Bacillus locisalis sp. nov., a new haloalkaliphilic species from hypersaline and alkaline lakes of China, Kenya and Tanzania

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Running title: *Bacillus locisalis* sp. nov.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain CG1^T, CG2, CG4, CG6, CG7, 103NT4 and WE1 are FR714930, FR714931, FR714932, FR714933, FR714934, X92163 and X92164, respectively.

Abstract

A polyphasic taxonomic study was performed on seven *Bacillus*-like bacteria isolated from three hypersaline and alkaline lakes located in China, Kenya and Tanzania. All strains were moderately halophilic and alkaliphilic, Gram positive, motile rods. The DNA G+C content from the seven isolates ranged from 42.2 to 43.4 mol% and their major fatty acid was anteiso-$C_{15:0}$. Strain CG1^T, selected as representative strain of the isolates, possesses *meso*-diaminopimelic acid in the cell wall peptidoglycan, MK-7 as the predominant menaquinone and diphosphatidyl glycerol, phosphatidylglycerol and phosphatidylethanolamine as the major polar lipids. Comparative 16S rRNA gene sequence analysis indicated that the isolates belonged to the genus *Bacillus*. The seven isolates shared 97.7-99.9% 16S rRNA gene sequence similarity, and formed a branch that was distinct from the type strains of the recognized species of the genus *Bacillus*. They were most closely related to *Bacillus agaradhaerens* DSM 8721^T (92.6-93.8% 16S rRNA sequence similarity). DNA–DNA hybridization values between the seven isolates were 85-100%. According to the polyphasic characterization, the strains represent a novel species, for which the name *Bacillus locisalis* sp. nov. is proposed. The type strain is CG1^T (CCM 7370^T = CECT 7152^T = CGMCC 1.6286^T = DSM 18085^T).

Key words: *Bacillus locisalis* sp. nov., New species, 16S rRNA gene analysis, Taxonomy, Polyphasic study, Hypersaline lakes, Soda lakes

Scope of the paper: Systematics

Haloalkaliphilic bacteria are extremophilic microorganisms that are widely distributed in different hypersaline and alkaline habitats with a variable (up to saturation) salt concentration and high pH values. The genus *Bacillus* was proposed by Cohn in 1872 [6] and since then it has undergone substantial taxonomic changes. Currently, this genus groups near 200 species [10] with some of them having a moderately halophilic and alkaliphilic/alkalitolerant response, such is the case of *B. oshimensis* (from soil in Japan) [29], *B. saliphilus* (from algal mat from a mineral pool in Italy) [24], *B. chagannorensis* (from a soda lake in China) [3], *B. aurantiacus* (from an extremely shallow soda lake in Hungary) [2], and *Bacillus polygoni* (from indigo balls in Japan) [1].

In the present study, we report the discovery of a novel moderately halophilic, alkaliphilic *Bacillus* species during a study of bacterial diversity in hypersaline habitats using a culture-dependent approach. Seven bacterial strains were isolated from water and sediment samples from hypersaline and alkaline lakes located in three different countries: China, Kenya and Tanzania. The taxonomic status of the isolates was determined using a polyphasic approach.

Strains 103NT4 and WE1 were isolated in 1988 following the methodology described by Duckworth et al. [8]. Strain 103NT4 was isolated from orange-coloured soda crusts surrounding a warm soda seep brine (35°C) located on the northern shore of Lake Natron (Tanzania) (2º08’ S, 36º00’ E, pH 10.5, conductivity 35 mS cm^{-1}), while strain WE1 was isolated from a sediment sample from the eastern shore of Lake Elmenteita, in the Kenyan section of the East African Rift Valley (0º25’ S, 36º15’ E, pH 10.5, conductivity 12.7 mS cm^{-1}) [8]. The other five strains were isolated from water (CG1^{T} and CG2) and sediment (CG4, CG6 and CG7) samples taken from Lake Chagannor, during an expedition in September 2003. This lake is situated near a soda works, 120 km south of Mandulatu

