Alteromonas litorea sp. nov., a slightly halophilic bacterium isolated from an intertidal sediment of the Yellow Sea in Korea

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A Gram-negative, motile, non-spore-forming, rod-shaped bacterium, designated strain TF-22^T, was isolated from an intertidal sediment in Korea. This organism grew optimally at 30–37 °C and in the presence of 2–5 % (w/v) NaCl. It did not grow without NaCl or in the presence of more than 14 % (w/v) NaCl. Strain TF-22^T was characterized chemotaxonomically as having ubiquinone-8 as the predominant respiratory lipoquinone and C_{16:0}, C_{16:1} ω7c and/or iso-C_{15:0} 2-OH and C_{18:1} ω7c as the major fatty acids. The DNA G + C content of strain TF-22^T was 46.0 mol%. Phylogenetic analyses based on 16S rDNA sequences showed that strain TF-22^T falls within the γ-subclass of the *Proteobacteria* and forms a coherent cluster with *Alteromonas macleodii* and *Alteromonas marina*. Levels of 16S rDNA similarity between strain TF-22^T and the type strains of two *Alteromonas* species were in the range 98.1–98.6 %. The level of DNA–DNA relatedness between strain TF-22^T and the type strains of two *Alteromonas* species was 15.7–18.5 %. Therefore, on the basis of phenotypic properties, phylogeny and genomic distinctiveness, strain TF-22^T should be placed in the genus *Alteromonas* as a novel species, for which the name *Alteromonas litorea* sp. nov. is proposed. The type strain is TF-22^T (=KCCM 41775^T = JCM 12188^T).

The genus *Alteromonas* was proposed by Baumann *et al.* (1972) with four *Alteromonas* species. Previously, there were at least 21 species with validly published names within the genus *Alteromonas*. However, the majority of these species have been reclassified as belonging to other genera, i.e. *Marinomonas*, *Pseudoalteromonas* or *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), and only two *Alteromonas* species, *Alteromonas macleodii* (Baumann *et al.*, 1972) and *Alteromonas fuliginea* (Romanenko *et al.*, 1994), remained in this genus. However, *A. fuliginea* was found to belong to *Pseudoalteromonas citrea* on the basis of DNA–DNA hybridization (Ivanova *et al.*, 1998). Phylogenetic analysis based on 16S rDNA sequences showed that *A. fuliginea* falls within the evolutionary radiation encompassed by the genus *Pseudoalteromonas*, not that encompassed by the

genus *Alteromonas* (Yoon *et al.*, 2003). Another *Alteromonas* species, *Alteromonas marina*, has recently been described (Yoon *et al.*, 2003). In this study, we describe a slightly halophilic bacterial strain, TF-22^T, which was considered to be an *Alteromonas*-like organism on the basis of the results of 16S rDNA sequence comparison. Accordingly, the aim of the present work was to establish the exact taxonomic position of strain TF-22^T by using a polyphasic approach involving phenotypic, genetic and chemotaxonomic analyses.

Strain TF-22^T was isolated from an intertidal sediment, collected from Daepo Beach, Yellow Sea of Korea, by using the dilution plating technique on marine agar 2216 (MA; Difco). Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. The flagellum type was determined by transmission electron microscopy using cells from exponentially growing cultures. The Gram reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions.

The GenBank accession number for the 16S rDNA sequence of strain TF-22^T is AY428573.

Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with anaerobically prepared MA. Growth in the absence of NaCl was investigated in trypticase–soy broth lacking NaCl. Growth at various NaCl concentrations (0.5–15 %, w/v) was investigated in marine broth (MB; Difco) or trypticase–soy broth. Growth at various temperatures was measured on MA at 4–45 °C. Catalase and oxidase activities and hydrolysis of casein, starch, Tween 20, Tween 40, Tween 60 and Tween 80 were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was examined on MA plates with the substrate concentrations described previously (Cowan & Steel, 1965). Hydrolysis of aesculin and gelatin and nitrate reduction were determined as described by Lanyi (1987) with the modification that artificial sea water was used. The artificial sea water

contained (per litre distilled water): 23.6 g NaCl, 0.64 g KCl, 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.94 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Bruns *et al.*, 2001). H_2S production was tested as described previously (Bruns *et al.*, 2001). The API ZYM system (bioMérieux) was used to determine the activity of some enzymes. Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested as described by Baumann & Baumann (1981). Other physiological and biochemical tests were performed with the API 20E and API 20NE systems (bioMérieux).

