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Bacillus coreaensis sp. nov.: a xylan-hydrolyzing bacterium isolated from the soil of Jeju Island, Republic of Korea

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A xylan-degrading bacterium, designated as MS5^T strain, was isolated from soil collected from the Jeju Island, Republic of Korea. Strain MS5^T was Gram-stain-positive, aerobic, and motile by polar flagellum. The major fatty acids identified in this bacterium were iso-$C_{15:0}$ (32.3%), $C_{16:0}$ (27.3%), and anteiso-$C_{15:0}$ (10.2%). A similarity search based on the 16S rRNA gene sequence revealed that the strain belongs to the class *Bacilli* and shared the highest similarity with the type strains *Bacillus beringensis* BR035^T (98.7%) and *Bacillus korlensis* ZLC-26^T (98.6%) which form a coherent cluster in a neighbor-joining phylogenetic tree. The DNA G+C content of strain MS5^T was 43.0 mol%. The major menaquinone was MK-7 and the diagnostic diamino acid in the cell-wall peptidoglycan was meso-diaminopimelic acid. The DNA-DNA relatedness values between strain MS5^T and two closely related species, *B. beringensis* BR035^T and *B. korlensis* ZLC-26^T, were less than 70%. DNA-DNA relatedness analysis and 16S rRNA sequence similarity, as well as phenotypic and chemotaxonomic characteristics suggest that the strain MS5^T constitutes a novel *Bacillus* species, for which the name *Bacillus coreaensis* sp. nov. is proposed. The type strain is MS5^T (=DSM25506^T=KCTC13895^T).

Keywords: xylanase, *Bacillus coreaensis*, DSM25506, KCTC-13895

Introduction

The genus *Bacillus* was first described by Cohn in 1872; it is one of the largest bacterial genera, comprising more than 240 species (Euzéby, 2013). The type species is *Bacillus subtilis*. *Bacillus* species are widely distributed in diverse environments such as water, soil, and air. The genus *Bacillus* includes Gram-stain-positive, aerobic or facultative aerobic, spore-forming, and rod-shaped bacteria which are members of the class *Bacilli.* The class *Bacilli* is one of the main groups in soil. Members of the genus *Bacillus* have iso-$C_{15:0}$ and anteiso-$C_{15:0}$ as the major fatty acids (Kämpher, 2002) and MK-7 as the major menaquinone (Kim *et al.*, 2014). The DNA G+C content of *Bacillus* is 32–66 mol% (Logan *et al.*, 2004). Since *Bacillus* species produce diverse bioactive compounds and commercial enzymes, they have been widely studied.

Xylan is a wide variety of highly complex polysaccharides composed of xylose units which are found in the cell walls of most plant and algal species. Xylan hydrolysis can be applied in many industries, e.g., neutraceuticals, textiles, paper, pulp industries (Collins *et al.*, 2005). Although xylanases have been characterized from various sources including filamentous fungi, yeast, marine algae, crustaceans, protozoans, insects, snails, and bacteria (Collins *et al.*, 2005; Chi *et al.*, 2012), bacteria (particularly, *Bacillus* strains) are known to the most important source for commercial applications owing to efficient secretion of enzyme and rapid cell growth (Nagar *et al.*, 2010). However, for a more efficient degradation of xylan, it is crucial to find new xylanases with excellent activity and stability. In this study, we report a novel *Bacillus* species with a thermostable xylanase, isolated from the soil of Jeju Island, Republic of Korea, and designated as MS5^T.

Materials and Methods

Bacterial strains

Strain MS5^T was isolated from a soil sample collected from the Jeju Island, which is located at 126°08′–126°58′ of east longitude and 36°06′–33°00′ north latitude, Republic of Korea. Series of dilutions (10^{-1}–10^{-5}) of soil sample prepared in 200 µl solutions were smeared on Luria-Bertani (LB) agar plate containing 0.2% xylan azure (w/v). To isolate a strain producing thermostable enzyme, the plates were incubated at 40°C for 48 h and xylan degradation was detected by blue zone around the colonies. One bacterium exhibiting strong xylan-hydrolyzing activity among the twelve xylanase-positive colonies was selected and designated as strain MS5^T. The strain was routinely cultured on LB or Marine Broth 2216 (Difco) agar plates. After 1 day incubation in LB broth at 40°C, the MS5^T stock culture was frozen with 20% glycerol (w/v) at -80°C. Reference strains, *B. beringensis* BR035^T (DSM 22571^T) and *B. korlensis* ZLC-26^T (NRRL B-51302^T) were obtained from the The Leibniz Institute DSMZ and ARS Culture Collection (NRRL), respectively.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MS5^T is JN578481.

