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A Major *Pseudomonas aeruginosa* Clone Common to Patients and Aquatic Habitats

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The genomic relatedness of 573 *Pseudomonas aeruginosa* strains from environmental and clinical habitats was examined by digesting the genome with the rare-cutting enzyme *Spe*I. Thirty-nine strains were collected from environmental habitats mainly of aquatic origin, like rivers, lakes, or sanitary facilities. Four hundred fifty strains were collected from 76 patients with cystic fibrosis (CF) treated at four different centers, and 25 additional clinical isolates were collected from patients suffering from other diseases. Twenty-nine *P. aeruginosa* isolates were collected from the environment of one CF clinic. Thirty strains from culture collections were of environmental and clinic origin. A common macrorestriction fingerprint pattern was found in 13 of 46 CF patients, 5 of 29 environmental isolates from the same hospital, in a single ear infection isolate from another hospital, and 8 of 38 isolates from aquatic habitats about 300 km away from the CF clinic. The data indicate that closely related variants of one major clone (called clone C) persisted in various spatially and temporally separated habitats. Southern analysis of the clonal variants with six gene probes and two probes for genes coding for rRNA revealed almost the same hybridization patterns. With the exception of the phenotypically rapidly evolving CF isolates, the close relatedness of the strains of the clone was also shown by their identical responses in pyocin typing, phage typing, and serotyping. Besides clone C, three other *P. aeruginosa* clones were isolated from more than one clinical or environmental source.

The amount of genetic variation in bacterial populations varies greatly among species. The range extends from pathogenic species like mycobacteria, which exhibit little diversity at the genetic level, to soil organisms like streptomycetes, which are highly variable (16). Most other bacteria are within these extremes. In human and animal pathogens, such as *Escherichia coli*, *Neisseria meningitidis*, and *Haemophilus influenzae*, the number of clones in the natural populations appeared to be relatively small, and disease is caused by a small proportion of the total number of existent clones (25).

Pseudomonas aeruginosa is an organism found ubiquitously in nature at low frequency. It may be recovered from water (17), soil, and plants, although its main habitat remains controversial (3, 7, 24). Commonly, *P. aeruginosa* is detected in great amounts in sewage contaminated by humans and animals (20). *P. aeruginosa* is also an opportunistic pathogen for patients with a local or general defect in their immune defense and a major cause for serious nosocomial infection. In cystic fibrosis (CF), an inherited disease, these bacteria chronically colonize the lung and are a major factor for morbidity and mortality (15, 18).

We examined the *P. aeruginosa* strains of one-third of the colonized patient population at a Hannover CF clinic (46 patients) by macrorestriction analysis of the bacterial chromosomes. Thirty percent of the patients were harboring clonal variants of the same strain (23, 27). The predominance of a common *P. aeruginosa* clone in the CF patient population may reflect nosocomial spread or selective advantage of the clone in colonizing CF airways. Alternatively, the major clone could also be overrepresented in soil and aquatic habitats, and then the frequency of *P. aeruginosa* clones in habitats associated with human disease would simply indicate the distribution of clones in the environment.

To settle this question, we wanted to survey the distribution of *P. aeruginosa* strains outside the specialized habitat of the lungs of CF patients, and we randomly collected *P. aeruginosa* strains from different locations. We analyzed strains from the inanimate environment of our CF clinic, patient isolates from other hospitals (CF and non-CF patients), and strains from sanitary facilities and aquatic environments collected in a distant geographic area. Additionally, we examined *P. aeruginosa* strains of nonclinical origin and International Antigen Type strains purchased from the American Type Culture Collection (ATCC).

The same clone was found in Hannover CF patients, in the Hannover Medical School, in a non-CF patient from another geographic area, and in moist and aquatic environments not related to medical or disease habitats. The fragment pattern similarity between strains was confirmed by hybridization with various gene probes. Phenotypical typing methods such as phage typing, pyocin typing, and serotyping were used to support the similarities found on the molecular level.

