Paenibacillus woosongensis sp. nov., a xylanolytic bacterium isolated from forest soil

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A novel xylan-degrading bacterium, YB-45^T, was isolated from forest soil. The organism is a facultatively anaerobic, Gram-variable, motile, endospore-forming, rod-shaped bacterium. It grew optimally at 37 °C and pH 7.5 in the presence of 3% (w/v) NaCl. The predominant cellular fatty acids were anteiso-C_{15:0}, iso-C_{15:0} and C_{16:0}. The DNA G + C content was 51.7 mol% and the predominant menaquinone was MK-7. Growth was observed with many carbohydrates, including xylan, as sole carbon sources. Strain YB-45^T produces a wide variety of hydrolytic enzymes, such as xylanase, cellulase, amylase, β -mannanase, β -mannosidase, β -xylosidase, α -galactosidase, β -galactosidase and β -glucosidase. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain YB-45^T belongs to the genus *Paenibacillus*, sharing sequence similarity that was <96%. It was related most closely to *Paenibacillus jamilae* DSM 13815^T, with 95.7% sequence similarity. On the basis of morphological, chemotaxonomic, physiological and phylogenetic properties, strain YB-45^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus woosongensis* sp. nov. is proposed. The type strain is YB-45^T (=KCTC 3953^T=DSM 16971^T).

Xylan is a heterogeneous and abundant polysaccharide in nature: it is composed of (1,4)-linked β -D-xylosyl residues and is the major component of hemicelluloses in monocotyledon cell walls (Timell, 1967). Xylanases (β -1,4-D-xylan xylanohydrolase and β -1,4-D-xylan xylohydrolase), produced by a wide range of micro-organisms, are a group of xylanolytic enzymes that hydrolyse xylan to xylan oligosaccharides and xylose, and are used in the pulp industry and in the production of biofuels (Viikari *et al.*, 1994; Lamed *et al.*, 1988). The genus *Paenibacillus* was originally proposed by Ash *et al.* (1993) [the name was validly published by Ash *et al.* (1994)] and its members are facultative anaerobes or strict aerobes that produce spores in swollen sporangia and have DNA G + C contents in the range 45–54 mol%. It contains some species that are able to degrade xylan (Zamost *et al.*, 1991; Morales *et al.*, 1995; Hespell, 1996; Nielsen & Sorensen, 1997; Aÿ *et al.*, 1998; Lee *et al.*, 2000; Velázquez *et al.*, 2004; Rivas *et al.*, 2005).

During the screening of xylan-degrading bacteria, strain YB-45^T was isolated from forest soil at Daejeon in Korea. For the isolation, soil samples were diluted serially with 0.85% (NaCl, w/v) saline solution. Aliquots of each serial dilution were spread on Luria–Bertani agar (Difco) supplemented with 0.5% xylan (from oat spelt; Sigma)

and incubated at 37 °C for 1–2 days. A colony with a conspicuous halo was isolated and the isolate (YB-45^T) was routinely grown aerobically on tryptic soy broth (TSB; Difco) for 1–2 days at 37 °C except where indicated otherwise. Cultures in liquid medium were incubated at 37 °C in a rotary shaker at 150 r.p.m. Cell growth was monitored by measuring OD₆₀₀. Cell biomass of strain YB-45^T was obtained from TSB cultures at 37 °C. Any requirement for and tolerance of NaCl (0–10%) were determined in tryptic soy agar (TSA; Difco). To test for growth on 0% NaCl, this compound was omitted from the TSB. Growth was tested in TSB at different temperatures (4–60 °C) and pH values (5.0–10.0). Cell morphology was studied by using light microscopy and transmission electron microscopy. Motility was investigated in wet mounts under a light microscope (E600; Nikon) at 12 and 36 h. Flagellation was investigated with transmission electron microscopy, using cells from the exponential growth phase. The cells were mounted on Formvar-coated copper grids and negatively stained with 1% potassium phosphotungstate (pH 7.0); the grids were examined in a Phillips 201 transmission electron microscope operated at 80 kV. The presence of endospores was investigated by using the Schaeffer–Fulton staining method (Smibert & Krieg, 1981). Gram staining was determined by using a bioMérieux Gram Stain kit according to the manufacturer's instructions. Catalase activity was determined from bubble

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production in a 3% (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by using oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine with a Bactident Oxidase strip (Merck). The hydrolysis of compounds was checked on TSA according to the methods described by Cowan & Steel (1965), Lanyi (1987) and Smibert & Krieg (1994). Nitrate reduction and the hydrolysis of tyrosine, Tweens 20 and 80, aesculin, casein, starch and gelatin were investigated according to the method of Lanyi (1987). Carbon-source utilization tests, acid-production tests and additional physiological tests were performed by using API 50CH, API 50CHB and API 20NE galleries according to the instructions of the manufacturer (bioMérieux). Growth under anaerobic conditions was determined in an anaerobic chamber (H₂: CO₂: N₂, 5: 10: 85; Mart Microbiology) after 2 days incubation at 37 °C on TSA.

