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

I. J. Carrasco¹, M. C. Márquez¹, Y. Xue², Y. Ma², D. A. Cowan³, B. E. Jones⁴, W. D. Grant⁵ and A. Ventosa¹

¹Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

²State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, 100080 Beijing, China

³Department of Biotechnology, University of the Western Cape, Bellville 7535, Cape Town, South Africa

⁴Genencor International, Leiden, The Netherlands

⁵Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, UK

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Author for correspondence: Antonio Ventosa, Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain. Tel. +34 95 455 6765; Fax +34 95 462 81 62. E-mail: ventosa@us.es

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CG15^T is XXXXXXXX.

A Gram-positive, moderately halophilic, sporeforming bacterium, designated strain CG15^T, was isolated from the soda Lake Chagannor in Inner Mongolia Autonomous Region, China. The cells of this strain were motile short rods, with ellipsoidal, terminal and deforming endospores. Strain CG15^T, a facultatively anaerobic bacterium, grew between pH 5.75 and 11.0 (optimal growth at pH 8.5), between 6-40°C (optimal growth at 37°C) and at salinities of 3-20% (w/v) total salts, growing optimally at 7% (w/v) salts. On the basis of 16S rRNA gene sequence analysis, strain CG15^T was shown to belong to the genus *Bacillus* within the phylum *Firmicutes*; based on the comparison of the 16S rRNA gene sequences, it showed the closest phylogenetic similarity with the species *Bacillus saliphilus* (96.0%). The DNA G+C content of this new isolate is 53.8 mol%. The major cellular fatty acids of strain CG15^T were anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0}, and its polar lipid pattern 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 is A1 γ , with meso-diaminopimelic acid as the diagnostic diamino acid. On the basis of polyphasic evidence from this study, a new species of the genus *Bacillus*, *Bacillus chagannoriensis* sp. nov. is proposed, with strain CG15^T (=DSM 18086^T =CCM XXX^T = AS XXX^T = CECT XXX^T) as the type strain.

Moderately halophilic bacteria that grow optimally in media containing 3 to 15% (w/v) salts are widely distributed among many hypersaline habitats. They are attracting interest because this group of bacteria has great biotechnological potential for the production of compatible solutes or hydrolytic enzymes (Margesin & Schinner, 2001). They are represented by a very heterogeneous physiological group, which includes both Gram-positive and Gram-negative microorganisms. Aerobic sporeforming moderately halophilic Gram-positive rods are also taxonomically diverse and have been isolated from saline environments such as soils and aquatic habitats (Arahal and Ventosa, 2002). The species of the genus *Bacillus* are also taxonomically very diverse and on recent years some of them have been reclassified as members of novel genera or transferred to other genera. The genus *Bacillus* contains at least six phylogenetically distinct groups on the basis of molecular analyses of 16S rRNA gene sequences (Ash et al., 1991; Stackebrandt & Liesack, 1993; Spring et al., 1996; Wainø et al., 1999; Schlesner et al., 2001). In nature, salinity often goes together alkalinity. Alkaline saline lakes represent a unique habitat with high pH and variable (up to saturation) salt concentration. This paper describes a novel alkali-tolerant, moderately halophilic Gram-positive bacterium, strain CG15^T, isolated from Lake Chagannor, an alkaline, saline lake in Inner Mongolia, China.

The sampling was carried out during an expedition in September 2003 to Lake Chagannor (43°21'N 113°08'E). At the time of sampling, the temperature of the water was 17°C, the pH 9.5 and the conductivity was 21.3 mS cm⁻¹. Strain CG15^T was isolated by diluting a water sample in sterile 10% (w/v) salt solution and then plating it on alkaline saline medium and incubated at 37°C aerobically. The alkaline saline isolation medium contained (g l⁻¹): glucose, 10.0; peptone, 5.0 (Difco); yeast extract (Difco), 5.0; KH₂PO₄, 1.0; MgSO₄ x 7H₂O, 0.2; NaCl, 80; Na₂CO₃, 20; agar, 20. The salts NaCl and

Na₂CO₃ were autoclaved separately and added to the organic components at 60°C (Duckworth *et al.*, 1996). The strain was subsequently purified three times and it was maintained on the same alkaline saline medium and at -80°C on this medium supplemented with 30% (v/v) glycerol.