Phylogenetic analysis

The isolate was cultured in LB broth at 37°C for 24 h; genomic DNA was extracted by using a genomic DNA extraction kit (Promega Co.). The 16S rRNA gene was PCR-amplified with bacterial universal primers (27F; 5′-AGAGTTT GATCCTGGCTCAG-3′ and 1492R; 5′-TACCTTGTTACG ACTT-3′, Baker *et al.,* 2003); the amplified double-stranded DNA was cloned into the pGEM-T easy vector (Promega Co.). The nucleotide sequencing was performed on an Applied Biosystems 3730xl DNA Analyzer. The 16S rRNA gene sequences of type strains related to the MS5^T strain were collected from the EzTaxon server (Chun *et al*., 2007) to construct a phylogenetic tree. Multi-alignment between related strains was determined using Clustal W software (Thompson *et al*., 1994) and 5′ and 3′ gaps were edited in BioEdit (Hall, 1999). Neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods in the PHYLIP suit program (Felsenstein, 1993) were used to construct the phylogenetic tree. The bootstrap value was calculated with data restructured approximately 1,000 times; values are noted at the nodes (Fig. 1). The evolutionary distance matrix was estimated according to Kimura's 2-parameter model (Kimura, 1983).

Phenotypic characteristics

The isolate was cultured in LB plate at 37°C for 24 h for phenotypic characterization. Cell morphology, size, and motility were examined by using phase-contrast microscopy with BX51 microscope (Olympus). Gram staining was performed with a gram stain kit (BD Diagnostics), according to the manufacturer's protocols. Flagella were observed by transmission electron microscopy (JEM1010, JEOL) after negative staining with 1% (w/v) phosphotungstic acid. Biochemical characteristics and enzyme production were determined using the API 20NE, API Staph, and API ZYM strip (bioMérieux), according to the manufacturer's protocols. Growth of strain MS5^T at different temperatures (0, 4, 10, 20, 25, 35, 40, 45, and 50°C), pH values (pH of 4–11, in 1-pH increments), and NaCl concentrations (0–15%, w/v, in 1% increments) were investigated on LB agar plates for up to 4 days. Growth under anaerobic conditions was tested in an anaerobic jar system (GasPak system; BBL). Strain MS5^T was streaked onto Marine Broth 2216 agar plates for antibiotic susceptibility testing and paper discs containing 30 μl of antibiotics (100 μg/μl) were placed on the plate. Plates were incubated at 37°C for 24 h, and clearance zones around the paper disc demonstrated the inhibitory activity of the antibiotics.

Chemotaxonomy

Major respiratory quinones were analyzed by performing reverse-phase high performance liquid chromatography (HPLC) using strain MS5^T on Marine Broth 2216 agar plate (Komagata and Suzuki, 1987). The isomer type of diaminopimelic acid in the peptidoglycan was analyzed by the thin layer chromatographic method described by Staneck and Roberts (1974). A cellular fatty acid methyl ester (FAME) mixture was prepared from the MS5^T cells grown on Marine Broth 2216 agar plate for 48 h by methyl esterification (Miller and Berger, 1985) and analyzed by gas chromatography using the Microbial Identification software package (Sasser, 1990).

DNA-DNA hybridization and DNA base composition

DNA-DNA relatedness between strain MS5^T and the most closely related strains, *B. beringensis* BR035^T and *B. korlensis* ZLC-26^T, was determined by DNA-DNA hybridization. Genomic DNA was prepared according to a modification of the procedure of Wilson (1987). DNA-DNA hybridizations were performed in three replicates at 40°C according the method described by Ezaki *et al.* (1989). The DNA G+C content of strain MS5^T was analyzed by reverse-phase HPLC as described previously (Mesbah *et al*., 1989).

