Alteromonas hispanica sp. nov., a polyunsaturated-fatty-acid-producing, halophilic bacterium isolated from Fuente de Piedra, southern Spain

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Strain F-32^T, which produces exopolysaccharides and contains polyunsaturated fatty acids, was isolated from a hypersaline water sample collected from Fuente de Piedra (southern Spain). Phylogenetic analyses indicated conclusively that the strain in question belonged to the genus *Alteromonas*. Phenotypic tests showed that it could be assigned to the genus *Alteromonas* although it had a number of distinctive characteristics: it is moderately halophilic, growing best with 7.5–10% w/v NaCl; it grows at 4 °C and produces H₂S; it does not grow with D-cellobiose, D-fructose, D-galactose, D-glucose or lactose as sole sources of carbon and energy; and its fatty-acid profile is typical of *Alteromonas* but it also contains a large amount of an unusual acid with three double bonds [18:3 ω 6c (6, 9, 12); 5.01%, w/v]. The major isoprenoid quinone is Q₈. The DNA G + C content is 46.3 mol%. The phylogenetic, phenotypic and genetic properties of strain F-32^T place it within a novel species, for which the name *Alteromonas hispanica* sp. nov. is proposed. The type strain is F-32^T (= CECT 7067^T = LMG 22958^T).

The genus *Alteromonas* was isolated and named by Baumann *et al.* (1972) [description emended by Gauthier *et al.* (1995) and later by Van Trappen *et al.* (2004)] and originally contained a phylogenetically and phenotypically heterogeneous group of Gram-negative, heterotrophic, marine bacteria, motile by a single polar flagellum. Many of its species, however, have gradually been reclassified into other genera such as *Marinomonas*, *Pseudoalteromonas* and *Shewanella* (Van Landschoot & de Ley, 1983; MacDonell & Colwell, 1985; Coyne *et al.*, 1989; Gauthier *et al.*, 1995; Sawabe *et al.*, 2000; Ivanova *et al.*, 2000, 2001). Nowadays *Alteromonas* comprises only four recognized species: *Alteromonas macleodii* (Baumann *et al.*, 1972, 1984; Gauthier *et al.*, 1995; Yi *et al.*, 2004), *Alteromonas marina* (Yoon *et al.*, 2003), *Alteromonas stellipolaris* (Van Trappen *et al.*, 2004) and *Alteromonas litorea* (Yoon *et al.*, 2004). Together with the genera *Glaciecola* (Bowman *et al.*, 1998) and *Aestuariibacter* (Yi *et al.*, 2004), it is included within the family *Alteromonadaceae* (Ivanova *et al.*, 2004).

The members of the *Alteromonadaceae* are Gram-negative, rod-shaped, motile bacteria that do not form endospores or microcysts. They are chemo-organotrophs, have a respiratory metabolism and use oxygen as electron acceptor. They do not denitrify or have dihydrolase activity. All the species require Na⁺ for growth and in most of them the major isoprenoid quinone is Q₈. The major fatty acids are 16:0, 16:1 ω 7c and 18:1 ω 7c. All the species have been isolated from marine habitats (coastal sea waters and marine invertebrates). The family is a member of the *Gammaproteobacteria* with the following nucleotide sequence characteristics: 304 (A), 734 (A), 736 (T), 770 (T), 809 (A). The type genus is *Alteromonas* (Ivanova *et al.*, 2004).

Van Trappen *et al.* (2004) made the last emended description of *Alteromonas*, which was based on Gauthier *et al.* (1995), when they discovered that members of the genus were prosthecate, budding bacteria. In addition to the traits reported for the family, the genus also includes bacteria which are catalase- and oxidase-positive, unpigmented and not luminescent. Species of the genus do not usually grow at 4 °C, do not accumulate poly- β -hydroxybutyrate (PHB) and require a sea-water base for growth but not organic growth factors. *A. macleodii*, *A. marina* and *A. stellipolaris* produce buds and prostheca when they grow at low temperatures (12–20 °C) for 3 or more days in complex media with added sea salts. The G + C content of the DNA is 44 to 47 mol%. The type species is *A. macleodii*.

Published online ahead of print on 15 July 2005 as DOI 10.1099/ij.s.0.63809-0.

