *Ssp*I, and *Nhe*I. λ oligomers as size markers were applied to the first and last lanes of each gel. Pulses were linearly increased from 1 to 40 s (panel A) or 1 to 18 s (panel B). Serotyping, phage typing, and pyocin typing yielded the following results: strain PACF11A had an NT O serotype, was pyocin type 2, and had the phage lysis pattern 7, 31, 119x, 352, 1214, F7, Col11, Col21; strain PACF11B had an NT O serotype, was pyocin type 6, and had the phage lysis pattern 7, 21, 31, 352, M4, Col11; and strain PACF11C had an NT O serotype, was pyocin type 1a, and had the phage lysis pattern 7, 16, 1214, M4, Col11.

the restriction digest of a reference strain that had been applied on both gels.

RESULTS

Genome fingerprinting of *P. aeruginosa* by FIGE for epidemiological purposes. (i) Principles of the technique. Agarose gel electrophoresis allows the separation of DNA molecules of up to 50 kbp. FIGE (3), like the other recently developed pulsed-field gel electrophoresis techniques (1, 6, 18), permits the easy characterization of very large fragments of DNA. It involves the use of modified submarine horizontal gel electrophoresis equipment in conjunction with a switching unit to control the alternating electric field. We can routinely separate and visualize, on the same gel, DNAs ranging from 5 to 800 kbp.

For genome fingerprinting, the *P. aeruginosa* chromosome is cleaved with restriction endonucleases whose recognition sequences occur less than 100 times in the bacterial genome. According to our experience, up to 50 fragments can be reliably resolved within an 18-cm lane.

Figures 1 and 2 display typical results of FIGE analysis of *P. aeruginosa* chromosomes. The genomic DNA of isolates from two CF patients was cleaved with five different restriction endonucleases that cut only rarely. The number of fragments increased in the order *Spe*I, *Dra*I, *Xba*I, *Ssp*I, *Nhe*I. Single digests with *Dra*I yielded about 40 fragments. Most *Dra*I fragments clustered in the range of 30 to 130 kbp. Fragments smaller than 30 kbp and larger than 130 kbp were obtained as pure species.

(ii) DNA relatedness. Pairs of *P. aeruginosa* strains were classified to be unrelated if the *Dra*I fingerprint of the bacterial chromosome differed by more than 20 bands. Strains were judged to be related if the pattern of *Dra*I fragments was either identical or differed by up to six fragments. Differences of 7 to 20 bands were not detected between any of the analyzed *Dra*I fingerprints.

Evaluation of DNA relatedness by comparing genome fingerprints was independent of the choice of restriction endonuclease. For pairs of unrelated strains, different fingerprints were obtained with each of the five restriction enzymes *Spe*I, *Dra*I, *Xba*I, *Ssp*I, and *Nhe*I (Fig. 1). On the other hand, related strains yielded identical or very similar patterns for each restriction enzyme (Fig. 2).

(iii) Reproducibility. Eighty-eight *P. aeruginosa* isolates were analyzed twice by genome fingerprinting. The *Dra*I digests revealed identical patterns of restriction fragments on separate FIGE gels for each of the 88 pairs. Fifteen strains were also subjected to a more rigorous test of the stability of the genome fingerprint. The physical genome analysis was performed with second subcultures, which had been stored at −70°C, and with the primary subcultures, which had been maintained on nonaerated agar slants for 6 to 36 months. In all analyzed cases, isolates of the same origin yielded identical patterns, suggesting that genome fingerprinting is a reliable technique that gives reproducible data.

(iv) Comparison of phage typing, pyocin typing, and genome fingerprinting. Table 1 shows a comparison of the results of genome fingerprinting with the results of phage and pyocin typing for *P. aeruginosa* isolates. In general, the degree of DNA relatedness as judged from the genome fingerprint correlated with the degree of similarity in the phage and pyocin patterns. Strains with identical *Dra*I fingerprints differed in fewer responses to pyocins and bacteriophages than did strains with unrelated fingerprints. However, a significant proportion of the strains would have been misclassified if the evaluation of strain identity had been based on phage or pyocin typing alone (Table 1); e.g., among the group of unrelated *Dra*I fingerprints, 9% of randomly assigned pairs of strains exhibited the same pyocin type.

