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ORIGINAL PAPER

# *Bacillus mesophilus* sp. nov., an alginate-degrading bacterium isolated from a soil sample collected from an abandoned marine solar saltern

**Yan-Xia Zhou · Guo-Hong Liu · Bo Liu · Guan-Jun Chen · Zong-Jun Du**

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**Abstract** A novel Gram-stain positive, endospore-forming bacterium, designated SA4$^T$, was isolated from a soil sample collected from an abandoned marine solar saltern at Wendeng, Shandong Province, PR China. Cells were observed to be rod shaped, alginase positive, catalase positive and motile. The strain was found to grow at temperatures ranging from 15 to 40 °C (optimum 35 °C), and pH 5.0–11.0 (optimum pH 8.0) with 0–7.0 % (w/v) NaCl concentration (optimum NaCl 3.0 %). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain SA4$^T$ belongs to the genus *Bacillus* and exhibits 16S rRNA gene sequence similarities of 96.6, 96.5, 96.3 and 96.2 % with *Bacillus horikoshii* DSM 8719$^T$, *Bacillus acidicola* 105-2$^T$, *Bacillus shackletonii* LMG 18435$^T$ and *Bacillus pocheonensis* Gsoil 420$^T$, respectively. The menaquinone was identified as MK-7 and the major polar lipids were identified as diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The major fatty acids detected were anteiso-$C_{15:0}$ (22.3 %), iso-$C_{15:0}$ (22.6 %), iso-$C_{16:0}$ (14.8 %) and iso-$C_{14:0}$ (14.7 %). The DNA G+C content was determined to be 42.4 mol %. Phenotypic, chemotaxonomic and genotypic properties clearly indicated that isolate SA4$^T$ represents a novel species within the genus *Bacillus*, for which the name *Bacillus mesophius* sp. nov. is proposed. The type strain is SA4$^T$ (=DSM 101000$^T$ =CCTCC AB 2015209$^T$).

**Keywords** Alginate-degrading bacterium · *Bacillus mesophilus* sp. nov. · Marine solar saltern · Soil

## Introduction

The genus *Bacillus* is composed of rod shaped, endospore-forming bacteria that are members of the phylum *Firmicutes* and are phylogenetically and phenotypically heterogeneous according to their distribution in various environments (Claus and Berkeley 1986). The majority of established species are mesophiles, and prefer neutral pH and low salt concentrations (Kosowski et al. 2014); however, some can also grow in alkaline environments (Denizci et al. 2010; Nielsen et al. 1995). In the course of screening for polysaccharide-degrading bacteria, we isolated a Gram-stain positive, endospore-forming, alkaliphilic strain,

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Y.-X. Zhou · G.-J. Chen · Z.-J. Du (✉)
College of Marine Science, Shandong University at Weihai, Weihai 264209, People's Republic of China
e-mail: duzongjun@sdu.edu.cn

G.-H. Liu · B. Liu (✉)
Agricultural Bio-Resource Institute, Fujian Academy of Agricultural Sciences, Fuzhou, Fujian 350003, People's Republic of China
e-mail: fzliubo@163.com

designated SA4$^T$, from a soil sample collected from an abandoned marine solar saltern at Wendeng, PR China. This new isolate can degrade alginate, which is the most abundant polysaccharide in brown algae and is expected to become a renewable bioenergy resource (Obataa et al. 2015). Thus, the isolate has potential ability to produce novel alginases that are resistance to the alkaline conditions encountered during the processing of biomass for bioenergy generation from alginates. In this paper, we report the taxonomic characterisation of strain SA4$^T$. On the basis of phenotypic characteristics, phylogenetic analysis and 16S rRNA data, the strain SA4$^T$ is considered to represent a novel species of the genus *Bacillus*.

