Marinobacterium litorale sp. nov. in the order *Oceanospirillales*

Hana Kim,[1] Yoe-Jin Choo,[1] Jaeho Song,[1] Jung-Sook Lee,[2] Keun Chul Lee[2] and Jang-Cheon Cho[1]

[1]Division of Biology and Ocean Sciences, Inha University, Incheon 402-751, Republic of Korea

[2]Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea

Correspondence
Jang-Cheon Cho
chojc@inha.ac.kr

A bacterial strain named IMCC1877^T was obtained from surface seawater collected near the coast of Deokjeok island (Yellow Sea), using a standard dilution-plating method. The strain was Gram-negative, chemoheterotrophic and facultatively anaerobic, requiring NaCl, and cells were motile rods with a single polar flagellum. Colonies on marine agar were very small (average diameter 0.1 mm). Based on 16S rRNA gene sequences, the most closely related species to strain IMCC1877^T was *Marinobacterium stanieri* (93.7 % sequence similarity to the type strain). Phylogenetic analyses based on 16S rRNA gene sequences showed that this marine isolate belonged to the order *Oceanospirillales* and formed an independent phyletic line within the clade forming the genus *Marinobacterium*. The DNA G+C content of the strain was 60.7 mol% and the predominant constituents of the cellular fatty acids were $C_{18:1}\omega7c$ (36.6 %), $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (26.7 %) and $C_{16:0}$ (24.3 %). Based on the taxonomic data, only a distant relationship could be established between strain IMCC1877^T and other *Marinobacterium* species; the strain therefore represents a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium litorale* sp. nov. is proposed. The type strain is IMCC1877^T (=KCTC 12756^T=LMG 23872^T).

The genus *Marinobacterium* was first described by González *et al.* (1997) and the genus currently comprises the four species *Marinobacterium georgiense* (González *et al.*, 1997), *Marinobacterium stanieri* (Satomi *et al.*, 2002), *Marinobacterium jannaschii* (Satomi *et al.*, 2002) and *Marinobacterium halophilum* (Chang *et al.*, 2007). *Marinobacterium stanieri* and *Marinobacterium jannaschii* were transferred into the genus *Marinobacterium* from *Pseudomonas* (Baumann *et al.*, 1983) and *Oceanospirillum* (Bowditch *et al.*, 1984), respectively. In the course of studies to reveal the microbial community structure inhabiting coastal areas of the Yellow Sea, a novel *Marinobacterium*-like strain, designated IMCC1877^T, was isolated from coastal water collected off Deokjeok island. Phenotypic characterization and phylogenetic analysis based on 16S rRNA gene sequences showed that strain IMCC1877^T represents a novel species in the genus *Marinobacterium*.

Strain IMCC1877^T was isolated from a surface seawater sample collected at a coastal region near Deokjeok island, Korea, using a standard dilution-plating method on a marine agar 2216 (MA; Difco) plate. After incubating the agar plate at 20 °C for 1 month, strain IMCC1877^T was initially isolated, further purified as single colonies and stored as 10 % (v/v) glycerol suspensions in liquid nitrogen. After determining the optimum growth temperature of the strain, cultures were grown and maintained routinely on MA at 30 °C.

The extraction of DNA, amplification of the 16S rRNA gene and sequencing of the PCR products were performed as described previously (Cho & Giovannoni, 2003) and resulted in the determination of an almost-complete 16S rRNA gene sequence (1508 bp) of the strain. To determine the phylogenetic relationships of strain IMCC1877^T, its 16S rRNA gene sequence was aligned against approximately 52 500 aligned reference 16S rRNA gene sequences using the ARB package (Ludwig *et al.*, 2004), and only 1248 unambiguously aligned nucleotide positions were used for phylogenetic analyses in the ARB package and PAUP* version 4.0 beta 10 (Swofford, 2002). Comparative 16S rRNA gene sequence analyses in the ARB database showed that the closest related type strain of a species with a validly published name to strain IMCC1877^T was *Marinobacterium stanieri* ATCC 27130^T (93.7 % sequence similarity), followed by *Marinobacterium halophilum* KCTC 12240^T (93.5 %), *Marinobacterium georgiense* ATCC 700074^T (92.9 %), *Marinobacterium jannaschii* IFO 15466^T (92.5 %) and *Neptunomonas naphthovorans* (92.1 %). To clarify the phylogenetic position of the strain further,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC1877^T is DQ917760.

