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Pseudomonas aeruginosa displays an epidemic population structure

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Summary

Bacteria can have population structures ranging from the fully sexual to the highly clonal. Despite numerous studies, the population structure of *Pseudomonas aeruginosa* is still somewhat contentious. We used a polyphasic approach in order to shed new light on this issue. A data set consisting of three outer membrane (lipo)protein gene sequences (*oprI*, *oprL* and *oprD*), a DNA-based fingerprint (amplified fragment length polymorphism), serotype and pyoverdine type of 73 *P. aeruginosa* clinical and environmental isolates, collected across the world, was analysed using biological data analysis software. We observed a clear mosaicism in the results, non-congruence between results of different typing methods and a microscale mosaic structure in the *oprD* gene. Hence, in this network, we also observed some clonal complexes characterized by an almost identical data set. The most recent clones exhibited serotypes O1, 6, 11 and 12. No obvious correlation was observed between these dominant clones and habitat or, with the exception of some recent clones, geographical origin. Our results are consistent with, and even clarify, some seemingly contradictory results in earlier epidemiological studies. Therefore, we suggest an epidemic population structure for *P. aeruginosa*, comparable with that of *Neisseria meningitidis*, a superficially clonal structure with frequent recombinations, in which occasionally highly successful epidemic clones arise.

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Introduction

Pseudomonas aeruginosa is noted for its metabolic versatility and its exceptional ability to adapt to and colonize a wide variety of ecological environments (water, soil, rhizosphere, animals) (Goldberg, 2000). It is also known for its capacity to cause disease in cystic fibrosis, burn, cancer and ventilated intensive care patients. Infections caused by *P. aeruginosa* are difficult to treat because of its inherent resistance to antibiotics. There seems to be a consensus about the fact that *P. aeruginosa* clinical isolates are genotypically, chemotaxonomically and functionally indistinguishable from environmental isolates. Römling *et al*. (1994) reported that the most frequently (28%) identified clone in cystic fibrosis patients was also detected at a relatively high frequency (21%) in aquatic environments, suggesting a common recent origin of these strains. Rahme *et al*. (1995) demonstrated the infectivity of a *P. aeruginosa* strain in both plant and animal models. Foght *et al*. (1996) observed that *P. aeruginosa* strains isolated from a gasoline-contaminated aquifer were indistinguishable, by molecular biological techniques, from clinical isolates. Alonso *et al*. (1999) reported that both oil-contaminated soil isolates and clinical isolates of *P. aeruginosa* show pathogenic and biodegradative properties. However, the population structure of *P. aeruginosa* is still under discussion. Denamur *et al*. (1993) and Picard *et al*. (1994) suggested that the population structure of *P. aeruginosa* was panmictic, but highlighted the need for caution in inferring bacterial population structure from any single class of genetic marker. Comparative sequencing of six genes in 19 environmental and clinical *P. aeruginosa* isolates revealed a high frequency of recombination and a net-like population structure (Kiewitz and Tümmler, 2000). Ruimy *et al*. (2001) used random-amplified polymorphic DNA (RAPD) typing to study the genetic diversity of *P. aeruginosa* pneumonia, bacteraemia and environmental isolates. They concluded that

the studied population underwent epidemic clonality with a high rate of genetic recombination and that bacteraemia and pneumonia are not caused by specific clones. Recently, Lomholt *et al*. (2001) observed an epidemic population structure for a *P. aeruginosa* population isolated mainly from patients with keratitis and their environment. They found evidence for an epidemic clone that is pathogenic to the eye and is characterized by a distinct combination of virulence factors. The above-mentioned studies were, however, somewhat biased, as the studied *P. aeruginosa* populations were often sampled in a relatively small region (mainly one country) and/or focused on a single pathology or specific environment and/or were analysed by only one method. With the call of van Belkum (1996) and Vandamme *et al*., 1996) for a polyphasic approach in mind, we integrated all phenotypic and genotypic data available to us in a consensus type of clustering to study aspects of the population genetics and epidemiology of *P. aeruginosa*. A data set, consisting of the nucleotide sequences of three outer membrane protein genes (*oprI*, *oprL* and *oprD*), amplified fragment length polymorphism (AFLP) pattern analysis, serotype and pyoverdine type, was combined for 73 *P. aeruginosa* isolates, collected from 18 countries, from clinical and environmental habitats. The *oprI* (249 bp) and *oprL* (504 bp) genes are coding for the outer membrane lipoproteins I (Cornelis *et al*., 1989a) and L (Lim *et al*., 1997) of *P. aeruginosa.* The *oprI* gene is conserved among the fluorescent pseudomonads and was found to be useful as a complementary phylogenetic marker for the classification of rRNA group I pseudomonads (De Vos *et al*., 1998). The *oprL* gene is conserved in *P. aeruginosa* and has proved to be a useful detection and identification target molecule (De Vos *et al*., 1997; Pirnay *et al*., 2000; Jaffe *et al*., 2001). The *P. aeruginosa oprD* gene (1323 or 1329 bp) codes for a specialized pore protein, OprD, which allows selective permeation of basic amino acids and their structural analogues such as the carbapenem antibiotic imipenem (Trias and Nikaido, 1990). Analysis of the *oprD* gene from 55 clinical and environmental isolates revealed important sequence variability and a microscale mosaic structure resulting from multiple recombinational events (Pirnay *et al*., 2002).

AFLP analysis is a genotyping method based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion (Vos *et al*., 1995). This technique has proved to be highly discriminatory and reproducible, which allows the compilation of standardized patterns in a database (Janssen *et al*., 1996; Savelkoul *et al*., 1999).

Pseudomonas aeruginosa strains have been divided into serotypes since 1926 (Aoki, 1926). Since then, many investigators have formed their own serotyping schemata, which has made serological study of *P. aeruginosa* very confusing. Hence, since its publication in 1983 (Liu *et al*., 1983), most European and American researchers have used the international serogrouping schema for *P. aeruginosa*, comprising 17 groups based on the heat-stable major somatic antigens, for clinical serotyping. The serotyping of *P. aeruginosa* strains, using this standardized schema, allows us to compare the evolutionary relationships between our isolates, provided by the two DNA-based techniques, with earlier published epidemiological data.

To satisfy their need for iron, pseudomonads generally produce high-affinity fluorescent peptidic siderophores, called pyoverdines (PVDs) (Meyer, 2000). In *P. aeruginosa*, three PVDs (I, II and III), easily differentiated by isoelectric focusing (IEF), have been observed so far, only one being produced by a given strain (Cornelis *et al*., 1989b; Meyer *et al*., 1997). The combined results obtained in this study are in agreement with earlier epidemiological studies and clearly indicate that the population structure of *P. aeruginosa* is epidemic.