## Materials and methods

### Bacterial strains, isolation and cultivation

The strain SA4$^T$ was isolated from a soil sample (temperature, pH and salinity of 31.9 °C, 8.01 and 2.71 %, respectively) collected from an abandoned marine solar saltern at Weihai, Shandong Province, PR China (122°0′21.59"E, 36°69′52.82"N) during October 2013. For culture, the soil sample was suspended in sterilised water, serially diluted, spread on marine 2216 agar (MA; Becton–Dickinson) directly without heat shock and incubated at 30 °C for 48 h. Pure cultures were obtained by several successive single colony isolations. The strain was stored both on MA slants at 4 °C and as suspensions in marine 2216 broth (MB; Becton–Dickinson) with 20 % (v/v) glycerol at −80 °C. The reference strains *Bacillus horikoshii* DSM 8719$^T$, *Bacillus acidicola* DSM 14745$^T$ and *Bacillus shackletonii* LMG 18435$^T$ were obtained from the culture collections and used as controls in the phenotypic tests.

### Phenotypic and physiological characterisation

A single colony of each strain was inoculated in 10 ml MB medium and incubated for 3 days at 30 °C before being subjected to phase-contrast light microscopy (Olympus BX-90). Strain SA4$^T$ was cultured over the temperature range 5–50 °C (at intervals of 5 °C) and the pH range 5.0–13.0 (at intervals of 1.0 pH unit) in MB. Growth at various NaCl concentrations was tested in NaCl-free artificial seawater medium supplemented with 5.0 g peptone, 1.0 g yeast extract and various concentrations of NaCl (final concentration: 0–10 % (w/v), using increments of 1.0 %) (Yang and Cho 2008). Cell density in MB was determined from the optical density at 600 nm with a spectrophotometer (UV-2550, Shimadzu, Japan) after incubation for up to 3 days. Growth under anaerobic conditions was determined after incubation in an anaerobic jar containing $H_2/CO_2/N_2$ (5:5:90, by vol.) on MA at 30 °C for 7 days. The Gram staining and the KOH lysis test were carried out according to the methods described by Gregersen (1978) and Smibert and Krieg (1994). Motility was examined on motility agar (Chen et al. 2007). Oxygen requirement, activities of catalase, urease and oxidase, hydrolysis of casein, starch and aesculin, nitrate reduction, the Voges–Proskauer test, indole and $H_2S$ production were determined by the conventional methods described by Cowan and Steel (1965) and Smibert and Krieg (1994). Alginate lyase activity was detected by the formation of clear zones around colonies on MA plates containing 1 % (w/v) sodium alginate after flooding with diluted Lugol solution (Akagawa and Yamasato 1989; Schlesner et al. 1990). The utilisation of sole carbon and nitrogen sources was determined according to the method described by Gao et al. (1994). API 20E and API 50CH kits (bioMérieux) were used according to the manufacturer's instructions except that the NaCl concentration was adjusted to 3.0 % (w/v) for strain SA4$^T$ and that the pH was adjusted to 5.0 for *Bacillus acidicola* DSM 14745$^T$.

### Chemotaxonomic characterisation

Biomass of strain SA4$^T$ and reference strains was harvested from cultures after incubation on MA medium at 30 °C for 48 h, when the bacteria were in the logarithmic growth phase. For cell wall chemotaxonomy, the peptidoglycan diamino acid test was carried out according to Schleifer (1985). The amino acid composition of the peptidoglycan hydrolysate (4 M HCl, 16 h, 100 °C) was determined by TLC, 2D-TLC and gas chromatography/mass spectrometry GC/MS. Menaquinones were analysed as described by Collins et al. (1977), using reverse-phase HPLC (Groth et al. 1996). Extraction and analysis of polar lipids by 2D-TLC was performed according to Minnikin et al. (1984) by the DSMZ identification service.

For determination of cellular fatty acids, the isolate was harvested after cultivation on MA at 30 °C for 48 h, the references strains were cultivated on MA. The experiments were performed according to the standard protocol of the Sherlock Microbial Identification System version 6.0 (MIDI), analysed by GC (model 7890; Agilent) and identified using the TSBA6 database of the Microbial Identification System (Sasser 1990).

