

Marinobacterium lutimaris sp. nov., isolated from a tidal flat

Jeong Myeong Kim, Se Hee Lee, Ji Young Jung and Che Ok Jeon

Department of Life Science, Chung-Ang University, Seoul 156-756, Republic of Korea

Correspondence
Che Ok Jeon
cojeon@cau.ac.kr

A Gram-staining-negative, moderately halophilic bacterium, designated strain AN9^T, was isolated from a tidal flat of the Taean coast in South Korea. Cells were catalase- and oxidase-positive short rods that were motile by means of a single polar flagellum. Growth of strain AN9^T was observed at 15–40 °C (optimum, 25–30 °C) and at pH 6.0–8.0 (optimum, pH 6.5–7.5). Strain AN9^T contained ubiquinone Q-8 as the predominant isoprenoid quinone and C_{10:0} 3-OH (31.7%), C_{18:1} ω7c (24.8%), C_{16:0} (14.7%) and summed feature 3 (comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH, 10.72%) as the major fatty acids. The G + C content of the genomic DNA of strain AN9^T was 58 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain AN9^T was related to members of the genus *Marinobacterium* and was related most closely to *Marinobacterium litorale* IMCC1877^T (96.8% similarity). On the basis of chemotaxonomic and molecular data, strain AN9^T is considered to represent a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium lutimaris* sp. nov. is proposed. The type strain is AN9^T (=KACC 13703^T =DSM 22012^T).

Vast tidal flats, known as getbol, are found along the Yellow Sea coast of the Korean peninsula. These habitats alternately undergo flooding with seawater and exposure to the atmosphere. Getbols provide valuable areas for microorganisms and marine animals and play an important role in the restoration of human-impacted coastal ecosystems. In the past few years, numerous microbial species have been isolated from the getbols of the Yellow Sea (Yi & Chun, 2004a, b, c; Baik *et al.*, 2005; Kim *et al.*, 2008a; Yoon *et al.*, 2008, 2009). The genus *Marinobacterium*, a member of the *Gammaproteobacteria*, was proposed by González *et al.* (1997) with the description of *Marinobacterium georgiense*, and many *Marinobacterium* species have been isolated from marine sediments (Chang *et al.*, 2007; Kim *et al.*, 2007, 2009a, b; Huo *et al.*, 2009). At the time of writing, the genus *Marinobacterium* comprises nine recognized species. In the course of investigations into the microbial communities inhabiting tidal flats of South Korea, a novel, moderately halophilic, *Marinobacterium*-like bacterium, designated strain AN9^T, was isolated from a tidal flat of the Yellow Sea. Based on data from the present polyphasic study, strain AN9^T is considered to represent a novel species of the genus *Marinobacterium*.

Strain AN9^T was isolated from getbol of Taean in South Korea by using a modification of the procedure described by Kim *et al.* (2008b). Briefly, the sediment was dispersed

in 0.9% (w/v) saline. The resultant suspension was serially diluted in 10-fold steps by the addition of 1 ml of the previous dilution to 9 ml saline, and 0.1-ml aliquots from each diluted suspension were spread on marine agar 2216 (MA; Difco) plates. The plates were then incubated under aerobic conditions at 25 °C for 5 days. Colonies were selected randomly and crude lysates containing genomic DNA from colonies were prepared by boiling a small amount of cell material in 100 µl of 5% Chelex 100 solution (Bio-Rad) for 10 min. The supernatant was prepared by centrifugation at 15 000 g for 10 min, and PCR amplification of 16S rRNA genes from the crude lysates was performed by using the universal primers described by Lu *et al.* (2006). The amplicons were double-digested with restriction enzymes *Hae*III and *Hha*I. Restriction fragment length polymorphism (RFLP) patterns were analysed on 2.5% MetaPhore agarose (BioWhittaker) gels; colonies were grouped according to their RFLP patterns and representative PCR products containing distinct RFLP patterns were sequenced. The resulting 16S rRNA gene sequences were analysed by using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) within the GenBank database. From this analysis, a novel *Marinobacterium*-like strain, AN9^T, was selected for further phenotypic and phylogenetic analysis. The strain was routinely grown aerobically on MA at 30 °C for 3 days, except where indicated otherwise. The strain was stored at –80 °C in marine broth (Difco) supplemented with 10% (v/v) glycerol.

The 16S rRNA gene sequence (1427 nt) of strain AN9^T was checked manually for quality and gaps. Sequence similarity

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AN9^T is FJ230839.

