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Comparison of Arbitrarily Primed PCR and Macrorestriction (Pulsed-Field Gel Electrophoresis) Typing of *Pseudomonas aeruginosa* Strains from Cystic Fibrosis Patients

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Arbitrarily primed PCR fingerprinting was carried out on 43 *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) patients. Seventeen major groups of strains that coincided with groups also distinguished by macrorestriction (pulsed-field gel electrophoresis) typing were identified. Our results illustrated that a CF patient can carry more than one strain and can carry a given strain for long periods of time and that strains can evolve by changes in drug resistance or other phenotypic traits during long-term colonization. The arbitrarily primed PCR method is recommended for first-pass screening of *P. aeruginosa* isolates from CF patients, especially when many strains are to be typed, because of its sensitivity and efficiency.

Most cystic fibrosis (CF) patients suffer from chronic and ultimately fatal pulmonary infections caused by strains of *Pseudomonas aeruginosa*. The first strains to colonize these patients often resemble strains from other body sites, whereas those recovered later tend to be mucoid, nonmotile, and/or nonchemotactic and/or to lack lipopolysaccharide side chains, and they are often resistant to multiple antibiotics (2, 4, 7, 9, 10, 14). The present comparison of arbitrarily primed PCR (AP-PCR) (17, 20) and the macrorestriction typing method (6, 14) was designed to prepare for more detailed analyses of *P. aeruginosa* populations in chronically infected CF patients—in particular, analyses of the complexity of *P. aeruginosa* populations and test of the role of infection from other CF patients versus the role of infection from other sources in colonization and disease. These issues are important in light of the unusual features of strains recovered from CF patients, the debilitating effects of *P. aeruginosa* infections on patient health, and the possibilities for interperson spread in CF clinics, support groups, and summer camps.

Of the many phenotypic and DNA-based typing methods used during the last decade to analyze *P. aeruginosa* isolates from CF patients (11, 12), the macrorestriction method may be the most discriminatory. In this method, bacteria are embedded in agarose plugs and lysed in situ, and then their genomic DNA is digested with restriction enzymes that cleave the chromosome infrequently and electrophoresed in pulsed-field gels (6, 14). Macrorestriction typing, in particular, indicated that (i) independent *P. aeruginosa* isolates are usually distinguishable from one another, (ii) a number of different strains can colonize CF patients, (iii) a given strain can be carried for months or years, (iv) a patient can carry two or more strains, and (v) individual strains tend to evolve during carriage (6, 14). Macrorestriction typing is laborious, however, especially for mucoid *P. aeruginosa* strains, since their mucoidy makes it difficult to estimate bacterial cell titers from culture optical densities. Accurate estimates of cell density are essential at the agarose embedding step to help ensure clear restriction fragment patterns and thus accurate strain typing. The labor-intensive nature of macrorestriction typing prompted us to seek a more efficient alternative, especially for analyses of *P. aeruginosa* populations that potentially could involve hundreds of isolates. It seemed that the newer AP-PCR, or randomly amplified polymorphic DNA (RAPD), fingerprinting method (17–20) might meet this need. In this method, DNA synthesis is primed at low stringency with single oligonucleotides of arbitrarily chosen sequence from sites in genomic DNA to which the oligonucleotide is fortuitously matched or almost matched, and strain-specific arrays of amplified DNA fragments are obtained from pairs of closely spaced sites. It is one of the easiest of typing methods, because the template DNA need not be of high molecular weight, double stranded, or highly purified, and only nanogram quantities of DNA are required. Tests with one or two primers are generally sufficient to distinguish unrelated strains, although higher sensitivity can be achieved when warranted by using more primers and thereby testing for sequence divergence in a larger fraction of the genome (3, 15, 17–20). The results presented here indicate that AP-PCR results are concordant with macrorestriction typing for CF *P. aeruginosa* isolates and that AP-PCR can serve as an efficient and sensitive means of high-throughput typing of such strains.

