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Paenibacillus donghaensis sp. nov., a Xylan-degrading and Nitrogen-fixing Bacterium Isolated from East Sea Sediment

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A Gram-positive and endospore-forming strain, JH8^T, was isolated from deep-sea sediment and identified as a member of the genus *Paenibacillus* on the basis of 16S rRNA gene sequence and phenotypic analyses. According to a phylogenetic analysis, the most closely related species was *Paenibacillus wynnii* LMG 22176^T (96.9%). Strain JH8^T was also facultatively anaerobic and grew optimally at 20–25°C. The major cellular fatty acid was anteiso-$C_{15:0}$, and the DNA G+C content was 53.1 mol%. The DNA-DNA relatedness between the isolate and *Paenibacillus wynnii* LMG 22176^T was 7.6%, indicating that strain JH8^T and *P. wynnii* belong to different species. Based on the phylogenetic, phenotypic, and chemotaxonomic characteristics, strain JH8^T would appear to belong to a novel species, for which the name *Paenibacillus donghaensis* sp. nov. is proposed (type strain=KCTC 13049^T=LMG 23780^T).

Keywords: Deep-sea sediment, nitrogen-fixing, *Paenibacillus donghaensis*, polyphasic taxonomy, xylan

The genus *Paenibacillus* has been proposed to accommodate groups of aerobic or facultatively anaerobic rod-shaped, endospore-forming bacteria based on 16S rRNA gene sequence analyses [1]. Thus, according to this proposal, the members of "group 3" within the genus *Bacillus* were transferred to the genus *Paenibacillus*, making it currently comprised of 83 type species and 2 subspecies [5]. Since the genus *Paenibacillus* has the ability to excrete many kinds of enzyme that degrade natural biopolymers, such as agar [32], chitin [11, 14], glucan [28], and other polysaccharides [23], several of these enzymes, including extracellular xylanase [9, 15], glucanase [21], and chitinase [10], were recently purified and characterized.

During the course of screening for biopolymer-degrading bacteria from bottom sediments sampled from the East Sea, Korea, several novel strains were isolated. Accordingly, the present study conducted phylogenetic, phenotypic, genotypic, and chemotaxonomic analyses to determine the taxonomic position of isolate JH8^T, exhibiting xylan-degrading ability. On the basis of the results, strain JH8^T is proposed as a novel species, *Paenibacillus donghaensis* sp. nov.

East Sea bottom sediments were retrieved at a depth of 200 to 500 m, and then serially diluted with filtered deep-sea water. Thereafter, an aliquot of each dilution was spread on a 100-fold diluted Marine agar (Difco) containing 1% xylan and incubated at 17°C for 3 to 10 days. The colonies showing a halo on the medium were selected as xylan-degrading bacteria, and further purified by transferring onto new plates, followed by additional incubation for 3 days at 25°C. The cultures were then stored at −80°C in a Tryptic soy broth (TSB; Difco) supplemented with 25% (v/v) glycerol, and strain JH8^T was routinely grown on a Tryptic soy agar (TSA) or TSB at 25°C for the phenotypic analyses.

The flagellation was examined using a transmission electron microscope (Philips CM20) after negative staining with 1% phosphotungstic acid, using cells grown on TSA at 25°C. The shape of the spores was determined by phase-contrast microscopy (Nikon 80*i*), and the Gram staining performed using BD Gram stain kits according to the manufacturer's instructions (BD, U.S.A.) and the nonstaining method as described by Buck [3]. The catalase activity was determined based on bubble production in 3% (v/v) H_2O_2 and the oxidase activity determined using an Orientation test kit (bioMérieux). In addition, the acid

