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ORIGINAL PAPER

Pontibaca salina sp. nov., isolated from marine sediment

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Abstract

A Gram-stain-negative, oval or short rod-shaped, non-motile, aerobic bacterium, designated strain S1109L^T, was isolated from a marine sediment in Weihai, PR China. Cells were oxidase positive and catalase positive. Growth of strain S1109L^T occurred at 10–40 °C (optimum, 30–33 °C), pH 6.5–10.0 (optimum, 7.0–8.0) and in the presence of 1–21% (optimum, 4–6%) (w/v) NaCl. 16S rRNA gene sequence phylogeny indicated that strain S1109L^T was associated with the genus *Pontibaca* of the family *Rhodobacteraceae* because it showed the highest sequence similarity to *Pontibaca methylaminivorans* KCTC 22497^T (97.5%). The average nucleotide identity (ANI) and the digital DNA–DNA hybridization (dDDH) scores between strain S1109L^T and *Pontibaca methylaminivorans* KCTC 22497^T were 74.6% and 18.7%. The major cellular fatty acids of strain S1109L^T were $C_{19:0}$ cyclo ω8c and $C_{18:1}$ ω7c. The polar lipids profiles of strain S1109L^T were phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and two unidentified lipids. Strain S1109L^T contained ubiquinone-10 as the major respiratory quinone. The genomic DNA G + C content was 55.9 mol%. On the basis of the evidence presented in this study, strain S1109L^T is considered to represent a novel species of the genus *Pontibaca*, for which the name *Pontibaca salina* sp. nov. is proposed. The type strain of is S1109L^T (= KCTC 82411^T = MCCC 1H00441^T).

Keywords *Pontibaca salina* · Prokaryotic taxonomy · 16S rRNA gene · Genome

Abbreviations

MCCC	Marine culture collection of China
KCTC	The Korean collection for type cultures
ANI	Average nucleotide identity
GGDC	Genome-to-genome distance calculator
dDDH	Digital DNA–DNA hybridization
MEGA	Molecular evolutionary genetics analysis
MIDI	Microbial identification system
HPLC	High-performance liquid chromatography
TLC	Thin layer chromatography
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
DPG	Diphosphatidylglycerol
MA	Marine agar 2216
MB	Marine broth 2216

Introduction

The genus *Pontibaca*, a member of the family *Rhodobacteraceae*, was described by Kwang Kyu et al. (Kim et al. 2010). *Pontibaca methylaminivorans*, the only member of the genus *Pontibaca*, was isolated from coastal sediment of the East Sea, Korea. The aim of the present work was to report on the taxonomic characterization of a *Pontibaca*-like bacterial strain, S1109L^T, which was isolated from marine sediment sample collected from Weihai.

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Materials and methods

Isolation of the strain and culture conditions

Strain S1109L^T was isolated from marine sediment sample collected from Weihai, PR China (37° 33′ 57.60″ N, 122° 7′ 38.80″ E). The sample, after being appropriately diluted with sterile seawater, was coated on marine sandwich agar plate for cultivation at 28 °C. The marine sandwich agar plate was prepared with marine agar 2216 (MA; Becton Dickinson) as the bottom layer, the growth-promoting bacterium *Roseibacterium beibuensis* MCCC 1F00103^T, which can provide growth promoting effect, as the middle layer and 0.1 × marine agar 2216 (MA; Becton Dickinson) as the top layer (the method has not been published). Strain S1109L^T was isolated and purified by sub-culture on MA plates. Strain S1109L^T was routinely cultured on MA at 30 °C (certified to be optimum) and preserved in marine broth 2216 (MB) containing glycerol (20%, v/v) at − 80 °C.

