Curr Microbiol (2009) 58:511–515
DOI 10.1007/s00284-009-9355-5

Marinobacterium marisflavi sp. nov., Isolated from a Costal Seawater

Hana Kim · Hyun-Myung Oh · Seung-Jo Yang · Jung-Sook Lee · Jae-Sang Hong · Jang-Cheon Cho

Received: 10 October 2008 / Accepted: 3 January 2009 / Published online: 3 February 2009

Abstract A marine bacterium designated strain IMCC4074^T was isolated from surface seawater collected off Incheon Port, the Yellow Sea, and subjected to a polyphasic taxonomy. The strain was Gram-negative, chemoheterotrophic, slightly halophilic, strictly aerobic, and motile rods. Based on 16S rRNA gene sequence comparisons, the strain was most closely related to *Marinobacterium litorale* KCTC 12756^T (93.9%) and shared low 16S rRNA gene sequence similarities with members of the genus *Marinobacterium* (91.8–93.9%) and the genus *Neptunomonas* (93.4%) in the order *Oceanospirillales*. Phylogenetic analyses showed that this marine isolate formed an independent phyletic line within the genus *Marinobacterium* clade. The DNA G+C composition of the strain was 56.0 mol% and the predominant constituents of the cellular fatty acids were $C_{16:0}$ (28.0%), $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH (19.3%), $C_{18:1}$ ω7c (17.8%), and $C_{17:1}$ cyclo (12.5%), which differentiated the strain from other *Marinobacterium* species. Based on the taxonomic data collected in this study, only a distant relationship could be found between strain IMCC4074^T and other members of the genus *Marinobacterium*, thus the strain represents a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium marisflavi* sp. nov. is proposed. The type strain of *Marinobacterium marisflavi* is IMCC4074^T (= KCTC 12757^T = LMG 23873^T).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC4074^T is EF468717.

H. Kim · H.-M. Oh · S.-J. Yang · J.-S. Hong · J.-C. Cho (✉)
Division of Biology and Ocean Sciences, Inha University, Incheon 402-751, Republic of Korea
e-mail: chojc@inha.ac.kr

J.-S. Lee
Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea

The genus *Marinobacterium* [8, 11], recently proposed as a member of the order *Oceanospirillales* in the *Gammaproteobacteria*, accommodates Gram-negative, chemoheterotrophic, and rod-shaped bacteria that are motile by one or two flagella. At present, the genus *Marinobacterium* comprises six validly published species names; *M. georgiense* [8] as the type species of the genus, *M. stanieri* [1, 19], *M. jannaschii* [2, 19], *M. halophilum* [3], *M. litorale* [11], and *M. rhizophilum* [12]. All members of the genus *Marinobacterium* have been isolated from marine habitats including pelagic and littoral seawaters, tidal flats, and costal rhizosphere. During studies on the diversity of microorganisms inhabiting coastal areas of the Yellow Sea, a bacterium designated strain IMCC4074^T was isolated from a surface seawater sample collected off Incheon Port. Strain IMCC4074^T was considered to be a novel *Marinobacterium*-like organism based on phenotypic characterization and 16S rRNA gene phylogenetic analysis. On the basis of this study, we propose that strain IMCC4074^T represents a novel species in the genus *Marinobacterium*, for which the name *Marinobacterium marisflavi* sp. nov. is proposed.

A sample of coastal seawater was collected, at a depth of 1 m, off Incheon Port, the Yellow Sea (37°19′N, 126°40′E). Strain IMCC4074^T was isolated using a standard spread plating method to spread 100 μl of seawater sample on marine agar 2216 (MA; Difco) that was incubated for 1 month at 20°C. The strain was purified as single colonies and stored as 10% (v/v) glycerol suspensions at

−70°C. After its optimum growth temperature had been determined, cultures of strain IMCC4074^T were routinely grown aerobically on MA at 30°C for phenotypic characterization, unless otherwise noted.

