Bacillus chagannorensis sp. nov., a moderate halophile from a soda lake in Inner Mongolia, China

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A Gram-positive, moderately halophilic, spore-forming bacterium, designated strain CG-15^T, was isolated from a soda lake, Lake Chagannor, in the Inner Mongolia Autonomous Region, China. The cells were found to be motile short rods with ellipsoidal, terminal and deforming endospores. Strain CG-15^T, a facultatively anaerobic bacterium, grew at pH 5.8–11.0 (optimally at pH 8.5), at 6–40 °C (optimally at 37 °C) and at salinities of 3–20 % (w/v) total salts (optimally at 7 % w/v). On the basis of the results of 16S rRNA gene sequence analysis, strain CG-15^T was shown to belong to the genus *Bacillus* (phylum *Firmicutes*), showing the greatest phylogenetic similarity with respect to *Bacillus saliphilus* (96.0 %). The DNA G + C content of the novel isolate was found to be 53.8 mol%. The major cellular fatty acids of strain CG-15^T were anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0}, and its polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three different unidentified phospholipids. The analysis of the quinones showed that MK-7 was the major menaquinone. The peptidoglycan type was A1γ, with meso-diaminopimelic acid as the diagnostic diamino acid. On the basis of the data from this polyphasic study, strain CG-15^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus chagannorensis* sp. nov. is proposed. The type strain is CG-15^T (=CCM 7371^T=CECT 7153^T=CGMCC 1.6292^T=DSM 18086^T).

Moderately halophilic bacteria that grow optimally in media containing 3–15 % (w/v) salts are widely distributed in hypersaline habitats. These organisms are of considerable interest because of their biotechnological potential in the production of compatible solutes and/or hydrolytic enzymes (Ventosa *et al.*, 1998). Moderate halophiles constitute a very heterogeneous physiological group that includes both Gram-positive and Gram-negative microorganisms. Aerobic, spore-forming, moderately halophilic, Gram-positive rods are also taxonomically diverse and have been isolated from saline environments such as soils

and aquatic habitats (Arahal & Ventosa, 2002). The introduction of molecular methods, especially the use of 16S rRNA gene sequencing, has had a major impact on *Bacillus* taxonomy and has resulted in a splitting up of the genus. Thus, in recent years some species have been reclassified as members of novel genera or transferred to other genera. The genus *Bacillus* includes a large number of species and contains at least six phylogenetically distinct groups on the basis of molecular analyses of 16S rRNA gene sequences (Ash *et al.*, 1991; Spring *et al.*, 1996; Schlesner *et al.*, 2001). In nature, salinity is often associated with alkalinity. Alkaline saline lakes represent a unique habitat with a high pH and a variable (up to saturation) salt concentration. In this paper, we describe a novel alkali-tolerant, moderately halophilic, Gram-positive bacterium, strain CG-15^T, isolated from Lake Chagannor, an alkaline saline lake in Inner Mongolia, China.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CG-15^T is AM492159.

A phase-contrast photomicrograph of cells of strain CG-15^T is available with the online version of this paper.

Sampling was carried out during an expedition to Lake Chagannor (43° 21' N 113° 08' E) in September 2003. At the time of sampling, the temperature of the water was 17 °C, the pH was 9.5 and the conductivity was 21.3 mS cm⁻¹. Strain CG-15^T was isolated by diluting a water sample in sterile 10 % (w/v) salt solution, plating this on alkaline saline medium and incubating it aerobically at 37 °C. The alkaline saline isolation medium contained the following (g l⁻¹): glucose, 10.0; peptone (Difco), 5.0; yeast extract (Difco), 5.0; KH₂PO₄, 1.0; MgSO₄·7H₂O, 0.2; NaCl, 80; Na₂CO₃, 20; and agar, 20. The salts NaCl and Na₂CO₃ were autoclaved separately and added to the organic components at 60 °C (Duckworth *et al.*, 1996). The pH of this medium was adjusted to 9.0. The strain was subsequently purified three times by plating it on the same medium. The strain was maintained on the same alkaline saline medium and at -80 °C on this medium without agar and supplemented with 30 % (v/v) glycerol.

