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Discriminatory Power of Three DNA-Based Typing Techniques for *Pseudomonas aeruginosa*

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We assessed the capacity of three DNA typing techniques to discriminate between 81 geographically, temporally, and epidemiologically unrelated strains of *Pseudomonas aeruginosa*. The methods, representing powerful tools for hospital molecular epidemiology, included hybridization of restricted chromosomal DNA with *toxA* and genes coding for rRNA (rDNA) used as probes and macrorestriction analysis of *Spe*I-digested DNA by pulsed-field gel electrophoresis. The probe typing techniques were able to classify all strains into a limited number of types, and the discriminatory powers were 97.7 and 95.6% for *toxA* and rDNA typing, respectively. Strains that were indistinguishable on the basis of both *toxA* and rDNA types defined 12 probe type homology groups. Of these, one contained five strains, three contained three strains each, and eight groups were represented by two strains each. Strains in 10 of the homology groups had the same O serotype. *Spe*I macrorestriction patterns discriminated between all strains with at least four band differences, which corresponded to a similarity level of 85%. Fifteen pairs of strains were similar at a level of >75% and differed by only four to seven bands. Of these pairs, 11 belonged to the same probe type homology group, indicating their clonal relatedness. We conclude that macrorestriction analysis of *P. aeruginosa* with *Spe*I provides the best means of discrimination between epidemiologically unrelated strains. However, DNA probe typing with either *toxA* or rDNA reveals information on the strain population structure and evolutionary relationships.

Apart from its ubiquitous occurrence in nature (2, 3, 5), *Pseudomonas aeruginosa* is a commonly isolated nosocomial pathogen (18) which is able to survive in hospital moist environments (35) and to colonize the gastrointestinal tracts of healthy subjects (29). It also persists in bronchial secretions of patients with compromised respiratory function (24). Moreover, *P. aeruginosa* causes the highest attributable mortality in patients with ventilator-associated pneumonia (14), and bloodstream infections in immunocompromised hosts have a 60% or greater mortality (19). For hospital epidemiology, subtyping techniques that define clonal relationships between individual isolates (23) are warranted in order to recognize nosocomial transmission and hence gaps in infection control practice. These techniques should not only be sensitive enough to identify outbreak strains but also be sufficiently specific to discriminate between clonal variants of the species that may be overrepresented in nature and therefore expected to predominate in the patient population.

Investigations of the nosocomial epidemiology of *P. aeruginosa* have long been hampered by the inadequate discriminatory capacity of commonly applied phenotypic markers, such as those used in serotyping, phage typing, and bacteriocin typing (6, 16). With the advent of DNA-based techniques, inherently stable bacterial markers have become available. The majority of these approaches compare isolates by generating type-specific DNA fragment patterns after restriction endonuclease cleavage (restriction endonuclease analysis). In order to facilitate interpretation of complex banding patterns after electrophoretic separation, a few fragments can be highlighted by hybridization to specific gene probes or may be generated a priori by rarely cutting restriction enzymes. Both approaches are currently employed for *P. aeruginosa* in epidemiological investigations (8, 22). Gene probes available for this purpose either hybridize to species-specific coding regions (20, 30) or are more broadly applicable, such as *Escherichia coli* genes coding for rRNA (rDNA) which hybridize to repetitive rDNA operons of a large number of bacteria (ribotyping) (15, 32). Resolution of large DNA fragments (macrorestriction analysis) is accomplished by pulsed-field gel electrophoresis techniques, such as field inversion gel electrophoresis and contour-clamped homogeneous electric field (CHEF) electrophoresis (8). These techniques identify variations in the electrophoretic mobilities of large restriction fragments and avoid the need for hybridization with previously labeled DNA probes.

Recently, a multicenter international trial for the typing of *P. aeruginosa* compared phenotypic and genotypic methods principally for their reproducibility with sets of isolates predominantly from cystic fibrosis patients (13). However, it was not possible in that study to address the key issue of the ability of novel genetic methods such as pulsed-field gel electrophoresis and use of universal (rDNA) and specific (*toxA*) probes to distinguish unequivocally between clearly distinct strain populations.

