Blastopirellula cremea sp. nov., isolated from a dead ark clam

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Strain LHWP2^T, a novel, aerobic, budding, motile and ovoid bacterium belonging to the phylum *Planctomycetes*, was isolated from a dead ark clam (*Scapharca broughtonii*) from the south coast of Korea. Strain LHWP2^T grew optimally at 30 °C, in the presence of 4 % (w/v) NaCl, and at pH 7. The predominant cellular fatty acids were $C_{16:0}$, $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ (summed feature 8) and $C_{18:1}\omega9c$. The major isoprenoid quinone was menaquinone-6 (MK-6). The dominant polar lipid was identified as phosphatidylglycerol. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the novel strain was most closely related to *Blastopirellula marina* DSM 3645^T, with a 16S rRNA gene sequence similarity of 94.1 %. The genomic DNA G+C content of strain LHWP2^T was 49.5 mol%. Strain LHWP2^T was distinguished from *B. marina* DSM 3645^T based on its optimum salinity, acid production from substrates, assimilation of substrates and DNA G+C content. Overall, these phenotypic, genotypic and phylogenetic data suggest that strain LHWP2^T should be classified as a novel species belonging to the genus *Blastopirellula*, for which the name *Blastopirellula cremea* sp. nov. is proposed. The type strain is LHWP2^T (=KACC 15559^T=JCM 17758^T).

The phylum *Planctomycetes* of the domain Bacteria is a distinct group of budding and peptidoglycan-free bacteria (Fuerst, 2005). At the time of writing, the family *Planctomycetaceae* in the phylum *Planctomycetes* includes the genera *Blastopirellula* (Schlesner, *et al.*, 2004), *Gemmata* (Franzmann & Skerman, 1984), *Isosphaera* (Giovannoni, *et al.*, 1987), *Pirellula* (Schlesner & Hirsch, 1987), *Planctomyces* (Gimesi, 1924), *Rhodopirellula* (Schlesner, *et al.*, 2004), *Schlesneria* (Kulichevskaya, *et al.*, 2007), *Singulisphaera* (Kulichevskaya, *et al.*, 2008), *Zavarzinella* (Kulichevskaya, *et al.*, 2009), *Aquisphaera* (Bondoso, *et al.*, 2011) and *Telmatocola* (Kulichevskaya, *et al.*, 2012). Among the genera in the family *Planctomycetaceae*, the genus *Blastopirellula* was proposed by Schlesner *et al.* (2004) after the transfer of *Pirellula marina* based on its taxonomic heterogeneity. A novel *Planctomycetes*-like bacterial strain LHWP2^T was isolated during an analysis of the bacterial community in an ark clam farm in Gangjin Bay, South Korea. In this study, the novel strain LHWP2^T was characterized using a polyphasic approach based on its phenotype, genotype, chemotaxonomy and phylogeny.

Strain LHWP2^T was isolated from a dead ark clam (*Scapharca broughtonii*) collected from a cage-cultured ark clam farm in Gangjin Bay in the south coastal region of the Korean peninsula. The ark clam sample was homogenized in sterilized 1× PBS and spread on four marine agar (MA; BBL) plates containing penicillin. After incubation for about 1 month, three colonies were observed and one colony was subcultured on MA medium. Strain LHWP2^T was repeatedly subcultured under aerobic conditions on MA medium at 25 °C to obtain a pure culture. The isolate was suspended in marine broth (MB; BBL) containing 40 % glycerol (v/v) and stored at −80 °C for long-term preservation.

Growth at different temperatures (4, 15, 20, 25, 30, 37, 45 and 65 °C) was examined in MA medium. The optimum pH range (pH 4.0−10.0, with intervals of 1.0 pH unit) for growth was determined in MB medium, where the pH of the MB medium was adjusted using 10 mM MES (for pH 4, 5 and 6) or 10 mM TAPS (for pH 7, 8, 9 and 10). The NaCl tolerance test was performed using media containing all the constituents of MB medium except NaCl, which was supplemented at various concentrations [0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 15 % (w/v)]. Growth was

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LHWP2^T is JF748733.

