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Bacillus daqingensis sp. nov., a Halophilic, Alkaliphilic Bacterium Isolated from Saline-Sodic Soil in Daqing, China[§]

Shuang Wang[1,2], Lei Sun[2], Dan Wei[2], Baoku Zhou[2], Junzheng Zhang[3], Xuejia Gu[2], Lei Zhang[2], Ying Liu[2], Yidan Li[2], Wei Guo[4], Shuang Jiang[5], Yaqing Pan[6], and Yufeng Wang[2*]

[1]*Heilongjiang Academy of Agricultural Sciences Postdoctoral Programme, Harbin 150086, P. R. China*
[2]*Institute of Soil Fertilizer and Environment Resources, Heilongjiang Academy of Agricultural Sciences/Key Lab of Soil Environment and Plant Nutrition of Heilongjiang Province/Heilongjiang Fertilizer Engineering Research Center/Scientific Observing and Experimental Station of Heilongjiang Farmland Conservation and Agricultural environment, Ministry of Agriculture, Harbin 150086, P. R. China*
[3]*School of Food Science and Engineering, Harbin Institute of Technology, Harbin 150001, P. R. China*
[4]*Institute of rural energy, Heilongjiang Academy of Agricultural Sciences, Harbin 150086, P. R. China*
[5]*Editorial Department of Journal of Northeast Agricultural University, Harbin 150030, P. R. China*
[6]*Pesticides Center, Heilongjiang Academy of Agricultural Sciences, Harbin 150086, P. R. China*

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An alkaliphilic, moderately halophilic, bacterium, designated strain X10-1^T, was isolated from saline-alkaline soil in Daqing, Heilongjiang Province, China. Strain X10-1^T was determined to be a Gram-positive aerobe with rod-shaped cells. The isolate was catalase-positive, oxidase-negative, non-motile, and capable of growth at salinities of 0–16% (w/v) NaCl (optimum, 3%). The pH range for growth was 7.5–11.0 (optimum, pH 10.0). The genomic DNA G+C content was 47.7 mol%. Its major isoprenoid quinone was MK-7 and its cellular fatty acid profile mainly consisted of anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$, $C_{16:0}$, and iso-$C_{16:0}$. The peptidoglycan contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The predominant polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylglycerol. Phylogenetic analysis based on 16S rRNA gene sequences showed that X10-1^T is a member of the genus *Bacillus*, being most closely related to *B. saliphilus* DSM 15402^T (97.8% similarity) and *B. agaradhaerens* DSM 8721^T (96.2%). DNA-DNA relatedness to the type strains of these species was less than 40%. On the basis of the phylogenetic, physiological, and biochemical data, strain X10-1^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus daqingensis* sp. nov. is proposed. The type strain is X10-1^T (=NBRC 109404^T =CGMCC 1.12295^T).

*For correspondence. E-mail: wangshuang0726@163.com
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Introduction

Since the aerobic, endospore-forming, obligately alkaliphilic bacterium *Bacillus alcalophilus* was first isolated by Vedder in 1934, many alkaliphilic *Bacillus* species have been described from different habitats. They distribute in not only common soil, sea, and feces (Cho *et al*., 2008) but also specific environments such as hot spa (Li *et al*., 2002), deserts (Zhang *et al*., 2011b), clinical samples, insect gut (Yumoto *et al*., 2011), and even spacecraft-assembly facilities (Vaz-Moreira *et al*., 2012). The different environmental adaptation mechanisms of various alkaliphilic *Bacillus* strains could be indicated by research of cytochrome content (Yumoto *et al*., 1997), lipid composition (Clejan *et al*., 1986) and cell wall composition (Aono and Horikoshi, 1983). Therefore, the genus *Bacillus* is considered a metabolically and ecologically diverse group. Numerous alkaliphiles of the genus *Bacillus* have been isolated not only from alkaline soils but also form soils of neutral pH (Horikoshi, 2006; Yumoto, 2007). At the time of writing, more than 20 alkaliphilic *Bacillus* species have been identified (Zhang *et al*., 2011a), which were isolated from various alkaline environments. These *Bacillus* strains are the focus of much research to investigate their physiological adaptation to high pH, and to perhaps utilize their enzymes for industrial purposes (Horikoshi, 1971, 1999; Yamamoto *et al*., 1972; Hayashi *et al*., 1988).