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production from carbohydrates, single-carbon-source assimilation, and additional physiological characteristics were determined using API 50CH, API 20E, API 20NE, and API ID 32 (bioMérieux) according to the manufacturer's instructions. The tolerance to NaCl was measured on a nutrient agar (Difco) containing 0–14% (w/v) NaCl, where the plates were incubated at 25°C for 10 days. The temperature range for growth was determined on TSA after incubation for 6 days at 0, 4, 10, 15, 20, 25, 30, 35, 37, 40, 45, and 50°C. Anaerobic growth was performed in TSB by using Hungate anaerobic culture tubes with nitrogen gas and incubation at 25°C. The nitrogen-fixing ability was determined by growth in 50 ml of a nitrogen-free medium (DSMZ medium No. 3) in a 500-ml Erlenmeyer flask, where the medium contained 5.0 g/l glucose, 5.0 g/l mannitol, 0.1 g/l $CaCl_2 \cdot 2H_2O$, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 5.0 mg/l $Na_2MoO_4 \cdot 2H_2O$, 0.9 g/l K_2HPO_4, 0.1 g/l KH_2PO_4, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 5.0 g/l $CaCO_3$, and 1 ml of a trace element mixture/l (SL-6, DSMZ medium No. 27) that contained 0.1 g/l $ZnSO_4 \cdot 7H_2O$, 0.03 g/l $MnCl_2 \cdot 4H_2O$, 0.3 g/l H_3BO_3, 0.2 g/l $CoCl_2 \cdot 6H_2O$, 0.01 g/l $CuCl_2 \cdot 2H_2O$, and 0.02 g/l $NiCl_2 \cdot 6H_2O$. Growth was determined after 2 weeks at 25°C in a shaking incubator. A pair of primer systems, PolF and PolR [19], were used to amplify the *nifH* gene. The PCR was run for 35 cycles with a DNA thermal cycler (MJ Mini; Bio-Rad, U.S.A.) using the following thermal protocol: denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min, and an extension at 72°C for 1 min, with the final cycle including an extension for 7 min. The PCR products were separated by electrophoresis on an agarose gel (0.8%, w/v) and stained with ethidium bromide. The band size was determined by comparison with a 100-bp ladder standard (Elpis Biotech, South Korea).

The cells were found to be Gram-positive, rod-shaped (0.7×4.8 μm), and motile based on peritrichous flagella (Fig. 1A). Strain JH8^T also produced ellipsoidal spores in sporangia in the subterminal region of the cell (Fig. 1B). The colonies grown on TSA at 25°C were nonpigmented, circular, slightly convex, and cream-colored, with a diameter of 1.0–1.5 mm. The isolate was facultatively anaerobic, showing similar growth under both aerobic and anaerobic conditions. Strain JH8^T was able to grow between 4 and 30°C, yet not at 0 or 35°C after 6 days. The optimal growth temperature was between 20 and 25°C. While growing in 0–3% (w/v) NaCl, it did not grow in 0.001% (w/v) lysozyme. Growth was also observed within a pH range of 6 to 10 after 7 days of incubation at 25°C. The phenotypic characteristics of the closest phylogenetic relatives are compared in Table 1, where strain JH8^T showed positive for the Voges-Proskauer test and citrate utilization, yet could not

Fig. 1. Morphology of strain JH 8^T.
Negatively stained transmission electron micrograph (**A**), and photomicrograph of sporangia and vegetative cells (**B**) of *Paenibacillus donghaensis* JH8^T.

Table 1. Characteristics that differentiate *P. donghaensis* sp. nov. from its closest relatives in the genus *Paenibacillus*.

Characteristic	1	2	3	4	5	6	7
Voges-Proskauer test	+	–	–	ND	ND	–	–
Nitrate reduction	–	+	–	+	+	–	–
Casein hydrolysis	–	–	+	ND	ND	–	–
Starch hydrolysis	+	+	–	ND	ND	+	+
Oxidase	–	–	–	–	–	–	+
Citrate utilization	+	–	–	ND	ND	ND	-
Catalase	–	+	+	+	+	+	+
Acid from carbohydrates							
L-Rhamnose	+	v1	–	–	–	–	–
Glycerol	–	v1	+	v2	+	–	–
D-Ribose	+	v1	–	+	–	+	+
N-Acetyl-glucosamine	–	+	+	+	+	+	+
Arbutin	+	v1	+	+	+	+	–
Glycogen	+	+	+	+	+	+	–
D-Sorbitol	–	+	v2	–	–	–	–
D-Arabitol	–	–	+	–	–	–	–
D-Mannitol	+	+	+	–	+	+	–
D-Melezitose	–	v1	+	–	+	+	–
Methyl-α-D-glucopyranoside	–	–	v2	+	+	+	+
Inulin	–	v1	+	+	v2	+	–
Xylitol	–	v1	v2	–	–	–	–
D-Lyxose, D-Tagatose	–	–	v2	–	–	–	–
L-Fucose	–	–	–	v2	–	+	–
Gluconate	–	v1	–	–	v2	+	–

