Note

Marinobacter litoralis sp. nov., a moderately halophilic bacterium isolated from sea water from the East Sea in Korea

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A Gram-negative, motile, non-spore-forming and moderately halophilic rod-shaped strain, SW-45^T, was isolated from sea water of the East Sea in Korea. The organism grew optimally at 30–37 °C and grew at 4 and 46 °C. It grew in the presence of 0.5–18 % (v/w) NaCl, with an optimum of 2–7 % NaCl. Strain SW-45^T was chemotaxonomically characterized by having ubiquinone-9 (Q-9) as the major respiratory lipoquinone and C_{16:0}, C_{18:1ω9c} and C_{16:1ω9c} as the predominant fatty acids. The DNA G + C content was found to be 55 mol%. Phylogenetic analysis based on 16S rDNA sequences showed that strain SW-45^T forms a coherent cluster with the clade comprising the two *Marinobacter* species. 16S rDNA sequence similarities between strain SW-45^T and the *Marinobacter* species was 94.9 % to *Marinobacter hydrocarbonoclasticus* DSM 8798^T and 95.3 % to *Marinobacter aquaeolei* DSM 11845^T. Levels of DNA–DNA relatedness between strain SW-45^T and the type strains of *M. hydrocarbonoclasticus* and *M. aquaeolei* were respectively 4.3 and 5.5 %. On the basis of phenotypic properties, phylogeny and genomic data, strain SW-45^T (= KCCM 41591^T = JCM 11547^T) should be placed in the genus *Marinobacter* as a member of a novel species, for which the name *Marinobacter litoralis* sp. nov. is proposed. As part of this study, the major respiratory lipoquinone of *M. hydrocarbonoclasticus* and *M. aquaeolei* was also found to be Q-9.

The genus *Marinobacter* was proposed by Gauthier *et al.* (1992) with one species, *Marinobacter hydrocarbonoclasticus*. This species was isolated from sea water (Gulf of Fos, French Mediterranean coast) near a petroleum refinery outlet (Al-Mallah *et al.*, 1990; Gauthier *et al.*, 1992). The second species of the genus, *Marinobacter aquaeolei*, was described by Huu *et al.* (1999). *M. aquaeolei* was isolated from an oil-producing well on an offshore oil/gas platform near a coastal town in southern Vietnam (Huu *et al.*, 1999). Both *Marinobacter* species are halophilic and degrade or utilize various hydrocarbons (Al-Mallah *et al.*, 1990; Gauthier *et al.*, 1992; Huu *et al.*, 1999). The genus *Marinobacter* accommodates Gram-negative, aerobic, motile and rod-shaped

bacteria. Phylogenetic analyses based on 16S rDNA sequences have shown that the genus falls within the γ -subclass of the *Proteobacteria* (Anzai *et al.*, 2000; Huu *et al.*, 1999; Yakimov *et al.*, 1998). Members of the genus are characterized chemotaxonomically by having C_{16:0}, C_{18:1ω9c}, C_{16:1ω9c} and C_{12:0} 3-OH as the predominant fatty acids and by having a DNA G + C content of 53–56 mol% (Gauthier *et al.*, 1992; Huu *et al.*, 1999).

In the course of screening taxonomically useful micro-organisms present in a variety of natural resources of Korea, a Gram-negative, moderately halophilic and rod-shaped bacterial strain (SW-45^T) was isolated from sea water at Jungdongjin beach on the East Sea in Korea. Strain SW-45^T was considered to be a *Marinobacter*-like strain from the result of 16S rDNA sequence analysis. To determine its exact taxonomic position, strain SW-45^T was subjected to a detailed polyphasic taxonomic investigation.

Abbreviations: FAME, fatty acid methyl ester; MA, marine agar 2216; MB, marine broth 2216.

The GenBank accession number for the 16S rDNA sequence of strain SW-45^T is AF479689.

