Lysinibacillus alkalisoli sp. nov., isolated from saline–alkaline soil

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Abstract

A Gram-stain-positive, aerobic bacterial strain, designated Y2A20T, with peritrichous flagella was isolated from the top layer saline–alkaline soil, Hangjin Banner, Inner Mongolia, northern China. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain Y2A20T formed a stable clade with ‘Lysinibacillus jejuensis’ N2-5T. Strain Y2A20T shared highest 16S rRNA gene sequence similarity with ‘L. jejuensis’ N2-5T (97.4 %), but lower 16S rRNA gene sequence similarities with all other type strains (<97.0 %). The major polar lipids of strain Y2A20T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two unknown aminophospholipids and three unknown phospholipids. Menaquinone-7 was the predominant menaquinone, while iso-C15:0, anteiso-C17:0 and iso-C16:0 were the major cellular fatty acids. Its genomic DNA G+C content was 39.0 mol%. DNA–DNA hybridization revealed that strain Y2A20T showed 26±5 % genomic DNA relatedness with its closest relative, ‘L. jejuensis’ N2-5T. The results of physiological and biochemical tests allowed the discrimination of strain Y2A20T from its phylogenetic relatives. Lysinibacillus alkalisoli sp. nov. is therefore proposed with Y2A20T (=CGMCC 1.15760T=KCTC 33825T) as the type strain.

The genus Lysinibacillus in the family Bacillaceae (phylum Firmicutes) was first described by Ahmed et al. [1]. Lysinibacillus boronitolerans was designated as the type species of the genus. The characteristic main fatty acids of this genus are iso-C15:0, anteiso-C15:0, anteiso-C17:0, C16:1ω7c alcohol and iso-C16:0. The main quinone is menaquinone-7 (MK-7). Most of the type strains in the genus Lysinibacillus were isolated from soil environments [2–7], but some also from organic solvent [8, 9], fermented food [10, 11], surface water [12], sludge [13], livestock waste [14, 15], compost [16], leaves [17], forest humus [18] and river sediment [19]. Most Lysinibacillus strains are characteristically halotolerant [3, 11]. At the time of writing, the genus Lysinibacillus is composed of 27 species with valid published names.

During an investigation into the saline–alkaline soil microbial community, a bacterial strain was isolated from a saline–alkaline soil sampled from farmland in Hangjin Banner–alkaline soil, Hangjin Banner, Inner Mongolia, northern China. By using the 10-fold dilution method on Luria–Bertani agar (LB: tryptone 10 g l−1, yeast extract 5 g l−1, NaCl 10 g l−1, agar 20 g l−1, pH 7.0). After growth in LB medium at 30 °C for 2 days in the dark, cells of strain Y2A20T were harvested for extraction of genomic DNA and amplification of the 16S rRNA gene with a previously described protocol [20]. After ligation into pMD19-T vector (TaKaRa) following the manufacturer’s instructions, the amplified 16S rRNA gene fragment was sequenced. The almost-complete 16S rRNA gene sequence (1347 nt) was compared with available DNA sequences in GenBank by using the BLAST tool (http://blast.ncbi.nlm.nih.gov) to determine the strain’s approximate taxonomic affiliation. After a multiple alignment of the data by CLUSTAL X software [21], phylogenetic analysis was performed using the software package MEGA version 6.1 [22]. Phylogenetic trees were then reconstructed with the neighbour-joining [23], maximum-likelihood [24] and minimum-evolution [25, 26] algorithms. The neighbour-joining tree used maximum composite likelihood distance [22], the maximum-likelihood tree used Tamura–Nei model [22], the minimum-evolution tree used Kimura’s two-parameter model [22] and each of them was reconstructed with the complete deletion option. Bootstrap analysis (1000 replications) was used to assess the stability of the tree topology [27]. Similarities between strain Y2A20T and its relatives were evaluated by using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; [28]). Strain Y2A20T shared a 16S rRNA gene similarity of 97.4 % with ‘Lysinibacillus jejuensis’ N2-5T, and less than 97 % similarity with all other type strains. The neighbour-joining phylogenetic tree revealed that strain Y2A20T was clustered with ‘L.
jejuensis’ N2-5T with a bootstrap value of 98% (Fig. 1). This affiliation and high bootstrap values were also supported by the maximum-likelihood and minimum-evolution phylogenetic trees (Figs S1 and S2, available in the online Supplementary Material). Therefore, ‘L. jejuensis’ N2-5T was selected as the reference strain for further biochemical and chemotaxonomic analyses.