In order to characterize phenotypically this isolate, standard phenotypic tests were performed. The Gram stain reaction was carried out using the method described by Dussault (1955). For the determination of cellular morphology and motility, a culture from liquid alkaline saline medium was examined by light microscopy under phase-contrast microscope. The morphology of colonies, their size and pigmentation were observed on the alkaline saline solid medium with different salt concentrations after 2 days of incubation. Growth at different concentrations of salts was determined on the 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 the alkaline saline liquid medium at pH values ranging from 4.5 to 11.0 using the appropriate biological buffers, Na₂HPO₄/NaH₂PO₄ (below pH 8.0), Na₂CO₃/NaHCO₃ (pH 8.0-10.0) and Na₂HPO₄/NaOH (pH 11), as described previously (Gomori, 1955). The pH was readjusted after sterilization; growth was scored as optical density at 600 nm. The temperature range for growth was determined at temperatures between 15 and 50°C. Catalase was tested by adding 3% H₂O₂ to culture plates. The oxidase reaction was performed on filter paper moistened with 1% (w/v) aqueous solution of *N, N, N', N'*-tetramethyl-*p*-phenyldiamine. Other tests shown in Table 1 or included in the species description were carried out following the methodology described previously (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; García *et al.*, 1987).

Strain CG15^T was Gram-positive, motile and facultatively anaerobic. Cells were rods with a width of 0.6–0.7 µm and length of 2.0–3.0 µm, forming short curved chains (Fig. 1). Ellipsoidal endospores were formed at terminal position in swollen sporangia, as found in the phylogenetically closely related *Bacillus* species. When grown for 2 days at 37 °C on the alkaline saline medium, the colonies were circular with entire margins, yellow-orange pigmented and with a diameter of 1 mm. 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. It grew between pH 5.75 and 11.0, and optimally in media with pH 8.5. Other phenotypic features are included on the species description.

Genomic DNA from strain CG15^T was prepared using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the forward primer 16F27 and the 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 the closely related bacteria and the alignment was confirmed and checked against both primary and secondary structure of the 16S rRNA molecule using the alignment tool of the ARB software package. The phylogenetic trees were constructed using three different methods: maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987), algorithms integrated in the ARB software for phylogenetic inference. The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Figure 2.

Almost-complete 16S rRNA gene sequence of strain CG15^T was obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. No sequences available in the public databases exhibited more than 96.04% similarity with the sequence of strain CG15^T. Comparative analysis of 16 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 CG15^T formed a phyletic group with *Bacillus saliphilus* DSM 15402^T (96.04% similarity), *Bacillus agaradhaerens* DSM 8721^T (94.04%) and *Bacillus clarkii* DSM 8720^T (93.48%) (Fig. 2). Neighbour-joining, maximum likelihood and distance methods resulted in highly similar tree topologies and only maximum-parsimony results are shown. No other known bacteria shared more than 93% sequence similarity with this new isolate. Based on the sequence divergence, it was evident that strain CG15^T constituted a different taxon separated from other *Bacillus* species. DNA-DNA hybridization between strain CG15^T and the nearest phylogenetic neighbours was not attempted since strains differing by more than 3% at the 16S rRNA gene sequence are unlikely to exhibit more than 70% relatedness at the 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 CG15^T was 53.8 mol%. This value is within the range for *Bacillus* but is not similar to those of the species phylogenetically closely related (Table 1).

