Marinobacterium profundum sp. nov., a marine bacterium from deep-sea sediment

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A Gram-stain-negative, rod-shaped and motile strain, designated PAMC 27536^T, was isolated from deep-sea sediment in the East Sea, Korea. Analysis of the 16S rRNA gene sequence of the strain showed an affiliation with the genus *Marinobacterium*. Phylogenetic analyses revealed that strain PAMC 27536^T was related most closely to *Marinobacterium rhizophilum* CL-YJ9^T with a 16S rRNA gene sequence similarity of 98.5 % and to other members of the genus *Marinobacterium* (94.0–91.7 %). Genomic relatedness analyses between strain PAMC 27536^T and *M. rhizophilum* KCCM 42386^T gave an average nucleotide identity of 85.6 % and an estimated DNA–DNA hybridization of 24.6 % using the genome-to-genome distance calculator, indicating that they represent genomically distinct species. Cells of strain PAMC 27536^T grew optimally at 25–30 °C and pH 7.0–7.5 in the presence of 3 % (w/v) sea salts. The major cellular fatty acids were $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$, and $C_{16:0}$. The major isoprenoid quinone was Q-8. The genomic DNA G+C content was 56.1–57.2 mol%. Based on the phylogenetic, chemotaxonomic, genomic and phenotypic data presented, a novel species with the name *Marinobacterium profundum* sp. nov. is proposed, with PAMC 27536^T (=KCCM 43095^T=JCM 30410^T) as the type strain.

The genus *Marinobacterium* was established by González *et al.* (1997) for a marine bacterium isolated from lignin-rich pulp mill waste, with *Marinobacterium georgiense* as the type species. Phylogenetic analyses of 16S rRNA and *gyrB* gene sequences have improved the classification of members of the genus *Marinobacterium*, with the transfer of *Pseudomonas stanieri* (Baumann *et al.*, 1983) and *Oceanospirillum jannaschii* (Bowditch *et al.*, 1984) to *Marinobacterium stanieri* and *Marinobacterium jannaschii*, respectively (Satomi *et al.*, 2002). The genus *Marinobacterium* belongs to the family *Alteromonadaceae*, and members of this genus contain $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$, and $C_{16:0}$ as the major fatty acids and Q-8 as the major respiratory quinone (González *et al.*, 1997; Kim *et al.*, 2008; Chimetto *et al.*, 2011; Alfaro-Espinoza & Ullrich, 2014). The genomic DNA G+C content of *Marinobacterium* species with validly published names ranges from 54.9 to 62.5 mol% (González *et al.*, 1997; Huo *et al.*, 2009). In addition to the aforementioned species, ten *Marinobacterium* species have been isolated from marine habitats, including coastal seawaters (*M. litorale*, Kim *et al.*, 2007; *M. marisflavi*, Kim *et al.*, 2009a), tidal flats (*M. halophilum*, Chang *et al.*, 2007; *M. lutimaris*, Kim *et al.*, 2010), roots of salt-tolerant plants (*M. rhizophilum*, Kim *et al.*, 2008; *M. mangrovicola*, Alfaro-Espinoza & Ullrich, 2014), sediments (*M. nitratireducens* and *M. sediminicola*, Huo *et al.*, 2009; *M. maritimum*, Kim *et al.*, 2009b) and coral (*M. coralli*, Chimetto *et al.*, 2011). Here, we isolated a bacterial strain (PAMC 27536^T) during a study to screen for methanol-utilizing bacteria associated with the biogeochemical processes of deep-sea sediments, and performed a polyphasic analysis to determine the taxonomic position of the strain.

A sediment core was collected using a box corer on R/V *Araon* in the East Sea (35.90° N 129.77° E; water column depth of 840 m) in July 2013. A surface sample of the sediment core amended with methanol (Sigma; final concentration of approximately 1 %) was incubated at 20 °C for 30 days to enrich methanol-utilizing bacteria. Subsequently, the methanol-amended sediment sample was diluted approximately 50-fold with autoclaved 3 % (w/v)

Abbreviation: ANI, average nucleotide identity.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the draft genome of strain PAMC 27536^T are KR047788 and BCNS01000000, respectively.

One supplementary table is available with the online Supplementary Material.

