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NOTE

Lysinibacillus jejuensis sp. nov., Isolated from Swinery Waste[§]

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A Gram-positive, endospore-forming, rod-shaped bacterium, designated strain N2-5^T, was isolated from swinery waste collected in Jeju, Republic of Korea. A phylogenetic analysis based on 16S rRNA gene sequences demonstrated that strain N2-5^T formed a phyletic group within the phylum *Firmicutes* with less than 97.0% similarities to members of the genus *Lysinibacillus*, its nearest phylogenetic neighbors. The highest levels of sequence similarity to the isolate were observed against *Lysinibacillus xylanilyticus* XDB9^T (96.8%), *Lysinibacillus macroides* LMG 18474^T (95.6%), and *Lysinibacillus parviboronicapiens* BAM-582^T (95.6%). The organism grew optimally at 30°C and pH 7 and in the presence of 1–3% (w/v) NaCl. Strain N2-5^T was chemotaxonomically characterized by possessing menaquinone-7 (MK-7) as the major menaquinone, and iso-$C_{15:0}$ (54.9%), iso-$C_{17:1}\omega10c$ (12.0%), and $C_{16:1}\omega7c$ alcohol (11.8%) as the predominant fatty acids. The genomic DNA G+C content of the novel strain was 43.3 mol% and the cell-wall peptidoglycan was type A4α. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. Based on its phenotypic properties and phylogenetic data, strain N2-5^T (=DSM 28310^T =KCTC13837^T) represents a novel species in the genus *Lysinibacillus*, for which the name *Lysinibacillus jejuensis* sp. nov. is proposed.

Keywords: *Firmicutes*, *Lysinibacillus jejuensis* sp. nov., swinery waste

The genus *Lysinibacillus* in the family *Bacillaceae* (phylum *Firmicutes*) was first described by Ahmed *et al.* (2007) with *Lysinibacillus boronitolerans*, *Lysinibacillus fusiformis*, and *Lysinibacillus sphaericus*. *Lysinibacillus boronitolerans* was designated as the type species of the genus. This genus contains one novel species and two former *Bacillus* species which were reclassified on the basis of their polyphasic taxonomic characteristics, particularly their cell-wall peptidoglycan structure. At the time of writing, the genus *Lysinibacillus* is composed of six species with valid published names: *L. parviboronicapiens* (Miwa *et al.*, 2009), *L. xylanilyticus* (Lee *et al.*, 2010), *L. sinduriensis*, *L. massiliensis*, *L. odyssey* (Jung *et al.*, 2011), *L. macroides* (Coorevits *et al.*, 2012), and *L. mangiferahumi* (Yang *et al.*, 2012). All members of the genus *Lysinibacillus* have been isolated from soil environments.

A large number of novel bacterial strains were isolated while we were investigating a culturable aerobic bacterial community in a swinery field waste site located in Jeju, Republic of Korea. One of these isolates, designated N2-5^T, appeared to be a member of the genus *Lysinibacillus* in the family *Bacillaceaes* and became a subject of taxonomic investigation. In this study, we conducted phylogenetic (16S rRNA), phenotypic, genotypic, and chemotaxonomic analyses to determine the precise taxonomic position of this strain.

Strain N2-5^T was isolated from swinery waste collected in Jeju, Republic of Korea. The waste sample was suspended in 0.85% NaCl, serially diluted, spread on tryptic soy agar (TSA; Difco, USA) and incubated at 30°C for 2 days. One of the representative colonies, strain N2-5^T, was cultured routinely on TSA at 30°C and preserved in a glycerol solution (20%, w/v) at -70°C. The reference strains used in this study were *L. xylanilyticus* KCTC 13423^T, *L. macroides* KACC 16627^T, *L. parviboronicapiens* KCTC 13154^T, *L. sphaericus* KCTC 3346^T, *L. fusiformis* KCTC 3454^T, and *L. boronitolerans* KACC 15323^T.

