Lysinibacillus xylanilyticus sp. nov., a xylan-degrading bacterium isolated from forest humus

Chang Soo Lee, Yong-Taek Jung, Sooyeon Park, Tae-Kwang Oh and Jung-Hoon Yoon

Correspondence

Jung-Hoon Yoon
jhyoon@kribb.re.kr

Korea Research Institute of Bioscience and Biotechnology (KRIIBB), PO Box 115, Yusong, Taejeon, Republic of Korea

A novel xylan-degrading bacterium, designated XDB9^T, was isolated from forest humus collected from Gyeryong Mountain in Korea. Cells were Gram-positive, aerobic, motile and endospore-forming rods. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that strain XDB9^T was most closely related to members of the genus *Lysinibacillus*. 16S rRNA gene sequence similarities between strain XDB9^T and the type strains of species of the genus *Lysinibacillus* ranged from 98.0 to 98.5 %. The cell-wall peptidoglycan type of strain XDB9^T was A4₂, which is based on L-Lys–D-Asp. Strain XDB9^T contained iso-C_{15:0} and C_{16:1}ω7c alcohol as the major fatty acids and MK-7 as the predominant menaquinone. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The DNA G + C content was 37.2 mol%. The DNA–DNA hybridization results and differential phenotypic properties showed that strain XDB9^T could be distinguished from recognized species of the genus *Lysinibacillus*. It was concluded that strain XDB9^T represents a new taxon for which the name *Lysinibacillus xylanilyticus* sp. nov. is proposed. The type strain is XDB9^T (=KCTC 13423^T=CCUG 57438^T).

The genus *Lysinibacillus* was proposed by Ahmed *et al.* (2007) by the description of one novel species and the reclassification of two *Bacillus* species on the basis of a polyphasic taxonomic study, and especially with regard to characteristics such as cell-wall peptidoglycan structure. At the time of writing, the genus *Lysinibacillus* was composed of four species with validly published names: *Lysinibacillus boronitolerans*, *Lysinibacillus fusiformis* and *Lysinibacillus sphaericus* (Ahmed *et al.*, 2007) and *Lysinibacillus parviboronicapiens* (Miwa *et al.*, 2009).

During the course of a study on the diversity of xylan-degrading bacteria from natural environments, a novel bacterial strain, XDB9^T, was isolated from forest humus. Xylan, a polymer of β-1,4-D-xylopyranosyl units, is one of the most ubiquitous polysaccharides in nature and a main constituent of hemicellulose, which is found in the cell walls of plants (Timmell, 1967). Xylan-degrading bacteria have been isolated in order to screen for xylanases, such as β-1,4-D-xylan xylanohydrolase and β-1,4-D-xylan xylohydrolase, which are useful in the food and pulp industries (Coughlan & Hazlewood, 1993).

For the screening of xylan-degrading bacteria, forest humus was collected from Gyeryong Mountain, Taejeon,

Korea. The samples were serially diluted in 0.85 % (w/v) saline solution and then aliquots of each serial dilution were spread onto solid medium containing 0.5 % (w/v) birch wood xylan (Sigma) and 1.5 % (w/v) Noble agar (Difco). The medium was incubated for 7 days at 25 °C under aerobic conditions. One of the isolates, XDB9^T, was selected and subcultivated on trypticase soy agar (TSA; Difco) at 30 °C for further study. *L. boronitolerans* JCM 21713^T, *L. fusiformis* KACC 10903^T and *L. sphaericus* KCTC 3346^T were used as reference strains for phenotypic characterization, cellular fatty acid analysis and DNA–DNA hybridization. *L. parviboronicapiens* KCTC 13154^T was also used as a reference strain for phenotypic characterizations and DNA–DNA hybridization.

To investigate the xylan-degrading activity of strain XDB9^T, Congo red (Wood *et al.*, 1988) and dinitrosalicylic acid (DNS) methods (Miller, 1959) were used. For the DNS test, strain XDB9^T was cultured and suspended in 100 ml of 50 mM potassium phosphate buffer (pH 6.5) and the inoculum density was adjusted to a McFarland standard of 0.5. Birch wood xylan (1 g) was added and the mixture was incubated for 6 h at 30 °C. A 100 μl aliquot of the mixture was then transferred into an Eppendorf tube, 1 ml DNS reagent was added and the tube was heated in a boiling water bath for 10 min. Finally, the absorbance was measured at 575 nm with a spectrophotometer (DU800; Beckman Coulter).

Abbreviation: DNS, dinitrosalicylic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain XDB9^T is FJ477040.