Epidemiology of the *P. aeruginosa* infection at a CF center. Typing analyses were performed on 835 clinical isolates of *P. aeruginosa*. Throat swabs positive for *P. aeruginosa* were obtained from 22 siblings with CF and 72 unrelated CF patients who were regularly attending the CF outpatient clinic at the Medizinische Hochschule Hannover. Sixty percent of the whole patient cohort were colonized with *P. aeruginosa*. The mean age of *P. aeruginosa* carriers was 13.3

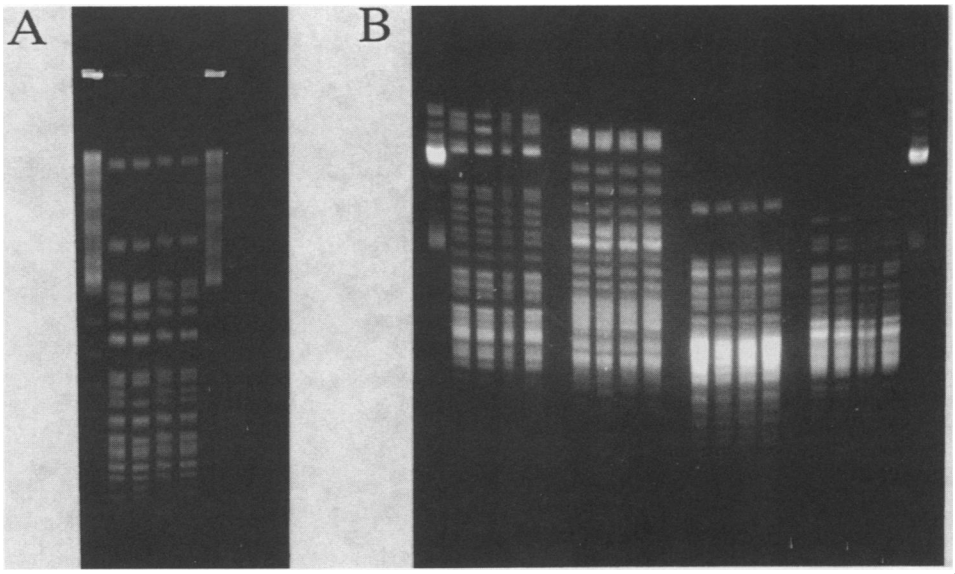

FIG. 2. Course of the colonization of a CF patient with closely related *P. aeruginosa* strains. The four strains were isolated from throat swabs at the onset of colonization (PACF127D1) and after 6 months (PACF127D2), 13 months (PACF127D3), and 17 months (PACF127D4) of colonization. Panels A and B display (from left to right) the FIGE analyses of the four *P. aeruginosa* chromosomes digested with (A) *Spe*I and (B) *Dra*I, *Xba*I, *Ssp*I, and *Nhe*I. λ oligomers as size markers were applied on the first and last lanes of each gel. Pulses were linearly increased from 2 to 40s (panel A) and from 1 to 15s (panel B). Serotyping, phage typing, and pyocin typing revealed the following results: all strains were of O serotype 9 and pyocin type 1f; strain PACF127D1 had the phage lysis pattern 7, 21, 68, 73, 119x, 352, 1214, F7, M4, Col11, Col21; strain PACF127D2 had the phage lysis pattern 7, 21, 24, 68, 73, 119x, 352, 1214, F7, F8, M4, Col11, Col21; strain PACF127D3 had the phage lysis pattern 7, 21, 68, 73, 119x, 352, 1214, F7, M4, M6, Col11, Col21; and strain PACF127D4 had the phage lysis pattern 7, 21, 68, 119x, 352, 1214, F7, M4, Col11, Col21.

years. Of the colonized CF patients, 63% were harboring one strain, 26% were harboring two strains, and 10% were harboring three strains. Five different strains were isolated from one patient.

A specific serotype was detected in only 40% of the isolates. Of the cultures, 41% were serologically polyagglutinable (PA) and 19% were nontypable (NT). This finding corresponds to reports from the literature (9, 14) that PA and NT strains of *P. aeruginosa* are frequently isolated from CF patients. A total of 33 CF patients in our cohort were exclusively harboring strains of defined serotype, 42 patients were colonized with NT and/or PA strains, and mixtures of NT/PA and serotype-specific strains were found in 19 patients.

