Bacillus daliensis sp. nov., an alkaliphilic, Gram-positive bacterium isolated from a soda lake

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A Gram-positive, alkaliphilic bacterium, designated strain DLS13^T, was isolated from Dali Lake in Inner Mongolia Autonomous Region, China. The isolate was able to grow at pH 7.5–11.0 (optimum at pH 9), in 0–8 % (w/v) NaCl (optimum at 2 %, w/v) and at 10–45 °C (optimum at 30 °C). Cells of the isolate were facultatively anaerobic, spore-forming rods with peritrichous flagella. The predominant isoprenoid quinone was MK-7 and its cell wall peptidoglycan contained *meso*-diaminopimelic acid. The major polar lipids consisted of phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. The major cellular fatty acids were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{15:0}$. The genomic DNA G+C content of the isolate was 43.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain DLS13^T was a member of the genus *Bacillus* and most closely related to *Bacillus saliphilus* DSM 15402^T (96.9 % similarity). The DNA–DNA relatedness value between strain DLS13^T and *B. saliphilus* DSM 15402^T was 38.7 ± 1.9 %. Comparative analysis of genotypic and phenotypic features indicated that strain DLS13^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus daliensis* sp. nov. is proposed; the type strain is DLS13^T (=CGMCC 1.10369^T=JCM 17097^T=NBRC 107572^T).

Natural alkaline environments, in particular soda lakes, which are distributed worldwide, contain large numbers of alkaliphiles from diverse lineages (Jones *et al.*, 1998; Martins *et al.*, 2001). Alkaliphilic *Bacillus* species, as the major group of alkaliphiles, have attracted much attention with regard to their physiological adaptation to high pH (Padan *et al.*, 2005) and biotechnological potential in the production of hydrolytic enzymes (Horikoshi, 1999). The genus *Bacillus* is phenotypically and phylogenetically heterogeneous. Based on comparative 16S rRNA sequence analysis, Ash *et al.* (1991) and Nielsen *et al.* (1994) divided *Bacillus* species into six rRNA groups, with the majority of alkaliphilic *Bacillus* species assigned to rRNA groups 1 and 6. Their studies have provided a firm basis for the subsequent taxonomic arrangement of the bacilli. In recent years, many novel alkaliphilic *Bacillus* species, e.g. *Bacillus bogoriensis* from Bogoria soda lake in Kenya (Vargas *et al.*, 2005), *Bacillus chagannorensis* from Lake Chagannor in China (Carrasco *et al.*, 2007), *Bacillus aurantiacus* from soda ponds in Kiskunság National Park, Hungary (Borsodi *et al.*, 2008), *Bacillus beveridgei* from Mono Lake in USA (Baesman *et al.*, 2009), *Bacillus marmarensis* from mushroom compost in Turkey (Denizci *et al.*, 2010) and *Bacillus nanhaiisediminis* from the South China Sea (Zhang *et al.*, 2011), have been described. During the course of studying the phylogenetic diversity of Dali Lake (43° 17′ N 116° 38′ E), the alkaliphilic strain DLS13^T was isolated from a water sample and appeared to represent a novel species of the genus *Bacillus*. In this paper, the phenotypic and genotypic characteristics of this alkaliphilic strain are presented.

Dali Lake is located at 1226 m above sea-level in Xilin Gol of Inner Mongolia Autonomous Region, China. It is a typical soda lake with a pH value of 9.6 and salinity of 0.6 %. Strain DLS13^T was obtained by enrichment in Horikoshi medium I (Horikoshi, 1999) at 37 °C followed by serial dilution plating of enrichment cultures on solid Horikoshi medium I until a pure colony was obtained. Strain DLS13^T was maintained in liquid Horikoshi medium I supplemented with 30 % (v/v) glycerol at −80 °C. Horikoshi medium I contained the following: 10 g glucose, 5 g polypeptone, 5 g yeast extract, 1 g K_2HPO_4, 0.2 g $MgSO_4 . 7H_2O$, 50 g NaCl, 900 ml distilled water, to which 100 ml 10 % Na_2CO_3 solution was added aseptically after autoclaving. The pH of Horikoshi medium I was 10.0. *Bacillus saliphilus* DSM 15402^T, *B. chagannorensis* CGMCC 1.6292^T and *Bacillus agaradhaerens* DSM 8721^T were used as reference strains for comparative phenotypic studies. Unless otherwise stated, strains were grown in modified Horikoshi medium I at