While screening halophilic bacteria and archaea in a soda meadow saline soil from Daqing, China (Wang *et al*., 2010, 2011), an aerobic, Gram-positive, moderately halophilic, yellow colony-forming bacterium designated strain X10-1^T was isolated. In the current study we carried out phenotypic, genotypic, chemotaxonomic, and phylogenetic analyses of strain X10-1^T, and found that it was affiliated with the genus *Bacillus* and that this isolate represents a novel species of this genus.

Materials and Methods

Isolation of strains and culture conditions

Strain X10-1^T was isolated from saline-alkaline soil in Daqing, China (46°34′N 125°07′E). The isolation procedure was the same as that described previously (Wang *et al*., 2010, 2011), using modified S-G agar medium (Sehgal and Gibbons, 1960) containg 10% NaCl (w/v). DSM medium 752 (DSMZ, http://www.dsmz.de) was used for cultivation and maintenance

of the strain. Unless otherwise indicated, morphological and physiological characterization of strains was performed on DSM medium 752. Strain X10-1^T was preserved at -70°C in a glycerol suspension (20%, v/v). This organism was then deposited to the China General Microbiological Culture Collection (=CGMCC 1.12295^T) and the National Institute of Technology and Evaluation Biological Resource Center, Japan (=NBRC 109404^T).

Phenotypic and biochemical characteristics

Cellular morphology was examined by optical microscope (Olympus BX5-1), and transmission electron microscopy (Hitachi H-7650) was performed with negative staining according to Bouchotroch *et al*. (2001). Gram staining was performed according to Dussault (1955) and motility was assessed using semi-solid agar. The presence of endospores was investigated using the Schaeffer-Fulton staining method (Murray *et al*., 1994). Growth under various NaCl, pH, and temperature regimens was determined in liquid DSM medium 752. Growth was monitored as changes in OD_{600}. Catalase activity was detected by the production of bubbles after the addition of a drop of 3% (v/v) H_2O_2. Oxidase activities, nitrate and nitrite reduction, hydrolysis of starch, gelatin, casein and aesculin, production of indole and H_2S, methyl red and Voges-Proskauer tests were performed as recommended by Smibert and Krieg (1994) using media supplemented with 3.0% NaCl. Hydrolysis of Tween 20, 40, 60 or 80 was examined as described by Harrigan and McCance (1976). Utilization of different compounds as sole carbon and energy sources and determination of acid production from carbohydrates was examined according to the method of Ventosa *et al*. (1982) using medium containing 1% substrate in the basal medium. Resistance to antibiotics was examined by the agar-diffusion method using antibiotic-impregnated discs as described by Buczolits *et al*. (2002). Other tests (shown in Table 1 or included in the species description) were performed as described previously (Wang *et al*., 2010, 2011).

Chemotaxonomic characterization

Isolate X10-1^T and the type strains *Bacillus agaradhaerens* DSM 8721^T and *Bacillus saliphilus* DSM 15402^T were cultivated on DSM 752 agar (pH 9.5) for 2 days at 35°C. Fatty acids of strain X10-1^T and the type strains were extracted and analyzed according to the standard protocol of the

Table 1. Phenotypic properties of strain X10-1^T and type strains of closely related *Bacillus* species

Strains: 1, X10-1^T; 2, *B. saliphilus* DSM 15402^T; 3, *B. agaradhaerens* DSM 8721^T; 4, *B. chagannorensis* CGMCC 1.6292^T. +, Positive or present; –, negative or absent; W, weak reaction. All three strains are positive for nitrate reduction, resistant to neomycin (30 μg/disc), but susceptible to erythromycin (15 μg/disc) and chloramphenicol (30 μg/disc). Data were obtained in this study unless indicated otherwise.