Cell biomass of strain TF-22^T for respiratory lipoquinone analysis and for DNA extraction was obtained from MB cultures at 30 °C. For fatty acid methyl ester analysis, cell mass of strain TF-22^T was obtained from agar plates after

Table 1. Differential phenotypic characteristics of *A. macleodii*, *A. marina* and strain TF-22^T

+, Positive; –, negative; w, weakly positive; v, variable. All strains/species are polarly flagellated and rod-shaped and test positive for the following characteristics: motility, oxidase, hydrolysis of Tween 80, acid production from D-trehalose, and utilization of D-galactose, D-fructose, sucrose, maltose, lactose, acetate and glycerol. All strains/species test negative for the following characteristics: Gram stain, spore formation, growth at 45 °C, acid production from L-arabinose, D-mannose, L-rhamnose, adonitol, *myo*-inositol and D-sorbitol, and utilization of D-sorbitol, succinate, citrate and L-malate.

Characteristic	<i>A. macleodii</i> *	<i>A. marina</i>	Strain TF-22 ^T
Reduction of nitrate to nitrite	v(–†)	–	–
Hydrolysis of:			
Gelatin	v(+†)	+	+
Starch	v(+†)	+	+
Utilization of:			
D-Mannitol	v(+†)	–	–
L-Lactate	v(+†)	–	–
Acid production from:†			
D-Cellobiose	+	–	–
D-Galactose	+	–	+
D-Glucose	+	–	–
D-Fructose	+	v(–†)	–
Lactose	+	v(–†)	–
Sucrose	+	+	–
Maltose	+	–	+
D-Melezitose	+	–	–
D-Ribose	+	v(+†)	–
D-Xylose	+	–	–
Melibiose	+	w	–
D-Mannitol	+	–	–
Enzyme activity (with API ZYM system):†			
Leucine arylamidase	+	w	+
Valine arylamidase	+	–	–
Growth at:			
4 °C	–	+	–
40 °C	v(+†)	+	+
DNA G+C content (mol%)	45–46	44–45	46

*Data from Baumann *et al.* (1972).

†Data for the type strain from Yoon *et al.* (2003).

cultivation for 2 days at 30 °C on MA. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), except that ribonuclease T1 was used with ribonuclease A. Isoprenoid quinones were extracted and analysed as described previously (Komagata & Suzuki, 1987), using reverse-phase HPLC. For quantitative analysis of cellular fatty acid compositions, a loop of cell mass was harvested and fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC.

16S rDNA was amplified by a PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rDNA and phylogenetic analysis were performed as described by Yoon *et al.* (2003). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded, and the remaining three values were used to calculate similarity values. The DNA relatedness values quoted are the means of the three values.

Morphological, physiological and biochemical characteristics are shown in Table 1 or are given in the species description (see below). Strain TF-22^T had an unsaturated ubiquinone with eight isoprene units (ubiquinone-8) as the predominant respiratory lipoquinone. *A. macleodii* DSM 6062^T and two strains of *A. marina* were found to contain ubiquinone-8 as the predominant respiratory lipoquinone (Yoon *et al.*, 2003). Strain TF-22^T had a cellular fatty acid profile containing large amounts of straight-chain, unsaturated and hydroxy fatty acids (Table 2). This fatty acid profile was similar to those of *A. macleodii* DSM 6062^T and *A. marina* KCCM 41638^T (Table 2). Strain TF-22^T contained C_{16:0}, C_{16:1} ω7c and/or iso-C_{15:0} 2-OH and C_{18:1} ω7c as the major fatty acids (Table 2). The DNA G + C content of strain TF-22^T was 46.0 mol%.

An almost complete 16S rDNA sequence of strain TF-22^T comprising 1456 nt (approx. 96 % of the *Escherichia coli* 16S rRNA sequence) was determined directly after PCR amplification. Phylogenetic analyses based on 16S rDNA sequences showed that strain TF-22^T forms a coherent cluster with *A. macleodii* and *A. marina*. In a phylogenetic tree based on the neighbour-joining algorithm, strain TF-22^T joined the cluster comprising *A. macleodii* and *A. marina* by bootstrap analysis at a confidence level of 100 % (Fig. 1). Similar tree topology was found in the trees generated with the maximum-likelihood and maximum-parsimony algorithms (data not shown). Strain TF-22^T exhibited 16S rDNA similarity levels of 98.1 and 98.6 %, respectively, to the type strains of *A. macleodii* and *A. marina*

Table 2. Cellular fatty acid composition (%) of *A. macleodii* DSM 6062^T, *A. marina* KCCM 41638^T and strain TF-22^T

