Marinobacter flavimaris sp. nov. and *Marinobacter daepoensis* sp. nov., slightly halophilic organisms isolated from sea water of the Yellow Sea in Korea

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Two Gram-negative, motile, non-spore-forming and slightly halophilic rods (strains SW-145^T and SW-156^T) were isolated from sea water of the Yellow Sea in Korea. Strains SW-145^T and SW-156^T grew optimally at 37 and 30–37 °C, respectively, and in the presence of 2–6 % (w/v) NaCl. Strains SW-145^T and SW-156^T were chemotaxonomically characterized as having ubiquinone-9 as the predominant respiratory lipoquinone and C_{16:0}, C_{18:1ω9c}, C_{16:1ω9c} and C_{12:0} 3-OH as the major fatty acids. The DNA G + C contents of strains SW-145^T and SW-156^T were 58 and 57 mol%, respectively. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains SW-145^T and SW-156^T fell within the evolutionary radiation enclosed by the genus *Marinobacter*. The 16S rRNA gene sequences of strains SW-145^T and SW-156^T were 94.8% similar. Strains SW-145^T and SW-156^T exhibited 16S rRNA gene sequence similarity levels of 94.3–98.1 and 95.4–97.7 %, respectively, with respect to the type strains of all *Marinobacter* species. Levels of DNA–DNA relatedness, together with 16S rRNA gene sequence similarity values, indicated that strains SW-145^T and SW-156^T are members of two species that are distinct from seven *Marinobacter* species with validly published names. On the basis of phenotypic properties and phylogenetic and genotypic distinctiveness, strains SW-145^T (=KCTC 12185^T=DSM 16070^T) and SW-156^T (=KCTC 12184^T=DSM 16072^T) should be placed in the genus *Marinobacter* as the type strains of two distinct novel species, for which the names *Marinobacter flavimaris* sp. nov. and *Marinobacter daepoensis* sp. nov. are proposed.

The genus *Marinobacter* was proposed by Gauthier *et al.* (1992) with a single species, *Marinobacter hydrocarbonoclasticus*. The second species, *Marinobacter aquaeolei*, was described by Huu *et al.* (1999). Recently, five further species, *Marinobacter litoralis* (Yoon *et al.*, 2003), *Marinobacter lipolyticus* (Martín *et al.*, 2003), *Marinobacter excellens* (Gorshkova *et al.*, 2003), *Marinobacter lutaoensis* (Shieh *et al.*, 2003) and *Marinobacter squalenivorans* (Rontani *et al.*, 2003), have been added to the genus *Marinobacter*. Members of the genus *Marinobacter* have been isolated from marine environments, saline soil and coastal hot springs. In this study, we describe two Gram-negative, slightly halophilic strains, SW-145^T and SW-156^T, isolated from sea water at Daepo Beach of the Yellow Sea in Korea. The two organisms

were considered to be *Marinobacter*-like strains on the basis of 16S rRNA gene sequence comparisons. Accordingly, the aim of the present study was to determine the exact taxonomic positions of strains SW-145^T and SW-156^T by means of a polyphasic characterization that included determination of phenotypic properties and a detailed phylogenetic analysis based on 16S rRNA gene sequences and genotypic relatedness.

Strains SW-145^T and SW-156^T were isolated by using the dilution plating technique on marine agar 2216 (MA) (Difco). Cell morphology was examined using light microscopy (Nikon E600) and transmission electron microscopy. The latter was used to examine flagellum type in cells from exponentially growing cultures. The Gram reaction was determined using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber using MA and MA supplemented with nitrate, both of which had been prepared anaerobically.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SW-145^T and SW-156^T are AY517632 and AY517633, respectively.

Details of the fatty acid compositions of the novel strains and related species are available as supplementary material in IJSEM Online.

Growth at various NaCl concentrations was investigated in marine broth 2216 (MB) (Difco) or trypticase soy broth (Difco). Growth in the absence of NaCl was investigated in trypticase soy broth lacking NaCl. Growth at various temperatures (4–50 °C) was measured on MA. Catalase and oxidase activities and hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and nitrate reduction were determined as described previously (Lanyi, 1987) with the modification that artificial sea water was used. The artificial sea water contained (l⁻¹ distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Levring, 1946). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA plates using the substrate concentrations described previously (Cowan & Steel, 1965). H₂S 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). Tests for the utilization of various substrates were performed as described previously (Yurkov *et al.*, 1994).