The purpose of the current investigation was to test three established DNA-based typing techniques for *P. aeruginosa* in parallel by a blinded design to determine the discriminatory power and reproducibility and, moreover, to provide empirically based guidelines for the interpretation of results obtained by these methods.

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MATERIALS AND METHODS

Strains. Seventy-seven strains of *P. aeruginosa* were selected from isolates formerly submitted for type identification to the Laboratory of Hospital Infection

at the Central Public Health Laboratory, London, United Kingdom. Each strain originated from a single hospital in the United Kingdom or one of 10 other countries. They were selected as representative of distinct strains of *P. aeruginosa* and were geographically, epidemiologically, and temporally unrelated (Table 1). To obscure their origins, all strains were given a four-digit code before they were shipped to the investigators. In addition, four American Type Culture Collection (ATCC) strains were included to complete the set of unrelated strains.

DNA fingerprinting. (i) *toxA* typing. DNA from *P. aeruginosa* was extracted by a rapid nontoxic lysis column purification method on an anion-exchange resin (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. DNAs from single strains (2.5 μg) were digested with each of the restriction endonucleases *Bgl*II, *Sal*I, and *Xho*I and electrophoresed in adjacent lanes in 1.2% agarose gels with a constant voltage of 30 V for 16 h. The gels were stained with ethidium bromide and inspected by UV transillumination (302 nm). Subsequently, the DNA fragments were transferred to nylon membranes (Hybond N+; Amersham, Braunschweig, Germany) and hybridized to the *toxA* probe. The probe consisted of a 2.7-kbp *P. aeruginosa* exotoxin A gene, comprising the entire structural gene and a 741-bp noncoding sequence (33). The probe was radiolabeled with [α-^{32}P]CTP to a specific activity of 10^9 dpm/μg.

(ii) rDNA typing. The *E. coli* rDNA gene probe hybridizes with four rDNA operons conserved at different parts of the *P. aeruginosa* genome (11, 27). Extracted chromosomal DNA was digested with *Pvu*II and treated as described for *toxA* typing. Hybridization was carried out with a radiolabeled 7.5-kbp *Pst*I-*Bam*HI fragment from plasmid pKK 3535 containing the entire *E. coli* rDNA operon *rrnB*.

(iii) Macrorestriction analysis. *P. aeruginosa* strains were grown overnight in broth culture to a density of 10^9 CFU/ml. Cells were sedimented by centrifugation, washed in 5 ml of 10 mM Tris–1 M NaCl (pH 7.5), and resuspended in 900 μl of the same buffer. A 500-μl volume of this suspension was mixed with an equal volume of 1.3% low-melting-point agarose and solidified in molds. Agarose plugs were then transferred to lysis buffer containing 0.05% proteinase K, 1% sodium laurylsarcosine, and 0.5 M EDTA (pH 9.6), and cells were lysed at 56°C for approximately 16 h. The agarose plugs or inserts were subsequently equilibrated in TE (10 mM Tris, 1 mM EDTA [pH 7.6]) and stored at 4°C until use. All plugs were then equilibrated with restriction buffer, and the DNA was digested by the rare-cutting enzyme *Spe*I during overnight incubation at 37°C. Electrophoresis was carried out in 1% (wt/vol) agarose gels (SeaKem; FMC BioProducts, Rockland, Maine) and running buffer containing 0.5× TBE (10× TBE consists of 108 g of Tris, 42 g of boric acid, 50 ml of 0.5 M EDTA, and *ad* 1l [pH 7.5]) with a CHEF DRII drive module (Bio-Rad, Richmond, Calif.). Running conditions consisted of two ramps in sequence (ramp A consisted of an initial switch time of 0.5 s, a final switch time of 25 s, and a run time of 20 h; ramp B consisted of an initial switch time of 30 s, a final switch time of 60 s, and a run time of 4 h). The voltage was 200 V for both ramps, and the temperature was kept constant at 14°C. Fragments were stained with ethidium bromide and photographed.

Serotyping. The strains were serotyped by slide agglutination with O-specific antisera representative of the International Antigenic Typing Scheme (25).

