**Alkalibacillus almallahensis** sp. nov., a halophilic bacterium isolated from an inland solar saltern

Azahara Pérez-Davó, Margarita Aguilera, Alberto Ramos-Cormenzana and Mercedes Monteoiva-Sánchez

Department of Microbiology, Faculty of Pharmacy, University of Granada, Campus Universitario de Cartuja s/n 18071, Granada, Spain

A halophilic, Gram-staining-positive, non-motile, endospore forming rod-shaped bacterial strain, S1LM8\(^T\), was isolated from a sediment sample collected from an inland solar saltern located in La Malahá, Granada (Spain). Growth was observed in media containing 7.5–30 % total salts (optimum 15 % total salts), at pH 7–10 (optimum pH 8) and at 15–50 °C (optimum 35–38 °C). The predominant isoprenoid quinone was MK-7. It contained A\(_1\gamma\) -type peptidoglycan with meso-diaminopimelic acid as the diagnostic diamino acid. The major cellular fatty acids were anteiso-C\(_{15:0}\), iso-C\(_{15:0}\), anteiso-C\(_{17:0}\) and iso-C\(_{16:0}\). The G+C content of its genomic DNA was 38.2 mol%. The affiliation of strain S1LM8\(^T\) with the species of the genus *Alkalibacillus* was determined by 16S rRNA gene sequence comparison. The most closely related species were *Alkalibacillus halophilus* YIM 012\(^T\) with 99.8 % similarity, *Alkalibacillus salilacus* BH163\(^T\) with 99.8 % similarity and *Alkalibacillus flavidus* ISL-17\(^T\) with 98.1 % similarity between their 16S rRNA gene sequences. However, DNA–DNA relatedness between the novel isolate and the related species of the genus *Alkalibacillus* was less than 34 %. Based on the phylogenetic, phenotypic and chemotaxonomic features, a novel species, *Alkalibacillus almallahensis* sp. nov. is proposed. The type strain is S1LM8\(^T\) (= CECT 8373\(^T\)=DSM 27545\(^T\)).

The last few years have seen increasing research on life in extreme environments and the description of isolates representing novel taxa. The reasons include improved culture methods, sampling from a wider range of geographically distinct sites and increased interest in understanding the influences of microbial communities in diverse environments around the world. There are a large number of recognized genera in the family *Bacillaceae* including taxa described as moderately halophilic or halotolerant, some of which are also alkaliophilic (i.e. *Amphibacillus*, *Bacillus*, *Gracilibacillus*, *Halobacillus*, *Filobacillus*, *Oceanobacillus* and *Virgibacillus*) (Arahal & Ventosa, 2002, Logan et al., 2009).

The genus *Alkalibacillus* was proposed by Jeon et al. (2005) based on a reclassification of *Bacillus haloalkalophilus* as *Alkalibacillus haloalkalophilus* and at the time of writing contains six species, *Alkalibacillus haloalkalophilus* (Fritze, 1996), *Alkalibacillus salilacus* isolated from a salt lake (Jeon et al., 2005), *Alkalibacillus filiformis* isolated from water of a mineral pool (Romano et al., 2005), *Alkalibacillus halophilus* isolated from hypersaline soil (Tian et al., 2007), *Alkalibacillus silvisoli* isolated from non-saline forest soil (Usami et al., 2007) and *Alkalibacillus flavidus* isolated from marine solar saltern (Yoon et al., 2010). Members of the genus *Alkalibacillus* are spore-forming long rods, Gram-stain-variable, catalase-positive, urease-negative, with spherical endospores and swollen sporangia, strictly aerobic and moderately halophilic, which can be differentiated from members of phylogenetically related genera, i.e. *Filobacillus*, on the basis of peptidoglycan A\(_1\gamma\) -type peptidoglycan with meso-diaminopimelic acid and 16S rRNA gene sequence similarity (Jeon et al., 2005). This genus has been of special industrial interest, particularly for enzyme production (Fritze, 1996).

In this study, a novel halophilic bacterium, designated S1LM8\(^T\), was isolated from a sediment sample collected from an inland solar saltern located in La Malahá, (Granada, southern Spain). La Malahá (37° 6’ 5” N 3° 43’ 21” W) is a town in Granada, the name of which derives from the Arabic word ‘al-Mallahá’, meaning ‘factory of salt’. Strain S1LM8\(^T\), was characterized by a polyphasic approach, including phylogenetic analysis based on 16S rRNA gene sequences, genomic relatedness and chemotaxonomic and phenotypic properties. The results obtained in this study indicated that S1LM8\(^T\) is a member of the genus *Alkalibacillus*, but it is clearly distinguishable from all species of the genus *Alkalibacillus* with validly published names.