16S rRNA gene sequencing and phylogenetic analyses

The 16S rRNA gen was amplified by PCR from cell lysate using the universal oligonucleotide primers 27F (5′-AGAGTTTGATC(A/C)TGGCTCAG-3′) and 1492R (5′-TACGG(C/T)TACCTTGTTACGACTT-3′) (Liu et al. 2014). The amplified production was purified and ligated into the vector pMD18-T (TaKaRa). The ligation product was transformed into *Escherichia coli* DH5α cells (TransGen Biotech). The clones of transformed *Escherichia coli* DH5α, which can grow on LB agar containing 0.1% ampicillin, were sequenced by GBI Co., Ltd (Qingdao, PR China). Sequences were compared with the available 16S rRNA gene sequences using BLAST search program (http://www.ncbi.nlm.nih.gov/blast.cgi) and EzBioCloud sever (https://www.ezbiocloud.net/identify). The 16S rRNA gene sequence of strain S1109L^T and other related strains were aligned using CLUSTAL_X (Thompson et al. 1997). The phylogenetic trees of 16S rRNA gene were constructed using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms supported by bootstrap test with 1000 replications in MEGA 7.0 software. (Kimura 1980; Kumar et al. 2016). Evolutionary distance matrices were computed using the Kimura's two-parameter model (Kimura 1980).

Genome extraction and analysis

Genomic DNA of strain S1109L^T was extracted and purified by using a TaKaRa miniBEST universal genomic DNA extraction kit following the instruction's manual. Whole genome sequencing of strain S1109L^T was performed by Beijing Novogene Bioinformatics Technology using the Illumina HiSeq PE150 platform. All good quality paired reads were assembled using SOAP de novo version 2.04 software (Li, 2010). The average nucleotide identity (ANI) values and the digital DNA–DNA hybridization (dDDH) values were analyzed using the average nucleotide identity (ANI) calculator (https://www.ezbiocloud.net/tools/ani) and genome-to-genome distance calculator (GGDC) (http://ggdc.dsmz.de/ggdc.php#). The genes of involved in S1109L^T metabolic pathways were annotated using KEGG database (Aziz, 2008) and the RAST Server (Kanehisa et al. 2016).

Physiology and chemotaxonomy

Considering the highest 16S rRNA gene sequence similarities to strain S1109L^T from EzBioCloud sever, *Pontibaca methylaminivorans* KCTC 22497^T (type species) and *Primorskyibacter marinus* KCTC 42952^T were chosen as reference strains for taxonomic analysis of strain S1109L^T. *Pontibaca methylaminivorans* KCTC 22497^T and *Primorskyibacter marinus* KCTC 42952^T were purchased from Marine Culture Collection of China and Korean Collection for Type Cultures, respectively.

Colony morphology was observed on MA after incubation at 30 °C for 3 days. Cell morphology was observed using light microscopy (E600, Nikon) and scanning electron microscopy (Nova NanoSEM450, FEI). The Gram staining was performed by using the bioMérieux Gram-stain kit as manufacturer's instructions. Motility was examined described by Gerhardt et al. (PhilippGerhardt et al. 1994) and Bowman et al. (Bowman 2000). The temperature range for growth was tested at 0, 4, 10, 15, 20, 25, 28, 30, 33, 37, 42 and 45 °C on MA. Growth at various concentrations of NaCl was examined using a medium containing 0.1% (w/v) yeast extract, 0.5% (w/v) peptone and 2.0% (w/v) agar and comprising 0.0–30.0% (w/v) NaCl (in increments of 1.0%), prepared with artificial seawater (0.32% $MgSO_4$, 0.12% $CaCl_2$, 0.07% KCl and 0.02% $NaHCO_3$, all w/v). The growth at pH range was measured in MB with pH 5.5 to 9.5 (in increments of 0.5 pH unit), using 20 mM MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5) and CAPSO (pH 9.0 and 9.5) for supplement during pH adjustment. Anaerobic growth was investigated on MA with or without 0.1% (w/v) KNO_3 in an anaerobic jar with an atmosphere of 10% H_2, 5% CO_2 and 85% N_2 for 14 days at 30 °C.