A supplementary figure is available with the online version of this paper.

determined after 7 and 14 days of incubation by measuring the turbidity of cultures (OD_{600}) using a spectrophotometer (SYNERGY MX; BioTek). The morphology of the colonies and cells were determined after incubation for 7 days on MA medium. Cell morphology was observed using a light microscope (ECLIPSE 50i; Nikon). Detailed morphologies were observed using a field emission electron microscope (SUPRA 55 VP; Carl Zeiss) installed at Jeju Center, Korea Basic Science Institute (KBSI). To prepare the samples for scanning electron microscopy, cells were fixed in 2.5 % (w/v) glutaraldehyde in 1 M PBS for 1 h and dehydrated using an ethanol series. The samples were dried using a critical point dryer (K850; Quorum Technologies) and ion sputter-coated with platinum (Q150T S; Quorum Technologies). Observations were made on a nylon filter. To prepare samples for transmission electron microscopy, Formvar-coated copper grids with a 200 mesh were floated on a droplet of the sample, followed by negative staining with 2 % uranyl acetate for 45 s, two washes with deionized water and air drying. Motility tests were conducted using semi-solid MA (Tittsler & Sandholzer, 1936). Growth was tested under anaerobic conditions using MA and MB medium in an anaerobic jar filled with mixed gases (N_2 : CO_2 : H_2, 90 : 5 : 5) for 1 week.

Strain LHWP2^T grew at pH 6.0–8.0 (optimum, pH 7.0), between 20 and 37 °C (optimum, 30 °C) and in the presence of 0–10 % (w/v) NaCl (optimum, 4 %). Cells were ovoid-shaped (0.6–1.5 μm × 0.6–1.4 μm) and formed cream, round and smooth colonies after 7 days on MA medium at 25 °C. Electron microscopic examination of cells detected the presence of buds, crateriform pits and flagella (Fig. 1). Based on flagellum observations and motility test, the cells were confirmed to be motile. Growth was not observed under anaerobic conditions.

Oxidase and catalase activity were assessed using 1 % (v/v) *p*-tetramethyl phenylenediamine (bioMérieux) and 3 % (w/v) hydrogen peroxide, respectively. Vitamin requirement tests were performed using modified M20 agar medium containing (g l^{-1}) agar (15), Casamino acid (1), glucose (1) and sea salt (62.5) (Schlesner, 1986), which was adjusted to pH 7.2 and incubated for 7 days with or without Wolin's vitamin solution containing (mg l^{-1}) biotin (0.02), folic acid (0.02), pyridoxine hydrochloride (0.1), riboflavin (0.05), thiamine (0.05), nicotinic acid (0.05), pantothenic acid (0.05), cyanocobalamin (0.001), *p*-aminobenzoic acid (0.05) and thioctic acid (0.05) (Wolin, *et al.*, 1963). API 20NE and API ZYM strips (bioMérieux) were used to characterize enzyme activities, according to the manufacturer's instructions. Acid production was tested with API 50CH strips using 50 CHB/E medium (bioMérieux), according to the manufacturer's instructions. Carbon and nitrogen source assimilation were tested using GN2 MicroPlates (Biolog) with inoculating fluid (Biolog), according to the manufacturer's instructions. Strain LHWP2^T was catalase- and oxidase-positive and vitamins were not required for growth. Strain LHWP2^T was positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, aesculin hydrolysis and *p*-nitrophenyl-β-D-galactopyranoside hydrolysis (based on API 20NE and API ZYM). It produced acid from glycerol, D-arabinose, L-arabinose, D-xylose, L-xylose, methyl β-D-xyloside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, gentiobiose, turanose, D-lyxose, D-fucose, L-fucose and 5-ketogluconate (API 50 CHB). Strain LHWP2^T assimilated the following carbon sources: β-cyclodextrin, mannan, Tween-40, amygdalin, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, *myo*-inositol, α-lactose, lactulose, D-mannitol, melezitose, melibiose, methyl α-D-galactoside, 3-methyl glucose, methyl β-D-glucoside, D-ribose, sedoheptulosan, sucrose, acetic acid, β-hydroxybutyric acid, α-ketovaleric acid, D-lactic acid methyl ester, L-lactic acid, D-fructose 6-phosphate, α-D-glucose-1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. It assimilated the following nitrogen sources: L-alaninamide, D-alanine, L-alanyl glycine, L-glutamic acid, L-pyroglutamic acid, L-serine, thymidine, uridine, adenosine-5′-monophosphate, thymidine-5′-monophosphate and uridine-5′-monophosphate (GN2 MicroPlate). The distinctive biochemical characteristics of strain LHWP2^T compared with *Blastopirellula marina* DSM 3645^T are shown in Table 1.