Samples were spread on MH complex medium supplemented with a balanced mixture of sea salts to give adequate salt
concentrations for growth of moderately halophilic strains. This medium contained (per litre) 10 g yeast extract (Difco), 5 g proteose-peptone number 3 (Difco) and 1 g glucose (Ventosa et al., 1982) and was supplemented with a balanced mixture of sea salts of Subov (1931). The pH was adjusted to 7.3 with 1 M NaOH. The medium was solidified with 20 g Bacto Agar (Difco) l⁻¹. The isolate was maintained and routinely grown aerobically on MH complex medium with 15 % (w/v) total salts at 35 °C except where indicated otherwise.

Genomic DNA was extracted according to the protocol of Marmur (1963). The 16S rRNA genes were amplified by PCR using primers 16F27 (5’-AGAGTTTGATCMTGGC-TCAG-3’) and 16R1525 (5’-AAGGAGGTGWTCCARC-C-3’) (Kharroub et al., 2011). PCRs were carried out under the conditions described by Saiki et al. (1988). PCR products were purified with a Microcon-100 concentrator (Amicon). The sequence was determined using an Applied Biosystems PRISM TaqDyeDeoxy DNA sequencer. Primers required for growth of strain S1LM8T were determined at 35 °C. The strain was cultured in MH medium containing the following concentrations of a balanced mixture of sea salts (Subov, 1931): 0.5, 3, 5, 7.5, 10, 15, 20, 25 and 30 % (w/v). Each 50 ml batch of medium was inoculated with 0.1 ml (approximately 10⁵ cells ml⁻¹) from an appropriate dilution of a 20 h culture of the micro-organism grown in MH medium containing 10 % (w/v) total salts. The cultures were incubated in a rotary shaker. Viable cells were determined from plate counts on solid MH medium at the appropriate salt concentration. Experiments were performed in triplicate. The pH growth range was determined in a

**Fig. 1.** Neighbour-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequences of strain S1LM8T and other related taxa. Branches with closed circles were also identified with the maximum-likelihood and neighbour-joining methods. Bootstrap values (expressed as percentages of 1000 replications) greater than 70 % are shown. Bar, 0.01 substitutions per nucleotide position. The sequence of *Brevibacillus fluminis* CJ71T was used as an outgroup.
similar way on MH medium by adjusting the final pH to values between pH 5 and 10 (at intervals of 0.5 pH units) adjusted with HCl or NaOH buffered with NaHPO₄/Na₂HPO₄ solutions. The temperature range was determined as described above by incubating the strain at temperatures from 4 to 55°C (4, 10, 15, 20, 25, 30, 32, 35, 38, 40, 45, 50 and 55°C). Anaerobic growth in the presence of L-arginine was tested as described by Hartmann et al. (1980). Anaerobic growth was determined by incubation in an anaerobic chamber at 37°C for 5 days on MH containing 20% salt solution (Subov, 1931); anaerobic conditions were prepared under an atmosphere generation system (AGS; Oxoid). Tests for catalase, oxidase, hydrolysis of gelatin, starch and Tween 80 were conducted according to the methods of Kharrourb et al. (2008) and test for hydrolysis of casein according to the method of Berd (1973). Additionally, the API 20E strips (bioMérieux) were used for phenotypic characterization. DNA hydrolysis was tested according to the methods of Smibert & Krieg (1994). The utilization of carbohydrates, sugar alcohols, amino acids and organic acids and the acids production from carbohydrates and sugar alcohols were carried out as described by Arahal & Ventosa (2002). Susceptibility to antibiotics was assayed as described by Mata et al. (2002). The closely related strains of species of the genus *Alkalibacillus*, *A. salilacus* DSM 16460ᵀ, *A. haloalkaliphilus* DSM 5271ᵀ, *A. filiformis* DSM 15448ᵀ, *A. halophilus* KCTC 3990ᵀ, *A. silvisoli* DSM 18495ᵀ and *A. flavidus* KCTC 13258ᵀ were used as reference strains.

