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Investigation of Persistent Colonization by *Pseudomonas aeruginosa*-Like Strains in a Spring Water Bottling Plant

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Ninety-seven strains, producing a fluorescent pigment under UV light and/or a green diffusive pigment on cetrimide-naladixic acid agar, were isolated from a spring water bottling plant. These strains were presumptively identified as *Pseudomonas aeruginosa*, but they could not be confirmed as strains of this species nor identified by the API 20NE identification system. The isolates and reference strains were clustered by computer-assisted whole-cell protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The numerical analysis of the protein electrophoregrams resulted in the formation of four clusters at a similarity level of 80% and two unclustered type strains. One cluster included strains isolated during a 4-month period and reference strains of several biotypes of *P. fluorescens*. The remaining isolates formed another cluster with a very high similarity level, which included two groups of strains based on biochemical characterization by the API 20NE Test System. Strains were typed by random amplified polymorphic DNA (RAPD)-PCR and two different RAPD patterns were obtained, corresponding to each biochemical profile. This persistent colonization seems to be caused by a single species present in the bottling system, with two clonal origins, not related to *P. aeruginosa* or to any of the other type strains tested. Partial 16S rDNA sequence of a representative strain of one cluster of isolates had a level of similarity of 99.3% with *P. alcaligenes*. This study shows that characteristics similar to *P. aeruginosa* on cetrimide-naladixic acid agar can be exhibited by several groups of fluorescent pseudomonads that do not belong to this species, clearly showing that confirmation tests must be performed before a decision regarding the water quality is made.

Bottled mineral and spring waters are characterized by an autochthonous microbial flora (8, 12, 18), but in rare instances bottled water can acquire allochthonous bacteria that enter the bottling system through equipment used to pump or transport the water from the source to the bottling plant or during the bottling process itself (2, 31). Bacteria introduced into the water could be important from a public health point of view if they are pathogenic or if they can multiply in water and become persistent, modifying the autochthonous microbial flora of the water.

Bottled spring waters are groundwaters from sources that are not recognized, within the European Community, as natural mineral waters because application for recognition as a natural mineral water has not been made or because these waters do not meet the conditions stipulated by the European Directive for mineral waters (1). In many countries, spring waters are tested under the same legislation as mineral bottled waters and must fulfill all quality parameters demanded of bottled mineral waters; consequently, they must be free of *Pseudomonas aeruginosa* in any 250-ml sample. The presence of *P. aeruginosa* is unacceptable because this species is an opportunistic pathogen, has been implicated in foodborne and waterborne diseases, and is now considered a primary infectious agent (6, 24, 31). This organism is also capable of multiplying abundantly in low-nutrient water (10, 19) and can, therefore, colonize bottled waters. Lately *P. aeruginosa* has been suggested as a surrogate indicator for the presence of other opportunistic pathogens (9).

The detection of *P. aeruginosa* is usually performed by membrane filtration on a selective solid medium, and it is presumptively identified by the production of pyocyanin and a diffusive fluorescent pigment. However, *P. aeruginosa* may also occasionally produce atypical colonies on isolation media, and even nonpigmented strains have been found (11). Moreover, strains of fluorescent *Pseudomonas* forming colonies on the isolation media are usually isolated from natural mineral waters (7). The use of pigmentation for identification of these organisms can, therefore, be erroneous. Some isolates of *P. aeruginosa* are difficult to identify on the basis of phenotypic characteristics, and alternate methods for the rapid identification of these organisms have been implemented (14).

During a 5-year period, several routine samples on cetrimide agar with nalidixic acid produced colonies that resembled *P. aeruginosa* at a spring water bottling plant in central Portugal. These strains were presumptively considered to be *P. aeruginosa* but were not identified by the API 20NE Test System as strains of this species. Stringent maintenance eliminated these organisms for several weeks to months, but they then reappeared periodically until they were definitely eliminated from the water plant after this period. The absence of organisms producing colonies similar to *P. aeruginosa* in water taken directly from the borehole, and their disappearance from the spring water bottling plant after the 5-year period, suggested that these organisms were allochthonous.

