**Lysinibacillus alkaliphilus** sp. nov., an extremely alkaliphilic bacterium, and emended description of genus *Lysinibacillus*

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A novel aerobic, alkaliphilic, Gram-staining-positive, endospore-forming bacterium, strain OMN17T, was isolated from a typical sandy loam soil under long-term OMN fertilization (half organic manure N plus half mineral N fertilizer) in northern China and was subjected to a polyphasic taxonomic study. The best growth was achieved at 30 °C and pH 8–10 in medium containing 0.5 % (w/v) NaCl. The cell-wall peptidoglycan of strain OMN17T was type A4z; (L-Lys–Gly–D-Asp) and the cell-wall sugars were ribose, traces of galactose and arabinose. The only respiratory quinone found in strain OMN17T was menaquinone 7 (MK-7). The major cellular fatty acids were iso-C15 : 0 and anteiso-C15 : 0. The major polar lipids were found to be phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Phylogenetic analysis of strain OMN17T based on the 16S rRNA gene sequence showed that the strain was most closely related to *Lysinibacillus halotolerans* (97.8 %), *Lysinibacillus sinduriensis* (97.5 %), *Lysinibacillus chungkukjangi* (97.4 %) and *Lysinibacillus xylanilyticus* (97.0 %). The DNA–DNA hybridization results indicated that this strain was distinct from other species of the genus *Lysinibacillus*, the degree of relatedness being 21.8 ¡ 0.2 % with *L. halotolerans*, 45.6 ¡ 1.8 % with *L. sinduriensis*, 33.7 ¡ 1.2 % with *L. chungkukjangi* and 23.7 ¡ 0.7 % with *L. xylanilyticus*. The DNA G+C content of strain OMN17T was 38.1 mol%. The phenotypic, chemotaxonomic and genetic analyses identified strain OMN17T as a novel species of the genus *Lysinibacillus*, for which the name *Lysinibacillus alkaliphilus* sp. nov. is proposed. The type strain is OMN17T (=DSM 28019T= CCTCC AB 2014073T). An emended description of the genus *Lysinibacillus* is also provided.

The genus *Lysinibacillus* belongs to the family *Bacillaceae* of the phylum *Firmicutes*, and was proposed for two former species of the genus *Bacillus*, *Bacillus fusiformis* and *Bacillus sphaericus*, and a newly described species, *Lysinibacillus boronitolerans* (Ahmed et al., 2007). At the time of writing, however, the genus *Lysinibacillus* is unique among the family *Bacillaceae* as it is characterized by a special A4z-type (Lys–Asp) cell-wall peptidoglycan structure. At the time of writing, the genus *Lysinibacillus* consisted of eighteen species with validly published names (http://www.bacterio.net/lysinibacillus.html) with *Lysinibacillus boronitolerans* as the type species. Members of this genus have been isolated from environments such as different types of soil (Ahmed et al., 2007; Miwa et al., 2009; Liu et al., 2013), surface water (Kämpfer et al., 2013), fermented soybean food (Kim et al., 2013), and inner tissues of plants and human beings (Glazunova et al., 2006; Duan et al., 2013). In this study, during an investigation of the diversity of bacterial population of typical sandy loam soil under long-term fertilization, an endospore-forming, Gram-staining-positive bacterium, strain OMN17T, was isolated and found to have morphological properties consistent with the genus *Lysinibacillus*.

Strain OMN17T was isolated from a typical sandy loam soil that was part of the State Experimental Station for Agro-Ecology, Fengqiu country (35° 00’ N 114° 24’ E), Henan province, China. The soil had been described previously by the authors (Zhao et al., 2014). A standard dilution-plating technique was used to isolate the strain on nutrient...
agar (5 g peptone, 3 g meat extract, 1 l distilled water; pH 7.2–7.4) at 30 °C for 2 days. Strain OMN17<sup>T</sup> was maintained on trypticase soy agar [TSA; trypticase soy broth (TSB) supplemented with 15 g Bacto Agar (Difco)] at 4 °C and stored in 20 % (v/v) glycerol suspensions at −20 °C.