Interpretation of restriction fragment patterns. *toxA* types or rDNA types were considered to be distinct if patterns differed by one or more bands. Macrorestriction pattern types were determined by including all fragments of >21 kbp. Their profiles were reproduced by highly exact tracing onto acetate transparencies. The transparencies were photocopied, and each lane was cut out and compared for all band positions with all other lanes, with a lambda *Hin*dIII size marker (Boehringer GmbH, Mannheim, Germany) as a reference. Patterns with a high degree of homology were reevaluated by electrophoresis in adjacent lanes of the same gel. The banding patterns were compared with the aid of a grid that divided each lane into 70 distinct sections, and the percent similarity was calculated according to the Dice equation (4). The discriminatory power of each typing method was assessed by the modified index of diversity suggested by Hunter and Gaston (12).

Reproducibility and statistical analysis. After the initial investigation, 35 strains of the panel were once again coded and resubmitted by the Central Public Health Laboratory. The second coding was carried out to test for technical reproducibility and the ability to reidentify strains by the applied methods. Thirty-five strains were required to have an 80% power of detection of any difference of 95% reproducibility at the overall 5% significance level. The statistical significance of observed differences between mean similarity values was determined by the Student *t* test for continuous data. The results of the blinded analysis were documented and kept until the breaking of the code.

RESULTS

***toxA* typing.** All strains of *P. aeruginosa* showed a *toxA*-reactive fragment pattern. Hybridization with the *toxA* probe produced 10, 13, and 29 different fragment patterns after restriction digestion with *Bgl*II, *Sal*I, and *Xho*I, respectively. Each pattern consisted of one to four bands and comprised variable as well as constant fragments. For four isolates, the constant fragments were missing after restriction digestion with any of the three enzymes, and these patterns were termed atypical *toxA* patterns. Nonetheless, atypical *toxA* patterns still represented interpretable fingerprints, and therefore *toxA* typing achieved a typeability of 100%. After combining the restriction patterns of all three enzymes, we obtained 49 different *toxA* types for all 81 strains. The most commonly encountered types had frequencies of 9.9, 8.6, 4.9, and 3.7%, which gave a discriminatory power of 97.7% (Table 2).

rDNA typing. Polymorphic fragments of ribosomal coding regions were generated by the frequently cutting enzyme *Pvu*II (7, 10). All isolates showed a probe-reactive fragment pattern that consisted of 7 to 28 variable bands of different intensities, and a typeability of 100% was achieved. Comparison of the patterns classified 81 isolates into 32 rDNA types with frequencies of 13.6, 11.1, 6.2, and 4.9% for the four most common rDNA types. A discriminatory power of 95.6% was calculated for this technique (Table 3).

When the two probe typing procedures were combined, the discrimination achieved was increased compared with that obtained by a single technique. Thirty-one strains previously assigned to 8 *toxA* types and 53 strains in 14 common rDNA types could be further differentiated by this approach. It also became apparent that some strains had the same *toxA* or rDNA type and could not be differentiated further by the combination of the two methods. Clusters of these strains were termed probe type homology groups (PTHGs). Table 4 shows that 63 groups were identified in total, and 51 of these were unique. Of the 12 PTHGs which contained more than one strain, one group was represented by five strains, three groups contained three strains, and eight were restricted to two strains each.

Macrorestriction analysis. Macrorestriction patterns generated by enzyme *Spe*I consisted of 17 to 37 (mean, 25.9) clearly discernible bands of between 21 and 650 kbp. Autodegradation of DNA rendered four strains of the panel untypeable even after repeated testing. Thus, typeability was 95.1%. Each of the remaining strains could be distinguished visually by a unique macrorestriction pattern, and a pairwise comparison of the most-similar strains electrophoresed in adjacent lanes revealed differences in at least four bands, corresponding to a Dice coefficient of similarity of approximately 0.85 (Fig. 1). Consequently, all strains could be distinguished from each other at a level of 85% similarity.