Strain SW-45^T was isolated from sea water by the dilution-plating technique on marine agar 2216 (MA) (Difco). Cell biomass for respiratory lipoquinone analysis and for DNA extraction was obtained from cultures in marine broth 2216 (MB) (Difco) at 30 °C. All strains were cultivated on a horizontal shaker at 150 r.p.m. For fatty acid methyl ester (FAME) analysis, cell mass of strain SW-45^T and reference strains was obtained from agar plates after growing for 3 days at 30 °C on MA. The reference strains were *M. hydrocarbonoclasticus* DSM 8798^T and *M. aquaeolei* DSM 11845^T, which were obtained from the DSMZ. Cell morphology was examined by light microscopy (Nikon) and transmission electron microscopy (TEM). Flagellum type was examined by TEM using cells from exponentially growing culture. 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. Catalase activity was determined by bubble production in 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. Hydrolysis of aesculin and nitrate reduction were determined as described by Lanyi (1987). Hydrolysis of casein, gelatin, starch, Tween 80 and urea was determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was examined on MA plates with concentrations of substrates described previously (Cowan & Steel, 1965). Additional enzyme activities were determined by using the API ZYM system (bioMérieux). Acid production from carbohydrates was determined as described by Leifson (1963). Tests for utilization of various substrates were performed as described previously (Gauthier *et al.*, 1992). Growth on MA with or without nitrate that was prepared anaerobically was determined after incubation in an anaerobic chamber. Anaerobic growth on acetate, citrate and succinate (sodium salts) and glucose with or without nitrate was determined in a synthetic medium (Gauthier *et al.*, 1992) omitting peptone. Growth at various NaCl concentrations was investigated on MA or in MB. Growth at various temperatures was measured on MA at 4–55 °C.

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. Respiratory lipoquinones were 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 FAMES were prepared and identified following the instructions of the Microbial Identification System (MIDI). The 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 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 was performed using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. The purified sequencing reaction mixtures were electrophoresed automatically using an Applied Biosystems model 377 automatic DNA sequencer. 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 analysis. Phylogenetic trees were inferred by using three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods in the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated with the algorithm of Jukes & Cantor (1969) with the program DNADIST. The stability of relationships was assessed by a bootstrap analysis based on 1000 resampling of the neighbour-joining dataset by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells.

Cells of strain SW-45^T were rod-shaped, measuring approximately 0.5–0.8 × 1.5–3.0 µm (Fig. 1), and stained Gram-negative. Strain SW-45^T was motile by means of a single polar flagellum. No spores were observed. Colonies on MA were cream-coloured, smooth, circular to slightly irregular, low-convex and 1.0–2.0 mm in diameter after 3 days cultivation at 30 °C. Strain SW-45^T grew over a temperature range of 4–46 °C with an optimum of 30–37 °C. Strain SW-45^T grew optimally at pH 7.0–8.5 and no growth was observed at pH 4.5. It grew in the presence of 0.5–18% (w/v) NaCl and optimally in the presence of 2–7% (w/v)

Fig. 1. TEM of a cell of strain SW-45^T cultivated for 3 days on MA. Bar, 1 µm.

NaCl. Growth did not occur in the absence of NaCl or in more than 19 % NaCl. Strain SW-45^T did not grow under anaerobic conditions on MA or on MA or synthetic medium with nitrate. Anaerobic growth did not occur in the presence of nitrate with acetate, citrate, succinate or glucose as well as in the absence of nitrate with acetate, citrate, succinate or glucose as sole carbon and energy sources. Strain SW-45^T showed oxidase and urease activities and weak catalase activity. Tween 80 was hydrolysed but aesculin, casein, gelatin, hypoxanthine, starch, tyrosine and xanthine were not. Nitrate was reduced to nitrite. No acid was produced from the sugars used in this study. L-Glutamate and succinate were utilized as sole carbon and energy sources. Enzymes present in strain SW-45^T and *M. hydrocarbonoclasticus* DSM 8798^T when assayed with the API ZYM system are described in Table 1. The phenotypic properties of strain SW-45^T are summarized in Table 1, together with those of *M. hydrocarbonoclasticus* and *M. aquaeolei*.

Strain SW-45^T contained ubiquinone-9 (Q-9) (approx. 92 %) as the major respiratory lipoquinone. The major component found in *M. hydrocarbonoclasticus* DSM 8798^T and *M. aquaeolei* DSM 11845^T was also Q-9. The cellular fatty acid profile of strain SW-45^T was characterized by large amounts of saturated and unsaturated fatty acids

(Table 2) and was similar to those of *M. hydrocarbonoclasticus* DSM 8798^T and *M. aquaeolei* DSM 11845^T (Table 2). Similar fatty acid patterns for the two *Marinobacter* species were reported by Huu *et al.* (1999). However, the two species exhibit differences in the proportion of fatty acids between this study and the study of Huu *et al.* (1999), which may be caused by different cultivation conditions, e.g. cultivation media. The DNA G+C content of strain SW-45^T was 55 mol%.