Abbreviations: EPS, exopolysaccharide; PHB, poly- β -hydroxybutyrate; PUFA, polyunsaturated fatty acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Alteromonas hispanica* F-32^T is AY926460.

In this study we describe strain F-32^T of *Alteromonas*, for which we propose the name *Alteromonas hispanica*. This strain is the only representative of the genus *Alteromonas* identified so far that has been isolated from an inland hypersaline habitat and produces polyunsaturated fatty acids (PUFAs) at a relatively high incubation temperature (32 °C), which contradicts the notion that only barophilic and psychrophilic marine species are able to produce significant levels of PUFAs (Nogi *et al.*, 1998; Russell & Nichols, 1999).

The strain studied here was isolated in 1998 from a hypersaline water sample taken from Fuente de Piedra (Málaga, southern Spain), an inland, hypersaline wetland, during a wide research programme aimed at discovering novel halophilic bacteria for biotechnological purposes (Martínez-Cánovas

et al., 2004; Quesada *et al.*, 2004). Strain F-32^T was isolated using MY medium (Moraine & Rogovin, 1966), supplemented with 10 % w/v marine salts (Rodríguez-Valera *et al.*, 1981). The strain was kept and routinely grown in MY medium, with the addition of 7.5 % w/v marine salts for optimum growth. Strain F-32^T was originally characterized phenotypically by Martínez-Cánovas *et al.* (2004) by means of 135 tests. Its flagellation pattern was determined in this work by transmission electron microscopy of negatively stained cells. The phenotypic data are given in the species description. Table 1 shows the main phenotypic differences between strain F-32^T and the other four species of the genus *Alteromonas*. The same table contains the G + C content of strain F-32^T estimated from the midpoint value (T_m) of the DNA thermal denaturation profile, as described in Martínez-Cánovas *et al.* (2004).

Table 1. Phenotypic characteristics and DNA G + C content distinguishing *Alteromonas hispanica* from the other species of *Alteromonas*

Data from Baumann *et al.* (1972, 1984), Gauthier *et al.* (1995), Ivanova *et al.* (2004), Van Trappen *et al.* (2004), Yi *et al.* (2004), Yoon *et al.* (2003, 2004) and from this study. ND, Not determined; (+) the majority of the strains are positive; W, weakly positive reaction.

Characteristic	F-32 ^T	<i>A. macleodii</i>	<i>A. marina</i>	<i>A. stellipolaris</i>	<i>A. litorea</i>
Origin of the type strain	Hypersaline water, Fuente de Piedra (Málaga, southern Spain)	Sea water	Sea water, East Sea in Korea	Antarctic sea water	Intertidal sediment, Yellow Sea of Korea
Cell size (µm)	1–2 × 0.75	1.8–2.5 × 0.9–1.3	2.5–4 × 1–1.2	2–7 × 0.4	2–4 × 0.9–1.2
Bud/prosthecum	+	+	+	+	ND
Pigmentation	Cream	Cream	Cream	Brown diffuse	Cream
PHB	+	–	ND	–	ND
EPS	+	ND	–	ND	ND
NaCl concentration for growth (% w/v)					
Range	7.5–15	ND	2–15	1–10	2–14
Optimum	7.5–10	ND	2–5	ND	2–5
Sea-salt concentration for growth (% w/v)					
Range	3–20	ND	ND	ND	ND
Optimum	5–15	ND	ND	ND	ND
Growth at 4 °C	+	–	+	+	–
Acid from:					
Glucose	–	+	–	–	–
Maltose	+	+	–	ND	+
Sucrose	–	+	+	W	–
D-Trehalose	–	+	+	+	+
H ₂ S from cysteine	+	ND	–	–	–
Growth on:					
D-Cellobiose	–	+	ND	(+)	ND
D-Fructose	–	+	+	+	+
D-Galactose	–	+	+	+	+
D-Glucose	–	+	+	+	+
Lactose	–	+	+	(+)	+
D-Mannitol	+	+	–	+	–
L-Serine	–	–	ND	W	ND
G + C content (mol%)	46.3	44.9–46.4	44–45	43–45	46

Fig. 1. Electron micrographs of negatively stained preparations of cells of strain F-32^T showing (a) a polar flagellum (F) and (b) prostheca (P) and buds (B). Cells were stained with 1 % w/v uranyl acetate in 0.4 % w/v sucrose. Bars, 0.3 μm.