Strains from the same PTHG showed a significantly higher degree of similarity in their macrorestriction pattern (mean similarity, 75.1%; confidence interval, 63.8 to 86.4%) than pairwise comparisons of 30 randomly chosen strains of the same serotype (mean similarity, 54.3%; confidence interval, 46.3 to 62.3%; $P < 0.01$), which may indicate relatedness in the overall genomic organization of strains with identical *toxA* and rDNA types. In fact, of the 15 pairs that had macrorestriction similarities of between 75 and 85%, 11 were from the same PTHG. More surprisingly, most strains of the same PTHG had the same serotype (Table 5).

Reproducibility. On repeated testing, all 35 strains could be unambiguously assigned to the same type pattern as that previously generated by macrorestriction analysis. *toxA* typing and rDNA typing were likewise reproducible. Breaking of the code and comparison with results obtained by blinded testing disclosed that all strains were epidemiologically unrelated and that all had been correctly identified after repeated testing.

DISCUSSION

Most of the conventional techniques for subtyping *P. aeruginosa* are based on phenotypic characteristics. They classify

TABLE 1. Origins of *P. aeruginosa* strains and typing results

Strain code	Hospital, center, or geographic source	Serotype	DNA typing pattern			
			toxA type[a]	rDNA type	PTHG	PFGE[b]
4587	Boston, UK[c]	II	10.08.09 T14	R6	5	1
4785	Kings College	4	04.06.11 T10	R7	6	2
5072	Charing	3	01.11.23 T3	R6	13	3
5670	Scunthorpe, UK	II	10.08.04 T16	R13	14	4
5757	Toulouse, France	1	01.01.01 T1	R8	2	5
6338	Studi	12	02.02.02 T17	R9	15	6
6563	Blackburn, UK	3	01.01.01 T1	R20	16	7
6582	Spain	II	01.01.03 T8	R14	17	8
6666	Birmingham, UK	9	03.01.01 T9	R2	7	9
6734	Austria	1	01.01.01 T1	R4	18	10
6898	Royal Free	II	04.02.04 T18	R13	19	11
6920	Worthing, UK	10	05.03.05 T2	R3	1	12
6950	Southport, UK	12	06.04.00 T4	R10	3	13
6954	Glasgow, UK	4	04.06.11 T10	R7	6	14
6973	Slovenia	6	01.07.07 T19	R21	20	15
6992	Netherlands	6	06.02.08 T20	R2	21	16
7027	Hong Kong	11	05.08.05 T2	R11	22	17
7083	Bury, UK	1	08.08.09 T21	R11	23	18
7186	Truro, UK	II	04.08.10 T5	R5	4	19
7193	Milton Keynes	4	09.08.11 T22	R4	24	20
7215	Bath, UK	11	05.03.05 T2	R3	1	21
7256	Hammersmith	6	06.02.12 T23	R2	25	22
7272	Cumbria, UK	6	04.08.05 T24	R22	26	23
7306	Queen Elizabeth II	6	06.09.13 T25	R15	27	24
7336	Oxford, UK	6	06.03.14 T11	R1	9	25
7433	Leicester, UK	1	03.10.15 T12	R2	8	26
7464	Leeds, UK	11	07.06.16 T26	R16	28	27
7622	Coventry, UK	6	07.11.07 T3	R1	10	Untyp.[d]
7627	Croydon, UK	4	01.01.01 T1	R9	29	28
7726	Sheffield, UK	II	09.02.18 T27	R7	30	29
7790	Nuneaton, UK	9	01.11.19 T28	R23	31	30
7793	Tyne & Wear, UK	1	03.01.20 T29	R1	32	31
7819	Whitechapel	6	04.06.11 T6	R24	33	32
7868	Winchester, UK	11	02.11.17 T30	R1	34	33
7874	West Yorkshire, UK	3	07.03.22 T31	R2	35	34
7916	Bristol, UK	II	10.08.10 T13	R17	36	35
7948	Durham, UK	11	10.08.17 T7	R25	37	36
8045	Norwich, UK	6	06.08.23 T32	R1	38	37
8049	Pilgrim	II	11.14.29 T33	R11	39	38
8101	Stourbridge, UK	11	10.08.17 T7	R18	11	39
8103	St. Mary's	3	07.08.17 T34	R1	40	40
8113	Conquest	II	10.08.09 T4	R6	5	41
8122	Mid-glamorgan	1	03.10.15 T12	R2	8	42
8131	Hull, UK	II	06.03.09 T35	R26	41	43
8159	Italy	11	05.03.05 T2	R27	42	44
8166	Redditch, UK	9	03.01.01 T9	R2	7	45
8184	Queen Mary's	II	04.08.10 T5	R5	4	46
8188	Grantham, UK	3	07.11.17 T3	R28	43	47
8202	St. Thomas	10	05.03.05 T2	R3	1	48
8212	Clwyd, UK	3	Atyp 1 T46	R12	44	49
8214	Llanelli	6	Atyp 2 T47	R29	45	50
8237	St. Bart's	10	05.03.05 T2	R3	1	51
8238	Park Royal, UK	4	01.01.01 T1	R12	46	52
8277	Durham, UK	1	01.01.01 T1	R8	2	53
8297	Portsmouth, UK	11	10.08.17 T7	R18	11	54
8314	Southhampton, UK	3	01.01.24 T36	R9	47	55
8318	Sutton, UK	6	01.01.23 T3	R1	10	Untyp.
8321	Edmonton, UK	II	04.08.11 T5	R5	4	56
8357	Plymouth, UK	II	10.02.10 T13	R5	48	57
8362	St. George's	3	03.11.23 T37	R4	49	58
8364	University College	3	01.02.18 T38	R1	50	59
8375	Gt. Ormond	11	03.02.17 T39	R30	51	60
8382	Whippscross	10	05.03.05 T2	R3	1	61
8386	Wexham, UK	1	09.11.10 T40	R1	52	62
8390	North Devonshire	8	Atyp 3 T48	R4	53	63
8400	New Cross	10	01.01.01 T1	R31	54	64
8405	Bangor, UK	8	Atyp 4 T49	R12	55	65