Results

Sequence analysis of oprI*,* oprL *and* oprD *genes*

The *oprI*, *oprL* and *oprD* sequences of the studied *P. aeruginosa* population were aligned and clustered using UPGMA. Alleles were assigned numbers according to their position in the alignment (Fig. 1). The *oprI* and *oprL* genes showed sequence variability comparable with that of housekeeping genes, as was to be expected because both genes code for a structural outer membrane lipoprotein. In the dendrograms (UPGMA), based on the similarity of the *oprI* and *oprL* genes of the studied *P. aeruginosa* population, supplemented with other members of the rRNA group I pseudomonads (Fig. 2), *P. aeruginosa* forms a sharply delineated species. Strains LMG 10643 and, to a lesser extent, strains LMG 5031 and Br680 diverge from the rest. With the exception of isolate LMG 10643, all mutations in *oprI* and *oprL* were silent, often occurring at the third position of the codon. The *oprD* gene, on the other hand, showed high sequence variability, a mosaic structure and multiple non-silent mutations, typical of a gene that is under strong selection for diversity (Fig. 1). The *oprD* gene of strains LMG 10643 and Lw1048 could not be amplified by polymerase chain reaction (PCR).

AFLP analysis

The AFLP patterns of the *P. aeruginosa* strains and one *Pseudomonas pseudoalcaligenes* strain were normalized and clustered using UPGMA. By applying the criteria for differentiation of *P. aeruginosa* by AFLP (Speijer *et al*., 1999), which were based on the criteria for pulsed-field gel

electrophoresis (Tenover *et al*., 1995), five clusters of related isolates (with ≥80% homology) were identified (Fig. 3).

Serotype determination

Sixty-one out of the 73 strains could be serotyped. Six strains were non-agglutinable, and six were polyagglutinable. The predominant serotypes were O1 (12.3%, 9/73), 6 (10.9%, 8/73), 11 (15.1%, 11/73) and 12 (9.6%, 7/73) (Table 1).

Pyoverdine typing by IEF

A majority of the *P. aeruginosa* strains (37/73) produced or were growth stimulated by type II PVD (Table 1). Fifteen isolates produced type I PVD and 14 type III. A few isolates failed to produce enough PVD to allow analysis by IEF. The presence of the receptor for a pyoverdine was therefore determined by a growth stimulation assay. In some cases, growth was stimulated by more than one PVD (Table 1). When this was the case, the pyoverdine that gave the strongest growth stimulation was considered as the cognate one, and the others are indicated between brackets.

Combined analysis

The data obtained from sequence analysis, AFLP analysis, serotyping and PVD typing of the 73 *P. aeruginosa* isolates was combined and analysed using BIONUMERICS biological data analysis software. In the dendrogram from the composite data set (Fig. 4), we identified a limited number of phylogenetic groups with ≥80% similarity. Some subclusters even showed >90% similarity. We also observed unique isolates, some of which diverged considerably from the rest of the population. There is also evidence that the relation among the isolates was distorted by recombination. We observed a network of relationships between all analysed parameters (Table 1) and non-congruence between experiments (Fig. 5).

Discussion

The observation of clones in many bacterial populations has led to the assumption that bacteria reproduce clonally. It was long supposed that point mutations are the major source of genetic variation in bacteria, whereas recombinational exchanges were considered to be rare. This view has changed in recent years. Maynard Smith *et al*. (1993) used multilocus enzyme electrophoresis (MLEE) to demonstrate that bacterial population structure ranges from the panmictic or fully sexual, with random association between loci (e.g. *Neisseria gonorrhoeae*), to one that is clonal, with non-random association of alleles, resulting in the frequent recovery of only a few of the possible multilocus genotypes (e.g. *Salmonella enterica*). Intermediate types of population structure were also reported. *Neisseria meningitidis*, for example, displays what the authors have called an 'epidemic' structure. Although the population is sexual in the long term, some epidemic clones show significant association between loci. Recently, Feil *et al*. (2001) used multilocus sequence typing (MLST) (Maiden *et al*., 1998) to examine the extent and significance of recombination in six bacterial pathogens. In four species (*N. meningitidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*), they observed a lack of congruence between gene trees, supported by high ratios of recombination to point mutation. In contrast, for *Haemophilus influenzae* and pathogenic isolates of *Escherichia coli*, there was some congruence between gene trees, suggesting lower rates of recombination.

In this work, data obtained by four different typing methods, performed on a large batch of unrelated clinical and environmental *P. aeruginosa* isolates, are combined using biological data analysis software in order to get some insights into the population structure of *P. aeruginosa*.

The lack of congruence among experiments (Fig. 5) is most easily explained as the consequence of multiple recombinational events that have eliminated the phylogenetic signal in each tree. This view is supported by the observation of a microscale mosaic structure in the *oprD* gene (Fig. 1), which supplies direct evidence for recombination. The non-congruence between the AFLP dendrogram and the trees based on sequence analysis could be expected, as sequence diversity is generally caused by single nucleotide polymorphisms, whereas differences in macrorestriction fragment patterns are mainly the result of insertions and/or deletions (Kiewitz and Tümmler, 2000). Although bacterial species in which recombination appears to be common are naturally transformable, there seems to be no obvious correlation between the degree of recombination and the transformability of species. Feil *et al*. (2001) showed that the naturally transformable *H. influenzae* showed by far greater congruence between gene trees than the non-transformable species *S. aureus* and *S. pyogenes*. They suggested that recombinational exchanges in *S. aureus* and *S. pyogenes* are presumably mediated by phage transduction, the effect of which is as great as that of transformation. *P. aeruginosa* is considered not to be competent for natural transformation. In this context, it is interesting that Ripp *et al*. (1994) suggested that environmentally endemic bacteriophages are formidable transducers of naturally occurring microbial communities of *P. aeruginosa*.

