Genetic heterogeneity of *Pseudomonas aeruginosa* clinical isolates revealed by esterase electrophoretic polymorphism and restriction fragment length polymorphism of the ribosomal RNA gene region

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Summary. The intra-species differentiation of *Pseudomonas aeruginosa* was analysed by comparing the polymorphism of esterases by conventional polyacrylamide-agarose gel electrophoresis, the physicochemical properties of the variants of the major esterase P_3 and the restriction fragment length polymorphism of ribosomal RNA gene regions (ribotyping) to O-serotyping for several panels of strains selected from among a series of 257 clinical isolates and two references strains, (ATCC nos. 10145 and 27853). The electrophoretic variation of four main kinds of esterase (P_1 – P_4) and 11 additional esterases distinguished by their spectra of hydrolytic activity with synthetic substrates and by their sensitivity to di-isopropyl-fluorophosphate, allowed the discrimination of 67 zymotypes. Thirty-two esterase P_3 variants were characterised by their pI, electrophoretic mobilities and titration curve analyses. They were distributed into two groups which, by these molecular criteria, seem to be distantly related. Combination of the patterns resulting from *Hind*III, *Eco*RI and *Bcl*I restriction endonuclease digestions allowed the discrimination of 33 ribotypes among 134 strains. The strains exhibiting esterase P_3 variants of group 2 presented a distinct ribotype and belonged to serotype O12. They could constitute a distinct group within the species. For the majority of the strains, the absence of correlation between zymotype, ribotype and serotype argues for a high level of heterogeneity within *P. aeruginosa* and indicates that the parallel use of the first two methods represent a potential tool for epidemiological study.

Introduction

The epidemiological fingerprinting of many pathogenic bacterial species is studied routinely by conventional phenotypic tests. The conventional characters do not provide an adequate basis with which to evaluate the heterogeneity of the species and the genetic distance between strains. This distance can be estimated by enzyme electrophoretic polymorphism^{1,2}—however, it has been recognised that the use of a single electrophoretic technique reflects incompletely the allelic variation of enzymes. To improve the detection of cryptic enzyme variations, previously we have used polyacrylamide agarose gel electrophoresis and thin-layer iso-electric focusing (IEF) in parallel runs, and proposed a two-dimensional electrophoretic profile (2-DEP).³ We have also evaluated the mol-

ecular relationship between the enzyme electrophoretic variants by comparative titration curve.^{4–6} On the other hand, it has been established that the parallel use of molecular typing systems studying different classes of genetic variation improves the intra-species differentiation of bacteria. The analysis of DNA restriction fragment length polymorphism (RFLP) is very sensitive to small changes in DNA organisation.⁷ The study of variations in the ribosomal DNA (rDNA) gene regions (ribotyping)^{8–10} has the advantage that it examines several sequences of the genome that encode a function distinct from that of enzymes. Consequently, the combining of ribotyping and enzyme electrophoresis has provided useful information on the genetic relatedness of strains within a bacterial species.^{11–13}

During the last 20 years, the prevalence of *Pseudomonas aeruginosa*, a major opportunist pathogen, has increased in nosocomial infections. Precise epidemiological analyses are necessary for the prevention and control of these infections. The main epidemiological markers remain phenotypic, including API 20NE

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profile, O serotype, pyocin type, phage type, lysogenic state and antibiotic susceptibility. In earlier reports we proposed a typing method based upon the variation of four esterases, P_1 – P_4 , characterised by polyacrylamide agarose gel electrophoresis¹⁴ and used this method in comparison with RFLP or the rDNA gene region for epidemiological analysis of strains obtained from cystic fibrosis (CF) patients originating from a single hospital.¹⁵ The present work utilised a large panel of 257 *P. aeruginosa* clinical isolates obtained in various hospitals and from several sites of infection (and from CF and non-CF patients) and compared the results obtained by three molecular typing methods: esterase electrophoresis in polyacrylamide agarose gel; IEF of the electrophoretic variants of the main esterase P_3 with the evaluation of the molecular relationships between these variants by titration curves; and RFLP of rDNA.