MATERIALS AND METHODS

Bacterial strains. The *P. aeruginosa* strain collection examined consisted of the following: 420 isolates from 46 CF patients and 4 isolates from burn patients at the Hannover Medical School; 29 isolates from sanitary facilities at a Hannover CF clinic; 10 isolates from CF patients in Copenhagen, Denmark; 10 isolates from CF patients in Magdeburg, Germany; 10 isolates from CF patients in Halle, Germany; 6 isolates from blood culture from Tel-Hashomer, Israel; 15

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strains from various infections from Heidelberg, Germany (urinary tract infections, ear infections, tracheobronchitis, and wounds); ATCC 33348 to ATCC 33364 (International Antigen Type serotypes); ATCC 14886, ATCC 15522, ATCC 21472, ATCC 21776, ATCC 33818, and ATCC 33988 (environmental isolates); ATCC 15691, ATCC 10145; Deutsche Sammlung für Mikroorganismen (DSM) 1117 (= ATCC 27853), DSM 1128 (= ATCC 9027), DSM 939 (= ATCC 15442), DSM 288, and DSM 1253; 1 environmental isolate from Oxford, England; and 38 environmental isolates from Mülheim, Germany (rivers, lakes, and sanitary facilities in households).

Methods. Bacterial strains were grown overnight at 37°C in tryptone broth (1% [wt/vol] casein hydrolysate, 0.5% [wt/vol] NaCl [pH 7.5]). Cells were centrifuged at 1,400 × *g* for 10 min. The pellet was suspended in SE buffer (75 mM NaCl, 25 mM EDTA [pH 7.4]) to a final cell concentration of 3×10^9 cells per ml. The suspension was mixed with 2% (wt/vol) low-melting-point agarose, pipetted into wells, and solidified. The cells embedded in the agarose plugs were incubated overnight in ES (0.5 M EDTA, 1% [wt/vol] *N*-lauroylsarcosine, 0.5 mg of proteinase K per ml [pH 9.5]) at 56°C. Agarose plugs were equilibrated with TE (10 mM Tris, 10 mM EDTA [pH 7.4]) and stored at 4°C until used. Prior to restriction digestion, half of a plug was washed three times in 1 ml of restriction enzyme buffer (New England Biolabs buffer 2), and digestion was performed in 60 μl of restriction enzyme buffer with 10 U of *Spe*I, 1 mM dithiothreitol, and 20 μg of bovine serum albumin per ml overnight at 37°C (13). Separation of the fragments was performed in a home-built crossed-field gel electrophoresis apparatus (26) under the following conditions: 1% (wt/vol) agarose gel, 0.25× Tris-borate-EDTA (TBE) buffer, 5.3 V/cm, and linear ramping from 5 to 25 s for 20 h and 5 to 60 s for 17 h. *P. aeruginosa* PAO *Spe*I-digested DNA, three λ ladders, and λ *Bst*EII digests were applied to each gel as standards. Fragment patterns on the same gel were compared visually. Different gels were compared by using a computer program for cluster analysis (11, 14). Only if the pattern of the reference strain *P. aeruginosa* PAO was recognized as being identical in different gels by the program was a gel-to-gel comparison of the other fragment patterns performed. If *Spe*I fragment patterns appeared to be identical, the respective restriction digestions of the chromosomes were rerun in adjacent lanes in a further crossed-field gel electrophoresis experiment.

Blotting was done with 0.4 M NaOH onto an Amersham HyBond N^+ membrane without pretreatment of the gel. Probes were labeled nonradioactively with digoxigenin for hybridization by using the Boehringer Mannheim random primer labeling kit. Hybridization (6) and detection of signals (1) were done as described elsewhere. The nonradioactively labeled probes were stored at −20°C in hybridization buffer for at least 2 years.

Phage typing was performed with the routine set of 20 bacteriophages described by Asheshov (2); for the pyocin typing, the modified spotting method of Fyfe et al. (9) was used. Serotyping was done by slide agglutination with Pasteur Diagnostika (Munich, Germany) antisera.