Hence, the population structure of *P. aeruginosa* is not fully sexual. In the dendrogram based on the comparison of the composite data set (Fig. 4), we clearly observed

oprD

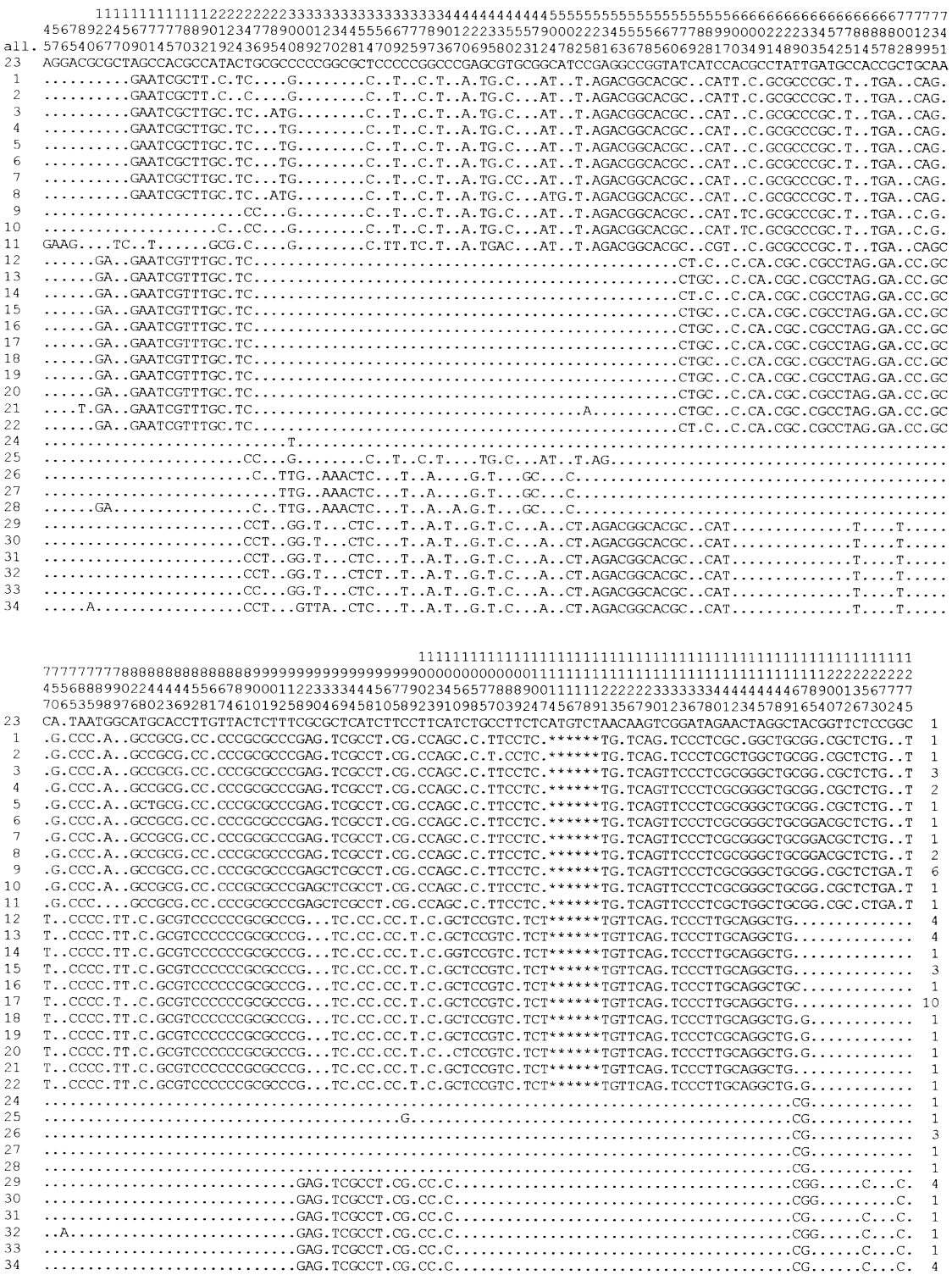

Fig. 1. Polymorphic sites detected in the different alleles of the *oprI*, *oprL* and *oprD* genes of 73 *P. aeruginosa* isolates. Only the sites that differ from the sequence of the allele of PAO1 (no. 23) are shown. Alleles are arranged according to their positions in the dendrogram of an UPGMA alignment. The number of representatives for each allele is shown on the right. Defective *oprD* mutations and alleles with unusually high sequence variability are excluded. Gaps are represented by *.

oprL

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                 1112222233333334444444
                41330347812234790123557
                07257401281465568792091
Allele 14       CCTCCGCCCACCCTGCCATTTCC 29
Allele  1       ..........T.......C.C..  4
Allele  2       ..................C.C..  2
Allele  3       .....A....T.......C.C..  1
Allele  4       ..................C.CT.  1
Allele  5       ....T............C.C..  1
Allele  6       ...T.............C.C.T  2
Allele  7       .T.............T.......  2
Allele  8       ...............T.......  7
Allele  9       ..............AT.......  1
Allele 10       ........T......T.......  1
Allele 11       ...T...........T.......  1
Allele 12       ............T..........  1
Allele 13       ...........T..........  2
Allele 15       .......T..............  6
Allele 16       ..........T...........  5
Allele 17       ......T...............  1
Allele 18       T.....................  1
Allele 19       ..................G.....  1
Allele 20       ..C......G.T.C..T.CCC..  1
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oprI

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                11111
                12789
                10435
Allele 1        ATTAC 61
Allele 2        ....T  8
Allele 3        ...G.  1
Allele 4        GCC..  2
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Fig. 1. *cont.*

seven distinct clonal complexes (CCs) with ≥80% similarity. Most CCs contain strains from geographically and ecologically different sites, suggesting high rates of migration and a remarkable nutritional versatility, acquired through recombination or another evolutionary mechanism. CC A, for example, contains a blood, wound and urine isolate from three distant cities in the USA, a blood isolate from Congo, a sputum, throat and plant rhizosphere isolate from Belgium and a burn wound isolate from Turkey. The variability within each geographic region was nearly as great as within the whole population. In Belgium and The Netherlands, for example, members of CCs A, B, C, E, F and G were isolated.