phylogenetic trees were generated by neighbour joining (Saitou & Nei, 1987) with the Kimura two-parameter model (Kimura, 1980), maximum parsimony (Fitch, 1971) and maximum likelihood (Felsenstein, 1981), and the resulting neighbour-joining and maximum-parsimony trees were subjected to bootstrap analyses based on 1000 resamplings. In all phylogenetic trees, strain IMCC1877^T and the type strains of the four *Marinobacterium* species formed a monophyletic clade within the order *Oceanospirillales*. This monophyletic clade was supported by 80 and 60 % bootstrap proportions, respectively, in the neighbour-joining and maximum-parsimony trees (Fig. 1). Phylogenetic inferences and low 16S rRNA gene sequence similarity (92.5–93.7 %) between strain IMCC1877^T and the type strains of the four *Marinobacterium* species indicated that strain IMCC1877^T represents a novel species within the genus *Marinobacterium*. Although there is no 'official' classification of the *Bacteria* (Staley & Krieg, 1984), the 2nd edition of *Bergey's Manual of Systematic Bacteriology* (Bowman & McMeekin, 2005) and the NCBI taxonomy browser (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) classify the genus *Marinobacterium* as a member of the order *Alteromonadales*. The phylogenetic analyses in this study, however, strongly suggest the reclassification of the genus *Marinobacterium* into the order *Oceanospirillales* (Garrity *et al.*, 2005).

Phenotypic and chemotaxonomic characterizations were carried out as described in previous studies (Cho & Giovannoni, 2004; Smibert & Krieg, 1994) using MA as the basal medium at 30 °C. Cell morphology was examined by energy-filtering transmission electron microscopy (LIBLA120; Carl Zeiss) and phase-contrast and epifluorescence microscopy (Nikon 80i). Anaerobic growth was tested on MA at 30 °C using both the MGC anaerobic system and the AnaeroPACK·Anaero (Mitsubishi Gas Chemical Company). Colony morphology, size and colour were examined from cultures grown aerobically on MA at 30 °C for 3 days. The catalase test was performed by addition of 3.0 % hydrogen peroxide to fresh colonies and oxidase activity was determined using Kovacs' solution (Kovacs, 1956). Biochemical tests and carbon-source utilization tests were carried out on API 20NE (bioMérieux) and API ZYM (bioMérieux) (biochemical tests) and Biolog GN2 microplates (carbon utilization), with artificial seawater (ASW) (l^{-1}: 25.0 g NaCl, 1.0 g $MgCl_2.6H_2O$, 5.0 g $MgSO_4.7H_2O$, 0.7 g KCl, 0.15 g $CaCl_2.2H_2O$, 0.5 g NH_4Cl, 0.1 g KBr, 0.27 g KH_2PO_4, 0.04 g $SrCl_2.6H_2O$, 0.025 g H_3BO_3) following the manufacturers' instructions. The following antibiotics were tested: ampicillin (10 μg), chloramphenicol (25 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 μg), rifampicin (50 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg). The DNA G+C content was analysed by using HPLC with a Discovery C18 column (5 μm, 15 cm × 4.6 mm; Supelco)(Mesbah *et al.*, 1989). Quinone content was analysed using reversed-phase HPLC analysis (Komagata & Suzuki, 1987). Cellular fatty acid methyl esters