Fatty acids analysis was performed using the MIDI system (Microbial Identification System). Cells were cultured on the alkaline saline medium at 37°C for 24 h. This analysis was carried out by the Identification Service of the Belgian Co-ordinated Collections of Microorganisms, Laboratory of Microbiology of Gent (BCCM/LMG), Gent, Belgium. The cellular fatty acid profile of strain CG15^T was characterized by the 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 fatty acids found in the cell membranes of *Bacillus* species (Albert *et al.*, 2005). In order to complete the chemotaxonomic characterization of strain CG15^T the analysis of polar lipids, quinones and peptidoglycan of the cell wall was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three different phospholipids of unknown structure. Strain CG15^T contained MK-7 as the major menaquinone and a cell wall type based on meso-diaminopimelic acid. The major fatty acid profile, respiratory lipoquinone, major polar lipids and peptidoglycan type of the cell wall of strain CG15^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, 2006b).

The characteristics that differentiate strain CG15^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, fatty acid and polar lipids composition can be used to distinguish this strain from the phylogenetically related taxa (Table 1). Therefore, on the basis of these polyphasic taxonomic data, we propose that the novel strain represents a

new species of the genus *Bacillus*, for which the new name *B. chagannoriensis* sp. nov. is proposed.

Description of *Bacillus chagannoriensis* sp. nov.

Bacillus chagannoriensis (cha.ga.nno.ri.en'sis N.L. adj. *chagannoriensis* pertaining to the soda lake Chagannor, inner Mongolia, China).

Cells are Gram-positive, sporeforming rods, 0.6–0.7 by 2.0–3.0 μm . Motile. Ellipsoidal endospores are produced at a terminal position in swollen sporangia. Colonies are circular and entire, 1 mm in diameter and yellow-orange pigmented on alkaline saline medium after 2 days of cultivation. Facultatively anaerobic. Alkalitolerant and moderately halophilic, growing over a wide range (3 to 20% w/v) of salt concentrations, with optimal growth at 7% (w/v) salts. No growth in the absence of NaCl. Grows at 6–40°C (optimal at 37°C) and pH 5.75 to 11.0 (optimal at pH 8.5). Catalase positive and oxidase negative. Nitrate is reduced to nitrite. Casein, gelatin, Tween 80 and starch are not hydrolyzed; DNA and aesculine are hydrolyzed. H₂S is not produced. Indole and phenylalanine deaminase tests are negative. Phosphatase negative. The following compounds are utilized as sole carbon and energy sources: acetate, aesculin, amygdalin, D-cellobiose, citrate, formate, fumarate, glycerol, hippurate, piruvate, starch 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, D-trehalose, D-xylose, butanol, dulcitol, *myo*-inositol, propanol, D-sorbitol, and xylitol. Susceptible to bacitracin (10 U) and vancomycin (30 μg). Resistant to cephalotin (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_{17:1 ω 5c} (0.5%), and C_{18:0} (0.3%). The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and three different unidentified phospholipids. The major isoprenoid quinone is MK-7. The peptidoglycan type is A1 γ , with meso-diaminopimelic acid as the diagnostic diamino acid.

The type strain is CG15^T (=DSM 18086^T =CCM XXX^T =AS XXX^T =CECT XXX^T) isolated from Lake Chagannor in Inner Mongolia, China.

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References

- Albert, R. A., Archambault, J., Rosselló-Mora, R., Tindall, B. J. & Matheny, M. (2005).** *Bacillus acidicola* sp. nov., a novel mesophilic, acidophilic species isolated from acidic *Sphagnum* peat bogs in Wisconsin. *Int J Syst Evol Microbiol* **55**, 2125-2130.
- Arahal, D.R. & Ventosa, A. (2002).** Moderately halophilic and halotolerant species of *Bacillus* and related genera. In *Applications and Systematics of Bacillus and Relatives*, pp. 83-99. Edited by R. Berkeley, M. Heyndrickx, N. Logan & P. De Vos. Oxford: Blackwell.
- Duckworth, A.W., Grant, W.D., Jones, B.E. & van Steenberg, R. (1996).** Phylogenetic diversity of soda lake alkaliphiles. *FEMS Microbiol Ecol* **19**, 181-191.
- Dussault, H.P. (1955).** An improved technique for staining red halophilic bacteria. *J. Bacteriol* **70**, 484-485.
- Felsenstein, J. (1981).** Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Fitch, W. M. (1971).** Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Gomori, G. (1955).** Preparation of buffers for use in enzyme studies. *Methods Enzymol* **1**, 138-146.
- Heyrman, J., Vanparrys, B., Logan, N. A., Balcaen, A., Rodriguez-Diaz, M., Felske, A. & De Vos, P. (2004).** *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli*