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Four supplementary figures are available with the online version of this paper.

30 °C for 20 h. Modified Horikoshi medium I contained the optimal NaCl concentration for each of the respective strains and the following: 10 g glucose, 5 g polypeptone, 5 g yeast extract, 1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 900 ml distilled water, to which 100 ml 3 % Na_2CO_3 solution was added aseptically after autoclaving. The pH of the modified Horikoshi medium I was 9.0. Solid media were prepared by the addition of 2 % agar.

Cellular morphology and motility were examined by phase-contrast and transmission electron microscopies after growth on modified Horikoshi medium I at 30 °C for 24 h. Gram staining was performed as described by Gerhardt *et al.* (1981), in parallel with the KOH lysis method (Gregersen, 1978). Flagella were demonstrated using negative staining and transmission electron microscopy. Endospores were observed by heating cultures at 100 °C for 10 min. Salt requirement for growth was tested by using modified Horikoshi medium I with NaCl concentrations ranging from 0 to 20 % (w/v) at intervals of 1 % (w/v) and at 25 % (w/v) NaCl. The pH range for growth was tested at intervals of 0.5 pH unit in modified Horikoshi medium I adjusted to various pH values after sterilization: pH 7–9 (adjusted by adding $NaHCO_3$), pH 9–11 (adjusted by adding Na_2CO_3) and pH 11–12 (adjusted by adding NaOH) (Nogi *et al.*, 2005). Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, 40, 45 and 50 °C) was determined in modified Horikoshi medium I. General biochemical tests (including the presence of oxidase and catalase, urease activity, H_2S production, nitrate reduction, indole production, methyl red test, Voges–Proskauer reaction, glucose oxidation/fermentation test, and hydrolysis of Tween 20, 40, 60 and 80, aesculin, gelatin, starch and casein) were performed according to previously described methods (Smibert & Krieg, 1981). Susceptibility to antibiotics was tested by spreading bacterial suspensions on agar plates of modified Horikoshi medium I and then placing paper discs (7 mm in diameter and 1 mm in thickness) impregnated with antibiotics on the surface of the plate. After incubation for 48 h, a clear zone of growth inhibition around the disc indicated sensitivity to the antibiotic (Romano *et al.*, 1993). Utilization of carbon sources was determined by growth in modified Horikoshi medium I containing 1 g yeast extract l^{-1} and 1 g polypeptone l^{-1} with a variety of substrates (added at 0.5 %, w/v); controls were grown without addition of any substrate. Growth was monitored by turbidity at OD_{600} and acid production was evaluated by measuring the pH value of cultures with a pH meter. Variation of the pH value by more than 0.5 compared to that of the controls indicated that acids were produced. All tests were carried out in triplicate. Cells of strain DLS13^T were Gram-positive, rod-shaped with peritrichous flagella and formed a terminal, oval endospore (see Supplementary Fig. S1, available in IJSEM Online). Colonies on modified Horikoshi I agar plates with 2 % (w/v) NaCl were yellow and circular after cultivation at 30 °C for 24 h. Growth occurred at pH 7.5–11.0, in NaCl concentrations of 0–8 % (w/v) and at temperatures of 10–45 °C. Other phenotypic properties are presented in the species description and Table 1.

