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Use of a Pilin Gene Probe To Study Molecular Epidemiology of *Pseudomonas aeruginosa*

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Strains of *Pseudomonas aeruginosa* from patients with cystic fibrosis (CF) are unusual. The majority have a rough lipopolysaccharide (LPS) which renders them nontypeable by conventional typing systems based on a serological reaction with the O polysaccharide of smooth LPS. We developed a new typing scheme using a pilin gene probe as a marker for hybridization with endonuclease-digested genomic DNA from *P. aeruginosa*. Twenty-one different restriction fragment length polymorphism (RFLP) types were found among 249 isolates. RFLP type 7 was recovered only from patients with thermal burns (9 of 14 isolates) in both Vancouver, British Columbia, and Edmonton, Alberta, Canada. None of the other RFLP types showed a clear predilection for disease state or environmental niche. Multiple morphologically different isolates from individual patients with CF were studied; each isolate in 33 of 40 sputum samples had an identical RFLP type, despite considerable LPS serotype heterogeneity. Sequential isolates from 23 patients were studied; in 10 isolates there was a clear change in both the RFLP type and the LPS serotype. We conclude that patients with CF usually harbor a single *P. aeruginosa* RFLP type in their sputa, but that one strain can replace another as the predominant colonizing type.

Pseudomonas aeruginosa has a peculiar propensity to colonize and infect the respiratory tract of patients with cystic fibrosis (CF) (33). Once acquired, these bacteria are almost never eradicated (33). Attempts to prevent colonization have been futile. In order to devise effective strategies to prevent respiratory tract colonization by *P. aeruginosa*, a basic understanding of its epidemiology would be invaluable.

Studies of the epidemiology of *P. aeruginosa* in patients with CF have been severely hampered by the imprecision of the existing methods that are used to determine the serotypes of strains, which are based on an agglutination reaction between bacterial lipopolysaccharide (LPS) and type-specific rabbit antisera (6). This serotyping scheme works well for clinical isolates of *P. aeruginosa* which possess smooth LPSs (23). However, *Pseudomonas* isolates from patients with CF are unusual; the majority are rough LPS strains that are deficient in the O-polysaccharide side chain, which is responsible for the serotyping reaction (9). Most of such strains are sensitive to the bactericidal effect of normal human serum and are nontypeable, autoagglutinable, or agglutinable by multiple typing sera (9, 10, 26, 27). Moreover, we and others (19, 21) have demonstrated that isolates with different LPS serotypes often prove to be clonally related when examined by gene probing and restriction fragment length polymorphism (RFLP) techniques. Therefore, conventional serotyping methods do not offer the discriminatory power necessary to distinguish among isolates and provide answers to basic epidemiological questions.

We have recently developed a method for determining the RFLP types of *P. aeruginosa* isolates. This method appears to be strain specific, reproducible, and able to determine the RFLP types of most isolates from patients with CF. The method is based on hybridization between a radioactive *Pseudomonas* pilus gene probe (21) and electrophoresed chromosomal DNA from clinical *P. aeruginosa* isolates. Using this method, we have recently shown (21) that serial isolates of *P. aeruginosa* from a patient with CF had identical RFLP patterns and identical pilin gene sequences, even though there was substantial heterogeneity in the phenotypic characteristics of the isolates. This method was further evaluated on sequential cultures from individual patients with CF and on multiple *P. aeruginosa* isolates from individual sputum samples. The results of these investigations are reported here.

MATERIALS AND METHODS

Patients and clinical specimens. Most specimens for culture of *P. aeruginosa* were obtained from patients with CF who were cared for at the Cystic Fibrosis Assessment Clinic at British Columbia's Children's Hospital. CF was diagnosed in all patients by conventional methods, and patients were seen by the clinic physicians at regular intervals or as required for the assessment of deterioration in their clinical status. Samples of sputum or pharyngeal secretions (if sputum could not be produced) were obtained and cultured at each clinic visit, and all isolates of *P. aeruginosa* were saved.