In order to characterize strain CG-15^T phenotypically, standard phenotypic tests were performed. The Gram-stain reaction was carried out using the method described by Dussault (1955). To determine cellular morphology and motility, a culture from liquid, alkaline saline medium was examined by light microscopy under a phase-contrast microscope. The morphology, size and pigmentation of colonies were observed on solid, alkaline saline medium at

various salt concentrations after 2 days incubation. Growth at different concentrations of salts was determined on isolation medium containing 0, 0.5, 1, 3, 5, 7, 10, 15, 20, 25 or 30 % (w/v) total salts. The pH range for growth was determined on liquid, alkaline saline medium at pH values ranging from 5.0 to 11.5, using the biological buffers Na₂HPO₄/NaH₂PO₄ (below pH 8.0), Na₂CO₃/NaHCO₃ (pH 8.0–10.0) and Na₂HPO₄/NaOH (pH 11), as described by Gomori (1955). The pH was readjusted after sterilization; growth was scored as an optical density at 600 nm. The temperature range for growth was determined at temperatures between 6 and 45 °C. Catalase activity was tested by adding 3 % H₂O₂ to culture plates. The oxidase reaction was performed on filter paper moistened with a 1 % (w/v) aqueous solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. Other tests (shown in Table 1 or included in the species description) were carried out according to methodologies described previously (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; García *et al.*, 1987).

Strain CG-15^T was Gram-positive, motile and facultatively anaerobic. The cells were rods 0.6–0.7 µm wide and 2.0–3.0 µm long and formed short, curved chains (see Supplementary Fig. S1, available in IJSEM Online). Ellipsoidal endospores were formed at the terminal position in swollen sporangia, as is the case in the phylogenetically closely related *Bacillus* species. When

Table 1. Characteristics used to distinguish strain CG-15^T from phylogenetically related *Bacillus* species

Strains: 1, CG-15^T (*Bacillus chagannorensis* sp. nov.; data from this study); 2, *B. saliphilus* DSM 15402^T (Romano *et al.*, 2005); 3, *B. agaradhaerens* DSM 8721^T (Nielsen *et al.*, 1995; Lim *et al.*, 2006a, c); 4, *B. clarkii* DSM 8720^T (Nielsen *et al.*, 1995; Lim *et al.*, 2006a, c). +, Positive; –, negative; w, weak; ND, no data available.

Characteristic	1	2	3	4
Cell morphology	Short rods	Cocci	Rods	Rods
Colony pigmentation	Yellow–orange	Yellow	White	Cream-white to dark yellow
Endospore formation	+	–	+	+
Motility	+	–	+	ND
Oxidase	–	+	–	–
Catalase	+	w	+	+
Hydrolysis of:				
Gelatin	–	+	–	+
Casein	–	–	–	+
Starch	–	–	+	–
Phenylalanine deamination	+	–	–	–
NaCl range (% w/v)	3–20	1–25	0.5–16	0.5–16
Optimum NaCl conc. (% w/v)	7	16	ND	ND
Temperature range (°C)	6–40	4–50	10–45	15–45
Optimum temperature (°C)	37	37	30	30
pH range	5.8–11.0	7–10	>7.0	>7.0
Optimum pH	8.5	9.0	≥10	>10.0
DNA G+C content (mol%)	53.8	48.4	39.2	42.4
Major fatty acids	anteiso-C _{15:0} (40 %), iso-C _{15:0} (21 %), anteiso-C _{17:0} (11 %)	anteiso-C _{15:0} (89 %), iso-C _{15:0} , C _{17:0}	anteiso-C _{15:0} (40 %), iso-C _{15:0} (25 %)	anteiso-C _{15:0} (36 %), iso-C _{15:0} (18 %), iso-C _{16:0} (11 %)

grown for 2 days at 37 °C on the alkaline saline medium, the colonies produced were circular with entire margins, yellow–orange in colour and 1 mm in diameter. This isolate was moderately halophilic and alkali-tolerant, growing in media containing 3–20% (w/v) salts and optimally in media containing 7% (w/v) salts. No growth was observed in the absence of NaCl. Strain CG-15^T grew at pH 5.75–11.0 (optimally in media at pH 8.5). Other phenotypic features are included in the species description.

