Halobacillus sediminis sp. nov., a moderately halophilic bacterium isolated from a solar saltern sediment

Su-Jin Kim,¹ Jae-Chan Lee,² Song-Ih Han¹ and Kyung-Sook Whang¹,²

¹Department of Microbial & Nano Materials, College of Science & Technology, Mokwon University, 88 Doanbuk-ro, Seo-gu, Daejeon 302-318, Republic of Korea
²Institute of Microbial Ecology and Resources, Mokwon University, 88 Doanbuk-ro, Seo-gu, Daejeon 302-318, Republic of Korea

A Gram-staining-positive, moderately halophilic bacterium, designated strain NGS-2T, was isolated from sediment of a solar saltern pond located in Shinan, Korea. Strain NGS-2T was a strictly aerobic, non-motile rod that grew at pH 5.0–10.0 (optimum, pH 8.0), at 10–30 °C (optimum, 28 °C) and in the presence of 1–20 % (w/v) NaCl (optimum, 10 % NaCl). Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain NGS-2T belonged to the genus Halobacillus, with sequence similarity of 98.4–95.8 % to existing type strains, showing the highest sequence similarity to Halobacillus dabanensis D-8T (98.4 %), H. litoralis SL-4T (98.4 %), H. trueperi SL-5T (98.2 %), H. faecis IGA7-4T (98.2 %), H. profundus IS-Hb4T (98.1 %) and H. mangrovi MS10T (98.0 %). The major polar lipids were phosphatidylglycerol, phosphatidylethanolamine, phosphatidyl-N-methylethanolamine and an unknown glycolipid. The cell-wall peptidoglycan was based on l-Orn–d-Asp, the predominant isoprenoid quinone was menaquinone 7 (MK-7) and the major fatty acids were anteiso-C₁₅: 0 and anteiso-C₁₆: 0 as the major fatty acids and a cell-wall peptidoglycan type based on L-Orn–D-Asp. The DNA G+C content of the novel isolate was 45.0 mol%. Levels of DNA–DNA relatedness between strain NGS-2T and the type strains of 12 other species of the genus ranged from 32 to 3 %. On the basis of the polyphasic analysis conducted in this study, strain NGS-2T represents a novel species of the genus Halobacillus, for which the name Halobacillus sediminis sp. nov. is proposed. The type strain is NGS-2T (=KACC 18263T=NIBRC 110639T).

The genus Halobacillus was proposed by Spring et al. (1996) and, at the time of writing, the genus comprised 18 species with validly published names (Parte, 2014): Halobacillus halophilus (the type species), H. trueperi and H. litoralis (Spring et al., 1996), H. salinus (Yoon et al., 2003), H. karajensis (Amoozegar et al., 2003), H. localis (Yoon et al., 2004), H. yeomjioni (Yoon et al., 2005), H. aidingensis and H. dabanensis (Liu et al., 2005), H. kuroshimensis and H. profundi (Hua et al., 2007), H. campisalis (Yoon et al., 2007), H. faecis (An et al., 2007), H. alkaliophilus (Romano et al., 2008), H. mangrovi (Soto-Ramirez et al., 2008), H. seohaensis (Yoon et al., 2008) and H. naozhouensis and H. salsuginis (Chen et al., 2009). Since 2009, no further species of the genus Halobacillus have been proposed. Members of the genus Halobacillus are Gram-staining-positive, motile or non-motile, spore-forming rods and are clearly differentiated from other related genera by having MK-7 as the predominant menaquinone, one or more of anteiso-C₁₅: 0, iso-C₁₅: 0, C₁₆: 0 and iso-C₁₆: 0 as the major fatty acids and a cell-wall peptidoglycan type based on L-Orn–D-Asp (Spring et al., 1996; Shida et al., 1997; Yoon et al., 2001; Nunes et al., 2006), with the exception of H. campisalis ASL-17T (Yoon et al., 2007), H. seohaensis KCTC 13145T (Yoon et al., 2008) and H. naozhouensis KCTC 13234T (Chen et al., 2009), the cell-wall type of which is based on meso-diaminopimelic acid. These spore-forming moderate halophiles are found mostly in marine or hypersaline environments such as salt-erns, salt flats, evaporation ponds, salt lakes, soda lakes and subsurface salt formations. The aim of present study was to determine the exact taxonomic position of a halophilic bacterial strain, NGS-2T, by using a polyphasic characterization, including phenotypic properties, lipid analyses, phylogenetic analysis based on 16S rRNA gene sequences and genotypic relatedness.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NGS-2T is AB971838.