To characterize strain OMN17<sup>T</sup> phenotypically, standard phenotypic tests were performed according to general protocols (Gordon et al., 1974; Lányi, 1987). Cellular morphology and motility were examined by light microscopy (Axio imager A1; Zeiss) and transmission electron microscopy (H-7650; Hitachi) using cells from the exponential phase. The morphology, size and pigment of colonies were observed on TSA plates after 2 days at 30 °C. Growth in TSB (BBL, Becton Dickinson) at 4 °C, 10 °C, 15 °C, 20 °C, 30 °C, 37 °C, 45 °C and 55 °C in an aerobic incubator was evaluated by measuring the turbidity of the broth by spectrophotometry after 1–7 days. Growth ability was tested at 30 °C in TSB adjusted to pH 4.0–13.5 at increments of 0.5 pH units by using 1 M hydrochloric acid and 10 % sodium hydroxide solutions. Growth in the absence of NaCl and in the presence of 1–10.0 % (w/v) NaCl at 0.5 % intervals was investigated at 30 °C in TSB. The Gram reaction was performed using the standard Gram-staining method (Smibert & Krieg, 1994). After incubation at 30 °C for 7 days (decline phase), spore formation was determined by staining with malachite green, as described by Conn et al. (1957). The chemotaxonomic characteristics of strain OMN17<sup>T</sup> were determined using cells cultured on TSA. Catalase activity was determined on freshly growing colonies using 5 % (v/v) hydrogen peroxide solution. The oxidase reaction was performed on filter paper moistened with a 1 % (v/v) aqueous solution of N,N,N′,N′-tetramethyl-p-phenylenediamine. Anaerobic growth of the cells was studied for 7 days at 30 °C in anaerobic agar (Claus & Berkeley, 1986). Hydrolysis of Tween 80 was determined as described by Cowan & Steel (1965). Metabolic properties and enzyme activities were tested using API 20 E, API 20 NE, API 50 CHB/E and API ZYM systems (bioMérieux), and the Biolog GP2 MicroPlate system (Biolog), according to the manufacturers’ instructions. All physiological assays were repeated three times.

For analysis of peptidoglycan structure, whole cells of strain OMN17<sup>T</sup> were hydrolysed (4 M HCl, 100 °C, 16 h) and the hydrolysates were subjected to TLC on cellulose plates according to the method of Schumann (2011). For analysis of the cell-wall sugars, the cells were hydrolysed (0.5 M H<sub>2</sub>SO<sub>4</sub>, 100 °C, 2 h) and the sugars were analysed by TLC on cellulose plates, following Schumann (2011). For polar lipids and respiratory quinones analysis, cell mass of strain OMN17<sup>T</sup> was harvested from medium DSM 92 after incubation for 24 h at 28 °C (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium92.pdf). Polar lipids were extracted from 100 mg of freeze-dried cell material and were separated by two-dimensional silica gel TLC (no. 818 135; Macherey-Nagel) as described by Tindall et al. (2007). For the analysis of the cellular fatty acids, biomass of strain OMN17<sup>T</sup> and four closely related strains were harvested from TSA after incubation for 24 h at 28 °C (exponential stage) according to the standard MIDI protocol. Fatty acid methyl esters were obtained from 40 mg cells and separated using the Sherlock Microbial Identification System (TSBA6, version 6.1; MIDI). Respiratory quinones were extracted from 100 mg freeze-dried cells and were separated by TLC on silica gel (no. 805 023; Macherey-Nagel). Ultraviolet-absorbing bands were removed from the plate and further analysed by HPLC (LDC Analytical; Thermo) with a reverse phase column (2 mm × 125 mm, 3 μm, RP18; Macherey-Nagel). Respiratory quinones were detected at 269 nm. Analysis of peptidoglycan structure, cell-wall sugars, polar lipids and respiratory quinones was carried out by the Identification Service of the DSMZ, Braunschweig, Germany.