The isolation and initial characterization of the set of CF *P. aeruginosa* strains from which the 43 isolates used here were drawn has been described previously (14). In brief, many *P. aeruginosa* strains were obtained from the sputa of patients with acute bronchopulmonary infection attending the Hopital Erasme (Brussels, Belgium) CF clinic over a 20-month period. The strains were characterized by macrorestriction typing and tested for susceptibilities to 14 different antibiotics (14) (see Table 1). Twenty-two major types were identified by macrorestriction of 122 isolates from 18 patients, and 13 of these types included minor subtypes that differed from one another by one or a few restriction fragments. *P. aeruginosa* isolates from a given patient often seemed to be closely related but distinguishable by resistance profile, macrorestriction subtype, or both, which probably reflects the persistence and continual evolution of individual strains in response to host defenses,

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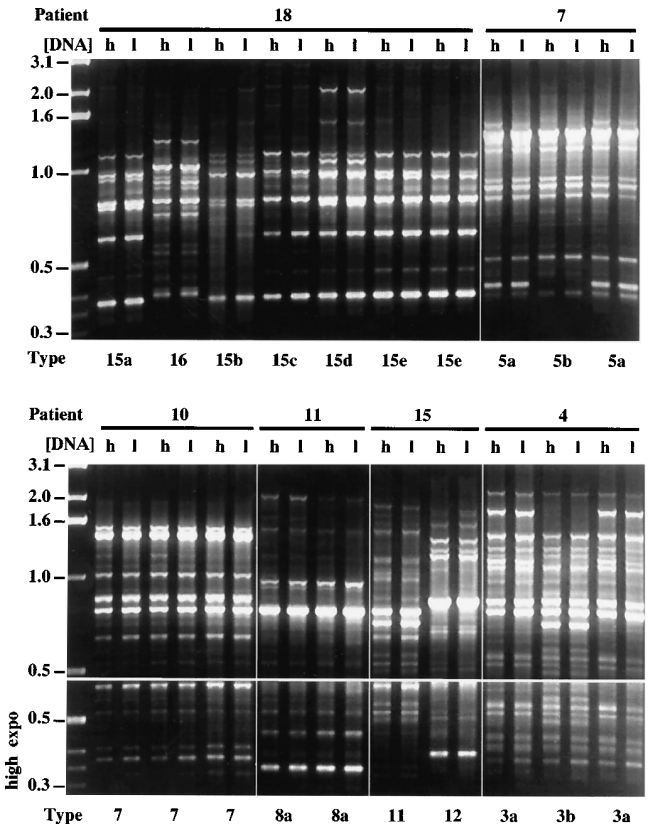

FIG. 1. Sensitivity and reproducibility of AP-PCR fingerprinting of *P. aeruginosa* strains. Presented here are the results of duplicate tests using 20 (h) and 4 (l) ng of template DNA with primer 10514. The patient numbers refer to those in reference 14 and in Table 1. Type corresponds to the fingerprint patterns defined in Table 1 (footnote *e*). Size markers (in kilobases) (1-kb ladder; Gibco BRL) are indicated on the left of each panel. The order of AP-PCR profiles matches that in Table 1, except for patient 18, for whom the profiles of isolates 3 and 4 precede those of isolates 1 and 2. Equivalent reproducibility was obtained with primer 14306 (Fig. 2). High expo, high exposure during photography of the ethidium bromide-stained gel to reveal bands not evident in the exposure suited for the more intensely stained larger fragments in the upper part of the gel.