Colonies used for the analysis of prostheca and buds were grown on MY (7.5 % w/v) for 7 days at 12 °C, as recommended by Van Trappen *et al.* (2004) (Fig. 1). Exopolysaccharide (EPS) and PHB granules were observed in cells grown on the same medium after 1 day's incubation at 32 °C (Fig. 2). Transmission electron micrographs were taken using the methods described by Bouchotroch *et al.* (2001).

Phylogenetic analyses were made according to Bouchotroch *et al.* (2001). We determined the near-complete 16S rRNA gene sequence of strain F-32^T (1479 bp). The sequence obtained was compared to reference 16S rRNA gene sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Centre of Biotechnology Information database using the BLAST search. Phylogenetic analysis was made using the software MEGA version 3.0 (Kumar *et al.*, 2004) after multiple data alignments by CLUSTAL_X (Thompson *et al.*, 1997). Distances and clustering were determined using the neighbour-joining and maximum-parsimony methods. The stability of clusters was ascertained by performing a bootstrap analysis (1000 replications). The phylogenetic tree obtained by neighbour-joining is shown in Fig. 3. The maximum-parsimony algorithm gave a similar result (data not shown). Our results indicate conclusively that strain F-32^T belongs to the genus *Alteromonas*, showing 95.3–97.4 % similarity to the other four species within the genus. As expected, the next closest neighbours were the other members of the family *Alteromonadaceae*, *Glaciecola* (Ivanova *et al.*, 2004) and *Aestuariibacter* (Yi *et al.*, 2004). The nucleotide-sequence characteristics of the family *Alteromonadaceae*, 304 (A), 734 (A), 736 (T), 770 (T), 809 (A) (Ivanova *et al.*, 2004), were present in strain F-32^T.

The fatty acids and quinones were analysed at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) in a culture of strain F-32^T made at 32 °C in

Fig. 2. Electron micrographs of thin-section preparations of cells of strain F-32^T showing (a) EPS and PHB granule, (b) bud formations (B) and (c) prostheca (P). Thin-section preparations were stained with lead citrate and 1 % w/v uranyl acetate. Bars, 0.3 μm.

Fig. 3. Phylogenetic relationship between *Alteromonas hispanica* F-32^T and the other species of the genus *Alteromonas*, together with members of the family *Alteromonadaceae* and other related gammaproteobacteria. The tree was constructed using the neighbour-joining algorithm. Only bootstrap values above 50% are shown (1000 replications). Bar, 2% estimated sequence divergence.

MY 7.5% w/v (see Table 2). The fatty-acid profile of strain F-32^T was typical of *Alteromonas* species, with a predominance of 16:0, 16:1 ω 7 c and 18:1 ω 7 c , but it also contains large amounts of 16:0 N alcohol, 17:0 10-methyl, 18:0 and an unusual unsaturated fatty acid [18:3 ω 6 c (6, 9, 12)]. PUFAs are rare in mesophilic bacteria and have not been

found so far in any *Alteromonas* species. The predominant respiratory quinone was Q₈ (ubiquinone 8, 96.5%; ubiquinone 7, 3.5%).

In conclusion, polyphasic analyses demonstrate that the new isolate belongs to a consistent taxon and represents a novel

Table 2. Major fatty acids in the *Alteromonas* species

Data from this work and from Van Trappen *et al.* (2004) and Yoon *et al.* (2004). —, Not detected or less than 1%.