Strain YB-45^T was isolated on Luria–Bertani plates and showed high levels of xylanolytic activity. Within 48 h on TSA at 37 °C, the colonies produced were visible (1–2 mm diameter), light beige to greyish, low convex, circular with slightly irregular edges, translucent and glossy. The strain was facultatively anaerobic and the cells were motile rods (0.5–0.7 µm wide and 2.5–5.0 µm long). Growth was observed at temperatures ranging from 28 to 47 °C; optimum growth occurred at 37 °C and pH 7.5. The cells were Gram-variable, catalase-positive and oxidase-negative, did not reduce nitrate to nitrite and produced single, ellipsoidal, terminal endospores in swollen sporangia. Strain YB-45^T differed from phylogenetically related species in that it was capable of growth at 47 °C. Other physiological and biochemical characteristics are summarized in Table 1 and the species description.

Table 1. Phenotypic characteristics that differentiate strain YB-45^T from related members of the genus *Paenibacillus*

Taxa: 1, strain YB-45^T; 2, *P. jamilae*; 3, *P. polymyxa*; 4, *P. azotofixans*; 5, *P. peoriae*; 6, *P. kribbensis*; 7, *P. terrae*; 8, *P. brasiliensis*. All species formed swollen sporangia and were positive for anaerobic growth, catalase, acid production from arbutin, D-cellobiose, D-fructose, D-glucose, glycogen, maltose, D-mannose, D-mannitol and salicin and growth at pH 5.6. All species were negative for oxidase and growth in the presence of 5% NaCl. Data for *P. jamilae*, *P. polymyxa*, *P. azotofixans*, *P. peoriae*, *P. kribbensis* and *P. terrae* are from Yoon *et al.* (2003) and data for *P. brasiliensis* are from von der Weid *et al.* (2002). NT, Not tested; +, positive; –, negative; w, weak; v, variable.

Characteristic	1	2	3	4	5	6	7	8
Spore shape*	E	E	O	O	O	E	E	E
Nitrate reduction	–	+	+	–	+	+	+	+
Hydrolysis of:								
Casein	+	+	+	–	+	+	+	+
Gelatin	–	+	+	–	+	+	+	v
Starch	+	+	+	–	+	+	+	+
Tween 80	–	NT	NT	NT	NT	+	w	NT
Utilization of:								
L-Arabinose	+	NT	NT	NT	NT	+	–	–
D-Fructose	+	NT	NT	NT	NT	+	–	NT
D-Ribose	+	NT	NT	NT	NT	+	–	NT
D-Xylose	+	NT	NT	NT	NT	+	–	–
Citrate	–	–	–	–	+	w	w	+
Succinate	–	NT	–	NT	+	w	w	–
Acid production from:								
L-Arabinose	+	+	+	–	+	+	+	–
Gentiobiose	+	NT	+	+	+	+	+	v
Glycerol	–	+	+	–	v	w	+	–
D-Ribose	+	+	+	–	+	+	+	+
Trehalose	+	+	+	+	–	+	+	v
Turanose	w	+	+	+	v	–	+	v
D-Xylose	+	+	+	–	+	+	+	–
Methyl β-D-xyloside	+	–	+	–	+	+	w	–
Methyl α-D-mannoside	+	NT	–	–	+	w	+	–
2-Ketogluconate	–	NT	–	+	–	–	–	–
5-Ketogluconate	–	NT	–	+	–	–	w	–
Growth with 2% NaCl	+	+	NT	v	NT	+	+	+
Growth at 47 °C	+	–	–	–	–	–	–	–
Optimum growth temp. (°C)	37	30	30	30–37	30	30–37	30	30–32
DNA G + C content (mol%)	51.7	41	43–46	48–53	45–47	48	47	NT

*Abbreviations: E, ellipsoidal; O, oval.