The 16S rRNA gene was amplified from genomic DNA extracts using universal 27F and 1522R primers (Weisburg *et al.*, 1991) and purified as described previously (Yoon *et al.*, 2003). The purified PCR product was sequenced by Genotech (Seoul, Korea). The full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR, USA). Sequences of related taxa were obtained from the GenBank database and EzTaxon server (Chun *et al.*, 2007). Multiple alignments were performed using the CLUSTAL X program (version 1.83) (Thompson *et al.*, 1997) and gaps were edited in the BioEdit program (Hall, 1999). The sequences were aligned manually against representatives of the family *Bacillaceaes* using the 16S rRNA secondary structure of the *Escherichia coli* sequence (Brosius *et al.*, 1978). The regions available for all sequences (positions 46–173 and 212–1431; based on the *Escherichia coli* numbering system) showed

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unambiguous alignment and were used to reconstruct phylogenetic tree. The phylogenetic tree was constructed based on the neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum parsimony (Kluge and Farris, 1969) algorithms using MEGA version 5.0 (Tamura *et al*., 2011). Bootstrapping analysis based on 1,000 resamplings was used to evaluate tree topology (Felsenstein, 1985).

Gram-staining was performed using a Gram-stain kit (BD Science, USA) according to the manufacturer's instructions. Cell morphology was examined by light microscopy (Nikon, Japan) and scanning electron microscopy (SEC, Korea). A motility test was carried out on tryptic soy broth (TSB; Difco, USA) containing 0.5% agar. Growth at 4, 10, 15, 20, 25, 30, 37, 40, and 45°C was measured on TSA. The pH range for growth was investigated on TSA adjusted to pH 4.0–11.0 in increments of 0.5 pH units using 1 M HCl or 1 M NaOH. Salt tolerance was tested by a 7 days incubation on TSA supplemented with 1–10% (w/v in increments of 1%) NaCl. Catalase activity was observed by bubble production in a 3% solution (v/v) of hydrogen peroxide, and oxidase activity was determined by the oxidation of 1% (w/v) tetramethyl *p*-phenylenediamine (Merck, USA). Hydrolysis of casein, starch, Tween 20, 40, 60, and 80 was performed on TSA using substrate concentrations described previously (Cowan and Steek, 1965). DNase activity was examined using DNase test agar (Difco) with methyl green. Growth under anaerobic conditions was determined after a 4 weeks incubation in an AnaeroPack (Oxoid, UK) on TSA. Other biochemical tests were carried out using the API 20E, API 20NE, API ZYM, and API 50CH kits (bioMérieux, UK) according to the mamufacturer's instructions. Utilization of different carbon sources was assessed using GP2 microplates (Biolog, USA) according to the mamufacturer's instructions. Susceptibility to antibiotics was tested on TSA plates using antibiotic discs (BBL, USA) containing the following: ampicillin (10 μg), cephalothin (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), erythromycin (15 μg), kanamycin (30 μg), lincomycin (2 μg), neomycin (30 μg), novobiocin (30 μg), penicillin G (10 IU), polymyxin B (300 IU), streptomycin (10 μg), and tetracycline (30 μg).

Fatty acid methyl esters were prepared according to the standard protocol of the Sherlock Microbial Identification System (MIDI; version 6.1) (Sasser, 1990) and analyzed by gas chromatography (GC 7890A; Agilent, USA) with TSBA6 library. The major respiratory quinones and DNA G+C content were analyzed by the Korean Culture Center of Microorganisms (KCCM; Korea), using reverse-phase high performance liquid chromatography (Tamaoka and Komagata, 1984). Polar lipids were extracted according to the procedure described by Minnikin *et al*. (1984) and analyzed by two-dimensional thin-layer chromatography (TLC) with appropriate detection reagents (Minnikin *et al*., 1984; Komagata and Suzuki, 1987). Cell-wall peptidoglycans were extracted and purified as described by Schleifer and Kandler (1972). The amino acid content of the hydrolyzed cell-wall peptidoglycan was determined using an automated amino acid analyzer (Pickering Lab., USA).

A 16S rRNA gene sequence analysis revealed that strain N2-5^T (1,410 bp) belonged to the genus *Lysinibacillus*, and

