

Paenibacillus marinisediminis sp. nov., a Bacterium Isolated from Marine Sediment[§]

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A Gram-negative, nonmotile, endospore-forming, rod-shaped bacterial strain LHW35^T, which belonged to the genus *Paenibacillus*, was isolated from marine sediment collected from the south coast of the Republic of Korea. A phylogenetic analysis of 16S rRNA gene sequences indicated that strain LHW35^T was most closely related to *Paenibacillus taiwanensis* G-soil-2-3^T (97.2% similarity). The optimal growth conditions for strain LHW35^T were 37°C, pH 6.0, and 0% (w/v) NaCl. The main isoprenoid quinone was menaquinone-7 (MK-7) and the major polyamine was spermidine. The diamino acid present in the cell-wall peptidoglycan was meso-diaminopimelic acid. The major fatty acids were anteiso-C_{15:0} and C_{16:0}. The polar lipids were phosphatidylethanolamine, phosphatidylglycerol, unidentified aminophospholipids, unidentified phospholipids, and unidentified polar lipids. A DNA-DNA hybridization experiment using the type strain of *P. taiwanensis* indicated <40% relatedness. The DNA G+C content was 45.0 mol%. Based on these phylogenetic, genomic, and phenotypic analyses, strain LHW35^T should be classified as a novel species within the genus *Paenibacillus*, for which the name *Paenibacillus marinisediminis* sp. nov. is proposed. The type strain is LHW35^T (=KACC 16317^T =JCM 17886^T).

Keywords: bacterial taxonomy, marine sediment, *Paenibacillus marinisediminis* sp. nov.

Introduction

The genus *Paenibacillus* belongs to the family *Paenibacillaceae*

(De Vos *et al.*, 2009) in the phylum *Firmicutes*. The comparative 16S rRNA sequence analyses conducted by Ash *et al.* (1993) led to a proposal that rRNA group 3 bacilli should be separated from the genus *Bacillus* and transferred to the genus *Paenibacillus*. This proposal was subsequently published in the Validation Lists (No. 51) of the International Journal of Systematic Bacteriology (Ash *et al.*, 1994) and the bacterium *Paenibacillus polymyxa* was assigned as the type species (Judicial Commission of the International Committee for Systematics of Prokaryotes, 2005). The members of the genus *Paenibacillus* comprise 144 species of rod-shaped endospore-forming bacteria, which are distributed widely in different environments [based on the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.cict.fr)]. The present study examined the taxonomic position of a novel bacterial strain isolated from marine sediment, LHW35^T, using a polyphasic taxonomy approach based on phylogenetic, genotypic, and phenotypic analyses.

Materials and Methods

Bacterial strains

Strain LHW35^T was isolated from a marine sediment (34° 88'37"N, 127°92'13"E) collected from the bay of Kangjin, Republic of Korea using a dilution-plating technique with pleuropneumonia-like organism agar (PPLOA; Difco, Korea) at 37°C. A colony was subcultured repeatedly to obtain a pure culture. To facilitate its long-term preservation, strain LHW35^T was suspended in marine broth 2216 (MB; Difco) containing 40% glycerol and stored at -80°C. Five type strains, i.e., *Paenibacillus taiwanensis* G-soil-2-3^T (KCTC 13628), *P. dendritiformis* T168^T (DSM 18844), *P. alvei* DSM 29^T (KCTC 3623), *P. assamensis* GPTSA 11^T (KCTC 13627), and *P. thiaminolyticus* NBRC 15656^T (KCTC 3764), were obtained from KCTC (Korean Collection for Type Cultures) or DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and used for comparative analyses.