(43º16’ N 112º55’ E, pH 10.5, conductivity 202 mS cm^{-1}), on the Inner Mongolian steppe, northwest of Beijing, China. The water samples were diluted in sterile 10% (w/v) marine salts (g l^{-1}): NaCl, 78; $MgCl_2$ x $6H_2O$, 13; $MgSO_4$ x $7H_2O$, 20.3; $CaCl_2$, 0.33; KCl, 2; $NaHCO_3$, 0.07; NaBr, 0.23 [28], plating on alkaline saline medium and incubating at 37ºC aerobically. The alkaline saline isolation medium contained (g l^{-1}): glucose, 10.0; peptone (Difco), 5.0; yeast extract (Difco), 5.0; KH_2PO_4, 2.0; $MgSO_4$ x $7H_2O$, 0.4; NaCl, 80; Na_2CO_3, 20. The salts NaCl and Na_2CO_3 were autoclaved separately and added to the organic components at 60ºC. The pH of this medium was adjusted to pH 10. When it was necessary, the medium was solidified by adding 2.0% (w/v) agar. The sediments (0.1g) were suspended in 10% (w/v) marine salts. The suspensions were vortexed for 1 min, allowed to settle, serially diluted in 10% (w/v) marine salts and then spread-plated in duplicate on alkaline saline medium followed by aerobic incubation at 37ºC. The strains were subsequently purified three times by plating on the same medium and maintained on the same alkaline saline medium and at -80ºC on this medium without agar and supplemented with 30% (v/v) glycerol. In addition to the seven isolates, *Bacillus agaradhaerens* DSM 8721^T was obtained from the Deustche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and cultivated at 37 ºC on alkaline saline medium. This bacterium was used as reference for comparative phenotypic and chemotaxonomic studies.

The phylogenetic position of the seven isolates was determined by complete 16S rRNA gene sequence analysis. Genomic DNAs were prepared using the method described by Marmur [19]. PCR amplifications of the 16S rRNA gene were carried out with the forward primer 16F27 and the reverse primer 16R1488. Sequencing was performed using an automated DNA sequencer model 3130XL (Applied Biosystems). Identification of

phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server version 2 (http://www.eztaxon.org/; [5]). The 16S rRNA gene sequences were aligned with the published sequences of closely related bacteria. The alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package [18]. The phylogenetic trees were constructed using three different methods: maximum-likelihood [11], maximum-parsimony [13] and neighbour-joining [25], algorithms integrated in the ARB software for phylogenetic inference. The robustness of the topology in the phylogenetic trees was evaluated by bootstrap analyses [12] of the neighbour-joining method based on 1000 resamplings. 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 Fig. 1.

Almost-complete 16S rRNA gene sequences of the seven isolates (1441 nucleotides) were obtained and used for initial BLAST and EzTaxon searches in GenBank and phylogenetic analysis. Comparative 16S rRNA gene sequence analysis revealed that the seven isolates have the closest phylogenetic affiliation with the genus *Bacillus.* A tree constructed by neighbour-joining analysis clearly showed that the seven isolates grouped together with 97.7-99.9% 16S rRNA gene sequence similarity among themselves. This cluster was separated from one formed by some other *Bacillus* species with 100 bootstrap support (Fig. 1). The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to those of the tree constructed by neighbour-joining analysis (data not shown). The nearest known relative of the isolates was *Bacillus agaradhaerens* DSM 8721^{T}, with values of 16S rRNA gene sequence similarity comprised between 92.6 and 93.8%. For determination of the DNA base composition of the seven