Characteristic	1	2	3	4
Colony colour	Yellow	Yellow	White	Yellow-orange
Cell size (μm)	0.4–0.6×1.0–2.0	0.8–0.9	0.4–0.8×1.0–2.7	0.6–0.7×2.0–3.0
Cell morphology	Rods	Cocci	Rods	Rods
Spore formation	–	–	+	+
Motility	–	–	+	+
Oxidase	–	+	–	–
Catalase	+	W	+	–
Urease	–	+	–	–
DNAse	–	–	–	+
Hydrolysis of:				
Casein	+	–	–	–
Gelatin	+	+	–	–
Starch	+	–	+	–
NaCl concentration for growth (% w/v)				
Minimum	0	>0	0.5	3
Optimum	3	16	5	7
Maximum	16	25	16	20
pH for growth:				
Range	7.5–11.0	7.0–10.0	>7.0	5.8–11.0
Optimum	10.0	9.0	10.0	8.5
Acid production from:				
D-Glucose	+	–	+	–
D-Fructose	–	–	+	–
D-Mannitol	+	–	+	–
Melibiose	+	–	–	–
L-Arabinose	+	–	–	–
Inositol	+	–	–	–
Susceptible to (per disc)				
Novobiocin (30 μg)	–	+	+	–
Tetracycline (30 μg)	–	–	+	–
Streptomycin (10 μg)	–	+	–	–
Penicillin G (10 IU)	+	–	+	–
Kanamycin (30 μg)	+	–	+	–
Bacitracin (0.04 IU)	+	+	–	+
DNA G+C content (mol%)	47.7	48.4[a]	39.5[b]	53.8[c]

[a] Data taken from R omano *et al*. (2005), [b] Data taken from Nielsen *et al*. (1995), [c] Data taken from Carrasco *et al*. (2007).

Microbial Identification System (Microbial ID Inc.) (Sasser, 1990; Kämpfer and Kroppenstedt, 1996). Isoprenoid quinones were extracted and purified according to Collins *et al.* (1987), and purified menaquinones were identified by reverse-phase high performance liquid chromatography (Wu *et al.*, 1989). Polar lipids were extracted as described by Minnikin *et al.* (1984) and were then identified by two-dimensional thin layer chromatography. The cell-wall diamino acid type of strain X10-1^T was determined from whole-cell hydrolysates as described by Hasegawa *et al.* (1983).

Molecular characterization

Genomic DNA from strian X10-1^T was isolated using the method described by Marmur (1961). The G+C content of the DNA was determined by means of the thermal denaturation method (Marmur and Doty, 1962) with DNA from *Escherichia coli* AS 1.365 as a reference.

To determine the taxonomic status of the isolate, the 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R according to a previously published protocol (Reysenbach *et al.*, 2000). The PCR product was ligated to the T-vector and transformed into *E. coli* Top 10 for purification and sequencing. The 16S rRNA gene sequences of species closely related to strain X10-1^T were retrieved from the GenBank database using the BLAST program (http:/www.ncbi.nlm.nih.gov/blast/) to determine an approximate phylogenetic affiliation. Phylogenetic analysis was performed using MEGA version 5 (Tamura *et al.*, 2011) and the PHYLIP software package (Felsenstein, 2005) after multiple alignments of the data were performed using CLUSTAL_X version 2 software (Larkin *et al.*, 2007). Evolutionary distances were computed using distance options according to Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were inferred using the neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Eck and Dayhoff, 1966; Fitch, 1971), and minimum-evolution methods (Rzhetsky and Nei, 1992). The resultant tree topology was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

DNA-DNA hybridization

DNA-DNA hybridization experiments between strain X10-1^T and its nearest phylogenetic neighbours were performed using the thermal denaturation and renaturation method of De Ley *et al.* (1970), modified by Huß *et al.* (1983). On the basis of 16S rRNA gene sequence similarity, *B. saliphilus* DSM 15402^T and *B. agaradhaerens* DSM 8721^T were chosen for DNA-DNA hybridization experiments.

