Alteromonas marina sp. nov., isolated from sea water of the East Sea in Korea

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Two Gram-negative, motile, non-spore-forming and moderately halophilic rods (strains SW-47^T and SW-49) were isolated from sea water of the East Sea in Korea and subjected to a polyphasic taxonomic study. The two strains grew optimally between 30 and 37 °C, and grew at 4 and 44 °C but not at temperatures above 45 °C. They grew optimally in the presence of 2–5 % (w/v) NaCl, but did not grow in the absence of NaCl. Strains SW-47^T and SW-49 had ubiquinone-8 (Q-8) as the predominant respiratory lipoquinone and C_{16:1} ω7c and/or iso-C_{15:0} 2OH, C_{16:0} and C_{18:1} ω7c as the major fatty acids, which are consistent with the corresponding data for *Alteromonas macleodii*. The DNA G + C contents of strains SW-47^T and SW-49 were 45 and 44 mol%, respectively. Strains SW-47^T and SW-49 showed a high level of 16S rDNA sequence similarity (99.9 %) and a mean level of DNA–DNA relatedness of 96.5 %. Phylogenetic analyses based on 16S rDNA sequences showed that the two strains form a coherent cluster with *A. macleodii*. Strains SW-47^T and SW-49 exhibited levels of 16S rDNA sequence similarity of 99.3 and 99.1 %, respectively, with *A. macleodii* DSM 6062^T and of less than 89.4 % with other species used in the phylogenetic analyses. *Alteromonas fuliginea* CIP 105339^T was found to be more closely related to the genus *Pseudoalteromonas* than to the genus *Alteromonas*. On the basis of phenotypic properties and phylogenetic and genomic data, strains SW-47^T and SW-49 represent a new species of the genus *Alteromonas*, for which the name *Alteromonas marina* (type strain SW-47^T = KCCM 41638^T = JCM 11804^T) is proposed.

The genus *Alteromonas* was proposed by Baumann *et al.* (1972) and included four species, *Alteromonas macleodii*, *Alteromonas communis*, *Alteromonas vaga* and '*Alteromonas marinopraesens*'. Initially, the genus *Alteromonas* accommodated strictly aerobic, non-spore-forming, straight or curved rods that were motile by means of polar flagella (Baumann *et al.*, 1972). There were at least 21 validly described species within the genus *Alteromonas*. However, the majority of these species have been reclassified in other genera, namely, *Marinomonas*, *Pseudoalteromonas* and *Shewanella* (Coyne *et al.*, 1989; Gauthier *et al.*, 1995; Ivanova *et al.*, 2000, 2001; MacDonell & Colwell, 1985;

Sawabe *et al.*, 2000; van Landschoot & De Ley, 1983). There are two validly described *Alteromonas* species, *A. macleodii* (Baumann *et al.*, 1972) and *Alteromonas fuliginea* (Romanenko *et al.*, 1994), at the time of writing. Phylogenetic analyses based on 16S rDNA sequence data showed that *A. macleodii* falls within the γ-subgroup of the class *Proteobacteria* (Anzai *et al.*, 2000). Recently, we isolated two moderately halophilic bacterial strains, SW-47^T and SW-49, from the East Sea in Korea. The bacteria were considered to be *Alteromonas*-like organisms as a result of a 16S rDNA sequence comparison. Accordingly, the aim of the present work was to establish the exact taxonomic positions of strains SW-47^T and SW-49, using a combination of phenotypic characterization, detailed phylogenetic analyses (based on 16S rDNA sequences) and genomic relatedness. In this study, the type strain of *A. fuliginea*, the 16S rDNA sequence of which has not been determined before, was subjected to 16S rDNA sequencing and then analysed phylogenetically.

The GenBank accession numbers for the 16S rDNA sequences of *Alteromonas marina* SW-47^T and SW-49 and *Alteromonas fuliginea* CIP 105339^T are AF529060, AF529061 and AF529062, respectively.

A light micrograph of strain SW-47^T and a phylogenetic tree constructed using a larger dataset are available in IJSEM Online.