NaCl solution. An aliquot (100 µl) of the sediment slurry was spread on the basal agar medium (per litre distilled water: 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2 . 6H_2O$, 5.94 g $MgSO_4 . 7H_2O$, 1.3 g $CaCl_2 . 2H_2O$, 0.2 g $NaNO_3$, 0.2 g NH_4Cl, 15 g agar, 0.05 g yeast extract; Bruns *et al.*, 2001) supplemented with methanol as sole carbon source (final concentration 0.5 %) and the plate was incubated at 20 °C under aerobic conditions for 10 days. Colonies were picked and checked for growth on marine agar 2216 (MA; Difco) as a conventional cultivation medium. Strain PAMC 27536^T was isolated and subsequently streaked onto MA. The purification procedure was repeated four times. The strain was maintained on MA at 20 °C and preserved in marine broth 2216 (MB; Difco) supplemented with 30 % (v/v) glycerol at 80 °C.

M. rhizophilum KCCM 42386^T (Kim *et al.*, 2008) was obtained from the Korean Culture Center of Microorganisms (KCCM) for comparisons of the phenotypic and chemotaxonomic characteristics with those of strain PAMC 27536^T. Growth of strain PAMC 27536^T and *M. rhizophilum* KCCM 42386^T was tested on MA at various temperature and salinity conditions (see below). Unless otherwise specified, all characteristics of strain PAMC 27536^T and *M. rhizophilum* KCCM 42386^T were based on cultures grown aerobically on MA at 30 °C for 3–5 days. Under these conditions, both strains appeared to be in mid- to late-exponential phases of growth.

PCR amplification of the 16S rRNA gene and direct sequencing of purified PCR products were performed as described by Hwang *et al.* (2015). The almost-complete 16S rRNA gene sequence (1474 bp) of strain PAMC 27536^T was obtained and analysed using a BLAST search against the GenBank and EzTaxon-e databases (Altschul *et al.*, 1990; Kim *et al.*, 2012). The 16S rRNA gene sequences of closely related taxa obtained from the GenBank database were aligned using the RDP aligner (Cole *et al.*, 2014) based on secondary structures. Phylogenetic analysis was performed using the program MEGA 6.0 (Tamura *et al.*, 2013). Distance matrices were calculated according to the Jukes and Cantor model (Jukes & Cantor, 1969). Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-likelihood (Felsenstein, 1981) methods using bootstrap analyses based on 1000 replications.

Phylogenetic analyses based on the 16S rRNA gene sequence showed that strain PAMC 27536^T was affiliated with the genus *Marinobacterium* (Fig. 1). Strain PAMC 27536^T was related most closely to *M. rhizophilum* CL-YJ9^T (98.5 % 16S rRNA gene sequence similarity) and next to the type strains of other *Marinobacterium* species with validly published names (94.0–91.7 %). The tree topologies inferred using the neighbour-joining, minimum-evolution and maximum-likelihood algorithms revealed that strain PAMC 27536^T formed a robust clade with *M. rhizophilum* CL-YJ9^T supported by a high bootstrap value of 100 % with all three tree-making methods (Fig. 1).

Phenotypic characteristics of strain PAMC 27536^T were tested in duplicate along with *M. rhizophilum* KCCM 42386^T, with repeat experiments on different days. Gram-staining was performed using a Gram-stain kit (Sigma). Cell morphology was examined by transmission electron microscopy (EX2; JEOL). Anaerobic growth was tested in an anaerobic jar (BBL) containing an AnaeroPak (Mitsubishi Gas Chemical) at 25 °C for 5 days. The temperature range for growth was examined by the ability to form colonies on MA with incubation at 4, 10–30 (in increments of 5 °C), 32, 34, 37 and 42 °C. The pH range (pH 5.0–11.0 at intervals of 0.5 pH units) for growth was determined by assessing turbidity measured as OD_{600} in pH-buffered MB (Hwang & Cho, 2008) using citric acid-phosphate buffer for pH 5.0, MES for pH 5.5–6.5, MOPS for pH 7.0–7.5, AMPD for pH 8.0–9.5 and CAPS for pH 10.0–11.0 (each at a final concentration of 50 mM at 25 °C), for up to 2 weeks. Salt tolerance was determined by assessing turbidity measured as OD_{600} at 25 °C using synthetic ZoBell broth (per litre distilled water: Bacto peptone, 5 g; yeast extract, 1 g; ferric citrate, 0.1 g) supplemented with 0–6 (at intervals of 1 %), 8, 10 and 12 % (w/v) sea salts (Sigma).