Five supplementary figures and a supplementary table are available with the online Supplementary Material.
Strain NGS-2T was isolated from sediment of a crystallizing pond at a solar saltern (34° 59’ 47.95” N 126° 10’ 33.83” E) in Shinan, Korea, during studies focused on the isolation of halophilic bacteria, and constituted a single isolate out of a total of 122 isolates from 15 genera. The pH and salinity of the soil sediment of the saltern pond were pH 8.3 and 10 % NaCl. The major chemical components of the soil and salt were NaCl, KCl and MgCl₂. The strain was isolated by diluting a sediment sample in sterile 3 % (w/v) NaCl and plating on MA medium (Difco), followed by aerobic incubation at 28 °C for 3 days. The medium was adjusted to pH 7.0 with 1 M NaOH. The strain was subsequently purified by plating three times on MA at 28 °C for 3 days and maintained on the same medium. The strain was stored at 80 °C on this medium without agar and supplemented with 20 % (v/v) glycerol. In order to characterize strain NGS-2T phenotypically, the isolate was routinely grown aerobically on MA for 3 days at 28 °C and pH 7.0, except where indicated otherwise. The number of halophilic bacterial colonies in sediment on MA supplemented with 10 % (w/v) NaCl (4.17 × 10⁶ c.f.u. ml⁻¹) was greater than in salt water (6.03 × 10⁴ c.f.u. ml⁻¹).

The morphology of the isolate was observed by Gram staining and transmission electron microscopy (CM20; Philips) and motility was observed by phase-contrast microscopy (DM500; Leica) using cells from exponentially growing cultures and the hanging-drop method. The presence of endospores was determined by a specific endospore-staining test, using malachite green (Schaeffer and Fulton endospore stain kit; Sigma). Gram staining was performed by the Burke method (Murray et al., 1994). Catalase and oxidase activities, nitrate reduction, hydrolysis of aesculin, casein, DNA, gelatin, hypoxanthine, l-tyrosine, starch, Tween 80, xanthine and urea, the Voges–Proskauer test and production of indole were tested as recommended by Cowan & Steel (1965), Lányi (1987) and Smibert & Krieg (1994) with substrate concentrations of 0.01 % (w/v). Acid production from carbohydrates was determined as described by Leifson (1963); all suspension media were supplemented with 10 % (w/v) NaCl. All carbon or nitrogen sources were sterilized by filtration before adding to the medium. Cell suspension was performed in 10 % (w/v) NaCl. Additional biochemical characteristics were determined using API 20NE (bioMérieux) supplemented with 10 % (w/v) NaCl at 28 °C. The remaining biochemical and nutritional tests were conducted using traditional methods and followed the recommended minimal standards for describing novel taxa of aerobic, endospore-forming bacteria (Logan et al., 2009). Enzyme activities were tested using the API ZYM kit according to the manufacturer’s instructions (bioMérieux). To determine the optimal temperature and pH for growth of strain NGS-2T, broth cultures in laboratory-made marine broth (MB) were incubated at 4, 10, 15, 20, 28, 37, 45 and 55 °C and at pH 5–11 (at intervals of 0.5 pH units). Media at pH <6, 6–9 and >9 were obtained by using sodium acetate/acetic acid, Tris/HCl and glycine/NaOH buffers, respectively. Growth in the presence of 0, 1, 2, 3, 5, 7, 9, 10, 12, 13, 15, 20 and 25 % (w/v) NaCl was tested in MB at pH 7.0. Growth was monitored by turbidity at OD₆₀₀ by using a spectroscopic method (model UV-1650PC; Shimadzu). Anaerobic growth was determined by incubation in the BBL GasPak Anaerobic System (Difco) for 5 days at 30 °C on MA.

