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Computer-aided comparison of protein electrophoretic patterns for grouping and identification of heterotrophic bacteria from mineral water

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A.C. FERREIRA, P.V. MORAIS, C. GOMES AND M.S. DA COSTA. 1996. The microflora of a natural mineral water was studied immediately after bottling (T0) and after 7 d storage (T7) during 6 months, and isolates were clustered by SDS-PAGE of whole-cell protein profiles. Isolates from each cluster were further characterized by API 20NE, fatty acid composition and quinone profiles. The numerical analysis of the electrophoregrams of all bacteria isolated from the mineral water formed 15 clusters and five unclustered strains. Except for five minor clusters, all clusters were composed of strains isolated over several months. The numerical analysis of the electrophoregrams of bacteria isolated immediately after bottling formed 15 clusters while after 7 d storage only four of these populations could be isolated, indicating that populations present in the mineral water were stable and that changes occurring after bottling probably resulted from a selection process. Only one unclustered strain was identified simultaneously by all the systems, as *Sphingomonas paucimobilis*. The monitoring of the aquifer and the bottling system, and the construction of a large database with bacteria of the autochthonous flora allows the detection of alterations in the aquifer by changes in the microflora.

INTRODUCTION

Natural bottled mineral waters cannot be treated by disinfection, filtration or pasteurization and, therefore, are not free from bacteria. As a consequence bottled still mineral waters usually have a large population of heterotrophic bacteria (Buttiaux and Boudier 1960; Schwaller and Schmidt-Lorenz 1980; Morais and da Costa 1990; Mavridou 1992; Hunter 1993) present in different metabolic states (Oger *et al.* 1987; Pedersen and Ekendahl 1990; Ferreira *et al.* 1994), that are able to survive and/or multiply for extended periods of time with very low concentration of nutrients.

EC directives (Anon. 1980) established that the 'revivable total counts of natural mineral waters may only be that resulting from natural increase in the bacteria content which it had at the source' and therefore, heterotrophic plate counts (HPC) are mandatory immediately after bottling. Although several studies showed that multiplication of bacteria originating from the aquifer and/or bottling system and present in the bottle immediately after bottling, occurred during storage, the precise identification of these bacteria has not been determined in most cases (Oger *et al.* 1987; Bischofberger *et al.* 1990; Mavridou 1992; Ferreira *et al.* 1994).

Several workers have investigated the microbial flora of bottled water and found that the majority of culturable bacteria are oxidative and stain Gram-negative (Schwaller and Schmidt-Lorenz 1980; Quevedo-Sarmiento *et al.* 1986; Manaia *et al.* 1990; Morais and da Costa 1990; Guillot and Leclerc 1993). However, further characterization has been difficult or misleading, and isolates have rarely been identified to the species level. Moreover, there are no reference methods for the identification and characterization of these microorganisms. A few simplified groups of biochemical tests for their identification have been suggested (Spino 1985; Holmes *et al.* 1986), but most isolates from deep aquifers have only been physiologically characterized by different rapid identification systems. The problem is that these rapid identification systems have been developed for the identification of pathogenic species and as a consequence often fail to identify

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environmental isolates (Balkwill *et al.* 1989; Amy *et al.* 1992). Using these systems, most of the strains have been assigned to the genus *Pseudomonas* sensu lato or to the *Flavobacterium-Cytophaga-Flexibacter* group, even though these groups are heterogeneous and have been recently split in several new genera and species (Willems *et al.* 1989, 1990), or have been simply grouped in biotypes.

The purpose of the present study was the characterization, by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) of whole-cell protein profiles, API 20NE, fatty acid composition and quinone profile, of bacterial strains isolated from bottled mineral water immediately after bottling and after 7 d storage, during a 6 month period. The polyphasic approach was used to obtain physiological data and identify the culturable chemoheterotrophic bacteria of a natural mineral water and examine the diversity and stability of the bottling system in the plant and the bottled water, over this period. The different methodologies were also compared in the identification of mineral water isolates. Strains isolated immediately after bottling and after 7 d storage at 22°C and at 37°C were characterized permitting the study of the influence of storage in the selection process of the bacterial populations that occurs in the bottle.