Catalase and oxidase tests were performed according to the methods described by Smibert & Krieg (1994) and Cappuccino & Sherman (2002), respectively. Hydrolysis of casein, starch, Tween 80, xanthine and hypoxanthine, and deaminase activities of ornithine, lysine and arginine were investigated as described by Hansen & Sørheim (1991). H_2S production was tested as described by Bruns *et al.* (2001). In addition, other enzyme activities using the API ZYM and API 20NE kits (bioMérieux) and acid production using the API 50CH kit (bioMérieux) were assayed according to the manufacturer's instructions except that the cell suspension was prepared as described by Hwang *et al.* (2009). Utilization of various substrates as sole carbon and energy sources was tested using the basal broth medium supplemented with yeast extract (per litre distilled water: 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2 . 6H_2O$, 5.94 g $MgSO_4 . 7H_2O$, 1.3 g $CaCl_2 . 2H_2O$, 0.2 g $NaNO_3$, 0.2 g NH_4Cl, 0.05 g yeast extract; Bruns *et al.*, 2001) with a final concentration of 0.4 % carbon source. Carbon utilization was scored as negative when growth was equal to, or less than, that in the negative control with no carbon source. Growth was measured by monitoring changes in the OD_{600} at 25 °C for 3 weeks.

Cells of strain PAMC 27536^T were rods approximately 0.7–1.0 µm wide and 1.9–2.4 µm long (Table 1). Strain PAMC 27536^T grew optimally at 25–30 °C and pH 7.0–7.5 in the presence of 3 % (w/v) sea salts and was positive for catalase and oxidase activities. Other physiological and biochemical characteristics of strain PAMC 27536^T are given in the species description and Table 1. Strain PAMC 27536^T could be phenotypically distinguished from *M. rhizophilum*

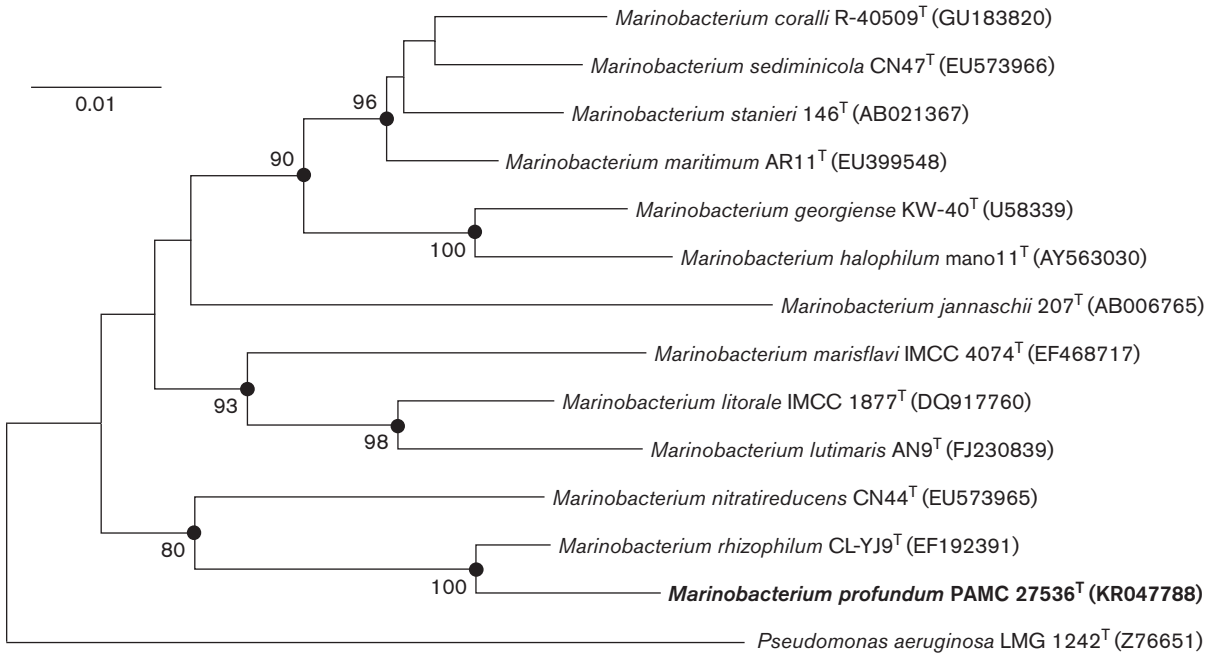

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain PAMC 27536^T and related species on the basis of 16S rRNA gene sequences. Only bootstrap values above 70 % are shown (1000 resamplings) at branch points. Filled circles indicate that the corresponding nodes were also obtained in both the maximum-likelihood and the minimum-evolution trees. *Pseudomonas aeruginosa* LMG 1242^T (Z76651) was used as an outgroup. Bar, 0.01 nt substitutions per site.