Clones are transient and, over time, recombination will obliterate the evidence of association. The nearly identical data set of the members of subclusters a, c, d, e and g (Table 1), resulting in ≥90% homology in the composite data dendrogram (Fig. 4), is evidence of a recent, explosive increase in these clones. These recent clones exhibit serotypes O1, 6, 11 and 12. This observation is in agreement with earlier epidemiological studies. A study of the serotypes of 2952 *P. aeruginosa* isolates showed a predominance of serotypes O1, 6 and 11 (Bert and Lambert-Zechovsky, 1996), and serotypes O11 and 12 are frequently associated with multidrug-resistant epidemic strains (Farmer *et al*., 1982; Grattard *et al*., 1993; Elaichouni *et al*., 1994; Richard *et al*., 1994; Tassios *et al*., 1998; Dubois *et al*., 2001). Serotyping of 7089 *P. aeruginosa* strains, isolated in 16 Belgian hospitals in the period from 1977 to 1986, revealed a steady increase in *P. aeruginosa* O12 isolates from 2% in 1982 to 22% in 1986 (Allemeersch *et al*., 1988). The majority of these O12 isolates showed the same distinctive pyocin and phage types, suggesting a high degree of homogeneity within the O12 strains in Belgium. A multicentre European study provided evidence for a common O12 *P. aeruginosa* strain in Europe (Pitt *et al*., 1989). Yet, not all O12 isolates belong to clone c. Serotype O12 clinical isolates Bo546 and Br680 are positioned far away from clone c in the composite data dendrogram (Fig. 4). Evidence of genotypic heterogeneity among *P. aeruginosa* serotype O12 outbreak isolates has been reported (Bingen *et al*., 1996).

Although CCs are globally distributed, recent clones are, logically, less widespread (Table 1). Clone d, for example, consists of 10 isolates of a major clone (called clone C) common to patients and aquatic environments in Germany, previously identified by Römling *et al*. (1994). The occasional clustering of strains of distant geographical origin in recent clones (e.g. strain PAO29 in clone c) illustrates the efficient dispersal of *P. aeruginosa* clones, probably aided by increased mobility of the human population.

The close genetic relation among the isolates of each clone was also detected by AFLP analysis. This shows that AFLP can be used, for example in clinical settings, to recognize epidemic *P. aeruginosa* clones over the short term (10 to maybe hundreds of years).

No significant correlation could be made between the type of PVD produced and the habitat. Recently, De Vos *et al*. (2001) reported a prevalence of type II PVD isolates in cystic fibrosis patients, but suggested that their might be a correlation between the pyoverdine type and the (clinical) origin of the *P. aeruginosa* isolates. The fact that several *P. aeruginosa* strains are able to use more than one PVD type (Table 1) could be the result of recombinational events involving PVD receptors. Strain LMG 10643 did not produce, nor was able to use, any of the three PVDs. This, together with the aberrant *oprI* and *oprL* sequences, makes us doubt that this isolate is a true *P. aeruginosa*. The clustering of isolates with different serotypes is not necessarily the result of recombinational events. Kobayashi *et al*. (1994) demonstrated that antipseudomonal drugs were able to induce changes in serotype, and possible evidence of a bacteriophage-mediated

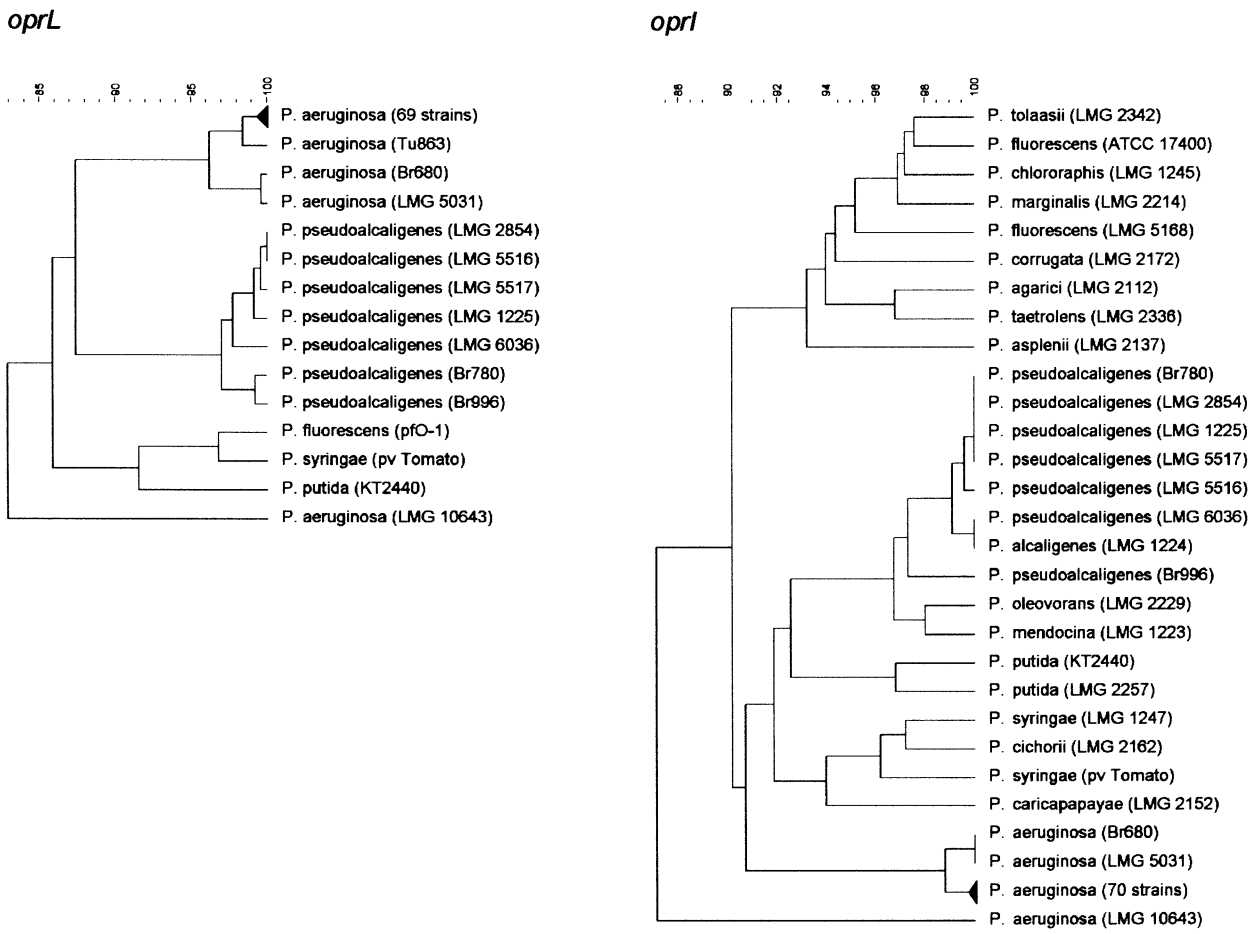

Fig. 2. Sequence similarity trees (UPGMA) based on the comparison of the *oprI* and *oprL* nucleotide sequences of the 73 studied *P. aeruginosa* isolates, supplemented with members of the rRNA group I pseudomonads. Sequences of *P. aeruginosa* and *P. pseudoalcaligenes* isolates were determined in this study, *P. fluorescens* (pf0-1), *P. syringae* (pv. tomato) and *P. putida* (KT2440) sequences were retrieved from the unfinished genomic sequence database (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi) using BLASTN software; all other sequences were retrieved from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/). Percentages of similarity are shown above the dendrogram.

serotype O5 to O16 conversion (Newton *et al*., 2001) was found in the clustering of reference strain PAO1 and strain LMG 14083 in CC B (Fig. 4).