Cell biomass for respiratory lipoquinone analysis and for DNA extraction was obtained from MB cultures at 30 °C. *M. hydrocarbonoclasticus* DSM 8798^T, *M. aquaeolei* DSM 11845^T, *M. litoralis* KCCM 41591^T, *M. lipolyticus* SM19^T and *M. excellens* KMM 3809^T were used as reference strains for DNA–DNA hybridization. Cell mass for DNA extraction from reference strains was obtained from MB cultures at 30 °C. Respiratory lipoquinones were analysed as described previously (Komagata & Suzuki, 1987) using reversed-phase HPLC. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that ribonuclease T1 was used together with ribonuclease A. The DNA G + C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. For fatty acid methyl ester analysis, a loop of cell mass of strain SW-145^T and of strain SW-156^T was harvested from agar plates after incubation for 3 days at 30 °C on MA. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). 16S rRNA genes were amplified by a PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA genes 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 relatedness values. The DNA relatedness values quoted are the means of the three values.

Strains SW-145^T and SW-156^T were similar in terms of most morphological, cultural, physiological and biochemical characteristics. However, strain SW-145^T grew at 4 °C, whereas strain SW-156^T did not. Strain SW-145^T grew in the presence of 20 % NaCl, but strain SW-156^T did not. D-Fructose, glycerol and D-gluconate were utilized by strain SW-145^T, but not by strain SW-156^T. There were also differences in lipase activity (C14) and nitrate reduction between the two strains (Table 1). Strains SW-145^T and SW-156^T are differentiated from other *Marinobacter* species by means of some phenotypic characteristics, including tolerance of NaCl, temperature and pH for growth and the ability to utilize some substrates (Table 1). Other phenotypic characteristics are shown in Table 1 or are given in the species descriptions. Strains SW-145^T and SW-156^T contained ubiquinone-9 (approx. 85 and 96 %, respectively) as the predominant respiratory lipoquinone. Strains SW-145^T and SW-156^T had cellular fatty acid profiles containing large amounts of straight-chain, unsaturated and hydroxyl fatty acids. The major fatty acids detected in strain SW-145^T were C_{16:0} (26.7 %), C_{18:1ω9c} (17.4 %), C_{16:1ω9c} (10.2 %), C_{12:0} 3-OH (10.5 %) and C_{12:0} (9.1 %). Strain SW-156^T contained the following major fatty acids: C_{16:0} (24.8 %), C_{18:1ω9c} (24.3 %), C_{16:1ω9c} (12.8 %), C_{12:0} 3-OH (9.4 %) and C_{12:0} (7.1 %). These profiles were similar to those of the type strains of *Marinobacter* species described previously, although there were some differences in the compositions of some fatty acids (see the supplementary table in IJSEM Online). Two fatty acids, C_{16:1ω7c} and C_{19:0}, were detected in *M. excellens* KMM 3809^T, but not in strains SW-145^T, SW-156^T or other *Marinobacter* species. However, the two fatty acids were detected from the type strains of *M. hydrocarbonoclasticus*, *M. aquaeolei* and *M. litoralis* in the study by Gorshkova *et al.* (2003). This observation may have been caused by different experimental conditions, e.g. cultivation conditions or analytical equipment. The predominant respiratory lipoquinone and the cellular fatty acid profiles of strains SW-145^T and SW-156^T are consistent with those of *Marinobacter* species except *M. lutaoensis* (Yoon *et al.*, 2003; Gorshkova *et al.*, 2003; Shieh *et al.*, 2003). *M. lutaoensis* contained ubiquinone-8 as the predominant ubiquinone and iso-C_{15:0} as the major fatty acid (Shieh *et al.*, 2003). The DNA G + C contents of strains SW-145^T and SW-156^T were 58 and 57 mol%, respectively.