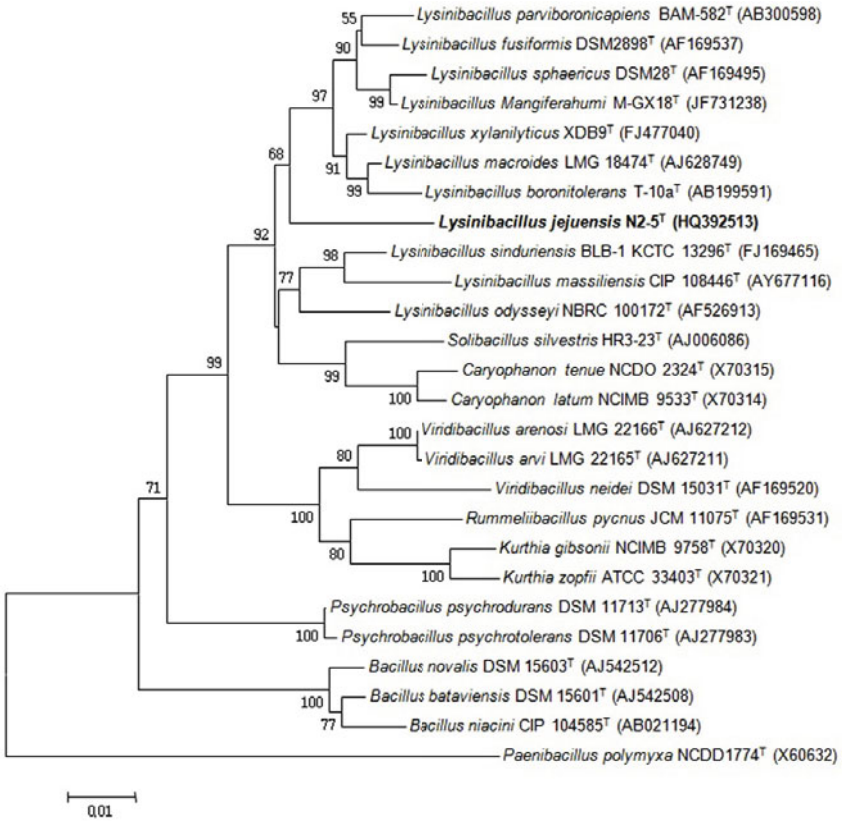

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain N2-5^T and some other related taxa. GenBank accession nos. are given in parentheses. Bootstrap value (>50%) based on 1,000 replications are shown. *Paenibacillus polymyxa* NCDO 1774^T was used as an out-group. Bar, 0.01 nucleotide substitutions per nucleotide position.

Table 1. Differential characteristics of strain N2-5^T from related species in genus *Lysinibacillus*

Strains: 1, *L. jejuensis* N2-5^T; 2, *L. xylanilyticus* KCTC 13423^T; 3. *L. macroides* KACC 16627^T; 4. *L. parviboronicapiens* KCTC 13454^T; 5, *L. fusiformis* KCTC 3454^T; 6, *L. sphaericus* KCTC 3346^T; 7, *L. boronitolerans* KACC 15323^T. All data were obtained in this study.
+, Positive; -, negative. All strains were positive for catalase, but negative for H_2S production.

Characteristic	1	2	3	4	5	6	7
Growth at/with							
10°C	+	+	+	+	-	+	-
40°C	-	+	+	-	+	+	+
6% NaCl	-	-	-	+	+	-	+
pH range for growth	6.5–10.0	5.0–9.0	7.0–9.0	6.0–9.0	6.0–10.0	6.0–10.1	5.5–9.5
Voges-Proskauer test	+	-	+	+	-	-	+
Hydrolysis of:							
Aesculin	+	+	-	+	-	-	+
Gelatin	-	+	-	-	+	+	-
Urea	-	-	-	+	+	-	+
Utilization of :							
Citrate	-	+	-	-	+	+	+
D-Galactose	-	-	-	-	-	+	-
D-Xylose	+	+	+	-	-	-	-
L-Arabinose	-	+	+	-	-	+	-
Maltose	+	+	-	+	-	-	-
Mannose	-	-	+	-	-	+	-
Pyruvate	+	-	-	-	+	+	
Ribose	+	-	-	-	+	+	+
Resistance to :							
Erythromycin	+	+	-	+[a]	-	+	
Gentamicin	+	-	+	-	-	+	+
Novobiocin	+	+	+	-	-	-	+
Tetracycline	+	+	+	-	-	+	
Streptomycin	+	+	+	+	+	+	-
DNA G+C mol%	43.3	37.2[b]	38.2[b]	38.7[b]	37.2[b]	37.2[b]	36.5[b]

[a] Asterisk represents different response from data reported previously (Miwa *et al.*, 2009).
[b] DNA G+C content data from Lee *et al.* (2010), Coorevits *et al.* (2012), Ahmed *et al.* (2007), and Miwa *et al.* (2009).