Catalase activity was measured by bubble production after dripping 3% (v/v) aqueous hydrogen peroxide solution onto fresh colonies. Oxidase activity was determined using the bioMérieux oxidase reagent kit according to the manufacturer's protocol. Hydrolysis of starch, casein, alginate, CM cellulose and Tweens (20, 40, 60 and 80) were

tested as described by Smibert and Krieg (Smibert and Krieg 1994). Hydrolysis of DNA was tested on DNase test agar (BD Diagnostics), with the modification that artificial seawater was used for the preparation of media. Other physiological and biochemical characteristics were investigated using API 20NE, API 50CHB, API ZYM strips (all from bioMérieux) and the Biolog GEN III MicroPlate kit according to the instruction manual (except salinity of the suspension medium, which was adjusted to 5.0%).

Susceptibility to antibiotics was measured on MA using the diffusion plate technique with the following procedure (Du et al. 2014). The cell suspension (McFarland standard 0.5) was evenly coated on MA; Commercial antibiotic discs (Hangzhou Binhe Microorganism Reagent Co., Ltd) were placed on to the surface; and zones of growth inhibition were registered following CLSI standards after 48 h of incubation at 30 °C.

The quinones and polar lipids were extracted as Minnikin described (Minnikin, 1984). The quinones were separated by a silica-gel thin-layer chromatography (TLC, Merck) plate and tested by HPLC (Kroppenstedt and Reiner 1982; Hiraishi et al. 1996). The polar lipids were separated by two-dimensional TLC on silica gel 60 F254 plates (Merck) using chloroform/methanol/water (65:25:4, by vol.) for the first dimension and chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) for the second dimension. Molybdatophosphoric acid, ninhydrin, a-naphthol and the spray reagent based on the Zinzadze reagent (Vaskovsky and Kostetsky 1968) were used to detect total lipids, aminolipids, glycolipids and phospholipids, respectively (Collins et al. 1980). Major cellular fatty acids were extracted as described (Eder 1995), identified by an Agilent 6890 N gas chromatograph and analyzed using the TSBA40 database of the Microbial Identification System (Sasser 1990).

Results

Phylogenetic analysis

Strain S1109L^T shared the highest sequence similarity with *Pontibaca methylaminivorans* KCTC 22497^T (97.5%) and *Primorskyibacter marinus* KCTC 42952^T (95.9%) by analysis of the complete 16S rRNA gene sequence. The neighbour-joining phylogenetic tree is given in Fig. 1. Neighbour-joining, maximum-likelihood and maximum-parsimony trees all showed that strain S1109L^T should be classified as a separate species of *Pontibaca* genus because of clustering with the closely related type strains *Pontibaca methylaminivorans* KCTC 22497^T (Fig. S1).

Only one 16S rRNA gene sequence (1479 bp) was detected at whole genome level and had 99.9% similarity to the cloned sequence (1427 bp). There were two base pairs of differences between the two sequences (12th and 1129th of the cloned sequence). The genome sequence of strain S1109L^T, consisting of 41 contigs, was 2,934,809 bp in length (N50 value of 221,932 bp) with 1099.7 × coverage. The genomic DNA G + C content was 55.9 mol%. The genome sequences of *Pontibaca methylaminivorans* KCTC 22497 ^{T} and *Primorskyibacter marinus* KCTC 42952^T were obtained from NCBI with GenBank accession numbers NZ_FTPS00000000.1 and NZ_QJSD00000000.1, respectively.

