Environmental gasoline-utilizing isolates and clinical isolates of *Pseudomonas aeruginosa* are taxonomically indistinguishable by chemotaxonomic and molecular techniques

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A total of 42 *Pseudomonas aeruginosa* strains was isolated previously from clinical sources (27 strains) and from a gasoline-contaminated aquifer (15 strains). Selected strains were subjected to taxonomic tests involving chemical and molecular biological techniques, including membrane fatty acid analysis, phage-sensitivity, growth temperature range, presence of plasmids, and PCR-amplification and sequencing of a species-specific 16S–23S rDNA internal transcribed spacer region. The clinical and environmental isolates formed a coherent taxonomic group with few distinguishing characteristics. Of the phenotypes observed, a consistent difference was the ability of the aquifer strains to utilize gasoline supplied in the gas phase as sole carbon source and, conversely, the inability of the clinical strains to do so. Fourteen of the 15 environmental strains possessed similar-sized cryptic plasmids. The clinical isolates either lacked detectable plasmids or contained plasmids of a different size. The observation that the clinical and environmental isolates of *P. aeruginosa* were taxonomically indistinguishable is discussed in terms of its relevance to environmental-regulatory guidelines because *P. aeruginosa*, a known opportunistic pathogen, is a prime candidate for use in bioremediation processes involving deliberate release of this organism to the environment.

Keywords: *Pseudomonas aeruginosa*, molecular taxonomy, environment, opportunistic pathogen, bioremediation

INTRODUCTION

Interest in the microbiological relationships of the aerobic pseudomonads during the 20th century has been driven by the recognition that several species of this group are associated with human infections. Relatively recently, however, it has been recognized that *Pseudomonas* species are important in the biodegradation of natural and anthropogenic toxic chemicals (Golovleva *et al.*, 1992). This, together with their demonstrated interactions with plants, beneficial and deleterious, has stimulated and broadened research into this group of organisms (Holloway, 1992). The potential benefits and risks of the environmental use and release of *Pseudomonas* species have been evaluated (Lindow, 1992).

Abbreviations: FAME, fatty acid methyl ester; LCDC, Laboratory Centre for Disease Control.

The difficulties of resolving the taxonomy of this genus have been reviewed (Palleroni, 1991, 1992). The latter review, dealing with RNA homology and phenotypic characteristics, describes the properties of the principal species of *Pseudomonas sensu stricto* (i.e. RNA Group I), which has been placed in the γ -branch of the Proteobacteria. Within *Pseudomonas sensu stricto*, three clusters are recognized: one containing species which are fluorescent and oxidase-positive, including *P. aeruginosa*, *P. putida* and *P. fluorescens*; a cluster of fluorescent phytopathogenic species including *P. syringae*; and a non-fluorescent cluster including *P. mendocina* and *P. stutzeri*. The latter two species are common soil inhabitants isolated under denitrification conditions and are rarely isolated from clinical sources. Some strains of *P. mendocina*, however, do produce alginate, a property associated with pseudomonads recovered from cystic fibrosis patients (Palleroni, 1991). Other RNA homology groups have been assigned

to new genera: Group II – *Burkholderia*; Group III – *Hydrogenophaga*, *Comamonas* and *Acidovorax*; Group IV – placed in the α -branch of the Proteobacteria; and Group V – *Xanthomonas* (Palleroni, 1993).

Our interest in the role of *Pseudomonas* species in hydrocarbon degradation arose from studies investigating the effects of crude oil composition and incubation temperature on the susceptibility of different crude oils to biodegradation (Westlake *et al.*, 1974). The inoculum used was derived from a soil sample which had been soaked over a period of years with diesel fuel. *Pseudomonas* species, identified on the basis of phenotypic characteristics and the production of fluorescent pigment, were almost always present in enrichments, with their incidence ranging from <10% to >40% of the bacterial population recovered by cultural techniques. Similarly, crude oil-degrading bacteria in marine water column and sediment samples obtained from the Straits of Juan de Fuca (Washington State, USA) were surveyed (Westlake & Cook, 1978), and sites which had not been impacted previously by spilled hydrocarbons usually yielded oil-degrading populations rich in *Pseudomonas* species. Subsequent investigations of such oil-degrading *Pseudomonas* species have focused on the types of hydrocarbons used for their growth (Foght & Westlake, 1988), and their molecular biological relationships (Foght & Westlake, 1991).