A transmission electron micrograph of a cell of strain AN9^T is available as supplementary material with the online version of this paper.

values between the new isolate and the type strains of related bacteria were evaluated by using the Nucleotide Similarity Search program (<http://147.47.212.35:8080>; Chun *et al.*, 2007) and the sequences were aligned by using the CLUSTAL W software program (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in the PHYLIP program package, version 3.6 (Felsenstein, 2002). The resulting tree topologies were evaluated by bootstrap analysis based on 1000 resampled datasets with the PHYLIP package.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain AN9^T formed a tight phyletic lineage with *Marinobacterium litorale* IMCC1877^T within the genus *Marinobacterium* with 100% bootstrap support (Fig. 1). The overall topologies of the maximum-likelihood and maximum-parsimony trees were essentially the same as that of the neighbour-joining tree (Fig. 1). Comparative 16S rRNA gene sequence analysis showed that strain AN9^T was related most closely to *M. litorale* IMCC1877^T (96.8% similarity); levels of 16S rRNA gene sequence similarity to the type strains of other *Marinobacterium* species were less than 94.2%.

Growth of strain AN9^T at different temperatures and pH was examined on MA at 5–45 °C at 5 °C intervals and in marine broth adjusted to pH 5.0–10.0 at 0.5 pH unit intervals. The pH was adjusted prior to sterilization by the addition of HCl or NaOH and was measured again after sterilization. Gram staining was performed by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Cell morphology and the presence of flagella



Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain AN9^T among related taxa. Bootstrap values are shown at nodes as percentages of 1000 replicates; only values >50% are shown. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Alteromonas macleodii* DSM 6062^T was used as an outgroup. Bar, 0.1 changes per nucleotide position.

were studied via phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) with 2-day-old cells grown on MA, as described by Jeon *et al.* (2004). Requirement for and tolerance of NaCl were determined in nutrient broth (NB; Difco) (3.0 g beef extract and 5.0 g peptone per litre distilled water) supplemented with modified artificial seawater [ASW; per litre distilled water: 0–15% (w/v) NaCl at 0.5% intervals, 5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl and 1.3 g CaCl₂] (Kahng *et al.*, 2009). Oxidase activity was tested based on oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck), and catalase activity was evaluated based on the production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution (Smibert & Krieg, 1994). Nitrate reduction was assessed according to the method of Lányi (1987) and acid production from carbohydrates was tested as described by Leifson (1963). Antibiotic susceptibility tests were performed in duplicate by using filter-paper discs (diameter, 8 mm) containing the following antibiotics: ampicillin (10 µg), polymyxin B (100 U), streptomycin (50 µg), penicillin G (10 IU), gentamicin (30 µg), chloramphenicol (100 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), carbenicillin (100 µg) and novobiocin (50 µg). Additional enzyme activities and biochemical characteristics were determined by using API ZYM and API 20NE kits (bioMérieux) and utilization or oxidation of carbon sources was determined by using the GN MicroPlate system (Biolog) according to the instructions of the manufacturers. Growth was assessed on MA under anaerobic (with 4–10% CO₂) conditions by using the GasPak Plus system (BBL) at 30 °C for 15 days.

When tested on MA, growth of strain AN9^T was observed at 15–40 °C (optimum, 25–30 °C), at pH 6.0–8.0 (optimum, pH 6.5–7.5) and in the presence of 1–10.0% (w/v) NaCl (optimum, 2–5%). Cells were Gram-staining-negative, strictly aerobic, short motile rods (0.6–0.8 µm wide and 1.2–1.4 µm long) with a single polar flagellum (see Supplementary Fig. S1 in IJSEM Online). Phenotypic characteristics were in accordance with those of members of the genus *Marinobacterium*, but some characteristics allowed the differentiation of strain AN9^T from closely related *Marinobacterium* species (Table 1).

Isoprenoid quinones were analysed by HPLC (model LC-20A; Shimadzu), as described by Komagata & Suzuki (1987), with the chromatograph equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel). For analysis of fatty acid methyl esters, cells of strain AN9^T were harvested from MA plates after incubation at 30 °C for 3 days. Analysis was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc.). The DNA G+C content of strain AN9^T was determined by reversed-phase HPLC (GROM-SIL 100 ODS-2FE; GROM) according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone of strain AN9^T was ubiquinone 8

Table 1. Differential phenotypic characteristics between strain AN9^T and the type strains of *Marinobacterium* species

Strains: 1, AN9^T; 2, *M. litorale* IMCC1877^T (data from Kim *et al.*, 2007); 3, *M. georgiense* KW-404^T (González *et al.*, 1997); 4, *M. nitratireducens* CN44^T (Huo *et al.*, 2009). All strains are positive for the following characteristics: motility with one polar flagellum, oxidase and catalase, growth at 40 °C, sensitivity to streptomycin and utilization of propionic acid, citric acid and succinic acid. +, Positive; -, negative; w, weakly positive.