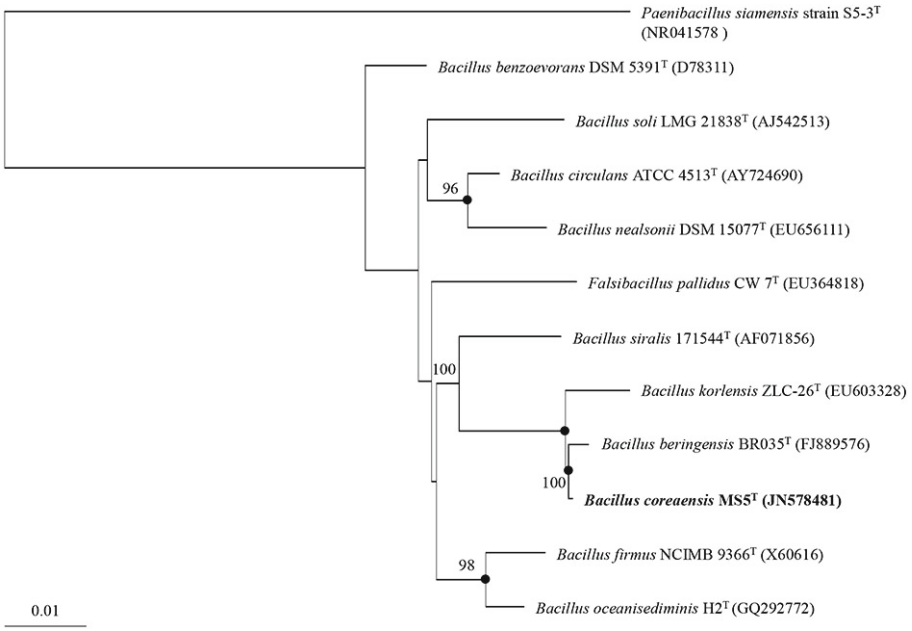

Fig. 1. A neighbor-joining (NJ) tree based on almost complete 16S rRNA gene sequences showing the relationships between strain MS5^T and other *Bacillus* species. Evolutionary distances were determined according to the Kimura's 2-parameter model. Closed circle represents that the corresponding branches were recovered in the neighbor-joining (NJ), maximum-parsimony (MP), and maximum likelihood (ML) trees. Bootstrap values (>70%) based on 1,000 replicates are listed as percentages at nodes. Nucleotide sequence accession numbers are given in parentheses. *Paenibacillus siamensis* strain S5-3^T (NR041578) was used as the outgroup. Scale bar, 0.01 substitutions per 100 nucleotides.

Results and Discussion

Phylogenetic analyses

The MS5^T 16S rRNA gene shared high sequence similarity with *B. beringensis* BR035^T (98.7%), *B. korlensis* ZLC-26^T (98.6%), *B. firmus* NCIMB9366^T (96.8%), *B. oceanisediminis* H2^T (96.7%), *B. benzoevorans* DSM5391^T (96.6%), *B. circulans* IAM12462^T (96.5%), and *B. siralis* 171544^T (96.4%). The 16S rRNA gene sequence similarities to other *Bacillus* type strains with validly published names were less than 96.4%. A neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) based on 16S rRNA revealed that MS5^T forms a coherent cluster with *B. beringensis* BR035^T, supported by a bootstrap value of 100% (Fig. 1). The 16S rRNA phylogenetic trees constructed by the NJ, MP, and ML methods were similar in topology.

Table 1. Phenotypic characteristics of strain MS5^T and its closely related type strains of genus *Bacillus*. Strains: 1, strain MS5^T (Data from this study); 2, *B. beringensis* BR035^T (Yu *et al*., 2011); 3, *B. korlensis* ZLC-26^T (Zhang *et al*., 2009). Symbols : +, positive; -, negative; w, weak positive; v, variable.

Characteristic	1	2	3
Source	Soil sample	Seawater	Soil sample
Flagella	Polar flagellum	Polar flagellum	Peritrichous flagella
Colony color	Yellowish beige	White	Cream-yellow
G+C content	43.0	37.8	38.2
Growth at:			
pH 10	-	+*	+*
4°C	-	+*	-*
45°C	+	-*	+*
8% NaCl	-	+*	+*
Production of:			
Alkaline phosphatase	+	-*	+*
Esterase (C4)	+	-*	+*
Esterase (C8)	+	-*	+*
α-Chymotrypsin	+	-*	+*
α-Galactosidase	+	-*	-*
β-Galactosidase	+	-*	-*
β-Glucuronidase	+	-*	+*
N-Acetyl-β-glucosaminase	+	-*	-*
Assimilation of:			
D-Glucose	+	-*	-*
L-Arabinose	+	-*	-*
D-Mannitol	w	-*	-*
Fermentation of:			
Erythritol	v	-*	-*
L-Arabinose	w	-*	+*
D-Ribose	+	-*	+*
D-Xylose	+	-*	+*
D-Galactose	w	-*	+*
D-Glucose	+	-*	+*
D-Fructose	+	-*	+*
L-Rhamnose	+	-*	+*
Inositol	-	-*	+*
D-Mannitol	+	-*	+*
Methyl-α-D-mannopyranoside	v	-*	-*
Methyl-α-D-glucopyranoside	+	-*	+*
Amygdalin	+	-*	+*
D-Melibiose	+	-*	+*
Xylitol	w	-*	-*
Potassium gluconate	w	-*	+*