Genomic DNA from strain CG-15^T was prepared using the method described by Marmur (1961). The 16S rRNA gene was amplified by a PCR with forward primer 16F27 and reverse primer 16R1488. Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (model 3100; Applied Biosystems). The 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig *et al.*, 2004). The 16S rRNA gene sequence was aligned with the published sequences of closely related bacteria and the alignment was then confirmed and checked against both primary and secondary structures of the 16S rRNA molecule (using the alignment tool of the ARB software package). Phylogenetic trees were constructed using three different algorithms within the ARB software for phylogenetic inference: maximum likelihood (Felsenstein, 1981), maximum parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987). The 16S rRNA gene

sequences used for the phylogenetic comparisons were obtained from the GenBank database, and their strain designations and accession numbers are shown in Fig. 1.

An almost-complete 16S rRNA gene sequence (1467 bp) was obtained for strain CG-15^T and was used for initial BLAST searches in GenBank and for phylogenetic analysis. No sequences available in the public databases exhibited more than 96.0% similarity with the sequence of strain CG-15^T. A comparative analysis of 16S rRNA gene sequences showed that our isolate was a member of the phyletic group classically defined as the genus *Bacillus* and was associated with 'Bacillus group 1' (Ash *et al.*, 1991; Schlesner *et al.*, 2001). The phylogenetic analysis, based on the maximum-parsimony algorithm, revealed that strain CG-15^T formed a phyletic group with *Bacillus saliphilus* DSM 15402^T (96.0% sequence similarity), *Bacillus agaradhaerens* DSM 8721^T (94.0%) and *Bacillus clarkii* DSM 8720^T (93.5%) (Fig. 1). Neighbour-joining, maximum-likelihood and distance methods resulted in highly similar tree topologies, so only the maximum-parsimony results are presented. No other known bacteria shared more than 93% sequence similarity with the isolate. Very recently, a novel species of the genus *Bacillus*, *Bacillus salarii*, was described by Lim *et al.* (2006c). This species, also isolated from a salt lake in China, is phylogenetically related to *B. agaradhaerens* DSM 8721^T, *Bacillus vedderi* DSM 9768^T and *B. clarkii* DSM 8720^T, but shares very low 16S rRNA gene

Fig. 1. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain CG-15^T and related species. Accession numbers for the sequences used in this study are shown in parentheses. *Sporosarcina ureae* DSM 2281^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

sequence similarity (less than 93 %) with them. The 16S rRNA sequence similarity between strain CG-15^T and *B. salarius* is 90.9 %, clearly indicating that they are not related organisms. On the basis of the sequence divergence, it was evident that strain CG-15^T constituted a different taxon with respect to recognized *Bacillus* species. DNA–DNA hybridization between strain CG-15^T and the nearest phylogenetic neighbours was not attempted because strains with 16S rRNA gene sequences that differ by more than 3 % are unlikely to exhibit more than 70 % relatedness at whole-genome level (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002; Stackebrandt & Ebers, 2006).

The G + C content of the genomic DNA was determined from the midpoint value of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979). The G + C content of the DNA of strain CG-15^T was 53.8 mol%. This value is within the range for the genus *Bacillus* but is higher than those of *B. saliphilus*, *B. agaradhaerens* and *B. clarkii* (Table 1).