Materials and methods

Bacterial strains

A total of 257 clinical *P. aeruginosa* strains was studied. They were collected from patients in nine hospitals located in eight cities in France (table I). All were isolated from pathological samples (urine, stool, blood, trachea or sputum) and were collected between 1984 and 1990. Fifty-six strains were from CF patients. Isolates were identified as *P. aeruginosa* based upon typical morphology, positive oxidase reaction, ability to produce pigments, growth at 42°C and biochemical tests (API-20 NE system; API, les Balmes, France). O serotyping was performed with O-antisera (Diagnostic Pasteur, Marne la Coquette, France). The strains belonged to 16 of the 17 serogroups of the international serotyping scheme.¹⁶ Two reference strains also studied were the type strain of the species, ATCC 10145 and strain ATCC 27853.

Esterase electrophoretic polymorphism

Bacteria were grown for 18 h at 37°C in broth¹⁷ with constant shaking and then harvested by centrifugation. Bacterial pellets were washed with 60 mM Tris-glycine buffer, pH 8.7, resuspended in the same buffer and disrupted by sonication for 18 min at 4°C. Crude extract supernates containing 40–60 mg of protein/ml, were stored at –20°C until used.¹⁸

Conventional electrophoresis

Horizontal slab gel electrophoresis was performed according to the method of Uriel¹⁹ in a composite polyacrylamide-agarose gel (7% and 1.4%, respectively) in a discontinuous Tris-glycine buffer, pH 8.7, at constant voltage (7 V/cm) until the bromophenol blue marker had run 13 cm. The relative mobility (M_F value) is the distance moved by the esterase band as a

percentage of the distance moved by the dye front. M_F values were compared by running bacterial extracts in contiguous tracks on the same gel.³

Iso-electric focusing

Thin-layer iso-electric focusing in polyacrylamide gels ($T = 5\%$, $C = 3\%$)²⁰ was performed at 4°C with carrier ampholytes of pH range 4–6.5 (Pharmalyte; Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Experiments were run at a constant power of 25 W for 2 h at 4°C. Under these conditions the samples reached their equilibrium position (pI position) after 90 min and a further condensation of the zone occurred after 30 min. The pH gradient was measured systematically for each gel after the elution of the carrier ampholytes from a zone where no sample was applied. Iso-electric points (pI) of esterases were determined by interpolation from this curve.

Titration curves by combined iso-electric focusing-electrophoresis

The technique employed was that described by Righetti *et al.*⁴ Briefly, as a first step, a pH gradient was formed extending from 3.5 to 10, the sample was then applied in a groove running all along the gradient and electrophoresis was performed perpendicular to this gradient. Sigmoidal pH-mobility curves approximating to titration curves were revealed by specific staining procedures for the esterases with α -naphthyl acetate as a substrate, as described below.

Esterase staining

Esterases were stained on the gel^{21,22} with the following specific substrates: α -naphthyl acetate, β -naphthyl acetate, α -naphthyl propionate, β -naphthyl propionate, α -naphthyl butyrate, β -naphthyl butyrate and indoxyl acetate (Sigma). The sensitivity of esterases to di-isopropylfluorophosphate (DFP) (10^{-3} M) was also tested.

RFLP of rDNA regions

Bacterial DNA was prepared as described previously.⁹ DNA (5 μ g) was digested with *Hae*III, *Hinf*I, *Taq*I, *Hind*III, *Eco*RI, and *Bcl*I restriction endonucleases (Boehringer Mannheim, Germany) according to the manufacturer's specifications and analysed by electrophoresis on submarine agarose 0.8% gels containing ethidium bromide. DNA fragment size-marker Raoul I (Appligene, Strasbourg, France) was used. Separated DNA restriction fragments were transferred to a nylon membrane (Gene Screen Plus, New England Nuclear Products, Boston, MA, USA) by the method of Southern.²³ Ribosomal 16+23 S RNA from *Escherichia coli* (Boehringer) was labelled by random oligopriming with a mixture of hexanucleo-

Table I. Geographical location of the 257 *P. aeruginosa* clinical isolates

Town	Hospital	Number of strain
Paris	Beaujon hospital	76
Paris	Robert Debré hospital	48
Compiègne	General hospital	9
Amiens	Regional hospital	5
Abbeville	General hospital	5
Rennes	Pontchaillon hospital	22
Lyon	E. Herriot hospital	44
Marseille	Salvator hospital	29
Tours	Bretonneau hospital	19

tides (Pharmacia) and cloned M-MLV reverse transcriptase (Bethesda Research Laboratory, Gaithersburg, MD, USA) in the presence of [32 P] dCTP (2' deoxycytidine-5'-triphosphate, 800 Ci/mmol; Amersham). Hybridisation, washing and autoradiographic procedures were as described previously.⁹