Strains SW-47^T and SW-49 were isolated by the dilution-plating technique on marine agar 2216 (MA) (Difco). Cell biomass of strains SW-47^T and SW-49 and *A. macleodii* DSM 6062^T for respiratory lipoquinone analysis and for DNA extraction was obtained from cultures in marine broth 2216 (MB) (Difco) at 30 °C. Cell biomass of *A. fuliginea* CIP 105339^T was produced in MB at 30 °C. All strains were cultivated on a gyratory shaker at 150 r.p.m. For fatty acid methyl ester (FAME) analysis, cell mass of strains SW-47^T and SW-49 and *A. macleodii* DSM 6062^T was obtained from agar plates after cultivation for 3 days at 30 °C on MA. *A. macleodii* DSM 6062^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and *A. fuliginea* CIP 105339^T was obtained from the Collection de l'Institut Pasteur (CIP), Paris, France. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (TEM). Flagellation was examined using TEM with cells from exponentially growing cultures. The cells were negatively stained with 1 % (w/v) phosphotungstic acid and, after air-drying, the grids were examined by using a model CM-20 transmission electron microscope (Philips). Gram reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Growth at various NaCl concentrations was investigated after 14 days incubation in MB. Growth at various temperatures was investigated after 14 days incubation on MA at 4–50 °C. Growth under anaerobic conditions was determined after incubation for 28 days in an anaerobic chamber with MA that had been prepared anaerobically using nitrogen. Catalase and oxidase activities and hydrolysis of casein and starch were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin was determined according to the method of Lanyi (1987), with the addition of 3 % (w/v) NaCl. Hydrolysis of hypoxanthine, tyrosine, xanthine and Tween 80 was tested on MA plates with the substrate concentrations described previously (Cowan & Steel, 1965). Hydrolysis of gelatin and nitrate reduction were studied as described previously (Cowan & Steel, 1965) with a modification that artificial sea water (Levring, 1946) was used. Hydrolysis of birchwood xylan (Sigma) was tested on solid marine salts basal medium (Baumann & Baumann, 1981) supplemented with 0.5 % (w/v) xylan as sole carbon source. H₂S production was tested as described previously (Bruns *et al.*, 2001). Utilization of substrates as sole carbon and energy sources was tested as described by Baumann & Baumann (1981). Acid production from carbohydrates was determined according to Leifson (1963). Enzyme activity was determined by using the API ZYM system (bioMérieux).

Respiratory lipoquinones were analysed as described previously (Komagata & Suzuki, 1987) using reversed-phase HPLC. For quantitative analysis of cellular fatty acid compositions, a loopful of a culture grown on MA was harvested and FAMEs were extracted and prepared by the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). 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. The G+C content of the DNA was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

16S rDNA was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). The PCR product was purified with a QIAquick PCR purification kit (Qiagen). Sequencing of the purified 16S rDNA PCR product was performed as described previously (Yoon *et al.*, 2003). Alignment of sequences was carried out with CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analyses. Phylogenetic trees were inferred by using three different calculation methods, i.e. the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) algorithms implemented in the PHYLIP software package version 3.5 (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated with the algorithm of Jukes & Cantor (1969) with the DNADIST program. The stability of grouping was assessed by a bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP software package. 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. Of the values obtained, the highest and lowest values in each sample were excluded and the remaining three values were used for calculation of the similarity values. DNA relatedness values are the mean of three values.

Strains SW-47^T and SW-49 were Gram-negative, non-spore-forming bacteria. Cells of the two strains were rod-shaped, measuring approximately 1.0–1.2 µm in width by 2.5–4.0 µm in length after 3 days cultivation at 30 °C on MA. The two strains were motile by means of a single polar flagellum [a light micrograph of strain SW-47^T is available as supplementary data in IJSEM Online (<http://ijs.sgmjournals.org>)]. Colonies on MA were circular, smooth, raised and cream in colour and 2.0–3.0 mm in diameter after incubation for 2 days at 30 °C. Strains SW-47^T and SW-49 grew optimally at 30–37 °C; they grew at 4 °C and their maximum growth temperature was 44 °C. The strains grew well over a broad pH range, pH 5.5–9.0, with an optimum between pH 7.0 and 8.0; they grew at pH 5.0 but not at pH 4.5. Both strains grew optimally in the presence of 2–5 % (w/v) NaCl and did not grow without NaCl or in the presence of >15 % NaCl. The two strains did not grow under anaerobic conditions on MA. Strains SW-47^T and SW-49 showed catalase and oxidase activities but no urease activity. Aesculin, casein, gelatin, hypoxanthine, starch, Tween 80 and tyrosine were hydrolysed, but xanthine and xylan were not. H₂S was not produced and nitrate was not

reduced to nitrite or nitrogen. The following enzymes were present in strains SW-47^T and SW-49, when assayed using the API ZYM system: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Results for utilization of and acid production from different substrates are shown in Table 1 or are given in the species description (see below). The phenotypic properties of strains SW-47^T and SW-49 are summarized in Table 1, together with those of *A. macleodii*. As shown in Table 1, there are differences between strains SW-47^T and SW-49 and *A. macleodii* in some characteristics.

