Marinobacter bryozoorum sp. nov. and *Marinobacter sediminum* sp. nov., novel bacteria from the marine environment

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Two marine, Gram-negative, aerobic, halophilic strains, designated KMM 3657^T and KMM 3840^T, were isolated and found to be phylogenetically closely related to each other, showing 96.6% 16S rRNA gene sequence similarity. Both strains are members of the genus *Marinobacter* in the γ -Proteobacteria (94.7–98.0% 16S rRNA gene sequence similarity). Strain KMM 3657^T and *Marinobacter lipolyticus* SM19^T were closely related, with 98.0% sequence similarity. The novel strains shared generic physiological and chemotaxonomic properties with *Marinobacter* species, but differed in their temperature range for growth, inability to grow in 20% NaCl and at >43 °C, metabolic properties and fatty acid composition. On the basis of phenotypic and phylogenetic analysis data, it is proposed that the strains represent two novel species, *Marinobacter bryozoorum* sp. nov., with the type strain KMM 3840^T (=50-11^T=DSM 15401^T), and *Marinobacter sediminum* sp. nov., with the type strain KMM 3657^T (=R65^T=DSM 15400^T).

The genus *Marinobacter* was described by Gauthier *et al.* (1992) and currently comprises eight species, four of which were described in 2003 [*Marinobacter excellens* (Gorshkova *et al.*, 2003), *Marinobacter litoralis* (Yoon *et al.*, 2003), *Marinobacter lipolyticus* (Martín *et al.*, 2003) and *Marinobacter lutaoensis* (Shieh *et al.*, 2003)] and two in 2004 [*Marinobacter daepoensis* and *Marinobacter flavimaris* (Yoon *et al.*, 2004)]. The type species is *Marinobacter hydrocarbonoclasticus* (Gauthier *et al.*, 1992). In the course of studying the diversity of halophilic bacteria associated with the marine environment, strains KMM 3657^T (=R65^T) and KMM 3840^T (=50-11^T) were isolated from a sediment sample obtained from Peter the Great Bay, Sea of Japan, Russia, and from a *Bryozoan* specimen collected in the Bering Sea, August 1991, respectively. Aliquots of the diluted sediment sample and homogenized internal tissues of the *Bryozoan* specimen were spread on agar plates of sea water medium (SWM), containing (l⁻¹): 5.0 g peptone, 2.5 g yeast extract, 1.0 g glucose, 0.2 g K₂HPO₄, 0.05 g

MgSO₄, 500 ml sea water, 500 ml distilled water and 15.0 g agar. Samples were incubated for 7 days at 28 °C. Each colony was streaked and cultivated on nutrient medium containing sodium ions or natural sea water. Strains KMM 3657^T and KMM 3840^T, deposited in the Collection of Marine Micro-organisms (KMM), Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia, were stored at –80 °C in liquid nutrient medium supplemented with 20% (v/v) glycerol. Strains were routinely grown on marine 2216 agar (MA; Difco), marine broth (MB; Difco) and SWM. Standard tests, Gram-reaction evaluation and determination of oxidase, catalase, amylase, caseinase, chitinase and gelatinase activities were carried out as described by Smibert & Krieg (1994). Growth at different temperatures (4–50 °C) and pH values (pH 5.0–10.0) was examined on MA and MB media, respectively. Requirement and tolerance of various NaCl concentrations were determined using SWM prepared on the artificial sea water base supplemented with the appropriate NaCl concentrations (0, 0.5, 1, 3, 5, 8, 10, 15, 18, 18.5, 20, 25 and 30%, w/v). Leifson's medium was used to test acid production from carbohydrates with 1% (w/v) of each compound (Leifson, 1963). In addition, biochemical tests were performed using the API

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of KMM 3657^T and KMM 3840^T are AJ609270 and AJ609271, respectively.