Reproducibility of the results

Electrophoretic relationships between the strains were established by numerous replicate runs comparing enzyme bands side by side on the same gel. The reproducibility of the results was confirmed in several ways. For pI values, all the samples coalesced as a single line after IEF when they were loaded at different positions on the gel, and samples remained in the same line with no additional bands being observed when IEF was continued for an additional 30-min period to condense the focused region. All values of both pI and M_F were tested by comparing each new strain with reference strains in all electrophoretic runs; 4–12 extracts of the same strain were tested and had the same pI in IEF and the same electrophoretic re-

lationship in CE. Reproducibility of the rDNA RFLP results was established by multiple runs comparing separated DNA fragments side by side on the same gel.

Results

Esterase electrophoretic polymorphism

Conventional electrophoresis. Among the 259 *P. aeruginosa* strains, four main kinds of esterase bands were identified by their activity toward the synthetic substrates and by their sensitivity or resistance to DFP (table II). They were numbered P_1 – P_4 in order of decreasing mobility toward the anode. Eleven additional esterase bands (AE) were observed which were present in some strains only.

Each kind of esterase showed a number of electrophoretic variants (table III). The genetic diversity coefficients²⁴ were 0.13, 0.72, 0.78 and 0.02 for esterases P_1 , P_2 , P_3 and P_4 , respectively. The polymorphic esterase P_3 showed eight mobility variants corresponding to electrophoretic steps from $M_F \approx 44$ to $M_F \approx 58$. Together, the combination of the electrophoretic variants of the main esterases and the presence of additional bands allowed the identification of 67 zymotypes among the 259 *P. aeruginosa* strains (table III). Some zymotypes were observed more frequently than others. Zymotype 29 was the most common and was found in 62 strains. Zymotypes 56, 8, 44, 51 and 34 were observed in 32, 22, 19, 14 and 13 strains respectively. Other zymotypes were observed in nine strains or less.

Iso-electric focusing. A panel of 74 strains was selected on the basis of the data obtained with the esterase electrophoretic typing, i.e., one or two strains from each zymotype, to study the esterase P_3 electrophoretic polymorphism further by iso-electric focusing

Table II. Characteristics of esterase bands of *P. aeruginosa*

Esterase	Substrates hydrolysed*							DFP (10^{-3} M)
	α NA	α NP	α NB	IA	β NA	β NP	β NB	
P_1	+	+	–	–	++	++	+	R
P_2	–	–	–	–	+	+	+	S
P_3	+++	+++	++	++	++	++	+	S
P_4	–	–	–	–	–	+	+	R
AE 1	+	+	–	+	+	+	–	R
AE 2	+	+	–	–	+	+	–	S
AE 3	+	+	–	–	–	–	–	S
AE 4	–	+	+	+	+	+	+	R
AE 5	+	+	–	+	++	++	++	S
AE 6	–	–	–	+	–	–	–	R
AE 7	+	+	+	–	+	+	+	S
AE 8	+	–	+	–	–	–	–	R
AE 9	+	–	–	–	–	–	–	R
AE 10	–	–	–	–	+	+	+	R
AE 11	–	–	–	–	–	–	+	S

α NA, α -naphthyl acetate; α NP, α -naphthyl propionate; α NB, α -naphthyl butyrate; IA, indoxyl acetate; β NA, β -naphthyl acetate; β NP, β -naphthyl propionate; β NB, β -naphthyl butyrate; R, resistant; S, sensitive; AE, additional esterase.

* Relative intensity of staining: +++ > ++ > +; undetected –.

Table III. Distribution of the 259 *P. aeruginosa* strains studied among the 67 zymotypes resulting from esterase electrophoresis