Characteristic	1	2	3	4
Colony colour*	CW	BM	T	Y
Growth at/in:				
4 °C	-	-	+	-
10 % NaCl	+	-	+	-
Hydrolysis of Tween 80	+	w	+	-
Nitrate reduction	-	-	-	+
Gelatinase	-	-	+	-
Arginine dihydrolase	-	-	+	-
Carbon source utilization				
D-Fructose	-	-	+	+
D-Mannose	-	-	+	-
Glycerol	-	-	+	+
L-Serine	+	+	-	+
D-Glucose	-	-	+	+
L-Aspartic acid	+	-	+	+
Sucrose	-	-	-	+
Formic acid	+	+	+	-
DNA G+C content (mol%)	58.0	60.7	54.9	62.5

*BM, Beige-milky; CW, creamy white; T, translucent; Y, yellow.

(Q-8). The predominant cellular fatty acids were C_{10:0} 3-OH (31.7%), C_{18:1ω7c} (24.8%), C_{16:0} (14.7%), summed feature 3 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH, 10.7%), C_{12:0} (8.5%), C_{12:0} 2-OH (7.0%) and C_{10:0} (2.5%). The DNA G+C content of strain AN9^T was 58 mol%. The major lipoquinone, major fatty acids and DNA G+C content were in accordance with those of members of the genus *Marinobacterium* (Chang *et al.*, 2007; Huo *et al.*, 2009; Kim *et al.*, 2007, 2009a, b).

The physiological, biochemical and phylogenetic characteristics of strain AN9^T thus support its description as representing a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium lutimaris* sp. nov. is proposed.

Description of *Marinobacterium lutimaris* sp. nov.

Marinobacterium lutimaris (L. n. *lutum* mud; L. gen. n. *maris* of the sea; N.L. gen. n. *lutimaris* of sea mud).

Cells are Gram-staining-negative, motile rods, 0.6–0.8 μm wide and 1.2–1.4 μm long. Colonies on MA are creamy white, convex and round with entire margins. Growth occurs at 15–40 °C (optimum, 25–30 °C) and at pH 6.0–8.0 (optimum, pH 6.5–7.5). Oxidase- and catalase-positive.

Negative for nitrate reduction, indole production, arginine dihydrolase, glucose fermentation, aesculin hydrolysis, gelatinase and β-galactosidase. Positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and α-glucosidase activities but negative for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannose and α-fucosidase. Weak enzyme activities are observed for esterase (C4), β-glucosidase and naphthol-AS-BI-phosphohydrolase (API ZYM). Positive for Biolog GN2 MicroPlate system substrates glycogen, Tweens 40 and 80, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D- and L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-pyrroglutamic acid, L-serine and putrescine. Other organic substrates included in Biolog GN2 microplates are not utilized. The major isoprenoid quinone is Q-8. The major cellular fatty acids are C_{10:0} 3-OH, C_{18:1ω7c} and C_{16:0}. Resistant to carbenicillin, but sensitive to penicillin G, polymyxin B, streptomycin, gentamicin, tetracycline, neomycin, kanamycin, ampicillin, lincomycin, chloramphenicol and oleandomycin. The DNA G+C content of the type strain is 58.0 mol% (HPLC).

The type strain, AN9^T (=KACC 13703^T =DSM 22012^T), was isolated from a tidal flat of the Taean coast in South Korea.

Acknowledgements

This research was supported by a Chung-Ang University Research Scholarship Grant in 2008.

References

- Baik, K. S., Seong, C. N., Kim, E. M., Yi, H., Bae, K. S. & Chun, J. (2005). *Hahella ganhwensis* sp. nov., isolated from tidal flat sediment. *Int J Syst Evol Microbiol* **55**, 681–684.
- Chang, H. W., Nam, Y. D., Kwon, H. Y., Park, J. R., Lee, J. S., Yoon, J. H., An, K. G. & Bae, J. W. (2007). *Marinobacterium halophilum* sp. nov., a marine bacterium isolated from the Yellow Sea. *Int J Syst Evol Microbiol* **57**, 77–80.
- Chun, J., Lee, J. H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- Felsenstein, J. (2002). PHYLIP (phylogeny inference package), version 3.6a. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- González, J. M., Mayer, F., Moran, M. A., Hodson, R. E. & Whitman, W. B. (1997). *Microbulbifer hydrolyticus* gen. nov., sp. nov., and *Marinobacterium georgiense* gen. nov., sp. nov., two marine bacteria from a lignin-rich pulp mill waste enrichment community. *Int J Syst Bacteriol* **47**, 369–376.