The 16S rRNA gene of strain LHWP2^T was amplified by colony PCR using a PCR pre-mix (WIZBIO) and two universal primers (8F and 1492R) (8F, 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R, 5′-AAGGAGGTGATCCAGCCGC-3′). Colony PCR was initiated by denaturation

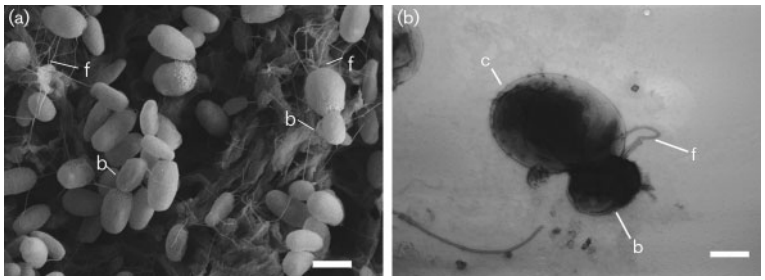

Fig. 1. Scanning (a) and transmission (b) electron micrographs of strain LHWP2^T captured via field emission electron microscopy. Cells were grown in MA medium for 7 days. b, Bud; f, flagellum; c, crateriform pit. Bars, 1 μm (a); 500 nm (b).

Table 1. Differential biochemical and physiological characteristics of strain LHWP2^T compared with its closely related taxon, *Blastopirellula marina* DSM 3645^T

Both strains were positive for acid production from D-arabinose, L-arabinose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, gentiobiose, turanose, D-lyxose, D-fucose and L-fucose on an API 50 CHB/E test strip; assimilation of β-cyclodextrin, mannan, Tween-40, amygdalin, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, *myo*-inositol, α-lactose, lactulose, D-mannitol, melezitose, melibiose, methyl α-D-galactoside, 3-methyl glucose, methyl β-D-glucoside, D-ribose, sedoheptulosan, acetic acid, α-ketovaleric acid, D-lactic acid methyl ester, L-lactic acid, L-alaninamide, D-alanine, L-alanyl-glycine, L-glutamic acid, L-pyroglutamic acid, L-serine, thymidine, adenosine-5′-monophosphate, thymidine-5′-monophosphate, uridine-5′-monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate on a GN2 MicroPlate; alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities on an API ZYM test strip; and aesculin ferric citrate hydrolysis and *p*-nitrophenyl-β-D-galactopyranoside on an API 20NE test strip. +, Positive; −, negative; ND, no data.