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain X10-1^T is HM598403.

Results and Discussion

Morphology and physiological characteristics

Cells of strain X10-1^T were Gram-positive, rod-shaped (0.4–0.6 × 1.0–2.0 μm), and non-motile. Endospores were not observed, as with some other bacilli such as *Bacillus beijingensis*, *Bacillus ginsengi* (Qiu *et al.*, 2009), *Bacillus foraminis* (Tiago *et al.*, 2006), and *Bacillus thermoamylovorans* (Combet-Blanc *et al.*, 1995). Colonies were smooth, circular, slightly raised, and yellow after 48 h of cultivation at 35°C on DSM medium 752. Strain X10-1^T grew in DSM medium 752 supplemented with 0–16% (w/v) NaCl, and optimum growth occurred at 3% NaCl. Growth was observed at temperatures of 10–50°C, with optimal growth occurring at 35°C. The optimal pH was10.0 (range pH 7.5–11.0), which confirmed that strain X10-1^T is an alkaliphilic bacterium. The growth and phenotypic characteristics that serve to distinguish strain X10-1^T from the type strains of closely related *Bacillus* species are shown in Table 1. Strain X10-1^T could be clearly distinguished phenotypically from *B. saliphilus* DSM 15402^T by cellular morphology, optimum NaCl concentration, pH for growth, and its ability to hydrolyze starch and casein, and could be distinguished from *B. agaradhaerens* DSM 8721^T and *B. chagannorensis* CGMCC 1.6292^T by spore formation, motility, and its inability to hydrolyze starch, gelatin, and casein.

Table 2. Cellular fatty acid content (%) of strain X10-1^T and type strains of closely related *Bacillus* species

Strains: 1, X10-1^T; 2, *B. saliphilus* DSM 15402^T; 3, *B. agaradhaerens* DSM 8721^T; 4, *B. chagannorensis* CGMCC 1.6292^T.

Fatty acid	1	2	3	4
$C_{12:0}$	–	–	3.40	–
iso-$C_{14:0}$	2.22	2.97	2.68	3.03
$C_{14:0}$	1.08	0.62	–	0.75
$C_{14:0}$ 2-OH	–	0.55	–	–
iso-$C_{15:0}$	15.35	5.23	21.21	15.66
anteiso-$C_{15:0}$	36.35	61.20	34.54	43.72
iso-$C_{16:0}$	7.29	6.62	2.40	10.39
$C_{16:1}\omega 11c$	–	–	2.62	–
$C_{16:0}$	12.32	3.41	19.38	4.91
iso-$C_{17:0}$	5.27	1.27	4.21	3.00
anteiso-$C_{17:0}$	16.90	15.55	6.42	13.49
anteiso-$C_{17:1}$ A	–	0.67	–	1.34
$C_{18:0}$	2.23	–	3.15	–
Summed Feature 3	0.98	1.53	–	2.69

All data are based on the result of this study. Values are percentages of total fatty acid and lower than 0.5% are not shown; –, not detected. Summed features represent groups of two or three fatty acids that could not be separated by GLC (gas-liquid chromatography) with the MIDI system; summed feature 3 contains $C_{16:1}\ \omega 7c$ and/or $C_{16:1}\ \omega 6c$.