Fatty acid methyl esters were analysed by GC/MS according to the instructions of the Microbial Identification system (MIDI; Microbial ID). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), using HPLC apparatus fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM). Methanol:2-propanol (2:1, v/v) was used as the mobile phase and quinone was detected at 270 nm. The peptidoglycan structure was elucidated by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The qualitative analyses of amino acids and peptides in peptidoglycan hydrolysates were carried out as described by Schleifer (1985) and Schleifer & Kandler (1972) using paper chromatography (Rhuland *et al.*, 1955). Quantitative analysis of amino acids in the total hydrolysate was performed by using GC and GC/MS as described by MacKenzie (1987) and Hasegawa *et al.* (1983). The N terminus of the interpeptide bridge was determined by dinitrophenylation according to Schleifer (1985). The DNA G+C content (mol%) was determined by reversed-phase HPLC using the method of Tamaoka & Komagata (1984). The cellular fatty acid profile of the strain was characterized by the presence of saturated fatty acids such as anteiso-C_{15:0} (40.0%), iso-C_{15:0} (16.2%), C_{16:0} (12.7%) and iso-C_{16:0} (8.1%) as the major fatty acids on TSA, and this pattern was similar to those of phylogenetically closely related species of the genus *Paenibacillus*. However, the proportions of anteiso-C_{15:0} and iso-C_{15:0} were lower and higher, respectively, than those reported for other *Paenibacillus* species (Table 2). Strain YB-45^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, and the predominant isoprenoid quinone was MK-7. The DNA G+C content of strain YB-45^T was 51.7 mol%. The major fatty acid profile, the isoprenoid quinone and the DNA G+C content are typical of the members of the group classically defined as constituting the genus *Paenibacillus* (Ash *et al.*, 1993).

The 16S rRNA gene was amplified by a PCR using the Eubac 27F and 1492R primers (DeLong, 1992) and the PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing of the purified 16S rRNA gene was performed by using an ABI PRISM BigDye Terminator cycle sequencing kit, as recommended by the manufacturer (Applied Biosystems), with five primers (337F, 785F, 1225F, 518R and 1100R). The purified sequencing-reaction mixtures were electrophoresed automatically, using an Applied Biosystems model 377 automatic DNA sequencer. The 16S rRNA gene sequence of strain YB-45^T was aligned with those of *Paenibacillus* species by using the CLUSTAL W program (Thompson *et al.*, 1994). Sequence similarity values were computed by using SIMILARITY MATRIX version 1.1 (Ribosomal Database Project II; Cole *et al.*, 2003). Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Phylogenetic trees were inferred by using three tree-making algorithms, i.e. the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969)

Table 2. Cellular fatty acid compositions (%) of the type strains of some *Paenibacillus* species and strain YB-45^T

Strains: 1, YB-45^T; 2, *P. polymyxa* DSM 36^T; 3, *P. azotofixans* DSM 5976^T; 4, *P. peoriae* DSM 8320^T; 5, *P. kribbensis* JCM 11465^T; 6, *P. terrae* JCM 11466^T. –, Not detected.

Fatty acid	1	2	3	4	5	6
Saturated fatty acids						
C _{11:0} 2-OH	–	0.4	–	–	–	–
C _{14:0}	1.9	0.7	5.0	1.1	1.9	1.3
C _{15:0}	1.5	0.5	2.2	2.6	1.7	2.2
C _{16:0}	12.7	9.1	15.5	6.3	10.2	9.1
C _{16:0} N alcohol	–	–	0.4	0.1	–	–
C _{17:0}	0.3	–	–	0.4	0.5	0.3
C _{18:0}	–	1.1	0.3	0.1	–	–
Unsaturated fatty acids						
C _{16:1} ω11 <i>c</i>	4.2	–	–	–	0.3	0.3
C _{17:1} ω6 <i>c</i>	–	0.7	–	–	–	–
C _{18:1} ω5 <i>c</i>	–	–	1.8	–	–	–
Branched fatty acids						
iso-C _{13:0}	0.2	–	1.3	0.4	–	0.1
anteiso-C _{13:0}	–	–	1.8	0.3	–	0.2
iso-C _{14:0}	2.4	0.6	4.7	2.2	1.2	1.1
iso-C _{15:0}	16.2	5.5	8.7	8.7	10.4	6.5
anteiso-C _{15:0}	40.0	49.9	45.4	56.4	52.4	62.3
iso-C _{16:0}	8.1	7.7	5.3	7.4	6.4	4.5
iso-C _{17:0}	5.7	7.0	1.1	5.9	6.3	3.8
iso-C _{17:0} 3-OH	–	–	2.9	–	–	–
anteiso-C _{17:0}	4.5	16.7	2.1	7.9	8.8	8.3
iso-C _{18:1} H*	–	–	0.8	–	–	–
Summed feature 4†	0.3	–	1.0	–	–	–

*The double bond position (indicated by a capital letter) is unknown.