Species: 1, *P. donghaensis* (data from this study); 2, *P. wynnii* [24]; 3, *P. borealis* [4, 19]; 4, *P. odorifer* [2]; 5, *P. graminis* [2]; 6, *P. macquariensis* [4, 16, 19, 27]; 7, *P. antarcticus* [19]. All the species produced acid from galactose, D-glucose, D-fructose, amygdalin, salicin, cellobiose, maltose, mannose, melibiose, sucrose, trehalose, D-raffinose, β-gentiobiose, and D-turanose. None of the species produced acid from erythritol, D-arabinose, D-fucose, L-xylose, adonitol, L-sorbose, dulcitol, inositol, L-arabitol, methyl-α-D-mannopyranoside, 2-ketogluconate, or 5-ketogluconate. All the species were negative for the production of H_2S, gelatin hydrolysis, and growth at 50°C. For *P. donghaensis*, the results are scored as positive or negative, whereas for the other species, the results are scored as follows: +, >90% strains positive; –, <10% strains positive; v1, 26–74%; v2, 11–89% strains positive; ND, not determined.

produce catalase and acid from *N*-acetyl-glucosamine. Since the nitrogenase reductase gene (*nifH*) encodes key enzymes in the nitrogen-fixing pathways in various microorganisms [33], the present study attempted to detect this gene in strain JH8^T to determine the strain's nitrogen-fixing capability. Based on PCR amplification using PolF and PolR [22], *nifH* (about 360 bp) was clearly amplified in strain JH8^T, as in *Paenibacillus wynnii* LMG 22176^T [24]. In addition, strain JH8^T grew well in a nitrogen-free liquid medium.

The G+C content of the chromosomal DNA was determined as described by Mesbah *et al.* [17] using reverse-phase HPLC. Cellular fatty acids profiles were determined for the strains grown on a TSA for two days at 25°C, where the cellular fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System [18]. The fatty acids were then analyzed by gas chromatography (Hewlett Packard 6890) using the Microbial Identification software package [26].

The G+C content of strain JH8^T was 53.1 mol%, placing it within the range previously observed for members of the genus *Paenibacillus* [27]. The fatty acids found in strain JH8^T are given in Table 2 and compared with the values available for phylogenetically related species of the genus *Paenibacillus* [24]. The predominant fatty acid component was anteiso-branched saturated $C_{15:0}$ (54.63%), which matched with the other members of the genus *Paenibacillus* [27]; however, the second major fatty acid found in strain JH8^T was iso-$C_{16:0}$ (13.5%), for which the value was significantly higher than those previously reported for the other type strains (Table 2).

For the phylogenetic analysis of strain JH8^T, DNA was extracted using a genomic DNA extraction kit (Solgent Co. Ltd., South Korea). The 16S rRNA gene was then amplified from the chromosomal DNA using the universal bacterial primer set 27F and 1492R, and the purified PCR products were sequenced by Solgent Co. Ltd. (Daejeon, South Korea) [20]. The full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR), and the 16S rRNA gene sequences for related taxa obtained from the GenBank database. Multiple alignments were then performed using the Clustal_X program [30], gaps edited using the BioEdit program [8], and the evolutionary distances calculated using the Kimura two-parameter model [12]. A phylogenetic tree was constructed based on the neighbor-joining method [25] using the MEGA 3 Program [13] with bootstrap values based on 1,000 replications [7].

Table 2. Comparison of fatty acid compositions of *Paenibacillus donghaensis* strain JH8^T and phylogenetically related species of the genus *Paenibacillus*.