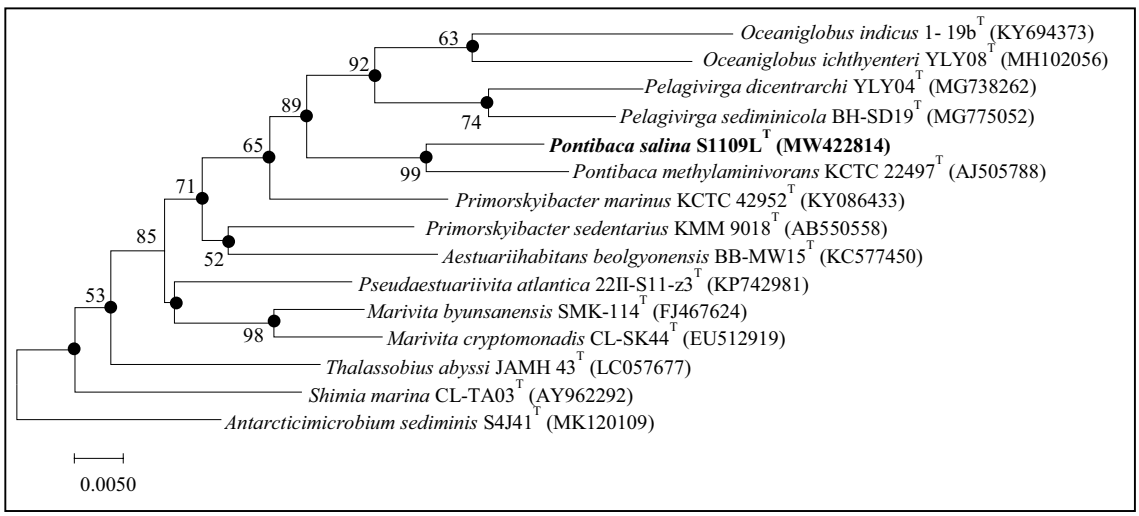

Fig. 1 Neighbour-joining phylogeny reconstructed with 16S rRNA gene sequences showing the position of strain S1109L^T among related taxa. The strain characterized in this study is shown in bold type. Only bootstrap values (percentages of 1000 resamplings) above 50% are shown. *Antarcticimicrobium sediminis* S4J41^T (MK120109) is used to root the tree. Solid circles indicate the corresponding nodes are also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.005 substitutions per nucleotide position

The ANI values between strain S1109L^T and *Pontibaca methylaminivorans* KCTC 22497^T and *Primorskyibacter marinus* KCTC 42952^T were 74.6% and 72.3%, respectively. The dDDH values between strain S1109L^T and *Pontibaca methylaminivorans* KCTC 22497^T and *Primorskyibacter marinus* KCTC 42952^T were 18.7% and 19.3%, respectively. Both the ANI and dDDH values are below the cutoff values for species differentiation (Richter and Rosselló-Móra 2009; Meier-Kolthoff et al. 2013). These data support that strain S1109L^T represents a novel species of the genus *Pontibaca*.

Sequencing of the draft genome of strain S1109L^T allowed genomic analyses. Strain S1109L^T contained 168 genes for carbohydrate metabolism and 117 genes for amino acid metabolism. The genome of strain S1109L^T contained 2 genes, *proA* and *proB*, for carbapenem biosynthesis. Carbapenem antibiotics, relatively resistant to hydrolysis by most β-lactamases, are generally considered to have broad-spectrum antibacterial effect. To test whether strain S1109L^T have carbapenem biosynthesis, strain S1109L^T was inoculated on MA, with carbapenem-sensitive *Escherichia coli* KCTC 25922^T and carbapenem-resistant *Escherichia coli* (CR-Eco) on left side and right side, respectively. The results showed that no growth inhibition occurred in *Escherichia coli* KCTC 25922^T around the growth lawn of S1109L^T after 48 h of incubation at 30 °C (Fig. S2). These indicated that there was no or inadequate carbapenem biosynthesis in strain S1109L^T to inhibit the growth of *Escherichia coli* KCTC 25922^T.

Phenotypic characteristics

Phenotypic characteristics of strain S1109L^T are displayed in Table 1 and in the species description.

Strain S1109L^T was found to be sensitive to penicillin (10 μg), ampicillin (10 μg), carbenicillin (100 μg), cefotaxime sodium (30 μg), ceftriaxone (30 μg), clarithromycin (15 μg), polymyxin B (300 μg), chloramphenicol (30 μg) and resistant to gentamycin (10 μg), tobramycin (10 μg), kanamycin (30 μg), neomycin (30 μg), tetracycline (30 μg), ofloxacin (5 μg), streptomycin (10 μg), norfloxacin (10 μg) and vancomycin (30 μg).