DNA extraction, amplification of the 16S rRNA gene, and sequencing of the PCR products were performed as described previously [4] and resulted in the determination of an almost-complete 16S rRNA gene sequence (1,487 bp) of strain IMCC4074^T. To determine the phylogenetic relationship of strain IMCC4074^T to other members of the γ-proteobacteria, the 16S rRNA gene sequence of the strain was carefully aligned using the ARB software package [14]. Unambiguously aligned 1186 nucleotide positions, determined from 33 γ-proteobacterial 16S rRNA gene sequences, were used for phylogenetic analyses in PAUP* 4.0 beta 10 [21]. Comparative 16S rRNA gene sequence analyses in the ARB database showed that the most closely related species with respect to strain IMCC4074^T was *M. litorale* KCTC 12756^T (93.9% sequence similarity), followed by *M. stanieri* ATCC 27130^T (93.6%), *Neptunomonas naphthovorans* ATCC 700637^T (93.4%), *M. georgiense* ATCC 700074^T (93.2%), *M. halophilum* KCTC 12240^T (93.1%), *M. rhizophilum* KCCM 42386^T (92.6%), and *M. jannaschii* IFO 15466^T (91.8%). To define the phylogenetic position of the strain clearer, phylogenetic trees were generated by the neighbor-joining [18] method with Jukes–Cantor distance [10], maximum parsimony [7], and maximum likelihood [6] algorithms. Robustness of the neighbor-joining and maximum parsimony trees was examined by bootstrap analyses based on 1,000 resamplings of the sequences. In all phylogenetic trees generated using three different treeing algorithms (Fig. 1), strain IMCC4074^T formed a robust monophyletic clade with uncultured bacteria VH-PA5-10 (95.0% sequence similarity [22]) and PC-FL10-8 (95.0% sequence similarity [22]). In the phylogenetic trees, the strain fell within the evolutionary radiation occupied by the genus *Marinobacterium* and the *Marinobacterium* clade was supported by a bootstrap value of 63% (Fig. 1). The phylogenetic position of strain IMCC4074^T located within the *Marinobacterium* clade and the low 16S rRNA gene sequence similarity (91.8–93.9%) between the strain and the type strains of *Marinobacterium* species suggested that the strain be classified as a new species within the genus *Marinobacterium*.

Temperature (4–42°C) and pH (4.0–12.0) ranges for growth were determined on MA. The NaCl concentrations for growth were determined in NaCl-free artificial seawater medium [5] supplemented with 5.0 g peptone, 1.0 g yeast extract, and various concentrations of NaCl (0–15%, w/v). Cellular morphology and size were observed by phase-contrast and epifluorescence microscopy (Nikon 80i) and transmission electron microscopy (CM200; Philips). Motility was tested using wet mounts of exponential-phase cells. The presence of poly-β-hydroxybutyrate (PHB) granules was checked using the Nile Blue A staining method described in Ref. [17]. Growth under anaerobic conditions was tested using both the MGC anaerobic system (Mitsubishi Gas Chemical Company, Inc.) and the Anaerocult C mini (EM Science). Colony morphology, size, and color were examined in cultures grown aerobically on MA at 30°C for 3 days. The catalase test was performed by the addition of 3.0% hydrogen peroxide to fresh colonies and oxidase activity was determined using Kovacs' solution [13]. Other biochemical tests were carried out on API 20NE and API ZYM system (bioMérieux). Carbon source assimilation was tested using Biolog GN2 microwell plates with artificial seawater medium [5] according to the manufacturer's instructions except that the plates were incubated for 1 week. Ten different kinds of

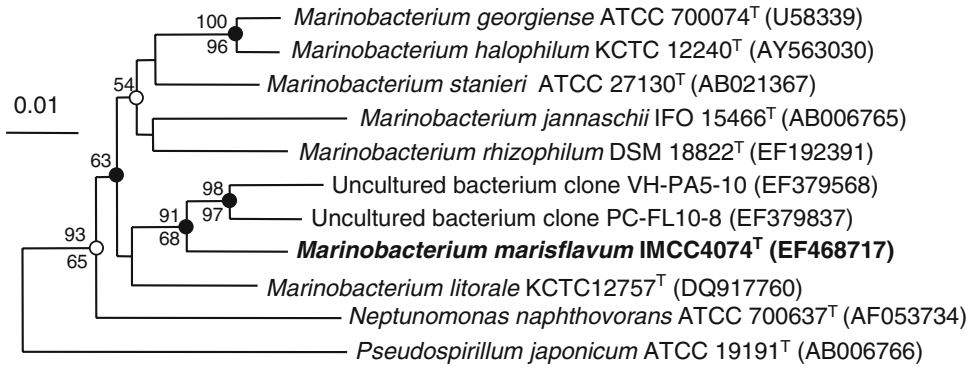

Fig. 1 A neighbor-joining 16S rRNA gene phylogenetic tree showing relationships between strain IMCC4074^T and representatives of the class γ-proteobacteria. Bootstrap proportions >50% from both neighbor-joining (above nodes) and maximum parsimony (below nodes) are shown. The filled circles and open circles at each node indicate the nodes recovered reproducibly by all treeing methods and the node's two treeing methods, respectively. Scale bar = 0.01 substitution per nucleotide position

antimicrobial agents (listed under Description of *Marinobacterium marisflavi* sp. nov., below) were tested by the diffusion plate method [9] on MA incubated for 4 days at 30°C. The DNA G+C content was analyzed by HPLC after digestion of the DNA with nuclease P1, using a Discovery C18 reverse-phase column [15]. The quinone content was analyzed using reverse-phase HPLC according to Minnikin et al. [16]. For analysis of cellular fatty acid methyl esters, cells of strain IMCC4074^T and *M. georgiense* KCTC 12422^T were grown on MA at 30°C for 3 days. Cellular fatty acid profiles were analyzed according to the instructions for the MIDI Sherlock Microbial Identification System (Microbial ID, Inc.).