†Summed feature 4 represents iso-C_{17:1} I and/or anteiso-C_{17:1} B, which could not be separated by GLC using the MIDI system.

methods available in PHYLIP, version 3.65 (Felsenstein, 2005). Evolutionary distance matrices for the neighbour-joining method were calculated by using the algorithm of the Kimura two-parameter model (Kimura, 1980) with the DNADIST program. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed with the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs in the PHYLIP package. The almost-complete 16S rRNA gene sequence (1511 nt) of strain YB-45^T was obtained; the 16S rRNA gene sequence search performed using BLAST revealed that strain YB-45^T exhibited low levels of similarity (not more than 96%) with respect to the recognized species of *Paenibacillus*, which is sufficient to indicate that strain YB-45^T represents a novel species of a genus (Stackebrandt & Goebel, 1994). The levels of 16S rRNA gene sequence similarity between strain YB-45^T and *Paenibacillus jamilae* DSM 13815^T, *Paenibacillus terrae* JCM 11466^T, *Paenibacillus polymyxa* DSM 36^T, *Paenibacillus azotofixans* DSM 5976^T, *Paenibacillus peoriae* DSM 8320^T, *Paenibacillus brasiliensis* DSM 14914^T and *Paenibacillus kribbensis* JCM 11465^T were 95.7, 95.5, 95.5, 95.4, 95.3,

95.2 and 95.2 %, respectively. The phylogenetic tree (Fig. 1) constructed by using neighbour-joining analysis showed that strain YB-45^T was affiliated to the genus *Paenibacillus* and formed a distinct phylogenetic line (68.4 % bootstrap support) with respect to closely related species. The topologies of the phylogenetic trees constructed by using the maximum-likelihood and maximum-parsimony algorithms were similar to that of the tree constructed with neighbour joining (data not shown).

Therefore, on the basis of the data presented, we propose that strain YB-45^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus woosongensis* sp. nov. is proposed.

Description of *Paenibacillus woosongensis* sp. nov.

Paenibacillus woosongensis (woo.song.en'sis. N.L. masc. adj. *woosongensis* of Woosong, the Korean name for the university in Korea at which the organism was isolated).

Cells are xylanolytic, Gram-variable, motile, aerobic or facultatively anaerobic, endospore-forming (ellipsoidal, terminal) rods measuring 0.5–0.7 × 2.5–5.0 µm. Colonies on TSA medium are circular with slightly irregular edges, low convex, light beige to greyish, translucent, glossy and usually

1–2 mm in diameter within 48 h at 37 °C. Growth occurs at 28–47 °C (optimum, 37 °C) and from pH 6.0 to 10.6 (optimum, pH 7.5). Grows at salinities of 0–3 % (w/v) NaCl (optimum, 3 % NaCl). No growth occurs in the presence of >5 % NaCl. Oxidase-negative and catalase-positive. Nitrate is not reduced to nitrite. Aesculin, casein and starch are hydrolysed; tyrosine, Tween 80, gelatin and urea are not hydrolysed. L-Arabinose, CM-cellulose, D-fructose, galactose, gluconate, glucose, D-ribose, starch, xylan and D-xylose are utilized; 5-keto-D-gluconate, D-arabinose, rhamnose and xylitol are utilized weakly as sole carbon and energy sources. 2-Keto-D-gluconate, citrate, glycerol, sorbitol, sorbose and succinate are not utilized as sole carbon and energy sources. Acid is produced from amygdalin, arabinose, arbutin, D-cellobiose, D-fructose, D-galactose, gentiobiose, gluconate, D-glucose, glycogen, inulin, D-lactose, D-lyxose, maltose, D-mannose, melibiose, methyl β-D-xylopyranoside, N-acetylglucosamine, raffinose, D-ribose, salicin, sucrose, trehalose, turanose and D-xylose; acid is produced weakly from xylitol. Acid is not produced from 2-keto-D-gluconate, 5-keto-D-gluconate, D-adonitol, arabinol, dulcitol, erythritol, fucose, gluconate, glycerol, inositol, melezitose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, D-sorbitol, L-sorbose, D-tagatose or L-xylose. Xylanase, cellulase, amylase, β-mannanase, β-mannosidase, β-xylosidase, α-galactosidase, β-galactosidase and β-glucosidase are produced. The

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain YB-45^T and related taxa. Bootstrap percentages (based on 1000 replications) are shown where >50 %. *Bacillus subtilis* NCIMB 3610^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acid is anteiso-C_{15:0}. The DNA G+C content of the type strain is 51.7 mol%.

The type strain, YB-45^T (=KCTC 3953^T=DSM 16971^T), was isolated from forest soil at Daejeon in Korea.

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