The major isoprenoid quinone of strain S1109L^T was ubiquinone-10 (Q-10), which was the same as that found in *Pontibaca methylaminivorans* KCTC 22497^T and *Primorskyibacter marinus* KCTC 42952^T. The major polar lipids detected in strain S1109L^T were phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and two unidentified lipid (L1-2). The polar lipid profile of strain S1109L^T was in line with that detected in *Pontibaca methylaminivorans* KCTC 22497^T and similar to that major lipids in *Primorskyibacter marinus* KCTC 42952^T, but diphosphatidylglycerol (DPG) was only found in

Table 1 Differential phenotypic and genotypic characteristics between strain S1109L^T and closely related strains of the genus *Pontibaca*

Characteristic*	1	2	3
Cell size (μm):			
Width	0.4–0.5	0.7–1.3	0.2–0.4
Length	0.5–1.0	0.9–1.6	0.6–2.2
Optimum NaCl for growth (%, w/v)	4.0–6.0	2.0–3.0	3.0
Optimum temperature for growth (°C)	20–33	30	30
Optimum pH for growth	7.0–8.0	7.0–8.0	7.0–7.5
DNase	−	ND	+
Hydrolysis of casein	+	−	−
Hydrolysis of starch	−	−	+
API 20NE test:			
Hydrolysis of nitrate reduction	−	+	+
Aesculin	−	−	+
Enzyme activities (API ZYM):			
Naphthol-AS-BI-phosphohydrolase	+	+	−
Acid phosphatase	−	−	+
α-Glucosidase	−	−	+
Acid production (API 50CHB):			
D-fructose	+	−	−
D/L-arabinose	−/−	+/+	+/+
D-ribose	−	+	+
D/L-xylose	−/−	+/+	+
D-galactose	−	+	+
D-glucose	−	+	+
D-mannose	−	+	−
L-rhamnose	−	+	−
Cellobiose	−	+	+
Lactose	−	+	−
Oxidation of (Biolog GEN III):			
L-alanine		−	−
L-arginine	+	−	−
L-glutamic acid	+	−	+
L-histidine	+	−	+
Methylpyruvate	−	+	−
L-lactic acid	+	−	−
α-Hydroxy-butyricacid	+	−	−
Acetoacetic acid	+	−	+
Propionic acid	+	−	−
DNA G + C content (mol%)	55.9	64.8[a]	57.8[b]

Strains: 1, S1109L^T; 2, *Pontibaca methylaminivorans* KCTC 22497^T 3, *Primorskyibacter marinus* KCTC 42952^T. All data are from this study unless otherwise indicated. +, Positive; −, Negative; ND, no data. All strains were Gram-stain-negative, short rod-shaped, non-motile, aerobic and had activities for oxidase, catalase, urease and leucine arylamidase. Glucuronamide, β-hydroxy-D,L-butyric acid, acetic acid and formic acid can be oxidized.

*Data from [a]Kim et al. (Kim et al. 2010); [b]Wang et al. (Wang et al. 2018)

Primorskyibacter marinus KCTC 42952^T. Further detailed polar lipid images of different specific stains are given in Fig. S3.

The major fatty acids (> 10%) detected in strain S1109L^T were $C_{19:0}$ cyclo ω8c and $C_{18:1}$ ω7c. The percentages of the fatty acid profile were similar to those in *Pontibaca methylaminivorans* KCTC 22497^T. However, the presence of $C_{16:0}$ was a distinct characteristic of the fatty acid composition of *Pontibaca methylaminivorans* KCTC 22497^T. Unlike in strain S1109L^T and *Pontibaca methylaminivorans* KCTC 22497^T, $C_{18:1}$ ω7c and $C_{18:1}$ ω7c 11-methyl were major fatty acids in *Primorskyibacter marinus* KCTC 42952^T. Differences in the fatty acid profiles of strain S1109L^T, *Pontibaca methylaminivorans* KCTC 22497^T and *Primorskyibacter marinus* KCTC 42952^T are listed in Table 2.