Characteristic	Strain LHWP2^T	*B. marina* DSM 3645^T
Colony pigmentation	Cream–white	Greyish to brownish-white*
Cell size (μm)	0.6–1.5 × 0.6–1.4	0.7–1.5 × 1.0–2.0†
Optimum temperature (°C)	30	27–33†
Temperature growth range (°C)	20–37	ND
pH growth range	6.0–8.0	ND
Growth without NaCl	+	−†
Optimum salinity (%, w/v)	4	1
DNA G+C content (mol%)	49.5	57.4†
Acid production from (API 50CH):		
Glycerol	+	−
Methyl β-D-xyloside	+	−
Starch	−	+
5-Ketogluconate	+	−
Assimilation of (GN2 MicroPlate):		
Maltose	−	+
Maltotriose	−	+
Methyl β-D-galactoside	−	+
Methyl α-D-glucoside	−	+
Raffinose	−	+
Salicin	−	+
Sucrose	+	−
Xylitol	−	+
β-Hydroxybutyric acid	+	−
Lactamide	−	+
D-Malic acid	−	+
Succinic acid monomethyl ester	−	+
Propionic acid	−	+
Succinamic acid	−	+
Succinic acid	−	+
N-Acetyl L-glutamic acid	−	+
Glycyl L-glutamic acid	−	+
Putrescine	−	+
2,3-Butanediol	−	+
2′-Deoxyadenosine	−	+
Inosine	−	+
Uridine	+	−

*Data from Schlesner *et al.* (2004).
†Data from Schlesner (1986).

at 95 °C for 10 min. Reactions were amplified for 30 cycles as follows: denaturation at 95 °C for 1 min, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 10 min. The PCR product was purified using a QIAquick PCR Purification kit (Qiagen) and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) with an automated DNA analyser system (PRISM 3730XL

DNA analyser; Applied Biosystems), according to the manufacturer's instructions. The quality of the 16S rRNA gene sequences was confirmed using the BioEdit program (Hall, 1999) and sequence fragments were assembled using the SeqMan program (DNASTAR) to obtain an almost full-length 16S rRNA gene sequence. The 16S rRNA gene sequence of strain LHWP2^T was compared with those of other type strains using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The 16S rRNA sequences of the isolate and its relatives were aligned using CLUSTAL W (Thompson, *et al.*, 1994). Phylogenetic trees were constructed using MEGA5 (Tamura, *et al.*, 2011) and the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods with 1000 randomly selected bootstrap replicates. Phylogenetic analyses showed that strain LHWP2^T was closely related to *B. marina* DSM 3645^T (94.1 % 16S rRNA gene sequence similarity), *Rhodopirellula baltica* SH 1^T (87.8 %) and *Pirellula staleyi* DSM 6068^T (87.3 %). The phylogenetic trees showing the relationships between strain LHWP2^T and related strains are illustrated in Fig. 2. As previously reported (Stackebrandt & Goebel, 1994), 16S rRNA gene sequence similarities of less than 97 % with closely related species indicated that strain LHWP2^T represented a novel species.

Genomic DNA from strain LHWP2^T was extracted using a G-spin Genomic DNA Extraction kit (iNtRON Biotechnology). The DNA G+C content was determined using SYBR Gold I (Invitrogen) and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA from *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T and *Ruminococcus obeum* ATCC 29174^T were used as calibration references. The DNA G+C content of strain LHWP2^T was 49.5 mol%.