Continued on following page

TABLE 1—*Continued*

Strain code	Hospital, center, or geographic source	Serotype	DNA typing pattern			
			toxA type[a]	rDNA type	PTHG	PFGE[b]
8414	Newcastle, UK	6	01.01.03 T8	R2	56	66
8420	Hope, UK	1	01.13.15 T15	R14	57	67
8440	Carmarthen, UK	6	10.08.03 T41	R17	58	68
8623	High Wycombe, UK	PA[e]	07.06.06 T6	R19	12	Untyp.
8722	Belfast, UK	12	06.04.00 T4	R10	3	69
8723	Harold Wood, UK	3	07.06.06 T6	R19	12	70
8734	Stoke/Trent	6	06.03.14 T11	R1	9	71
8735	Birmingham, UK	1	01.01.01 T1	R8	2	72
8756	France	12	06.04.00 T4	R10	3	73
8757	Newcastle, UK	9	06.03.09 T42	R32	59	Untyp.
292	ATCC	11	04.01.26 T43	R16	60	74
9721	ATCC	6	07.11.27 T4	R1	61	75
15442	ATCC	1	01.13.15 T1	R2	62	76
27853	ATCC	6	06.12.28 T45	R15	63	77

[a] Numbers separated by periods indicate fragment patterns generated by enzymes *Bgl*I, *Sal*I, and *Xho*I, respectively. Atyp 1 through 4, restriction patterns lacking conserved fragments.
[b] PFGE, pulsed-field gel electrophoresis type.
[c] UK, United Kingdom.
[d] Untyp., untypeable.
[e] PA, polyagglutinable.

strains into a limited number of types and when used alone are able to identify similarities between strains. However, it is often necessary to combine methods such as O serotyping with phage typing or to add bacteriocin typing in order to obtain maximal discrimination between apparently related strains. The reproducibility of phage typing, in particular, is poor (5, 13), and that of bacteriocin typing may be inadequate for some strains, such as those from cystic fibrosis patients (6, 13).

Chromosomal fingerprinting with DNA probes or macrorestriction analysis by pulsed-field gel electrophoresis has been successfully applied to the subtyping of *P. aeruginosa* (8, 31). An array of different probes has been proposed so far. They include virulence genes such as the 3′ pilin coding region from strain PAK (30), *toxA* (21), *algD* and *lasA* (17), and *plcSR* (34) and a broad-spectrum probe of *E. coli* rDNA (for ribotyping) (1, 7, 9, 10). Only recently, some of these techniques, phenotypical as well as DNA-based methods, have been critically evaluated (13). However, strains investigated in that study were mainly from cystic fibrosis patients and thus cannot be regarded as representative of epidemiologically unrelated hospital strains.