Phenotypic properties of strain NGS-2T were consistent with its assignment to the genus Halobacillus (Spring et al., 1996; Shida et al., 1997; Yoon et al., 2001; Nunes et al., 2006). It was a Gram-staining-positive, endospore-forming bacterium, but motility was not observed, with no flagella (Figs S1 and S2, available in the online Supplementary Material). Colonies were yellow, circular and convex with entire margins when grown for 3 days at 28 °C on MA. Cells were rods, 0.8–1.2 μm wide and 2.2–2.5 μm long (Fig. S1). Strain NGS-2T was able to grow at 10–30 °C, at pH 5.0–10.0 and in the presence of 1.0–20.0 % (w/v) NaCl. Optimal growth was observed at 28 °C, at pH 8.0 and with 10 % (w/v) NaCl. Growth was also observed on Luria–Bertani (LB) agar and trypticase soy agar (TSA) (both from Difco). Cells were catalase- and oxidase-positive. Other phenotypic features are included in the species description, and characteristics that differentiate strain NGS-2T from related type strains of species of the genus Halobacillus are summarized in Table 1.

Genomic DNA from strain NGS-2T was prepared by using the method described by Tamaoka & Komagata (1984). The 16S rRNA gene was amplified by PCR with the forward primer 27F and the reverse primer 1492R (DeLong, 1992). Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (ABI 3730XL; Applied Biosystems). The full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequence was aligned with published sequences of closely related bacteria with CLUSTAL W 2.0 software (Larkin et al., 2007), Gaps at the 5’ and 3’ ends of the alignment were omitted in further analyses. Phylogenetic trees were reconstructed by using three different methods: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms within the MEGA6 program (Tamura et al., 2013). Evolutionary distance matrices for the neighbour-joining method were calculated using the algorithm of Kimura’s two-parameter model (Kimura, 1980). To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed (Felsenstein, 1985). The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and GenBank accession numbers are shown in Fig. 1.

To determine genomic relatedness, DNA–DNA hybridization was performed using the modified method of Ezaki et al. (1989). Probe labelling for DNA–DNA hybridization

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Table 1. Differential phenotypic characteristics of strain NGS-2T and other type strains of the genus *Halobacillus*

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<td>D-Trehalose</td>
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<td>DNA G + C content</td>
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<td>42.1</td>
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<td>46.5</td>
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<td>39.3</td>
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*E, Ellipsoidal; s, spherical.
†C, Central; l, lateral; st, subterminal.
‡Data from this study.

Strains: 1, NGS-2T; 2, *H. dabanensis* JCM 12772T (unless indicated, data from Liu et al., 2005); 3, *H. litoralis* KCTC 3687T (Spring et al., 1996); 4, *H. faecis* KCTC 13121T (An et al., 2007); 5, *H. profundi* JCM 14154T (Hua et al., 2007); 6, *H. mangrovi* CECT 7206T (Soto-Ramirez et al., 2008); 7, *H. kurajensis* DSM 14948T (Amoozegar et al., 2003); 8, *H. campisalis* KCTC 13144T (Yoon et al., 2007); 9, *H. yeomjeoni* KCTC 3957T (Yoon et al., 2005); 10, *H. salinus* KCTC 3842T (Yoon et al., 2003); 11, *H. halophilus* KCTC 3685T (Claus et al., 1983; Spring et al., 1996). All strains share the following characteristics: positive for activity of catalase and oxidase; negative for nitrate reduction and urease activity. +, Positive; –, negative; w, weakly positive.
was conducted by using the non-radioactive DIG-High Prime system (Roche); hybridized DNA was visualized using the DIG luminescent detection kit (Roche) and the level of DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). Isolation of DNA was performed using the method of Saito & Miura (1963) and determination of the DNA G+C content were performed by HPLC (SPD-10AV; Shimadzu), as described by Mesbah et al. (1989).

The almost-complete 16S rRNA gene sequence (1502 bp) of strain NGS-2T was obtained and used for initial BLAST searches of the GenBank database and for phylogenetic analysis. The 16S rRNA gene sequences of related taxa were obtained from the GenBank and EzTaxon-e servers and the identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved by using the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012). Strain NGS-2T was shown to belong to the genus Halobacillus, showing the highest 16S rRNA gene sequence similarity to H. dabanensis D-8T (98.4 %), H. litoralis SL-5T (98.2 %), H. faecis IGA7-4T (98.2 %), H. profundis IS-Hb4T (98.1 %), H. mangrovi MS10T (98.0 %), H. kuroshimensis IS-Hb7T (98.0 %), H. trueperi SL-5T (98.2 %), H. faecis IGA7-4T (98.2 %), H. profundis IS-Hb4T (98.1 %), H. mangrovi MS10T (98.0 %), H. karajensis MA-2T (97.9 %), H. yeomjeoni MSS-402T (97.9 %), H. campisalis ASL-17T (97.9 %), H. locisalis MSS-155T (97.7 %), H. salinus HSL-3T (97.6 %), H. aidingensis AD-6T (97.6 %) and H. halophilus DSM 2266T (97.5 %). 16S rRNA gene