20NE and API ZYM test kits (bioMérieux) according to the manufacturer's instructions, except that the cultures were suspended in 3% (w/v) NaCl solution. The isolates were also characterized using the Biolog GN MicroPlate panel. Strains were grown on MA medium at 28 °C for 24 h and the microtitre plates were inoculated with cells suspended in 3% (w/v) NaCl solution. Results were read automatically with a spectrophotometer after 24, 48 and 96 h and for up to 7 days incubation at 28 °C. The DNA G + C content was determined as described by Marmur & Doty (1962) with the modification of Owen *et al.* (1969). Fatty acid methyl esters were analysed using the standard procedure of the Microbial Identification system (Microbial ID) and compared to the fatty acid database. For fatty acid methyl ester analysis, bacteria were grown on MA for 3 days at 28 °C. Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and sequencing of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced directly using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The Applied Biosystems 310 DNA Genetic Analyzer was used for electrophoresis of the sequence reaction products. The 16S rRNA gene sequences were aligned manually with nucleotide sequences obtained from GenBank and EMBL. The method of Jukes & Cantor (1969) was applied to calculate evolutionary distances. Phylogenetic dendrograms were reconstructed according to the method of De Soete (1983) and the neighbour-joining method contained in the PHYLIP package (Felsenstein, 1993). The accession numbers of the reference strains used in the phylogenetic analysis are shown in Fig. 2. Automated ribotyping of the isolates was accomplished using the RiboPrinter (Qualicon) system (Allerberger & Fritschel, 1999) and *Eco*RI as the restriction enzyme for cutting the genomic DNA. The patterns were compared to those included in the DSMZ RiboPrint database.

The marine isolates KMM 3840^T and KMM 3657^T were Gram-negative, aerobic, oxidase- and catalase-positive, non-pigmented, halophilic bacteria. The strains studied were motile, rod-shaped organisms that differed slightly in their cell morphology. Cells of KMM 3840^T were ovoid, 1.0–1.3 µm long and 0.4–0.5 µm in diameter (Fig. 1a), whereas those of KMM 3657^T were long rods, 1.8–2.5 µm long and 0.3–0.4 µm in diameter (Fig. 1b). Isolates KMM 3840^T and KMM 3657^T required sodium ions for growth and grew in 1.0–18.0% (w/v) NaCl at 7–42 °C and 0.5–18.0% (w/v) NaCl at 4–42 °C, respectively.

According to 16S rRNA gene sequence analysis, strains KMM 3840^T and KMM 3657^T were closely related to each other phylogenetically (96.6% sequence similarity) and showed moderate to close gene sequence relatedness to some *Marinobacter* type strains, i.e. *M. lipolyticus* SM19^T, *M. hydrocarbonoclasticus* DSM 8798^T and *Marinobacter aquaeolei* VT8^T (sequence similarity values of 96.6, 96.1 and 96.1% to KMM 3840^T and 98.0, 96.4 and 96.6% to

Fig. 1. Scanning electron micrographs of strains KMM 3840^T (a) and KMM 3657^T (b). Bars, 2 µm.

KMM 3657^T, respectively). The sequence divergence values of >2% obtained for the novel isolates and previously described *Marinobacter* type strains exceeded the value accepted as criteria for the delineation of different species (Stackebrandt & Goebel, 1994). The phylogenetic position of the two isolates was identical using two different treeing algorithms. Fig. 2 displays the dendrogram based on neighbour-joining analysis (Felsenstein, 1993). Ribotyping analysis (Fig. 3), which served as a basis for molecular identification, revealed individual patterns for the two novel isolates, thus confirming the differences between strain KMM 3657^T and *M. lipolyticus* DSM 15157^T.

The physiological and genomic characteristics of strains KMM 3840^T and KMM 3657^T were compared with those of other species of *Marinobacter* (Table 1). Some biochemical characteristics and carbon sources for growth were similar between the novel isolates and other *Marinobacter* species, but many differences were found that allowed them to be distinguished from each other (Table 1). Both strains examined could be distinguished from related *Marinobacter* species in their maximal growth temperature, salinity

Fig. 2. Dendrogram, constructed using 16S rRNA gene sequences (Felsenstein, 1993), showing the phylogenetic positions of the novel members of the genus *Marinobacter*. Numbers indicate the percentage of bootstrap samplings, derived from 500 resamplings. Bar, 5% sequence divergence. More distantly related members of the γ -Proteobacteria served as a root. Numbers in parentheses are GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences.

range for growth, narrow spectrum of organic substrates that could be utilized as sole carbon sources, weak or negative enzymic reactions and DNA G+C content (Table 1). Other reactions obtained by API 20NE, API ZYM and Biolog are indicated in the species description.

The fatty acid compositions of the isolates are shown in Table 2. The fatty acids $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{16:1}\omega 9c$ and $C_{18:1}\omega 9c$ have been reported to be predominant in the cellular fatty acid compositions of known *Marinobacter* species (Spröer *et al.*, 1998; Nguyen *et al.*, 1999; Martín *et al.*,

2003; Yoon *et al.*, 2003). Strain KMM 3657^T possessed significant amounts of $C_{16:1}\omega 7c$ (15.87%), whereas strain KMM 3840^T differed from other *Marinobacter* strains by having a high proportion of $C_{18:1}\omega 9c$ and a low level of $C_{16:1}\omega 9c$ (Table 2). The DNA G+C content of KMM 3657^T was 56.5 mol%, whereas that of strain KMM 3840^T was found to be slightly higher (59.6 mol%) than those of the other *Marinobacter* strains, which range between 55 and 57 mol%.