We set out to assess the discriminatory capacity of the currently most successful DNA fingerprinting techniques for *P. aeruginosa* (7, 13, 28) and chose *toxA* and rDNA typing as well as *Spe*I macrorestriction analysis for comparison. All three methods had never before been tested in parallel on a large panel of unrelated strains.

TABLE 2. Frequency of *toxA* types among epidemiologically unrelated strains of *P. aeruginosa*[a]

toxA type(s)	No. of strains	Frequency (%)
T1	8	9.88
T2	7	8.64
T3	4	4.94
T4	3	3.70
T5	3	3.70
T6	3	3.70
T7	3	3.70
T8	2	2.47
T9	2	2.47
T10	2	2.47
T11	2	2.47
T12	2	2.47
T13	2	2.47
T14	2	2.47
T15	2	2.47
T16–T49	1[b]	1.23[c]
Total	81	100

[a] Overall discriminatory power, 0.9769.
[b] One strain each of these 34 types.
[c] The frequency for each of the 34 types.

TABLE 3. Frequency of rDNA types among epidemiologically unrelated strains of *P. aeruginosa*[a]

rDNA type(s)	No. of strains	Frequency (%)
R1	11	13.58
R2	9	11.11
R3	5	6.17
R4	4	4.94
R5	4	4.94
R6	3	3.70
R7	3	3.70
R8	3	3.70
R9	3	3.70
R10	3	3.70
R11	3	3.70
R12	3	3.70
R13	2	2.47
R14	2	2.47
R15	2	2.47
R16	2	2.47
R17	2	2.47
R18	2	2.47
R19	2	2.47
R20–R32	1[b]	1.23[c]
Total	81	100

[a] Overall discriminatory power, 0.9565.
[b] One strain each of these 13 types.
[c] The frequency for each of the 13 types.

TABLE 4. PTHGs as defined by a combination of *toxA* and rDNA type patterns: frequency among epidemiologically unrelated strains of *P. aeruginosa*[a]

toxA-rDNA type(s)	No. of strains	Frequency (%)
1 (T2 R3)	5	6.17
2 (T1 R8)	3	3.70
3 (T4 R10)	3	3.70
4 (T5 R5)	3	3.70
5 (T14 R6)	2	2.47
6 (T10 R7)	2	2.47
7 (T9 R2)	2	2.47
8 (T12 R2)	2	2.47
9 (T11 R1)	2	2.47
10 (T3 R1)	2	2.47
11 (T7 R18)	2	2.47
12 (T6 R19)	2	2.47
13–63	1[b]	1.23[c]
Total	81	100

[a] Overall discriminatory power, 0.9917.
[b] One strain each of these 51 types.
[c] Frequency for each of the 51 types.

Typeability, reproducibility, and ease of interpretation. Overall typeabilities of 100 and 95.5% were achieved by *toxA* and rDNA typing and by macrorestriction analysis, respectively. Moreover, double-blind repeated testing of 35 strains allowed unambiguous identification of all strains, thus demonstrating a high degree of technical reproducibility and chromosomal stability of *P. aeruginosa*.

toxA fragment patterns are relatively easy to interpret because of the low number of bands. Furthermore, visual comparison between gels is facilitated by the presence of constant bands that can be used as internal standards to allow normalization of slight gel-to-gel variations. Interpretation of rDNA types using *Pvu*II restriction is occasionally more cumbersome because of the variability of band intensities. Since bands with mainly constant locations are most discriminative, faint bands can render type designation difficult. Employing restriction enzymes that cut less frequently reduces the complexity of band profiles (as well as the discriminatory power of ribotyping) but does not overcome ambiguities imposed by band in-