Ridgway *et al.* (1990) used conventional phenotypic methods, hydrocarbon-degrading capabilities, and analysis of protein banding patterns to show that 86.9% of 244 identified bacterial types isolated from a shallow coastal aquifer contaminated with unleaded gasoline (petrol) were *Pseudomonas* species. Eighty-nine isolates identified as *P. aeruginosa* were divided into 16 groups forming two distinct clusters based on whole-cell protein banding patterns. The hydrocarbon utilization of both clusters of *P. aeruginosa* isolates was restricted to low-molecular-mass aromatic components such as *p*-xylene, ethyl benzene and naphthalene, although one group in each cluster also grew on selected alkanes such as *n*-hexane and methylcyclopentane.

Analysis of the bacterial isolates present before and during bioremediation of this gasoline-contaminated aquifer used RFLP of genomic DNA in addition to protein banding patterns (Hook *et al.*, 1992). Hydrocarbon-degrading abilities appeared predominantly in *P. aeruginosa* strains pre-bioremediation, whereas hydrocarbon-degrading strains isolated during active bioremediation were predominantly *P. mendocina*, with a smaller number of *P. aeruginosa* isolates.

The presence of *P. aeruginosa* and *P. mendocina* in bioremediation flora raises regulatory concerns because the former species is a frequent opportunistic pathogen in nosocomial and pulmonary infections in cystic fibrosis patients (Goldberg, 1992), and the latter species recently has been associated with human disease (Aragone *et al.*, 1992). In addition, Rahme *et al.* (1995) have reported the pathogenic versatility of a *P. aeruginosa* strain which demonstrated infectivity in both plant and animal models.

While conventional taxonomy may produce ambiguous results, the additional data provided by molecular and chemotaxonomic tests can be used unequivocally to assign isolates to or exclude isolates as *P. aeruginosa*. In this paper we report taxonomic data comparing *P. aeruginosa* obtained from clinical sources and a gasoline-contaminated aquifer (Ridgway *et al.*, 1990) which provide evidence that *P. aeruginosa* strains isolated from these two sources are indistinguishable by current molecular biological techniques.

METHODS

Bacterial isolates and chemotaxonomic identification. The *P. aeruginosa* strains used in this study and their sources of isolation are shown in Table 1. A total of 27 clinical strains isolated from patients at different times and from geographically diverse sources between 1971 and 1993 were examined. Twenty strains were selected from archival collections at the Laboratory Centre for Disease Control (LCDC), Ottawa, and seven from the University of Alberta, Edmonton (generous gifts of W. Paranchych, Department of Biological Sciences). Fifteen environmental *P. aeruginosa* strains isolated from different sample wells on different dates from a gasoline-contaminated aquifer in Orange County California (Ridgway *et al.*, 1990) were selected for characterization. Reference strains of *P. aeruginosa* included ATCC 17933, 19660 and 27853.

The environmental strains were previously identified using conventional biochemical methods (Ridgway *et al.*, 1990), DNA cluster analysis and protein banding patterns (Hook *et al.*, 1992). The University of Alberta clinical isolates had been previously subjected to phenotypic tests and serological typing (Speert *et al.*, 1989). The LCDC clinical isolates were identified as *P. aeruginosa* using analysis of cell-membrane lipids by standard fatty acid methyl ester (FAME) techniques (Rolla & Lehtonen, 1988). In the current study, environmental strains 28–37, inclusive, were subjected to FAME analysis by the same methods. Cells were cultured, saponified, methylated and analysed by GLC using the Microbial Identification System and Library Generation System software (Microbial ID, Inc.). Results were compared to the reference *P. aeruginosa* strains (ATCC 17933, 19660 and 27853) in the database.

Three environmental isolates (strains 38, 41 and 42) and three clinical isolates (strains 9, 24 and 25) were tested in this study for the presence of pili using standard sensitivity tests with four pili-specific bacteriophage (Pf1, PO4, B9 and F116; Farinha *et al.*, 1994), with *P. aeruginosa* strain PAK serving as a positive control.

Two commercially available hydrocarbon-degrading isolates, *P. putida* NCIB 9816 (pWW60) and (pWW61) (Yen & Serdar, 1988) and *P. putida* ATCC 33015 [TOL(pWW0)], were used as sources of reference plasmids for hybridization and as molecular mass markers.