Phylogenetic and genomic analysis

Genomic DNA was extracted from strain LHW35^T using a G-spinTM Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea). The 16S rRNA gene was then amplified by PCR using a PCR pre-Mix (Wizbio, Korea) and universal bacterial 16S ribosomal RNA gene primers (8F, 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492R, 5'-GGT TAC CTT GTT ACG ACT T-3') (Lane *et al.*, 1985; Suzuki and Giovannoni, 1996), as described previously (Roh *et al.*, 2008). The 16S rRNA gene was sequenced using an automated DNA

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analyzer system (PRISM 3730XL DNA analyzer, Applied Biosystems, USA). An almost full-length 16S rRNA gene sequence was obtained using SeqMan (DNASTAR). EzTaxon-e was used to identify phylogenetic neighbors and to calculate pairwise 16S rRNA nucleotide sequence similarities (Kim *et al.*, 2012). The 16S rRNA gene sequences of strain LHW35^T and its phylogenetic neighbors were aligned using the SILVA Incremental Aligner (SINA) (Pruesse *et al.*, 2012). Phylogenetic trees were then constructed using MEGA5 (Tamura *et al.*, 2011) with the neighbor-joining (NJ) (Saitou and Nei, 1987), maximum-parsimony (MP) (Kluge and Farris, 1969), and maximum-likelihood (ML) (Felsenstein, 1981) methods based on 1,000 randomly selected bootstrap replicates. A DNA-DNA hybridization (DDH) experiment was performed using the fluorometric method described by Ezaki *et al.* (1989) to determine the genetic relatedness between strain LHW35^T and *Paenibacillus taiwanensis* KCTC 13628^T. All of the reactions were repeated five times. The highest and lowest of the five values were ignored and the DDH value was calculated using the three remaining values. The genomic DNA G+C content was determined using a real-time PCR thermocycler and SYBR Gold I (Invitrogen, USA), as described previously by Gonzalez and Saiz-Jimenez (2002). Genomic DNAs from *Ruminococcus obeum* ATCC 29174^T, *Ruegeria pomeroyi* DSS-3^T, and *Escherichia coli* K12 were used as the calibration references.

Nucleotide sequence accession number

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain LHW35^T is JF748731.

Morphological, physiological, and biochemical characterization

Bacterial growth was evaluated on brain-heart infusion agar (BHIA; Difco), Columbia agar (Difco), and marine agar 2216 (MA; Difco). Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, 45, and 65°C) was examined by culturing in MB. Growth at different pH values (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) was examined by culturing in MB where the pH was adjusted using 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 4.0, 5.0, and 6.0) or 10 mM N-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) (pH 7.0, 8.0, 9.0, and 10.0). NaCl tolerance was tested by

culturing in a medium containing all of the constituents of MB except NaCl, which was supplemented with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 15% (w/v) NaCl. The turbidity of each culture was measured at OD₆₀₀ with a UV/VIS spectrophotometer (SYNERGY MX, BioTek, USA). The cell morphology was observed using a light microscope (ECLIPSE 50i, Nikon, Japan). Anaerobic growth on BHIA was examined by culturing the bacteria for 7 days in an anaerobic chamber at 37°C (Coy Laboratory Products, USA) with an atmosphere containing N₂:CO₂:H₂ (90:5:5). Gram staining, the catalase and oxidase activity, and starch hydrolysis were tested using the methods described by Benson (2002). Motility was examined on semi-solid agar plates (Tittsler and Sandholzer, 1936). Endospore formation was examined using the method described by Schaeffer and Fulton (1933). The hydrolysis of DNA and Tween 20 were tested as described by Gonzalez *et al.* (1978). Hydrolysis of carboxymethylcellulose was determined as described by Percival Zhang *et al.* (2006). Tyrosine hydrolysis was assessed based on the appearance of clear zones around colonies grown on MA containing L-tyrosine (0.5 g/L). API 20NE and API ZYM strips (bioMérieux, France) and a Biolog Gram-negative (GN) plate were used to test the enzyme activities and sole carbon substrate utilization capacities, according to the manufacturer’s instructions.