For analysis of the 16S rRNA gene sequence, bacterial DNA was extracted using a Bacterial DNA kit (D3350; OMEGA) according to the manufacturer’s instructions. The 16S rRNA gene sequence was PCR-amplified using universal primers (27f: 5′-AGAGTTTGTATCCTGGCTCAG-3′ and 1492r: 5′-TACGCTACCTTGTACGATT-3′) according to the methods of Timke et al. (2005) and the amplification product was directly sequenced automatically using an Applied Biosystems DNA sequencer (model 377) and software provided by the manufacturer. Multiple alignments of data and phylogenetic analysis were performed using MEGA software (version 5.0) (Tamura et al., 2011). Distances were calculated using Kimura’s two-parameter method (Kimura, 1980) and clustering with the neighbour-joining, minimum-evolution and the maximum-likelihood algorithms. Bootstrap values were determined based on 1000 replications. The neighbour-joining tree is shown in Fig. 1. The minimum-evolution and maximum-likelihood trees are available as Figs S1 and S2, available in the online Supplementary Material. The DNA G+C content of strain OMN17<sup>T</sup> was determined as described by Mesbah et al. (1989) using a reversed-phase HPLC. DNA–DNA hybridization was carried out as described by De Ley et al. (1970) using a UV/VIS spectrophotometer (UV1201; Rayleigh).

Cells of strain OMN17<sup>T</sup> were Gram-staining-positive, rods (1.2–1.4 μm wide and 2.5–4.4 μm long), occurring singly or in pairs. They were motile by means of several peritrichous flagella (Fig. S3). Subterminal ellipsoidal endospores were observed in unswollen sporangia (Fig. S4). The results of the other physiological and biochemical analyses are summarized in Table 1 and the species description. Compared with the closest relatives, it was observed that strain L. chungkukjangi KCTC 16626<sup>T</sup> and L. xylanilyticus KCTC 13423<sup>T</sup> could grow slightly at 10 °C on TSB medium, but strain OMN17<sup>T</sup>, L. halotolerans ACCC 00718<sup>T</sup> and L. sinduieriensis KCTC 13296<sup>T</sup> could not grow at such low temperature. Growth pH range also differentiated strain OMN17<sup>T</sup> from its closest relatives; for example, OMN17<sup>T</sup> could grow at pH 12.0, while the other four strains did not tolerate such extreme pH levels. Other various physiological
and biochemical characteristics also supported the distinctiveness of strain OMN17T from its close relatives (Table 1).

One-dimensional and two-dimensional TLC of the total hydrolysate of the peptidoglycan (4 M HCl, 16 h at 100 °C) revealed the presence of the amino acids lysine, glycine, alanine, aspartic and glutamic acid. The approximate molar ratio of the amino acids was 1.2 Ala, 0.6 Gly, 0.8 Asp, 1.0 Glu, 0.8 Lys. The identity of all amino-acid derivatives was confirmed by their gas chromatographic retention times and their mass spectra. Peptides that were stable under these conditions for hydrolysis (e.g., L-Lys-D-Asp) could not be detected. The peptides L-Ala-D-Glu, L-Lys-Gly and L-Lys-D-Ala were detected by two-dimensional TLC of the partial hydrolysate of the peptidoglycan (4 M HCl, 45 min at 100 °C). Dinitrophenylation revealed that aspartic acid represents the N-terminus of the interpeptide bridge. On the basis of these results, the following peptidoglycan type of strain OMN17T was concluded: A42 (l-Lys–Gly–D-Asp).

The only respiratory quinone of strain OMN17T was MK-7. The peptidoglycan and the quinone of strain OMN17T were in agreement with those of numerous species of the genus Lysinibacillus, including the type species, L. boronitolerans (Ahmed et al., 2007). However, these characteristics were significantly different from closely related genus Bacillus (Claus & Berkeley, 1986). The major fatty acid (>10 %) compounds of strain OMN17T were iso-C15_0 (53.4 %) and anteiso-C15_0 (15.4 %). The major compounds, fatty acids iso-C15_1_0 and anteiso-C15_0, support allotting strain OMN17T to the genus Lysinibacillus. However, some qualitative differences in fatty acid content could be observed between strain OMN17T and its phylogenetically closest relatives. In particular, the relatively larger percentage of fatty acid iso-C15_0 could distinguish strain OMN17T clearly from L. halotolerans, L. sinduriensis and L. chungkukjangi. Moreover, strain OMN17T could be differentiated from L. xylanilyticus by the relative larger percentage of fatty acid anteiso-C15_0, the presence of iso-C17_0 and the absence of iso-C17_0 (Table S1). The polar lipids detected in strain OMN17T were diphasphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol (Fig. S5). The presence of diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol has also been reported for L. halotolerans (Kong et al., 2014) and L. xylanilyticus (Lee et al., 2010). Conversely, strain OMN17T differs from L. chungkukjangi (Kim et al., 2013) and L. sinduriensis (Jung et al., 2012), by the absence of several unknown phospholipids. The cell-wall sugars of strain OMN17T were ribose, and traces of galactose and arabinose.