Cultural conditions. Clinical strains were revived from lyophilized stocks and grown at 37 °C in Tryptic Soy Broth (TSB; Difco) or on Plate Count Agar (Difco). The environmental strains were maintained on HCMM2 agar (Ridgway *et al.*, 1990), prepared with Noble Agar (Difco) and incubated at 22 °C in a sealed chamber containing gasoline vapour.

To test the ability of isolates to use gasoline vapours as sole carbon and energy source, duplicate HCMM2 agar plates were inoculated by streaking: one plate was incubated in the chamber with gasoline vapours while the duplicate was incubated in a

Table 1. Sources of *P. aeruginosa* isolates

Isolates were from: AB, Alberta; BC, British Columbia; MN, Manitoba; NB, New Brunswick; NS, Nova Scotia; ON, Ontario; and PQ, Province of Quebec, in Canada; and OH, Ohio, in the United States. For environmental isolates, the year of isolation is given. Repository institution: LCDC, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada; UA, University of Alberta, Department of Biological Sciences, Edmonton, Alberta, Canada; OCWD, Orange County Water District, CA, USA.

Isolate no.	Clinical isolates	Institution
1	Burn patient (BC)	LCDC
2	Urine (PQ)	LCDC
3	Ankle ulcer (AB)	LCDC
4	Bronchial secretion (PQ)	LCDC
5	Peritoneal fluid (PQ)	LCDC
6	Sputum (NB)	LCDC
7	Urine (PQ)	LCDC
8	Throat (BC)	LCDC
9	Cystic fibrosis (ON)	LCDC
10	Bottled water (MA)	LCDC
11	Cystic fibrosis (BC)	LCDC
12	Cystic fibrosis (BC)	LCDC
13	Cystic fibrosis (BC)	LCDC
14	Cystic fibrosis (ON)	LCDC
15	Cystic fibrosis (ON)	LCDC
16	Cystic fibrosis (ON)	LCDC
17	Cystic fibrosis (NS)	LCDC
18	Cystic fibrosis (NS)	LCDC
19	Cystic fibrosis (NS)	LCDC
20	Cystic fibrosis (NS)	LCDC
21	Burn (OH)	UA
22	Cystic fibrosis (AB)	UA
23	Burn (BC)	UA
24	Cystic fibrosis (AB)	UA
25	Burn (BC)	UA
26	Burn (BC)	UA
27	Cystic fibrosis (AB)	UA
Environmental isolates		
28	Aquifer, well no. 8, 1987	OCWD
29	Aquifer, well no. 8, 1987	OCWD
30	Aquifer, well no. 8, 1987	OCWD
31	Aquifer, well no. 8, 1987	OCWD
32	Aquifer, well no. 8, 1987	OCWD
33	Aquifer, well no. 8, 1987	OCWD
34	Aquifer, well no. 8, 1987	OCWD
35	Aquifer, well no. 8, 1987	OCWD
36	Aquifer, well no. 2, 1987	OCWD
37	Aquifer, well no. 30, 1986	OCWD
38	Aquifer, well no. 2, 1987	OCWD
39	Aquifer, well no. 30, 1986	OCWD
40	Aquifer, well no. 8, 1987	OCWD
41	Aquifer, well no. 8, 1987	OCWD
42	Aquifer, well no. 8, 1987	OCWD

sealed plastic bag in another laboratory, as a 'no added carbon' control. Growth was assessed after 3 and 7 d incubation at 22 °C by observing colonial growth on the plates incubated with gasoline vapours. The corresponding control plates incubated without any added carbon showed no growth in any trials.

For temperature-range testing, selected isolates (strains 22–25, 28, 34, 37, 41 and 42) were inoculated into TSB and incubated at 4, 10, 22, 30, 37 and 42 °C with aeration. Growth was assessed at intervals for a suitable incubation period (from 18 h to 14 d) by visually inspecting the turbidity of the medium.