KCCM 42386^T based on the temperature and salinity ranges for growth, ability to utilize melibiose as sole carbon and energy source and inability to produce α-glucosidase in the API ZYM assay (Table 1). In addition, acid production from 12 carbohydrates in the API 50CH assay gave different results for the two strains (Table 1).

The fatty acid methyl esters in whole cells of strain PAMC 27536^T and *M. rhizophilum* KCCM 42386^T grown on MA at 30 °C for 5 days were analysed by GC (Agilent Technologies 7890B) according to the instructions of the Microbial Identification System (MIDI; version 6.2) with the TSBA6 database. Genomic DNA of strain PAMC 27536^T was

Table 1. Differential characteristics between strain PAMC 27536^T and *M. rhizophilum* KCCM 42386^T

Data were obtained in this study, unless otherwise indicated. +, Positive; −, negative.

Characteristic	PAMC 27536^T	*M. rhizophilum* KCCM 42386^T
Temperature range (°C) for growth (optimum)	4–32 (25–30)	10–34 (25–30)
Salinity range (%, w/v) for growth (optimum)	1–4 (3)	1–6 (3)
pH range for growth (optimum)	6.0–9.0 (7.0–7.5)	6.0–9.0 (7.0)
α-Glucosidase	−	+
Acid production from:		
D-Adonitol, D-arabitol, L-arabitol, cellobiose, D-glucose, inositol, inulin, methyl α-D-glucopyranoisde, trehalose, turanose, xylitol	−	+
Raffinose	+	−
Utilization as sole carbon and energy source:		
N-Acetyl-D-glucosamine	−	+
Melibiose	+	−
DNA G+C content (mol%; HPLC analysis)	56.1 (57.2)*	61†

*Whole genome analysis.
†Data from Kim *et al.* (2008).

extracted using a commercial kit (DNeasy Blood & Tissue kit; Qiagen). The genome sequence of strain PAMC 27536^T was determined using an Illumina MiSeq sequencer and assembled using CLC Genomics Workbench 7.0 (Qiagen) at the ChunLab (Seoul, Korea). The draft genome of strain PAMC 27536^T was deposited at DDBJ/EMBL/GenBank under accession number BCNS01000000. A draft genome sequence of *M. rhizophilum* DSM 18822^T was retrieved from GenBank under accession number ARJM00000000 (5 360 582 bp with 68 contigs and DNA G+C content of 58.5 mol%). The degree of genome-based relatedness between strain PAMC 27536^T and *M. rhizophilum* DSM 18822^T was estimated by both an average nucleotide identity (ANI) value, following the BLAST-based ANI calculation method described by Goris *et al.* (2007), and the genome-to-genome distance calculation method described by Auch *et al.* (2010). The genomic DNA G+C content of strain PAMC 27536^T was also determined by both HPLC analysis (Tamaoka & Komagata, 1984) carried out by the identification service of the KCCM and whole genome analysis calculated from the draft genome.

The major fatty acids (>10 %) of strain PAMC 27536^T were $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$ (42.7 %), $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$ (20.9 %), and $C_{16:0}$ (19.1 %; Table S1, available in the online Supplementary Material), which are typically found as major components in members of the genus *Marinobacterium* (Kim *et al.*, 2008; Chimetto *et al.*, 2011; Alfaro-Espinoza & Ullrich, 2014). No major differences were found in the fatty acid profiles of strain PAMC 27536^T and *M. rhizophilum* KCCM 42386^T (Table S1).

The draft genome of strain PAMC 27536^T contained 5 637 742 bp in 226 contigs with a DNA G+C content of 57.2 mol% and coverage of 169×. The ANI value between strain PAMC 27536^T and the type strain of *M. rhizophilum* was 85.6 %, which is below the proposed cut-off ANI values of 95–96 % for delineating bacterial species (Goris *et al.*, 2007; Richter & Rosselló-Móra, 2009). Consistently, the mean DNA–DNA hybridization value estimated by genome-to-genome distance calculation was 24.6±2.4 % between strain PAMC 27536^T and the type strain of *M. rhizophilum*, indicating that PAMC 27536^T is a member of a separate species of the genus *Marinobacterium* (Rosselló-Mora & Amann, 2001).