RESULTS AND DISCUSSION

For the detection of relatedness, the chromosomes were cleaved with *Spe*I. This enzyme is known to cut the *P. aeruginosa* genome into 30 to 40 pieces (22, 23). The fragments were separated by pulsed-field gel electrophoresis, and the fragment patterns of different strains were then screened for close similarity. Rare-cutter sites are distributed throughout the genome, and identical macrorestriction digests are a criterion for close relatedness of chromosomes. Likewise, changes of genome organization like insertions and deletions of >5 kbp, gross chromosomal rearrangements, or point mutations within the restriction site are visualized by shifts of some fragments in the fingerprint as an indicator for genome diversity. Thus, stability as well as flexibility of a chromosome are detectable. Whole-genome analysis will not indicate correlations only if the property of interest is encoded by an extrachromosomal element or a limited number of genes dissipated on the chromosome. Intragenic recombination within genes of interest could also be responsible for a lack of correlation. The absence of a connection between esterase electrophoretic pattern and *rrn* restriction fragment length polymorphism within over 250 *P. aeruginosa* strains was demonstrated recently (8).

We characterized more than 500 *P. aeruginosa* isolates by their macrorestriction fingerprints. Many different patterns, indicating the extensive versatility of the species, were seen. Isolates of *P. aeruginosa* with identical fragment patterns were regarded as clones. When two patterns differed in up to six bands from each other, they were regarded as clonal variants. This definition of relatedness refers to the overall similarity in genomic organization and was corroborated by Southern blot hybridization of *Spe*I digests with eight gene probes (*algR*, *alg60*, lipase, exotoxin A, pilin, origin of replication, 23S RNA, and the whole operon of genes coding for rRNA [whole rDNA operon]) evenly distributed on the *P. aeruginosa* PAO genome map (22). The probes almost always recognized bands with identical molecular weights if the *Spe*I fingerprints were similar, and the few band shifts in the autoradiogram corresponded to the low, but existing, variability of the fragment pattern (Fig. 1b and Fig. 2).

Strains with more than six *Spe*I band differences were never found to be clonally related. In cases in which fragment size distributions of two fingerprints were similar overall but not identical (Fig. 3), Southern analysis showed that most bands of similar size were unrelated.

An example of clonal variants is shown in Fig. 1a and 2. The so-called clone C had been isolated from three different habitats. The corresponding hybridization pattern is shown in Fig. 1b. All probes but exotoxin A, lipase, and some rDNA probes detected fragments of the same size in the various isolates. Similarity between strains was further confirmed by identical pyocin type and serotype (Table 1). The phage lysis patterns were similar in isolates of non-CF origin. The CF isolates were not informative because *P. aeruginosa* strains gradually lose their susceptibility to phages during their colonization of the lungs of CF patients (19).

In addition to the 420 isolates from the CF patients from the Hannover clinic, we examined three panels of 10 isolates from randomly chosen CF patients from CF clinics in Copenhagen, Halle, and Magdeburg. Isolates with identical fragment patterns were identified within each set from the same location: one pair in Halle, two pairs in Magdeburg, and two pairs out of six typeable strains in Copenhagen. Four strains from Copenhagen were not typeable because of an endogenous nuclease. These data confirm the earlier findings in our CF clinic that unrelated CF patients may harbor clonal variants of the same strain. Members of a clone were isolated exclusively from the same clinic but not from any of the other three CF clinics, and correspondingly we conclude that a strong association of a particular clone with CF airway disease does not seem to exist. The geographic or political separation of the four clinics and different living conditions of the different areas may explain the prevalence of different clones at each clinic. Copenhagen is located in another country altogether, and Halle and Magde-