- Huo, Y. Y., Xu, X. W., Cao, Y., Wang, C. S., Zhu, X. F., Oren, A. & Wu, M. (2009). *Marinobacterium nitratreducens* sp. nov. and *Marinobacterium sedimnicola* sp. nov., isolated from marine sediment. *Int J Syst Evol Microbiol* **59**, 1173–1178.
- Jeon, C. O., Park, W., Ghiorse, W. C. & Madsen, E. L. (2004). *Polaromonas naphthalenivorans* sp. nov., a naphthalene-degrading bacterium from naphthalene-contaminated sediment. *Int J Syst Evol Microbiol* **54**, 93–97.
- Kahng, H. Y., Chung, B. S., Lee, D. H., Jung, J. S., Park, J. H. & Jeon, C. O. (2009). *Cellulophaga tyrosinoydans* sp. nov., a tyrosinase-producing bacterium isolated from seawater. *Int J Syst Evol Microbiol* **59**, 654–657.
- Kim, H., Choo, Y. J., Song, J., Lee, J. S., Lee, K. C. & Cho, J. C. (2007). *Marinobacterium litorale* sp. nov., in the order Oceanospirillales. *Int J Syst Evol Microbiol* **57**, 1659–1662.
- Kim, Y. G., Jin, Y. A., Hwang, C. Y. & Cho, B. C. (2008a). *Marinobacterium rhizophilum* sp. nov., isolated from the rhizosphere of the coastal tidal-flat plant *Suaeda japonica*. *Int J Syst Evol Microbiol* **58**, 164–167.
- Kim, J. M., Le, N. T., Chung, B. S., Park, J. H., Bae, J.-W., Madsen, E. L. & Jeon, C. O. (2008b). Influence of soil components on the biodegradation of benzene, toluene, ethylbenzene, and o-, m-, and p-xylenes by the newly isolated bacterium *Pseudoxanthomonas spadix* BD-a59. *Appl Environ Microbiol* **74**, 7313–7320.
- Kim, S.-J., Park, S.-J., Yoon, D.-N., Park, B.-J., Choi, B.-R., Lee, D.-H., Roh, Y. & Rhee, S.-K. (2009a). *Marinobacterium maritimum* sp. nov., a marine bacterium isolated from Arctic sediment. *Int J Syst Evol Microbiol* **59**, 3030–3034.
- Kim, H., Oh, H. M., Yang, S. J., Lee, J. S., Hong, J. S. & Cho, J. C. (2009b). *Marinobacterium marisflavi* sp. nov., isolated from a coastal seawater. *Curr Microbiol* **58**, 511–515.
- Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Lányi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Leifson, E. (1963). Determination of carbohydrate metabolism of marine bacteria. *J Bacteriol* **85**, 1183–1184.
- Lu, S., Park, M., Ro, H.-S., Lee, D. S., Park, W. & Jeon, C. O. (2006). Analysis of microbial communities using culture-dependent and culture-independent approaches in an anaerobic/aerobic SBR reactor. *J Microbiol* **44**, 155–161.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt. Washington, DC: American Society for Microbiology.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Yi, H. & Chun, J. (2004a). *Hongiella mannitolivorans* gen. nov., sp. nov., *Hongiella halophila* sp. nov. and *Hongiella ornithinivorans* sp. nov., isolated from tidal flat sediment. *Int J Syst Evol Microbiol* **54**, 157–162.
- Yi, H. & Chun, J. (2004b). *Nocardioides ganghwensis* sp. nov., isolated from tidal flat sediment. *Int J Syst Evol Microbiol* **54**, 1295–1299.
- Yi, H. & Chun, J. (2004c). *Nocardioides aestuarii* sp. nov., isolated from tidal flat sediment. *Int J Syst Evol Microbiol* **54**, 2151–2154.
- Yoon, J. H., Kang, S. J., Jung, Y. T. & Oh, T. K. (2008). *Aestuariicola saemankumensis* gen. nov., sp. nov., a member of the family Flavobacteriaceae, isolated from tidal flat sediment. *Int J Syst Evol Microbiol* **58**, 2126–2131.
- Yoon, J. H., Kang, S. J., Lee, J. S. & Oh, T. K. (2009). *Lutimaribacter saemankumensis* gen. nov., sp. nov., isolated from a tidal flat of the Yellow Sea. *Int J Syst Evol Microbiol* **59**, 48–52.