Zymotype	P ₁ (M _F)	P ₂ (M _F)	P ₃ (M _F)	P ₄ (M _F)	AE (M _F)	Number of strains	Serotype
1			44	26	60 (4)	1	13
2		63	44	26		1	12
3		65	44	26		1	12
4		66	44	26		2	12
5	63		44	26		1	12
6	66	52	46	26		1	9
7	65	55	46	26		1	11
8	66	55	46	26		22	1, 7, 8, 10, 11, 15
9	66	55	46	26	32 (6)	1	U
10	66	55	46	30		1	6
11		57	46	26	55 (5)	1	11
12	66	60	46	26		2	8, 10
13	66	60	46	30		1	U
14	66		46	26	60 (4)	2	4, U
15	66		46	26	55 (5)	1	11
16	66		46	30	55 (5)	1	3
17	66	55	48	26		3	9, 10, U
18	66	56	48	26		9	1, 4, 5, 11, U
19	66	57	48	26		1	10
20	65	60	48	26		1	U
21	66		50	26		6	3, 4, 9
22	63	54	50	26		1	5
23	66	54	50	26		2	1
24	66		50	26/55		1	1
25	66		50	26/60		1	U
26	66		50	26	52 (9)	1	3
27	66		50	26	54 (9)	1	6
28	66		50	26	56 (9)	1	U
29	66		52	26		62	1, 3, 6, 11, 12, 13, 14, U
30	66	35	52	26		2	12
31	66	48	52	26		5	5, 6
32	66	48	52	26	35 (1)	1	U
33	66	55	52	26		1	U
34	66	56	52	26		13	1, 3, 6, 9, 12, 16
35	66	56	52	26	35 (1)	1	10
36	66	56	52	26	35 (2)	2	5, 16
37	66	56	52	26	40 (2)	1	U
38	66	56	52	26	10 (8)	1	4
39	70	56	52	26/60		1	1
40		56	52	26		3	1, 3, 4
41	66	57	52	26		1	9
42	63		55	26	70 (7)	1	11
43	66	47	55	26		1	16
44	66		55	26		19	1, 3, 6, 10, 11, 16, U
45	66/42		55	26		1	U
46		35	55	26		1	7
47		56	55	26		1	16
48	66	50	55	26		1	4
49	66	56	55	26		2	1, 3
50	66	60	55	26		2	2, 6
51	66		56	26		14	3, 6, 11, 15, 16, U
52	66		56	26	32 (1)	1	6
53	66		56	26	35	1	U
54	66		56	26/52		1	15
55	66	50	56	26		3	1
56	66	52	56	26		32	1, 2, 3, 6, 8, 9, 16, U
57	66	52	56	26	35	1	U
58	66	52	56	26	35 (2)	1	6
59			56	26		1	U
60	66	53	56	26		1	1
61	66	60	56	26		4	11
62	66	52	58	26	35 (1)	3	1, 6, 16
63	66	60	58	26		1	11
64	66			26	48 (3)	1	15
65	66	55		26		1	1
66	66			26	52 (5)	1	6
67	66			26	56 (10) + 52 (11)	1	U

AE, additional esterase—the AE are numbered (in brackets) as in table II; U, unserotypable strain.

and titration curve analysis. On iso-electric focusing, 13 allelic esterase P₃ variants were detected with iso-electric points varying from pH 4.9 to pH 5.5.

Two-dimensional electrophoretic profile. By plotting mobility values *versus* pI for all the esterase P₃ variants, a two-dimensional profile (2-DEP)^{3,6} was obtained

Fig. 1. Two-dimensional electrophoretic profile of the 32 esterase P_3 electromorphs produced by the 74 *P. aeruginosa* strains. The groups of electromorph 1 and 2 are indicated (see results). The electromorphs used for the titration curve analyses are labeled 1–8.

(fig. 1). This representation allowed discrimination of 32 variants or electromorphs. These variants may be classified into two distinct electrophoretic groups. Group 1 was defined by mobilities ranging from $M_F \approx 46$ to $M_F \approx 57$ and by pI from 4.9 to 5.2 and encompassed 27 variants. Group 2 was defined by mobilities ranging from $M_F \approx 44$ to $M_F \approx 46$ and pI from 5.3 and 5.5 and encompassed five variants. The number of electromorphs detected by the 2-DEP analysis was at least two-fold higher than that obtained when M_F and pI were considered separately.

Titration curves. Crude bacterial extracts were submitted to electrophoresis perpendicularly to a pre-formed stable carrier ampholyte pH gradient in order to obtain mobility curves which were revealed by specific staining. Electromorphs 2 (pI 5.125, $M_F \approx 46$) and 5 (pI 5, $M_F \approx 52$) (fig. 2) gave a titration pattern in which titration curves were indistinguishable in the acidic part of the gradient but separated in the basic part. This pattern is suggestive of a single substitution where a neutral amino acid in the former electromorph is replaced by an acidic amino acid in the latter.⁴ Results were similar with variants 2 and 4, 4 and 6, 3 and 4, and 2 and 3, suggesting the same type of amino-acid substitution between these various electromorphs.