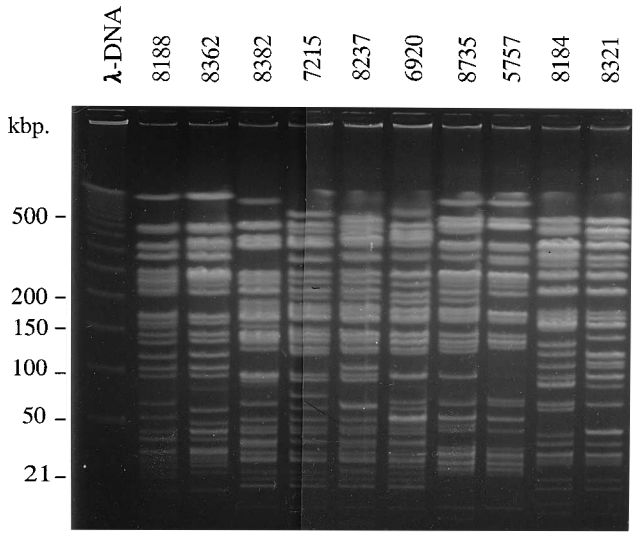

FIG. 1. Representative macrorestriction patterns of unrelated isolates showing a high degree of similarity. Strains 8188 and 8362 were from different PTHGs; strains 8382, 7215, 8237, and 6920 were all from PTHG I; strains 8735 and 5757 were from PTHG II; and strains 8184 and 8321 were from PTHG IV. Positions of λ DNA size markers are indicated on the left.

TABLE 5. IATS[a] serotypes and macrorestriction analysis of *P. aeruginosa* strains from the same PTHG

PTHG	Strain	O serotype	% Similarity to indicated strain as determined by MRA[b]				
I			6920	7215	8202	8237	8382
	6920	10		84.4	82.2	81.0	74.1
	7215	11			63.8	85.4	70.9
	8202	10				70.1	55.2
	8237	10					75.4
	8382	10					
II			5757	8277	8735		
	5757	1		79.6	86.4		
	8277	1			84.4		
	8735	1					
III			6950	8722	8756		
	6950	12		84.9	55.6		
	8722	12			83.9		
	8756	12					
IV			7186	8184	8321		
	7186	II		84.0	85.7		
	8184	II			81.1		
	8321	II					
V			4587	8113			
	4587	II		44.5			
	8113	II					
VI			4785	6954			
	4785	4		85.4			
	6954	4					
VII			6666	8166			
	6666	9		66.0			
	8166	9					
VIII			7433	8122			
	7433	1		72.0			
	8122	1					
IX			7336	8734			
	7336	6		75.0			
	8734	6					
X			8101	8297			
	8101	11		66.0			
	8297	11					
XI			7622	8318			
	7622	6		Untyp.[c]			
	8318	6	Untyp.				
XII			8623	8723			
	8623	PA[d]		Untyp.			
	8723	3	Untyp.				

[a] IATS, International Antigenic Typing Scheme (25).
[b] MRA, macrorestriction analysis.
[c] Untyp., untypeable.
[d] PA, polyagglutinable.

tensities (unpublished observation). DNA macrorestriction patterns produced by *Spe*I digestion, though also complex, were less subjective than *Pvu*II rDNA types. Interpretable bands of >21 kbp were abundant and stained evenly in corresponding gel regions. Furthermore, most strains were clearly discernible if the intervals between band clusters as well as the number of bands were taken into account. In our hands, this technique allowed visual discrimination of 77 strains without computer support. Nevertheless, analysis of larger culture collections will require digitalized data management as visual comparison becomes virtually impossible.

Discriminatory power. Restriction endonuclease analysis based on DNA probes recognizes an altered distribution of restriction sites within or in the vicinity of the probe-reactive sequence and therefore ignores differences in other parts of the genome. Mutations causing such differences are more likely to accumulate in noncoding regions than within transcribed genes. The discriminatory capacity of this method is therefore limited and depends largely on the mutational frequency within the target sequence. Accordingly, *toxA* typing and rDNA typing are capable only of classifying strains of *P. aeruginosa* into a finite number of distinct probe types. We identified 49 *toxA* types and 32 rDNA types among 81 unrelated strains. Similarly, the International *Pseudomonas aeruginosa* Typing Study Group described 54 *toxA* types among 200 strains (13), and Ogle and Vasil recognized 66 distinct *toxA* types among 251 unrelated strains (21). By rDNA typing, Blanc et al. identified 8 to 14 different rDNA types after restriction digestions with either *Bam*HI, *Eco*RI, *Cla*I, or *Pst*I, and combinations of all four enzymes distinguished 33 different rDNA types among 55 unrelated isolates, giving a discriminatory power of 95.8% (1).