The genomic DNA G+C content was determined using the thermal denaturation method [29] using DNA from Escherichia coli K12 as a standard reference. DNA–DNA hybridization was carried out to assess the genomic DNA relatedness between strain Y2A20T and ‘L. jejuensis’ N2-5T by using the optical renaturation rate method described by De Ley et al. [30] with the modifications described by Huss et al. [31]. The genomic DNA G+C content of strain Y2A20T was 39.0 mol%, within the values recorded for all Lysinibacillus strains (35.9–43.2%) [1, 15], but much lower than that of ‘L. jejuensis’ N2-5T (43.3 mol%; 15). The DNA–DNA hybridization value of strain Y2A20T with ‘L. jejuensis’ N2-5T was 26±5%, below the threshold value (70%) recommended for defining a novel species [32, 33].

For cellular fatty acid analysis, cells of strains Y2A20T and ‘L. jejuensis’ N2-5T were grown on trypticase soy agar (TSA; Difco) at 30°C and were harvested at the same stage during the exponential growth period (2 days). Fatty acid methyl esters were prepared and identified by following the instructions of the Microbial Identification system (MIDI) as described by Sasser [34]. Polar lipids were extracted, and examined by two-dimensional TLC as described previously [35]. Menaquinones were extracted with chloroform/methanol (2 : 1, v/v) solution and analysed as described by Komagata and Suzuki [36] using HPLC. The cellular fatty acids of

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**Fig. 1.** Phylogenetic tree reconstructed by the neighbour-joining algorithm based on 16S rRNA gene sequences. *Paenibacillus polymyxa* NCDO1774T was used as the outgroup strain. Numbers on branch nodes are bootstrap values greater than 50% (of 1000 resamplings). Bar, 0.01 substitutions per nucleotide position.
strain Y2A20\textsuperscript{T} and ‘L. jejuensis’ N2-5\textsuperscript{T} are listed in Table 1. The major fatty acids of strain Y2A20\textsuperscript{T} were iso-C\textsubscript{15:0} 3-OH, iso-C\textsubscript{16:0} and iso-C\textsubscript{17:0} \textsubscript{3-OH} in line with those of ‘L. jejuensis’ N2-5\textsuperscript{T}. However, some fatty acid proportions were different, i.e. strain Y2A20\textsuperscript{T} had higher proportions of iso-C\textsubscript{16:0} and iso-C\textsubscript{17:0} \textsubscript{3-OH} and lower proportions of C\textsubscript{16:1} \textsubscript{ω7c} alcohol and iso-C\textsubscript{17:1} \textsubscript{ω10c} than ‘L. jejuensis’ N2-5\textsuperscript{T}. The predominant isoprenoid quione of strain Y2A20\textsuperscript{T} was MK-7 (88.1 %), similar to those of ‘L. jejuensis’ N2-5\textsuperscript{T} [15] and other Lysinibacillus strains [1]. Furthermore, small amounts of MK-6 (5.1 %) and MK-8 (6.8 %) were detected in strain Y2A20\textsuperscript{T}. The polar lipids of strain Y2A20\textsuperscript{T} consisted of diphosphatidyglycerol, phosphatidylglycerol, phosphatidyethanolamine, two unknown aminophospholipids and three unknown phospholipids (Fig. S3). The profile of major polar lipids of strain Y2A20\textsuperscript{T} was generally similar to that of ‘L. jejuensis’ N2-5\textsuperscript{T} [15]. The cellular fatty acid and polar lipid profiles both supported the affiliation of strain Y2A20\textsuperscript{T} to the genus Lysinibacillus.