When electromorphs 4 (pI 5, $M_F \approx 51$) and 8 (pI 5.15, $M_F \approx 51$) were titrated together, the pattern was suggestive of single substitution of a neutral amino acid in variant 5 by a basic amino acid in variant 9.⁴ Titration curves of variant 1 (pI 5.5, $M_F \approx 44$, group 1) with variant 2 (group 2) could not be interpreted in

Fig. 2. Comparative titration curves of 1, electromorph 2; 2, electromorph 6 of esterase P_3 ; → indicates the groove in which the crude enzyme extracts were placed.

terms of simple substitution. However, the two curves exhibited patterns that could evoke gross molecular similarity between them.

RFLP of rDNA regions

RFLP of the rDNA regions was studied with a panel of 134 strains selected on the basis of the data

Fig. 3. A representative RFLP pattern of *P. aeruginosa* rDNA regions obtained by digestion with *Hind*III enzyme. Lanes 1 and 4, pattern c; 2, 3 and 5, pattern a; 6, pattern d.

obtained with the esterase electrophoretic typing, i.e., one or two strains of each zymotype plus 59 strains belonging to the most frequent zymotype 18. Among the six restriction endonucleases tested, *Hind*III, *Eco*RI and *Bcl*I were retained for the size and the number of fragments which were obtained. *Hind*III digestion produced four-to-six rDNA fragments from 0.5 to 37 kb in size (fig. 3). Their combination allowed recognition of nine rDNA RFLP patterns within the 134 *P. aeruginosa* strains (fig. 4A). *Eco*RI digestion produced two-to-four rDNA fragments ranging in size from 4.5 to 19 kb. Their combination allowed recognition of 11 rDNA RFLP patterns (fig. 4B). *Bcl*I digestion gave four-to-five rDNA fragments from 1.4 to 20 kb in size. Their combination indicated nine rDNA RFP patterns (fig. 4C).

A combination of patterns resulting from two or three digestions was used to define a ribotype. Thus, 20–25 distinct ribotypes were obtained with the combinations of two endonucleases, and 33 ribotypes were differentiated with the combination of three endonucleases (table IV). Some ribotypes were isolated more frequently than others. The most common ones

were ribotype B (found in 49 strains) and ribotype D (19 strains). Ribotypes A, C, E, G, I, L, M, N, P, S, T, U, V, X and Y encompassed two-to-six strains only. Sixteen ribotypes were represented by single isolates.

Comparison of esterase electrophoretic typing, ribotyping and serotyping

Esterase electrophoretic typing and serotyping did not appear to correlate, as within each of the serogroups, the strains exhibited various zymotypes, while several serogroups were found in each of the more frequently occurring zymotypes (table III). The same result was obtained when ribotyping and serotyping were compared. However, the strains of zymotypes 2–5 belonged to ribotype P and serogroup O12.

Combination of esterase typing and ribotyping allowed the delineation of 89 types among the 134 strains studied (table IV); each type was designated as the association of a given zymotype to a given ribotype. The most common type was type 29/B, found in 34 strains. The type strain of the species, ATCC 10145 exhibited type 29/A, which was less frequent. The

Fig. 4. RFLP rDNA patterns of *P. aeruginosa* strains: schematic representation of (A) *Hind*III restriction pattern, (B), *Eco*RI restriction pattern, (C), *Bcl*I restriction pattern (numbered as in table IV) of DNA from strains after hybridising to ³²P-labelled *E. coli* rDNA probe.

Table IV. Distribution of the 259 *P. aeruginosa* strains studied among the 33 ribotypes resulting from the combination of the RFLP rDNA profiles obtained with *Hind*III, *Eco*RI and *Bcl*I enzymes: the zymotypes exhibited by the strains are indicated