The typing data revealed that the 72 unrelated CF patients were colonized with different strains. Clusters of a specific pyocin type were not observed. A nosocomial spread of organisms did not occur even though the patients had been regularly admitted to the CF center once or twice a year for a 2-week course of intravenous antipseudomonal antibiotics. The absence of transmission of *P. aeruginosa* strains may be attributed to the strict hygiene measures observed at this CF clinic. During hospitalization, CF patients are accommodated in separate rooms in a ward for infectious diseases, and after a patient is discharged the room is disinfected. Our epidemiological data are in contrast to those reported by the Danish CF center, where clusters of distinct epidemiological types of *P. aeruginosa* were found among small groups of CF patients (2 to 10 individuals) (10) and epidemic spreading of antibiotic-resistant organisms occurred (13).

Typing of isolates from CF siblings. In eight families with two or three affected children, one to three *P. aeruginosa* strains were present in all CF siblings. *Dra*I digests of strains isolated from the siblings revealed either identical (five families) or closely related (three families) fingerprints. *P. aeruginosa* strains from different families were distinct from each other. An example of FIGE analysis is displayed in Fig. 3, which shows the pattern of the *Dra*I restriction fragments

TABLE 1. Correlation of *Dra*I genome fingerprints of *P. aeruginosa* strains with phage and pyocin typing[a]

*Dra*I fingerprint comparison for pairs of strains	% Frequency of different responses to the main pyocin types									% Frequency of different responses in bacteriophage lysis							
	0	1	2	3	4	5	6	7	8	0	1 or 2	3 or 4	5 or 6	7 or 8	9 or 10	11 or 12	13 or 14	15–20
Identical patterns	90	10								40	28	22	10					
One to six different *Dra*I fragments	8	86	4	2						9	34	43	8	6				
More than 20 different *Dra*I fragments	9	14	17	15	14	12	10	8	1	1	9	18	29	17	13	8	4	1

[a] A total of 500 randomly assigned pairs from each subgroup with identical, related, and unrelated *Dra*I fingerprints were analyzed. The pairs were chosen from a collection of 835 isolates from 94 CF patients. Of the 835 strains, 182 were isolated from the 13 families with CF siblings.

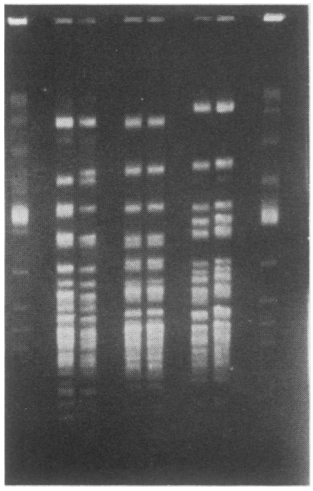

FIG. 3. FIGE of *Dra*I restriction fragments of *P. aeruginosa* chromosomes isolated from three pairs of CF siblings. Left- and rightmost lanes: λgt10 oligomers as size markers. Pulses were linearly increased from 2 to 23 s.

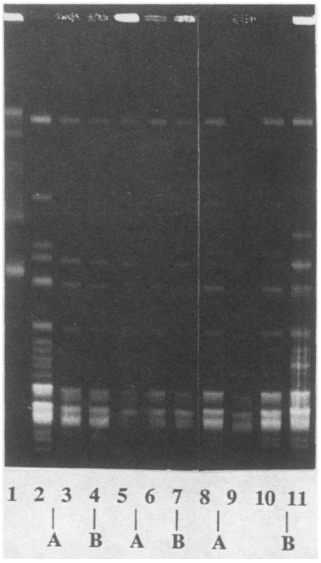

FIG. 4. FIGE analysis of the course of the *P. aeruginosa* colonization in two CF siblings. From left to right: lane 1, λgt10 oligomers; lanes 2 to 11, *P. aeruginosa* chromosomes digested with *Dra*I (lanes 2 and 3, onset of colonization, patient A; lane 4, onset of colonization, patient B; lanes 5 and 6, 6 months of colonization, patient A; lane 7, 6 months of colonization, patient B; lanes 8 and 9, 14 months of colonization, patient A; lanes 10 and 11, 14 months of colonization, patient B). Pulses were linearly increased from 2 to 23 s.

of the *P. aeruginosa* chromosomes from three pairs of CF siblings. Pyocin typing and the less informative serotyping (see above) confirmed the results of genome fingerprinting. Of the pairs of strains with identical or related *Dra*I fingerprints isolated from CF siblings in a family, 62% exhibited the same pyocin type and 35% differed by one pyocin response (Table 1). In contrast, phage typing indicated some degree of heterogeneity. Strains which were identical in the pattern of *Dra*I and *Spe*I restriction fragments differed by up to four responses in the phage lysis pattern.