isolates the DNAs were extracted and purified by the method of Marmur [19] and the G+C contents of the DNAs were determined in triplicate from the midpoint value of the thermal denaturation profile [20] by using the equation of Owen and Hill [24]. The genomic DNA G+C contents of the seven isolates ranged from 42.2 to 43.4 mol% (Table S1). These values are within the range for *Bacillus* but are higher than that of *Bacillus agaradhaerens* DSM 8721^T (39.5 mol%). DNA-DNA hybridization was carried out to evaluate the genomic DNA relatedness between the seven isolates, following the competition procedure of Johnson [16], described in detail elsewhere [21]. The hybridization temperature was 46.4 ºC, which was within the limit of validity for the filter method [7] and the percentage of hybridization was calculated according to Johnson [16]. The values presented were based on a minimum of four replicates. The values of DNA-DNA hybridization between strain CG1^T and the other six isolates ranged between 85% and 99%. These values are clearly higher than 70%, cut-off generally accepted for species delineation and support the placement of the seven isolates as the same genotypic species [27].

In order to phenotypically characterize the isolates and, following the minimal standards for describing new genera and species of aerobic, endospore-forming bacteria recommended by Logan et al. [17], standard phenotypic tests were performed. The Gram stain reaction was carried out using the method described by Dussault [9]. Cell morphology and motility were studied by phase-contrast microscopy. 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 alkaline saline medium containing 0, 0.5, 1, 3, 5, 7, 10, 15, 20, 25 or 30% (w/v) NaCl. The pH range for growth was determined on the alkaline saline liquid medium at pH values ranging from 7.0 to 12.5, with increments of 0.5 pH units, using the

appropriate biological buffers, Na_2HPO_4/NaH_2PO_4 (below pH 8.0), $Na_2CO_3/NaHCO_3$ (pH 8.0-10.0) and Na_2HPO_4/NaOH (pH 11), as described previously [15]. 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 6 and 50ºC. Catalase was tested by adding 3% H_2O_2 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*-phenylendiamine. Sporulation was tested on the alkaline saline solid medium supplemented with 5 mg l^{-1} $MnSO_4$ (Merck). Utilization of various substrates as sole carbon and energy sources, or carbon, nitrogen and energy sources, were determined using a basal medium with the following composition (g l^{-1}): yeast extract (Difco), 0.01; KNO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$ x $7H_2O$, 0.2; $(NH_4)_2HPO_4$, 1.0; NaCl, 80; Na_2CO_3, 20. To this liquid medium a 0.1% (w/v) filter-sterilized substrate was added. Carbohydrates were used at a final concentration of 0.2% (w/v). When amino acids were used as substrate the basal medium contained neither KNO_3 nor $(NH_4)_2HPO_4$. A growth test was considered positive when the OD_{600} reached or exceeded a value of 0.3 after 4 days at 37 °C. Other tests shown in Table S1 or included in the species description were carried out following methodologies described previously [14, 23, 28]. Unless otherwise indicated the tests were carried out in the alkaline saline medium (pH 10) and incubated at 37ºC in sealed containers to minimise evaporation. The seven isolates studied in this work were very similar in their phenotypic characteristics, although some differences were observed between them (Table S1 and species description).

Fatty acids were determined for the seven isolates, as well as for the reference strain *Bacillus agaradhaerens* DSM 8721^T using the MIDI system (Microbial Identification System). All the strains were grown on alkaline saline medium, pH 10 at 37ºC, for 48 h.

This analysis was carried out by the Identification and Characterization Service of the CECT (Valencia, Spain). Anteiso-$C_{15:0}$ was the predominant compound although slight variation was observed between the compositions of the seven isolates (Table 1).

Analysis of peptidoglycan of the cell wall, quinones and polar lipids content of strain CG1^T, selected as representative strain of the isolates, was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The cell biomass for these analyses was obtained by cultivation on the alkaline saline medium (pH 10) at 37ºC, for 48 h. Strain CG1^T possessed a cell wall peptidoglycan of type A1γ (*meso*-Dpm, directly cross-linked; A31; http://www.dsmz.de/microorganisms/main.php?content_id=35) and contained MK-7 (98%) as the predominant menaquinone, with MK-6 (2%) present in minor amounts. The polar lipids of this strain consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, four phospholipids and an aminophospholipid of unknown structure (Fig. S1). The results obtained from these chemotaxonomic analyses were consistent with the results from the phylogenetic analysis that suggest that our isolates may belong to the genus *Bacillus* [4].