the sequence was deposited in the GenBank database (accession number HQ392513). A sequence similarity calculation using the EzTaxon server (http://www.EzTaxon.org/; Chun *et al.*, 2007) indicated that the closest relatives of strain N2-5^T were *L. xylanilyticus* XDB9^T (96.8%), *L. macroides* LMG 18474^T (95.6%), *L. parviboronicapiens* BAM-582^T (95.6%), *L. sphaericus* C3-41^T (95.6%), *L. fusiformis* NRBC 15717^T (95.5%), and *L. boronitolerans* T10a^T (95.4%). Strain N2-5^T formed a distinct subline within the cluster comprising members of the genus *Lysinibacillus* in a neighbor-joining phylogenetic tree based on 16S rRNA gene sequences (Fig. 1). In phylogenetic trees reconstructed using maximum-likelihood and maximum-parsimony algorithms, strain N2-5^T also fell within the clade encompassed by the genus *Lysinibacillus*.

Cells of strain N2-5^T were Gram-positive, aerobic, endospore-forming, and rod-shaped, with overall dimensions of 0.7–1.0 μm (width) × 2.5–6.0 μm (length), as assessed by 2 day cultures grown at 30°C on TSA. Colonies were circular to ovoid, smooth, low-convex, ivory and 2–4 mm in diameter after a 2 days incubation on TSA. The optimal temperature for growth was 30°C; growth occurred at 10–37°C, but not at 4 or 40°C. The pH range for growth was 6.0–10.0, with optimum at 7.0. It tolerated up to 3% NaCl. The strain was catalase- and oxidase-positive, but negative for nitrate reduction. Gelatin and starch were not hydrolyzed. Phenotypic and chemotaxonomic characteristics that distinguished strain N2-5^T from the type strains of other *Lysinibacillus* species

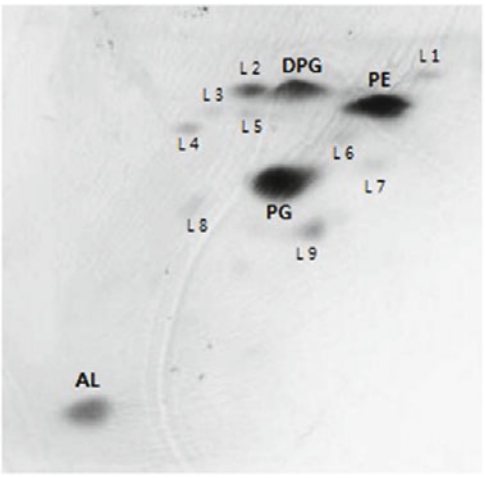

Fig. 2. Two dimensional thin-layer chromatogram sprayed with molybdatophosphoric acid reagent to identify total polar lipids of strain N2-5^T. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; AL, unknown aminolipid; L1–9, unknown lipids.

are listed in Table 1.

An analysis of cellular fatty acids revealed that the major fatty acids (>10% of the total) of the strain was iso-$C_{15:0}$ (54.9%), iso-$C_{17:1}\omega10c$ (12.0%), and $C_{16:1}\omega7c$ alcohol (11.8%), with minor amounts of iso-$C_{16:0}$ (5.7%), iso-$C_{17:0}$ (5.1%), and anteiso-$C_{15:0}$ (3.1%). The total fatty acid profile was similar to those of the type strains of *Lysinibacillus* species, although there were differences in the relative amounts of some fatty acids (Table 2). The predominant isoprenoid quinone detected in strain N2-5^T was menaquinone-7 (MK-7), which has been found in other *Lysinibacillus* species. The polar lipids found in strain N2-5^T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, unknown lipids (L1–L9), and aminolipids (Fig. 2). The polar lipid profile was consistent with the reported profile of other *Lysinibacillus* species (Ahmed *et al*., 2007; Coorevits *et al*., 2012). The G+C content of the genomic DNA was 43.3 mol%, which was significantly higher than the range reported for *Lysinibacillus* species (Ahmed *et al*., 2007). The peptidoglycan of strain N2-5^T contained the amino acids alanine, glutamic acid, aspartic acid, and lysine in an approximate molar ratio of 1.3 : 1.2 : 1.3 : 0.9. From these data, it was concluded that strain N2-5^T contained a type A4α peptidoglycan, based on L-Lys–D-Asp (Schleifer and Kandler, 1972), and was in agreement with that of the genus *Lysinibacillus*.