Fatty acid*	DSM 6062 ^T	KCCM 41638 ^T	TF-22 ^T
Straight-chain fatty acids			
C _{12:0}	2.5	2.9	2.7
C _{14:0}	2.5	2.6	3.6
C _{15:0}	2.5	2.8	2.1
C _{16:0}	23.8	21.2	20.0
C _{16:0} N alcohol	6.6	3.2	5.6
C _{17:0}	2.6	3.2	2.9
C _{18:0}	0.6	0.6	0.8
Branched fatty acids			
iso-C _{16:0}	1.1	0.7	0.9
anteiso-C _{17:0}	0.5	0.5	0.6
Unsaturated fatty acids			
C _{15:1} ω8c	1.7	1.1	1.0
C _{16:1} ω7c alcohol	4.3	1.9	5.8
C _{17:1} ω8c	4.3	5.6	3.6
C _{18:1} ω7c	9.9	11.8	12.5
Hydroxy fatty acids			
C _{10:0} 3-OH	1.5	1.3	1.4
C _{11:0} 3-OH	0.9	1.0	1.2
C _{12:0} 3-OH	1.2	1.1	1.1
C _{12:1} 3-OH	0.4	0.6	0.7
Methyl fatty acids			
C _{17:0} 10-methyl	2.9	1.5	4.5
C _{18:1} ω7c 11-methyl	ND	0.7	0.6
Summed feature†			
2	3.3	3.6	3.0
3	24.6	28.5	20.0
7	ND	0.5	0.4

ND, Not detected.

*Fatty acids representing less than 0.5 % in all strains were omitted.

†Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained one or more of iso-C_{16:1} I and/or C_{14:0} 3-OH. Summed feature 3 contained one or more of C_{16:1} ω7c and/or iso-C_{15:0} 2-OH. Summed feature 7 contained one or more of an unknown fatty acid of equivalent chain length 18.846, C_{19:0} cyclo ω10c and/or C_{19:1} ω6c.

(Fig. 1). Sequence similarities to all other species included in the phylogenetic analysis were less than 89.7 % (Fig. 1). DNA–DNA hybridization was performed to determine the genomic relatedness of strain TF-22^T to the type strains of two *Alteromonas* species with validly published names. Strain TF-22^T exhibited DNA–DNA relatedness levels of 15.7 and 18.5 %, respectively, to *A. macleodii* DSM 6062^T and *A. marina* KCCM 41638^T.

The 16S rDNA sequence analysis indicated that strain TF-22^T has the closest phylogenetic affiliation to the γ-Proteobacteria, particularly to the genus *Alteromonas* (Fig. 1). Chemotaxonomic analysis confirms the result of

Fig. 1. Neighbour-joining tree, based on 16S rDNA sequences, showing the phylogenetic positions of *A. litorea* TF-22^T and the representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. Bar, 0.01 substitutions per nucleotide position.

monothetic classification based on 16S rDNA sequence analysis. The predominant respiratory lipoquinone and cellular fatty acid profile of strain TF-22^T were found to be most similar to those of *Alteromonas* species (Yoon *et al.*, 2003). Accordingly, in view of these combined chemotaxonomic and phylogenetic analyses, strain TF-22^T is considered as a member of the genus *Alteromonas*. Strain TF-22^T is differentiated from *A. macleodii* and *A. marina* through some physiological and biochemical characteristics, as shown in Table 1. Levels of DNA–DNA relatedness, together with some differential phenotypic properties and phylogenetic distinctiveness, warrant the separation of strain TF-22^T from the two *Alteromonas* species described previously (Wayne *et al.*, 1987). Therefore, on the basis of the phenotypic, chemotaxonomic and phylogenetic data and genomic distinctiveness, we propose to include strain TF-22^T in the genus *Alteromonas* as a novel species, *Alteromonas litorea* sp. nov.

Description of *Alteromonas litorea* sp. nov.

Alteromonas litorea (li.to.re' a. L. adj. *litorea* of the shore).

Cells are rods, 0.9–1.2 × 2.0–4.0 µm on MA. Motile by means of a single polar flagellum. Colonies on MA are circular, smooth, raised and cream-coloured and 2.0–3.0 mm in diameter after 2 days incubation at 30 °C. Grows well at 25–37 °C with an optimum between 30 and 37 °C. Growth occurs at 10 and 43 °C, but not at 4 °C or above 44 °C. Optimal growth pH is between 7.0 and 8.0. Growth is observed at pH 5.5, but not at pH 5.0. Optimal growth occurs in the presence of 2–5% (w/v) NaCl. No

growth occurs in the absence of NaCl or in the presence of more than 14% (w/v) NaCl. Growth does not occur under anaerobic conditions on MA. Catalase-positive. Aesculin, casein, hypoxanthine, Tween 20, Tween 40, Tween 60 and tyrosine are hydrolysed. Xanthine is not hydrolysed. H₂S is not produced. Indole is not produced. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are absent. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Acid is not produced from D-raffinose. The predominant ubiquinone is ubiquinone-8. The major fatty acids are C_{16:0}, C_{16:1} ω7c and/or iso-C_{15:0} 2-OH and C_{18:1} ω7c. The DNA G + C content is 46.0 mol% (HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, TF-22^T (= KCCM 41775^T = JCM 12188^T), was isolated from an intertidal sediment in the Yellow Sea of Korea.

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