Bacterial strains. Specimens of respiratory tract secretions from patients with CF in Vancouver were plated onto sheep blood and MacConkey agar plates, and colonies of *P. aeruginosa* were identified by conventional methods (26). All morphologically different *Pseudomonas* colonies from each culture (classic, mucoid, enterobacter, rough, or dwarf) were saved and were characterized as described previously (31). In addition, multiple colonies (>10) were picked from primary isolation plates containing the cultures of sputum from five patients. All these isolates were characterized as described below. All isolates analyzed in this study are listed in Table 1. In addition to isolates from Vancouver and

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Edmonton, *P. aeruginosa* isolates from patients with CF were obtained from Minneapolis, Minn., and Morven Edwards (Baylor University, Houston, Tex.), Randall Irvin (University of Toronto, Toronto, Ontario, Canada), Noni MacDonald (University of Ottawa, Ottawa, Ontario, Canada), and Gerald Pier (Harvard University, Boston, Mass.) *P. aeruginosa* isolates were also obtained from the blood of patients with thermal burns from Vancouver, Edmonton, and San Antonio, Tex.; a neutropenic patient with a malignancy from Edmonton; and infected surgical incisions, tracheal secretions, and a corneal infection from patients from Edmonton.

P. aeruginosa strains were also obtained from vegetables and sink drains. Swabs from sink or bathtub drains or crushed bits of vegetables were placed in acetamide broth containing 5 g of NaCl, 1 g of $NH_4H_2PO_4$, 1 g of K_2HPO_4, and 20 g of CH_3CONH in 1 liter of distilled water (12). The broths were incubated at 37°C for 10 days and then subcultured onto PC agar medium (Columbia agar with 30 μg of phenanthroline [B.D.H. Chemicals, Toronto, Ontario, Canada] per ml and 30 μg of C-390 [Norwich Eaton Pharmaceuticals, Norwich, N.Y.] per ml), which is selective for the growth of *P. aeruginosa* (2). Any bacteria that were recovered were identified as *P. aeruginosa* by using API RAPID NFT strips (Analytab Products, St. Laurent, Quebec, Canada).

Serotyping methods. Serotypes of *P. aeruginosa* isolates were determined by using the International Antigenic Typing System (Difco Laboratories, Detroit, Mich.) as described previously (26). Isolates that were agglutinated by more than one typing serum were designated polytypeable, and those that agglutinated in nonimmune serum or in saline were designated autoagglutinable.

***P. aeruginosa* DNA preparation.** Genomic DNA was isolated from late-log-phase bacteria (32) by a variation of the method described by Coleman et al. (3). DNA concentrations were determined by the ethidium fluorescence assay of Morgan et al. (17).

RFLP typing. Determination of RFLP types was performed as described previously (21) by using a 1.2-kilobase *Hin*dIII restriction fragment containing the entire pilin gene from *P. aeruginosa* PAK (20). Restriction enzyme digestion of the chromosomal DNA was performed according to the recommendations of the manufacturer with *Pst*I and *Hin*dIII enzymes purchased from GIBCO Laboratories (Grand Island, N.Y.). The DNA was electrophoresed on agarose gels and transferred to nitrocellulose by the method of Southern (25). The digested DNA was then probed at 37°C with the PAK pilin gene, which was nick translated with ^{32}P as described previously (15). The sizes of probe-reactive fragments were determined by comparing them with parallel lanes containing molecular weight standards. The standard deviations for the fragment sizes were approximately 10% (e.g., 2.0 ± 0.2 kilobases).

RESULTS

Distribution and frequency of RFLP types among clinical and environmental isolates of *P. aeruginosa*. A total of 249 *P. aeruginosa* isolates were studied by the RFLP typing method. Among these were 210 isolates from 75 patients with CF (1 to 12 isolates per patient; average, 2.8 isolates). Isolates from patients with CF were obtained from Vancouver (162 isolates), Edmonton (8 isolates), Minneapolis (1 isolate), Toronto (5 isolates), Boston (13 isolates), Houston (9 isolates), and Ottawa (12 isolates). Fourteen *P. aeruginosa* isolates from burn wounds were obtained from patients in Vancouver (10 isolates), Edmonton (3 isolates), and San Antonio (1 isolate). Isolates from a patient with a malignancy (five isolates), from infected surgical incisions (two isolates), from tracheal secretions (eight isolates), and from a corneal infection (one isolate) were all obtained from Edmonton. Environmental isolates from a sink drain (one isolate) and from vegetables (eight isolates) were obtained from Vancouver. All isolates were stored at −70°C, and many were tested by restriction fragment analysis more than once.