sp. nov., *Bacillus botaviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. *Int J Syst Evol Microbiol* **54**, 47-57.

Heyrman, J., Rodriguez-Diaz, M., Devos, J., Felske, A., Logan, N. A. & De Vos, P. (2005). *Bacillus novalis* sp. nov., *Bacillus arvi* sp. nov. and *Bacillus humi* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **55**, 111-117.

Lim, J.-M., Jeon, C. O. & Kim, C. –J. (2006a). *Bacillus taeanensis* sp. nov., a halophilic Gram-positive bacterium from a solar saltern in Korea. *Int J Syst Evol Microbiol* **56**, 2903-2908.

Lim, J.-M., Jeon, C. O., Lee, J. C., Ju, Y. J., Park, D. J. & Kim, C. –J. (2006b). *Bacillus koreensis* sp. nov., a spore-forming bacterium, isolated from the rhizosphere of willow roots in Korea. *Int J Syst Evol Microbiol* **56**, 59-63.

Ludwig, W. & Strunk, O. (1996). ARB – a software environment for sequence data. <http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/ARB.ps>

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Molec Biol* **3**, 208-218.

Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109-118.

Nielsen, P., Fritze, D., & Priest, F. G. (1995). Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology* **141**, 1745-1761.

Owen, R. J. & Hill, L. R. (1979). The estimation of base compositions, base pairing and genome size of bacterial deoxyribonucleic acids. In *Identification Methods for*

Microbiologists, 2nd edn, pp. 217-296. Edited by F. A. Skinner & D. W. Lovelock.
London: Academic Press.

Priest, F. G., Goodfellow, M. & Todd, C. (1988). A numerical classification of the genus *Bacillus*. *J Gen Microbiol* **134**, 1847-1882.

Romano, I., Lama, L., Nicolaus, B., Gambacorta, A. & Giordano, A. (2005). *Bacillus saliphilus* sp. nov., isolated from a mineral pool in Campania, Italy. *Int J Syst Evol Microbiol* **55**, 159-163.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152-155.

Stackebrandt, E., Fredericksen, W., Garrity, G.M. & 10 other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043-1047.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846-849.

Ventosa, A., Quesada, E., Rodriguez-Valera, F., Ruiz-Berraquero, F. & Ramos-Cormenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. *J Gen Microbiol* **128**, 1959-1968.

Wieser, M., Worliczek, H., Kämpfer, P. & Busse, H.-J. (2005). *Bacillus herbersteinensis* sp. nov. *Int J Syst Evol Microbiol* **55**, 2119-2123.

Table 1. Characteristics useful to distinguish *Bacillus chagannoriensis* strain CG15^T from phylogenetically related *Bacillus* species. Data from Romano *et al.* (2005), Nielsen *et al.* (1995) and this study.

Symbols: +, positive; –, negative; W, weak; ND, not determined.