*Data from this study.
All stains are positive: Fermentation of glycerol, *N*-acetylglucosamine, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon (starch), glycogen, gentiobiose, and D-turanose. Production of acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-glucosidase, and β-glucosidase. Nitrate reduction and esculin hydrolysis. Growth at 0–7% (w/v) NaCl, pH 6.0–9.0, and 10–42°C.
All strains are negative: Fermentation of D-arabinose, D-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-mannose, L-sorbitol, dulcitol, D-sorbiol, D-lyxose, D-tagatose, D-fucose, L-fucose, potassium 2-ketogluconate, and potassium 5-ketogluconate. Production of gelatinase, urease, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-mannosidase, and α-fucosidase. Indole production. Growth at 9% (w/v) NaCl, pH 5.0, and 50°C.

Phenotypic characteristics

Strain MS5^T was Gram-stain-positive and aerobic; it formed smooth, circular, yellowish-beige colonies on LB agar plates after incubation at 37°C for 24 h. The colony was circular with a diameter of 0.8 mm. The bacterium is 0.4–0.7 μm wide, 0.9–1.4 μm long, and motile by means of a polar flagellum. Other phenotypic features of strain MS5^T are summarized in Table 1 with data from the phylogenetically closest relatives.

Biochemical characteristics

Strain MS5^T grew at 10–45°C with the optimal temperature between 30–37°C. Growth occurred in media containing 0–7% NaCl (w/v), with an optimal concentration of 0–2% NaCl (w/v). The pH for growth ranged from pH 6.0–9.0, with the optimal pH in the range of pH 7.0–8.0. The MS5^T strain was susceptible to ampicillin, thiostrepton, and chloramphenicol but resistant to kanamycin, apramycin, neomycin, paromomycin, and ribostamycin.

The strain can be clearly distinguished from the phylogenetically closest relative *B. beringensis* BR035^T (Yu *et al.*, 2011) in most of biochemical characteristics listed in Table 1. It was also distant from the *α/β*-galactosidase- and *N*-acetyl-*β*-glucosaminase-negative *B. korlensis* ZLC-26^T (Zhang *et al.*, 2009) by its inability to produce acid from L-arabinose, D-galactose, inositiol, and potassium gluconate, and thus further genotypic analyses would be useful to determine the relatedness between these strains and the newly described species.

The predominant isoprenoid quinone of strain MS5^T was MK-7, consistent with numerous members of the genus *Bacillus* (Claus and Berkeley, 1986). The diagnostic diamino acid in the cell-wall peptidoglycan of the strain was meso-diaminopimelic acid. The major fatty acids (≥5%) of strain MS5^T were iso-$C_{15:0}$ (32.3%), $C_{16:0}$ (27.3%), anteiso-$C_{15:0}$ (10.2%), $C_{14:0}$ (8.3%), and $C_{16:1}$ *ω*11*c* (6.9%), while those of *B. beringensis* BR035^T and *B. korlensis* ZLC-26^T were iso-$C_{15:0}$ (30.5%), anteiso-$C_{15:0}$ (20.2%), iso-$C_{14:0}$ (12.6%), and $C_{16:1}$ *ω*7*c* (9.9%), and iso-$C_{15:0}$ (34.4%), $C_{16:1}$ *ω*11*c* (18.1%), anteiso-$C_{15:0}$ (17.2%), and summed feature 3 comprising $C_{16:1}$*ω*7*c*/iso-$C_{15:0}$2-OH (5.4%), respectively. Other cellular fatty acids were present at significantly different levels among the three strains (Table 2).

DNA-DNA hybridization and DNA base composition

The DNA G+C content of strain MS5^T was 43.0 mol%, which also differed significantly from *B. beringensis* BR035^T (37.8 mol%: Yu *et al.*, 2011) and *B. korlensis* ZLC-26^T (38.2 mol%: Zhang *et al.*, 2009).

