

## *Marinobacterium halophilum* sp. nov., a marine bacterium isolated from the Yellow Sea

Ho-Won Chang,<sup>1,2</sup> Young-Do Nam,<sup>1,3</sup> Hyuk-Yong Kwon,<sup>1</sup> Ja Ryeong Park,<sup>1</sup> Jung-Sook Lee,<sup>1</sup> Jung-Hoon Yoon,<sup>4</sup> Kwang-Guk An<sup>2</sup> and Jin-Woo Bae<sup>1,3,5</sup>

Correspondence  
Jin-Woo Bae  
baejw@kribb.re.kr

<sup>1</sup>Biological Resource Center, KRIBB, Daejeon 305-806, Korea

<sup>2</sup>Department of Biology, Chungnam National University, Daejeon 306-764, Korea

<sup>3</sup>University of Science and Technology, Daejeon 305-333, Korea

<sup>4</sup>21 C Frontier Microbial Genomics and Applications Center, KRIBB, Daejeon 305-806, Korea

<sup>5</sup>Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Korea

A moderately halophilic, aerobic, Gram-negative bacterium was isolated from a tidal flat area of Dae-Chun, Chung-Nam, Korea. The strain, designated *mano11*<sup>T</sup>, comprised rod-shaped cells that were motile by means of polar flagella. It grew with 3–12 % NaCl and at 4–37 °C and pH 5.3–9.3. The predominant menaquinone present in this strain was MK-7 and diaminopimelic acid was not found in the cell-wall peptidoglycan. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain *mano11*<sup>T</sup> belongs to the genus *Marinobacterium*. Strain *mano11*<sup>T</sup> exhibited 92.8–98.3% 16S rRNA gene sequence similarity when compared with the type strains of three other species of the genus *Marinobacterium*. DNA–DNA hybridization between strain *mano11*<sup>T</sup> and *Marinobacterium georgiense* DSM 11526<sup>T</sup>, its closest relative in terms of 16S rRNA gene sequence similarity, was 13%. On the basis of the phenotypic, genetic and phylogenetic data, strain *mano11*<sup>T</sup> represents a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium halophilum* sp. nov. is proposed. The type strain is *mano11*<sup>T</sup> (=KCTC 12240<sup>T</sup> = DSM 17586<sup>T</sup>).

Halophilic bacteria can be found in habitats representing a wide range of salt concentrations, from marine biotopes to hypersaline environments (Ollivier *et al.*, 1994). The western and south-western coasts of the Korean peninsula consist primarily of tidal flats, which are also known as getbol (Kim *et al.*, 2004). Getbol are unique among other marine sediments as they are alternately undergoing flooding with seawater and exposure to the atmosphere (Kim *et al.*, 2005). Recently, a variety of bacterial species were isolated from Korean getbol and identified as phylogenetically novel micro-organisms (Baik *et al.*, 2005; Yi *et al.*, 2003; Yi & Chun, 2004; Yoon *et al.*, 2004). To elucidate the bacterial diversity of Korean getbol, we have searched for novel micro-organisms in these sediments. Among the different isolates obtained was a novel *Marinobacterium*-like strain, designated *mano11*<sup>T</sup>. In this study, the taxonomic position of this novel strain was determined using phenotypic, genetic and chemotaxonomic analyses.

Strain *mano11*<sup>T</sup> was isolated from a tidal flat area of Dae-Chun, Chung-Nam, Korea (36° 17' 45.2" N 126° 31' 9.5" E),