Not that long ago, the ability to identify clusters of isolates with an identical data set from different countries and habitats over a period of time would have been taken as evidence of a clonal structure. The multiple associations of serotypes O11 and 12 with infection and epidemics and the frequent recovery of only a few of all the possible serotypes (O1, 6 and 11) superficially suggest that the *P. aeruginosa* population is clonal. Our results show an epidemic population structure for *P. aeruginosa*, comparable with that of *N. meningitidis* (Feil *et al*., 2001), a population composed of a limited number of widespread clones, which originated, through selection, from a background of a large number of relatively rare and unrelated genotypes that are recombining at a high frequency. These adaptive clones are abundant and widespread in nature and are therefore expected to predominate in the patient population.

Future investigations should be directed at factors that play a role in the selective advantages of these highly successful clones in the environment as a whole, instead of restricting analysis to patients and the hospital environment. The cause of the association between virulence and fitness is still unclear (Groisman and Ochman, 1994).

It should be noted that MLST focuses exclusively on housekeeping genes because of selective neutrality. Analysis of these genes provides a more realistic impression of the effect of recombination. In contrast, *oprD* recombinants can be selectively favoured if, for example, they confer resistance to carbapenem antibiotics. However, we chose to include the *oprD* sequence data for the following reasons: (i) OprD-related resistance to carbapenems is mainly achieved by non-recombinational events such as point mutations (Pirnay *et al*., 2002); (ii) it provides direct

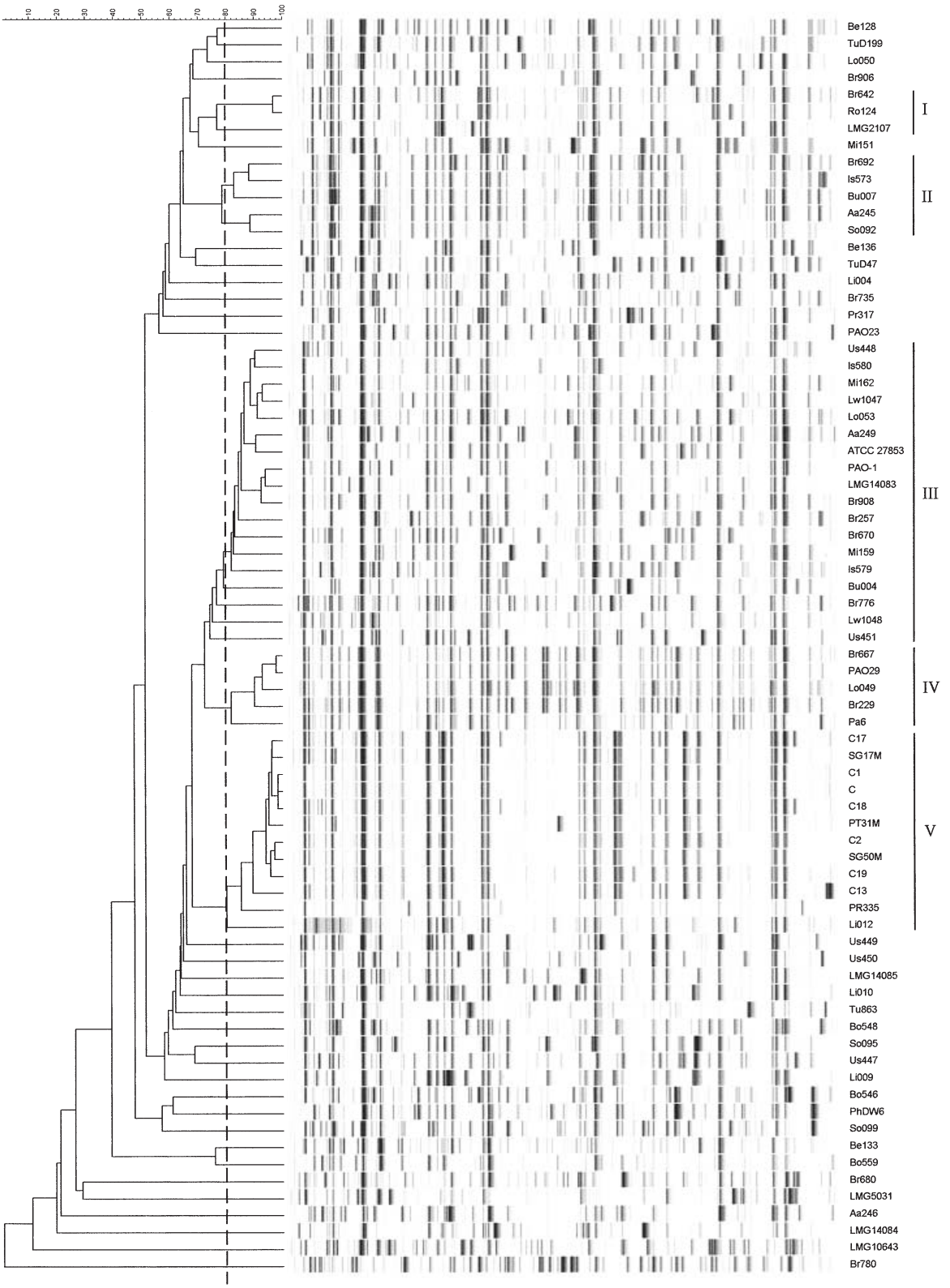

Fig. 3. Normalized AFLP patterns and dendrogram for 73 different *P. aeruginosa* isolates and one *P. pseudoalcaligenes* isolate (Br780). Cluster analysis was performed with BIONUMERICS software using the Pearson correlation and UPGMA. Percentages of similarity are shown above the dendrogram. Clusters with ≥80% similarity (according to Tenover *et al.*, 1995; Speijer *et al.*, 1999) are indicated by Roman numerals. Clusters I, II and III contain isolates with slightly <80% homology.

evidence for localized recombination in *P. aeruginosa*; and (iii) despite the high sequence variability, members of recent clones show identical *oprD* sequences, thus illustrating the stability of these clonal complexes. Although we did not perform conventional MLST, we feel that there is sufficient justification for concluding that *P. aeruginosa* displays an epidemic population structure. A more detailed and prospective study involving more clinical and environmental isolates from other parts of the world, as well as housekeeping gene sequence data, is currently under way. We also feel that the exchange of standardized data between laboratories and the creation of international reference databases of typed microorganisms should be encouraged. It will enable the efficient monitoring of changes in microbial populations.