A fatty acid analysis was performed using MIDI (Microbial Identification System). Cells were cultured on the alkaline saline medium at 37 °C for 24 h; the analysis was carried out by the Identification Service of the Belgian Co-ordinated Collections of Micro-organisms, Laboratory of Microbiology of Ghent (Ghent, Belgium). The cellular fatty acid profile of strain CG-15^T was characterized by the presence of branched fatty acids anteiso-C_{15:0} (39.7 %), iso-C_{15:0} (21.0 %) and anteiso-C_{17:0} (10.8 %) as the major fatty acids; these branched fatty acids are typical of the fatty acids found in the cell membranes of *Bacillus* species (Albert *et al.*, 2005). In order to complete the chemotaxonomic characterization of strain CG-15^T, the analysis of polar lipids, quinones and peptidoglycan in the cell wall was carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three different phospholipids of unknown structure. Strain CG-15^T contained MK-7 as the major menaquinone and possessed a cell wall type based on meso-diaminopimelic acid. The major respiratory lipoquinone, the polar lipids and the peptidoglycan type of the cell wall of strain CG-15^T were typical of those found in members of the genus *Bacillus* (Priest *et al.*, 1988; Heyrman *et al.*, 2004, 2005; Wieser *et al.*, 2005; Lim *et al.*, 2006a, b).

The characteristics that differentiate strain CG-15^T from other related *Bacillus* species are summarized in Table 1. The differences in some features, such as cell morphology, range and optimal salt concentration for growth and optimal pH for growth, as well as the genomic DNA G + C content or fatty acid composition, can be used to distinguish this strain from phylogenetically related taxa (Table 1). Therefore, on the basis of the taxonomic data from this polyphasic study, strain CG-15^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus chagannorensis* sp. nov. is proposed.

Description of *Bacillus chagannorensis* sp. nov.

Bacillus chagannorensis (cha.gan.no.ren'sis. N.L. masc. adj. *chagannorensis* pertaining to Lake Chagannor).

Cells are Gram-positive, motile, spore-forming rods 0.6–0.7 by 2.0–3.0 µm in size. Ellipsoidal endospores are produced at a terminal position in swollen sporangia. Colonies are circular and entire, 1 mm in diameter and yellow–orange in colour on alkaline saline medium after 2 days cultivation at 37 °C. Facultatively anaerobic. Alkali-tolerant and moderately halophilic, growing over a wide range (3–20 %, w/v) of salt concentrations, with optimal growth at 7 % (w/v) salts. No growth occurs in the absence of NaCl. Grows at 6–40 °C (optimally at 37 °C) and pH 5.8–11.0 (optimally at pH 8.5). Catalase-positive and oxidase-negative. Nitrate is reduced to nitrite. Casein, gelatin, Tween 80 and starch are not hydrolysed; DNA and aesculin are hydrolysed. H₂S is not produced. Indole, phenylalanine deaminase and phosphatase tests are negative. The following compounds are utilized as sole carbon and energy sources: acetate, aesculin, amygdalin, D-cellobiose, citrate, formate, fumarate, glycerol, hippurate, pyruvate and sucrose. The following compounds are not utilized as sole carbon and energy sources: butyrate, D-arabinose, benzoate, ethanol, D-glucose, D-fructose, L-fucose, D-galactose, inulin, D-lactose, D-mannitol, maltose, D-mannose, D-melibiose, propionate, L-raffinose, D-ribose, salicin, trehalose, D-xylose, butanol, dulcitol, *myo*-inositol, propanol, D-sorbitol, starch and xylitol. Sensitive to bacitracin (10 U) and vancomycin (30 µg). Resistant to cephalothin (30 µg), kanamycin (30 µg), nalidixic acid (30 µg), penicillin G (10 U), streptomycin (30 µg) and tetracycline (30 µg). Cellular fatty acids are anteiso-C_{15:0} (39.7 %), iso-C_{15:0} (21.0 %), anteiso-C_{17:0} (10.8 %), iso-C_{16:0} (6.8 %), C_{16:0} (6.0 %), iso-C_{17:0} (5.2 %), iso-C_{14:0} (2.4 %), C_{14:0} (0.8 %), iso-C_{17:1ω5c} (0.5 %) and C_{18:0} (0.3 %). Polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three different unidentified phospholipids. The major isoprenoid quinone is MK-7. The peptidoglycan type is A1γ, with meso-diaminopimelic acid as the diagnostic diamino acid.

The type strain, CG-15^T (=CCM 7371^T=CECT 7153^T=CGMCC 1.6292^T=DSM 18086^T), was isolated from Lake Chagannor in Inner Mongolia, China.

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