Cell morphology and motility were examined by light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). The Gram reaction was performed using the bioMérieux Gram-stain kit according to the manufacturer's instructions. Growth at various temperatures (4, 10, 15, 20, 25, 30, 37, 40 and 45 °C) was measured on TSA. The pH range for growth was determined in trypticase soy broth (TSB; Difco) adjusted to various pH values (pH 4.0–10.0, in increments of 0.5 pH units) by the addition of HCl or Na₂CO₃. Growth at various NaCl concentrations (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 %, w/v) was investigated using TSB prepared according to the formula of the Difco medium except that NaCl was excluded. Oxidase and catalase activities and hydrolysis of casein, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine and xanthine were tested on appropriate media and substrates as described by Cowan & Steel (1965). Hydrolysis of aesculin and reduction of nitrate were examined as described by Lányi (1987). Utilization of various substrates, enzyme activities and other physiological and biochemical properties were tested by using the API 20E, API 50CH and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The cells were suspended in AUX medium to inoculate the API 50CH system. Resistance to antibiotics was assessed by spreading bacterial suspension on TSA and applying paper discs impregnated with the following (µg per disc unless otherwise stated): polymyxin B (100 U), streptomycin (50), penicillin G (20 U), chloramphenicol (100), ampicillin (10), cephalothin (30), gentamicin (30), novobiocin (5), tetracycline (30), kanamycin (30), lincomycin (15), oleandomycin (15), neomycin (30) and carbenicillin (100). Boron tolerance was tested as described by Ahmed *et al.* (2007) at concentrations of 0, 50, 100 and 150 mM.

For cellular fatty acid analysis, cell biomass was harvested from TSA plates after cultivation for 24 h at 28 °C. Cell biomass of strain XDB9^T for the analyses of cell-wall components, isoprenoid quinones and polar lipids and for DNA extraction was obtained from cultures grown in TSB at 30 °C. The fatty acids were extracted and the fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The cell-wall peptidoglycan was extracted and purified as described by Schleifer & Kandler (1972). The peptidoglycan amino acids were determined using an automated amino acid analyser (L-8500A; Hitachi). Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analysed using reversed-phase HPLC equipped with a YMC ODS-A (250 × 4.6 mm) column. Polar lipids were extracted and identified by two-dimensional TLC followed by spraying with appropriate detection reagents as described by Minnikin *et al.* (1984). Extraction of chromosomal DNA and PCR-mediated amplification and sequencing of the 16S rRNA gene sequence were performed as described previously (Yoon *et al.*, 1996, 1998, 2003).

The 16S rRNA gene sequence was aligned with sequences retrieved from EMBL by using CLUSTAL_X (Thompson *et al.*,

1997) and edited by using BioEdit (Hall, 1999). Phylogenetic trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) algorithms with MEGA version 3.1 (Kumar *et al.*, 2004). The stability of relationships in the neighbour-joining tree was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replications. The DNA G + C content was determined as described by Tamaoka & Komagata (1984) with the modification that, after hydrolysis of the DNA, the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and 96-well plates (Nunc). Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were quoted as DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain XDB9^T are given in the species description and in Table 1. The 16S rRNA gene sequence of strain XDB9^T determined in this study consisted of 1349 nucleotides and showed the closest affiliations to those of members of the genus *Lysinibacillus* (Fig. 1). In the phylogenetic trees based on 16S rRNA gene sequences, strain XDB9^T formed an evolutionary lineage within the cluster comprising species of the genus *Lysinibacillus*, clustering most closely with *L. boronitolerans* 10a^T with a bootstrap resampling value of 88 % (Fig. 1). The relationship between strain XDB9^T and *L. boronitolerans* 10a^T was also found in the trees constructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). 16S rRNA gene sequence similarity values between strain XDB9^T and the type strains of species of the genus *Lysinibacillus* ranged from 98.0 % (*L. sphaericus* NRRL B-23268^T) to 98.5 % (*L. fusiformis* NRRL NRS-350^T and *L. boronitolerans* 10a^T).

Quantitative analysis of the cell-wall peptidoglycan showed that strain XDB9^T and *L. boronitolerans* JCM 21713^T had similar molar ratios of alanine, aspartic acid, glutamic acid and lysine (approximately 1.43:0.43:1.0:0.33 and 1.45:0.65:1.0:0.45, respectively). The smaller amounts of lysine and aspartic acid were due to the presence of the stable peptide L-Lys–D-Asp. From these data, it was concluded that strain XDB9^T had the peptidoglycan type A4_z, which is based on L-Lys–D-Asp, as described by Schleifer & Kandler (1972). This supported the result of the phylogenetic analysis, which showed an affiliation of strain XDB9^T to the genus *Lysinibacillus*, because the A4_z peptidoglycan type is a key marker used to discriminate the genus *Lysinibacillus*, as well as the genus *Kurthia*, from other members of *Bacillus* group 2 (Shaw & Keddie, 1983; Stackebrandt *et al.*, 1987; Claus & Fritze, 1989; Ash *et al.*, 1991; Rheims *et al.*, 1999; Nakamura *et al.*, 2002). The predominant isoprenoid quinone detected in strain XDB9^T was menaquinone-7 (MK-7), which is the same as that