Almost complete 16S rRNA gene sequences of strains SW-145^T and SW-156^T comprising, respectively, 1495 and 1496 nt (approx. 96 % of the *Escherichia coli* 16S rRNA gene sequence) were determined in this study. Strains SW-145^T and SW-156^T were found to have the highest 16S rRNA gene sequence similarity values to members of the *γ-Proteobacteria*. The level of 16S rRNA gene sequence similarity between strains SW-145^T and SW-156^T was 94.8 %. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that strains SW-145^T and SW-156^T fell within the radiation of the cluster comprising *Marinobacter* species (Fig. 1). Similar tree topologies were

Table 1. Differential phenotypic characteristics of strains SW-145^T and SW-156^T and other *Marinobacter* species

Strains/species: 1, strain SW-45^T; 2, strain SW-56^T; 3, *M. hydrocarbonoclasticus* (data from Gauthier *et al.*, 1992; Yoon *et al.*, 2003); 4, *M. aquaeolei* (Huu *et al.*, 1999); 5, *M. litoralis* (Yoon *et al.*, 2003); 6, *M. lipolyticus* (Martín *et al.*, 2003); 7, *M. excellens* (Gorshkova *et al.*, 2003); 8, *M. lutaoensis* (Shieh *et al.*, 2003). +, Positive; –, negative; w, weak; ND, not determined. The following tests were positive for all species: motility, catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl- β -glucosaminidase. The following tests were negative for all species: cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase, α -fucosidase and utilization of D-cellobiose, D-mannose and sucrose.

Characteristic	1	2	3	4	5	6	7	8
Flagellation	Single polar	Single polar	Single polar or none	Single polar	Single polar	ND	Single polar	Single or several polar
Urease	–	–	–	+	+	ND	ND	ND
Lipase (C14)*	+	–	–	–	–	ND	ND	ND
Valine arylamidase*	–	–	w	+	–	ND	ND	ND
Nitrate reduction to nitrite	+	–	+	+	+	–	+	–
Nitrite reduction to N ₂	–	–	+	–	–	–	+	–
Hydrolysis of:								
Aesculin	–	–	ND	–	–	ND	ND	ND
Gelatin	–	–	–	+	–	–	–	–
Starch	–	–	–	ND	–	–	+	–
Tween 80	+	+	ND	+	+	+	+	ND
Growth at/in:								
pH 5.0	–	–	–	+	+	+	–	+
0 % NaCl	–	–	–	+	–	–	–	–
20 % NaCl	+	–	+	+	–	–	–	–
4 °C	+	–	–	–	+	–	–	–
Utilization of:								
D-Glucose	–	–	–	–	–	+	–	+
D-Fructose	+	–	–	–	–	+	+	ND
D-Mannitol	–	–	–	–	–	+	+	+
L-Glutamate	–	–	+	+	+	–	–	+
D-Gluconate	+	–	–	–	–	+	–	ND
Succinate	+	+	+	+	+	–	+	ND
DL-Alanine	–	–	–	–	–	–	–	+ (L-)
L-Arginine	–	–	–	–	–	ND	ND	–
Aspartate	–	–	–	–	–	–	–	+
Temperature for growth (°C):								
Maximum	45	45	45	50	46	40	41	< 60
Optimum	37	30–37	32	30	30–37	37	20–25	45
Predominant ubiquinone	Q-9	Q-9	Q-9	Q-9	Q-9	ND	Q-9	Q-8
DNA G + C content (mol%)	58	57	53	56	55	57	55–56	63.5

*Enzyme activity (API ZYM system; bioMérieux) was not determined for *M. lipolyticus*, *M. excellens* or *M. lutaoensis*.

also found in the trees generated with maximum-parsimony and maximum-likelihood algorithms (data not shown). Strain SW-145^T exhibited the highest level of 16S rRNA gene similarity to the type strain of *M. lipolyticus* (98.1 %) and similarity was 94.3–96.3 % with respect to the type strains of other *Marinobacter* species. Strain SW-156^T exhibited the highest level of 16S rRNA gene sequence similarity to the type strains of *M. aquaeolei* and *M. hydrocarbonoclasticus* (97.7 and 97.2 %, respectively) and similarity was 95.4–96.8 % with respect to the type strains of other *Marinobacter* species. Levels of 16S rRNA gene

sequence similarity between strains SW-145^T and SW-156^T and other species used in the phylogenetic analysis were less than 91.3 %. DNA–DNA hybridization was performed to elucidate the genetic relationships between strains SW-145^T and SW-156^T and between the two strains and the type strains of five *Marinobacter* species, including those with more than 97 % similarity in 16S rRNA gene sequences. The mean level of DNA–DNA relatedness between strains SW-145^T and SW-156^T was 6.3 % when their DNAs were used individually as labelled DNA probes for cross-hybridization, which indicates that the two strains are

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic positions of strains SW-145^T and SW-156^T within the radiation of representatives of the γ -Proteobacteria. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. Scale bar, 0.01 substitutions per nucleotide position.

members of different genomic species (Wayne *et al.*, 1987). Strains SW-145^T and SW-156^T exhibited levels of DNA–DNA relatedness, respectively, of 6–12 and 7–13% with respect to *M. hydrocarbonoclasticus* DSM 8798^T, *M. aquaeolei* DSM 11845^T, *M. litoralis* KCCM 41591^T, *M. lipolyticus* SM19^T and *M. excellens* KMM 3809^T.