After strain Y2A20\textsuperscript{T} was grown on LB agar at 30 °C for 2 days, cells were harvested, air dried and negatively stained with 1 % (w/v) phosphotungstic acid. Its morphological features were examined by using transmission electron microscopy (Hitachi-7000). Gram-staining and endospore formation were investigated as described by Smibert and Krieg [37]. The temperature range for growth was determined in LB medium at 4, 10, 15, 20, 25, 30, 35, 37, 40, 45 and 50 °C. Growth at different pH values (pH 4.0–12.0 in 1.0 unit intervals) was assessed in LB medium adjusted with 10 % (w/v) HCl or NaOH solutions, after 2 days of incubation at 30 °C. NaCl tolerance was tested by using a modified LB broth amended with 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 % (w/v) NaCl. Cell motility was determined in LB medium containing 0.4 % agar [38]. Anaerobic growth was tested in LB medium supplemented with nitrate as described by Kim et al. [39]. Antibiotic sensitivity tests were performed using the diffusion method [40] on LB agar and Mueller–Hinton agar with filter-paper discs (8 mm diameter) containing one of the following antibiotics: ampicillin (10 µg), amoxicillin (10 µg), bacitracin (0.04 U), carbenicillin (100 µg), cefadroxil (30 µg), chloromycetin (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), novobiocin (30 µg), penicillin G (10 U), rifampicin (5 µg), roxithromycin (15 µg), spectinomycin (100 µg), streptomycin (10 µg), trimethoprim (5 µg), tetracycline (30 µg) and vancomycin (30 µg). The antibiotic activities of strain Y2A20\textsuperscript{T} were determined by the size of the inhibition zone according to Dong and Cai [38]. Oxidase and catalase activities were evaluated by adding oxidase reagent (bioMérieux) and 3 % hydrogen peroxide solution to fresh colonies [38]. Other biochemical characteristics were tested using API 20NE and API ZYM kits (bioMérieux) according to the manufacturer’s instructions. The reference strain ‘L. jejuensis’ N2-5\textsuperscript{T} was simultaneously tested under the same conditions. Cells of strain Y2A20\textsuperscript{T} were Gram-stain-positive and rod-shaped with several peritrichous flagella (Fig. S4). Cells of strain Y2A20\textsuperscript{T} were 0.6–1.0 µm in width and 1.3–2.6 µm in length, much smaller than those of ‘L. jejuensis’ N2-5\textsuperscript{T}. Strain Y2A20\textsuperscript{T} was resistant to bacitracin, carbenicillin, rifampicin and streptomycin, but sensitive to amoxicillin, ampicillin, cefadroxil, chloromycetin, erythromycin, gentamicin, kanamycin, novobiocin, penicillin G, roxithromycin, spectinomycin, tetracycline, trimethoprim and vancomycin. Other phenotypic features of strain Y2A20\textsuperscript{T} are listed in the species description and in Table 2.

On the basis of the phylogenetic, phenotypic, chemotaxonomic and morphological data, we propose that strain Y2A20\textsuperscript{T} should be classified as the type strain of a novel species within the genus Lysinibacillus, for which the name Lysinibacillus alkalisoli sp. nov. is proposed.

### Table 1. Fatty acid profile of strain Y2A20\textsuperscript{T} and its reference strain ‘L. jejuensis’ N2-5\textsuperscript{T}