Based on the phylogenetic, phenotypic and chemotaxonomic results mentioned above, strain S1109L^T represents a new species of the genus *Pontibaca*, for which the name *Pontibaca salina* sp.nov. is proposed.

Description of *Pontibaca salina* sp. nov.

Pontibaca salina (sa.li'na. N. L. fem. adj. *salina* salted, saline)

Cells are Gram-stain-negative, oval or short rod-shaped (0.4–0.5 × 0.5–1.0 μm) and non-motile. Colonies on MA are about 0.5–1 mm in diameter, circular, smooth, slightly convex, translucent and light-brown coloured after incubation for 3 days at 30 °C. Growth occurs at 10–40 °C (optimum, 30–33 °C), pH 6.5–10.0 (optimum, 7.0–8.0) and in the presence of 1–21% (optimum, 4–6%) (w/v) NaCl. Catalase and oxidase are positive. Nitrate is not reduced. Urea and casein are hydrolysed, but starch, aesculin, gelatin, CM cellulose, alginate, DNA and Tweens (20, 40, 60 and 80) are not. Activities of leucine arylamidase and naphthol-AS-BI-phosphohydrolase are present. Acid are produced from D-fructose and potassium 5-keto-gluconate. In carbon source oxidation tests, positive results are obtained for L-alanine, L-arginine, L-glutamic acid, L-histidine, L-serine, glucuronamide, L-lactic acid, α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, acetoacetic acid, propionic acid, acetic acid and formic acid. Ubiquinone-10 (Q-10) is the major isoprenoid quinone. The main fatty acids are $C_{19:0}$ cyclo ω8c and $C_{18:1}$ ω7c. The major polar lipids are phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and two unidentified lipids. The G + C content of the DNA is 55.9%.

The type strain, S1109L^T (= KCTC 82411^T = MCCC 1H00441^T), was isolated from a marine sediment collected from Weihai, PR China (37° 33′ 57.60″ N, 122° 7′ 38.80″ E).

The GenBank accession number for the 16S rRNA gene sequence of strain S1109L^T is MW422814. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAEIJD000000000.

Table 2 Fatty acid composition (%) of strain S1109L^T and closely related strains of the genus *Pontibaca*

Fatty acid	1	2	3
$C_{12:0}$	TR	3.3	–
$C_{14:0}$	TR	TR	TR
$C_{16:0}$	3.2	**15.3**	9.7
$C_{17:0}$	TR	TR	1.3
$C_{18:0}$	TR	1.2	1.7
$C_{19:0}$ cyclo ω8c	**49.1**	**39.7**	–
$C_{18:1}$ ω7c 11-methyl	3.8	4.2	**13.1**
C20:2 ω6,9c	1.4	3.5	–
$C_{10:0}$ 3-OH	2.8	2.4	–
$C_{12:0}$ 3-OH	2.0	3.1	TR
$C_{16:0}$ 2-OH	1.2	–	–
Summed Feature 3[†]	TR	1.6	0.5
Summed Feature 8[#]	**30.1**	**17.7**	**70.3**

Strains: 1, S1109L^T; 2, *Pontibaca methylaminivorans* KCTC 22497^T 3, *Primorskyibacter marinus* KCTC 42952^T. All data are from the present study. Major components (≥ 10.0%) are highlighted in bold. TR, trace amount (< 1.0%); –, not detected

[†]Summed feature 3: $C_{16:1}$ω7c/$C_{16:1}$ω6c

[#]Summed feature 8: $C_{18:1}$ ω7c

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Author contributions Strain S1109L^T was isolated by J-SB. Material preparation, experimental operation, data collection and analysis were performed by J-SB, SW and X-Z Song. The manuscript was written by J-SB. Project guidance and critical revision of manuscripts was performed by Z-JD. All authors read and approved the final manuscript.

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Data availability The GenBank accession number for the 16S rRNA gene sequence and whole-genome shotgun project of strain S1109L^T are MW422814 and JAEIJD000000000, respectively.

Declarations

Conflict of interests The authors declare that there is no conflict of interest.

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