The genomic DNA G+C content of strain PAMC 27536^T was 56.1 mol% as determined by HPLC analysis, which was similar to that based on calculation from the draft genome of the strain (57.2 mol%; Table 1). The DNA G+C content of strain PAMC 27536^T was lower than that of its phylogenetically closest relative, *M. rhizophilum* KCCM 42386^T (61 mol%; Kim *et al.*, 2008), but nevertheless was within the range of other *Marinobacterium* species (54.9–62.5 mol%; González *et al.*, 1997; Huo *et al.*, 2009).

Although strain PAMC 27536^T was isolated from a methanol-enriched sediment sample, growth was not detected in the medium employed here when supplemented with methanol as sole carbon source (final concentration of 0.1 or 0.4 %). However, the draft genome of PAMC 27536^T exhibits the presence of genes encoding the biosynthesis of alcohol dehydrogenase and a coenzyme pyrroloquinoline quinone (PQQ) (data not shown), which play a key step in methylotrophic metabolism (i.e. oxidation of methanol to formaldehyde; Witthoff *et al.*, 2013), suggesting the potential of this strain as a methanol-utilizing bacterium.

The phylogenetic, chemotaxonomic, genomic and phenotypic data obtained in this study indicate that strain PAMC 27536^T should be assigned to a novel species in the genus *Marinobacterium*, for which the name *Marinobacterium profundum* sp. nov. is proposed.

Description of *Marinobacterium profundum* sp. nov.

Marinobacterium profundum (pro.fun′dum. L. neut. adj. *profundum* deep, living within the depth of the oceans).

Gram-stain-negative, strictly aerobic, motile rods approximately 0.7–1.0 μm wide and 1.9–2.4 μm long. After 7 days on MA at 30 °C, colonies are creamy white, circular, convex and approximately 0.5 mm in diameter. Grows at 4–32 °C (optimum, 25–30 °C) and at pH 6.0–9.0 (optimum, pH 7.0–7.5). Growth occurs at sea-salt concentrations of 1–4 % (w/v) (optimum, 3 %). Positive for oxidase and catalase. Starch, Tween 80 and hypoxanthine are hydrolysed. Casein, aesculin, gelatin and xanthine are not hydrolysed. Indole production, H_2S production, glucose fermentation and nitrate reduction are negative. Alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive; esterase lipase (C8) is weakly positive; *N*-acetyl-β-glucosaminidase, arginine dihydrolase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, urease and trypsin are negative. Acid is produced from L-arabinose, D-fructose, DL-fucose, glycerol, melibose (weakly), raffinose, L-rhamnose (weakly), potassium 2-ketogluconate and sucrose, but not from *N*-acetylglucosamine, D-adonitol, amygdalin, D-arabinose, DL-arabitol, arbutin, cellobiose, dulcitol, erythritol, aesculin ferric citrate, D-galactose, gentiobiose, D-glucose, glycogen, inositol, inulin, lactose, D-lyxose, maltose, D-mannitol, D-mannose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, melezitose, methyl β-D-xylopyranoside, potassium gluconate, potassium 5-ketogluconate, D-ribose, salicin, D-sorbitol, L-sorbose, starch, D-tagatose, trehalose, turanose, xylitol or DL-xylose. *N*-Acetyl-D-glucosamine, DL-aspartate, glycogen, melibiose, L-ornithine, L-proline, pyruvate and succinate are utilized as sole carbon and energy source, but acetate, L-arabinose, cellobiose, citrate, formate, D-fructose, D-galactose, D-glucose, glycerol, inositol, lactose, maltose, D-mannitol, D-mannose, methanol, raffinose, L-rhamnose, L-threonine, trehalose and

sucrose are not utilized. Major fatty acids are $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$, and $C_{16:0}$.

The type strain, PAMC 27536^T (=KCCM 43095^T=JCM 30410^T), was isolated from deep-sea sediment. The DNA G+C content of the type strain is 56.1 mol% (HPLC analysis) – 57.2 mol% (whole genome analysis).

Acknowledgements

We thank the crew and captain of R/V *Araon* for their excellent cooperation during the cruise. This work was supported by grants of the Korea Polar Research Institute (PP15010 and PE15080).

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