### Phylogenetic analysis of 16S rRNA gene sequence

For 16S rRNA gene sequencing and phylogenetic analysis, chromosomal DNA was extracted and purified according to standard methods (Hopwood et al. 1985). The 16S rRNA gene sequences were amplified by PCR with the universal primers 9F (5′-GAGTTT GATCCTGGCTCAG-3′) and 1542R (5′-GGAGAAA GGAGGTGATCCAGCC-3′) as described by Lee et al. (2001). The sequence of the amplified 16S rRNA gene was determined using a DNA sequencer (ABI 310 sequencer; Applied Biosystems). The resultant 16S rRNA gene sequence was compared with that of reference strains with validly published names using the EzTaxon-e server (http://www.eztaxon-e.ezbiocloud.net/; Kim et al. 2012). For determination of pairwise sequence similarity values, sequence data were calculated using the global alignment algorithm obtained through the EzTaxon-e server (Kim et al. 2012). After multiple alignments of data by CLUSTAL_X (Thompson et al. 1997), phylogenetic trees were constructed using the neighbour-joining (NJ) (Saitou and Nei 1987), maximum-parsimony (MP) (Fitch 1971) and maximum-likelihood (ML) (Felsenstein 1981) methods implemented with MEGA version 6 (Tamura et al. 2013). Evolutionary distances were computed according to the Juke–Cantor model (Jukes and Cantor 1969). The reliability of each branch was evaluated by bootstrap analysis based on 1000 replications (Felsenstein 1985). The 16S rRNA gene sequences used for the phylogenetic comparisons are shown in the ML phylogenetic tree with their strain designations and accession numbers.

For determination of DNA G+C content, genomic DNA was prepared using the method described by Hopwood et al. (1985). The G+C content was determined using the HPLC method (Mesbah et al. 1989).

## Results and discussion

### Phenotypic characteristics

Morphological features showed that cells of the strain SA4$^T$ are Gram-positive, motile and rod shaped. Ellipsoidal spore were observed to lie subterminally in unswollen sporangia (supplementary Fig. S1). Growth was observed at temperatures ranging from 15 to 40 °C, and pH 5.0–11.0 with 0–8.0 % (w/v) NaCl concentration. Optimum growth was found at 35 °C with pH 8.0 and 3.0 % (w/v) NaCl concentration. No growth was evident below 15 °C or pH 5.0 or above 9.0 % (w/v) NaCl. Strain SA4$^T$, *B. horikoshii* DSM 8719$^T$, *B. Acidicola* DSM 14745$^T$and *B. Shackletonii* LMG 18435$^T$ were found to be negative for nitrate reduction; all strains were found to produce acid from *N*-acetylglucosamine and trehalose, but not from D-arabinose, D-fucose, L-fucose or 5-ketogluconate. However, the Voges–Proskauer test was found to be positive for strain SA4$^T$, distinguishing it from the three reference strains. Other detailed physiological and biochemical characteristics of strain SA4$^T$ in comparison with its close phylogenetic neighbours are presented in Table 1.

### Molecular phylogenetic analysis

An almost complete 16S rRNA gene sequence (1501 bp, GenBank accession number KU324461) of strain SA4$^T$ was determined. Pairwise comparisons indicated SA4$^T$ to be closely related to *B. horikoshii* DSM 8719$^T$ (96.6 %), followed by *B. acidicola* 105-2$^T$ (96.5 %), *B. shackletonii* LMG 18435$^T$ (96.3 %) and *Bacillus pocheonensis* Gsoil 420$^T$ (96.2 %). In the ML tree, phylogenetic analysis placed strain SA4$^T$ in the genus *Bacillus* although its relationships *B. horikoshii* DSM 8719$^T$, *B. acidicola* 105-2$^T$ and others was not supported by high bootstrap values (Fig. 1). The topologies of the phylogenetic trees built using NJ and MP methods also support the conclusion that strain SA4$^T$ forms a stable clade although its relationships with the reference strains *B. horikoshii* DSM 8719$^T$, *B. acidicola* 105-2$^T$ and others remains to be clarified (supplementary Figs.S2 and S3). This analysis suggested that strain SA4$^T$ represents a distinct species within the genus *Bacillus*. The genomic DNA G+C content of strain SA4$^T$ was determined to be