TABLE 1. Distribution of RFLP types among strains of *P. aeruginosa*

RFLP type	Fragment size (kb)		No. (%) of strains[a]
	*Pst*I	*Hin*dIII	
1	2.0, 3.7	>20[b]	78 (31.3)
2	2.0, 3.7	3	24 (10.4)
3	0.6, 1.5	1.2	41 (16.5)
4	6.5	3	37 (14.9)
5	4	1.2	20 (8.0)
6	6	>20	5 (2.0)
7	1.7, 1.8	>20	9 (3.6)
8	2	1.5	5 (2.0)
9	2.3, 2.5	>20	7 (2.8)
10	>20	3.0, 3.2	6 (2.4)
11	1.8, 2.2	0.7, 0.9	1 (0.4)
12	1.8, 2.3	3.5	2 (0.8)
13	2.0, 3.7	2.1, 20	2 (0.8)
14	1.6, 2.4	>20	2 (0.8)
15	1.8, 2.5, 9.6	3.7, 20	1 (0.4)
16	2.3, 16	2.1, >20	2 (0.8)
17	1.4, 2.5	4	1 (0.4)
18	1.0, 2.5, 3.7	1.5, 3.0	1 (0.4)
19	2.6, 9.4	2.3, 20	1 (0.4)
20	0.9, 2.0	1.5	1 (0.4)
21	1.5, 3.7	>20	1 (0.4)
NT[c]			3 (1.2)

[a] A total of 249 strains were tested.
[b] Bands of >20 kilobases (kb) in size entered the gel; however, it was not possible to determine their precise size.
[c] NT, Nontypeable.

The characteristics and frequencies of the different RFLP types are summarized in Table 1 and Fig. 1. By using two restriction enzymes (*Pst*I and *Hin*dIII), 21 unique patterns were observed (Fig. 1). The restriction fragment patterns were repeated many times and found to be highly reproducible. Some patterns (e.g., type 15) contained three fragments that were larger than the probe, suggesting the possibility of incomplete digestion. One explanation for this phenomenon may be the fact that highly conserved nonpilin gene sequences at the ends of the 1.2-kilobase PAK probe sometimes occur more than 3 kilobases apart in other isolates (W. Paranchych and P. Sastry, unpublished data). When this occurs, it becomes possible for the PAK probe to hybridize with more than two fragments that are larger in size than the probe itself.

All but two of the *P. aeruginosa* isolates (0.8%), both of which were from patients with CF, were typeable by RFLP analysis. Types 1 to 5 were the most prevalent; isolates of these five types made up 81% of the total number of isolates tested. Types 6 to 10 were moderately prevalent, whereas types 11 to 21 were rare and probably arose from recent mutational events in one of the restriction enzyme recognition sites. The results obtained by RFLP analysis were highly reproducible and were stable after the isolates were stored frozen for at least 12 months. The isolates studied in

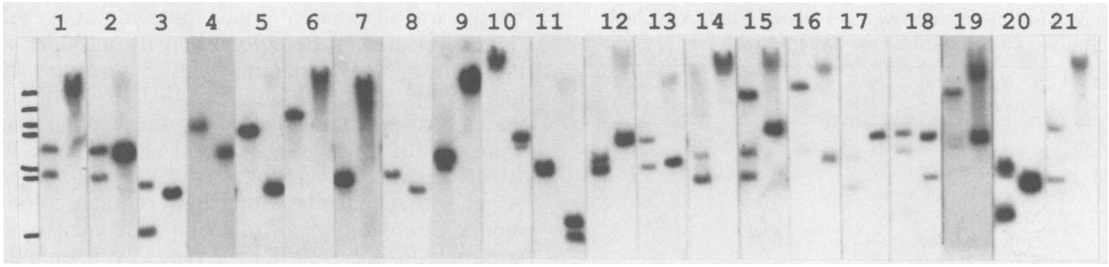

FIG. 1. RFLP types among strains of *P. aeruginosa*. Genomic DNAs were digested with *Pst*I and *Hin*dIII and subjected to Southern hybridization as described in the text. The blots were probed with a pilin gene containing a 1.2-kilobase *Hin*dIII fragment of *P. aeruginosa* PAK. The left lane of each RFLP type contains the *Pst*I digest, while the right lane contains the *Hin*dIII digest. Horizontal lines on the left represent mobilities of the following molecular size standards (in kilobases; from top to bottom, respectively): 23.7, 9.5, 6.7, 4.3, 2.3, 2.0, and 0.6.

this investigation were selected from several research collections, and the frequencies of RFLP types may not be representative of those in the general population of *P. aeruginosa* strains. There was no obvious geographic clustering of RFLP types. For instance, type 1 was found in patients with CF from Boston, Houston, Minneapolis, Ottawa, Toronto, and Vancouver. The rarer RFLP types (types 11 to 21) may be unique to specific geographic locations, but many more strains need to be analyzed to determine whether this is the case.