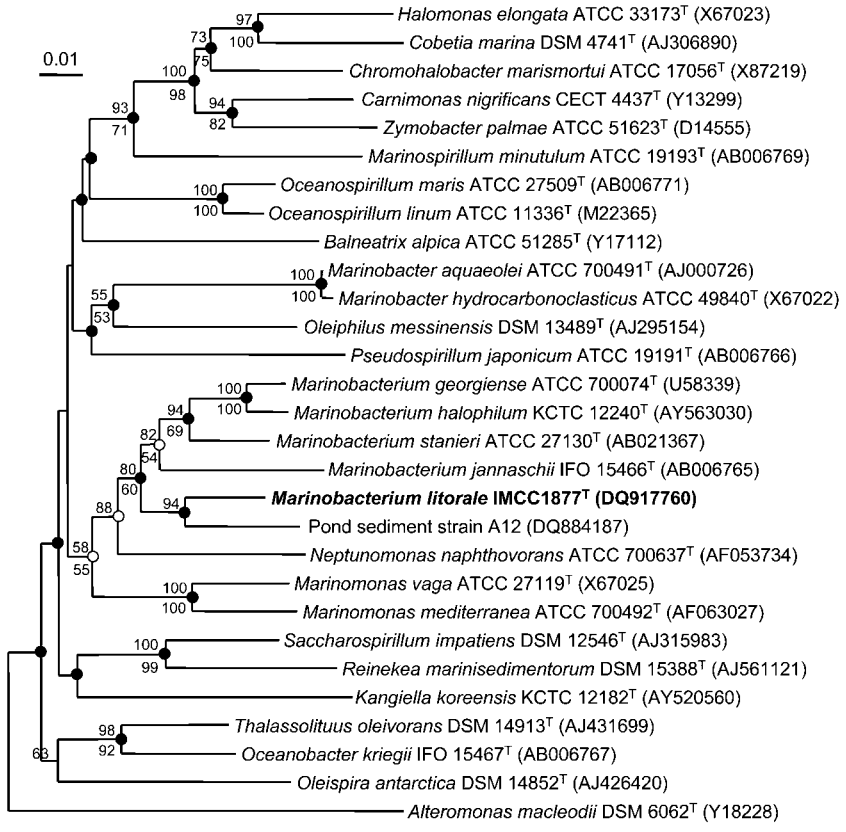

Fig. 1. Neighbour-joining 16S rRNA gene phylogenetic tree showing relationships among strain IMCC1877^T, *Marinobacterium* species and representatives of the order *Oceanospirillales*. Bootstrap proportions (over 50 %) from both neighbour joining (above nodes) and maximum parsimony (below nodes) are shown. Filled and open circles at each node respectively indicate nodes recovered reproducibly by all treeing methods or by two treeing methods. Bar, 0.01 substitutions per nucleotide position.

were prepared from a culture grown on MA at 30 °C for 3 days and analysed according to the instructions of the Microbial Identification System (MIDI).

Detailed morphological, physiological and biochemical characteristics of strain IMCC1877^T are given in the species description and in Table 1. In summary, the strain was a Gram-negative, motile rod that was chemoheterotrophic, facultatively anaerobic, requiring NaCl for growth, and was catalase- and oxidase-positive. Characteristics that differentiate strain IMCC1877^T from other *Marinobacterium* species are listed in Table 1. The DNA G+C content of strain IMCC1877^T was 60.7±0.5 mol%. The major isoprenoid quinone type detected was Q-8, which is a typical respiratory quinone found in the order *Oceanospirillales*. The major fatty acids found in strain IMCC1877^T were $C_{18:1}\omega7c$ (36.6 %), $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (26.7 %), $C_{16:0}$ (24.3 %) and $C_{10:0}$ 3-OH (5.2 %), which are generally in agreement with those in *Marinobacterium halophilum* (Chang *et al.*, 2007), but in different proportions.

In conclusion, based on combined phenotypic, chemotaxonomic and phylogenetic data, the organism represents a novel species within the genus *Marinobacterium*, for which the name *Marinobacterium litorale* sp. nov. is proposed.

Table 1. Characteristics that differentiate strain IMCC1877^T from type strains of *Marinobacterium* species

Strains: 1, strain IMCC1877^T (*Marinobacterium litorale* sp. nov.); 2, *Marinobacterium georgiense* ATCC 700074^T (data from González *et al.*, 1997); 3, *Marinobacterium jannaschii* IFO 15466^T (Bowditch *et al.*, 1984); 4, *Marinobacterium stanieri* ATCC 27130^T (Baumann *et al.*, 1983); 5. *Marinobacterium halophilum* KCTC 12240^T (Chang *et al.*, 2007). All strains are motile, rod-shaped and oxidase- and catalase-positive. All strains have Q-8 as the major quinone (data for *M. georgiense*, *M. jannaschii* and *M. stanieri* from Satomi *et al.*, 2002). +, Positive; −, negative; ND, no data available; PHB, poly-β-hydroxybutyrate.