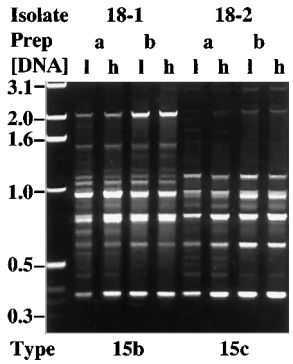

FIG. 2. Reproducibility of RAPD profiles obtained by using different DNA preparations. Primer 14306 was used here. Template DNA prep a was stored at −20°C for a year before use, whereas template DNA prep b was prepared on the day before use. 18-1 and 18-2 refer to the first two isolates from patient 18 in Table 1. Two DNA concentrations, 4 (l) and 20 (h) ng, were used to test for reproducibility, as for Fig. 1.

antibiotic treatment, and competition with other microbes. Isolates from different patients were usually easily distinguishable from one another, which probably reflects different independent colonization events. In addition, however, it seemed that the same or very closely related strains were recovered one or more times from several pairs of patients, consistent with their all attending the same CF clinic. The isolates exhibited many different resistance patterns, and these were not closely correlated with macrorestriction type.

To prepare DNA, 500 μl of an overnight, stationary-phase, Mueller-Hinton broth culture was spun for 1 min in a microcentrifuge, and the cell pellet was suspended in 200 μl of buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 50 mM glucose, and 10 mg of lysozyme per ml and incubated for 30 min at room temperature. Then 500 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 50 mM EDTA, 1% sodium dodecyl sulfate, 50 mg of proteinase K per ml) was added, and the solution was incubated for 3 h at 55°C. RNase was added to a final concentration of 20 μg/ml, and the mixture was incubated for 30 min at 55°C. The solution was extracted sequentially with equal volumes of phenol, phenol-chloroform-isoamyl alcohol (25:24:1), and chloroform-isoamyl alcohol (24:1). One-tenth of a volume of 3 M sodium acetate was added. The DNA was precipitated with 2 volumes of ethanol, washed with 70% ethanol, and dissolved in 200 μl of distilled water. An aliquot was electrophoresed in a Tris-acetate-buffered 1% agarose gel and stained with ethidium bromide to estimate the yield and verify the integrity of the DNA.

RAPD fingerprinting was carried out in a 25-μl volume containing about 20 or 4 ng of bacterial DNA, to test for reproducibility (h and l, respectively, in Fig. 1) (17, 18); 3 mM $MgCl_2$; 20 pmol of primer (see below); 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Division, Roche Molecular Systems, Branchburg, N.J.); 250 μM (each) dCTP, dGTP, dATP, and dTTP (Boehringer Mannheim, Indianapolis, Ind.) in 10 mM Tris-HCl (pH 8.3)–50 mM KCl; and 0.001% gelatin, all under a drop of mineral oil in a Perkin-Elmer (Norwalk, Conn.) model 480 thermal cycler. Two arbitrary primers, 10514 (5′-TGGTGGCCTCGAGCAAGAGAACGGAG) and 14306 (5′-GGTTGGGTGAGAATTGC) were used, with the following cycling parameters: 4 cycles of 94°C for 5 min, 40°C for 5 min, and 72°C for 5 min; then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and then 72°C for 10 min. After PCR, 8-μl aliquots were electrophoresed in 2% agarose gels containing 0.5 μg of ethidium bromide per ml and 1× Tris-acetate running buffer and photographed under UV light. A 1-kb DNA ladder (Gibco BRL, Gaithersburg, Md.) was used as a size marker in all gels. Gel photographs were processed for presentation by scanning with a Microtek Scanmaker 600ZS, using Adobe Photoshop 2.0 software (Adobe Systems, Inc., Mountain View, Calif.) and imported into Canvas 3.0.2 (Deneba Software, Miami, Fla.) running on a Macintosh Quadra 700 computer.