**Table 1** Characteristics used to distinguish strain SA4$^T$ from its close phylogenetic neighbours

| Characteristics | Strain SA4$^T$ | *B. horikoshii* DSM 8719$^T$ | *B. acidicola* DSM 14745$^T$ | *B. shackletonii* LMG 18435$^T$ |
|---|---|---|---|---|
| Temperature (°C) range | 15–40 | 10–40 | 15–45 | 15–55 |
| Optimum | 35 | 30 | 30 | 50–55 |
| pH range | 5.0–11 | 7.0–11 | 4.0–7.0 | 5.0–9.0 |
| Optimum | 8.0 | 8.0 | 5.0 | 7.0 |
| NaCl range | 0–7 % | 0–9 % | 0–2 % | 0–8.5 % |
| Optimum | 3 % | 0 | 0 | 0.5 % |
| Acid production from (using API 50 CH) | | | | |
| Glycerol | + | + | − | − |
| D-Xylose | + | − | + | − |
| Glucose | + | w | + | + |
| Fructose | + | + | + | w |
| Mannose | + | − | + | w |
| D-Mannitol | − | − | − | w |
| Aesculin | − | w | + | + |
| Cellobiose | − | − | − | + |
| Maltose | + | + | + | w |
| Sucrose | + | + | + | − |
| D-Tagatose | − | − | − | w |
| Citrate utilisation | − | − | + | − |
| ONPG hydrolysis | − | + | + | − |
| Gelatin hydrolysis | + | + | w | − |
| Voges–Proskauer test | + | − | − | − |
| Cell-wall composition | *meso*-DAP | *meso*-DAP[a] | *meso*-DAP[b] | *meso*-DAP[c] |
| Quinone | MK-7 | MK-7 | MK-7, MK-6, MK-5, MK-4 and MK-3. | MK-7 |
| Polar lipids | DPG, PE, PG | ND | DPG, PE, PG[b] | ND |
| Major fatty acids | iso-$C_{15:0}$, anteiso-$C_{15:0}$ | iso-$C_{15:0}$, anteiso-$C_{15:0}$ | iso-$C_{15:0}$, anteiso-$C_{15:0}$ | iso-$C_{15:0}$, anteiso-$C_{15:0}$ |
| DNA G+C content (mol %) | 42.4 | 41.1–42.0[a] | 43.2[b] | 36.8[c] |

Data were obtained in this study unless indicated otherwise

+, positive; −, negative; *w* weak, *ND* no data, *PG* phosphatidylglycerol, *DPG* diphosphatidylglycerol, *PE* phosphatidylethanolamine

Data taken from [a] Nielsen et al. (1995); [b] Albert et al. (2005); [c] Logan et al. (2004)

42.4 mol % whereas that of *B. horikoshii* DSM 8719$^T$ is 41.1 mol % (Nielsen et al. 1995).

## Chemotaxonomic characterisation

Analysis of the cell wall peptidoglycan showed that strain SA4$^T$ contains *meso*-diaminopimelic acid as the diagnostic diamino acid. The menaquinone was identified as MK-7. The polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, two aminophospholipids (APL) and seven unidentified phospholipids (supplementary Fig. S4). The major fatty acids present in strain SA4$^T$ (>10 % of the total fatty acids) were identified as anteiso-$C_{15:0}$ (22.3 %), iso-$C_{15:0}$ (22.6 %), iso-$C_{16:0}$ (14.8 %) and iso-$C_{14:0}$ (14.7 %). Marginal quantitative differences were observed in the profile of fatty acids compared with the close neighbour, *B. horikoshii* DSM 8719$^T$ (supplementary Table S1). The fatty acid profile of strain SA4$^T$ was similar to that of the reference strains tested, although there were differences in the