Chemotaxonomy

The isoprenoid quinones were extracted using chloroform:methanol (2:1, v/v), purified by one-dimensional thin-layer chromatography (TLC), and identified by high performance liquid chromatography, as described previously by Collins and Jones (1981). Polyamines were analyzed using high performance liquid chromatography, as described by Busse and Auling (1988). The cell-wall peptidoglycan was hydrolyzed with 6 N HCl at 100°C for 18 h and the diamino acids in the cell-wall peptidoglycan were identified using an automatic amino acid analyzer (ARACUS, membraPure, Germany). The cellular fatty acids analysis used strain LHW35^T and the five reference strains (*P. taiwanensis* KCTC 13628^T, *P. dendritiformis* DSM 18844^T, *P. alvei* KCTC 3623^T, *P. assamensis* KCTC 13627^T, and *P. thiaminolyticus* KCTC 3764^T), which were cultivated on BHIA at 30°C and harvested at the same physiological age after 24 h of incubation. The cellular fatty acids were then analyzed according to the

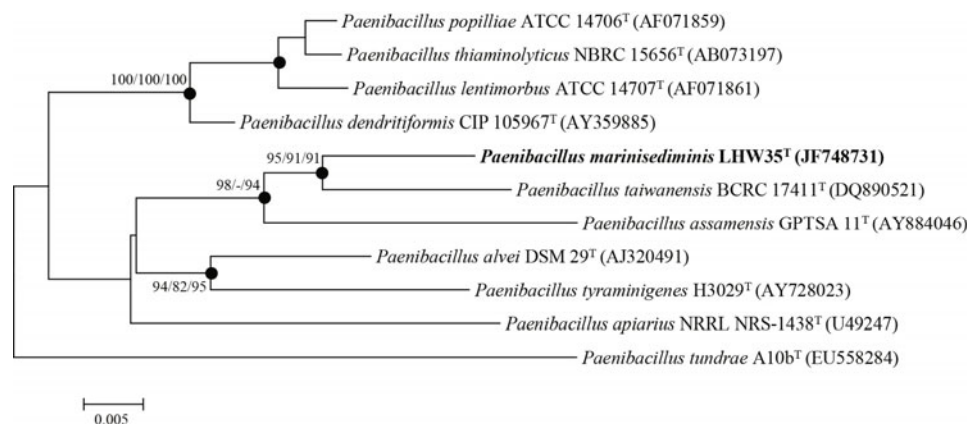


Fig. 1. Phylogenetic tree produced using the neighbor-joining (NJ) algorithm based on the 16S rRNA gene sequences of strain LHW35^T and the type strains of other closely related species. The closed circles represent nodes that were also recovered using the maximum-parsimony (MP) and maximum-likelihood (ML) methods. The numbers on the nodes indicate bootstrap values >70% calculated using the NJ/MP/ML probabilities, which are expressed as percentages of 1,000 replicates. *Paenibacillus tundrae* A10b^T was used as the out-group. Bar=0.005 accumulated changes per nucleotide.

Sherlock Microbial Identification System (MIDI, 1999) using the standard MIDI protocol (version 6.2), the TSBA6 database, and gas chromatography (Hewlett Packard 6890, USA). Polar lipids were extracted from strain LHW35^T and separated by two-dimensional TLC on silica gel plates (Merck, USA), according to the method of Minnikin *et al.* (1984). The separated polar lipid spots were identified by spraying each plate with 5% ethanolic molybdophosphoric acid (to

detect total polar lipids), molybdenum blue (to detect phospholipids), ninhydrin (to detect aminolipids), and α -naphthol-sulfuric acid (to detect glycolipids). The designations of the specific phospholipid spots were also confirmed by one-dimensional TLC using the following references: phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol (Sigma-Aldrich, USA).

Table 1. Characteristics of strain LHW35^T and other closely related species in the genus *Paenibacillus*

Data were obtained from the present study, except where indicated. All strains were positive for leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (based on API ZYM), and β -glucosidase (aesculin hydrolysis) (API 20NE), but negative for β -glucuronidase (API ZYM) and D-glucose fermentation, as well as the assimilation of L-arabinose, D-mannitol, capric acid, adipic acid, and phenylacetic acid (API 20NE).