Table 1. Differential phenotypic characteristics of strain XDB9^T and other *Lysinibacillus* species

Species: 1, *Lysinibacillus xylanilyticus* sp. nov. XDB9^T (data from this study); 2, *L. boronitolerans* JCM 21713^T (Ahmed *et al.*, 2007; this study); 3, *L. fusiformis* KACC 10903^T (Priest *et al.*, 1988; this study); 4, *L. parviboronicapiens* KCTC 13154^T (Miwa *et al.*, 2009; this study); 5, *L. sphaericus* KCTC 3346^T (Claus & Berkeley, 1986; this study). All species are Gram-positive and endospore-forming rods and give positive reactions in tests for motility, catalase activity, resistance to ampicillin, polymyxin B, cephalothin and lincomycin, and utilization of glucose, fructose, cellobiose, gluconate, 2-ketogluconate and 5-ketogluconate. All species are negative for production of H₂S and indole, reduction of nitrate and lysine decarboxylase and ornithine decarboxylase activities (data are taken from the reference species used in this study), NaCl requirement, resistance to carbenicillin, kanamycin, neomycin, oleandomycin, penicillin G and tetracycline, utilization of *N*-acetylglucosamine, adonitol, amygdalin, D-arabinose, D- and L-arabitol, arbutin, dulcitol, erythritol, D- and L-fucose, gentiobiose, glycerol, glycogen, inositol, inulin, lactose, D-lyxose, mannitol, melezitose, raffinose, salicin, sorbose, starch, D-tagatose, turanose, xylitol, L-xyllose, methyl α -D-glucoside, methyl α -D-mannoside and methyl β -D-xyloside and acid production from D-arabinose, inositol, melibiose, L-rhamnose and D-sorbitol. +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Cell size (μ m)	0.8–1.0 $\times$ 3.0–5.0	0.8–1.5 $\times$ 3.0–5.0	ND	0.5–1.0 $\times$ 2.0–5.0	0.6–1.0 $\times$ 1.5–5.0
Spore shape and position*	R, T, B	R/O, T, B	R, C/T, B	R, T, B	R, T, B
Growth at/with:					
10 °C	+	–	–	+	+
40 °C	+	+	+	–	+
pH 5.0	+	–	–	–	–
pH 9.5	–	+	+	–	+
6 % (w/v) NaCl	–	–	+	+	–
Boron tolerance (mM)	0	150	60†	50	75†
Voges–Proskauer test‡	–	+	–	+	–
Oxidase‡	+	+	+	–	+
Arginine dihydrolase‡	–	+	–	–	–
β -Galactosidase	–	–	–	–	+
Hydrolysis of:‡					
Aesculin	+	+	–	+	–
Gelatin	+	–	+	–	+
Urea	–	+	+	+	–
Resistance to:‡					
Chloramphenicol	–	–	+	+	–
Gentamicin	–	+	–	–	+
Novobiocin	+	+	–	–	–
Streptomycin	+	–	+	+	+
Utilization of:‡					
Aesculin	+	+	–	+	–
L-Arabinose	+	–	–	–	+
Citrate	+	+	+	–	+
D-Galactose	–	–	–	–	+
Maltose	+	–	–	–	–
Mannose	–	–	–	–	+
Melibiose	+	–	–	–	–
Rhamnose	+	–	–	–	–
Ribose	–	+	+	–	+
Sorbitol	+	–	–	–	–
Sucrose	+	–	–	–	–
Trehalose	–	–	–	–	+
D-Xylose	+	–	–	–	+

*B, Bulging; C, central; O, oval or slightly oval; R, round; T, terminal.

†Data from Ahmed *et al.* (2007).

‡Data were taken from this study for the reference species as described previously.

found in members of the genus *Lysinibacillus* (Ahmed *et al.*, 2007; Miwa *et al.*, 2009). The cellular fatty acid profile of strain XDB9^T is shown in Table 2 together with those of

three *Lysinibacillus* species, also analysed in this study, and *L. parviboronicapiens* BAM-582^T, analysed by Miwa *et al.* (2009). The major fatty acids (>10 % of total fatty acids)

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain XDB9^T and some other related taxa. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Paenibacillus polymyxa* NCDO 1774^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid contents of strain XDB9^T and the type strains of species of the genus *Lysinibacillus*

Strains: 1, *Lysinibacillus xylanilyticus* sp. nov. XDB9^T; 2, *L. boronitolerans* JCM 21713^T; 3, *L. fusiformis* KACC 10903^T; 4, *L. parviboronicapiens* BAM-582^T; 5, *L. sphaericus* KCTC 3346^T. —, Not detected or not described. Data for taxa 1–3 and 5 are from this study; data for taxon 4 are from Miwa *et al.* (2009). Fatty acids that represented <1.0% in all strains are omitted.