It was important to demonstrate that the colonies isolated in the bottling plant during this period were not *P. aeruginosa*, because the presence of this species could be indicative of a lack of good manufacturing procedures and could promote the growth of other potential opportunistic pathogenic organisms (12). Moreover, it was also important to trace the origin and the clonal structure of the colonization by rapid methods of identification. Therefore, in this study we used computerized analysis of whole-cell protein patterns (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) as a routine procedure to recognize and differentiate the organisms iso-

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TABLE 1. List of isolates and reference strains examined in this study organized according to SDS-PAGE groups

SDS-PAGE cluster	Species name (no. of strains)	Strain designation[a]
A1	*P. aureofaciens* (3)	LMG 1245^T, LMG 5832t1 (*P. fluorescens* biotype E), LMG 5832t2 (*P. fluorescens* biotype E)
	P. chlororaphis (1)	LMG 5004^T
	P. fluorescens (2)	LMG 1799, ATCC 13525^T (biotype A)
	P. aureofaciens-P. fluorescens (isolates) (7)	MAS78, P1CN1, P1L1, P1L2, P1MG1, P3CN5, P3CN6
	P. marginalis (2)	LMG 2210^T, LMG 5170 (*P. fluorescens* biotype B)
	P. tolaasii (1)	LMG 2342^T
A2	Isolates (13)	0491A2, 0391A8, 0491A3, 0191A6, 0191A8, 0191A10, 0391A12, 1188T1, 0391A21, 0191A1, 0391A7, 0391A23, 1290T12
B	*P. putida* (6)	LMG 2232, LMG 2258, ATCC 12633^T (biotype A), LMG 2257^T (biotype A), LMG 5835 (biotype A), LMG 1246 (biotype B)
C	Isolates (84)	0491A11, 0391A21, 1188T12, 1188T11, 1188T14, 1188T15, 1188T9, 1190T4, 1190T8, 1190T5, 1188T8, 1190T6, 1190T7, 0391A17, 0391A18, 1190T2, 1190T3, 1288T2, 1288T3, 1288T1, 0391A7, 1188T16, 1188T17, 1188T18, 1188T13, 1188T10, 0391A1, 0391A2, 0191A5, 0191A4, 0391A8, 0391A10, 0391A3, 0391A11, 0191A9, 0391A6, 0391A9, 0391A4, 0191A7, 0191A1, 0491A13, 0291A1, 0291A2, 1190T1, 1188T2, 0492A3, 0492A4, 0492A1, 0492A2, 0492A6, 0391A9, 0391A10, 0391A27, 0391A28, 0391A28, 0391A29, 0391A19, 0391A25, 0391A30, 0491A8, 0491A9, 0492A7, 0492A8, 1290T9, 1290T10, 1290T2, 1290T1, 1290T4, 1288T4, 1188T19, 1290T5, 1290T6, 1290T7, 0391A26, 0391A20, 0491A6, 0491A5, 0492A1, 0491A7, 0491A17, 0491A16, 0491A15, 0491A14, 0491A12
Unclustered	*P. resinovorans* (1)	LMG 2274^T
D	*P. aeruginosa* (5)	ATCC 10145^T, ATCC 27853, LMG 5033, LMG 5827, LMG 9009
	P. aeruginosa (isolates) (20)	BV3CN3, BV3L6a, DZ-15B, MAS18.2, MAS19.1, MAS39.2, MAS46, MAS58, Pa1, Pa2, Pa3, Pa4, Pa6, Pa7, Pa8, Pa9, Pa10, Pa11, Pa12, Pa13
Unclustered	*P. alcaligenes* (1)	LMG 1224^T

[a] Origins of the strains: LMG, Culture Collection of the Laboratorium voor Microbiologie, Gent, Belgium; ATCC, American Type Culture Collection, Rockville, Md.; MAS, isolates from residual waters; BV, PCN, PL, and PMG, isolates from bottled waters; Pa and DZ, clinical isolates, Lisbon, Portugal.

lated on cetrimide agar with nalidixic acid during the 5-year period, because this technique provides a reliable measure of genomic relatedness comparable to DNA-DNA hybridization in several bacterial groups (4, 22) and permits the comparison of large numbers of bacterial strains and the construction of databases to identify organisms.