Experimental procedures

Bacterial strains and growth conditions

A total of 73 *P. aeruginosa* clinical and environmental isolates, collected worldwide, mainly in the late 1980s and 1990s, with some earlier isolates, were examined. The geographical origin, isolation site and time of all *P. aeruginosa* isolates are listed in Table 1.

The *P. aeruginosa* strains used in this study were kindly provided by: Dr A. T. McManus, US Army Institute of Surgical Research, TX, USA; Dr L. Ménesi, General Hospital St Istvan, Budapest, Hungary; Dr A. Vanderkelen, Queen Astrid Military Hospital, Neder-Over-Heembeek, Belgium; Dr J. A. Clark, Queen Mary's University Hospital, London, UK; Dr A. F. Vloemans, Rode Kruis Ziekenhuis, Beverwijk, The Netherlands; Dr T. Taddonio, University of Michigan, MI, USA; Dr A. Radke, Klinik für Verbrennungs- und Plastische Wiederherstellungschirurgie, Aachen, Germany; Professor R. Konigova, Charles University Hospital, Prague, Czech Republic; Dr R. G. Tompkins, Burns Institute, Shriners Hospital for Children, Boston, MA, USA; Dr B. Tümmler, Medizinische Hochschule, Hannover, Germany; Dr M. Caneira, Hospital de Santa Maria, Lisbon, Portugal; Professor A. Boudabous, Science Faculty, Tunis, Tunisia; Dr M. Mergeay, Environmental Technology Expertise Centre, Mol, Belgium; Dr A. E. Lim, Jr., St Scholastica's College of Health Sciences, Tacloban City, Philippines; Professor O. Hadjiiski, Scientific Institute of Emergency Medicine Pirogov, Sofia, Bulgaria; Professor K. Taviloglu, University of Istanbul, Istanbul, Turkey; Dr W. D. H. Hendriks, Zuiderziekenhuis, Rotterdam, The Netherlands; Dr G. Wauters, University of Louvain, Brussels, Belgium; Dr O. Vandenberg, Universitair Ziekenhuis St-Pieters, Brussels, Belgium. Strain PAO-1 was kindly provided by Dr C. K. Stover (PathoGenesis Corporation, Seattle, WA, USA). Strain ATCC 27853 was purchased from Gibson Laboratories. *P. aeruginosa* strains LMG 2107, 5031, 10643 and 14083–5 and *P. pseudoalcaligenes* strains LMG 1225, 2854, 5516, 5517 and 6036 were purchased from the BCCM/LMG bacteria collection. Unless otherwise indicated, strains were grown on Luria–Bertani broth medium (Gibco BRL Life Technologies) at 37°C on a rotary shaker (150 r.p.m.).

PCR and sequencing of the oprI*,* oprL *and* oprD *genes*

DNA was extracted from overnight *P. aeruginosa* cultures using the High Pure™ PCR template preparation kit (Roche Diagnostics) according to the manufacturer's guidelines.

The *oprI*, *oprL* and *oprD* genes were amplified by PCR, using the primers described in Table 2. PCR was performed in 200 μl microcentrifuge tubes. The PCR mixture (50 μl final volume) contained the following: 25.5 μl of sterile distilled water, 5 μl of 10× PCR buffer (500 mmol l^{-1} KCl and 100 mmol l^{-1} Tris-HCl, pH 8.3), 4 μl of a deoxynucleotide mixture (dGTP, dTTP, dATP and dCTP; 2 mmol l^{-1} each), 5 μl of $MgCl_2$ (2.5 mmol l^{-1}), 5 μl of a primer mixture (PS1/2 for *oprI*, PAL1/2 for *oprL* or pDF1/R1 for *oprD*; 10 μmol l^{-1} each), 5 μl of template DNA and 0.5 μl of AmpliTaq DNA polymerase (5 U μl^{-1}). All PCR reagents and primers were ordered from PE Applied Biosystems. The amplification was performed in a GeneAmp® PCR system 2400 (PE Applied Biosystems). The amplification programme was set at 50 cycles of denaturation at 94°C for 30 s, annealing at 50°C or 57°C, according to the primers (Table 2), for 30 s and elongation at 72°C for 1 min. For the amplification of *P. pseudoalcaligenes oprL* genes, the annealing temperature was lowered to 55°C. The reaction mixture was put on a 1.5% (w/v) agarose gel for electrophoresis and visualization of the PCR product after staining with ethidium bromide on a transilluminator. The DNA bands corresponding to the amplified *oprI*, *oprL* and *oprD* genes were excised from the agarose gel with a clean scalpel. DNA was extracted from the gel slice using the QIAEX II gel extraction kit (Westburg) according to the manufacturer's recommendations. Purified PCR fragment (5 μl) was used as a template in the sequencing reaction. PCR primers were used for sequencing. Sequencing of the coding and anticoding strand of the *oprD* PCR products necessitated two additional internal primers, pDF2 and pDR2 (Table 2). DNA sequencing used an ABI 377 automated fluorescence sequencer (PE Applied Biosystems) and the ABI Prism® BigDye™ Terminator cycle sequencing ready reaction kit (PE Applied Biosystems) as detailed in the manufacturer's protocols. The *oprD* gene of isolate Be128 was sequenced directly from genomic DNA. PCR and sequencing were performed in duplicate in order to be able to detect eventual PCR mistakes. Sequences were aligned and clustered using the unweighted pair group method using arithmetic averages (UPGMA) and BIONUMERICS software (Applied Maths).

AFLP

AFLP used an ABI 377 automated fluorescence sequencer (Applied Biosystems) and the AFLP microbial fingerprinting kit (Applied Biosystems) as detailed in the manufacturer's protocols. The enzymes used were T4 DNA ligase, *Eco*RI and *Tru*9I (all purchased from Roche Diagnostics). The primer pair used was *Eco*RI-0[FAM]/*Mse*I-C. GeneScan-500[ROX] internal standard (Applied Biosystems) was co-electrophoresed with each sample in order to allow an accurate calculation of fragment lengths and correction for variation rates and gel distortions. Normalization and fragment sizing were carried out using GENESCAN software (Applied Biosystems). Band patterns were imported into BIONUMERICS software for further normalization (background

Table 1. Properties of the isolates analysed in this study.