The characteristics that differentiate strain CG1^T from *Bacillus agaradhaerens* DSM8721^T are summarized in Table 2. The differences in some features, such as colony pigmentation, growth in anaerobic conditions, range and optimal salt concentration for growth, optimal temperature for growth, hydrolysis of starch, Voges-Proskauer test, as well as the genomic DNA G+C content, can be used to distinguish this strain from *Bacillus agaradhaerens* (Table 2). Therefore, the taxonomic data from polyphasic analysis clearly suggest that our isolates belong to the genus *Bacillus* and represent a new species of this genus, for which the new name *B. locisalis* sp. nov. is proposed.

Description of Bacillus locisalis sp. nov.

Bacillus locisalis (lo.ci.sa'lis. L. n. *locus* place, locality; L. gen. n. *salis* of salt; N.L. gen. n. *locisalis* from a salted place).

Gram-positive rods, 1.0 by 2.0–5.0 µm. Motile, oval endospores are produced at terminal and subterminal positions in swollen sporangia. Facultatively anaerobic. Colonies are orange, circular, opaque and entire on alkaline saline medium after 2 days of cultivation. Moderately halophilic, growing in a wide range (1 to 25% w/v) of salt concentrations, with optimal growth at 7-10% (w/v) NaCl. Grows at 10-45ºC (optimal at 37ºC) and pH 8-12 (optimal at pH 9-10). Oxidase negative and catalase positive. Acid is produced from D-fructose, D-glucose, D-maltose, D-mannitol, D-melibiose, D-ribose, D-trehalose and D-xylose. Acid is not produced from D-amygdaline, D-arabinose, L-citruline, dulcitol, DL-ethionine, glycerol, inulin, lactose, D-melezitose, *m*-inositol, and xylitol. Casein is not hydrolyzed. Indole production and Voges-Proskauer test are negative. D-fucose, D-fructose and D-glucose are utilized as sole carbon and energy sources. The following compounds are not utilized as sole carbon and energy sources: aesculin, butanol, *m*-inositol, sorbitol, xylitol and citrate. L-alanine and cysteine are utilized as sole carbon, nitrogen, and energy sources. L-phenylalanine and L-glutamine are not utilized as sole carbon, nitrogen, and energy sources. DNA base composition ranges from 42.2 to 43.4 mol%. The cell wall contains peptidoglycan of the *meso*-diaminopimelic acid type. Major isoprenoid quinone is MK-7. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, four phospholipids and an aminophospholipid of unknown structure. Additional characteristics of the strains are listed in Table S1. Cellular fatty acid composition is given in Table 1.

The habitats are saline and alkaline waters and soils.

The type strain is CG1^T (CCM 7370^T = CECT 7152^T = CGMCC 1.6286^T = DSM 18085^T), isolated from Lake Chagannor, in Inner Mongolia, China.

Description of the type strain.

The description of the type strain is the same as that of the species. The base composition of its DNA is 42.2 mol% G+C, as determined by the thermal denaturation method. Other characteristics are shown in Supplementary Table 1.

Acknowledgements

This study was supported by grants from the Quality of Life and Management of Living Resources Programme of the European Commission (Project "Multigenome access technology for industrial catalysts", QLK3-CT-2002-01972), Spanish Ministerio de Educación y Ciencia (BIO2009-10138 and CGL2010-19303) and Junta de Andalucía (PO6-CVI-01829).

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298 **Legend to Figure**

299 **Fig. 1.** Neighbour-joining tree, based on the 16S rRNA gene sequence comparison,
300 showing the relationship of strain CG1^T with related species. The accession numbers of the
301 sequences used in this study are shown in parentheses after the strain designation.
302 *Paenibacillus polymixa* NCDO 1774^T was used as an outgroup. Bootstrap values >70% are
303 shown. The scale bar represents 0.01 substitutions per nucleotide position.