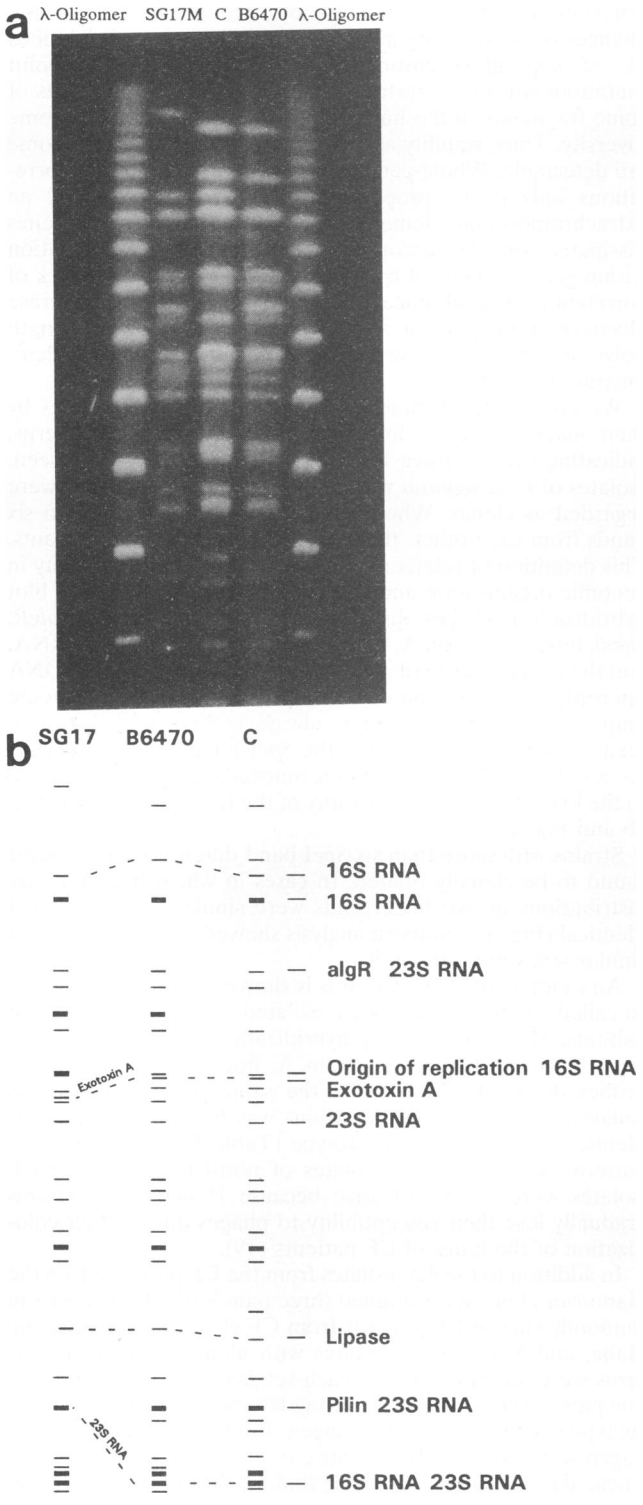

FIG. 1. (a) Clonal variants of strain C from different habitats. Fragments of *Spe*I-digested chromosomes were separated by using a CHEF DRII pulsed-field apparatus. SG17M is an environmental isolate from a river, C is a typical CF isolate (Hannover), and B6470 is an isolate from an ear infection (Heidelberg). Electrophoresis conditions: 200 V; cooling temperature, 10°C; buffer, 0.5× TBE; agarose concentration, 1.5%; two linear ramps, 5 to 30 s for 18 h and 5 to 70 s for 20 h at an angle of 120°. (b) Hybridization signals of various gene probes; the hybridization signals for clone C are marked. Dotted lines between the fragment patterns indicate changes in fragment detection by a probe in either of the other clonal variants.

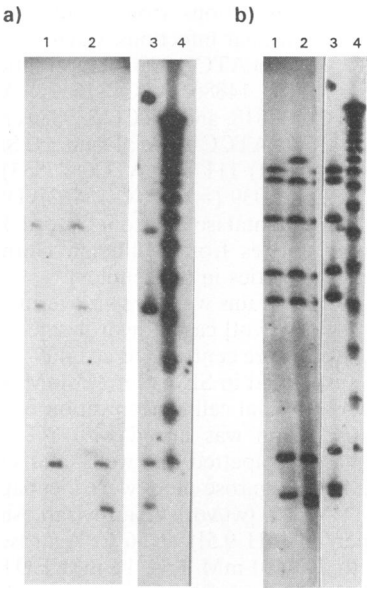

FIG. 2. Southern blot analysis of clonal variants. (a) Probing with pHF360 (part of the 23S RNA of *P. aeruginosa* [21]); (b) probing with pPA10 (contains whole rDNA operon of *P. aeruginosa* beside the first 810 bases of the 16S RNA [21]). Lanes: 1, SG17M (environmental isolate from Mülheim); 2, B6470 (ear infection isolate from Heidelberg); 3, clone C (CF isolate from Hannover); 4, λ-oligomer standard. Electrophoresis conditions are described in Materials and Methods.

burg are located in a part of Germany that belonged to the former German Democratic Republic, which until 1989 severely restricted the travel of its residents to West Germany.