Detailed results of morphological, physiological, and biochemical tests are given under Description of *Marinobacterium marisflavi* sp. nov. (below) and in Table 1. Strain IMCC4074^T was Gram-negative, chemoheterotrophic, strictly aerobic rods that are motile by a single polar flagellum (Fig. 2). The DNA G+C content (56.0 mol%) of strain IMCC4074^T was within the range of DNA G+C content of other *Marinobacterium* species (55–61 mol%). The major isoprenoid quinone type detected was Q-8, which is a typical respiratory quinone found in the genus *Marinobacterium*. However, several phenotypic characteristics including growth properties and enzyme activities differentiated strain IMCC4074^T from other *Marinobacterium* species (Table 1). The major fatty acids found in strain IMCC4074^T were generally similar to those of the existing *Marinobacterium* species. However, the presence of $C_{17:0}$ cyclo and $C_{19:0}$ cyclo ω8c in IMCC4074^T could serve as fatty acid signatures for differentiating the strain from other *Marinobacterium* species (Table 2). As shown by the low 16S rRNA gene sequence similarity,

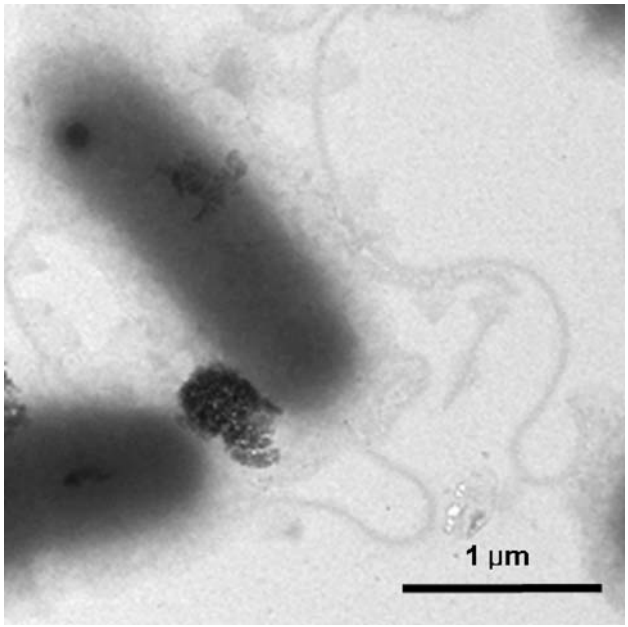

Fig. 2 Transmission electron micrograph of cells of strain IMCC4074^T. Bar = 2.0 μm

<97% (<93.9% [20]), to other *Marinobacterium* species, the distinct phylogenetic relationship (Fig. 1), and several differential phenotypic characteristics (Tables 1 and 2), strain IMCC4074^T was assigned to the genus *Marinobacterium* as a novel species. In conclusion, on the basis of the phenotypic characterization and the phylogenetic analysis, strain IMCC4074^T represents a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium marisflavi* sp. nov. is proposed.

Table 1 Characteristics that differentiate strain IMCC4074^T from other members of the genus *Marinobacterium*

Characteristic	1	2	3	4	5	6	7
Flagella	1 polar	1 polar	1 polar	1–2 polar	1 polar	–	1 polar
Growth at							
4°C	–	–	+	–	–	+	–
40°C	+	+	+	–	+	–	–
10% NaCl	–	–	+	ND	ND	+	–
Anaerobic growth	–	+	–	ND	ND	–	–
PHB accumulation	–	–	–	+	+	ND	+
Nitrate reduction	–	–	–	+	+	–	–
Gelatinase	–	–	–	+	–	–	–
Arginine dihydrolase	–	–	+	+	–	+	+
G + C mol%	56.0	60.7	54.9	56–57	55–57	ND	61.0