Ribotype	rDNA RFLP pattern			Zymotype	Number of strains
	<i>Hind</i> III	<i>Eco</i> RI	<i>Bcl</i> I		
A	a	a	a	29, 37, 65	3
B	a	b	a	6, 29, 31–35, 38, 52, 55–58, 60, 62, 67	49
C	c	b	c	29	2
D	b	a	a	8, 10, 12, 16, 23, 29, 39, 40, 44, 46, 47, 51, 56, 63	19
E	b	a	b	17–19	4
F	e	b	a	9, 29	1
G	c	c	a	26, 29, 30	4
H	a	b	f	29	1
I	d	b	a	21, 29, 48, 53, 61	6
J	d	d	a	27	1
K	f	e	a	22	1
L	b	b	a	1, 7, 14, 15, 29	6
M	d	b	b	24, 25, 34	3
N	d	b	e	28, 29	2
O	c	a	d	51	1
P	g	g	a	2–5	4
Q	c	a	a	20	1
R	a	b	b	29	1
S	h	b	a	29	2
T	d	a	a	29, 31, 43	3
U	a	c	a	36, 50	2
V	b	c	a	29, 45	4
W	c	h	h	49	1
X	c	f	a	29, 54; 56	3
Y	a	b	g	59, 61	2
Z	b	a	h	64	1
AA	b	f	i	29	1
AB	d	b	c	29	1
AC	d	i	b	29	1
AD	i	b	a	30	1
AE	b	j	b	13	1
AF	b	k	a	11	1
AG	d	c	a	41	1

The rDNA RFLP patterns are designated as in fig. 4.

distribution of the strains was identical among CF and non-CF patients.

Discussion

PAGE and IEF were complementary in their resolution of enzyme polymorphism and refined intra-species molecular relationships of *P. aeruginosa*. Indeed, the numbers of esterase P_3 variants detected by PAGE, IEF and on the 2-DEP were 8, 13 and 32, respectively. Results of this type have been observed previously for several bacterial enzymes.^{3,4} While analysis of the genetic structure of a bacterial population is generally based on the number of electrophoretically detected variants,^{2,25–27} the conjunction of findings obtained by 2-DEP and titration curves constitutes a novel approach to our understanding of the molecular relatedness of the various allozymes.⁶ For instance, the esterase P_3 variants belonging to groups 1 and 2 in fig. 1 were distantly related to each other in molecular terms as demonstrated by their titration curves, indicating a

phylogenetic divergence between these variants. In contrast, variants of group 1 appeared to be closely related. Similar conclusions were reached from the analysis of carboxylesterase B of *E. coli*⁶ and esterase β_a of *Providencia alcalifaciens*⁵. Interestingly, variants of group 2 were detected in a group of *P. aeruginosa* strains belonging to serogroup O12^{28,29} and originating from a single hospital. This divergence was confirmed by both esterase typing and ribotyping. Indeed, these strains exhibited specific zymotypes 2–5 and a distinct ribotype P corresponding to two very different rDNA RFLP patterns after *Eco*RI and *Hind*III digestions (table IV, fig. 4). Consequently, they could constitute a distinct group within the species. The level of intra-species molecular relatedness evaluated for esterase P_3 in terms of amino-acid substitution correlates with the divergence between the strains observed at the genotype level by ribotyping.

Electrophoretic typing of esterases within *P. aeruginosa* species represents a potential method for epidemiological analysis^{14,15} as 67 zymotypes were detected

among the 259 strains studied. This method is simple and rapid and might be used more extensively to type epidemiologically related strains, as for other nosocomial pathogens.^{30,31} The absence of correlation between zymotyping and ribotyping enhanced the discriminative power of each individual method in differentiating 89 types among the strains and indicates that the two methods could be used advantageously in parallel in a combined typing system—as was suggested previously.³² On the other hand, this system could be considered as a screening method before DNA-DNA hybridisation studies. The reference strain of the species, ATCC 10145, exhibited type 29/A, which was an infrequent type, while the 34 strains belonging to type 29/B appeared to be more representative of *P. aeruginosa*. Comparable results were previously obtained with the genus *Acinetobacter*.¹

Six endonucleases were tested in the study of rDNA RFLP. The *Hae*III digestion pattern visualised with ethidium bromide was composed of low molecular size fragments. This result is explained by the site of restriction of *Hae*III (GGCC), a four base pair cutter, and the G + C mol% of *P. aeruginosa* (67%) which is higher than that of many other bacteria (e.g., *E. coli* 50%; *Staphylococcus aureus* 34%). This illustrates the fact that before ribotyping, the restriction enzyme must be chosen from the GC mol% data. The number of ribotypes resulting from the use of three endonucleases³³ was higher than that obtained with two endonucleases (20–25). This fact indicates that several endonucleases must be used to enhance the discrimin-

ative power of ribotyping, as was demonstrated previously for the genera *Branhamella*³⁴ and *Yersinia*.¹³ On the other hand, some strains were found to be atypical with one endonuclease only, indicating that the genotypic diversity of *P. aeruginosa* species must be measured by the comparison of data obtained by several restriction enzymes.