Fatty acid	1	2	3	4	5	6
Straight-chain fatty acids:						
$C_{14:0}$	1.6	5.7	18.8	4.7	4.9	9.8
$C_{15:0}$	0.5	0.0	0.0	0.0	3.3	0.0
$C_{16:0}$	8.3	30.9	12.5	14.7	8.6	8.0
Unsaturated fatty acids:						
$C_{16:1}$ ω7c alcohol	0.4	0.0	0.0	0.0	0.0	2.4
$C_{16:1}$ ω11	0.7	10.1	<1.0	2.1	1.1	24.6
Branched-chain fatty acids:						
iso-$C_{14:0}$	5.7	6.3	4.6	4.3	1.7	2.8
iso-$C_{15:0}$	9.3	6.9	13.2	14.4	13.8	11.0
anteios-$C_{15:0}$	54.6	34.0	37.0	49.3	59.3	40.0
iso-$C_{16:0}$	13.6	4.4	9.0	5.5	1.7	1.4
iso-$C_{17:0}$	2.0	1.7	2.4	2.9	0.0	0.0
Anteios-$C_{17:0}$	2.3	0.0	2.3	2.3	<1.0	0.0

Species: 1, *P. donghaensis* (data from this study); 2, *P. wynnii* [23]; 3, *P. borealis* [23]; 4, *P. odorifer* [23]; 5, *P. macquariensis* [23]; 6, *P. antarcticus* [23].

The 16S rRNA gene sequence of strain JH8^T determined in this study was a continuous stretch of 1,434 bp, and has been deposited in the NCBI/EMBL/DDBJ under Accession No. EF079062. The resulting phylogenetic trees based on the neighbor-joining method, minimum evolution (data not shown), and maximum-parsimony (data not shown) revealed that strain JH8^T fell within the evolutionary radiation enclosed by the genus *Paenibacillus* and formed a cluster with the type strains *P. wynnii*, *P. borealis*, *P. odorifer*, *P. stellifer*, and *P. graminis*, and particularly with *P. wynnii* LMG 22176^T (Fig. 2). The relationship between this cluster and the remaining cluster of other *Paenibacillus* species was supported by a bootstrap resampling value of 94%. In particular, the relationship between strain JH8^T and *P. wynnii* was supported by a high bootstrap value (99%). In addition, a sequence similarity calculation showed that the closest relative of strain JH8^T was *P. wynnii* LMG 22176^T (96.9%), whereas the similarity values shown by JH8^T to the other *Paenibacillus* type strains were all under 97% (*Paenibacillus borealis* DSM 13188^T, 96.4%; *Paenibacillus odorifer* LMG 19079^T, 96.7%). Thus, based on the 16S rRNA gene sequence analyses, strain JH8^T appeared to be a new taxon, as it has been suggested that a 70% DNA reassociation defines a genomic species in bacterial strains with a less than 97% 16S rRNA gene sequence identity [29, 32].

To further verify the new taxon of strain JH8^T, DNA-DNA hybridizations were performed with *Paenibacillus wynnii* LMG 22176^T [24]. The DNA-DNA hybridizations were performed between JH8^T and the type strains of *P. wynnii* LMG 22176^T using photobiotin-labeled DNA probes and microdilution wells, as described by Ezaki *et al.* [6]. The hybridizations were performed with five replications for each sample, where the highest and lowest values obtained were excluded, and the means of the remaining three values were quoted as the DNA-relatedness value. As a result, the low DNA-DNA reassociation value of 7.6% between the two strains was low enough for strain JH8^T to be classified as a novel *Paenibacillus* species [32].