RFLP type 7 was only found in patients with thermal burns. Of the 14 *P. aeruginosa* burn wound isolates tested, 9 were of type 7. This RFLP type was recovered from patients from both Vancouver and Edmonton and was not found in any other clinical or environmental setting. None of the other RFLP types showed a similar predilection for disease state or environmental niche. Environmental isolates were of RFLP types 1, 2, 4, 8, and 12.

A total of 200 *P. aeruginosa* isolates from patients with CF were analyzed by LPS serotyping (Table 2). These data demonstrate the imprecision of this method for determining the serotypes of strains from this group of patients. Only 39.5% were typeable, and even these gave weak reactions compared with those of other clinical specimens and environmental isolates. Of the isolates that failed to agglutinate with a single antiserum, 50% were polytypeable, 5% were nontypeable, and 5.5% were autoagglutinable (Table 2). All 39 isolates from patients without CF were easily typeable with LPS antisera.

TABLE 2. Frequencies of LPS serotypes among *P. aeruginosa* strains from patients with CF

LPS serotype	No. (%) of strains[a]
1	18 (9.0)
2	1 (0.5)
3	8 (4.0)
4	1 (0.5)
5	2 (1.0)
6	33 (16.5)
7	0 (0)
8	0 (0)
9	1 (0.5)
10	11 (5.5)
11	3 (1.5)
12	0 (0)
13	0 (0)
14	1 (0.5)
15	0 (0)
16	0 (0)
Polytypeable	100 (50.0)
Nontypeable	10 (5.0)
Autoagglutinable	11 (5.5)

[a] A total of 200 strains were tested.

Studies on the heterogeneity of *P. aeruginosa* strains in individual patients with CF. In order to determine whether individual patients with CF carry single or multiple strains of *P. aeruginosa*, we typed multiple isolates from individual sputum samples and analyzed sequential isolates over periods of up to 7 years in patients from Vancouver. A total of 162 isolates from 32 patients (1 to 12 strains per patient; average, 5.1 strains) were studied. We analyzed multiple *P. aeruginosa* isolates from 40 separate sputum samples (2 to 7 strains per specimen; average, 2.6 strains). Despite a great phenotypic heterogeneity, multiple isolates recovered from each of these 40 sputum specimens had identical RFLP types in 33 of 40 (82.5%) cases. Sequential *P. aeruginosa* isolates were recovered from patients with CF over a 7-year period. An average of 4.0 specimens were obtained from each of 23 patients. From 1 to 6 isolates (average, 1.5 isolates) were obtained from each specimen. Isolates recovered from these sequential series had identical RFLP types in only 13 of 23 (56.5%) isolates. Of the 10 patients with changes in the predominant colonizing RFLP type, 8 patients had two different RFLP types and 2 patients had three different RFLP types. In those cases in which the RFLP type changed during sequential sampling, there was a clear shift in the LPS serotype as well. In four patients who were studied, changes in the RFLP type of the colonizing strain also coincided with shifts in the pilin size, as determined by Western immunoblotting (21).

Examples of multiple isolates from individual patients with CF are given in Table 3. These examples demonstrate that the two phenotypic characteristics (colonial morphology and LPS serotype) correlated very poorly with the RFLP types. Furthermore, serotypes of several of the isolates could not be determined by the conventional LPS serotyping method, but all were typeable by restriction fragment analysis.

DISCUSSION

Several different methods have been used to type bacteria for epidemiological investigations, including agglutination with LPS-specific antisera (5, 14, 16), phage sensitivity (10, 22, 23), plasmid analysis (11), antimicrobial susceptibility (4), and outer membrane protein analysis (1). Each of these methods has proven imperfect for the typing of *P. aeruginosa* strains from patients with CF. These strains are unusual among the pathogenic bacteria, as most of them

TABLE 3. Multiple *P. aeruginosa* isolates from individual sputum cultures from two patients with CF

Strain[a]	Colonial morphotype[b]	LPS serotype	RFLP type
BWHI			
1	Enterobacter	6	1
2	Mucoid	PT[c]	1
3	Mucoid	PT	1
4	Enterobacter	6	1
5	Enterobacter	6	1
TMAC			
1	Mucoid	6	4
2	Mucoid	6	4
3	Mucoid	PT	4
4	Mucoid	3/6[d]	4
5	Mucoid	PT	4
6	Mucoid	3/6	4
7	Dwarf	6	6
8	Mucoid	6/10	6

[a] All strains were isolated on 10 July 1986.
[b] As defined previously (29).
[c] PT, Polytypeable.
[d] 3/6, Agglutinated by both type 3 and 6 antisera.

possess a rough LPS (9); this renders them nontypeable with commercial antisera directed against LPS (6). Antimicrobial susceptibility often changes under the pressure of antibiotic therapy (7), rendering this method to be of limited usefulness (28). Outer membrane proteins of *P. aeruginosa* are remarkably conserved among strains (18), and analysis of their profiles, therefore, cannot be used for epidemiological purposes.