Strain	City	Country	Year	Source	Clonal complex ≥80%	Clonal complex ≥90%	AFLP group	Alleles of: *oprD*	Alleles of: *oprL*	Alleles of: *oprI*	Serotype	PVD type
Br680	Brussels	Belgium	1998	Burn				HSV	HSV	4	12	II
LMG 5031	Unknown	Puerto Rico	1961	Chinese evergreen				HSV	HSV	4	NT	II
Us448	San Antonio	USA	1993	Urine	A		III	13	4	1	9	II
Is580	Istanbul	Turkey	1997	Burn	A		III	16	18	1	3	II
ATCC 27853	Boston	USA	1971	Blood	A	a	III	13	7	1	6	II
Br257	Brussels	Belgium	1997	Plant rhizosphere	A	a	III	13	7	1	6	II
Mi159	Ann Arbor	USA	1997	Pressure sore	A		III	12	14	1	6	I (III)[a]
Br670	Brussels	Belgium	1998	Sputum	A		III	15	13	1	6	II
Br776	Brussels	Belgium	1998	Throat	A		III	18	8	1	6	II
Lw1048	Lwiro	Congo	2001	Blood	A		III	NA	8	1	6	II
Aa249	Aachen	Germany	1997	Burn	B		III	15	8	1	PA	I
Lo053	London	UK	1996	Burn	B		III	34	16	1	PA	II
Mi162	Ann Arbor	USA	1997	Burn	B		III	34	16	1	11	I[a]
Bu004	Budapest	Hungary	1997	Throat	B		III	26	8	2	NT	I
Lw1047	Lwiro	Congo	2001	Blood	B		III	15	14	2	NT	I
PA01	Melbourne	Australia	1955	Wound	B		III	23	14	1	5	I
LMG 14083	Unknown	Hungary	1958–65	Unknown	B		III	25	8	1	16	II
Is579	Istanbul	Turkey	1997	Burn	B		III	20	14	1	8	II
Br908	Brussels	Belgium	1999	Throat	B		III	26	11	1	8	III
Br667	Brussels	Belgium	1998	Burn	C	c	IV	9	15	1	12	III
PAO29	Karachi	Pakistan	1998	River water	C	c	IV	9	15	1	12	III
Lo049	London	UK	1996	Burn	C	c	IV	9	15	1	12	III
Br229	Brussels	Belgium	1997	Hospital environ.	C	c	IV	9	15	1	12	III
Pa6	Brussels	Belgium	1985	Urine	C	c	IV	9	15	1	12	III
Us447	San Antonio	USA	1993	Urine				9	15	1	4	I (III)[a]
Us451	San Antonio	USA	1993	Burn			III	10	3	1	4	III
Us449	San Antonio	USA	1993	Sputum				28	19	1	11	III
Us450	San Antonio	USA	1993	Burn				23	14	1	11	II
SG50M	Mülheim	Germany	1992	Swimming pool	D	d	V	17	14	1	1	II
SG17M	Mülheim	Germany	1992	River water	D	d	V	17	14	1	1	II[a]
C1	Hannover	Germany	1987	CF patient	D	d	V	17	14	1	1/13	II
C	Hannover	Germany	1989	CF patient	D	d	V	17	14	1	1/13	II
C18	Hannover	Germany	1989	Hospital environ.	D	d	V	17	14	1	1/13	II
C17	Hannover	Germany	1989	Hospital environ.	D	d	V	17	14	1	1/13	II
PT31M	Mülheim	Germany	1986	Drinking water	D	d	V	17	14	1	1/13	II

C19	Hannover	Germany	1989	CF patient	D	d	V	17	14	1	1/13	II[a]
C2	Hannover	Germany	1988	CF patient	D	d	V	17	14	1	1/13	II[a]
C13	Hannover	Germany	1985	CF patient	D	d	V	17	14	1	1	II
Pr335	Prague	Czech Republic	1997	Hospital environ.	D		V	12	14	2	1	–
Li012	Lisbon	Portugal	1997	CF patient	D		V	12	17	1	PA	NP
Br642	Brussels	Belgium	1998	Hospital environ.	E	e	–	3	16	1	1	–
Ro124	Rotterdam	The Netherlands	1997	Burn	E	e	–	3	16	1	1	–
LMG 2107	Canas	Puerto Rico	1938	Shallow well	E		–	5	1	1	1	–
Tu863	Tunis	Tunisia	1998	Ear				11	20	1	13	III
LMG 14085	Unknown	Hungary	1958–65	Unknown				13	14	2	15	III
Bo548	Boston	USA	1992	Burn				27	8	2	PA	–
Li010	Lisbon	Portugal	1997	CF patient				22	6	1	PA	–
Li009	Lisbon	Portugal	1997	CF patient				19	13	1	6	II
So095	Sofia	Bulgaria	1997	Burn				26	8	1	E[b]	III
Be128	Beverwijk	The Netherlands	1997	Sputum	F			34	14	1	11	–
TuD199	Tunis	Tunisia	1998	Sputum	F			6	1	1	11	–
Mi151	Ann Arbor	USA	1997	Burn	F			8	14	1	11	I(II,III)[a]
Br692	Brussels	Belgium	1998	Burn	G	g	II	30	14	1	11	II
Is573	Istanbul	Turkey	1997	Burn	G	g	II	29	14	1	11	II
Aa245	Aachen	Germany	1997	Burn	G	g	II	29	14	1	11	II
So092	Sofia	Bulgaria	1997	Burn	G		II	31	14	1	1	II
Bu007	Budapest	Hungary	1997	Burn	G		II	29	14	1	E[b]	II
Pr317	Prague	Czech Republic	1996	Burn				29	14	1	11	II
Be136	Beverwijk	The Netherlands	1996	Sputum				1	2	1	3	II
TuD47	Tunis	Tunisia	1998	Ascite				4	14	1	9	II
PAO23	Karachi	Pakistan	1998	River water				34	14	1	NT	III
Lo050	London	UK	1996	Burn				14	14	1	NT	II
Li004	Lisbon	Portugal	1997	CF patient				8	14	1	7	II
Br906	Brussels	Belgium	1999	Nose				2	5	1	6	III
Br735	Brussels	Belgium	1998	Burn				33	1	1	E[b]	II
Bo546	Boston	USA	1992	Burn				21	9	3	12	–
So099	Sofia	Bulgaria	1997	Burn				24	12	2	NT	–
PhDW6	Tacloban City	Philippines	1993	Wound				12	10	2	PA	II
Be133	Beverwijk	The Netherlands	1996	Burn				4	2	1	NT	III
Bo559	Boston	USA	1997	Burn				3	16	2	1	–
Aa246	Aachen	Germany	1997	Burn				32	6	1	11	II
LMG 14084	Bucharest	Romania	1960–64	Water				7	1	1	17	–
LMG 10643	Pusakanegara	Indonesia	1990	*Oryza sativa* leaves				NA	HSV	HSV	NT	NP