We also used a random amplified polymorphic DNA (RAPD) technique as a molecular typing technique to track the strains over the period of colonization by this bacterium and to understand its origin, persistence, and disappearance from the water bottling plant.

MATERIALS AND METHODS

Bacterial strains. All strains producing a diffusive green pigment and/or a fluorescent pigment on cetrimide agar with nalidixic acid (Institute Pasteur, Paris, France) during a 5-year period from the routine water analysis in a spring water bottling plant from central Portugal were isolated and purified. These are listed in Table 1. We also included 7 strains of *P. fluorescens-P. aureofaciens* and 7 strains of *P. aeruginosa* previously isolated from residual waters and bottled mineral water (17), 12 strains identified as *P. aeruginosa* isolated from clinical sources, 20 type or reference strains of *Pseudomonas* rRNA homology group I (20), and the type strains of *P. resinovorans* and *P. tolaasii.*

Reference strains and isolates were routinely grown on nutrient agar (Difco) and were stored at −80°C in nutrient broth containing 15% glycerol.

Phenotypic characterization and respiratory quinone analysis. All isolates were tested for the production of fluorescent pigment on Pseudo F (Difco) and pyocyanin on Pseudo P (Difco), casein hydrolysis on skim milk agar (Difco), and growth at 41.5°C in nutrient broth (Difco) after a 24-h incubation. Gram stain and cytochrome oxidase test (26) were performed on all strains, and the API 20NE Test System (API System, S.A., La Balme-les-Grottes, France) was used for biochemical characterization. Quinone profiles were determined in several strains having the same phenotypic characteristics by the method described by Tindall (28).

PAGE of whole-cell proteins. Test strains were grown on nutrient agar (Difco) for 48 h at 30°C. Whole-cell protein extracts were prepared as described previously (15). Supernatants were stored at −20°C for direct use and at −80°C for long-term storage. SDS-PAGE of whole-cell protein extracts was performed by small modifications of the procedure of Laemmli (16) as previously described (15) at 10°C with 16 mA constant current.

Protein electrophoretic patterns were scanned on an Ultrascan laser densitometer (LKB, Bromma, Sweden). An objective comparison and normalization of lanes on different gel slabs was achieved as described previously (30). The densitometric analysis, normalization and interpolation of the protein profiles, and construction of the database were accomplished by using the software package GelCompar W3.0 (29). Computation of the similarity between all pairs of traces was performed with the Pearson product moment correlation coefficient (r), and clustering was achieved by the unweighted-pair group method with arithmetic average algorithm. The numerical analysis was performed by using points 15 to 125 and 135 to 320 of a total of 400 for each interpolated trace.

Partial 16S rDNA sequence data and its analysis. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of PCR products were carried out as described previously (23). Purified PCR products were sequenced with the *Taq* Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed by using the Applied Biosystems 373A DNA Sequencer. The 16S rDNA sequences comprising ~450 nucleotides of the 5′ end of the 16S rDNA were aligned manually against representatives of the domain *Bacteria*. Similarity values were calculated by pairwise comparison of the sequence data.