**Fig. 1.** Rooted neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain NGS-2T and related bacteria in the genus Halobacillus and some other related taxa. Bootstrap values, expressed as percentages of 1000 replications, are given at branching points when >50 %. Filled and open circles at nodes indicate generic branches that were also recovered by using the maximum-likelihood and maximum-parsimony algorithms, and the maximum-likelihood algorithm, respectively. Gracilibacillus halotolerans NN1 was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.
sequence similarity to members of species of the genus _Halobacillus_ was 98.4–95.8 %. The neighbour-joining (Fig. 1), maximum-likelihood (Fig. S3) and maximum-parsimony (Fig. S4) methods resulted in highly similar neighbour-joining (Fig. S4) methods resulted in highly similar results. DNA–DNA hybridization with strain NGS-2^T^ was 45.0 mol%, which is relatively higher than those reported for other species of the genus _Halobacillus_ (Table 1).

For analysis of fatty acids, strain NGS-2^T^ was cultured on _Halobacillus_ (Spring et al., 1996; Shida et al., 1997; Yoon et al., 2001; Nunes et al., 2006).

Strain NGS-2^T^ shared similar chemotaxonomic characteristics with members of the genus _Halobacillus sensu stricto_ in terms of the predominant menaquinone, major fatty acids and peptidoglycan type, in which strain NGS-2^T^ is located in the cluster of cell-wall type based on _L-Orn–D-Asp_, which is a key marker that differentiates strain NGS-2^T^ and the genus _Halobacillus_ from other genera (Spring et al., 1996; Shida et al., 1997; Yoon et al., 2001; Nunes et al., 2006).

Therefore, on the basis of the results of this polyphasic taxonomic study, we propose that strain NGS-2^T^ represents a novel species of the genus _Halobacillus_, for which the name _Halobacillus sediminis_ sp. nov. is proposed.

**Description of _Halobacillus sediminis_ sp. nov.**

_Halobacillus sediminis_ (se.di.mi’nis. L. gen. n. sediminis of sediment).

Cells are Gram-staining-positive, non-motile rods. Endospores are ellipsoidal and are located in a central or subterminal position. Colonies are circular, convex with entire margins, yellow and 1.0–3.0 mm in diameter on MA supplemented with 10 % NaCl (w/v) after 3 days of incubation at 28 °C. Growth also occurs on LB agar and TSA. Grows at 10–30 °C (optimally at 28 °C), pH 5.0–10.0 (optimally at pH 8.0) and with 1–20 % (w/v) NaCl, with optimal growth at 10 % (w/v) NaCl. Catalase- and

Major fatty acids in strain NGS-2^T^ were anteiso-C_{15:0} (57.3 %) and anteiso-C_{17:0} (13.6 %). Moderate amounts of summed feature 4 (iso-C_{17:1} \_I \ and/or anteiso-C_{17:1} \_I B; 6.9 %), iso-C_{15:0} (6.7 %), iso-C_{17:0} (3.5 %) and C_{17:0} \_isoC \_alcohol (3.4 %) were also present (Table S1). These are common characteristic features of members of the genus _Halobacillus_ (Amoozegar et al., 2003; Yoon et al., 2003, 2004; Liu et al., 2005; An et al., 2007; Hua et al., 2007; Soto-Ramirez et al., 2008). The presence of anteiso-C_{15:0} at a relatively high percentage distinguished strain NGS-2^T^ from type strains of closely related species of the genus _Halobacillus_ (Table S1). The predominant isoprenoid quinone was MK-7 (97 %), with a minor amount of MK-8 (3 %), as in all known members of the genus _Halobacillus_ (Amoozegar et al., 2003; Yoon et al., 2003, 2004, 2005, 2007, 2008; Liu et al., 2005; An et al., 2007; Hua et al., 2007; Romano et al., 2008; Soto-Ramirez et al., 2008). Polar lipids of NGS-2^T^ comprised phosphatidylglycerol, phosphatidylyethanolamine, phosphatidyl-N-methylethanolamine, an unknown glycolipid, two unknown phospholipids and an unknown lipid (Fig. S5); this profile is similar to the complex lipid pattern reported for _H. alkaliphilus_ FPS^T^, with the presence of two phospholipids, phosphatidylglycerol and a glycolipid, but differentiated by the presence of phosphatidylyethanolamine and phosphatidyl-N-methylethanolamine (Romano et al., 2008). From the results of the cell-wall analysis, the peptidoglycan type of strain NGS-2^T^ was determined as _L-Orn–D-Asp_, which is a key marker that differentiates strain NGS-2^T^ and the genus _Halobacillus_ from other genera (Spring et al., 1996; Shida et al., 1997; Yoon et al., 2001; Nunes et al., 2006).