Taxa: 1, *Paenibacillus marinisediminis* sp. nov. LHW35^T; 2, *P. taiwanensis* KCTC 13628^T; 3, *P. dendritiformis* DSM 18844^T; 4, *P. alvei* KCTC 3623^T; 5, *P. assamensis* KCTC 13627^T; 6, *P. thiaminolyticus* KCTC 3764^T. Symbols: +, positive; -, negative.

Characteristics	1	2	3	4	5	6
Growth at:						
10°C	-	+ ^a	- ^b	- ^a	- ^d	- ^c
45°C	+	+ ^a	+ ^b	+ ^a	- ^d	+ ^e
pH 5.6	+	+ ^a	+ ^b	- ^c	- ^d	- ^e
Anaerobic growth	+	+ ^a	+ ^b	+ ^c	- ^d	+ ^e
Oxidase activity	-	- ^a	+ ^b	- ^c	+ ^d	+ ^e
API ZYM:						
Alkaline phosphatase	-	+	-	+	-	+
Esterase (C4)	-	+	+	+	+	+
Esterase lipase (C8)	+	+	-	+	+	+
Lipase (C14)	+	-	-	-	-	-
Valine arylamidase	+	-	+	+	+	-
Cystine arylamidase	-	+	+	+	+	+
Trypsin	-	+	+	+	-	+
α -Chymotrypsin	+	-	+	-	-	-
α -Galactosidase	-	-	-	-	+	-
β -Galactosidase	+	+	+	-	+	+
α -Glucosidase	-	+	+	+	+	-
β -Glucosidase	+	-	+	+	+	+
N-Acetyl- β -glucosaminidase	-	-	-	-	-	+
α -Mannosidase	-	+	-	+	+	-
α -Fucosidase	-	+	-	+	+	-
API 20NE:						
Reduction of nitrates	-	-	-	-	-	+
Indole production	-	-	+	-(+ ³)	-	+
l-Arginine dihydrolase	-	-	+	-	-	+
Urease	-	-	+	+	-	+
Protease (gelatin hydrolysis)	-	+	+	-	+	+
β -Galactosidase (PNPG hydrolysis)	+	+	+	+	-	-
Assimilation of:						
D-Glucose	-	+	+	-(+ ³)	+	+
D-Mannose	-	-	+	-	-	+
N-Acetyl-glucosamine	-	+	+	+	+	+
D-Maltose	-	+	+	+	+	+
Potassium gluconate	-	+	+	+	+	+
l-Malate	-	-	+	-	-	+
Trisodium citrate	-	-	+	-	-	+
G+C content (mol%)	45.0	44.6 ^a	55 ^b	45-47 ^c	41.2 ^d	52-54 ^e

^aData from Lee *et al.* (2007)

^bData from Tcherpakov *et al.* (1999)

^cData from Shida *et al.* (1997)

^dData from Saha *et al.* (2005)

^eData from Nakamura (1996)

Results and Discussion

Phylogenetic and genomic analysis

Phylogenetic trees based on the 16S rRNA gene sequences showed that strain LHW35^T was tightly clustered with the type strain of *Paenibacillus taiwanensis*, based on high bootstrap values (95, 91, and 91% in the NJ, MP, and ML trees, respectively) (Fig. 1). Analysis of the 16S rRNA gene sequences showed that strain LHW35^T was closely related to the type strain of *P. taiwanensis* (16S rRNA gene sequence similarity = 97.2%), *P. dendritiformis* (95.6%), *P. alvei* (95.5%), *P. assamensis* (95.3%), and *P. thiaminolyticus* (95.1%). Other type strains belonging to the genus *Paenibacillus* shared <95.0% 16S rRNA gene sequence similarity with strain LHW35^T. The DDH result for strain LHW35^T and *P. taiwanensis* KCTC 13628^T was 38.7%. The DNA G+C content of strain LHW35^T was 45.0 mol%, which is similar to that of the closely related strain *P. taiwanensis* KCTC 13628^T (44.6 mol%) (Lee *et al.*, 2007). These results show that strain LHW35^T is associated with the genus *Paenibacillus* and that it can be considered a distinct genospecies (Wayne *et al.*, 1987).