Characteristic	<i>Bacillus chagannoriensis</i> CG15 ^T	<i>Bacillus saliphilus</i> DSM 15402 ^T	<i>Bacillus agaradhaerens</i> DSM 8721 ^T	<i>Bacillus clarkii</i> DSM 8720 ^T
Cell morphology	Shorts rods	Cocci	Rods	Rods
Colony pigmentation	Yellow-orange	Yellow	White	Cream-white to dark yellow
Endospore formation	+	-	+	+
Motility	+	-	ND	ND
Oxidase	-	+	ND	ND
Hydrolysis of:				
Aesculin	+	ND	ND	ND
Gelatin	-	+	-	+
Casein	-	-	-	+
Starch	-	-	+	
Phenylalanine deamination	+	-	-	ND
NaCl range (% w/v)	3-20	0-25	0.5-16	0.5-16
Optimum NaCl (% 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	5.75-11	7-10	>7.0	>7.0
Optimum pH	8.5	9.0	10	>10.0
DNA G+C content (mol%)	53.8	48.4	39.5	43.0
Quinone composition	MK-7	MK-7(H ₄) (15%); MK-7(H ₂) (70%); DMK-7(H ₂) (15%)	ND	ND

Major fatty acids	Anteiso-C _{15:0} (40%) Iso-C _{15:0} (21%) Anteiso-C _{17:0}	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} (19%)
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Legend to figures

Fig. 1. Phase-contrast photomicrograph of cells of strain CG15^T, showing sporeforming rods. Bar, 10 μm.

Fig. 2. Maximum-parsimony tree, based on the 16S rRNA gene sequence comparison, showing the relationship of strain CG15^T with related species. The accession numbers of the sequences used in this study are shown in parentheses after the strain designation. *Sporosarcina ureae* was used as an outgroup. The scale bar represents 0.02 substitutions per nucleotide position.