Analysis of genetic relatedness by using a probe for a conserved gene obviates the problems of phenotypic variability; restriction fragment analysis permitted RFLP type determination of most isolates of *P. aeruginosa* that were nontypeable by earlier methods. This technique has already been shown by Ogle and co-workers (19) to be useful for RFLP type determination of *P. aeruginosa* strains from patients with CF by using a probe for another region of the *Pseudomonas* genome. Samadpour and co-workers (24) were able to distinguish among strains of *P. aeruginosa* by RFLP analysis with DNA probes for both the exotoxin A and pilin genes (24). In a related but somewhat different approach, Grothues et al. (8) examined RFLP types in *P. aeruginosa* strains by digesting chromosomal DNA with restriction endonucleases that cut only rarely and separated the large fragments by field inversion gel electrophoresis. They observed the total restriction fragment pattern, rather than just those fragments which hybridized with a specific probe.

With our new typing system, it is possible to assign almost all isolates an RFLP type. This provides a useful marker for epidemiological investigations. Although the typing scheme appears to have limited discriminatory power (94% of the strains made up 10 types), we were able to demonstrate multiple types in 7 of 40 individual sputum samples and a clear change in the predominant type during sequential sampling of sputum specimens from 10 of 23 patients. RFLP analysis with the exotoxin A probe appears to provide for greater discrimination among strains (19), but the types of all *P. aeruginosa* isolates may not be able to be determined by this method, since some lack the gene for exotoxin A (30).

Certain questions about the epidemiology of *P. aeruginosa* in patients with CF could not be answered previously by the use of conventional typing methods. Although patients appeared to be colonized with multiple strains of *P. aeruginosa*, as determined by LPS serotype (26), colonial morphology (26), and antimicrobial susceptibility (28) testing, it was not known whether these phenotypic variants were genetically related. Ogle et al. (19) have found, using RFLP analysis with a probe near the *P. aeruginosa* exotoxin A gene, that patients with CF were each colonized with a unique *Pseudomonas* strain and that each patient carried strains with only one RFLP type, even though there was considerable heterogeneity in colonial morphology, LPS serotype, and biotype. We also observed that there was substantial phenotypic heterogeneity among *P. aeruginosa* isolates from individual sputum samples from patients with CF and that the serotype frequently changed in isolates from serial sputum samples from the same patient. However, we also found that individual patients were often colonized with more than one RFLP type of *P. aeruginosa* and that in sequential cultures the type sometimes changed. More recent observations from Ogle et al. (J. W. Ogle, A. I. Vasil, and H. R. Rabin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, B141, p. 53) are in accord with our own. Multiple sequential isolates from individual patients revealed cocolonization with more than one RFLP type and a change in the colonizing type over time.

The means by which *P. aeruginosa* strains are acquired by patients with CF remain to be determined. Data suggesting that strains are spread from patient to patient (10, 22, 34) are not supported by convincing epidemiological evidence. Siblings who share the same strain (13, 26, 29) may have acquired it from a common environmental source. There is no clear evidence that these strains are acquired nosocomially. In descriptions of epidemics within CF clinics (10, 22, 34), isolates were typed by using agglutination with LPS antisera. The apparent identity among strains may represent agglutination of unrelated strains by sera with a high affinity for the exposed core or the rough portion of serotypically different LPS molecules. Studies from two North American summer camps for individuals with CF failed to demonstrate the spread of strains among the campers (27, 29). Finally, we failed to demonstrate sustained cross-colonization between patients with CF who shared a hospital room during admissions for treatment of pulmonary exacerbations (26).

We have described a method for determining the RFLP types of isolates of *P. aeruginosa* which could not be typed by conventional methods. Many critical questions regarding the epidemiology of *P. aeruginosa* in patients with CF remain to be answered. Using this method, we hope to determine how these peculiar strains are acquired and whether there is a risk of nosocomial transmission. Without answers to these fundamental questions, it will be difficult to design rational strategies for infection control or prevention of *P. aeruginosa* infections in patients with CF.

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