Cellular fatty acids and isoprenoid quinones

The cellular fatty acid profiles of strain X10-1^T and related species are shown in Table 2. Strain X10-1^T could also be distinguished from the reference strains on the basis of differences in the fatty acid composition, mainly because of the proportions of anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{16:0}$. These branched compounds are typically the major fatty acids found in *Bacillus* cell membranes (Kämpfer, 1994; Albert *et al.*, 2005). The fatty acid composition of the novel isolate was similar to those found in *B. saliphilus* DSM 15402^T, *B. agaradhaerens* DSM 8721^T, and *B. chagannorensis* CGMCC 1.6292^T, but there were differences in proportion. MK-7 was the major menaquinone component, which was

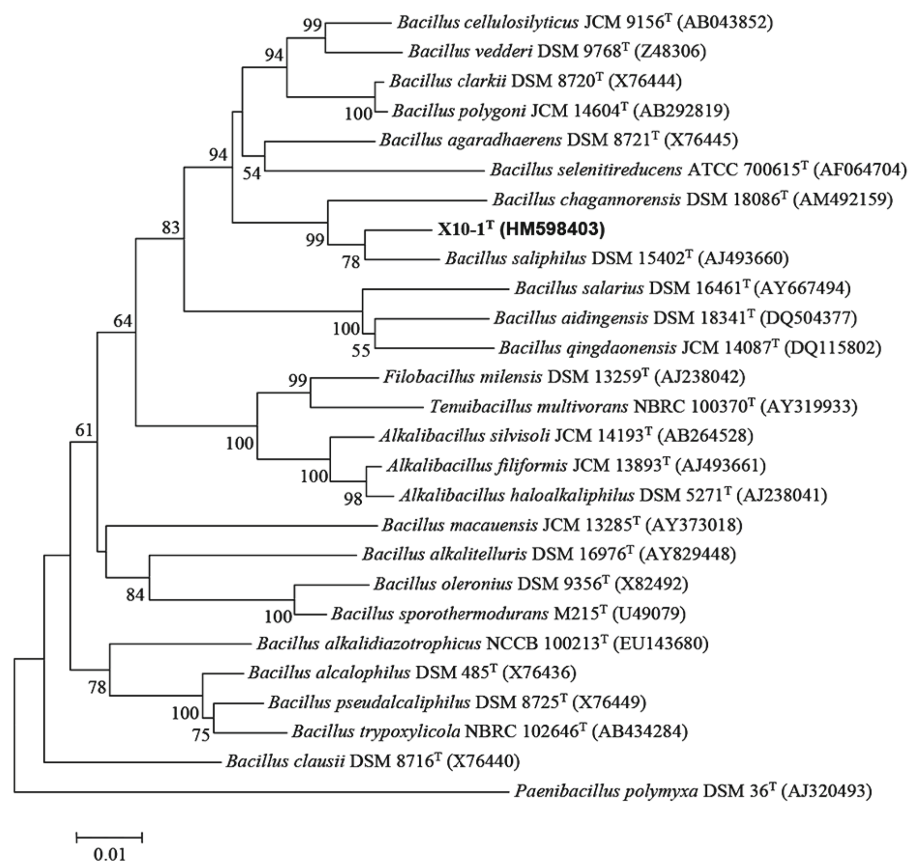

Fig. 1. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain X10-1^T and related taxa. Bootstrap values (%) are based on 1,000 replicates and are shown for branches with more than 50% bootstrap support. Bar, 0.01 changes per nucleotide position.

consistent with the description of members of the genus *Bacillus* (Claus and Berkeley, 1986). The polar lipid profile of strain X10-1^T was composed of the major amount of diphosphatidylglycerol, phosphatidylethanolamine, and phosphatidylglycerol, and minor ones of an unknown aminolipid and four unknown lipids. Strain X10-1^T contained *meso*-diaminopimelic acid as its diagnostic diamino acid.