All data were from the present study. Values are percentages of the total fatty acids. Fatty acids comprising <0.5 % of the total in both strains are not shown. ND: Not detected.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Y2A20\textsuperscript{T}</th>
<th>‘L. jejuensis’ N2-5\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso-C\textsubscript{15:0}</td>
<td>39.3</td>
<td>41.2</td>
</tr>
<tr>
<td>anteiso-C\textsubscript{15:0}</td>
<td>6.1</td>
<td>2.2</td>
</tr>
<tr>
<td>C\textsubscript{16:0}</td>
<td>1.9</td>
<td>ND</td>
</tr>
<tr>
<td>C\textsubscript{16:1ω7c} alcohol</td>
<td>6.1</td>
<td>15.1</td>
</tr>
<tr>
<td>iso-C\textsubscript{16:0}</td>
<td>12.2</td>
<td>11.4</td>
</tr>
<tr>
<td>C\textsubscript{16:1ω11c}</td>
<td>2.3</td>
<td>2.1</td>
</tr>
<tr>
<td>iso-C\textsubscript{17:0}</td>
<td>6.6</td>
<td>13.3</td>
</tr>
<tr>
<td>iso-C\textsubscript{17:1ω7c}</td>
<td>11.1</td>
<td>5.9</td>
</tr>
<tr>
<td>anteiso-C\textsubscript{17:0}</td>
<td>7.1</td>
<td>2.2</td>
</tr>
<tr>
<td>C\textsubscript{18:0}</td>
<td>0.8</td>
<td>ND</td>
</tr>
<tr>
<td>Summed feature 4*</td>
<td>1.9</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Summed feature 4\* consisted of iso-C\textsubscript{17:1ω7c} and/or anteiso-C\textsubscript{17:1ω7c}.

### DESCRIPTION OF LYSINIBACILLUS ALKALISOLI SP. NOV.

Lysinibacillus alkalisoli [al.ka.li.so’li. N.L. n. alkali (from Arabic al-qaliy), alkali; L. n. solum soil; N.L. gen. n. alkalisoli of alkaline soil, referring to the fact that the type strain was isolated from alkaline–saline soil].

Cells are Gram-stain-positive rods, 0.6–1.0 µm in width and 1.3–2.6 µm in length, with several peritrichous flagella. Ellipsoidal endospores are formed in a terminal position in swollen sporangium after 3 days of incubation. Colonies on LB agar at 30 °C for 48 h are circular with jagged margins, ivory-coloured, coarse, of low convexity and usually 2–3 mm in diameter. Grows at 10–45 °C (optimum, 30 °C), at pH 6.0–10.0 (optimum, pH 8.0–9.0) and with 0–8 % (w/v) NaCl (optimum, 1–2 %). Positive for activities of arginine dihydrolase, urease, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase; negative for activities of nitrile reductase, indole production, glucose fermentation, hydrolysis of...
Table 2. Differential characteristics between strain Y2A20T and ‘L. jejuniensis’ N2-5T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Y2A20T</th>
<th>‘L. jejuniensis’ N2-5T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth temperature (°C)</td>
<td>10–45</td>
<td>10–37*</td>
</tr>
<tr>
<td>Oxidase</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>API 20NE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Mannose assimilation</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Mannitol assimilation</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Malic acid assimilation</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Trisodium citrate assimilation</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>API ZYM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>39.0</td>
<td>43.3*</td>
</tr>
<tr>
<td>NaCl tolerance (% w/v)</td>
<td>0–8</td>
<td>1–3*</td>
</tr>
</tbody>
</table>

Data were from the present study except where indicated otherwise (“Kim et al. [15]). The two strains are positive for activities of catalase, arginine dihydrolase, urease, acid phosphatase, naphthol-AS-BI-phosphohydrolase, esterase (C4) and esterase lipase (C8). They are negative for activities of nitrate reduction, indole production, glucose fermentation, aesculin hydrolysis, β-galactosidase, lipase (C14), cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-mannosidase, and assimilation of D-glucose, L-arabinose, N-acetyl-glucosamine, maltose, potassium glutonate, capric acid, adipic acid and phenylacetic acid. +, Positive; −, negative.

The type strain, Y2A20T (=CGMCC 1.15760T=KCTC 33825T), was isolated from a top layer of saline–alkaline soil sample collected from Inner Mongolia, China. The genomic DNA G+C content of the type strain is 39.0 mol%.

References

Conflicts of interest
The authors declare that there are no conflicts of interest.

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