Typing by RAPD-PCR. Thirty-seven strains, representative of cluster C, defined by numerical analysis of protein electrophoregrams were cultivated in nutrient agar, and DNA was extracted by following the procedure of Pitcher et al. (21). Twelve 10-mer sequences (Operon Technologies, Inc., Alameda, Calif.) with various G+C contents were examined. PCR amplifications were performed in a 50-μl reaction volume with 5 μl of PCR-buffer 10× (Pharmacia Biotech), 200 μM each dNTP (Promega), 0.2 to 2 ng of template DNA per μl, 2.5 μM $MgCl_2$, 20 U of *Taq* DNA polymerase per ml (Pharmacia Biotech), and 3 ng of primer per μl (Operon Technologies Inc.).

Amplifications were performed in a thermocycler, model 9600 (Perkin-Elmer Cetus, Norwalk, Conn.) programmed for an initial denaturation of 5 min at 94°C

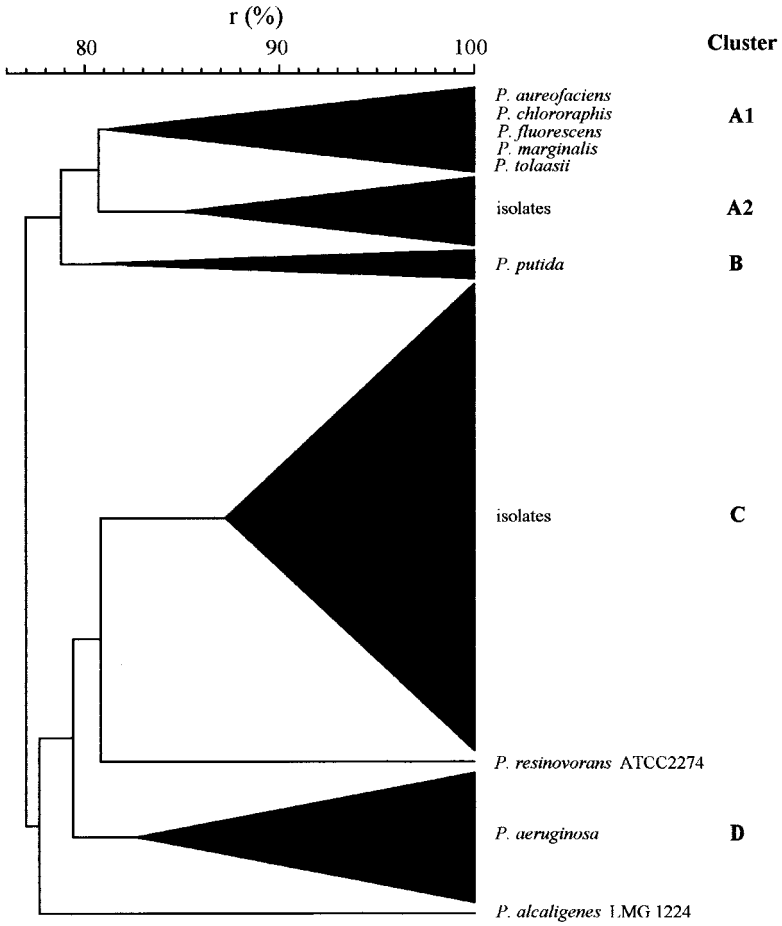

FIG. 1. Dendrogram based on unweighted-pair group average linkage of correlation coefficients (*r*) of protein patterns. The dendrogram was produced by using points 15 to 125 and 135 to 320 of the electrophoretic traces obtained for the strains listed in Table 1.

followed by 40 cycles of 1 min at 94°C, 1 min at 35°C, and 3 min at 72°C. Amplified DNA fragments were separated by electrophoresis on 2% agarose gels in TAE 1× buffer with 0.1 μg of ethidium bromide ml^{-1}. The gels were run for 3 h at 60 V and photographed under UV illumination with Polaroid 667 film.

RESULTS

PAGE of whole-cell proteins. The reproducibility of the technique was verified with *Psychrobacter immobilis* (LMG 1125, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium), and only gels with a level of similarity of 92% or higher were used for numerical analysis. The numerical analysis of whole-cell SDS-PAGE profiles resulted in the formation of four clusters at an 80% similarity level, and two type strains (*P. resinovorans* and *P. alcaligenes*) remained unclustered (Fig. 1).