The predominant respiratory lipoquinone found in strains SW-47^T and SW-49 was an unsaturated ubiquinone with eight isoprene units (Q-8) at peak ratios of approximately 94 and 96 %, respectively. In this study, *A. macleodii* DSM 6062^T was also found to contain Q-8 (approx. 92 %) as the predominant respiratory lipoquinone. Strains SW-47^T and

SW-49 had cellular fatty acid profiles containing large amounts of straight-chain, unsaturated and hydroxy fatty acids (Table 2). Strains SW-47^T and SW-49 contained C_{16:1} ω 7c and/or iso-C_{15:0} 2OH, C_{16:0} and C_{18:1} ω 7c as the major fatty acids (Table 2). These fatty acid profiles were similar to that of *A. macleodii* DSM 6062^T (Table 2). A similar fatty acid pattern for the type strain of *A. macleodii* was reported by Svetashev *et al.* (1995). However, there are differences in the proportion of fatty acids between this study and the study of Svetashev *et al.* (1995), which may be caused by different cultivation, extraction or analytical conditions. The DNA G + C contents of strains SW-47^T and SW-49 were 45 and 44 mol%, respectively.

The 16S rDNA sequences of strains SW-47^T and SW-49 and *A. fuliginea* CIP 105339^T determined in this study comprised 1489, 1489 and 1491 nt, respectively, corresponding to the region between positions 28 and 1524 by

Table 1. Differential phenotypic characteristics of strains SW-47^T and SW-49 and *A. macleodii*

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

Characteristic	SW-47 ^T	SW-49	<i>A. macleodii</i> *
Nitrate reduction 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	–	+	+
Lactose	–	+	+
Maltose	–	–	+
D-Melezitose	–	–	+
D-Ribose	+	–	+
D-Xylose	–	–	+
Melibiose	w	w	+
D-Mannitol	–	–	+
Enzyme activity (with API ZYM system)†:			
Leucine arylamidase	w	w	+
Valine arylamidase	–	–	+
Growth at 4 °C	+	+	–
Growth at 40 °C	+	+	v(+†)
G + C content (mol%)	45	44	45–46

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

†Data are for the type strain from this study.

Table 2. Cellular fatty acid compositions (as percentages of the total) of strains SW-47^T and SW-49 and *A. macleodii* DSM 6062^T

Fatty acids representing less than 0.5% in all strains were omitted. ND, Not detected; ECL, equivalent chain length. 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} 3OH. Summed feature 3 contained one or more of C_{16:1} ω7c and/or iso-C_{15:0} 2OH. Summed feature 7 contained one or more of an unknown fatty acid of ECL 18.846, C_{19:0} cyclo ω10c and/or C_{19:1} ω6c.

Fatty acid	SW-47 ^T	SW-49	DSM 6062 ^T
Straight-chain fatty acid:			
C _{12:0}	2.9	3.2	2.5
C _{14:0}	2.6	2.5	2.5
C _{15:0}	2.8	2.7	2.5
C _{16:0}	21.2	24.1	23.8
C _{16:0} N alcohol	3.2	1.7	6.6
C _{17:0}	3.2	3.3	2.6
C _{18:0}	0.6	0.7	0.6
Branched fatty acid:			
iso-C _{16:0}	0.7	1.0	1.1
anteiso-C _{17:0}	0.5	0.6	0.5
Unsaturated fatty acid:			
C _{15:1} ω8c	1.1	1.2	1.7
C _{16:1} ω7c alcohol	1.9	1.6	4.3
C _{17:1} ω8c	5.6	5.2	4.3
C _{18:1} ω7c	11.8	12.8	9.9
Hydroxy fatty acid:			
C _{10:0} 3OH	1.3	1.6	1.5
C _{11:0} 3OH	1.0	1.1	0.9
C _{12:0} 3OH	1.1	1.2	1.2
C _{12:1} 3OH	0.6	0.8	0.4
Methyl fatty acid:			
C _{17:0} 10-methyl	1.5	0.6	2.9
C _{18:1} ω7c 11-methyl	0.7	0.8	ND
Summed features:			
2	3.6	3.9	3.3
3	28.5	26.3	24.6
7	0.5	0.9	ND
Unknown fatty acid:			
14.959 (ECL)	0.4	ND	0.7