304 **Table 1.** Cellular fatty acid composition of the seven isolate strains and *B. agaradhaerens*
305 DSM 8712^T grown on alkaline saline medium (pH 10) at 37ºC, for 48 h. Data are
306 percentages of the total fatty acids. -, Values less than 0.5% in all strains; ND, Not detected

Fatty acids	Strains							
	CG1^T	CG2	CG4	CG6	CG7	WE1	103NT4	DSM 8712^T
Straight chain								
$C_{12:0}$	-	-	-	-	-	0.7	0.8	0.5
$C_{14:0}$	1.1	1.0	0.7	0.7	0.7	1.4	1.3	0.7
$C_{16:0}$	4.3	3.9	5.3	3.9	5.6	2.1	2.4	6.0
$C_{18:0}$	1.0	-	0.7	0.5	0.8	0.5	ND	-
Branched								
iso- $C_{14:0}$	3.6	3.1	4.5	3.9	4.6	9.0	5.3	1.0
iso-$C_{15:0}$	11.4	11.4	11.5	11.1	10.3	15.9	11.5	23.3
anteiso $C_{15:0}$	54.1	55.9	42.0	44.2	39.4	46.2	61.5	40.9
iso-$C_{16:0}$	3.8	3.4	6.1	5.3	6.7	4.9	3.7	3.3
iso-$C_{17:0}$	3.9	3.6	6.7	5.6	7.2	0.9	1.5	7.0
anteiso $C_{17:0}$	11.5	11.6	13.9	13.9	15.0	4.4	6.2	11.9
iso-$C_{18:0}$	ND	ND	-	-	0.7	ND	ND	ND
Unsaturated								
$C_{16:1}$ $\omega 7c$ alcohol	ND	ND	ND	ND	ND	2.0	ND	ND
$C_{16:1}$ $\omega 11c$	1.3	1.5	2.1	3.0	2.8	4.3	1.7	1.2
$C_{17:1}$ iso $\omega 10c$	1.7	2.0	3.7	4.9	4.0	3.8	1.7	1.5

$C_{18:1}$ $\omega 9c$	1.2	1.0	1.1	1.0	1.0	2.0	1.6	1.4

307 **Table 2.** Characteristics used to distinguish *Bacillus locisalis* strain CG1^T from *B.*

308 *agaradhaerens* DSM 8721^T (data from this study). +, positive; –, negative.

Characteristic	*Bacillus locisalis* strain CG1^T	*Bacillus agaradhaerens* DSM 8721^T
Sampling site	Water	Soil
Colony pigmentation	Orange	White
Anaerobic growth	+	-
NaCl range (%, w/v)	1-20	0-16
Optimum NaCl (%, w/v)	10	0.5
Optimum temperature (ºC)	37	30
Hydrolysis of starch	-	+
Voges-Proskauer test	-	+
Growth on[a]:		
Aesculin	-	+
D-Fucose	+	-
D-Melezitose	-	+
D-Raffinose	-	+
D-Ribose	-	+
Salicin	-	+
Sucrose	-	+
D-Trehalose	-	+
Butanol	-	+
Ethanol	+	-
Glycerol	-	+
Propanol	-	+
D-Sorbitol	-	+
Xylitol	-	+
Acetate	-	+
Citrate	-	+
Growth on[b]:		
L-Alanine	+	-
L-Arginine	+	-
L-Aspartate	+	-
L-Cysteine	+	-

L-Glutamine	-	+
L-Methionine	+	-
DNA G+C content (mol%)	42.2	39.2
Major fatty acids	Anteiso-$C_{15:0}$ (53%) Anteiso-$C_{17:0}$(13%) Iso-$C_{15:0}$ (13%)	Anteiso-$C_{15:0}$ (41%) Iso-$C_{15:0}$ (25%) Anteiso-$C_{17:0}$(12%)

[a] When supplied as the sole source of carbon and energy.

[b] When supplied as the sole source of carbon, nitrogen, and energy.