a. Siderotyped by PVD-induced growth stimulation. Non-cognate growth-stimulating PVDs are indicated between brackets.
b. Agglutinated with the antisera mix E (O2+5+15+16), but not with either of the separate monovalent antisera.
CF, cystic fibrosis; environ., environment; HSV, unusually high sequence variability; NA, no amplification in PCR; NP, no pyoverdin production or uptake; NT, non-typeable; PA, polyagglutination.


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Br680
LMG5031
Us448
Is580
ATCC 27853
Br257
Mi159
Br670
Br776
Lw1048
Aa249
Lo053
Mi162
Bu004
Lw1047
PAO-1
LMG14083
Is579
Br908
Br667
PAO29
Lo049
Br229
Pa6
Us447
Us451
Us449
Us450
SG50M
SG17M
C1
C
C18
C17
PT31M
C19
C2
C13
PR335
Li012
Br642
Ro124
LMG2107
Tu863
LMG14085
Bo548
Li010
Li009
So095
Be128
TuD199
Mi151
Br692
Is573
Aa245
So092
Bu007
Pr317
Be136
TuD47
PAO23
Lo050
Li004
Br906
Br735
Bo546
So099
PhDW6
Be133
Bo559
Aa246
LMG14084
LMG10643
a
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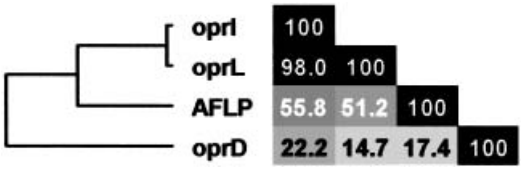

Fig. 5. Matrix of congruence values (percentages) between dendrograms based on the comparison of oprI, oprL and oprD sequences and AFLP patterns, and a dendrogram derived from that matrix. The congruence between experiments was calculated using the Pearson product-moment correlation coefficient.

subtraction, filtering: arithmetic average and band search: minimum profiling 0.5% relative to maximum value) and cluster analysis (similarity coefficient: Pearson correlation, dendrogram type: UPGMA, optimization: 0%, position tolerance: 1%, uncertain bands were ignored).

Serotyping

Isolates were serotyped by slide agglutination according to the international serogrouping schema for *P. aeruginosa* (Liu *et al*., 1983), using a panel of 16 type O monovalent antisera (Sanofi Diagnostics Pasteur).

Pyoverdine typing by IEF

PVD-IEF was carried out according to the method developed by Koedam *et al*. (1994), as described previously (Meyer *et al*., 1997). IEF was performed on Ampholine-PAG plates (pH 3.5–9.5; Pharmacia). The following reference strains were included: PAO-1, representative of PVD type I; ATCC 27853, representative of PVD type II; and strain Pa6, a clinical isolate representing PVD type III (Meyer *et al*., 1997).

Pyoverdine typing by PVD-induced growth stimulation

The effect on bacterial growth of each of the three known PVDs was tested as described previously (Meyer *et al*., 1997). Casamino acid agar (CAA) plates (Cornelis *et al*., 1992) supplemented with 0.5 mg ml^{-1} ethylenediaminedihydroxyphenylacetic acid (EDDHA) were homogeneously inoculated with 100 μl of a 1:10 diluted overnight bacterial culture at 37°C in CAA medium. Sterile filter paper disks (6 mm antibiotic disks; Institut Pasteur Productions) were impregnated with 20 μl of each filter-sterilized 1 mM aqueous solution of pyoverdine and placed on the surface of the agar. Plates were incubated at 37°C and scored after 24 h: no stimulation (no growth), slight stimulation (growth, diameter <10 mm) and good stimulation (thick growth, diameter >15 mm). Pyoverdines produced by the type strains PAO1 (PVD type I), ATCC 27853 (PVD type II) and Pa6 (PVD type III) were purified as described previously (Meyer *et al*., 1997).

Data analysis

The entire data set, consisting of *oprI*, *oprL* and *oprD* sequences, AFLP pattern, serotype and pyoverdine type of 73 *P. aeruginosa* isolates, was analysed and combined using BIONUMERICS (Applied Maths) biological data analysis software. Similarity values were taken from the individual experiments and multiplied by weights (AFLP: 35, *oprD*: 11, *oprL*: 6, serotype: 5, *oprI*: 2, and PVD type: 1). These weights were an educated guess and designed to compensate for the bias caused by the differences in discriminatory capacity between the experiments in this study. In other words, the weights are proportional to the supposed discriminatory capacity of the different typing methods. A dendrogram from the composite data set was obtained using UPGMA. Congruence between experiments was calculated using the Pearson product-moment correlation coefficient.

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Table 2. Primers for PCR and sequencing.

Primer	Gene	Sequence (5′ to 3′)	Temp. (°C)
PS1	*oprI*	ATGAACAACGTTCTGAAATTCTCTGCT	57
PS2	*oprI*	CTTGCGGCTGGCTTTTTCCAG	57
PAL1	*oprL*	ATGGAAATGCTGAAATTCGGC	57
PAL2	*oprL*	CTTCTTCAGCTCGACGCGACG	57
pDF1	*oprD*	ATGAAAGTGATGAAGTGGAGC	50
pDF2	*oprD*	AACCTCAGCGCCTCCCT	50
pDR1	*oprD*	CAGGATCGACAGCGGATAGT	50
pDR2	*oprD*	AGGGAGGCGCTGAGGTT	50

Fig. 4. Dendrogram (UPGMA) based on the comparison of the composite data set consisting of *oprI*, *oprL* and *oprD* nucleotide sequences, AFLP pattern, serotype and pyoverdine type of 73 *P. aeruginosa* isolates. Letters indicate clusters or clonal complexes with ≥80% (caps) and subclusters with ≥90% similarity. Some clusters contain isolates with slightly less homology. Percentages of similarity are shown above the dendrogram.

at the *Pseudomonas* 2001 conference, Brussels, Belgium, September 17–21.

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