MATERIALS AND METHODS

Strains and culture conditions

Bottled mineral water was taken monthly during 6 months directly from the filling line at a mineral water bottling plant in Central Portugal. The first sample of each batch was maintained at 4°C during transportation to the laboratory, examined within 3 h of bottling and designated immediately after bottling samples (T0). The other bottles of water were maintained in the dark at room temperature and examined after 7 d storage (T7). Isolations of water samples were performed immediately after bottling (T0) and after 7 d storage (T7) on R_2A medium (Reasoner and Geldreich 1985) at 22°C for 21 d and at 37°C for 5 d, as described previously (Ferreira *et al.* 1994).

The culture collection strains used in this study are listed in Table 1. All strains were maintained at −80°C in Nutrient Broth (Difco) containing 15% glycerol.

Characterization by SDS-PAGE

Ten bacterial strains from each sampling time (T0 and T7) and from the two recovery temperatures (22°C and 37°C) for a total of 200, were examined by SDS-PAGE of their whole-cell protein extracts.

The strains were grown on Nutrient Agar (NA; Difco) for 48 h at 30°C. The cells were harvested from the plates, centrifuged and heated at 100°C in the presence of β-mercaptoethanol and sodium dodecyl sulphate (SDS) to obtain whole-cell protein extracts using the procedure of Kiredjian *et al.* (1986). SDS-PAGE of whole-cell protein extracts was performed using the procedure of Kiredjian *et al.* (1986) at 10°C with a constant current of 16 mA and a final polyacrylamide content of 12% with 0·1% SDS.

After densitometer recording of the gel, the traces were normalized by including the protein extract of the strain *Psychrobacter immobilis* (LMG 1125), as a reference, four times in each gel. Computation of the similarity between all possible pairs of traces was performed using the Pearson product moment correlation coefficient (r) and clustering was achieved by unweighted pair group method with arithmetic averages (UPGMA) algorithm. The densitometric analysis, normalization and interpolation of the protein profiles, numerical analysis and construction of the database was per-

Table 1 Culture collection and type strains used in the study

Strain	Reference number
Acinetobacter sp.	LMG 1144*
Ac. calcoaceticus	ATCC 23055^T†
Ac. baumannii	ATCC 19606^T
Ac. lwoffii	ATCC 9957
Alcaligenes denitrificans	CCDB 1375‡
Alc. xylosoxydans subsp. *xylosoxydans*	ATCC 27061
Alteromonas putrefaciens	ATCC 8071^T
Aquaspirillum gracile	ATCC 19624^T
Caulobacter crescentus	ATCC 15252^T
Citrobacter freundii	ATCC 8090
Flavobacterium aquatile	LMG 4008^T
Fl. meningosepticum	ATCC 13253^T
Fl. mizutaii	LMG 8340^T
Flexibacter canadensis	LMG 8368^T
Pseudomonas aeruginosa	ATCC 27853
Ps. aureofaciens	LMG 5832
Ps. cepacia	ATCC 25416^T
Ps. diminuta	ATCC 11568^T
Ps. fluorescens	ATCC 13525
Ps. marginalis pv. *marginalis*	LMG 5170
Ps. putida	LMG 5835
Ps. testosteroni	ATCC 11996^T
Psychrobacter immobilis	LMG 1125
Sphingobacterium multivorum	LMG 8342^T
S. spiritivorum	DSM 2582§
Sphingomonas adhesiva	LMG 10922^T
S. capsulata	LMG 2830^T
S. paucimobilis	LMG 1227^T

*Laboratorium voor Microbiology, Gent, Belgium; †American Type Culture Collection, Rockville, MD, USA; ‡Culture Collection, Department of Biochemistry, Coimbra, Portugal; §Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

formed using the PC-Windows directed software package GelCompar 3.0 (Applied Maths, Kortrijk, Belgium) as described previously (Vauterin and Vauterin 1992).

Characterization by API 20NE identification system

The biochemical features of 103 strains from the clusters formed by the numerical analysis of whole-cell proteins by SDS-PAGE of the bacteria isolated from the mineral water, were examined using the API 20NE identification system (bioMérieux, La-Balme Les Grottes, France), according to the instructions of the manufacturer.