The almost-complete 16S rDNA sequence of 1496 nt of strain SW-45^T, representing approximately 96 % of the *Escherichia coli* 16S rRNA sequence, was determined directly following PCR amplification. Strain SW-45^T was found to have the highest 16S rDNA similarity to members of the γ -subclass of the *Proteobacteria*. Treeing analysis based on neighbour-joining algorithm showed that strain SW-45^T forms a coherent cluster with the two species of the genus *Marinobacter* (Fig. 2). This relationship was also found in trees generated with maximum-parsimony and maximum-likelihood algorithms (data not shown). Strain SW-45^T exhibited levels of 16S rDNA similarity of 94.9 and 95.3 %, respectively, to the type strains of *M. hydrocarbonoclasticus* and *M. aquaeolei*. Levels of 16S rDNA similarity between strain SW-45^T and other strains used in the phylogenetic analysis were less than 90.2 %. The genomic relatedness

Table 1. Differential phenotypic characteristics of strain SW-45^T, *M. hydrocarbonoclasticus* and *M. aquaeolei*

Data for reference species were from Gauthier *et al.* (1992) (*M. hydrocarbonoclasticus*) and Huu *et al.* (1999) (*M. aquaeolei*) unless otherwise stated. Tests positive for all species: catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl- β -glucosaminidase, nitrate reduction to nitrite and utilization of L-glutamate and succinate. Tests negative for all species: lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase and utilization of cellobiose, D-fructose, D-glucose, glycerol, D-mannitol, D-mannose, sucrose, D-gluconate, aspartate, DL-alanine, L-arginine and L-serine. W, Weak; ND, not determined.

Characteristic	Strain SW-45 ^T	<i>M. hydrocarbonoclasticus</i>	<i>M. aquaeolei</i>
Flagellation	Single polar	Single polar or none	Single polar
Urease	+	—	+
Valine arylamidase	—	W*	+
Nitrite reduction to N ₂	—	+	—
Hydrolysis of			
Aesculin	—	ND	—
Gelatin	—	—	+
Starch	—	—	ND
Tween 80	+	ND	+
Growth at/in:			
pH 5.0	+	—	+
0 % NaCl	—	—	+
20 % NaCl	—	+	+
4 °C	+	—	—
Temperature for growth (°C):			
Maximum	46	45	50
Optimum	30–37	32	30
DNA G+C content (mol%)	55	53	56

*Data from this study.

Table 2. Cellular fatty acid profiles of strain SW-45^T, *M. hydrocarbonoclasticus* DSM 8798^T and *M. aquaeolei* DSM 11845^T

Values are percentages of total fatty acid content. Fatty acids representing less than 0.4 % were omitted.

Fatty acid	Strain SW-45 ^T	DSM 8798 ^T	DSM 11845 ^T
Saturated fatty acids			
C _{10:0}	3.2	1.2	0.5
C _{12:0}	5.5	5.1	8.4
C _{14:0}	3.0	3.1	4.5
C _{15:0}	0.7	1.0	1.5
C _{16:0}	25.0	25.3	24.8
C _{17:0}	2.5	1.9	1.8
C _{18:0}	2.9	2.0	1.1
Unsaturated fatty acids			
C _{16:1} ω ₉ c	10.6	7.6	15.2
C _{17:1} ω ₈ c	0.9	2.2	2.3
C _{18:1} ω ₇ c	0.5	1.2	0.5
C _{18:1} ω ₉ c	30.3	32.5	19.7
Hydroxy fatty acids			
C _{12:0} 3-OH	9.6	8.4	9.8
10-Methyl-C _{16:0}	0.8	1.0	2.1
Summed feature 3*	2.6	5.1	4.3

*Summed feature 3 represents iso-C_{15:0} 2-OH and/or C_{16:1}ω₇c, which could not be separated by GLC with the MIDI system.

between strain SW-45^T and the type strains of *M. hydrocarbonoclasticus* and *M. aquaeolei* was respectively 4.3 and 5.5 %.

The result of 16S rDNA sequence comparison showed that strain SW-45^T has the closest phylogenetic affiliation to the γ-subclass of the *Proteobacteria*, particularly to the genus *Marinobacter*. Phylogenetic inference based on 16S rDNA sequences revealed that strain SW-45^T joins the evolutionary clade comprising two species of the genus *Marinobacter*,

M. hydrocarbonoclasticus and *M. aquaeolei*, with a bootstrap resampling value of 100 % (Fig. 2). This result suggests two possibilities for the classification of strain SW-45^T; as a member of the genus *Marinobacter* or as a member of a new genus. Chemotaxonomic analysis is required to determine the exact taxonomic status of strain SW-45^T or to support the result of monothetic classification based on 16S rDNA sequence analysis. However, chemotaxonomic data, except cellular fatty acid data, have scarcely been reported for members of the genus *Marinobacter*. The cellular fatty acid profile of strain SW-45^T was found to be similar to those of *Marinobacter* species reported by Huu *et al.* (1999) as well as in this study (Table 2). Strain SW-45^T also shows the presence of significant amount of the hydroxy fatty acid C_{12:0} 3-OH, which is a characteristic component of the fatty acid profiles of *Marinobacter* species. In this study, strain SW-45^T was found to have an identical respiratory lipoquinone to those of the type strains of the two *Marinobacter* species. All three strains contain Q-9 as the major respiratory lipoquinone. The results obtained from chemotaxonomic analysis make it reliable to assign strain SW-45^T to the genus *Marinobacter*. Accordingly, in view of the combined morphological, phenotypic, chemotaxonomic and phylogenetic data, strain SW-45^T should be classified as a member of the genus *Marinobacter*.