Nucleic acid isolation and PCR amplification. Total nucleic acids were isolated from strains 1–20 and strains 28–37, inclusive, using SDS lysis followed by phenol/chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). Aliquots of 100 ng total nucleic acids from each strain were used as the template in 50 µl PCR mixtures containing 200 µM each dNTP, 1 µM for each of primer PA1 and PA2 (PA1: 5'-TCC AAA CAA TCG TCG AAA GC-3', and PA2: 5'-CCG AAA ATT CGC GCT TGA AC-3'; Tyler *et al.*, 1995), 2.5 U *Taq* polymerase and 1× reaction buffer supplied by the manufacturer with 1.5 mM MgCl₂. PCR was performed in a PE 9600 (Perkin Elmer Cetus) thermocycler using an initial denaturation of 2 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C. Following amplification, samples were incubated at 72 °C for 10 min and then cooled to 4 °C. PCR mixtures were analysed by electrophoresis in 2% (w/v) agarose and stained with ethidium bromide (Sambrook *et al.*, 1989).

Sequence-based identification of environmental isolates 28–37 inclusive was performed by PCR amplification of a region of the 16S rRNA gene with the eubacterial broad-range 16S rRNA primers designated p13B/p93E (Relman *et al.*, 1990). The 5' and 3' ends of these primers correspond to positions 1390–1371 and 930–950, respectively, of the *Escherichia coli* 16S rRNA sequence. Amplified DNA products (both strands) were subsequently sequenced directly using an automated Applied Biosystems 373A DNA sequencer and standard dye terminator chemistry. Analysis of the sequences was carried out using PC/GENE release 6.6 (Intelligenetics). Alignment was performed using the CLUSTAL program and the *P. aeruginosa* 16S rRNA GenBank sequence PARRSAAED (Toschka *et al.*, 1988).

Plasmid isolation. Clinical isolates were grown overnight at 37 °C in TSB, and 0.5–1 ml culture was used for plasmid isolation. Plasmids were isolated from aquifer-derived strains either after growth in TSB or after growth for 2 or 3 d on HCM2 agar with gasoline vapours. Colonies were washed from the agar surface with 1–2 ml 3 mM phosphate buffer (pH 8), and 0.5–1 ml suspension was used for plasmid isolation.

A modified procedure based on the method of Kieser (1984) was used to isolate plasmids, by adding 500 µl 5 M potassium acetate solution immediately before the acid phenol/chloroform solution step. This method has been used successfully to isolate plasmids ranging from <4 kb to >115 kb from a variety of genera (Foght & Westlake, 1991). Plasmids were separated using horizontal gel electrophoresis in 0.7% agarose (UltraPurc, Gibco BRL) in Tris/EDTA-acetate buffer (Sambrook *et al.*, 1989). Restriction endonuclease digestions were performed according to the manufacturers' instructions.

The archetypal toluene-degradative plasmid pWW0 (TOL; Burlage *et al.*, 1989) was isolated from *P. putida* ATCC 33015 and labelled with non-radioactive digoxigenin-dUTP according to the manufacturer's instructions (Boehringer Mannheim). This plasmid was used to probe a Southern blot composed of the degradative reference plasmids plus cryptic plasmids from

selected clinical and environmental strains. Overnight hybridization at 65 °C was followed by washes in 0.5 × SSC (1 × SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate; Sambrook *et al.*, 1989) at 65 °C.

RESULTS

All clinical and environmental isolates used in this study were previously identified as *P. aeruginosa* using standard biochemical tests and traditional taxonomic identification procedures. In this study, the 20 isolates from the LCDC and 10 of the environmental isolates (strains 28–37) were confirmed as *P. aeruginosa* strains by molecular biological and chemotaxonomic techniques, namely: PCR amplification of *P. aeruginosa*-specific regions of the 16S–23S rDNA internal transcribed spacer (Tyler *et al.*, 1995); sequence analysis of a region of the 16S rRNA gene amplified by PCR (Relman *et al.*, 1990); and FAME analysis of cell membrane fatty acids. Additional conventional tests included growth on gasoline vapours as sole carbon source, the presence of plasmids (Table 2), and growth temperature range.

Molecular taxonomy

PCR amplification of the 16S–23S rDNA internal transcribed spacer region with primers PA1 and PA2 generated a 181 bp DNA fragment from environmental isolates 28–37 inclusive, and from each of the 20 LCDC clinical strains. Primers PA1 and PA2 target a species-specific region of the 16S–23S internal transcribed spacer of the ribosomal genes in *P. aeruginosa* ATCC 19660, as documented by Tyler *et al.* (1995) with 220 clinical isolates and 24 ATCC reference strains of *Pseudomonas*. The *P. aeruginosa*-specific 16S–23S spacer region sequences have been assigned GenBank accession numbers L28148 (Paer1) and L28149 (Paer2) and are described in more detail by Tyler *et al.* (1995).