The accuracy of inferences from DNA fingerprinting depends on the reproducibility of profiles. In our experience, if optimal concentrations of AmpliTaq polymerase and $MgCl_2$ are used, as was done here, the most serious source of nonreproducibility comes from inhibitors that remain in some DNA preparations. Accordingly, we used two different amounts of template DNA (20 and 4 ng). As illustrated in Fig. 1, we found that input DNA levels did not affect the profiles obtained. Equivalently high levels of reproducibility were seen with each of two primers (10514 and 14306; sequences are shown above) and with DNA preparations made at different times, although the intensities of certain large bands did vary with the DNA preparation, as illustrated in Fig. 2. Several 10-nucleotide primers that had been useful for our studies of other microbes (1, 3, 8, 16) did not give completely reproducible profiles with

TABLE 1. Comparison of RAPD and macrorestriction results for *P. aeruginosa* strains from CF patients

Patient[a]	Date of strain isolation (day.mo.yr)[b]	Resistance pattern[c]	No. of resistances	Fingerprint pattern			
				Macrorestriction[d]		AP-PCR[e]	
				XbaI	DraI	10514	14306
2	28.08.89	43	10	2a	2a	1	1
3	11.05.89	12	3	3b	3b	2	2
	25.08.89	4	1	3a	3a	2	2
4	09.03.90	4	1	4a	4a	3a	3a
	18.06.90	25	6	4b	4a	3b	3b
	22.06.90	25	6	4a	4b	3a	3a
6	09.02.90	1	0	6a	6b	4	4
	14.02.90	1	0	6b	6c	4	4
7	27.12.88	38	9	7a	7a	5a	5a
	03.10.89	16	4	7b	7a	5b	5a
	12.01.90	7	2	7c	7a	5a	5a
9	14.03.90	9	3	9a	9a	6	6
	18.04.90	42	10	9a	9b	6	6
10	06.03.90	4	1	10a	10a	7	7
	22.03.90	4	1	10b	10c	7	7
	29.03.90	8	3	10c	10b	7	7
11	27.02.90	4	1	11a	11a	8a	8a
	21.03.90	1	0	11b	11a	8a	8b
12	07.11.89	15	4	2a	2a	1	1
	08.11.89	15	4	2b	2b	1	1
14	29.05.89	4	1	14a	14a	9	9
	18.08.89	21	4	15a	15a	10a	10a
15	17.05.89	6	2	16a	16a	11	11
	26.07.89	4	1	17a	17a	12	12
16	19.06.89	28	4	18a	18a	13a	13a
	28.12.88	11	3	18b	18b	13a	13a
	19.12.89	10	3	18c	18c	13a	13a
	24.04.90	4	1	19a	19a	14	14
17	26.06.89	35	8	15d	15d	10a	10a
	09.08.89	37	9	15e	15d	10b	10a
	24.09.89	39	9	15f	15e	10b	10a
	09.10.89	4	1	10a	10a	7	7
	03.10.89	39	9	15d	15d	10a	10a
	02.11.89	47	11	15d	15e	10a	10a
	02.12.89	45	12	15d	15e	10a	10a
	20.12.89	40	10	15 f	15e	10b	10a
18	13.01.89	3	1	20a	20a	15b	15b
	07.02.89	1	0	20b	20b	15c	15b
	14.03.89	4	1	22a	22a	16	16
	23.05.89	3	1	21a	21a	15a	15a
	30.05.89	3	1	20b	20c	15d	15c
	23.07.90	22	4	20d	20a	15e	15d
	25.07.90	18	4	20e	20d	15e	15d

[a] Patient numbers match those in reference 14.

[b] Patients were typically in the CF clinic for 15 day periods of intravenous and aerosol antibiotic therapy that included dates of strain isolation.

[c] Resistances to the following antibiotics were scored: neomycin, netilmicin, sisomycin, streptomycin, tobramycin, amikacin, gentamicin, kanamycin, spectinomycin, piperacillin, ceftazidime, aztreonam, imipenem, and ciprofloxacin. The resistance patterns of isolates from patient 18 were as follows: 3, resistant to streptomycin; 1, susceptible to all antibiotics tested; 4, resistant to kanamycin; 22, resistant to neomycin, sisomycin, streptomycin, and aztreonam; 18, resistant to neomycin, streptomycin, kanamycin, and aztreonam. The exact set of resistances corresponding to other patterns and details of antibiotic sensitivity testing are available from M.J.S.