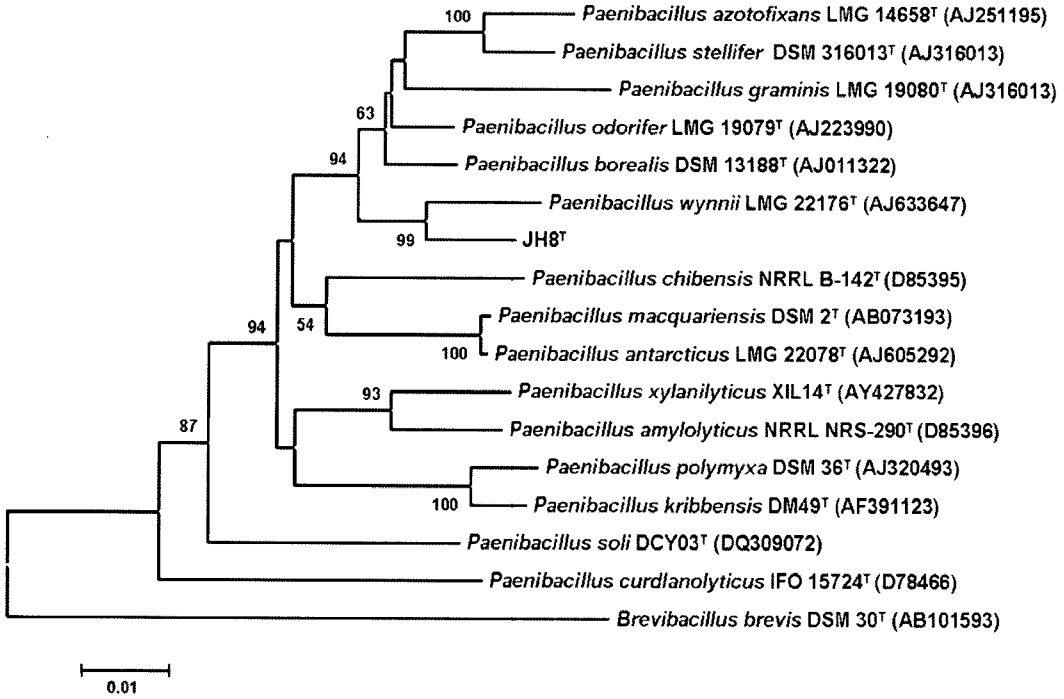

Fig. 2. Phylogenetic position based on neighbor-joining of the 16S rRNA gene sequence of strain JH8^T among related *Paenibacillus* species.

Bootstrap values are expressed as percentages of 1,000 replications and greater than 50% are shown at branch points. The *Brevibacillus brevis* sequence was used as an outgroup. The bar shows sequence divergence. Bar, 0.01 substitutions per nucleotide position.

The morphological, physiological, chemotaxonomic, and phylogenetic data showed that strain JH8^T belonged to the genus *Paenibacillus*, yet the DNA-DNA hybridization analysis clearly distinguished strain JH8^T from *Paenibacillus wynnii* LMG 22176^T [24]. Thus, based on the polyphasic evidence, it is proposed that strain JH8^T be assigned as the type strain for a novel species in the genus *Paenibacillus*, *Paenibacillus donghaensis* sp. nov.

Description of *Paenibacillus donghaensis* sp. nov.

Paenibacillus donghaensis (dong.ha.en'sis. N.L. masc. adj. donghaensis of Donghae, the Korean name for the East Sea, from which the strains were isolated).

Cells are rod-shaped (0.7×4.8 μm) and motile based on peritrichous flagella. Subterminal ellipsoidal spores are formed in sporangia. Colonies grown on TSA are nonpigmented, circular, slightly convex, and cream-colored. Cells are facultatively anaerobic and Gram-positive. Growth is not inhibited by the presence of 3% NaCl, yet is inhibited by 0.001% lysozyme. Growth occurs between 4 and 30°C, yet not at 0 or 35°C; optimal growth occurs between 20 and 25°C. Urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, indole production, and tryptophan deaminase are all negative. Acid is produced from L-arabinose, D-xylose, methyl-β-D-xylose, aesculin, D-lactose, D-melibiose, and amidon. Other pheonotypic characteristics of strain JH8^T are given in Table 1. The predominant fatty acid is anteiso-$C_{15:0}$ (54.6%).

The type strain is JH8^T (=KCTC 13049^T=LMG 23780^T); its G+C content is 53.1 mol%.

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