Analysis of isoprenoid quinones

The analysis of respiratory lipoquinones was performed on 34 strains from the clusters formed by the numerical analysis of whole-cell proteins by SDS-PAGE of the bacteria isolated from the mineral water and on the reference strains, as well. The strains were grown on NA for 48 h at 30°C, the lipoquinones were extracted from freeze-dried cells and purified by thin layer chromatography as described by Tindall (1989). The lipoquinones were separated on a Gilson model HPLC using a reverse phase (RP18) column (Spherisorb, S5, ODS2) with methanol : heptane (10 : 2 v/v) as the mobile phase and detected at 270 nm.

Analysis of fatty acid methyl esters

The fatty acids methyl esters (FAMEs) of 36 strains from the clusters formed by the numerical analysis of whole-cell proteins by SDS-PAGE of the bacteria isolated from the mineral water and the reference strains, were grown in Tryptic Soy Broth (BBL) at 28°C for 48 h ± 2 or in NA when growth was not achieved on the former medium. FAMEs were obtained and quantified using the protocol of the MIDI Identification System (MIDI; Microbial ID, Inc., Newark, DE, USA). The identification and quantification of FAMEs as well as the numerical analysis of the fatty acid profiles, were performed using the MIDI software package.

RESULTS

The reproducibility of SDS-PAGE was verified using *Psychrobacter immobilis*, and only the gels with a similarity of 93% or higher (mean 95%) were used for the numerical analysis.

The numerical analysis of the protein electrophoregrams of all the bacteria isolated throughout the sampling months, formed 15 clusters and five unclustered strains, at an 80% similarity level or higher (Fig. 1). Five of these clusters (A, B, C, D and E) represented major populations of this mineral water and constituted between 6·5% (cluster E) and 35·5% (cluster A) of the total bacteria isolated (Table 2). One population (cluster A) included strains isolated from all sampling months (except April) immediately after bottling and after 7 d storage and from both recovery temperatures.

The strains isolated immediately after bottling (T0) formed 15 different clusters (Fig. 2a) but the strains isolated after 7 d storage (T7) formed only four separate clusters (Fig. 2b), based on 80% similarity or higher. Except for populations A, B, C, H and strain C8-47 belonging to cluster L that were recovered both at T0 and T7, all the other populations were recovered only at T0. Nevertheless, populations recovered at T7 were always present at T0.

The numerical analysis of the protein patterns of the strains isolated from the mineral water and the reference strains, revealed that only population P could be identified as *S. adhesiva* based at an 85% similarity level, and unclustered strain C9-13 was identified as *S. paucimobilis*, at an 80% similarity level (Fig. 1). Population J and the reference strains of *Ps. marginalis*, *Ps. aureofaciens*, *Ps. putida* and *Ps. fluorescens*, had major bands of similar molecular weight and grouped at 82% similarity level. However, the differences in the remaining band pattern clearly distinguished them. A second cluster analysis was performed in which the principal protein band was omitted, revealing that the strains grouped at a similarity level of 75% (results not shown).

The clusters formed after numerical analysis of the SDS-PAGE protein patterns corresponded to the groups formed after the utilization of the API 20NE identification system. Each group had a characteristic and distinct physiological profile (Table 3). Only four populations and an unclustered strain could be identified at a level greater than 95% as *Alc. xylosoxydans* subsp. *denitrificans* (cluster E), CDC gr. IV C-2 (cluster F), *S. paucimobilis* (cluster L), *Ps. alcaligenes* (cluster M) and unclustered strain C9-13 as *S. paucimobilis*. Unclustered strain C4-47 was identified as *Sphingobacterium multivorum* but at a lower identification level.

The results of the quinone analysis showed that ubiquinone 8 was predominantly found (nine populations and two unclustered strains), ubiquinone 10 was the second most common type (five populations and two unclustered strains) and ubiquinone 9 was only found in one population (Table 3). Menaquinones were not found in the micro-organisms examined.