Preparation of the cell wall and determination of peptidoglycan composition were performed as described by Schleifer & Kandler (1972) with the modification that TLC on cellulose sheets was used instead of paper chromatography. Polar lipids were extracted with chloroform/methanol (1 : 2) and identified by two-dimensional TLC followed by spraying with the appropriate detection reagent (Romano *et al.*, 2001). Respiratory quinones were extracted with chloroform/methanol (2 : 1) from dried cells and purified on TLC (Collins *et al.*, 1977). The purified contents were analysed by reverse-phase HPLC (Groth *et al.*, 1996). Cellular fatty acids were analysed according to the standard protocol of the MIDI Microbial Identification system (Sasser, 1990) at the Identification Service of China General Microbiological Culture Collection Center (Beijing, China). Cell mass for analysis of cellular fatty acids was harvested after incubation on modified Horikoshi medium I at 30 °C for 16 h, when cells were in late exponential growth phase. Chromosomal DNA was extracted using a standard method (Marmur, 1961) and the genomic DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962). Strain DLS13^T contained *meso*-diaminopimelic acid as a diagnostic diamino acid in the cell-wall peptidoglycan. The polar lipid extract contained phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine (see Supplementary Fig. S2 in IJSEM Online). The major isoprenoid quinone of strain DLS13^T was MK-7. The cellular fatty acid profile of strain DLS13^T comprised anteiso-$C_{15:0}$ (39.84 %), anteiso-$C_{17:0}$ (13.3 %), iso-$C_{15:0}$ (12.39 %), $C_{16:1}\omega11c$ (8.99 %), $C_{16:1}\omega7c$ (5.11 %), $C_{16:0}$ (5.05 %), iso-$C_{17:0}$ (2.89 %), iso-$C_{16:0}$ (2.25 %), iso-$C_{14:0}$ (2.06 %), $C_{14:0}$ (0.88 %) and iso-$C_{17:1}\omega10c$ (0.78 %). The genomic DNA G+C content of strain DLS13^T was 43.9 mol%. These chemotaxonomic features of strain DLS13^T are typical of those found in members of the genus *Bacillus*.

The 16S rRNA gene of strain DLS13^T was amplified by PCR using the universal primers 27F (5′-AGAGTTTGATCA-TGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCA-GCC-3′) (Baker *et al.*, 2003). The nearly complete nucleotide sequence (1467 bp) was determined by direct sequencing and compared with available 16S rRNA gene sequences in GenBank using the program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identification of phylogenetic neighbours and calculation of levels of pairwise 16S rRNA gene sequence similarity were achieved by using the EzTaxon server 2.1 (http://www.eztaxon.org/; Chun *et al.*, 2007). Multiple alignment with sequences from closely related species was performed by using the program CLUSTAL W in MEGA5 (Tamura *et al.*, 2011). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees were constructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA5 (Tamura *et al.*, 2011). Topologies of the resultant trees were evaluated by

Table 1. Differential characteristics of strain DLS13^T and phylogenetically related *Bacillus* species

Strains: 1, DLS13^T; 2, *B. saliphilus* DSM 15402^T; 3, *B. chagannorensis* CGMCC 1.6292^T; 4, *B. agaradhaerens* DSM 8721^T. All strains are positive for Gram staining, acid production from glucose, fructose, galactose, mannose, sucrose and cellobiose, hydrolysis of aesculin and the Voges–Proskauer reaction. Negative for utilization of erythritol and galactitol, methyl red test, urease activity, and hydrolysis of Tweens 20, 40, 60 and 80. All strains have phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine as the predominant polar lipids, *meso*-diaminopimelic acid as diagnostic diamino acid and MK-7 as the main isoprenoid quinone. Data are from this study. +, Positive; −, negative; w, weak.