comparison with the *Escherichia coli* 16S rRNA gene sequence. The level of 16S rDNA similarity between strains SW-47^T and SW-49 was 99.9%. In a neighbour-joining phylogenetic tree based on 16S rDNA sequences, strains SW-47^T and SW-49 formed a coherent cluster that was supported by a bootstrap resampling value of 93.1% [Fig. 1; a phylogenetic tree constructed using a larger dataset is available in IJSEM Online (<http://ijs.sgmjournals.org>)]. This cluster joined to the evolutionary lineage of *A. macleodii*, and this relationship was supported by a bootstrap value of 100% (Fig. 1). This tree topology was also found in the trees generated with the maximum-likelihood and maximum-parsimony algorithms (data not shown). Strains SW-47^T

and SW-49 exhibited levels of 16S rDNA similarity of 99.3 and 99.1%, respectively, with *A. macleodii* DSM 6062^T (Fig. 1). Sequence similarities to all other taxa included in the phylogenetic analyses were less than 89.4% (Fig. 1). *A. fuliginea* CIP 105339^T was found to be phylogenetically only distantly related to *A. macleodii* DSM 6062^T and strains SW-47^T and SW-49, and formed a phylogenetic lineage within the radiation of the cluster comprising *Pseudoalteromonas* species. *A. fuliginea* CIP 105339^T showed levels of 16S rDNA similarity of 89.0–89.1% to *A. macleodii* DSM 6062^T and strains SW-47^T and SW-49. Sequence similarity values between *A. fuliginea* CIP 105339^T and the type strains of *Pseudoalteromonas* species were 91.2–99.7%. *A. fuliginea* CIP 105339^T showed high levels of 16S rDNA similarity, of 98.5–99.7%, with organisms in the clade including *Pseudoalteromonas haloplanktis*. Therefore, *A. fuliginea* may have to be reclassified as a member of the genus *Pseudoalteromonas* through additional taxonomic study.

Strains SW-47^T and SW-49 exhibited a mean level of DNA–DNA relatedness of 96.5%. This value indicates that strains SW-47^T and SW-49 are members of the same species. Strains SW-47^T and SW-49 exhibited levels of DNA–DNA relatedness of 26.8 and 24.5%, respectively, with *A. macleodii* DSM 6062^T, indicating that they are distinct from this recognized species (Wayne *et al.*, 1987).

In view of the results from the morphological, chemotaxonomic and phylogenetic analyses done in this study, it is appropriate that strains SW-46^T and SW-52 be classified in the genus *Alteromonas*. The level of DNA–DNA relatedness, together with differential phenotypic properties and phylogenetic data, justify a taxonomic discrimination of strains SW-47^T and SW-49 from *A. macleodii* (Wayne *et al.*, 1987). Therefore, on the basis of the data presented here, strains SW-46^T and SW-52 should be placed in the genus *Alteromonas* as members of a novel species, for which we propose the name *Alteromonas marina*.

Description of *Alteromonas marina* sp. nov.

Alteromonas marina (ma.ri'na. L. fem. adj. *marina* of the sea, marine).

Cells are rod-shaped, measuring 1.0–1.2 μm in width and 2.5–4.0 μm in length when grown on MA. Gram-negative. Non-spore-forming. 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. The optimal temperature for growth is 30–37 °C. Growth occurs at 4 and 44 °C but not above 45 °C. The optimal pH for growth is between pH 7.0 and 8.0. Growth is observed at pH 5.0 but not at pH 4.5. 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 15% NaCl. Growth does not occur under anaerobic conditions on MA. Catalase- and oxidase-positive. Urease-negative. Aesculin, casein, hypoxanthine and tyrosine are hydrolysed. Xanthine and xylan are not

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strains SW-47^T and SW-49 and representatives of some other related taxa based on 16S rDNA sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points.

hydrolysed. H₂S is not produced. 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 cysteine 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. Other characteristics are given in Table 1. The predominant respiratory lipoquinone is ubiquinone-8 (Q-8). The major fatty acids are C_{16:1} ω 7c and/or iso-C_{15:0} 2OH, C_{16:0} and C_{18:1} ω 7c. DNA G+C content is 44–45 mol% (HPLC). Isolated from sea water of Hwajinpo beach of the East Sea in Korea. Reference strain is SW-49 (=KCCM 41639=JCM 11805).

The type strain is SW-47^T (=KCCM 41638^T=JCM 11804^T). Its G+C content is 45 mol%.

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