Cell morphology and endospore formation were studied by using a phase-contrast optical microscope (standard 25; Zeiss) and Gram staining was performed according to the protocol of Dussault (1955). Transmission electron microscopy was carried out with cells grown in MH broth at 15% salts (w/v). Samples were negatively stained with 2% (w/v) uranyl acetate (30s) and washed with an acetic acid solution at 3% (w/v). The cell morphology and flagella were examined using a high-resolution transmission electron microscope at 80 kW (TEM 902; Zeiss).

Cells of strain S1LM8ᵀ were Gram-staining-positive, non-motile, sporulating rods approximately 8.0–10.0 μm long by 0.5–0.7 μm wide. Terminal endospores were observed (see Fig. S1, available in the online Supplementary Material). Colonies on agar plates were smooth, white, with a diameter that did not exceed 1 mm after 5 days of growth. In the presence of 20–30% total salts, colonies were light orange. Strain S1LM8ᵀ grew in media containing from 7.5 to 30% total salts, optimally at 15%. The range of temperatures for growth was 15–50°C, the optimum was 35–38°C, and the optimum pH for growth was pH 8.0 (pH range for growth was pH 7–10). The phenotypic characteristics of strain S1LM8ᵀ are summarized and compared with those obtained from type strains of related species of the genus *Alkalibacillus* in Table 1 and in the species description. Strain S1LM8ᵀ is able to grow in one of the broadest range of NaCl concentrations (7.5–30%) and at pH 10. Cells of S1LM8ᵀ

**Table 1. Differential characteristics of strain S1LM8ᵀ and type strains of related species of the genus *Alkalibacillus***

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell morphology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Long rods</td>
<td>White to light orange</td>
<td>Rods</td>
<td>Cream</td>
<td>Long rods</td>
<td>White to transparent</td>
<td>Long rods</td>
</tr>
<tr>
<td><strong>Gram reaction</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>NaCl range (%) (w/v)</strong></td>
<td>7.5–30</td>
<td>5–20</td>
<td>0–25</td>
<td>0–18</td>
<td>5.0–30.0</td>
<td>5.0–25.0</td>
<td>4.0–26</td>
</tr>
<tr>
<td><strong>pH range</strong></td>
<td>7.0–10.0</td>
<td>7.0–9.0</td>
<td>7.5–10.0</td>
<td>7.0–10.0</td>
<td>6.5–9.0</td>
<td>7.0–10.0</td>
<td>5.5–9.5</td>
</tr>
<tr>
<td><strong>Oxidase</strong></td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><strong>Reduction of nitrate to nitrite</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Hydrolysis of</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>w</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
<td>w</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Acid production from</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Fructose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>DNA G + C content (mol%)</strong></td>
<td>38.2</td>
<td>41.0</td>
<td>38.0</td>
<td>39.5</td>
<td>39.0</td>
<td>37.0</td>
<td>48.1</td>
</tr>
</tbody>
</table>
were non-motile like those of *A. filiformis* and its pigmentation was light orange when bacteria were grown with 20 % salts as distinct from the cream colour described for the rest of the species of the genus *Alkalibacillus*. Moreover, S1LM8T showed a terminal endospore and swollen sporangium, which is typical for this genus. An important and distinctive feature from the other species of the genus *Alkalibacillus* with validly published names was the production of acids from glycerol by S1LM8T.

For analysis of cellular fatty acids, isoprenoid quinones and peptidoglycan structure, cells were cultivated on MH complex medium supplemented with 15 % (w/v) total salts for 2 days at 35 °C under aerobic conditions. The fatty acids were extracted, methylated and analysed using the standard MIDI system (Sherlock version 6.1; Microbial Identification) (Sasser, 1990) and an Agilent 6890 GC. Isoprenoid quinones were analysed as described by Tindall (1990a, 1990b) by TLC on silica gel (art. no. 805023; Macherey-Nagel). UV-absorbing bands corresponding to the different quinone classes were removed from the plate and further analysed by HPLC. Analysis of the peptidoglycan structure was carried out according to the procedures of Schleifer & Kandler (1972), Schleifer (1985) and Schumann (2011).

The cellular fatty acid profile of strain S1LM8T was characterized by a large percentage of branched saturated fatty acids (Table S1). The major fatty acids were: anteiso-C15:0 (34.7 %), iso-C15:0 (26.7 %), anteiso-C17:0 (19.8 %) and iso-C16:0 (8.2 %), which were in accordance with the reported profiles of other species of the genus *Alkalibacillus* (Table S1). S1LM8T possessed A1γ-type peptidoglycan with meso-diaminopimelic acid as the diagnostic diamino acid, as in all species of the genus *Alkalibacillus*, which was in common with the great majority of endospore-forming, Gram-staining positive strains of the family *Bacillaceae*. The predominant isoprenoid quinone detected in strain S1LM8T was menaquinone-7 (MK-7).