On the basis of the phenotypic and phylogenetic data and genomic distinctiveness, strains SW-145^T and SW-156^T should be placed in the genus *Marinobacter* as two distinct novel species, for which the names *Marinobacter flavimaris* sp. nov. and *Marinobacter daeipoensis* sp. nov., respectively, are proposed.

Description of *Marinobacter flavimaris* sp. nov.

Marinobacter flavimaris (fla.vi.ma'ris. L. masc. adj. *flavus* yellow; L. gen. neut. n. *maris* of the sea; N.L. gen. masc. n. *flavimaris* of the Yellow Sea).

Cells are rods, 0.6–0.9 × 1.5–3.0 μ m. Gram-negative. Non-spore-forming. Colonies are cream-coloured, smooth, circular to slightly irregular, low convex and 1.0–2.0 mm in diameter after 3 days cultivation at 30 °C on MA. The optimal pH for growth is 7.0–8.0; growth occurs at pH 5.5 but not at pH 5.0. Optimal growth occurs in the presence of 2–6% (w/v) NaCl. Growth occurs in the presence of 20% (w/v) NaCl but not in the presence of more than 21% (w/v) NaCl. Growth occurs under anaerobic conditions on MA and MA supplemented with nitrate. Tweens 20, 40 and 60 are hydrolysed, but casein, hypoxanthine, tyrosine and xanthine are not. Acid is produced from D-fructose. Acid is not produced from the following sugars: adonitol, L-arabinose, D-cellobiose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-melezitose, melibiose, myo-inositol, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, D-trehalose and D-xylose. Glycerol, L-serine and hexadecane are not utilized as sole carbon and energy sources. The predominant respiratory lipoquinone is

ubiquinone-9. The major fatty acids are C_{16:0}, C_{18:1} ω 9c, C_{16:1} ω 9c and C_{12:0} 3-OH. The DNA G + C content is 58 mol% (determined by HPLC).

The type strain, SW-145^T (=KCTC 12185^T=DSM 16070^T), was isolated from sea water at Daepo Beach of the Yellow Sea in Korea.

Description of *Marinobacter daeipoensis* sp. nov.

Marinobacter daeipoensis (dae.po.en'sis. N.L. masc. adj. *daeipoensis* of Daepo, the beach where the type strain was isolated).

Cells are rods, 0.6–0.8 × 1.5–3.0 μ m. Gram-negative. Non-spore-forming. Colonies are cream-coloured, smooth, circular to slightly irregular, low convex and 1.0–2.0 mm in diameter after 3 days cultivation at 30 °C on MA. The optimal pH for growth is 7.0–8.0; growth occurs at pH 5.5 but not at pH 5.0. Optimal growth occurs in the presence of 2–6% (w/v) NaCl. Growth occurs in the presence of 18% (w/v) NaCl but not in the presence of more than 19% (w/v) NaCl. Growth occurs under anaerobic conditions on MA and MA supplemented with nitrate. Tweens 20, 40 and 60 are hydrolysed, but casein, hypoxanthine, tyrosine and xanthine are not. Acid is not produced from the following sugars: adonitol, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-melezitose, melibiose, myo-inositol, D-raffinose, L-rhamnose, D-ribose, D-sorbitol, sucrose, D-trehalose and D-xylose. Glycerol, L-serine and hexadecane are not utilized as sole carbon and energy sources. The predominant respiratory lipoquinone is ubiquinone-9. The major fatty acids are C_{16:0}, C_{18:1} ω 9c, C_{16:1} ω 9c and C_{12:0} 3-OH. The DNA G + C content is 57 mol% (determined by HPLC).

The type strain, SW-156^T (=KCTC 12184^T=DSM 16072^T), was isolated from sea water at Daepo Beach of the Yellow Sea in Korea.

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