The clusters formed after numerical analysis based on the fatty acid methyl esters profile of the strains were in agreement with the clusters formed by SDS-PAGE and the API 20NE system. Using the MIDI identification system only one unclustered strain (C1-12) and one population (L) were identified as *Acidovorax facilis* and *S. paucimobilis*, respectively, based on a similarity index greater than 0·8 (Table 3).

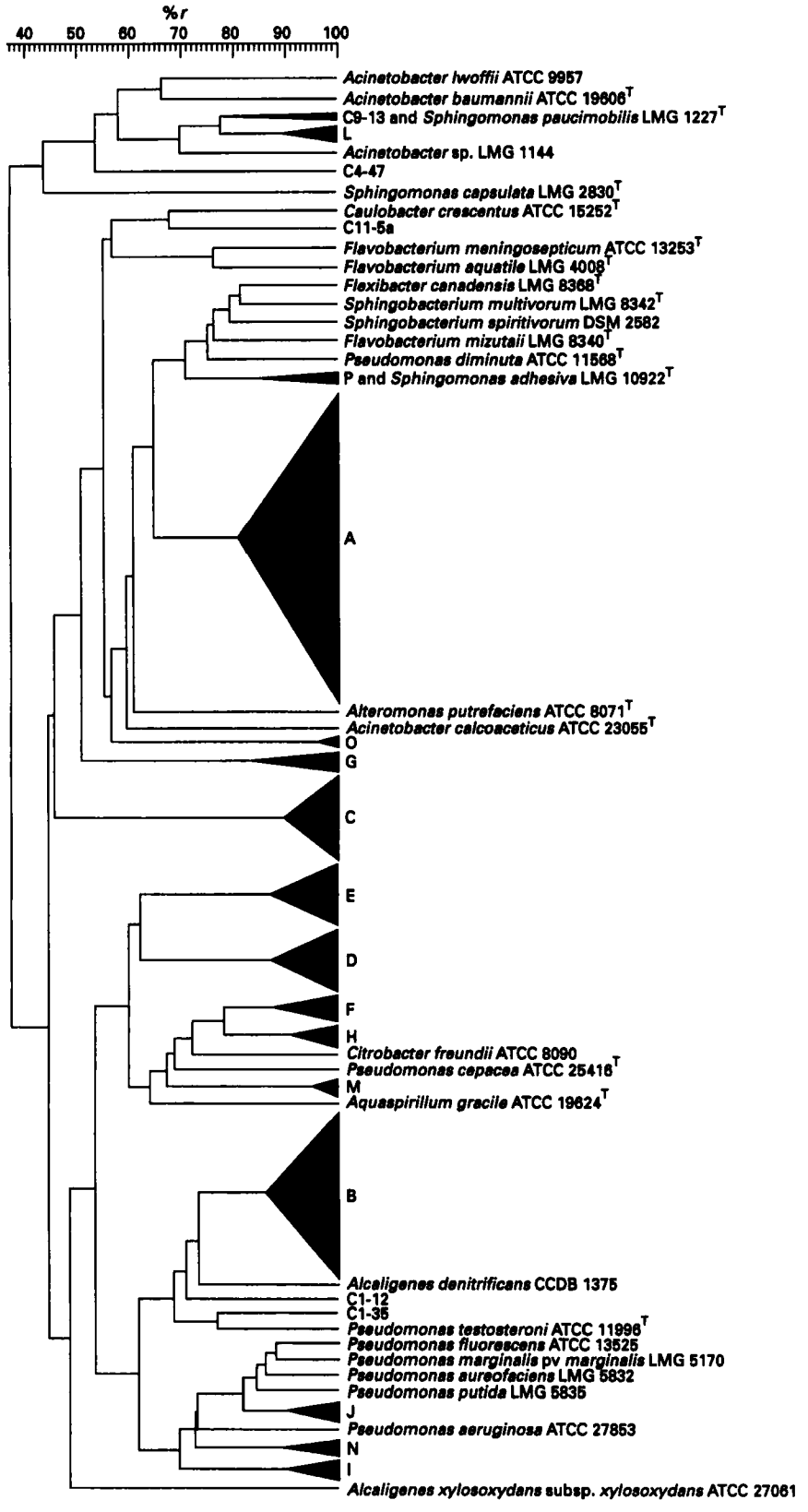

Fig. 1 Dendrogram of the numerical analysis based on bacterial whole-cell protein SDS-PAGE profiles of reference strains and of 200 strains isolated from a mineral water during 6 months, immediately after bottling (T0) and after 7 d storage (T7) at 37°C and 22°C. The percentage similarities were determined by the Pearson product-moment correlation coefficient and unweighted pair group average linkage clustering (UPGMA). The populations were formed at an 80% similarity level or higher