Fatty acid	F-32 ^T	<i>A. macleodii</i>	<i>A. marina</i>	<i>A. stellipolaris</i>	<i>A. litorea</i>
10:0 3-OH	1.20	1.5	1.3	—	1.4
11:0 3-OH	—	—	1.0	—	1.2
12:0	1.77	2.5	2.9	—	2.7
12:0 3-OH	1.06	1.2	1.1	—	1.1
14:0	2.07	2.5	2.6	—	3.6
15:0	—	2.5	2.8	—	2.1
15:1 ω 8 c	—	1.7	1.1	—	1.0
16:0	13.77	23.8	21.2	12.6	20.0
16:0 iso	—	1.1	—	—	—
16:0 N alcohol	7.35	6.6	3.2	—	5.6
16:1 ω 7 c alcohol	1.167	4.3	1.9	—	5.8
17:0	—	2.6	3.2	—	2.9
17:0 10-methyl	15.63	2.9	1.5	—	4.5
17:1 ω 8 c	—	4.3	5.6	9.4	3.6
18:1 ω 7 c	14.30	9.9	11.8	18.0	12.5
18:0	5.89	—	—	—	—
18:3 ω 6 c (6, 9, 12)	5.01	—	—	—	—
15:0 iso 2-OH/16:1 ω 7 c	22.10	24.6	28.5	27.3	20.0
16:1 iso I/14:0 3-OH	2.57	3.3	3.6	—	3.0

species within the genus *Alteromonas*, for which we propose the name *Alteromonas hispanica*.

Description of *Alteromonas hispanica* sp. nov.

Alteromonas hispanica (his.pa'ni.ca. L. fem. adj. *hispanica* Spanish).

The cells are straight rods, 1–2 µm long and 0.75 µm wide, appearing either singly or in pairs. They stain Gram-negative and are motile by one polar flagellum. They produce buds, prostheca and PHB. No spores are observed under any conditions. Colonies are cream coloured, round, convex and mucoid. EPS is produced. Growth pattern is uniform in a liquid medium. The bacterium is chemo-organotrophic and strictly aerobic, i.e. anaerobic respiration with nitrate, nitrite or fumarate is negative. Catalase and oxidase are positive. It produces acids from maltose but not from any of the following carbohydrates: adonitol, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, lactose, D-mannitol, mannose, D-melezitose, L-rhamnose, D-salicin, D-sorbitol, sorbose, sucrose and trehalose. It is moderately halophilic, capable of growing in NaCl concentrations of 7.5 to 15 % w/v (optimum 7.5–10 %). It does not require additional magnesium or potassium salts. It grows within the temperature range of 4 to 40 °C (optimum 32 °C) and at pH values between 5 and 10 (optimum 7–8). It shows positive activity for ONPG, phosphatase, selenite reduction, H₂S production from cysteine and hydrolysis of aesculin, casein, gelatin, Tween 20, Tween 80, starch and DNA. It is negative for nitrate and nitrite reduction, urease, lecithinase phenylalanine deaminase, gluconate oxidation, growth on cetrimide agar, growth on MacConkey agar, indole, methyl red, Voges–Proskauer and haemolysis. It grows in synthetic media supplemented with maltose and mannitol as sole sources of carbon and energy. It does not grow in synthetic media supplemented with the following sole sources of carbon and energy, or carbon, nitrogen and energy: aesculin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, D-mannose, D-melezitose, L-rhamnose, D-salicin, starch, D-trehalose, citrate, formate, fumarate, gluconate, lactate, malonate, propionate, succinate, adonitol, ethanol, *myo*-inositol, sorbitol, L-alanine, L-cysteine, L-histidine, DL-isoleucine, L-lysine, L-methionine, L-serine and L-valine. It is susceptible to amoxicillin (25 µg), ampicillin (10 µg), carbenicillin (100 µg), cefotaxime (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), polymyxin B (300 UI), rifampicin (30 µg), streptomycin (10 µg), sulphamide (250 µg), tobramycin (10 µg) and trimetoprim/sulphamethoxazol (1.25/23.75 µg). It is resistant to cefoxitin (30 µg). The principal fatty acids are (%): 16:0 N alcohol (7.35), 15:0 iso 2-OH/16:1ω7c (22.10), 16:0 (13.77), 17:0 10-methyl (15.63), 18:0 (5.89), 18:3ω6c (6, 9, 12) (5.01) and 18:1ω7c (14.30). DNA G+C content is 46.3 mol% (*T_m* method).

The type strain, F-32^T (= CECT 7067^T = LMG 22958^T), was isolated from a hypersaline water sample taken at Fuente de Piedra (Málaga, southern Spain).

Acknowledgements

This research was supported by grants from the Dirección General de Investigación Científica y Técnica (BOS2003-00498) and from the Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía, Spain. The authors are very grateful to Concepción Fernández and David Porcel for their expertise in the electron-microscope studies and to their colleague Dr J. Trout for revising the English text.

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