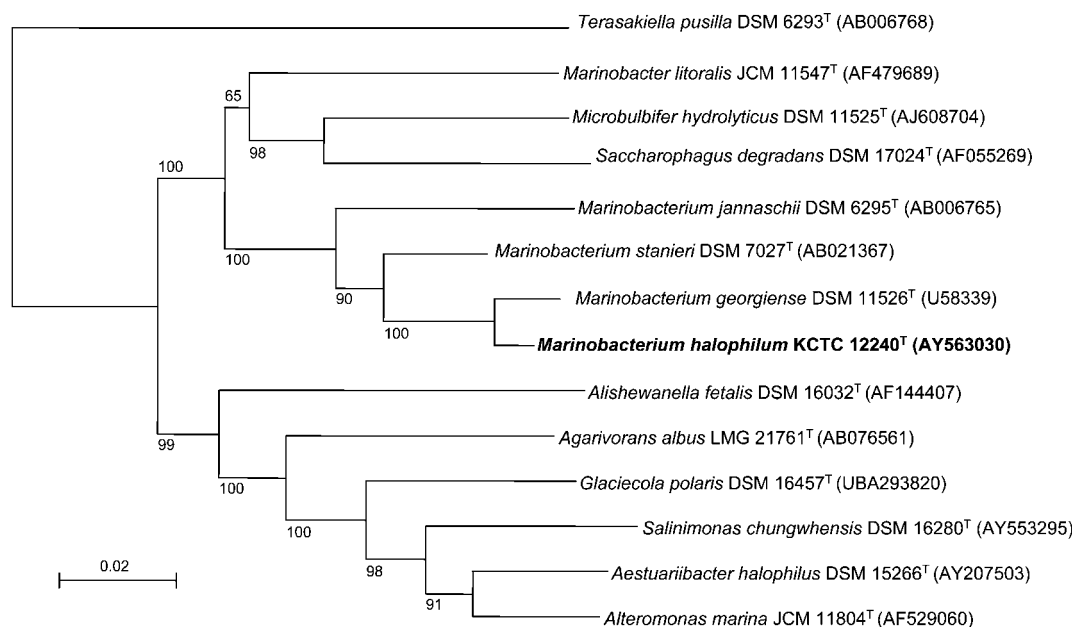
using the dilution plating technique. It was grown at 25 °C for 3 days on marine agar (Difco) plates or marine salts basal medium (MB; Baumann & Baumann, 1981) supplemented with various carbon sources. Its closest relative in terms of 16S rRNA gene sequence similarity, *Marinobacterium georgiense* DSM 11526<sup>T</sup>, which was used as a reference strain, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and grown under the same conditions. All phenotypic growth tests were carried out with the novel isolate and *M. georgiense* DSM 11526<sup>T</sup>. Bacterial cultures of the isolate and the reference strain were stored at –80 °C on MB containing 20 % glycerol. For morphological and physiological characterization, strain *mano11*<sup>T</sup> and the reference strain were generally cultivated in MB at 25 °C with shaking. API 20E, API 20NE and API ZYM test strips (bioMérieux) were used to analyse these bacterial strains biochemically and physiologically and other biochemical tests were performed using the methods and media described by Gordon *et al.* (1973). The ability to grow on various carbon sources was tested as described by Gonzalez *et al.* (1997). Catalase activity was determined by means of bubble production in a 3 % (v/v) H<sub>2</sub>O<sub>2</sub> solution. Oxidase activity was determined using an oxidase reagent

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain *mano11*<sup>T</sup> is AY563030.

(bioMérieux). Growth under anaerobic conditions was determined after incubation for 7 days in anaerobic GasPak jars (BBL) containing an atmosphere comprising N<sub>2</sub>/CO<sub>2</sub>/H<sub>2</sub> (80:10:10). Growth at various NaCl concentrations, temperatures and pH values was measured in MB. Cellular morphology and sporulation were investigated using microscopy (E600; Nikon). Cellular motility was observed for the novel isolate in fresh wet mounts of young bacterial cultures (grown in MB) by means of the hanging drop method. For observation using transmission electron microscopy, cells from exponentially growing cultures were negatively stained with 1% (w/v) phosphotungstic acid. After being air-dried, the grid was examined using a transmission electron microscope (H-7600; Hitachi). Isoprenoid quinones of the *mano11*<sup>T</sup> strain were extracted from 100 mg aliquots of freeze-dried cells according to methods described previously (Collins & Jones, 1981); they were then purified via preparative TLC (silica gel F254; Merck). The ubiquinone fraction was also analysed by HPLC (L-5000; Hitachi) using a reversed-phase column (YMC pack ODS-AM; YMC), as described previously (Shin *et al.*, 1996). Bacterial strains grown on marine agar for 3 days at 25 °C were used for the analysis of fatty acid methyl esters, which were extracted and prepared according to standard protocols provided by the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Chromosomal DNA was extracted and purified as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified by a PCR using two universal primers, as previously described (Stackebrandt *et al.*, 1993). Sequencing of the