Table 2 Constitution of the populations isolated from the mineral water during 6 months, immediately after bottling (T0) and after 7 d storage (T7) at 37°C and 22°C (the populations were formed after numerical analysis of the protein electrophoregrams based on 80% similarity and higher)

Cluster	Number of isolates	Sampling time	Isolates
A	71	T0	J1*, J2, J3, J4, J5, F1, F2, F3, F5, F6, F7, F8, F9
		T7	N21, N22, N23, N24, N25, N26, N27, N28, N29, N31, N33, N34, N35, N36, N37, N38, N39, N40, D21, D22, D23, D24, D25, D26, D27, D28, D29, D30, D31, D33, D34, D35, D36, D38, D39, D40, F21, F22, F23, F24, F26, F28, F29, F30, F32, F35, F36, M23, M31, M32, M33, M34, M35, M36, M37, M38, M39, M40
B	35	T0	N1, N7, N8, N9, A2, A4, A5, A8, A16, A18
		T7	D32, F25, F33, F34, F37, F38, F39, F40, A21, A22, A24, A25, A26, A27, A28, A29, A30, A31, A32, A33, A34, A35, A36, A37, A38
C	18	T0	M1, M5, M6, M7, M8, M10
		T7	N30, F31, M21, M22, M24, M25, M26, M27, M28, M29, M30, A23
D	14	T0	J7, J11, J12, J13, J14, J15, J16, J17, J18, J20, F14, F15, F17, F20
		T7	
E	13	T0	F11, F12, F13, F16, F18, F19, M11, M12, M14, M15, M17, M19, M20
		T7	
F	6	T0	N13, N15, N17, N18, N19, N20
		T7	
G	5	T0	N4, N5, N6, N10, N14
		T7	
H	5	T0	N12, N16
		T7	N32, A39, A40
I	5	T0	M13, M16, M18, A13, A20
		T7	
J	5	T0	A11, A12, A14, A15, A17
		T7	
L	5	T0	J8, J19, F4, A6
		T7	F27
M	4	T0	N3, A3, A7, A10
		T7	
N	4	T0	J6, J9, J10, F10
		T7	
O	3	T0	M2, M3, M9
		T7	
P	2	T0	A9, A19
		T7	
Unclustered			
C1-12	1	T0	N2
C1-35	1	T0	N11
C4-47	1	T7	D37
C9-13	1	T0	M4
C11-5a	1	T0	A1

*Strain designations are as follows; N, November; D, December; J, January; F, February; M, March; A, April; T0, immediately after bottling; T7, after 7 d storage; numbers 1–10 and 21–30 indicate that the bacteria were isolated from R_2A at 37°C; numbers 11–20 and 31–40 indicate that the bacteria were isolated from R_2A at 22°C.

Population B was identified as *Burkholderia solanacearum* and population N as *Bradyrhizobium japonicum*, based on a lower similarity index. Unclustered strain C9-13 was identified by the MIDI system as *Ps. saccharophila* by the TSBA Library