In three families, the CF siblings were colonized with closely related *P. aeruginosa* strains that differed by one to six bands in their fingerprint pattern. These cases allowed us to monitor the exchange of strains among the siblings. Strains were transmitted several times in these families during the 3-year study period. Figure 4 shows the course of *P. aeruginosa* colonization in one of these families. The two CF siblings had acquired *P. aeruginosa* at the same time. One strain, which was regularly found in the elder sister, was detected in throat swabs taken from her brother 14 months after the onset of colonization. In three of the five cases in which only one sibling was harboring *P. aeruginosa*, the two CF siblings lived in separate homes.

The colonization of CF siblings with identical or closely related strains is most probably explained by cross-infection within the family. Transmission of strains among unrelated CF patients treated at the same CF clinic was not observed. Hence, we conclude, in agreement with two earlier reports (4, 11), that cross-infection is rare unless contact is intimate and prolonged.

DISCUSSION

In recent years, several biological criteria have been assessed for typing *P. aeruginosa*, including colony morphology, pigmentation, metabolic activities, susceptibility to antimicrobial agents, and phage sensitivity. The two most often applied methods are serotyping (16) and pyocin typing (7).

O serotyping of *P. aeruginosa* is a reliable method. However, since only 16 serotypes are differentiated on a routine basis, its discriminatory power is no more than fair. The mucoid isolates from chronically colonized CF patients present a particular problem, because the majority of strains are either PA or NT (9, 14). Therefore, serotyping is of limited value for epidemiological studies of the *P. aeruginosa* infection in CF patients.

The discriminatory potential of pyocin typing is superior to that of serotyping. By using the spotting method (5), 105 main types and 25 subtypes can be identified by the patterns of inhibition observed on 13 indicator strains. However, in our study a significant portion of randomly assigned pairs of strains displayed the same pyocin type, even though their genome fingerprints were completely different.

Physical genome analysis of *P. aeruginosa* by digestion with restriction endonucleases that cut only rarely and subsequent FIGE turned out to be more sensitive than pyocin and serotyping. In particular, this technique had the advantage of clearly separating related from unrelated strains. Related strains differed by less than six *Dra*I bands, whereas unrelated strains differed by more than 20. According to our experience, one restriction digest with *Dra*I is sufficient for epidemiological purposes. If necessary, the sensitivity of the method can easily be increased by using a set of several rarely cutting restriction enzymes that reveal resolvable patterns of 20 to 100 fragments on pulsed-field agarose gels.

Conventional agarose gel electrophoresis is not as appropriate for fingerprint analysis of bacterial genomes. Since only DNA molecules of up to 50 kbp can be separated, frequently cutting restriction endonucleases have to be used that yield a nonresolvable smear of fragments on the agarose gel. In this case, single bands must be visualized by Southern blot hybridization experiments with specific probes.

Recently, Vasil and co-workers (12, 21) observed a restriction fragment heterogeneity in the region upstream of the exotoxin A gene of *P. aeruginosa*. A *Pst*I-*Nru*I fragment

derived from this hypervariable genomic site was successfully applied as a probe in epidemiological investigations of *P. aeruginosa*. The DNA probe was capable of distinguishing 90% of the tested strains with only two restriction enzymes (21).

Both Southern analysis of a hypervariable site and fingerprinting probe polymorphisms in the bacterial genome. However, the *Pst*I-*Nru*I fragment monitors only a single genomic site, whereas fingerprinting with endonucleases that cut only rarely allows multiple sites to be scanned at once. Hence, genome fingerprinting offers the unique advantage of classifying *P. aeruginosa* strains in terms of DNA relatedness (compare data in Table 1 and Fig. 1 and 2).

This report illustrates the usefulness of genome fingerprinting in bacteriological epidemiology. In addition to plasmid fingerprinting (19) and DNA probing of hypervariable sites (12), bacterial genome fingerprinting may become an important diagnostic tool for investigating outbreaks of nosocomial infection and for aiding effective infection control.

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