The absence of correlation demonstrated between serotyping, esterase typing and ribotyping argues for a high level of heterogeneity within the species unlike *E. coli*, where relationships within bacterial clones were established by the same techniques.¹¹ Ribotyping and esterase typing appeared to be more discriminative than serotyping, as 33 and 67 types, respectively, were determined with the first two techniques and 16 with the third. On the other hand, O serotyping is of limited value for epidemiological studies of *P. aeruginosa* infection in CF patients, as many strains are either polyagglutinable, autoagglutinable or non-typable.^{15,33,35} The variation of serotype in strains exhibiting identical esterase type and ribotype could represent a subtle genetic change within these strains, such as phage conversion, gene duplication or phase variation.³⁶ Consequently, the use of molecular typing methods such as esterase typing, ribotyping, genome fingerprinting by field inversion gel electrophoresis³⁵ and RFLP in the exotoxin A³⁶ or pili gene regions^{33,37} appear to be more advantageous than the phenotypic systems for *P. aeruginosa* strain differentiation.

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References

- Picard B, Goulet P, Bouvet PJM, Decoux G, Denis JB. Characterization of bacterial genospecies by computer-assisted statistical analysis of enzyme electrophoretic data. *Electrophoresis* 1989; **10**: 680–685.
- Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* 1986; **51**: 873–884.
- Goulet P, Picard B. A two-dimensional electrophoretic profile for bacterial esterases. *Electrophoresis* 1985; **6**: 132–135.
- Righetti PG, Krishnamoorthy R, Gianazza E, Labie D. Protein titration curves by combined isoelectric focusing-electrophoresis with hemoglobin mutants as models. *J Chromatogr* 1978; **166**: 455–460.
- Goulet P, Picard B, Krishnamoorthy R. An evaluation of allozyme amino acid substitutions for the study of molecular relationships in *Providencia* strains. *Ann Inst Pasteur/Microbiol* 1988; **139**: 689–702.
- Picard B, Goulet P, Krishnamoorthy R. A novel approach to study of the structural basis of enzyme polymorphism. Analysis of carboxylesterase B of *Escherichia coli* as model. *Biochem J* 1987; **241**: 877–881.
- Nei M, Li W-H. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc Natl Acad Sci USA* 1979; **76**: 5269–5273.
- Grimont F, Grimont PAD. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann Inst Pasteur/Microbiol* 1986; **137B**: 165–175.
- Picard-Pasquier N, Ouagued M, Picard B, Goulet P, Krishnamoorthy R. A simple, sensitive method of analyzing bacterial ribosomal DNA polymorphism. *Electrophoresis* 1989; **10**: 186–189.
- Stull TL, LiPuma JJ, Edlind TD. A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. *J Infect Dis* 1988; **157**: 280–286.
- Arthur M, Arbeit RD, Kim C *et al.* Restriction fragment length polymorphisms among uropathogenic *Escherichia coli* isolates: pap-related sequences compared with *rrn* operons. *Infect Immun* 1990; **58**: 471–479.
- Denny TP, Gilmour MN, Selander RK. Genetic diversity and relationships of two pathovars of *Pseudomonas syringae*. *J Gen Microbiol* 1988; **134**: 1949–1960.
- Picard-Pasquier N, Picard B, Heeralal S, Krishnamoorthy R, Goulet P. Correlation between ribosomal DNA polymorphism and electrophoretic enzyme polymorphism in *Yersinia*. *J Gen Microbiol* 1990; **136**: 1655–1666.
- Goulet P, Picard B. *Pseudomonas aeruginosa* isolate typing by esterase electrophoresis. *FEMS Microbiol Lett* 1991; **78**: 195–200.
- Denamur E, Picard B, Goulet P, Bingen E, Lambert N, Elion J. Complexity of *Pseudomonas aeruginosa* infection in cystic fibrosis: combined results from esterase electrophoresis and rDNA restriction fragment length polymorphism analysis. *Epidemiol Infect* 1991; **106**: 531–539.
- Liu PV, Matsumoto H, Kusama H, Bergan T. Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. *Int J Syst Bacteriol* 1983; **33**: 256–264.
- Lennox ES. Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* 1955; **1**: 190–206.
- Goulet P. An esterase zymogram of *Escherichia coli*. *J Gen Microbiol* 1973; **77**: 27–35.
- Uriel J. Méthode d'électrophorèse dans des gels d'acrylamide-agarose. *Bull Soc Chim Biol* 1966; **48**: 969–982.
- Vesterberg O, Svensson H. Isoelectric fractionation analysis and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in