After PCR amplification using the eubacterial broad-range primers p13B/p93E (Relman *et al.*, 1990), partial 16S rRNA gene sequences of 402–410 bases in length were determined for the 20 LCDC strains, environmental strains 28–37 inclusive, and reference strain *P. aeruginosa* ATCC 17933. The sequences were aligned with the GenBank *P. aeruginosa* sequence (PARRSAAED; Toschka *et al.*, 1988). The amplified sequences from the environmental isolates were identical to the reference *P. aeruginosa* sequences at all but 2 of >400 nucleotides in the targeted region. Representative results shown in Fig. 1 are from environmental strain 29; the other nine environmental strains yielded sequences identical to strain 29.

Conventional- and chemotaxonomy

Because the molecular taxonomic results confirmed that all isolates were *P. aeruginosa*, we looked for other characteristics to discriminate between environmental and clinical groups of strains. FAME analysis of cell membranes was also consistent with identification of all 20 LCDC isolates and the 10 environmental isolates tested as *P. aeruginosa*. Similarity indexes ranged from 0.66 to 0.93

against a database containing 152 *P. aeruginosa* isolates, including reference strains ATCC 17933, 19660 and 27853.

A range of standard physiological tests also failed to discriminate between the two groups of *P. aeruginosa* strains under study. The temperature ranges for growth of the gasoline-utilizing and clinical groups were found to be broadly similar. The exceptions were that none of the clinical strains tested grew well at 4 °C, whereas two of the environmental isolates grew well at 4 °C but less well at 42 °C. Some strains of both groups produced a green fluorescent pigment in TSB at 30 or 37 °C. In this study, none of the three representative environmental isolates (strains 38, 41 and 42) nor three clinical isolates (strains 9, 24 and 25) was sensitive to the four phage types tested (Pf1, PO4, B9 and F116).

Phenotypic and genotypic differences between clinical and environmental isolates

The most obvious distinguishing feature between the gasoline-utilizing and clinical groups of *P. aeruginosa* strains was that only those recovered from the gasoline-contaminated aquifers were capable of growing with gasoline vapours as the sole carbon and energy source (Table 2). Even with prolonged incubation, the clinical strains were incapable of utilizing gasoline.

Another difference between the two groups was the plasmid content of the strains. Fourteen of the 15 gasoline-utilizing *P. aeruginosa* strains contained similar-sized cryptic plasmids (Fig. 2) with an estimated size of 80 kb, even when the strains were isolated from different sampling wells (e.g. isolates 38 and 34 in lanes 1 and 2, Fig. 2). The aquifer plasmids had identical *Bam*HI restriction endonuclease digestion profiles, whereas the clinical strains' banding patterns were different (Fig. 3 and unpublished observations).

We questioned whether the sole significant difference between the clinical and gasoline-utilizing isolates was the presence of the distinctive plasmids described above. Degradation of toluene and *p*-xylene (present in gasoline) is encoded in several *Pseudomonas* species by an homologous family of plasmids typified by the archetypal TOL plasmid, pWW0 (Burlage *et al.*, 1989). Indeed, a gasoline-utilizing *P. putida* strain isolated from the same aquifer as the environmental strains described herein was found to harbour a TOL-like plasmid (Leddy *et al.*, 1995), and almost 75% of the original aquifer isolates utilized toluene as sole carbon and energy source (Ridgway *et al.*, 1990). A Southern hybridization experiment with plasmids from environmental strains 31–34 and clinical strains 1–10 showed that none had homology with the TOL plasmid pWW0 when used as a probe (results not shown). This result does not preclude the aquifer-derived plasmids from encoding aromatic hydrocarbon catabolism, but suggests that the environmental plasmids (and clinical plasmids) are distinct from the TOL plasmid pWW0.

Attempts to transfer the plasmids from environmental strains 38 and 42 to clinical strains 21 and 22 by

Table 2. Summary of taxonomic results

Results identical or equivalent to that obtained with reference *P. aeruginosa* are indicated by ✓. Significant growth on gasoline vapours as sole carbon source is indicated by +; – indicates no growth on gasoline.