Characteristic	1	2	3	4
Cell morphology	Rods	Cocci	Rods	Rods
Colony pigmentation	Yellow	Yellow	Yellow-orange	White
O_2 requirement	Facultively anaerobic	Strictly aerobic	Facultively anaerobic	Facultively anaerobic
Endospore formation	+	−	+	+
Motility	+	−	+	+
Growth temperature (°C):				
Range	10–45	10–50	10–40	10–45
Optimum	30	37	37	30
Growth pH:				
Range	7.5–11.0	7.0–10.0	6.0–11.0	7.5–11.0
Optimum	9	9	8.5	10
NaCl concentration for growth (%, w/v):				
Range	0–8	1–20	3–20	0–15
Optimum	2	15	7	8
Oxidase	+	+	−	−
Catalase	+	w	+	+
Nitrate reduction	−	−	+	−
Indole production	+	+	−	+
H_2S production	+	+	−	−
Hydrolysis of:				
Starch	−	−	−	+
Gelatin	−	+	−	−
Casein	+	−	−	+
Growth on:				
Lactose	+	+	−	+
Maltose	+	+	−	+
L-Arabinose	−	+	−	+
D-Ribose	+	−	−	+
L-Rhamnose	+	+	+	−
D-Xylose	+	−	−	+
D-Raffinose	+	+	−	+
Inulin	+	−	−	+
Salicin	+	−	+	+
Ethanol	−	+	−	−
Inositol	−	+	−	−
D-Mannitol	+	+	−	+
Sorbitol	−	−	−	+
DNA G+C content (mol%)	43.9	48.8	53.8	39.6

bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. A comparative analysis of 16S rRNA gene sequences revealed that strain DLS13^T was a member of the genus *Bacillus* and associated with rRNA group 6 of *Bacillus* (Nielsen *et al.*, 1994) (see Supplementary Fig. S3 in IJSEM Online). The closest phylogenetic relatives were *B. saliphilus* DSM 15402^T (sequence similarity of 96.9 %) and *B. chagannorensis* CGMCC 1.6292^T (sequence similarity of 95.2 %). The phylogenetic tree based on the maximum-likelihood algorithm (Fig. 1) showed that strain DLS13^T formed a phyletic group with *B. saliphilus* DSM 15402^T and *B. chagannorensis* CGMCC 1.6292^T within the genus *Bacillus* and was distantly related to other members of the genus. Similar tree topologies were obtained by the maximum-parsimony method (see Supplementary Fig. S4 in IJSEM Online). DNA–DNA hybridization experiments were carried out in triplicate by the spectrophotometric renaturation rate method (De Ley *et al.*, 1970) and a mean level of relatedness of 38.7 % (with ±1.9 % standard deviation) was obtained, indicating that strain DLS13^T was genotypically distinct from *B. saliphilus* DSM 15402^T.