Morphological, physiological, and biochemical characteristics

Strain LHW35^T was Gram-negative, nonmotile, endospore-forming, and rod-shaped (Supplementary data Fig. S1). LHW35^T grew on BHIA, Columbia agar, MA, and PPLOA, and it grew at 25–45°C, pH 4.0–8.0, and in the presence of 0–3% (w/v) NaCl. Optimal growth occurred at 37°C, pH

6.0, and with 0% NaCl. Strain LHW35^T grew in anaerobic conditions and was catalase-positive and oxidase-negative. The isolate hydrolyzed DNA and Tween 20, but did not hydrolyze starch, carboxymethylcellulose, and tyrosine. The other phenotypic characteristics of strain LHW35^T are included in the species description. The characteristics of strain LHW35^T are compared with those of other closely related members of the genus *Paenibacillus* in Table 1. Strain LHW35^T was distinguished from closely related *Paenibacillus* strains by its phenotypic characteristics, i.e., growth at 10°C, 45°C, and pH 5.6, as well as anaerobic growth, specific enzyme activities, and the assimilation of various carbon sources. These results suggest that strain LHW35^T can be differentiated from other *Paenibacillus* species.

Chemotaxonomy

Strain LHW35^T contained menaquinone-7 (MK-7) as the major isoprenoid quinone, which is also the case in the closely related type strains of *P. taiwanensis* and *P. assamensis* (Saha *et al.*, 2005; Lee *et al.*, 2007). The predominant polyamine in strain LHW35^T was spermidine. The diamino acid in the cell-wall peptidoglycan of strain LHW35^T was meso-diaminopimelic acid. The major cellular fatty acids identified in strain LHW35^T were anteiso-C_{15:0} (32.0%) and C_{16:0} (23.2%). The detailed fatty acid compositions of strain LHW35^T and the five reference strains are shown in Table 2. All of the strains contained anteiso-C_{15:0} and C_{16:0} as the main fatty acid components. The major polar lipids isolated from strain LHW35^T were phosphatidylethanolamine, phosphatidylglycerol, unidentified ninhydrin-positive phospholipids,

Table 2. Comparison of the fatty acid contents of strain LHW35^T and closely related species in the genus *Paenibacillus*

Taxa: 1, *Paenibacillus marinisediminis* sp. nov. LHW35^T; 2, *P. taiwanensis* KCTC 13628^T; 3, *P. dendritiformis* DSM 18844^T; 4, *P. alvei* KCTC 3623^T; 5, *P. assamensis* KCTC 13627^T; 6, *P. thiaminolyticus* KCTC 3764^T. All data were obtained in the present study. The values are the percentages of the total fatty acids. Fatty acids that represented <1% of the total fatty acids are not shown for any strains. Tr, Trace (<1%); -, not detected.

Fatty acids	1	2	3	4	5	6
C _{10:0}	6.5	1.5	2.9	13.9	2.6	1.6
C _{11:0} 2-OH	1.9	-	Tr	4.8	Tr	Tr
anteiso-C _{12:0}	1.3	-	-	-	-	-
C _{13:0}	-	-	-	1.1	-	-
C _{13:0} 2-OH	-	Tr	Tr	2.2	-	Tr
C _{13:1} at 12-13	-	-	-	1.1	-	-
C _{14:0}	2.6	2.7	2.1	2.1	3.8	2.8
iso-C _{14:0}	1.0	2.5	Tr	-	Tr	1.1
iso-C _{15:0}	5.5	9.8	3.7	9.6	6.4	3.0
anteiso-C _{15:0}	32.0	47.3	37.0	15.1	31.4	35.9
C _{15:1} ω8c	-	-	-	3.6	-	-
C _{16:0}	23.2	13.1	24.4	22.5	22.8	28.3
iso-C _{16:0}	1.7	7.0	5.3	2.0	1.8	4.7
C _{16:1} ω11c	4.3	2.0	Tr	2.8	14.2	3.5
iso-C _{17:0}	1.9	2.4	3.9	Tr	2.4	1.4
anteiso-C _{17:0}	5.2	4.5	13.0	-	3.9	8.4
iso-C _{17:1} ω10c	-	Tr	-	1.9	1.3	Tr
C _{18:0}	7.7	2.6	4.1	7.1	4.3	5.8
Summed feature 1 ^a	2.6	Tr	1.0	7.5	1.2	Tr
Summed feature 3 ^a	-	Tr	-	1.9	-	-
Summed feature 4 ^a	1.1	Tr	-	-	Tr	Tr