On the basis of the physiological and molecular properties, it is proposed that isolates KMM 3840^T and KMM 3657^T represent two novel species in the genus *Marinobacter*, *Marinobacter bryozoorum* sp. nov. and *Marinobacter sediminum* sp. nov., respectively.

Description of *Marinobacter bryozoorum* sp. nov.

Marinobacter bryozoorum [bry.o.zo.o'rum. N.L. gen. pl. n. *bryozoorum* of Bryozoa (bryozoans), marine invertebrate specimen, source of isolation].

Gram-negative, aerobic, heterotrophic, oxidase- and catalase-positive, motile and rod-shaped (1.0–1.3 μ m long and 0.4–0.5 μ m in diameter). Requires sodium ions for growth. Grows in 1.0–18.0% (w/v) NaCl at 7–42 °C. No growth observed in >18.5% NaCl or at >43 °C. Colonies on MA are non-pigmented, whitish, translucent and smooth,

Fig. 3. Diversity of normalized *EcoRI* ribotype patterns found within the novel members of the genus *Marinobacter* and their phylogenetic neighbours.

Table 1. Phenotypic characteristics of KMM 3840^T, KMM 3657^T and phylogenetically related *Marinobacter* species

Species/strains: 1, KMM 3840^T; 2, KMM 3657^T; 3, *M. hydrocarbonoclasticus*; 4, *M. lipolyticus*; 5, *M. aquaeolei*; 6, *M. litoralis*. Data were obtained from this study and the following references: Gauthier *et al.* (1992), Nguyen *et al.* (1999), Yoon *et al.* (2003) and Martín *et al.* (2003). All strains are positive for motility, catalase, oxidase, alkaline phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl- β -glucosaminidase (strain SM19^T was not tested). All strains are negative for arginine dihydrolase, lipase C14, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase (strain SM19^T was not tested), and utilization of D-fructose, D-mannitol, D-gluconate and L-arginine (strain SM19^T was not tested). +, Positive; –, negative; W, weak reaction; V, variable; ND, not determined.

Feature	1	2	3	4	5	6
Cell size (μm)	0.4–0.5 $\times$ 1.0–1.3	0.3–0.4 $\times$ 1.8–2.5	0.3–0.6 $\times$ 2.0–3.0	0.3–0.5 $\times$ 2.5–3.5	0.4–0.5 $\times$ 1.4–1.6	0.5–0.8 $\times$ 1.5–3.0
Nitrite reduction to N ₂	–	–	+	–	–	–
Growth in/at:						
0 % NaCl	–	–	–	–	+	–
20 % NaCl	–	–	+	–	+	–
4 °C	–	+	–	–	–	+
Maximum growth temperature (°C)	42	42	45	37	50	46
Hydrolysis of:						
Tween 80	–	W	+	+	+	+
Gelatin	–	–	V	–	+	–
Urease	–	–	–	ND	+	+
Utilization of:						
Adipate	+	–	+	ND	–	ND
Citrate	–	–	+	–	+	ND
D-Glucose	–	+	–	+	–	–
D-Mannose	+	+	+	–	+	+
Cellobiose	+	+	+	–	+	+
Sucrose	+	+	+	–	+	+
Glycerol	+	+	+	–	+	+
L-Serine	+	+	+	–	+	+
Malate	–	–	+	ND	ND	ND
Acetate	–	–	+	–	+	ND
Butyrate	–	–	+	ND	+	ND
DL-Lactate	–	–	+	–	+	ND
L-Glutamate	–	–	+	–	+	+
L-Proline	–	–	+	–	+	ND
L-Leucine	–	–	–	–	+	ND
L-Alanine	–	–	–	–	+	ND
API ZYM tests:						
Esterase C4	–	–	+	ND	+	+
Esterase lipase C8	W	W	+	ND	+	+
Leucine arylamidase	+	W	+	ND	+	+
Valine arylamidase	W	–	W	ND	+	–
Acid phosphatase	W	–	+	ND	+	+
DNA G + C content (mol%)	59.6	56.5	57.5	57.0	55.7	55