The discriminatory power of a typing system takes into account the homogeneity of distribution between types and is thus an objective measure of the chance that two unrelated strains will belong to two different types. Given that a typing method has enough specificity if the chance that two unrelated strains are grouped in the same subtype is <5%, the discriminatory power of *toxA* typing (97.7%) and rDNA typing (95.6%) is sufficient for discerning between unrelated strains. A combination of the results from both probe typing procedures increased the discriminatory power to 99.2% by further differentiating common *toxA* and rDNA types. Thereby, 63 probe type combinations were identified, 51 of which were represented by a single strain and 12 of which contained between five and two strains (Table 4).

The concordance of typing results obtained from two independent chromosomal loci led to the definition of PTHGs, which suggested a high degree of genetic homogeneity within a group. The biologic significance of this classification is underscored by the finding that most strains of the same PTHG had the same O serotype, indicating a group-specific relationship between genetic organization and the lipopolysaccharide O-antigen synthesis.

Macrorestriction analysis resolves restriction site polymorphisms throughout the genome and is able to provide insights into the chromosomal organization of a species (27). This technique revealed the best discrimination. All of the 77 typeable strains could be distinguished at similarity levels of <85%. Strains displaying the most-similar patterns differed by four or more bands (Fig. 1). On the basis of macrorestriction genome mapping, Grothues et al. defined six band differences for *Dra*I and *Xba*I digests as discriminative between clonally related and unrelated isolates (8). Fifteen pairs of epidemiologically unrelated strains studied here differed by four to seven bands, i.e., displaying between 85 and 75% similarity. Eleven of these pairs were assigned to the same PTHG, indicating a significant association between locus-specific restriction polymorphisms and the chromosomal architecture.

Two strains from PTHG II that originated from hospitals as distant as northern England and southern France showed seven band differences in the *Spe*I macrorestriction pattern but were overall highly similar (Fig. 1, strains 8735 and 5757). Since an epidemiological association seems unlikely, this high degree of genetic homology may more likely be explained by a common ancestral origin than simply by chance alone. Variants of a single clonal lineage are often isolated from the same reservoir (31), but our findings indicate that apparently clonally related strains may also be present in different hospitals over a broad geographic area. This is supported by the finding that strains belonging to a major clone were recently isolated from patients and the natural environment from different parts of Germany (28). An overrepresentation of clonally related *P. aeruginosa* strains in the patient population and hospitals may certainly compromise the value of any typing technique that is unable to quantify the degree of genetic relatedness between strains.

Other investigators (26) likewise reported up to seven differences for genetically associated isolates with the *Spe*I macrorestriction method but found fewer than three band differences in the majority of outbreak-related isolates. On the basis of our experience with outbreaks, we assume that in situations in which the temporal and spatial distribution of isolates does not allow for acquisition of multiple genetic alterations, fewer than four band differences in the *Spe*I profile of different isolates are suggestive of relatedness and hence transmission. Variations of four to seven bands seem to exclude direct transmission, but infection with a clonal variant of the same lineage is possible. With more than seven band differences, genetic relatedness becomes increasingly unlikely.

We conclude that macrorestriction analysis is the most powerful tool for the study of the hospital epidemiology of *P. aeruginosa* because of its high discriminatory capacity, good reproducibility, and ease of interpretation. Although *toxA* typing and rDNA typing are also indicative of clonal relatedness of strains, these techniques are less likely to discriminate between infections caused by variants of the same clonal lineage that persist in different geographic locales. Nevertheless, DNA fingerprinting results should be interpreted with caution, since genetic relatedness of strains alone does not allow the drawing of unequivocal conclusions about transmission of any bacterial organism in the absence of sound epidemiological data.

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