The 16S rRNA gene sequence (1472 bp) was obtained and subjected to similarity searches using the sequence matching tool of the NCBI BLAST program (http://www.ncbi.nlm.nih.gov), Ribosomal Database Project (http://rdp.cme.msu.edu) and EzBioCloud (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). Comparative 16S rRNA gene sequence analysis of strain OMN17T showed similarities of 97.8 %, 97.5 %, 97.4 % and 97.0 % with those of L. halotolerans, L. sinduriensis, L. chungkukjangi and L. xylanilyticus, respectively. The phylogenetic tree recreated based on 16S rRNA gene sequences of strain OMN17T and related species of the genus Lysinibacillus is shown in Fig. 1. In the phylogenetic tree based on the neighbour-joining algorithm, strain OMN17T formed an independent cluster with L. halotolerans, L. sinduriensis and L. chungkukjangi.
Table 1. Differential phenotypic characteristics of strain OMN17\textsuperscript{T} and the type strains of phylogenetically closely related species

<table>
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<th>Characteristic</th>
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<td>Temperature range for growth (°C)</td>
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<td>10–45</td>
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<td>pH range for growth</td>
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<td>w*</td>
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<td>w</td>
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<td>Enzyme activity</td>
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<td>Leucine arylamidase</td>
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<td>w*</td>
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<td>Trypsin</td>
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<td>–</td>
<td>w*</td>
<td>+</td>
<td>+</td>
<td>w*</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>38.1</td>
<td>36.4\textsuperscript{\dagger}</td>
<td>35.9\textsuperscript{b}</td>
<td>41.8\textsuperscript{c}</td>
<td>37.2\textsuperscript{d}</td>
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\*Different results compared with published data.

\(\dagger\)Data from: a, Kong et al. (2014); b, Jung et al. (2012); c, Kim et al. (2013); d, Lee et al. (2010).

Similar tree topologies were seen in the minimum-evolution and maximum-likelihood phylogenetic trees (Figs S1 and S2). The DNA G+C content of strain OMN17\textsuperscript{T} was 38.1 mol%. DNA–DNA hybridization studies showed relatively low relatedness values with \textit{L. halotolerans} ACCC 00718\textsuperscript{T} (21.8 ± 0.2 %, SD), \textit{L. sinduriensis} KCTC 13296\textsuperscript{T} (45.6 ± 1.8 %, SD), \textit{L. chungkukjangi} KACC 16626\textsuperscript{T} (33.7 ± 1.2 %, SD) and \textit{L. xylanilyticus} KCTC 13423\textsuperscript{T} (23.7 ± 0.7 %). The results of DNA–DNA hybridization as well as those of phylogenetic analysis showed that strain OMN17\textsuperscript{T} belongs to the genus \textit{Lysinibacillus}, but represents a novel species within this genus.

In recent years, fatty acids have been emphasized more; however, it is well known that their resolution is limited. The combination of 16S rRNA gene sequence data and phenotypic properties, including physiology, morphology and biochemistry, is still necessary for determining taxonomic arrangement (You \textit{et al.}, 2013). The significant differences in the cellular fatty acid profile and phenotypic properties can be used to distinguish strain OMN17\textsuperscript{T} from \textit{L. halotolerans}, \textit{L. sinduriensis}, \textit{L. chungkukjangi} and \textit{L. xylanilyticus}. Therefore, it is concluded that the isolate should be placed in the genus \textit{Lysinibacillus}. On the basis of the taxonomic data described above, strain OMN17\textsuperscript{T} represents a novel species of the genus \textit{Lysinibacillus}, for which the name \textit{Lysinibacillus alkaliphilus} sp. nov. is proposed.