To prepare samples for cellular fatty acid analysis, strain LHWP2^T and *B. marina* DSM 3645^T were cultivated on MA medium at 25 °C for 7 days. Quantitative analysis of the cellular fatty acid composition was performed by harvesting cells in the stationary phase, which was determined using liquid cultures and subjecting the cellular fatty acids to saponification, methylation and extraction, according to the protocol of the Sherlock Microbial Identification System (MIDI, 1999). The fatty acids were analysed by gas chromatography (6890; Hewlett Packard) and identified using the Microbial Identification software package (Sasser, 1990) with the TSBA 6.0 library. Isoprenoid quinones were extracted using chloroform : methanol (2 : 1, v/v), according to the method of Collins and Jones (1981a), purified by one-dimensional TLC on a silica gel 60 F254 plate (Merck) and identified by HPLC (Collins & Jones, 1981b). Polar lipids were extracted according to the method of Xin *et al.* (2000) and separated and identified using TLC on a silica gel 60 F254 plates (Merck). After separation, each component was identified by spraying the separate plates using appropriate detection reagents (Tindall, 1990), followed by identification via liquid chromatography (LC) (Ultimate 3000; Dionex) using an ion trap-mass spectrometer equipped with an electrospray ionization probe (HCT; Bruker), which was installed at the Jeju Center, KBSI, according to the protocol of Taguchi *et al.* (2005) with minor modifications. A reverse phase Hydrosphere C18 column (50 mm × 2.0 mm i.d., YMC) and an isocratic eluent [acetonitrile : methanol : 0.7 % (v/v) formic acid in water, 70 : 22 : 8, flow rate: 0.2 ml min^{-1}] were used for LC separation. The main cellular fatty acids identified in strain LHWP2^T were $C_{16:0}$ (34.3 %), $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ (summed feature 8; 24.1 %) and $C_{18:1}\omega9c$ (22.0 %) (Table 2). *B. marina* DSM 3645^T was closely related to strain LHWP2^T and it also contained $C_{16:0}$ and $C_{18:1}\omega9c$ as the main fatty acids (Schlesner, *et al.*, 2004). However, the $C_{18:0}$ and iso-$C_{19:1}$ fatty acids had significantly different features in strain LHWP2^T and *B. marina* DSM 3645^T. Strain LHWP2^T contained MK-6 as the main menaquinone, which was also the major quinone in *B. marina* DSM 3645^T (Schlesner, *et al.*, 2004). The polar lipids of strain LHWP2^T were phosphatidylglycerol (PG),

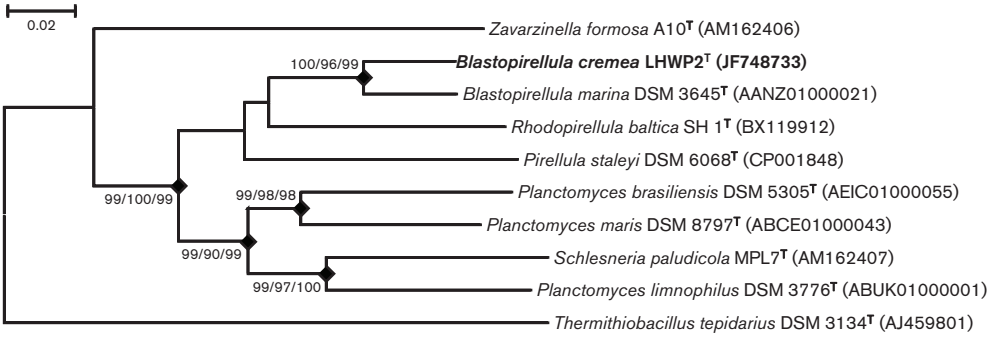

Fig. 2. Phylogenetic tree constructed using the neighbour-joining method based on the 16S rRNA gene sequences, showing the taxonomic position of strain LHWP2^T compared with closely related taxa in the family *Planctomycetaceae*. The numbers at the nodes indicate the bootstrap values (>70 %) calculated using the neighbour-joining/maximum-parsimony/maximum-likelihood probabilities based on 1000 replicates. Filled diamonds on the nodes indicate branches that also were recovered using the maximum-parsimony and maximum-likelihood methods. Bar, 0.02 substitutions per nucleotide position.

Table 2. Cellular fatty acid content (%) of strain LHWP2^T and *B. marina* DSM 3645^T

All data were obtained from the present study. The values represent the percentages of all fatty acids. −, Not detected.

Fatty acid	Strain LHWP2^T	*B. marina* DSM 3645^T
$C_{10:0}$	0.89	0.91
$C_{11:0}$ 2-OH	0.22	0.11
$C_{11:0}$ 3-OH	0.17	−
$C_{12:0}$ 3-OH	0.12	0.16
$C_{14:0}$	0.95	0.84
Summed feature 1*	0.24	0.26
iso-$C_{16:0}$	0.32	0.19
$C_{16:1}\omega11c$	1.91	1.21
Summed feature 3*	4.34	4.39
$C_{16:1}\omega5c$	0.35	0.31
$C_{16:0}$	34.32	39.04
iso-$C_{17:0}$	0.11	−
$C_{17:1}\omega8c$	0.97	1.47
$C_{17:1}\omega6c$	0.21	0.26
$C_{17:0}$	0.31	0.45
$C_{16:0}$ 2-OH	0.19	−
iso-$C_{18:0}$	0.32	−
$C_{18:1}\omega9c$	21.95	15.84
Summed feature 8*	24.13	23.19
$C_{18:0}$	6.37	10.55
iso-$C_{19:1}$	0.20	−
$C_{20:1}\omega7c$	0.60	0.44
$C_{20:0}$	0.22	0.24