Clinical isolate	Chemotaxonomic and molecular taxonomic tests				
	FAME	16S–23S spacer	Partial 16S rRNA sequence	Growth on gasoline	No. of plasmids observed
1	✓	✓	✓	–	0
2	✓	✓	✓	–	1
3	✓	✓	✓	–	2
4	✓	✓	✓	–	0
5	✓	✓	✓	–	1
6	✓	✓	✓	–	0
7	✓	✓	✓	–	1
8	✓	✓	✓	–	1
9	✓	✓	✓	–	0
10	✓	✓	✓	–	1
11	✓	✓	✓	–	NT
12	✓	✓	✓	–	NT
13	✓	✓	✓	–	NT
14	✓	✓	✓	–	NT
15	✓	✓	✓	–	NT
16	✓	✓	✓	–	NT
17	✓	✓	✓	–	NT
18	✓	✓	✓	–	NT
19	✓	✓	✓	–	NT
20	✓	✓	✓	–	NT
21	NT	NT	NT	–	0
22	NT	NT	NT	–	0
23	NT	NT	NT	–	1
24	NT	NT	NT	–	1
25	NT	NT	NT	–	1
26	NT	NT	NT	–	1
27	NT	NT	NT	–	1
Aquifer isolate					
28	✓	✓	✓	+	2
29	✓	✓	✓	+	1
30	✓	✓	✓	+	1
31	✓	✓	✓	+	1
32	✓	✓	✓	+	1
33	✓	✓	✓	+	1
34	✓	✓	✓	+	2
35	✓	✓	✓	+	1
36	✓	✓	✓	+	1
37	✓	✓	✓	+	1
38	NT	NT	NT	+	1
39	NT	NT	NT	+	0
40	NT	NT	NT	+	1
41	NT	NT	NT	+	1
42	NT	NT	NT	+	1

NT, Not tested.

PARRSAAED	GCATGTGGTTTAATTCGAAGCAACGCGAAGACCTTACCTGGCCTTGACA	50
ATCC 17933	T.....	48
Isolate no. 29	-----	43
PARRSAAED	TGCTGAGAACTTTCCAGAGATGGATTTGGTGCCTTCGGGAATCAGACACA	100
ATCC 17933		98
Isolate no. 29		93
PARRSAAED	GGTCTGCATGGCTGTCTCAGCTCGTGTCTGTGAGATGTGGGTTAAGTC	150
ATCC 17933		148
Isolate no. 29		143
PARRSAAED	CCGTAACGAGCGCAACCCCTTGCTCCTTAGTTACCAGCACCTCGGGTGGGCA	200
ATCC 17933		198
Isolate no. 29		193
PARRSAAED	CTCTAAGGAGACTGCCGGTGACAAACCGAGGAAGGTGGGGATGACGTCA	250
ATCC 17933		248
Isolate no. 29		243
PARRSAAED	AGTCATCATGGCCCTTACGGCNAGGGCTACACACCTGCTACAATGGTCGG	300
ATCC 17933	 C.....	298
Isolate no. 29	 C.....	293
PARRSAAED	TACAAAGGGTTGCGAAGCCGCGAGGTGGAGCTAATCCCATAAACCGATC	350
ATCC 17933	 C.....	348
Isolate no. 29	 C.....	343
PARRSAAED	GTAGTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTA	400
ATCC 17933		398
Isolate no. 29		393
PARRSAAED	GTAATCGTGAATCAGAATGTCAAGGTGAATACGTTCCCGGGCCTT	445
ATCC 17933		418
Isolate no. 29		410

Fig. 1. Alignment of partial 16S rRNA sequences from *P. aeruginosa* ATCC 17933 and environmental isolate 29 with reference GenBank sequence PARRSAAED (Toschka *et al.*, 1988). Asterisks indicate identity. The other nine gasoline-utilizing isolates yielded sequences identical to that of isolate 29.

Fig. 2. Agarose gel showing plasmids isolated from environmental and clinical strains. Lanes: 1–4, gasoline-utilizing isolates 38, 34, 42 and 41, respectively; 5–9, clinical isolates 3, 8, 9 and 25, respectively; 5, circular plasmid markers: TOL (pVW0), 115 kb; NAH2 (pVW60), 87 kb; and pVW61, approximately 60 kb. chr, Chromosomal DNA.

electroporation, selecting for acquisition of the ability to utilize gasoline vapours, were unsuccessful. Thus, we do not yet know whether the aquifer-derived plasmids

Fig. 3. *Bam*HI restriction digests of isolated plasmids. Lanes: 1, lambda DNA digested with *Hind*III (linear marker); 2, clinical isolate 25; 3–5, gasoline-utilizing strains 38, 42 and 41, respectively.

actually encode gasoline-degradative genes or whether a clinical isolate can become a degradative isolate through acquisition of a catabolic plasmid. Similarly, it remains to be established whether the environmental *P. aeruginosa* isolates have pathogenic potential as opportunists in human and plant infections through acquisition and/or expression of virulence genes.