Characteristic	1	2	3	4	5
Flagella	1 polar	1 polar	1–2 polar	1 polar	−
Growth at/in:					
4 °C	−	+	−	−	+
40 °C	+	+	−	+	−
10 % NaCl	−	+	ND	ND	+
Anaerobic growth	+	−	ND	ND	−
PHB accumulation	−	+	+	+	ND
Nitrate reduction	−	−	+	+	−
Gelatinase	−	+	+	−	−
Arginine dihydrolase	−	+	+	−	+
Carbon-source utilization					
D-Fructose	−	+	−	−	+
D-Glucose	−	+	−	−	−
D-Mannose	−	+	−	−	+
Glycerol	−	+	−	−	+
Formic acid	+	+	−	−	ND
Propionic acid	+	+	+	−	ND
Succinic acid	+	+	+	−	ND
L-Aspartic acid	−	+	+	−	ND
L-Leucine	−	−	+	−	ND
L-Phenylalanine	−	−	+	+	ND
L-Serine	+	−	+	+	ND
DNA G+C content (mol%)	60.7	54.9	56–57	55–57	ND

Description of *Marinobacterium litorale* sp. nov.

Marinobacterium litorale (li.to.ra′le. L. neut. adj. *litorale* of the seashore, a shallow-seawater dweller).

Cells are Gram-negative, motile, facultatively anaerobic, straight rods (1.2–2.0 μm long and 0.5–0.8 μm wide). Requires NaCl for growth. Colonies on MA are circular, smooth, convex, opaque, beige–milky in colour and 0.1 mm in diameter when observed after 2–3 days of incubation. Growth occurs at 8–42 °C, optimally at 30 °C, but not at 4 °C. Growth occurs at pH 5–12 and 1.0–7.5 % NaCl, optimally at pH 9.0 and 3.0–3.5 % NaCl. Oxidase and catalase are positive. Does not produce indole. Nitrate reduction, arginine dihydrolase, glucose fermentation, aesculin hydrolysis, gelatinase and β-galactosidase are negative. Urea is hydrolysed. Alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin and acid phosphatase activities are present. Based on the tests contained in the Biolog GN2 microplate, the following carbon substrates are utilized: pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, β-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, DL-lactic acid, propionic acid, quinic acid, succinic acid, bromosuccinic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, L-proline, L-serine, γ -aminobutyric acid and phenyl ethylamine. Utilizes the following carbon sources weakly: glycogen, Tween 80, i-erythritol, D-galacturonic acid, γ-hydroxybutyric acid, α-ketobutyric acid, malonic acid, D-saccharic acid, L-alanyl glycine, L-ornithine, putrescine and 2,3-butanediol. Dextrin, α-cyclodextrin, Tween 40, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, itaconic acid, α-ketovaleric acid, sebacic acid, succinamic acid, glucuronamide, L-asparagine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-threonine, DL-carnitine, urocanic acid, inosine, uridine, thymidine, 2-aminoethanol, glycerol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate and D-glucose 6-phosphate are not utilized as carbon

sources. Susceptible to ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, penicillin G, rifampicin, streptomycin and tetracycline, but resistant to vancomycin. The cellular fatty acids are composed of $C_{18:1}\omega7c$ (36.6 %), $C_{16:1}\omega7c$ and/or $C_{15:0}$ iso 2-OH (26.7 %), $C_{16:0}$ (24.3 %), $C_{10:0}$ 3-OH (5.2 %), $C_{12:0}$ (3.5 %), $C_{14:0}$ (1.2 %), $C_{18:0}$ (0.8 %) and $C_{10:0}$ (0.7 %). The major isoprenoid quinone type is Q-8. The DNA G+C content is 60.6±0.5 mol%.

The type strain, IMCC1877^T (=KCTC 12756^T=LMG 23872^T), was isolated from surface seawater of the coast of Deokjeok island, Yellow Sea, Korea.

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