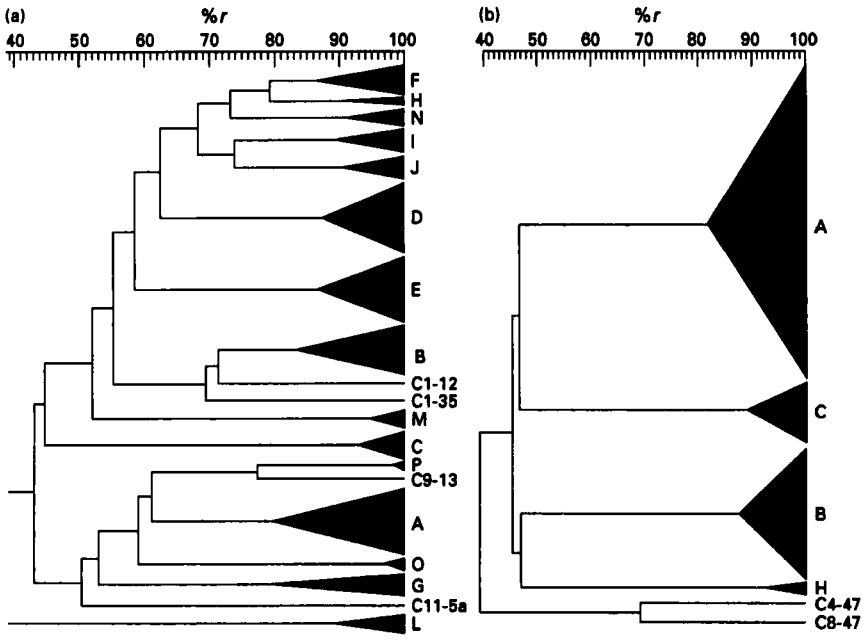

Fig. 2 Dendrogram of the cluster analysis based on bacterial whole-cell protein SDS-PAGE profiles of 100 strains isolated immediately after bottling (a), and of 100 strains isolated after 7 d storage (b), at 37°C and 22°C, from a mineral water during 6 months. The percentage similarities were determined by the Pearson product-moment correlation coefficient and unweighted pair group average linkage clustering (UPGMA). The populations were formed at an 80% similarity level or higher

Table 3 Phenotypic and chemotaxonomic characterization of the populations isolated from the mineral water

Cluster	API 20NE profile	Probable identification	Quinone profile	MIDI identification system	Similarity index	SDS-PAGE
A	1 460 0/20/24	—	10	—	—	—
B	1 2/351 557	—	8	*Burkholderia solanacearum*	0·718	—
C	1 000 004	—	8	—	—	—
D	1 253 557	—	8	—	—	—
E	1 200 477	*Alcaligenes xylosoxydans* subsp. *denitrificans*	8	—	—	—
F	0 200 477	CDC gr. IV C-2	8	—	—	—
G	1 430 244/5	—	8	—	—	—
H	1 244 004	—	8	—	—	—
I	0 047 704	—	10	—	—	—
J	1 357 555	—	9	—	—	—
L	0 463 30/44	*Sphingomonas paucimobilis*	10	*Sphingomonas paucimobilis*	0·883	—
M	1 040 464	*Pseudomonas alcaligenes*	8	—	—	—
N	1 600 004	—	10	*Bradyrhizobium japonicum*	0·546	—
O	1 010 204	—	8	—	—	—
P	0 462 1/264	—	10	—	—	*Sphingomonas adhesiva*
Unclustered						
C1-12	1 045 444	—	8	*Acidovorax facilis*	0·914	—
C1-35	0 245 555	—	8	—	—	—
C4-47	0 463 204	*Sphingobacterium multivorum*	10	—	—	—
C9-13	0 463 345	*Sphingomonas paucimobilis*	10	*Sphingomonas paucimobilis**	0·630	*Sphingomonas paucimobilis*
C11-5a	1 100 054	—	8, 9	—	—	—

*In the Clinical Database. In the TSBA Database this strain was identified as *Pseudomonas saccharophila* (0·578).
Populations were defined by SDS-PAGE analysis at 80% similarity or higher and characterized by API 20NE, quinone profile and fatty acid composition. Presumptive identification with API 20NE was considered at an identification of greater than 95%. Presumptive identifications based on MIDI analysis of FAMEs were considered with a similarity index greater than 0·5.

or as *S. paucimobilis* by the Clinical Library. However, this strain has ubiquinone 10 as a major quinone and hydroxy fatty acids characteristic of the genus *Sphingomonas*.