DISCUSSION

Although the *P. aeruginosa* strains isolated from aquifers were shown to have certain properties distinct from the clinical isolates (for example, the ability to grow on hydrocarbons and the presence of a common plasmid), they were indistinguishable from clinically isolated, known opportunistic pathogens at the level of molecular taxonomy.

It is likely that the taxonomic similarities to clinical *P. aeruginosa* strains are not limited to aquifer populations, since a recent study based on a genomic restriction digestion technique (Römling *et al.*, 1994) reported that the most frequently identified *P. aeruginosa* clone in cystic fibrosis patients (28%) was also detected at a relatively high frequency (21%) in aquatic environments from various geographic areas, including rivers, lakes and sanitary facilities. Römling *et al.* (1994) speculated on the likely widespread distribution of potentially pathogenic *P. aeruginosa* outside the clinic. Our results suggest that gasoline-contaminated environments harbouring degradative *P. aeruginosa* strains may be added to their list of reservoirs. The recent observation that at least some *P. aeruginosa* strains have pathogenic potential in both

animals and plants (Rahme *et al.*, 1995) emphasizes the need for caution when dealing with environmental *P. aeruginosa* isolates.

The observation that the two sources of *P. aeruginosa* yielded a biochemically and genetically coherent group of isolates suggests the importance of further investigation of the pathogenic potential of *P. aeruginosa* strains proposed for environmental release or *in situ* stimulation of hydrocarbon bioremediation (Jain *et al.*, 1992; Van Dyke *et al.*, 1993). Further studies are required to determine whether there are genetic or phenotypic markers useful for discriminating between strains for environmental monitoring of *P. aeruginosa* at bioremediation sites. Recent observations associating *P. mendocina* with human disease (Aragone *et al.*, 1992) also raise the same questions about environmental and clinical strains of this species.