[d] Macrorestriction pattern (type) assignments match those in reference 14. These assignments were based on the mobilities of the 22 to 35 fragments in the size range of 10 to 350 kb that typically were generated by either *Xba*I or *Dra*I digestion of *P. aeruginosa* genomic DNA and resolved by pulsed-field gel electrophoresis, as illustrated by Fig. 1 and 3 of reference 14. Pairs of isolates differing by more than four bands were considered to represent different major types, and these types are designated 2, 3, 4, etc. Pairs of isolates differing in fewer fragments were considered minor types of the same major type, and these minor types are designated a, b, c, etc., as in reference 14. Isolates of the same minor type often differed in their antibiotic resistance patterns, illustrating that even macrorestriction typing detects only a subset of all genomic DNA sequence variation.

[e] AP-PCR patterns were assessed as described in the legend to Fig. 1 and in accord with guidelines developed in reference 14. Two strains were assigned to different major types (1, 2, 3, etc.) if they differed by two or more prominent bands. Two strains were considered minor variant types (a, b, c, etc.) of a given major type if they differed by just one or two faint bands in the array of up to about a dozen RAPD fragments.

P. aeruginosa (data not shown; see also reference 5a), and accordingly we do not recommend the use of 10-nucleotide primers for this species.

Our AP-PCR tests with primers 10514 and 14306 described above showed (i) quite different profiles of RAPD products from *P. aeruginosa* isolates from different persons, (ii) closely related profiles for most separate isolates from a given patient, and (iii) profiles differing from one another by one or two weak bands for some isolates from a given patient (e.g., pattern 15a versus pattern 15b versus pattern 15c for patient 18). The isolates fell into 16 major groups that closely matched the grouping from macrorestriction data (Table 1). Minor subtypes were found among strains of the same major type from 15 patients (13 subtypes), whereas macrorestriction subtypes were found among the isolates from 11 of the patients (31 subtypes), which indicated that macrorestriction is more sensitive than AP-PCR. Our results also supported inferences from macrorestriction typing that the same or closely related strains had been isolated from several pairs of patients (i.e., patients 2 and 12, 10 and 17, and 14 and 17 in Table 1). This is in accord with their attending the same CF clinic during the same time period. It is not clear now, however, whether (or how often) these patients were in direct contact, nor do we have samples from all clinic patients during this time period, which might have helped assess whether the shared strains were in fact quite widespread.

The reproducibility of our AP-PCR tests indicated that the small differences in profiles of related isolates were due to genetic divergence, not PCR artifacts. We propose (i) that such differences usually reflect divergence from a recent common ancestor by mutation or by interstrain DNA transfer and (ii) that major differences in AP-PCR profiles or macrorestriction patterns usually indicate strains from quite unrelated lineages. Although AP-PCR with the two primers used here was less discriminant than macrorestriction typing for studies of closely related *P. aeruginosa* strains (a result reminiscent of recent studies of *Staphylococcus aureus* [13]), it easily distinguished strains likely to be from different lineages and thus likely to reflect different colonization events. The cloning of AP-PCR fragments that distinguish closely related strains (as described in reference 5) and characterization of corresponding genomic DNA sequences should allow us to assess the relative contributions of mutation and gene transfer from unrelated strains in *P. aeruginosa* population dynamics in CF patients. This, in turn, should contribute to our understanding of host-specific adaptation and virulence of these *P. aeruginosa* strains during long-term chronic infection.

We anticipate a continuing need for high-throughput, cost-effective typing of *P. aeruginosa* isolates from CF patients, their families, and other contacts over time, in particular to examine the complexity and stability of bacterial populations and to understand better how they adapt and evolve. The AP-PCR method seems to be well suited for such first-pass screening, and it could be followed by macrorestriction or other high-resolution examination of representative isolates when warranted. Such analyses should provide critical new insights into microbial population dynamics and disease, and they may contribute significantly to CF patient care.

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