Cluster B corresponded to *P. putida* and cluster D, formed by strains with very similar protein profiles and clearly distinct from the protein profiles of other species, corresponded to *P. aeruginosa*. Cluster A included all strains of the different biotypes of *P. fluorescens* and 13 isolates. Cluster A could be subdivided into two subclusters at the 82% similarity level, although when compared visually, the electrophoretic protein patterns of the two subclusters were similar. Subcluster A1 corresponded to the type and reference strains of the *P. fluorescens* group, while subcluster A2 was formed by strains isolated during a 4-month period, with the exception of one strain which was isolated 3 years earlier (Fig. 1).

Strains of *P. putida* species (biotypes A and B) formed cluster B, while cluster C, defined at the 87% similarity level, contained the remaining 84 strains isolated from the spring water plant during 5 years. Visual comparisons of the electrophoretic profiles of these isolates were almost identical.

Physiological characterization. All isolates from the spring water plant produced a fluorescent pigment and hydrolyzed casein but did not produce pyocyanin and were not able to grow at 41.5°C. Physiological characterization by API 20NE defined two groups of isolates. One group, including all strains of SDS-PAGE cluster A2 and 35% of the strains of SDS-PAGE cluster C, was identified by the API 20NE Test System as *P. aureofaciens* or *P. fluorescens*. This group was characterized by being capable of reducing NO_3^- to NO_2^- or to N_2 and assimilated all carbon sources tested except malate and adipate (Table 2). A second group was formed by 65% of strains of cluster C which did not use nitrate as terminal electron acceptor and utilized only 4 of the 12 carbon sources (Table 2). Strains of both clusters had ubiquinone 9 as the major respiratory quinone.

16S rDNA analysis. The alignment of the 16S rDNA partial sequence showed that strain 1190T2 as a representative of cluster C was most similar to *P. alcaligenes* (Table 3) and had only 94.2% sequence similarity with *P. aeruginosa.*

RAPD-PCR fingerprinting. Twelve 10-mer primer sequences were tested in the RAPD assay. Under the PCR conditions used, eight of the primers produced an informative banding

TABLE 2. Physiological characteristics of isolates of cluster C determined by API 20NE

RAPD group	Physiological test[a]																				
	NO_3^-	TRP	GLUF	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
A	−	−	−	+	−	−	+	−	+	−	−	−	−	−	−	+	−	+	+	+	+
B	+	−	−	+	−	−	+	−	+	+	+	+	+	−	+	+	−	+	+	+	+

[a] NO_3^-, nitrate reductase; TRP, tryptophanase; GLUF, fermentation of glucose; ADH, arginine dihydrolase; URE, urease; ESC, esculine hydrolysis; GEL, gelatine hydrolysis; PNPG, β-galactosidase; assimilation of: GLU, D-glucose; ARA, L-arabinose; MNE, D-mannose; MAN, mannitol; NAG, *N*-acetyl-glucosamine; MAL, maltose; GNT, D-gluconate; CAP, caprate; ADI, adipate; MLT, L-malate; CIT, citrate; PAC, phenylacetate; OX, oxidase.

pattern. The most discriminating primer, OPA03 (5′ AGTCA GCCAC 3′), was chosen to examine the clonal structure of the isolates of cluster C, as defined by SDS-PAGE analysis. Good reproducibility of the profile was produced with several test strains (Fig. 2). The profiles were compared visually, and two groups could be defined based on two very different patterns of major bands (Fig. 2). Both groups included strains isolated during the 5 years of colonization, but all the strains isolated during the first year had the same major band pattern (pattern A). Subgroups of each RAPD patterns group could be defined if less intense bands were taken into account.