Based on the phenotypic and phylogenetic analyses results, strain N2-5^T represents a new species in the genus *Lysinibacillus*, and the name *Lysinibacillus jejuensis* sp. nov., is proposed.

Description of *Lysinibacillus jejuensis* sp. nov.

Lysinibacillus jejuensis (je.ju.en′sis. N.L. fem. adj. *jejuensis* referring to Jeju Island in the Republic of Korea, where the type strain was isolated).

Cells are Gram-positive, motile, and endospore-forming rods (0.7–1.0 μm wide and 2.5–6.0 μm long). Colonies are ivory-colored, circular to ovoid, smooth, and low-convex on TSA. Optimal growth is observed at 30°C and at pH 7.0. Growth occurs in the presence of 1–3% NaCl, at 10–37°C and pH 6.0–10.0. No growth is observed below pH 5 or above pH 11. Anaerobic growth does not occur on TSA. The Voges-Proskauer (acetoin production) test is positive, but indole and H_2S production are negative. Casein is hydrolyzed, but starch, DNase, and Tweens 20, 40, 60, and 80 are not. API 50CH strips indicated that carbohydrates and acids are not produced from any substrate. D-xylose, maltose, pyruvate, and ribose were utilized for growth, but not sucrose, mannose, glycogen, fucose, maltose, rhamnose, or sorbitol. API ZYM was positive for acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), and lipase (C14), but negative for naphthol-AS-BI-phosphohydrolase, α–galactosidase, α-glucosidase, esterase lipase (C8), leucine arylamidases, valine-, and cystine, trypsin, a – glucosidase, β-glucuronidas, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The major fatty acids are iso-$C_{15:0}$ (54.9%), iso-$C_{17:1}\omega10c$ (12.0%), and $C_{16:1}\omega7c$ alcohol (11.8%). The predominant menaquinone is MK-7 and the DNA G+C content is 43.3 mol%. The cell wall peptidoglycan type is A4α, based on L-Lys-D-Asp. The polar lipid profile contains major amounts of diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine.

The type strain, N2-5^T (=DSM 28310^T =KCTC 13837^T), was isolated from swinery waste collected in Jeju, Republic of Korea.

The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N2-5^T is HQ392513.

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Table 2. Cellular fatty acid profiles of strain N2-5^T and the *Lysinibacillus* species type strains

Strains: 1, *L. jejuensis* N2-5^T; 2, *L. xylanilyticus* KCTC 13423^T; 3. *L. macroides* KACC 16627^T; 4. *L. parviboronicapiens* KCTC 13454^T; 5, *L. fusiformis* KCTC 3454^T; 6, *L. sphaericus* KCTC 3346^T; 7, *L. boronitolerans* KACC 15323^T. All data were obtained from cultures on TSA at 30°C for 2 days. Fatty acids that accounted for < 0.5% of the total fatty acids in all strains were excluded. -, not detected.

Fatty acid	1	2	3	4	5	6	7
$C_{14:0}$	-	1.5	0.5	-	-	-	0.6
iso-$C_{14:0}$	-	3.0	4.3	5.2	7.1	1.2	1.6
anteiso-$C_{15:0}$	3.1	16.1	5.3	14.9	5.3	11.0	29.3
iso-$C_{15:0}$	54.9	37.2	45.0	40.0	51.0	61.2	40.3
$C_{16:0}$	0.4	1.5	1.5	-	0.6	-	1.8
iso-$C_{16:0}$	5.7	7.3	16.8	13.6	9.2	3.3	6.7
$C_{16:1}\omega11c$	2.3	3.2	1.9	1.0	1.6	2.6	1.0
$C_{16:1}\omega7c$ alcohol	11.9	9.5	12.2	16.5	18.9	8.3	4.1
iso-$C_{17:0}$	5.1	4.5	5.2	2.8	2.1	3.8	3.5
anteiso-$C_{17:0}$	1.3	8.7	1.9	2.9	-	2.0	7.3
Iso-$C_{17:1}\omega10c$	12.1	2.9	1.3	0.9	2.5	2.6	0.6
$C_{18:1}\omega9c$	-	-	1.5	-	-	-	1.7
Summed feature 4[a]	2.6	4.2	1.1	1.2	0.8	2.6	1.3

[a] Summed feature represent groups of two or three fatty acids that could not be separated by ECL with the MIDI system. Summed feature 4 comprises iso-$C_{17:1}$ I and/or anteiso$C_{17:1}$ B.

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