amplified 16S rRNA gene and phylogenetic analysis were performed according to the methods described by Yoon *et al.* (1998). DNA–DNA hybridization was performed according to previously described methods (Ezaki *et al.*, 1989). The 16S rRNA gene sequence of *mano11*<sup>T</sup> was aligned with 13 reference sequences from the Ribosomal Database Project (Fig. 1), using the multiple sequence-alignment program CLUSTAL\_X (1.8) (Thompson *et al.*, 1997). Phylogenetic relationships between representatives of the genus *Marinobacterium* were determined using MEGA, version 2.1. Distance matrices were determined according to the assumptions described by Kimura (1980). These matrices were used to elaborate dendrograms using the neighbour-joining method (Saitou & Nei, 1987). To investigate the stability of the trees, a bootstrap analysis was performed. The consensus tree obtained was based on 1000 randomly generated trees.

The morphological, cultural, physiological and biochemical characteristics of strain *mano11*<sup>T</sup> and related species are shown in Table 1 and are provided in the species description (below). Strain *mano11*<sup>T</sup> grew at temperatures from 4 to 37 °C, but not at temperatures below 4 °C or above 37 °C; it grew at pH 5.3–8.8 but not at pH values below 4.1 or above 9.3. Growth was observed in the presence of 3–12% NaCl and very weak growth was observed at NaCl concentrations of 1–2%. No growth was detected at NaCl concentrations below 1% or above 15%. However, growth of *M. georgiense* DSM 11526<sup>T</sup> occurred at NaCl concentrations from 0.5 to 11.4% (Gonzalez *et al.*, 1997). The novel isolate did not



**Fig. 1.** Consensus phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain *mano11*<sup>T</sup>, type strains of different *Marinobacterium* species and representatives of related genera. The tree was constructed using the neighbour-joining method and *p*-distance. Bootstrap analyses were performed with 1000 repetitions and only values > 50% are shown. GenBank accession numbers are shown in parentheses. Bar, 0.02 substitutions per nucleotide position.

**Table 1.** Taxonomic characteristics of novel isolate mano11<sup>T</sup> and type strains of the genus *Marinobacterium*

Taxa: 1, *M. georgiense* DSM 11526<sup>T</sup>; 2, *M. jannaschii* DSM 6296<sup>T</sup> (data from Bowditch *et al.*, 1984); 3, *M. stanieri* DSM 7027<sup>T</sup> (Baumann *et al.*, 1983); 4, *M. halophilum* sp. nov. mano11<sup>T</sup>. Data for *M. georgiense* and strain mano11<sup>T</sup> are from this study. +, Positive reaction; –, negative reaction; ND, not determined. All of the taxa are motile by means of single polar flagellum, rod-shaped, aerobic, oxidase-positive, catalase-positive, unable to reduce nitrate, utilize citrate and possess quinone type Q-8.