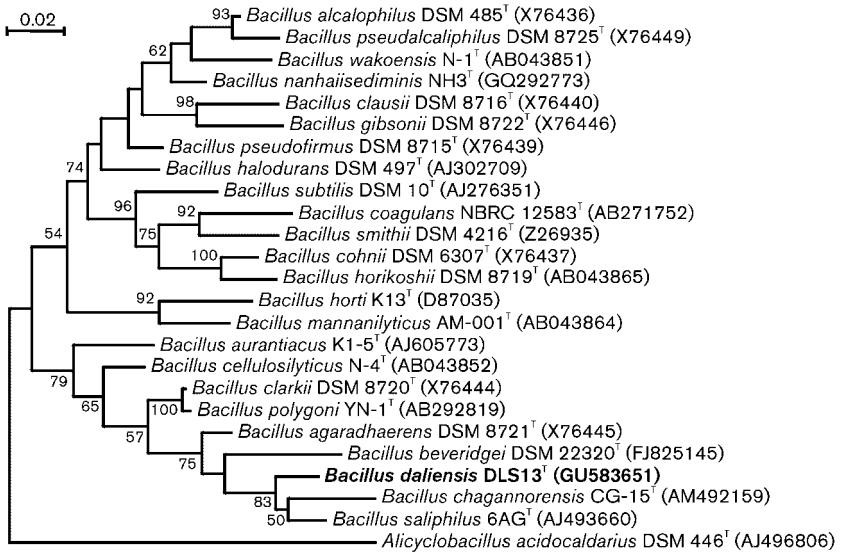

Fig. 1. Phylogenetic tree showing the relationship between strain DLS13^T and closely related species based on 16S rRNA gene sequences. Numbers at nodes represent the levels of bootstrap support (%) based on a maximum-likelihood analysis of 1000 resampled datasets, only values $\geqslant$50 % are given. Nucleotide sequence accession numbers are given in parentheses. Bar, 2 % sequence divergence.

The characteristics that differentiate strain DLS13^T from other related *Bacillus* species are summarized in Table 1. The differences in some features, such as cell morphology, casein hydrolysis, oxidase activity, optimal salt concentration and temperature for growth, as well as the DNA G+C content, can be used to distinguish this strain from phylogenetically related taxa (Table 1). Therefore, on the basis of the taxonomic data presented here, it is suggested that the isolate represents a novel species for which the name *Bacillus daliensis* sp. nov. is proposed.

Description of *Bacillus daliensis* sp. nov.

Bacillus daliensis (da.li.en′sis. N.L. masc. adj. *daliensis* of or belonging to Dali Lake, a soda lake in China).

Cells are Gram-positive rods with round ends, 2.5–3.5 μm in length and 0.4–0.5 μm in width, occurring singly or in pairs and motile by peritrichous flagella. Oval endospores form at terminal positions in swollen sporangia. Colonies are yellow, circular and 4–5 mm in diameter with regular margins after cultivation at 30 °C on modified Horikoshi medium I with 2 % (w/v) NaCl for 24 h. No diffusible pigments are produced. Facultatively anaerobic. Growth occurs between 10 °C and 45 °C, with optimum growth at 30 °C. The pH range for growth is 7.5–11.0, with optimum growth at pH 9.0. Grows in 0–8 % (w/v) NaCl, with optimum growth in 2 % (w/v) NaCl. Positive for hydrolysis of casein and aesculin, oxidase, catalase, H_2S production, indole production and the Voges–Proskauer test, but negative for hydrolysis of starch, gelatin and Tweens 20, 40, 60 and 80, urease activity, methyl red test and nitrate reduction. Grows and produces acids on D-glucose, D-fructose, D-galactose, D-mannose, L-rhamnose, D-xylose, D-ribose, sucrose, maltose, lactose, cellobiose, raffinose, salicin and D-mannitol as sole carbon sources. Glycerol and inulin can be utilized as sole carbon sources but no acid is produced. Unable to utilize L-arabinose, ethanol, inositol, erythritol, galactitol and sorbitol as sole carbon sources. Acids are produced fermentatively from D-glucose. Sensitive to ampicillin (10 μg), azithromycin (15 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), josamycin (15 μg), kanamycin (30 μg), kitasamycin (15 μg), medemycin (15 μg), neomycin (30 μg), nitrofurantoin (300 μg), oxacillin (1 μg), penicillin G (10 U), rifampicin (5 μg), spectinomycin (100 μg), spiramycin (15 μg), tetracycline (30 μg), tobramycin (10 μg) and vancomycin (30 μg). Resistant to bacitracin (10 U), gentamicin (30 μg), novobiocin (5 μg) and streptomycin (10 μg). The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{15:0}$. Phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine are the predominant polar lipids and *meso*-diaminopimelic acid is a diagnostic diamino acid in the cell-wall peptidoglycan. MK-7 is the predominant isoprenoid quinone.

The type strain is DLS13^T (=CGMCC 1.10369^T=JCM 17097^T=NBRC 107572^T), isolated from a sediment sample of Dali Lake in Inner Mongolia Autonomous Region, China. The genomic DNA G+C content of the type strain is 43.9 mol% (T_m).

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