^a Summed feature 1 comprised C_{13:0} 3-OH and/or iso-C_{15:1} H; summed feature 3 comprised C_{16:1} ω6c and/or C_{16:1} ω7c; summed feature 4 comprised iso-C_{17:1} I and/or anteiso-C_{17:1} B.

unidentified phospholipids, and unidentified polar lipids (Supplementary data Fig. S1). No glycolipids were detected.

Taxonomic conclusion

The results of the phylogenetic, genomic, and phenotypic analyses showed that strain LHW35^T belonged to the genus *Paenibacillus* and could be differentiated from the other type strains of *Paenibacillus*. Based on the polyphasic taxonomic analyses, strain LHW35^T represents a novel species in the genus *Paenibacillus*, for which the name *Paenibacillus marinisediminis* sp. nov. is proposed.

Description of *Paenibacillus marinisediminis* sp. nov.

Paenibacillus marinisediminis (ma.ri.ni.se.di'mi.nis. L. adj. marinus, of or belonging to the sea, marine; L. n. sediment-inis, sediment; N.L.gen. n. *marinisediminis*, of marine sediment).

The cells are Gram-negative, nonmotile, endospore-forming, and rod-shaped (1.0–1.3 µm × 0.4–0.5 µm). Colonies grown on BHIA are cream in color, circular, and approximately 2–5 mm in diameter. Growth occurs on PPLOA, BHIA, Columbia agar, and MA. The bacteria grow at 25–45°C (optimum = 37°C), pH 4.0–8.0 (optimum = 6.0), and in the presence of 0–3% (w/v) NaCl (optimum = 0%). The bacteria grow in anaerobic conditions and are catalase-positive and oxidase-negative. The bacteria hydrolyze DNA and Tween 20, but not starch, carboxymethylcellulose, or tyrosine. API ZYM strip tests indicate the presence of esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, and β-glucosidase activity, whereas alkaline phosphatase, esterase (C4), cystine arylamidase, trypsin, α-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activity are absent. API 20NE strip tests are positive for β-glucosidase (esculin hydrolysis) and β-galactosidase (PNPG hydrolysis), but negative for nitrate reduction (to nitrites or nitrogen), indole production, D-glucose fermentation, L-arginine dihydrolase, urease, and protease (gelatin hydrolysis), and for the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, L-malate, trisodium citrate, and phenylacetic acid. Biolog GN plate tests are positive for α-cyclodextrin, dextrin, D-fructose, α-D-glucose, α-D-lactose, maltose, D-mannose, D-melibiose, inosine, and uridine, but negative for glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, i-erythritol, L-fucose, D-galactose, gentiobiose, m-inositol, lactulose, D-mannitol, β-methyl-D-glucoside, D-psiocose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylofuranose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxy-phenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid,

sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyrroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, γ-aminobutyric acid, urocanic acid, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, D,L-α-glycerol phosphate, α-D-glucose-1-phosphate, and D-glucose 6-phosphate. The main isoprenoid quinone is menaquinone-7 (MK-7). The predominant polyamine is spermidine. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major cellular fatty acids are anteiso-C_{15:0} and C_{16:0}. The polar lipids are phosphatidylethanolamine, phosphatidylglycerol, unidentified amino-phospholipids, unidentified phospholipids, and unidentified polar lipids. The DNA G+C content is 45.0 mol%. The type strain, LHW35^T (=KACC 16317^T =JCM 17886^T), was isolated from marine sediment collected from the bay of Kangjin (Republic of Korea).

Acknowledgements

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