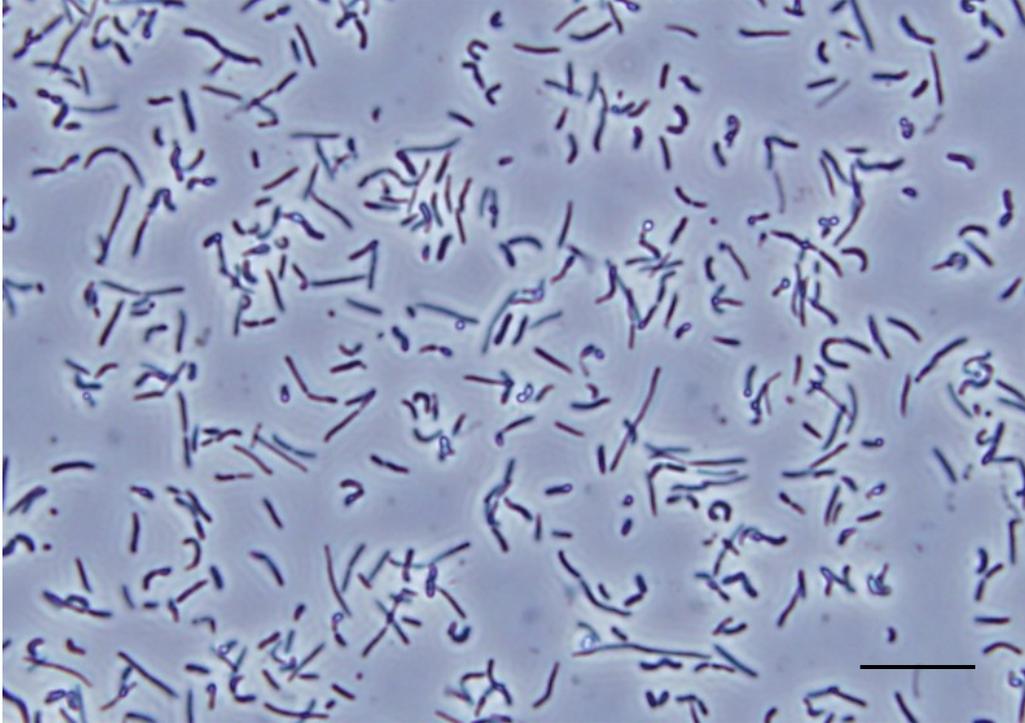
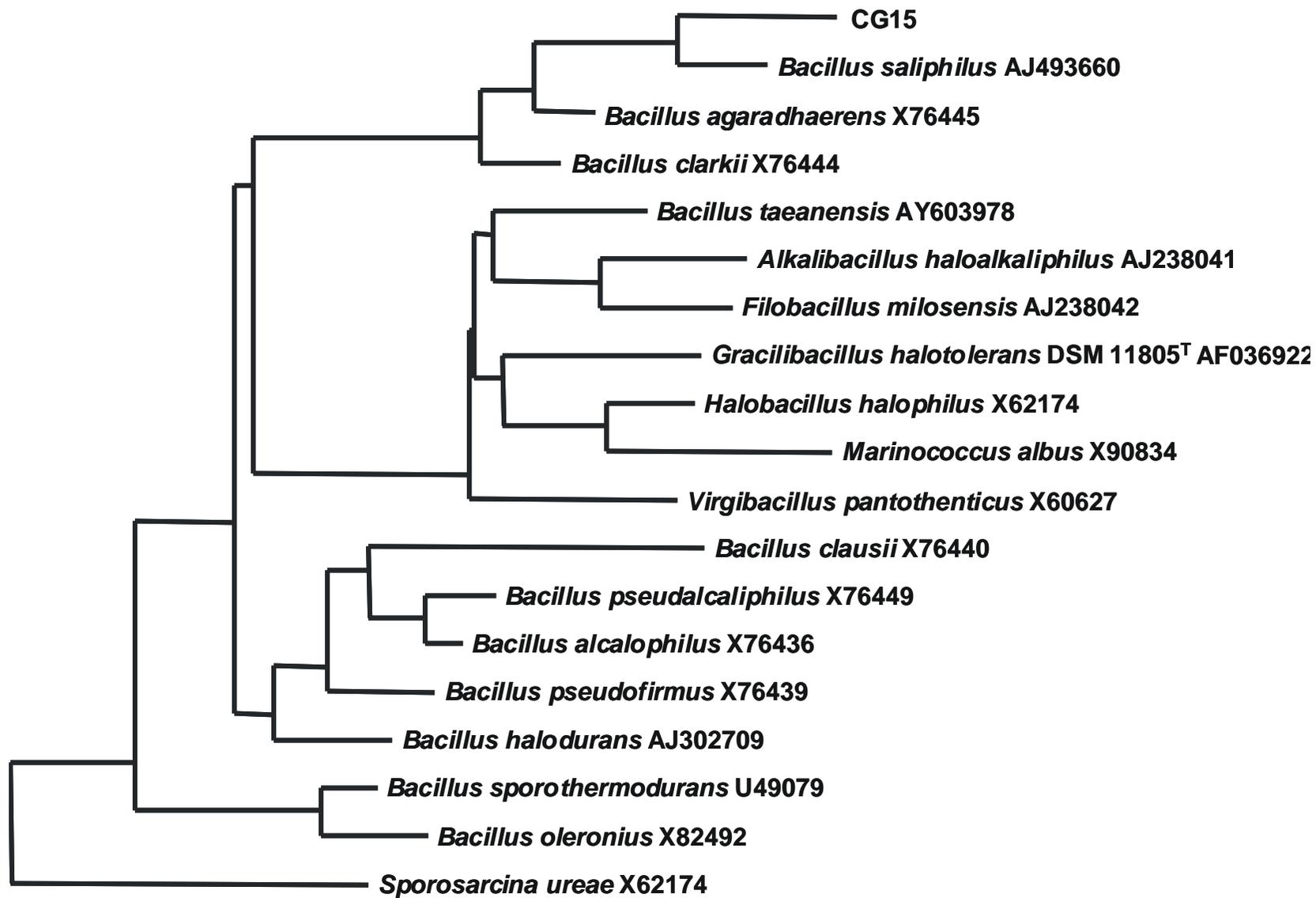


Figure 1.



0.02