DISCUSSION

Isolates examined in this study were grouped, by computer-assisted analysis of SDS-PAGE protein patterns, into subcluster A2 and cluster C, neither of which included the culture collection strains or the clinical strains of *P. aeruginosa* used for comparison.

Cluster A2 included only strains isolated during a very short period (4 months) and one strain isolated 3 years before; on the other hand, cluster C was formed by strains isolated during the 5 years of the persistent colonization. Strains of cluster A were identified as belonging to the "*P. fluorescens*" group, and strains of cluster C could not be identified in a similarity matrix that comprised species of authentic *Pseudomonas* rRNA group I (5, 20) because none of the type or reference strains were included in this cluster.

Nevertheless, all reference strains were differentiated with species-specific protein patterns, with the exception of *P. tolaasii*, which could not be distinguished from strains of *P. fluorescens*. Moreover, the classical biotypes of *P. fluorescens* tested (*P. chlororaphis*, *P. auerofaciens*, and *P. marginalis*) had similar protein patterns and were not distinguished from each other. The difficulty in providing a basis for distinguishing between *P. fluorescens* biotypes has been discussed previously (3, 25), and even for *P. chlororaphis* and *P. aureofaciens*, DNA-DNA hybridization values cast doubts on the convenience of maintaining two separate taxa (13). Strains of *P. putida* formed a distinct cluster that included strains of biotypes A and B, although some difficulty in distinguishing *P. putida* biotype B from *P. fluorescens* has been previously found (3, 27).

Biochemical characterization of strains of cluster A2 was in agreement with SDS-PAGE protein profile identification. Those strains were identified as *P. fluorescens* or *P. aureofaciens*, with an API 20NE profile identical to the type strain of *P. aureofaciens*, and had ubiquinone 9 as the major quinone. The absence of these strains in the water bottling plant during long periods suggests that they were not related to the persistent colonization and constitute a punctual contamination with a species present in the environment. This population was never again isolated, and its origin was not determined.

Cluster C included the majority of the strains isolated over the long-term colonization. Despite the long period of colonization by an apparently allochthonous organism, numerical analysis of the electrophoregrams clustered the strains at a very high similarity level. The partial sequence of 16S rDNA of a representative strain of cluster C showed high similarity with *P. alcaligenes*, but the type strain of this species was not included in the cluster formed by numerical analysis of protein electrophoregrams. Furthermore, physiological characteristics differentiate strains of cluster C from *P. alcaligenes*, as they produce fluorescent pigment, use glucose for growth, and do not grow at 41.5°C, and some strains do not denitrify, indicating that this strain may not belong to *P. alcaligenes.*

Although these results show that the two populations isolated were different from *P. aeruginosa*, their presence in the bottling system indicated that strains present in the environment were able, at certain times, to overcome the hygienic barriers of the bottling system.

As the strains of cluster C clustered at a very high similarity level, it suggested that they were genotypically very similar and could have the same origin. In spite of the homogeneity of the electrophoretic patterns, biochemical characterization indicated that these isolates were heterogeneous, and two groups could be defined based on biochemical characterization by the API 20NE Test System, one with the same biochemical profile as cluster A2 and a second group with a different profile.

In an attempt to understand the genetic diversity of cluster C, the isolates were typed by RAPD-PCR since only identical or very closely related strains produce the same spectrum of PCR products (32, 33). Two different RAPD patterns were formed, corresponding to the biochemical profiles. Apparently in this study, DNA polymorphism of the bacterial population of cluster C was reflected phenotypically in the API 20NE profiles but not in the SDS-PAGE patterns.