Strain SW-45^T can be differentiated from *M. hydrocarbonoclasticus* and *M. aquaeolei* by some physiological characteristics, such as tolerance of NaCl, temperature and pH for growth and the ability to hydrolyse certain substrates (Table 1). Strain SW-45^T did not grow in the presence of more than 19 % (w/v) NaCl, whereas *M. hydrocarbonoclasticus* and *M. aquaeolei* grow in the presence of 20 % (w/v) NaCl (Table 1). While strain SW-45^T was observed to grow at 4 °C, the minimum growth temperatures of *M. hydrocarbonoclasticus* and *M. aquaeolei* are respectively 10 and 13 °C (Gauthier *et al.*, 1992; Huu *et al.*, 1999). Strain SW-45^T and *M. hydrocarbonoclasticus* show differences in growth at pH values below 6.0;

Fig. 2. Neighbour-joining tree based on 16S rDNA sequence data showing the phylogenetic position of strain SW-45^T within the radiation of representatives of the γ-subclass of the *Proteobacteria*. Scale bar, 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points.

M. hydrocarbonoclasticus does not grow at pH values less than 6.0 (Gauthier *et al.*, 1992). However, the separate position of strain SW-45^T within the genus *Marinobacter* becomes much more distinct from the results of 16S rDNA sequence comparison (Fig. 2). Levels of 16S rDNA similarity (94.9–95.3 %) between strain SW-45^T and the type strains of *Marinobacter* species are low enough to exclude the possibility for assigning strain SW-45^T to one of the two *Marinobacter* species (Stackebrandt & Goebel, 1994). In addition, DNA–DNA similarity values between strain SW-45^T and the type strains of the two *Marinobacter* species were far below the threshold value suggested for species delineation (Wayne *et al.*, 1987). Therefore, on the basis of the phenotypic, phylogenetic and genomic evidence, strain SW-45^T should be placed in the genus *Marinobacter* as a novel species, for which we propose the name *Marinobacter litoralis* sp. nov.

Description of *Marinobacter litoralis* sp. nov.

Marinobacter litoralis (li.to.ra'lis. L. adj. *litoralis* of the shore).

Cells are Gram-negative, non-spore-forming rods, 0.5–0.8 × 1.5–3.0 µm. Motile by means of a single polar flagellum. 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. Optimal growth temperature is 30–37 °C. Grows at 4 and 46 °C, but not above 47 °C. Optimal pH for growth is 7.0–8.5; no growth occurs at pH 4.5. Optimal growth occurs in the presence of 2–7 % (w/v) NaCl. Growth occurs in the range 0.5–18 % (w/v) NaCl and not in the presence of more than 19 % (w/v) NaCl. No growth under anaerobic conditions on MA or on MA or synthetic medium with nitrate. Anaerobic growth does not occur in the absence of nitrate with acetate, citrate, succinate or glucose. Anaerobic growth does not occur in the presence of nitrate with acetate, citrate, succinate or glucose. Oxidase- and urease-positive. Catalase weakly positive. Tween 80 is hydrolysed, whereas aesculin, casein, gelatin, hypoxanthine, starch, tyrosine and xanthine are not. Nitrate is reduced to nitrite. 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, stachyose, sucrose, D-trehalose and D-xylose. L-Glutamate and succinate are utilized as sole carbon and energy sources. Cellobiose, D-fructose, D-glucose, glycerol, D-mannitol, D-mannose, sucrose, D-gluconate, aspartate, DL-alanine, L-arginine and L-serine are not utilized as sole carbon and energy sources. The following enzymes are present when assayed with the API ZYM test system: alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase. The following enzymes are absent when assayed with the API ZYM system: lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase,

β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase. The major respiratory lipoquinone is Q-9. The predominant fatty acids are C_{18:1}ω9c, C_{16:0}, C_{16:1}ω9c and C_{12:0} 3-OH. The DNA G+C content is 55 mol% (determined by HPLC).

The type strain, strain SW-45^T (=KCCM 41591^T =JCM 11547^T), was isolated from sea water at Jungdongjin beach on the East Sea in Korea.

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