Characteristic	1	2	3	4
Growth at 35 °C	+	–	+	+
Arginine dihydrolase	+	+	–	+
Production of:				
Acetoin	+	ND	ND	+
Gelatinase	+	+	–	–
Lipase	ND	–	–	–
Utilization of:				
Caprate	–	+	+	–
Citrate	+	+	+	+
Glucose	+	–	–	–
Mannitol	–	–	ND	–
Rhamnose	–	ND	ND	–
Sucrose	–	–	ND	–

grow under anaerobic conditions, gave a negative Voges–Proskauer test and showed catalase, oxidase and urease activities. Although *Marinobacterium jannaschii* DSM 6295<sup>T</sup> and *Marinobacterium stanieri* DSM 7027<sup>T</sup> were able to reduce nitrate to nitrite, strain mano11<sup>T</sup> could not. Colonies of strain mano11<sup>T</sup> were pale orange to yellow, whereas those of *M. georgiense* DSM 11526<sup>T</sup> were translucent. Strain mano11<sup>T</sup> grew on the following carbon sources: glucose, mannose, malate, hydroxybutyrate, citrate and phenylacetate. Substances not utilized for growth included sucrose, rhamnose, lactose, *N*-acetyl-D-glucosamine, L-arginine and glycine. Isolate mano11<sup>T</sup> hydrolysed Tween 80 but not chitin. The characteristics that differentiate the novel isolate from related species are shown in Table 1. The fatty acid methyl esters of mano11<sup>T</sup> were identified as C<sub>16:1</sub>ω7c (43.2%), C<sub>16:0</sub> (21.9%), C<sub>18:1</sub>ω7c (15.7%), C<sub>10:0</sub> 3-OH (7.6%), C<sub>12:0</sub> (4.6%), C<sub>10:0</sub> (4.3%) and C<sub>14:0</sub> (1.6%).

Phylogenetic trees based on 16S rRNA gene sequences from members of different genera of the *Alteromonadaceae* (the family that contains the genus *Marinobacterium*), showed that strain mano11<sup>T</sup> falls within a cluster comprising *Marinobacterium* species (Fig. 1). Strain mano11<sup>T</sup> exhibited 16S rRNA gene sequence similarities of 92.8–98.3% with respect to the type strains of three *Marinobacterium* species. DNA–DNA relatedness studies were performed to determine the genomic relationship between strain mano11<sup>T</sup> and *M. georgiense* DSM 11526<sup>T</sup>, its closest relative in terms of 16S rRNA gene sequence similarity. The mean level of DNA–DNA relatedness observed between these two strains was

13%. Therefore, taken together, the phylogenetic and DNA–DNA hybridization results suggest that strain mano11<sup>T</sup> represents a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium halophilum* sp. nov. is proposed.

### Description of *Marinobacterium halophilum* sp. nov.

*Marinobacterium halophilum* (ha.lo'phi.lum. Gr. n. *halos* salt; Gr. adj. *philos* loving; N.L. neut. adj. *halophilum* salt-loving).

Cells are rods with overall dimensions of 0.5–0.7 μm (width) and 2.1–3.0 μm (length) in 3-day-old cultures growing at 25 °C on marine agar plates. Gram-negative and motile. Colonies are pale orange to yellow, measure 2–3 mm in diameter and are smooth, round or slightly irregular in shape after 5 days culture on Luria–Bertani agar plates. Growth occurs in the presence of 3–12% NaCl, but no growth is observed in the absence of NaCl or when supplemented with 15% NaCl. Growth occurs at 45 °C and at pH 5.3–9.3. Casein and starch are hydrolysed. Acid is produced from sucrose, fructose, raffinose, mannitol, ribose, glycerol, mannose, lactose, glucose, maltose and trehalose. Acid is not produced from dulcitol, galactose, inulin, D-arabitol, rhamnose, arabinose, sorbitol or D-xylose. Using the API ZYM system, activity is detected for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. No activity is detected for lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Diaminopimelic acid is not present in the cell-wall peptidoglycan and the predominant quinone is Q-8. The predominant fatty acids are C<sub>16:1</sub>ω7c and C<sub>16:0</sub> (43.2 and 21.9%, respectively).

The type strain, strain mano11<sup>T</sup> (=KCTC 12240<sup>T</sup>=DSM 17586<sup>T</sup>), was isolated from a tidal flat area of Dae-Chun, Chung-Nam, Korea.

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