These results indicate that the bottling plant was initially colonized by only one bacterial clone of cluster C, as all strains isolated during the first year were identical. A second colonization appears to have occurred during the second year that did not substitute for the first one, and subsequently both

TABLE 3. 16S rDNA sequence similarity values based on partial sequence comparisons

Strain	% Similarity based on partial sequence analysis
Strain 1190T2 (ID95-356)	100.0
Pseudomonas alcaligenes	99.3
Pseudomonas azotoformans	98.3
Pseudomonas mendocina	97.8
Pseudomonas stutzeri	97.1
Pseudomonas putida	96.9
Pseudomonas fuscovaginae	96.1
Pseudomonas aeruginosa	94.2
Pseudomonas fluorescens	92.8

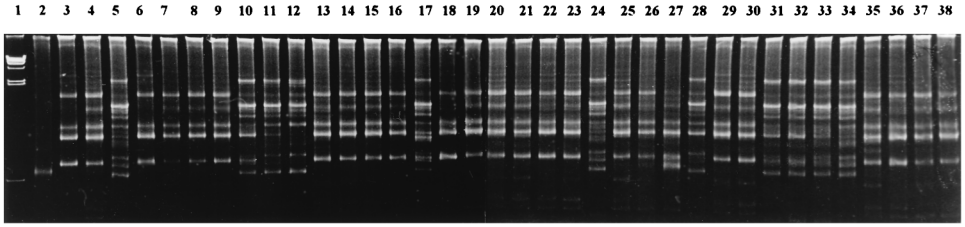

FIG. 2. RAPD-PCR genomic fingerprints for strains of cluster C defined by SDS-PAGE of whole-cell protein. Lane 1, *Hin*dIII-digested lambda DNA as a size marker, and lane 2, negative control (no DNA added). RAPD type A lanes: 3, 1188T3; 4, 1190T1; 6, 0291A1; 7, 1188T10; 8, 1188T16; 9, 1188T18; 10, 1290T9; 13, 1188T14; 14, 1188T9; 15, 1288T1; 16, 1190T1; 18, 1188T8; 19, 0391A7; 20, 1188T10; 21, 1188T13; 22, 1188T18; 23, 0491A14; 25, 0491A16; 26, 0492A1; 27, 0491A5; 30, 1288T4; 35, 0492A2; 36, 0492A5; 37, 0391A29; 38, 0391A25. RAPD type B lanes: 5, 0191A1; 11, 0191A6; 12, 0191A9; 17, 0391A17; 24, 0491A15; 28, 0391A20; 29, 1290T7; 31, 1290T1; 32, 1290T9; 33, 0492A7; 34, 0491A8. Lanes 4, 16, 7, and 20 represent the same strain amplified separately to illustrate the reproducibility of the patterns.

clones persisted for 4 years. The persistence of the same clone of strains, during 4 years, suggests that the strains colonized an environment in the bottling system that was not affected by the disinfection program. This persistence could have two explanations, either resistance of the strains to disinfection, although they disappeared after stringent maintenance of the water plant was implemented, or, most probably, colonization of a part of the plant less affected by the disinfection program where they could grow.

In this study, numerical analysis of SDS-PAGE of whole-cell protein patterns was successfully used to identify and to determine relationships among closely related strains, as has been shown previously (8, 30), and proved useful in identifying bacterial species in a simple ecological system such as a water bottling plant. The method was also able to establish the relationship between strains isolated over a long period of time, although additional characterization was useful in examining the clonal structure of the isolates, as was shown in this study using the API 20NE Test System and RAPD-PCR.

In conclusion, our results indicate that the persistent colonization was due to a single species that was not identified as any of the *Pseudomonas* species used for comparison. The species that colonized the system had two clonal origins. These two organisms persisted for 5 years in the bottling system in an area less affected by the disinfection program. This emphasizes the need for constant vigilance over bottling systems and bottled waters and also demonstrates the advantages of constructing a database on the autochthonous microbial flora of each bottled water, allowing quick identification of allochthonous bacteria. This work also shows that characteristics similar to *P. aeruginosa* on cetrimide agar-naladixic acid can be exhibited by several groups of fluorescent pseudomonads other than *P. aeruginosa* and emphasizes the need for confirmatory results before a decision regarding water quality is made.

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