Fatty acid (%)	1	2	3	4	5
Straight-chain					
C _{16:0}	1.2	1.8	1.3	4.1	1.8
C _{20:0}	—	—	6.7	—	—
Branched					
iso-C _{11:0}	8.8	—	—	—	—
iso-C _{14:0}	2.3	1.7	3.2	5.9	1.8
iso-C _{15:0}	50.7	41.9	42.6	37.4	42.0
anteiso-C _{15:0}	2.6	15.1	6.9	19.0	2.5
iso-C _{16:0}	6.3	9.3	14.7	5.9	11.5
iso-C _{17:0}	6.2	6.8	6.0	7.9	11.0
anteiso-C _{17:0}	1.3	7.1	2.6	9.6	1.2
iso-C _{17:1} ω10c	4.0	2.3	1.1	0.9	6.8
Unsaturated					
C _{16:1} ω7c alcohol	10.4	9.0	11.3	4.2	15.6
C _{16:1} ω11c	1.5	2.5	2.0	4.9	3.3
Summed feature 4*	1.3	2.6	1.1	2.7	1.6

*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 4 consisted of iso-C_{17:1}ω7c and/or anteiso-C_{17:1}.

were iso-C_{15:0} (50.7%) and C_{16:1}ω7c alcohol (10.4%). This fatty acid profile was similar to those of the type strains of species of the genus *Lysinibacillus*, although there were differences in the proportions of some fatty acids (Table 2). The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The DNA G+C content of strain XDB9^T was 37.2 mol%.

Mean DNA–DNA relatedness values between strain XDB9^T and the type strains of four *Lysinibacillus* species were in the range of 11–27%: *L. parviboronicapiens* KCTC 13154^T (11%), *L. sphaericus* KCTC 3346^T (16%), *L. fusiformis* KACC 10903^T (21%) and *L. boronitolerans* JCM 21713^T (27%). These values indicate that strain XDB9^T can be assigned to a distinct genomic species (Wayne *et al.*, 1987). Strain XDB9^T is distinguishable from other species of the genus *Lysinibacillus* on the basis of several phenotypic differences, including boron tolerance and hydrolysis and utilization of several substrates (Table 1). Strain XDB9^T is similar to other species of the genus *Lysinibacillus* in that the predominant fatty acid is iso-C_{15:0}, but it is distinguishable from them in that a significant amount of iso-C_{11:0} is also present (Table 2). Therefore, on the basis of differential phenotypic properties and phylogenetic and genetic distinctiveness, strain XDB9^T is sufficiently different from other *Lysinibacillus* species to be classified as a member of a novel taxon, for which the name *Lysinibacillus xylanilyticus* sp. nov. is proposed.

Description of *Lysinibacillus xylanilyticus* sp. nov.

Lysinibacillus xylanilyticus [xy.la.ni.ly'ti.cus. N.L. n. *xylanum* xylan; N.L. masc. adj. *lyticus* (from Gr. masc. adj.

lutikos) able to loosen, able to dissolve; N.L. masc. adj. *xylanilyticus* xylan-dissolving].

Cells are Gram-positive, motile and endospore-forming rods ($0.8\text{--}1.0 \times 3.0\text{--}5.0\ \mu\text{m}$). Colonies on TSA are dark yellow, opaque and circular with entire margins. Growth occurs at 10 and 40 °C (optimum 30 °C), but not at 4 or 45 °C, at pH 5.0 and 9.0, but not at pH 4.5 or pH 9.5 and with 0–5 % (w/v) NaCl. Casein is hydrolysed, but starch and Tweens 20, 40, 60 and 80 are not. Susceptible to boron and does not grow with 50, 100 or 150 mM boron. In assays with the API ZYM system, alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α - and β -galactosidases, β -glucuronidase, α - and β -glucosidases, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. The predominant menaquinone is MK-7. The major fatty acids (>10 % of total fatty acids) are iso-C_{15:0} and C_{16:1 ω 7c} alcohol. The cell-wall peptidoglycan type is A4 α . The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Other phenotypic properties are shown in Table 1.

The type strain, XDB9^T (=KCTC 13423^T=CCUG 57438^T), was isolated from forest humus of Gyeryong Mountain, Taejon, Korea. The DNA G + C content of the type strain is 37.2 mol%.

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