- connection with separation of myoglobins. *Acta Chem Scand* 1966; **20**: 820–834.
21. Lawrence SH, Melnick PJ, Weimer HE. A species comparison of serum proteins and enzymes by starch gel electrophoresis. *Proc Soc Exp Biol Med* 1960; **105**: 572–575.
 22. Uriel J. Caractérisation des cholinestérases et d'autres estérases carboxyliques après électrophorèse et immunoélectrophorèse en gélose. 1. application à l'étude des estérases du sérum humain normal. *Ann Inst Pasteur* 1961; **101**: 104–119.
 23. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; **98**: 503–517.
 24. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 1978; **89**: 583–590.
 25. Ochman H, Whittam TS, Caugant DA, Selander RK. Enzyme polymorphism and genetic population structure in *Escherichia coli* and *Shigella*. *J Gen Microbiol* 1983; **129**: 2715–2726.
 26. Selander RK. Protein polymorphism and the genetic structure of natural populations of bacteria. In: Ohta T, Aoki K (eds) *Population genetics and molecular evolution*. Berlin, Springer-Verlag. 1985: 85–106.
 27. Selander RK, Caugant DA, Whittam TS. Genetic structure and variation in natural populations of *Escherichia coli*. In: Ingraham JL et al. (eds) *Escherichia coli and Salmonella typhimurium*, cellular and molecular biology. Washington, D.C., American Society for Microbiology. 1987: 1625–1648.
 28. Pitt TL. Epidemiological typing of *Pseudomonas aeruginosa*. *Eur J Clin Microbiol Infect Dis* 1988; **7**: 238–247.
 29. Pitt TL, Livermore DM, Pitcher D, Vatopoulos AC, Legakis NJ. Multiresistant serotype O12 *Pseudomonas aeruginosa*: evidence for a common strain in Europe. *Epidemiol Infect* 1989; **103**: 565–576.
 30. Picard B, Gouillet P. Epidemiological complexity of hospital *Aeromonas* infections revealed by electrophoretic typing of esterases. *Epidemiol Infect* 1987; **98**: 5–14.
 31. Picard B, Bruneau B, Gouillet P. Demonstration of an outbreak of *Serratia marcescens* infections in a medical intensive care unit by esterase electrophoretic typing. *J Hosp Infect* 1988; **11**: 194–195.
 32. Griffith SJ, Nathan C, Selander RK et al. The epidemiology of *Pseudomonas aeruginosa* in oncology patients in a general hospital. *J Infect Dis* 1989; **160**: 1030–1036.
 33. Speert DP, Campbell ME, Farmer SW, Volpel K, Joffe AM, Paranchych W. Use of a pilin gene probe to study molecular epidemiology of *Pseudomonas aeruginosa*. *J Clin Microbiol* 1989; **27**: 2589–2593.
 34. Denamur E, Picard-Pasquier N, Mura C, Picard B, Orfila J, Krishnamoorthy R. Comparison of molecular epidemiological tools for *Branhamella catarrhalis* typing. *Res Microbiol* 1991; **142**: 585–589.
 35. Grothues D, Koopmann U, Von Der Hardt H, Tümmler B. Genome fingerprinting of *Pseudomonas aeruginosa* indicates colonization of cystic fibrosis siblings with closely related strains. *J Clin Microbiol* 1988; **26**: 1973–1977.
 36. Ogle JW, Janda JM, Woods DE, Vasil ML. Characterization and use of a DNA probe as an epidemiological marker for *Pseudomonas aeruginosa*. *J Infect Dis* 1987; **155**: 119–126.
 37. Pasloske BL, Joffe AM, Sun Q et al. Serial isolates of *Pseudomonas aeruginosa* from a cystic fibrosis patient have identical pilin sequences. *Infect Immun* 1988; **56**: 665–672.