For analysis of fatty acids, strain NGS-2^T^ was cultured on MA at 28 °C and cells were obtained at the late-exponential growth phase; reference strains were cultured under similar conditions. Cellular fatty acids were extracted and analysed by GC (6890N; Agilent Technologies) according to the standard protocol of the Sherlock Microbial Identification System (version 4.5; MIDI database TSBA40 4.10). For analysis of quinones and polar lipids, cells were harvested in the late-exponential phase and freeze-dried. Isoprenoid quinones were extracted, washed and hydrolysed with 0.5 M NaOH as described by Collins & Jones (1981). For polar lipid analysis, the cellular lipids were extracted, washed and hydrolysed with 0.5 M NaOH as described by Yabuuchi et al. (1990, 1999). The total lipids were separated on silica-gel plates by two-dimensional TLC with a solvent system composed of chloroform/methanol/water (65: 25: 4, by vol.) in the first direction and chloroform/methanol/acetic acid/water (80: 15: 12: 4, by vol.) in the second direction. To detect spots and their colour reaction, 5 % ethanolic molybdate-phosphoric acid, ninhydrin solution, Dittmer–Lester reagent and _2-naphtol_ reagent were used for all lipids, aminolipids, phospholipids and glycolipids, respectively. Preparation of cell walls and determination of the peptidoglycan structure were carried out by the methods described by Schleifer & Kandler (1972) with the modification that TLC on cellulose sheets was used instead of paper chromatography.
oxidase-positive. Nitrile is not reduced to nitrite. Indole is produced. Aesculin is hydrolysed but gelatin, casein, starch, urea, tyrosine, xanthine and hypoxanthine are not. Methyl red, Voges–Proskauer, H$_2$S production, lysine and ornithine decarboxylase, arginine dihydrolase and phenylalanine deaminase tests are negative. Utilizes aesculin, D-tagatose and D-arabitol, but not L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate or phenylacetate. Acid is produced from glycerol, D-glucose, D-fuctose, D-mannose, aesculin, maltose, trehalose and starch, but not from D- or L-arabinose, D-ribose, methyl β-D-mannopyranoside, D- or L-fucose, amygdalin, cellobiose, melibiose, sucrose, melezitose, L-xylene, raffinose, gentiobiose, turanose, 2- or 5-ketogluconate, erythritol, D-xylene, D-adonitol, methyl β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, D- or L-arabitol, inositol, D-mannitol, D-sorbitol, methyl β-D-glucopyranoside, N-acetyl-D-glucosamine, arbutin, L-salicin, lactose, inulin, glycogen, xylitol, gluconate, D-lyxose or D-tagatose. Enzyme activities are observed for esterase (C4), lipase (C14), acid phosphatase, lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, β- or γ-galactosidase, α- or β-glucosidase or α-fucosidase. The predominant isoprenoid quinone is menaquinone 7 (MK-7). The major fatty acids are anteiso-C$_{15:0}$ and anteiso-C$_{17:0}$. The cell-wall peptidoglycan type is L-Orn–D-Asp. Polar lipids are phosphatidylglycerol, phosphatidylethanolamine, phosphatidyl-N-methylethanolamine, an unknown glycolipid, two unknown phospholipids and an unknown lipid.

The type strain, NGS-2$^T$ (=KACC 18263$^T$=NBRC 110639$^T$), was isolated from a sediment of a solar saltern pond at Shinan, Korea. The DNA G+C content of the type strain is 45.0 mol%.

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**References**