2–3 mm in diameter. In addition to the phenotypic characteristics indicated in Table 1, the type strain does not produce caseinase, amylase or chitinase. Does not produce acid from glucose, sucrose, lactose, maltose, galactose, arabinose, rhamnose, *N*-acetylglucosamine, glycerol or mannitol (determined by Leifson's method). According to API 20NE, positive for nitrate reduction and adipate utilization, and negative for indole production,

arginine dihydrolase, urease production, aesculin, gelatin, β -galactosidase and utilization of D-glucose, D-mannitol, maltose, L-arabinose, D-mannose, *N*-acetylglucosamine, D-gluconate, caprate, L-malate, citrate and phenylacetate. Biolog GN MicroPlate tests were positive for utilization of Tween 40 and methyl pyruvate and negative for the other organic compounds included in the Biolog panel. The whole-cell fatty acid composition is given in Table 2.

Table 2. Fatty acid composition (%) of KMM 3840^T, KMM 3657^T and some *Marinobacter* species

Strains: 1, KMM 3840^T; 2, KMM 3657^T; 3, *M. hydrocarbonoclasticus* DSM 8798^T; 4, *M. litoralis* SW-45^T; 5, *M. aquaeolei* VT8^T; 6, *M. lipolyticus* SM19^T. Data for *Marinobacter* species from Nguyen *et al.* (1999), Yoon *et al.* (2003) and Martín *et al.* (2003). ND, Not determined.

Fatty acid	1	2	3	4	5	6
C _{12:0}	5.58	4.15	4.66	5.5	7.89	8.3
C _{12:0} 3-OH	10.68	8.04	7.74	9.6	9.94	11.3
C _{14:0}	0.81	0.92	2.24	3.0	2.60	0
C _{15:0}	0	0	1.36	0.7	2.24	1.0
C _{16:0}	17.45	21.78	23.59	25.0	22.62	28.5
C _{16:1} ω9c	3.67	13.28	7.40	10.6	11.62	10.5
C _{16:1} ω7c	0	15.87	0	0	0	ND
C _{16:1} ω7d/15 iso 2-OH	0.97	0	8.29	2.6	6.78	ND
C _{16:0} 10-methyl	0	0	2.78	0.8	2.84	4.0
C _{17:1} ω8c	0.53	2.79	3.60	0.9	4.54	2.9
C _{17:0}	1.36	1.31	3.43	2.5	3.78	3.6
C _{18:1} ω9c	47.51	16.12	29.44	30.3	19.82	13.9
C _{18:1} ω7c	0.78	2.91	0	0.5	0	2.3
C _{18:0}	4.24	2.20	1.87	2.9	1.44	2.7
C _{18:3} ω6c (6,9,12)	0	1.88	ND	ND	ND	ND
C _{19:0} cyclo ω10c/19ω6	4.91	0	ND	ND	ND	ND

Type strain is KMM 3840^T (= 50-11^T = DSM 15401^T). The DNA G + C content of the type strain is 59.6 mol%. Isolated from a *Bryozoa* specimen collected in the Bering Sea.

Description of *Marinobacter sediminum* sp. nov.

Marinobacter sediminum (se.di.mi'num. L. gen. pl. n. *sediminum* of sediments, pertaining to source of isolation).

Gram-negative, aerobic, heterotrophic, oxidase- and catalase-positive, motile and rod-shaped (1.8–2.5 µm long and 0.3–0.4 µm in diameter). Requires sodium ions for growth. Grows in 0.5–18.0 % (w/v) NaCl at 4–42 °C. No growth is observed in >18.5 % NaCl or at >43 °C. Does not produce gelatinase, caseinase, amylase or chitinase. Does not produce acid from glucose, sucrose, lactose, maltose, galactose, arabinose, rhamnose, *N*-acetylglucosamine, glycerol or mannitol. API results are negative for β-galactosidase, indole production, arginine dihydrolase, urease production, aesculin, gelatin and utilization of L-arabinose, D-mannitol, maltose, D-mannose, *N*-acetylglucosamine, D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate. Biolog tests were positive for Tween 40, and weakly positive for Tween 80 hydrolysis and utilization of D-glucose, α-ketobutyric acid and α-ketovaleric acid; the other organic substrates included in the Biolog panel are not utilized. The whole-cell fatty acid composition is indicated in Table 2.

The type strain is KMM 3657^T (= R65^T = DSM 15400^T). The DNA G + C content of the type strain is 56.5 mol%.

Isolated from marine coastal sediments obtained from Peter the Great Bay, Sea of Japan, Russia.

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