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REFERENCES

- Aragone, M. R., Maurizi, D. M., Clara, L. O., Estrada, J. L. N. & Ascione, A. (1992). *Pseudomonas mendocina*, an environmental bacterium isolated from a patient with human infective endocarditis. *J Clin Microbiol* **30**, 1583–1584.
- Burlage, R. S., Hooper, S. W. & Saylor, G. S. (1989). The TOL (pWW0) plasmid. *Appl Environ Microbiol* **55**, 1323–1328.
- Farinha, M. A., Conway, B. D., Glaser, L. M. G., Ellert, N. W., Irvin, R. T., Sherburne, R. & Paranchych, W. (1994). Alteration of the pilin adhesin of *Pseudomonas aeruginosa* PAO results in normal pilus biogenesis but a loss of adherence to human pneumocyte cells and decreased virulence in mice. *Infect Immun* **62**, 4118–4123.
- Foght, J. M. & Westlake, D. W. S. (1988). Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. *Can J Microbiol* **34**, 1135–1141.
- Foght, J. M. & Westlake, D. W. S. (1991). Cross hybridization of plasmid and genomic DNA from aromatic and polycyclic aromatic hydrocarbon-degrading bacteria. *Can J Microbiol* **37**, 924–932.
- Goldberg, J. B. (1992). Regulation of alginate volume in *Pseudomonas aeruginosa*. In *Pseudomonas Molecular Biology and Biotechnology*, pp. 75–82. Edited by E. Galli, S. Silver & B. Witholt. Washington, DC: American Society for Microbiology.
- Golovleva, L. A., Maltseva, O. V. & Solyanikova, I. P. (1992). Metabolism of foreign compounds in *Pseudomonas* species. In *Pseudomonas Molecular Biology and Biotechnology*, pp. 231–238. Edited by E. Galli, S. Silver & B. Witholt. Washington, DC: American Society for Microbiology.
- Holloway, B. W. (1992). *Pseudomonas* in the late twentieth century. In *Pseudomonas Molecular Biology and Biotechnology*, pp. 1–8. Edited by E. Galli, S. Silver & B. Witholt. Washington, DC: American Society for Microbiology.
- Hook, L. A., Bogardt, A. H., Hemmingsen, B. B., Odelson, D. A., Safarik, J., Phipps, D., Carl, P., Clark, D. & Ridgway, H. F. (1992). Cluster analysis of DNA and protein binding patterns as a means of genotypic identification of indigenous hydrocarbon-degrading bacteria. In *Gas, Oil and Environmental Biotechnology IV*, pp. 153–172. Edited by C. Akin, R. Markuszewski & J. Smith. Chicago: Institute of Gas Technology.
- Jain, D. K., Lee, H. & Trevors, J. T. (1992). Effect of additions of *Pseudomonas aeruginosa* UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil. *J Ind Microbiol* **10**, 87–93.
- Kieser, T. (1984). Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. *Plasmid* **12**, 19–36.
- Leddy, M. B., Phipps, D. W. & Ridgway, H. F. (1995). Catabolite-mediated mutations in alternate toluene degradative pathways in *Pseudomonas putida*. *J Bacteriol* **177**, 4713–4720.
- Lindow, S. E. (1992). Environmental release of pseudomonads: potential benefits and risks. In *Pseudomonas Molecular Biology and Biotechnology*, pp. 399–407. Edited by E. Galli, S. Silver & B. Witholt. Washington, DC: American Society for Microbiology.
- Palleroni, N. J. (1991). Introduction to the Family Pseudomonadaceae. In *The Prokaryotes*, 2nd edn, pp. 3071–3085. Edited by A. Ballows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.
- Palleroni, N. J. (1992). Present situation of the taxonomy of aerobic pseudomonads. In *Pseudomonas Molecular Biology and Biotechnology*, pp. 105–115. Edited by E. Galli, S. Silver & B. Witholt. Washington, DC: American Society for Microbiology.
- Palleroni, N. J. (1993). *Pseudomonas* classification. *Antonie Leenwenhoek* **64**, 231–251.
- Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899–1902.
- Relman, D. A., Loutit, J. S., Schmidt, T. M., Falkow, S. & Tompkins, L. S. (1990). The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. *New Engl J Med* **323**, 1573–1580.
- Ridgway, H. F., Safarik, J., Phipps, D., Carl, P. & Clark, D. (1990). Identification and catabolic activity of well-derived gasoline-degrading bacteria from a contaminated aquifer. *Appl Environ Microbiol* **56**, 3565–3575.
- Rolla, E. E. & Lehtonen, O. P. (1988). Optimal data processing procedure for automatic bacterial identification by gas-liquid chromatography. *J Clin Microbiol* **26**, 1745–1753.
- Römling, U., Wingender, J., Müller, H. & Tümmler, B. (1994). A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Appl Environ Microbiol* **60**, 1734–1738.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Speert, D. P., Campbell, M. E., Farmer, S. W., Volpel, K., Joffe, A. M. & Paranchych, W. (1989). Use of a pilin gene probe to study the molecular epidemiology of *Pseudomonas aeruginosa*. *J Clin Microbiol* **27**, 2589–2593.
- Toschka, H. Y., Hoepfl, P., Ludwig, W., Schleifer, K. H., Ulbrich, N. & Erdman, V. A. (1988). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Pseudomonas aeruginosa*. *Nucleic Acids Res* **16**, 2348.
- Tyler, S. D., Strathdee, C. A., Rozee, K. R. & Johnson, W. M. (1995). Oligonucleotide primers designed to differentiate pathogenic pseudomonads on the basis of the sequencing of 16S–23S genes coding for RNA internal transcribed spacers. *Clin Diagn Lab Immunol* **2**, 448–453.
- Van Dyke, M. I., Gulley, S. L., Lee, H. & Trevors, J. T. (1993).

Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil. *J Ind Microbiol* **11**, 163–170.

Westlake, D. W. S. & Cook, F. D. (1978). *Microbial Degradation of Hydrocarbons*. Publication no. EPA-600/7-78-148. Washington, DC: Office of Energy Mines and Minerals, Office of Research Development, US Environmental Protection Agency.

Westlake, D. W. S., Jobson, A., Phillippe, R. & Cook, F. D. (1974).

Biodegradability and crude oil composition. *Can J Microbiol* **20**, 915–928.

Yen, K.-M. & Serdar, C. M. (1988). Genetics of naphthalene catabolism in pseudomonads. *CRC Crit Rev Microbiol* **15**, 247–268.

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