Strains: 1, *M. marisflavi* IMCC4074^T; 2, *M. litorale* KCTC 12756^T [11]; 3, *M. georgiense* ATCC 700074^T [8]; 4, *M. jannaschii* IFO 15466^T [2]; 5, *M. stanieri* ATCC 27130^T [1]; 6, *M. halophilum* KCTC 12240^T [3]; 7. *M. rhizophilum* KCCM 42386^T [12]

Note: *ND* no data available. + positive, – negative

Table 2 Cellular fatty acid composition (%) of strain IMCC4074^T and related species in the genus *Marinobacterium*

Fatty acid	1	2	3	4	5
Saturated					
$C_{10:0}$	–	–	3.3	4.3	3.9
$C_{12:0}$	1.8	3.5	2.2	4.6	4.0
$C_{14:0}$	4.8	1.2	–	1.6	–
$C_{16:0}$	28.0	24.3	26.5	21.9	16.6
Unsaturated					
$C_{16:1}$ ω7c	–	–	–	43.2	40.3
$C_{18:1}$ ω7c	17.8	36.6	35.8	15.7	26.6
Cyclopropane acids					
$C_{17:0}$ cyclo	12.5	–	–	–	–
$C_{19:0}$ cyclo ω8c	2.8	–	–	–	–
Hydroxylated					
$C_{10:0}$ 3-OH	2.3	5.2	5.1	7.6	7.1
ECL 11.799	1.1	1.1	1.1	–	–
Summed feature 2[a]	4.3	–	–	–	–
Summed feature 3[b]	19.3	26.7	22.1	–	–

Strains: 1, *M. marisflavi* IMCC4074^T (this study); 2, *M. litorale* KCTC 12756^T [11]; 3, *M. georgiense* KCTC 12242^T (this study); 4, *M. halophilum* KCTC 12240^T [12]; 5, *M. rhizophilum* KCCM 42386^T [12]

Note: *ECL* equivalent chain length. Only fatty acids representing at least 1% of the total cellular fatty acids of at least one of the species are listed. All strains were grown on marine agar. (–) Trace amount or not detected

[a] Summed feature two comprises any combination of $C_{12:0}$ aldehyde, $C_{14:0}$ 3-OH, and/or iso-$C_{16:1}$

[b] Summed feature three comprises the combination of iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ ω7*c*

Description of *Marinobacterium marisflavi* sp. nov

Marinobacterium marisflavi (ma.ris.fla'vi. L. gen. neut. n. *maris* of the sea; L. neut. adj. *flavum* yellow; N.L. gen. n. *marisflavi* of the Yellow Sea)

Cells are Gram-negative, motile and straight rods (1.1–1.7 μm long and 0.4–0.7 μm wide). Colonies are smooth, circular, convex, opaque, light-beige in color, and 0.2–0.3 mm in diameter after 3 days of incubation at 30°C on MA. Growth occurs at 15–42°C (optimally at 30°C) but not below 10°C. Growth is observed at pH 5.0–11.0 and 1.5–7.5% NaCl, optimally at pH 7.0–8.0 and 2.5–3.0% NaCl. *Marinobacterium marisflavi* sp. nov. requires NaCl for growth, is slightly halophilic, and does not grow under anaerobic conditions. Oxidase is positive but catalase is negative. Urease, esculin hydrolysis, and β-galactosidase (substrate, *p*-nitrophenyl-β-D-galactopyranoside) are positive. Nitrate reduction, indole production, arginine dihydrolase, acid production from glucose fermentation, and gelatinase are negative. In the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, β-glucosidase, and acid phosphatase are present. However, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase (substrate, 2-naphthyl-β-D-galactopyranoside), β-glucuronidase, α-glucosidase, *N*-acetyl- β-glucosaminidase, α-mannosidase, and α-fucosidase are absent. The species oxidized a limited range of carbon substrates in Biolog GN2 microplates as the following: α-keto butyric acid, DL-lactic acid, propionic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, L-alanine, L-asparagine, L-aspartic acid, and inosine. *Marinobacterium marisflavi* sp. nov. is susceptible to ampicillin (10 μg), chloramphenicol (25 μg), erythromycin (15 μg), penicillin G (10 μg), rifampicin (50 μg), streptomycin (10 μg), etracycline (30 μg) and vancomycin (30 μg), but resistant to gentamicin (10 μg) and kanamycin (30 μg). The cellular fatty acid profiles of the type strain are listed in Table 2. The major isoprenoid quinone type is Q-8. The DNA G+C content is 56.0 mol%. The type strain, IMCC4074^T (=KCTC 12757^T = LMG 23873^T), was isolated from surface seawater of the Yellow Sea, Korea (37°19′N, 126°40′E).

Acknowledgment This study was supported by an Inha University Research Grant.

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