*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 1 comprised iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH; summed feature 3 comprised $C_{16:1}\omega7c$ and/or iso-C_{15} 2-OH; summed feature 8 comprised $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$.

an unidentified phospholipid (PL) and unidentified lipids (L1, L2 and Ls) (Fig. S1, available in IJSEM Online). PG was also the main polar lipid in *B. marina* DSM 3645^T (Schlesner, *et al.*, 2004). Overall, these results and the results of the chemotaxonomic analyses confirmed that strain LHWP2^T was closely related to the type strain of *B. marina*.

Strain LHWP2^T was distinct from *B. marina* DSM 3645^T based on its colony pigmentation, growth conditions, acid production from substrates, assimilation of substrates and fatty acid profile, as well as its 16S rRNA gene sequence dissimilarity. Thus, based on phenotypic, genotypic, chemotaxonomic and phylogenetic analyses, strain LHWP2^T represents a novel species, for which the name *Blastopirellula cremea* sp. nov. is proposed.

Description of *Blastopirellula cremea* sp. nov.

Blastopirellula cremea (cre′me.a. N.L. fem. adj. *cremea* cream–white, referring to the colour of the colonies).

Cells are aerobic, ovoid (0.6–1.5 μm × 0.6–1.4 μm) and motile, with buds, crateriform pits and flagella. Colonies formed on MA medium are cream, round, smooth and 0.1–0.4 mm in diameter. Growth occurs at pH 6.0–8.0 (optimum, pH 7.0), at 20–37 °C (optimum, 30 °C) and with 0–10 % (w/v) NaCl (optimum, 4 %). Catalase- and oxidase-positive. Vitamins are not required for growth. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, aesculin hydrolysis and *p*-nitrophenyl-β-D-galactopyranoside hydrolysis (from API 20NE and API ZYM). Produces acid from glycerol, D-arabinose, L-arabinose, D-xylose, L-xylose, methyl β-D-xyloside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, gentiobiose, turanose, D-lyxose, D-fucose, L-fucose and 5-ketogluconate (API 50CHB). Assimilates carbon from β-cyclodextrin, mannan, Tween-40, amygdalin, D-arabitol, arbutin, cellobiose, D-fructose, L-fucose, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, *myo*-inositol, α-lactose, lactulose, D-mannitol, melezitose, melibiose, methyl α-D-galactoside, 3-methyl glucose, methyl β-D-glucoside, D-ribose, sedoheptulosan, sucrose, acetic acid, β-hydroxybutyric acid, α-ketovaleric acid, D-lactic acid methyl ester, L-lactic acid, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. Assimilates nitrogen from L-alaninamide, D-alanine, L-alanyl glycine, L-glutamic acid, L-pyroglutamic acid, L-serine, thymidine, uridine, adenosine-5′-monophosphate, thymidine-5′-monophosphate and uridine-5′-monophosphate (GN2 MicroPlate). The major cellular fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$ (summed feature 8) and $C_{18:1}\omega9c$. The predominant quinone is MK-6. The polar lipids comprise phosphatidylglycerol, an unidentified phospholipid and unidentified lipids.

The type strain, LHWP2^T (=KACC 15559^T=JCM 17758^T), was isolated from a dead ark clam collected from the southern coastal region of Korea. The DNA G+C content of strain LHWP2^T is 49.5 mol%.

Acknowledgements

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