G+C content and phylogenetic analysis

The G+C content of the X10-1^T genomic DNA was 47.7 mol%. A partial 16S rRNA gene sequence (1,569 bp) was obtained from strain X10-1^T. Comparative 16S rRNA gene sequence analysis confirmed that strain X10-1^T was most closely related to members of the genus *Bacillus*. Strain X10-1^T exhibited the highest 16S rRNA gene sequence similarities to *B. saliphilus* DSM 15402^T (97.8%), *B. agaradhaerens* DSM 8721^T (96.2%), and *B. chagannorensis* CGMCC 1.6292^T (95.5%). The 16S rRNA gene sequence similarities to the type strains of all other *Bacillus* species with validly published names were below 95%. In the phylogenetic tree based on the neighbour-joining algorithm, strain X10-1^T was placed in a cluster within the genus *Bacillus* (Fig. 1). The topologies of the phylogenetic trees generated using the maximum-parsimony and minimum-evolution algorithms (Supplementary data Fig. S1) differed from that of the neighbour-joining method.

DNA-DNA hybridization

Low DNA-DNA relatedness was observed between strain X10-1^T and its closest relatives: 38.6% with *B. saliphilus* DSM 15402^T, and 22.6% with *B. agaradhaerens* DSM 8721^T. These values were clearly below the threshold of 70% DNA-DNA relatedness recommended for the delineation of bacterial species (Wayne *et al.*, 1987). These data suggest that strain X10-1^T represents a novel species within the genus *Bacillus*.

Taxonomic conclusion

The phylogenetic and chemotaxonomic analyses suggested that strain X10-1^T belonged to the genus *Bacillus*. However, phenotypic features, phylogenetic analysis, DNA base composition and DNA-DNA relatedness clearly indicated that strain X10-1^T could be distinguished from other *Bacillus* species. Based on the consensus of results mentioned above, we concluded that strain X10-1^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus daqingensis* sp. nov. is proposed.

Description of *Bacillus daqingensis* sp. nov.

Bacillus daqingensis (da.qing.en'sis. N.L. fem. adj. *daqingensis* pertaining to Daqing, north-east China, where the type strain was isolated).

Cells are Gram-positive straight rods, approximately 0.4–

0.6 μm wide and 1.0–2.0 μm long, non-motile, and non-sporulating. Colonies are circular and yellow. Cells are strictly aerobic and heterotrophic. Growth occurs at temperatures from 10–50°C (optimally at 35°C) and at NaCl concentrations of 0–16% (w/v) (optimally at 3%). pH range for growth is 7.5–11.0 (optimum, pH 10.0). Cultures are positive for catalase, and methyl red and Voges-Proskauer tests, and negative for oxidase, indole production, deamination of phenylalanine, and H_2S production. Nitrate is reduced to nitrite but nitrite reduction is negative. Cells produce β-galactosidase, arginine dihydrolase, lysine, and ornithine decarboxylase, but do not produce urease or tryptophan deaminase. Aesculin, casein, gelatin, starch, and lecithin are hydrolyzed, but *B. daqingensis* does not hydrolyze Tweens 20–80. The following substances are utilized as sole carbon and energy sources: glucose, sucrose, D-trehalose, arabinose, melibiose, mannitol, inositol, and amygdalin. Acid is produced from D-glucose, D-mannitol, L-arabinose, melibiose and inositol, but not from sucrose, D-fructose or D-sorbitol. Cells are sensitive to the following antibiotics (μg per disc, unless indicated otherwise): penicillin G (10 IU per disc), kanamycin (30), chloramphenicol (30), erythromycin (15), ampicillin(10), vancomycin (30) and bacitracin (0.04 IU per disc). Cells are resistant to novobiocin (30), tetracycline (30), streptomycin (10), neomycin (30), and gentamycin (10). The major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$, $C_{16:0}$, and iso-$C_{16:0}$. The detailed fatty acid profile is listed in Table 2. Polar lipids are diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. The predominant respiratory quinine is MK-7 and DNA G+C content is 47.7 mol%.

The type strain is X10-1^T (=CGMCC 1.12295^T =NBRC 109404^T), which was isolated from soda meadow saline soil in Daqing City, Heilongjiang Province of China.

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