Genomic DNA G+C content was determined from the mid-point value (Tm) of the thermal denaturation profile (Marmur & Doty, 1962). Tm was determined by the graphic method described by Ferragut & Leclerc (1976) and the DNA G+C content was calculated from this temperature by using the equation of Owen & Hill (1979). DNA–DNA hybridization between S1LM8T and closely related species was conducted following the method of Lind & Ursing (1998) with the modifications of Ziemke et al. (1998). The mean G+C content of strain S1LM8T was 38.2 mol% (Tm method), this is a low G+C content that was closest to those of *A. haloalcaliphilus* and *A. halophilus*. All these three species of the genus *Alkalibacillus* showed as common features growth with high salts and similar ranges of pH and temperatures for growth (Table 1). In general, DNA G+C contents could be associated with adaptation to different survival conditions (Rothschild & Mancinelli, 2001; Logan et al., 2009).

DNA–DNA hybridization experiments between strain S1LM8T and the related species of the genus *Alkalibacillus* revealed levels of relatedness of 33 % with *A. halophilus* KCTC 3990T, 17 % with *A. saliticus* DSM 16460T and 16 % with *A. flavidus* KCTC 13258T. These levels of DNA–DNA hybridization are low enough to classify strain S1LM8T as a representative of a novel species of the genus *Alkalibacillus*.

On the basis of the data presented, strain S1LM8T is considered to represent a novel species of the genus *Alkalibacillus*, for which the name of *Alkalibacillus almallahensis* sp. nov. is proposed.

**Description of *Alkalibacillus almallahensis* sp. nov.**

*Alkalibacillus almallahensis* (al.mal.lah.en’sis. N.L. masc. adj. *almallahensis* of al-mallah, a name of Arabic origin, which gave the name to the township of La Malahá, the place where the type strain was isolated).

Cells are Gram-staining-positive, strictly aerobic and non-motile. Terminal spherical endospores are observed in swollen sporangia. Cells are 8.0–10.0 μm long by 0.5–0.7 μm wide. Colonies are smooth, circular, white and about 1 mm or less in diameter after incubation for 5 days at 35 °C. In the presence of 20–30 % of total salts, colonies are light orange. Growth occurs in media containing 7.5–30 % total salts (w/v) with optimal growth at 15 % total salts (w/v). Growth occurs at pH 7.0–10.0, the optimal pH being 8.0. Optimal temperatures for growth are 35–38 °C. Growth is observed at temperatures in the range of 15–50 °C. Mg2+ is not required for growth. Catalase-positive and oxidase-positive. Nitrate and nitrite are not reduced. Negative for lysine decarboxylase, ornithine decarboxylase, arginine dehydrogenase, β-galactosidase and tryptophan deaminase. Hydrolysis of casein, gelatin, starch, urea, DNA and Tween 80 are negative. Voges–Proskauer and H2S production are negative. Utilizes citrate as sole carbon and energy source, but not D-glucose, lactose, D-fructose, D-galactose, maltose, L-arabinose, cellobiose, D-mannose, raffinose, L-rhamnose, melibiose, sucrose, sorbitol, inositol, mannitol, L-alanine, L-aspartate, L-lysine, glycine, succinate, malate or pyruvate. Acids are produced from glycerol but not from D-glucose, lactose, D-fructose, D-galactose, maltose, L-arabinose, cellobiose, D-mannose, raffinose, L-rhamnose, melibiose, sucrose, sorbitol, inositol and mannitol. Indole production is negative. The major fatty acids are anteiso-C15:0, iso-C15:0, anteiso-C17:0 and iso-C16:0 when grown on MH medium containing 15 % (w/v) total salts. A1γ-type peptidoglycan with meso-diaminopimelic acid. Menaquinone-7 (MK-7) is the predominant isoprenoid quinone detected.

The type strain is S1LM8T (= CECT 8373T = DSM 27545T), isolated from an inland solar saltern located in La Malahá, Granada (southern Spain). The DNA G+C content of the type strain is 38.2 mol% (determined by the Tm method).

**ACKNOWLEDGEMENTS**

This work was supported by grants of the Ministry of Science and Innovation (Spain) (project no. CGL2008-00447)
REFERENCES