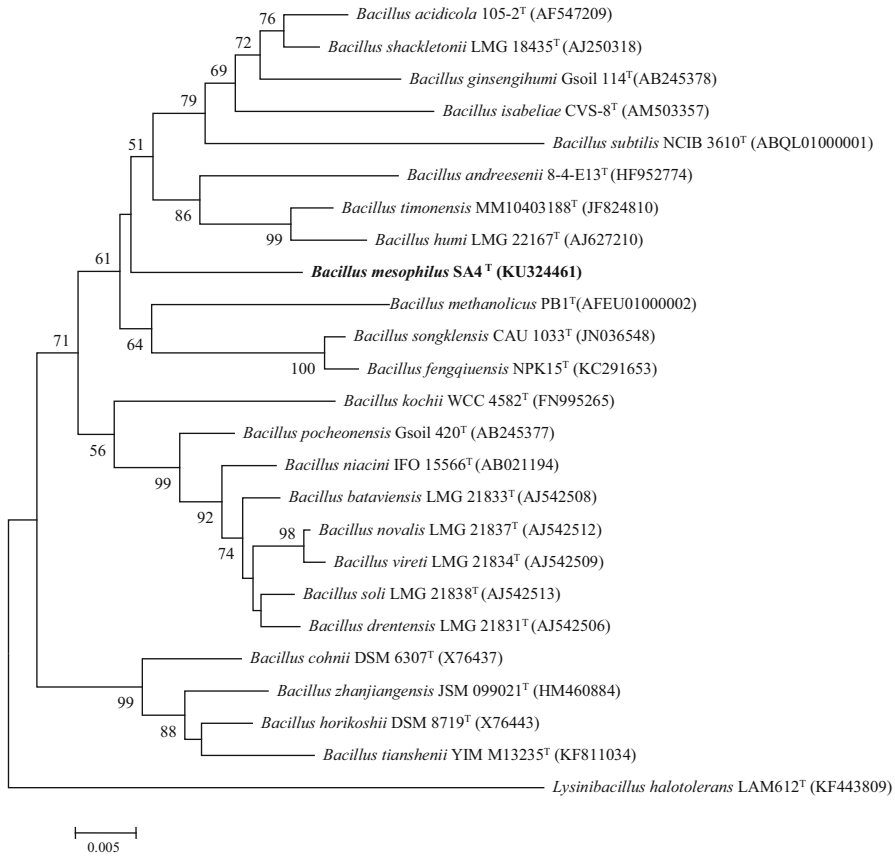


**Fig. 1** Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain SA4$^T$ and closely related species within the genus *Bacillus*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. *Bar*, 0.005 substitutions per site

proportions of some fatty acid components. These data are consistent with reports that iso- and anteiso-branched fatty acids of the 14–17 carbon series are typical of those found in the membranes of members of the genus *Bacillus* (Albert et al. 2005; Kämpfer 1994).

## Taxonomic conclusion

The 16S rRNA gene analysis showed that strain SA4$^T$ has 96.6 % identity with its close relative *B. horikoshii* DSM 8719$^T$, which is below the 98.7 % threshold suggested for DNA:DNA hybridization (Tindall et al. 2010; Meier-Kolthoff et al. 2013; Kim et al. 2014). The phenotypic and chemotaxonomic properties of strain SA4$^T$ distinguish it from *B. horikoshii* DSM 8719$^T$. Based on the present polyphasic analysis, strain SA4$^T$ is considered to represent a novel species within the genus *Bacillus*, for which the name *Bacillus mesophilus* sp. nov. is proposed.

## Description of *Bacillus mesophilus* sp. nov.

*Bacillus mesophilus* (me.so'phi.lus. Gr. adj. *mesos*, middle; Gr. n. *philos*, friend, loving; N.L. masc. adj. *mesophilus,* a mesophilic bacillus).

Cells are Gram-stain positive motile rods (0.5–0.8 × 2.0–5.0 μm) with a polar flagellum, occurring singly or in short chains. Ellipsoidal endospores are located sub-terminally. Colonies grown at 30 °C on MA for 48 h appear circular, pale pink in colour, flat, about 1–2 mm diameter. Growth occurs at 20–40 °C (optimum 35 °C), pH 5.0–11.0 (optimum pH 8.0), with 0–8.0 % (w/v) NaCl concentration (optimum 3.0 %). Aerobic; catalase and oxidase positive but urease negative. $H_2S$ and indole are not produced. Positive for gelatin and alginate hydrolysis, and Voges–Proskauer test. Negative for nitrate reduction, citrate utilisation, β-galactosidase (ONPG), tryptophan deaminase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase. Produces

acid from trehalose, sucrose, maltose, glycerol, D-xylose, glucose, fructose, mannose and *N*-acetylglucosamine, but not from other substrates. The major cellular fatty acids are anteiso-$C_{15:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{14:0}$. The respiratory quinone is MK-7. The DNA G+C content of the type strain is 42.4 mol %.

The type strain, SA4$^T$ (=DSM 101000$^T$=CCTCC AB 2015209$^T$), was isolated from a soil sample collected from a deserted saltworks at Wendeng, Shandong Province, PR China. The GenBank accession number of the 16S rRNA gene sequence of strain SA4$^T$ is KU324461.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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