Nosocomial spread was not limited to the CF clinics. Strains with identical fragment patterns were also detected in an intensive care unit in Tel-Hashomer. Three of the six available blood culture isolates showed identical *Spe*I fingerprints. In the Heidelberg clinic, two ear infection and four tracheobronchitis and wound isolates of the 15 strains of the panel had identical patterns.

To trace nosocomial spread in our CF clinic, we collected strains from sinks, tubs, and toilets in the outpatient clinic and in the ward during a 2-month study period. The two clones C and M were found in patients as well as in the moist habitats of the hospital settings (Table 1; Fig. 4). The detection rate of clone M in the clinic environment was not associated with its incidence in patients, probably because clone M was introduced into the clinic habitat only very recently and the chance for nosocomial transmission was probably low. The predominant clone C, however, was found at about the same frequency in the clinic environment as in the patients of our clinic.

We next compared strains from patients with different diseases. The Hannover CF strains were compared with four burn wound isolates from Hannover, six blood culture strains from Tel-Hashomer, and 15 isolates of various origins from Heidelberg. No close similarity was detected between the CF strains from the burn wound isolates from the same hospital and the blood culture strains from Tel-Hashomer. The two CF clones K and C, however, were also found in the strain collection from Heidelberg (Table 1; Fig. 1a and 4). Strain K

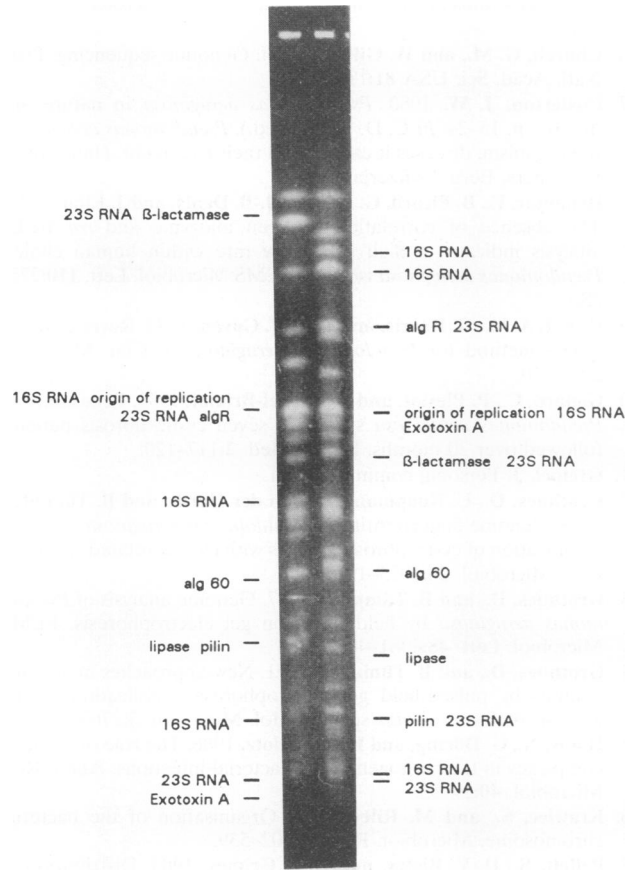

FIG. 3. Strain ATCC 33351 (left) and clone C of CF patients (right). These two strains are not regarded as clonal variants. Fragments of SpeI-digested chromosomes were separated by using a CHEF DRII apparatus. The fragments detected by various gene probes are indicated. Electrophoresis conditions are described in Material and Methods except that 0.5× TBE was used instead of 0.25× TBE.

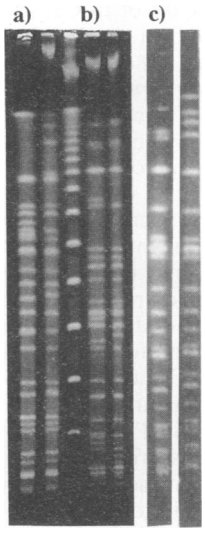

FIG. 4. Clonal variants J, K, and M of strains from different habitats. (a) Clone J. Lanes: left, CF patient isolate (Hannover); right, environmental isolate (Mülheim). (b) Clone K. Lanes: left, CF patient isolate (Hannover); right, wound infection isolate (Heidelberg). (c) Clone M. Lanes: left, CF patient isolate (Hannover); right, clinical environment isolate (Hannover). Electrophoresis conditions are described in Material and Methods.

was isolated from a wound infection, and strain C was isolated from a middle ear infection. Thus, CF clones were not unique for the disease but were also found in association with other illnesses.

