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-15T, 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-15T, 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-15T 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-15T were anteiso-C15 : 0, iso-C15 : 0 and anteiso-C17 : 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-15T represents a novel species of the genus Bacillus, for which the name Bacillus chagannorensis sp. nov. is proposed. The type strain is CG-15T (=CCM 7371T=CECT 7153T=CGMCC 1.6292T=DSM 18086T).

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

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 alkalitolerant, moderately halophilic, Gram-positive bacterium, strain CG-15T, isolated from Lake Chagannor, an alkaline saline lake in Inner Mongolia, China.
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-15T 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 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-15T 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-15T 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-15T from phylogenetically related Bacillus species

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Short rods</td>
<td>Cocci</td>
<td>Rods</td>
<td>Rods</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Yellow-orange</td>
<td>Yellow</td>
<td>White</td>
<td>Cream-white to dark yellow</td>
</tr>
<tr>
<td>Endospore formation</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Oxidase</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>W</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gelatin</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Casein</td>
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<td>−</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Starch</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Phenylalanine deamination</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>NaCl range (% w/v)</td>
<td>3–20</td>
<td>1–25</td>
<td>0.5–16</td>
<td>0.5–16</td>
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<tr>
<td>Optimum NaCl conc. (% w/v)</td>
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<td>16</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Temperature range (°C)</td>
<td>6–40</td>
<td>4–50</td>
<td>10–45</td>
<td>15–45</td>
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<tr>
<td>Optimum temperature (°C)</td>
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<td>37</td>
<td>30</td>
<td>30</td>
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<tr>
<td>pH range</td>
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<td>7–10</td>
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<tr>
<td>Optimum pH</td>
<td>8.5</td>
<td>9.0</td>
<td>≥10</td>
<td>&gt;10.0</td>
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<td>DNA G+C content (mol%)</td>
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<td>48.4</td>
<td>39.2</td>
<td>42.4</td>
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<td>Major fatty acids</td>
<td>anteiso-C₁₅ :₀ (40 %), iso-C₁₅ :₀ (21 %), anteiso-C₁₇ :₀ (11 %)</td>
<td>anteiso-C₁₅ :₀ (89 %), iso-C₁₅ :₀, C₁₇ :₀</td>
<td>anteiso-C₁₅ :₀ (40 %), iso-C₁₅ :₀ (25 %)</td>
<td>anteiso-C₁₅ :₀ (36 %), iso-C₁₇ :₀ (18 %), iso-C₁₆ :₀ (11 %)</td>
</tr>
</tbody>
</table>
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-15T 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-15T 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-15T 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-15T. 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-15T formed a phyletic group with Bacillus saliphilus DSM 15402T (96.0% sequence similarity), Bacillus agaradhaerens DSM 8721T (94.0%) and Bacillus clarkii DSM 8720T (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 salarius, was described by Lim et al. (2006c). This species, also isolated from a salt lake in China, is phylogenetically related to B. agaradhaerens DSM 8721T, Bacillus vedderi DSM 9768T and B. clarkii DSM 8720T, 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-15T and related species. Accession numbers for the sequences used in this study are shown in parentheses. Sporosarcina ureae DSM 2281T 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-15T 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-15T constituted a different taxon with respect to recognized Bacillus species. DNA–DNA hybridization between strain CG-15T 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-15T 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-15T was characterized by the presence of branched fatty acids anteiso-C15:0 (39.7 %), iso-C15:0 (21.0 %) and anteiso-C17:0 (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-15T, 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-15T 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-15T 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-15T 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-15T 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. Alkalitolerant 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-rafínose, D-ribose, salicin, trehalose, D-xyllose, butanol, dulcitol, myo-inositol, propanol, D-sorbitol, starch and xylose. 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-C15:0 (39.7 %), iso-C15:0 (21.0 %), anteiso-C17:0 (10.8 %), iso-C16:0 (6.8 %), C16:0 (6.0 %), iso-C17:0 (5.2 %), iso-C14:0 (2.4 %), C14:0 (0.8 %), iso-C17:0:5c (0.5 %) and C18:0 (0.3 %). Polar lipids are diphosphatidylglycerol, 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-15T (=CCM 7371T=CECT 7153T=CGMCC 1.6292T=DSM 18086T), was isolated from Lake Chagannor in Inner Mongolia, China.

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