DISCUSSION

In this study, isolates taken monthly during six consecutive months from the filling line of a mineral water bottling plant and analysed immediately after bottling (T0) and after 7 d storage (T7), were grouped by numerical analysis of whole-cell protein electrophoregrams. Strains recovered at the bottling plant (T0) formed 15 distinct clusters. These results showed the diversity of bacterial populations present in the mineral water at the bottling plant without dominant physiological types. Diversity of heterotrophic bacteria has also been found in several oligotrophic environments and seems to be a characteristic of the bacterial flora of deep groundwaters (Balkwill *et al.* 1989; Chapelle and Lovley 1990; Fredrickson *et al.* 1991).

Each cluster with the exception of the minor clusters F, G, J, O and P, were composed of strains isolated over several months, indicating that the major bacterial populations were stable over this period. Populations isolated only during 1 month (sampling time) probably constituted minor populations of the mineral water flora which were sometimes below the detection level or were detected as a consequence of changes that may occur in the bottling system due to maintenance.

The microbial diversity found in the water at the bottling plant decreased drastically after storage. Ten populations representing 65% of the flora of the mineral water were not isolated after storage because they did not survive or were unable to multiply. All the populations recovered after storage were already present in the water at the bottling plant, showing that bacteria in the bottled water originated from bacteria present in the mineral water aquifer and/or bottling system, that multiplied after bottling. These are populations able to survive in the bottle without the addition of nutrients. Multiplication of bacteria as it occurs after bottling, is probably the strategy used by these populations for adaptation and survival and is a strategy characteristic of bacteria from oligotrophic environments (Roszak and Colwell 1987; Morita 1990).

The microbial flora after storage was stable during the sampling period, since populations A, B and C made up 95% of the isolates, showing that the small variations in the flora of the mineral water observed during the experimental period, at the bottling plant, did not affect the major populations present in the bottled water. Therefore, the variations observed at T0 could not be interpreted as disruptions in the equilibrium of the mineral water flora. According to the EC legislation (Anon. 1980), the autochthonous flora of a mineral water aquifer should be stable and characteristic, as it appears to be the case in this water.

Computer-aided whole-cell protein SDS-PAGE was used for grouping all strains and a correlation was found between protein patterns and biochemical characteristics, as each SDS-PAGE cluster had a distinct API 20NE profile.

API identification systems are often used, although they are generally incapable of identifying aquatic bacteria (Balkwill *et al.* 1989; Morais and da Costa 1990; Fredrickson *et al.* 1991). In this study API 20NE was used to characterize, or if possible identify, the clusters formed by numerical analysis of the electrophoregrams of the isolates. Only strains of four clusters and one unclustered strain were identified as *Alc. xylosoxydans* subsp. *denitrificans* (cluster E), *S. paucimobilis* (cluster L and unclustered strain C9-13), *Ps. alcaligenes* (cluster M) and CDC gr. IV C-2 (cluster F). Only two identifications could be confirmed with the techniques used: one by API 20NE and FAMEs analysis (population L) and another by SDS-PAGE whole-cell protein, API 20NE and FAMEs analysis (unclustered strain C9-13), as *S. paucimobilis*. These identifications were in agreement with the isoprenoid quinone profiles of the strains. *Sphingomonas paucimobilis* together with *Ps. putida* were the only species common to several French mineral water sources (Guillot and Leclerc 1993) and are probably widespread in groundwater aquifers (Balkwill *et al.* 1989; Fredrickson *et al.* 1991; Amy *et al.* 1992). Moreover, based on numerical analysis of phenotypic characteristics, three clusters of bacteria of the genus *Pseudomonas* were also found in several French mineral waters (Elomari *et al.* 1995).

Each identification system used identified some of the isolates but only one cluster and an unclustered strain were identified simultaneously by all systems. The clusters did not match the type strains used, therefore more characteristics have to be used to ascertain whether or not populations of the mineral water resemble previously identified strains or are new species.

In addition, numerical analysis of whole-cell electrophoretic protein patterns of a large number of bacterial strains, stored as digitalized processed electrophoretic traces of representative strains on computer files, allows the construction of large databases. Databases constructed with previously identified strains and mineral water isolates, will allow the rapid identification of alterations in the aquifer or the bottling system by the occurrence of strains not usually present in the autochthonous flora. Moreover, the monitoring of the bottling system during 6 months allowed us to show the stability and the specific characteristics of the microflora of the mineral water.

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