We then included environmental *P. aeruginosa* strains that were unrelated to hospital settings or any disease in the comparative analysis of genome fingerprints to test for the putative selection of clones in clinical habitats. ATCC environmental strains showed no similarity to other isolates, but the strain collection from moist and aquatic habitats in Germany contained variants of the CF clones C and J (Table 1; Fig. 1a and 4). These two clones are the two most frequently occurring clones in the Hannover CF patients. Our major clone C constituted 28% of CF isolates and was detected at the similar frequency of 21% in the environmental isolates. It was found in a river, drinking water, the water of a swimming pool, the drain in a private household, and the bench of a tap in an abattoir. Thus, this clone seems to be a typical water organism. The other CF clone, J (Fig. 4), was detected in the airways of 4 of 46 analyzed patients and also in an intake into a stretch of standing water. The environmental strains had been isolated by different persons from various aquatic habitats 300 to 400 km west of Hannover.

In conclusion, the macrorestriction analysis of *P. aeruginosa* strains representing various bio- and pathovars revealed that almost all strains had different *Spe*I fingerprints. This variability of the overall genomic structure reflects the well-known versatility of the species. However, three strains with identical or very similar chromosomal architecture were isolated more than once. Thus, this study shows for the first time that clones of *P. aeruginosa* are prevalent in a broad geographic area. No strong association of a clone with a particular bio- or pathovar was observed, which is in contrast to findings in human pathogens like *E. coli*, *N. meningitidis*, or *Listeria monocyto-*

TABLE 1. Frequency and characteristics of *P. aeruginosa* clones found in different habitats

Source of isolates	% Clone				Pyocin type	Phage type(s)	Serotype
	C	K	J	M			
Hannover CF patients	28 (13/46)[a]	2 (1/46)	9 (4/46)	2 (1/46)	Of	Variable	1 or pa
Hannover clinic environment	17 (5/29)[b]			35 (10/29)	Of	PS7, PS21, (PS68); PS7, PS21, or no answer	ND[c]
Heidelberg patients	7 (1/15)[a]	7 (1/15)			Of	PS7, PS21, (1214)	1
Mülheim nonclinic environment	21 (8/38)[b]		3 (1/38)		Of	PS7, PS21, 1214	1

[a] Values in parentheses indicate number of patients with indicated clone/number of patients tested.
[b] Values in parentheses indicate number of isolates of clone indicated/total number of isolates.
[c] ND, not determined.

genes (23); e.g., outbreaks of listeriosis in various geographic areas had been caused by a clone with the same macrorestriction fingerprint pattern (5). There are also several reports of outbreak studies of *P. aeruginosa* (4, 10, 12). However, no intensive comparison of strains between different centers were made. The use of a common restriction enzyme, common separation conditions, and/or exchange of major strains seems to be useful.

The most frequently identified *P. aeruginosa* clone in our study was the major clone in CF patients (28%), and it was also detected at high frequency (21%) in the aquatic environment from various geographic areas. Because of the almost equal prevalence of this clone in diseased patients and the inanimate environment, no selection for the lungs of CF patients can be ascribed to the clone. Clone C was also isolated from an ear infection, which demonstrates that the pathogenicity of this clone is not restricted to CF. In addition to the most abundant clone C, the second most frequent clone in CF patients, clone J, was also isolated from the aquatic environment.

The finding of similar clones in various biovars shows that these clones must be widely distributed in the environment. A common recent origin of these strains or a stable genomic organization which is maintained in different habitats may be responsible for this phenomenon. This study shows that potentially infectious *P. aeruginosa* strains could be found almost everywhere. Whenever infections are traced, careful examination of the patient environment is necessary and should not be restricted to hospital settings. Potential reservoirs outside of clinics might play a role, which to date has not been adequately addressed.

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