Emended description of the genus \textit{Lysinibacillus}

The description is as given previously (Ahmed \textit{et al.}, 2007; Jung \textit{et al.}, 2012) with the following amendments. Major cellular fatty acids iso-C\textsubscript{15}:0 anteiso-C\textsubscript{15}:0 or iso-C\textsubscript{16}:0 are present. The respiratory quinone system contains MK-7 and/or MK-7 (H\textsubscript{2}) as the predominant quinone, with varying amounts of MK-3, MK-4, MK-5 and MK-6. The DNA G+C content is 35–44 mol%.

Description of \textit{Lysinibacillus alkaliphilus} sp. nov.

\textit{Lysinibacillus alkaliphilus} [\textit{al.kal.\'i}phil.\textit{us}. N.L. n. \textit{alkali} (from Arabic article \textit{al} the; Arabic n. \textit{qaliy} ashes of saltwort)
Forms circular colonies with crenate to fimbriate edges, flat elevation, opaque and granular surface texture, pale-brown in colour that are about 0.5–2 mm in diameter after 2 days incubation on TSA plate at 30 °C. Temperature range for growth is 20–45 °C, optimum growth occurred at 30 °C. pH range for growth is 5.5–12.0, with optimum pH 8.0–10.0. The NaCl tolerance range is 0–2 % (w/v). The preferred growth medium is TSA. Cells are aerobic and oxidase-negative but catalase-positive. Hydrolysis of Tween 80 and starch is negative. In the API 20 E and 20 NE strip (bioMérieux), \( \beta \)-nitrophenyl-\( \beta \)-D-galactopyranoside is not hydrolysed. Reactions for the Voges–Proskauer test, citrate utilization and hydrolysis of protease are positive. Negative reactions for gelatinase, assimilation of capric acid, \( \alpha \)-glucose, \( \alpha \)-d-mannitol and phenylacetic acid. Delayed and weak acid production without gas is observed from the following carbohydrates in the API 50 CH gallery using CHB suspension medium (bioMérieux): D-Adonitol, D- and L-arabitol, arbutin, dulcitol, D-fructose, D-mannitol, potassium gluconate, potassium 5-ketogluconate, D-ribose, D-sorbitol, L-sorbitose, D-tagatose, xyitol and L-xyllose. After incubation on a Biolog GP2 MicroPlate (Biolog) at 37 °C for 24 h, the following carbon sources are oxidized: acetic acid, L-alanine, L-asparagine, L-malic acid, L-glutamic acid, D-L-\( \alpha \)-glycerol phosphate, pyruvic acid, D-ribose, succinic acid, succinic acid monomethyl ester and D-xyllose. In API ZYM assays, reactions of esterase lipase (C8) and leucine arylamidase are weakly positive, but reactions of \( \alpha \)-chymotrypsin, cystine arylamidase, trypsin and valine arylamidase are negative. The cell-wall peptidoglycan type is A4\( \alpha \); \( \alpha \)-Lys–Gly–D-Val–L-Lys–Gly. The predominant polar lipids are diphasphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. The only respiratory quinone is MK-7. The major cellular fatty acids are iso-C\( _{15} \):\( _{0} \) and anteiso-C\( _{15} \):\( _{0} \).

The type strain, OMN17\( ^{T} \) (=DSM 28019\( ^{T} \)=CTCC AB 204073\( ^{T} \)), was isolated from a typical sandy loam soil under long-term OMN fertilization at the State Experimental Station for Agro-Ecology, Fengjiu county (35° 00' N 114° 24' E), Henan province, PR China. The DNA G+C content of the type strain is 38.1 mol%.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (nos 41101229 and 41371253) and the China Post-doctoral Science Foundation (no. 20110491468). The authors thank Professor Soon-Wo Kwon, Korean Agricultural Culture Collection (KACC) for kindly providing L. chungkukjangi KACC 16626\( ^{T} \) and Professor Jiabao Zhang, Institute of Soil Science, Chinese Academy of Sciences for providing sampling sites.

References