The DNA-DNA relatedness of strain MS5^T was 59.9% with *B. beringensis* BR035^T and 47.7% with *B. korlensis* ZLC-26^T. As a DNA–DNA hybridization value of 70% is generally accepted as the limit for species delineation (Wayne *et al.*, 1987), it can be concluded that strain MS5^T belongs to a single novel species.

Taxonomic conclusion

The strain MS5^T has many different biochemical and physiological characteristics as well as DNA G+C content and DNA-DNA relatedness from those of the phylogenetically closest relatives, *B. beringensis* BR035^T and *B. korlensis* ZLC-26^T. Thus, we conclude that the isolated strain is a novel species within the genus *Bacillus*; we propose the name *Bacillus coreaensis* sp. nov.

Table 2. Cellular fatty acid compositions (%) of strain MS5^T and its related *Bacillus* species. Strains: 1, strain MS5^T; 2, *B. beringensis* BR035^T; 3, *B. korlensis* ZLC-26^T. Values are percentages of total fatty acids. Symbols: –, not detected.

Fatty acid	1*	2*	3*
Straight-chain saturated			
$C_{14:0}$	8.3	2.0	3.1
$C_{15:0}$	1.4	3.2	-
$C_{16:0}$	27.3	1.2	4.0
Mono-unsaturated			
$C_{15:1}$*ω*6*c*	-	1.1	-
$C_{16:1}$*ω*11*c*	6.9	3.8	18.1
$C_{16:1}$*ω*7*c* alcohol	0.6	9.9	2.0
iso-$C_{16:1}$H	-	1.1	-
iso-$C_{17:1}$*ω*10*c*	-	0.6	1.0
Branched saturated			
iso-$C_{14:0}$	3.4	12.6	3.6
iso-$C_{15:0}$	32.3	30.5	34.4
iso-$C_{16:0}$	2.0	4.2	1.2
iso-$C_{17:0}$	2.9	2.5	3.8
anteiso-$C_{15:0}$	10.2	20.2	17.2
anteiso-$C_{17:0}$	1.5	2.9	1.0
Summed feature			
3 ($C_{16:1}$*ω*7*c* and/or iso-$C_{15:0}$2-OH)	0.4	1.2	5.4
4 (iso-$C_{17:1}$I and/or anteiso-$C_{17:1}$B)	-	2.1	0.7

*All of the data were obtained from the present study.

Description of *Bacillus coreaensis* sp. nov.

Bacillus coreaensis (corea.en'sis. N.L. masc. adj. *coreaensis* named after Corea, an alternate spelling of Korea).

Cells are Gram-stain-positive, mesophilic, aerobic, spore-forming, motile, 0.4–0.7 μm wide, and 0.9–1.4 μm long. Colonies are yellowish beige, opaque, convex, and round on MA plate after 24 h incubation at 40°C. Growth occurs at 10–45°C with optimum growth at 30–37°C but not at 4 or 50°C. Growth occurs at pH 6.0–9.0. Optimum pH for growth is pH 7.0–8.0. The NaCl concentration (w/v) for growth is 0–7% with optimum growth at 0–2%. Cells show susceptibility to ampicillin, thiostrepton, and chloramphenicol, but resistance to kanamycin, apramycin, neomycin, paromomycin, and ribostamycin. Positive for alkaline phosphatase, esterase (C4 and C8), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, nitrate reduction, and esculine hydrolysis, but negative for arginine dihydrolase, urease, gelatinase, lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α-mannosidase, α-fucosidase, and indole production. Utilizes D-glucose, L-arabinose, mannitol (weak), *N*-acetylglucosamine (weak), maltose, gluconate, and malic acid, but not capric acid, adipic acid, trisodium citrate, and phenylacetic acid. Fermentation from D-glucose, glycerol (weak), erythritol (very weak), L-arabinose (weak), D-ribose, D-xylose, D-galactose (weak), D-glucose, D-fructose, L-rhamnose, D-mannitol, methyl-α-D-mannopyranoside (variable), methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon (starch), glycogen, xylitol (weak), gentiobiose, D-turanose, and potassium gluconate (weak) are observed but D-arabinose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-mannose, L-sorbitol, dulcitol, inositol, D-sorbitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate, and potassium 5-ketogluconate are not fermented. The predominant menaquinone is MK-7. The cell-wall peptidoglycan contains meso-diaminopimelic acid. The major fatty acids are iso-$C_{15:0}$, $C_{16:0}$, and anteiso-$C_{15:0}$. The G+C content of strain MS5^T is 42.3 mol%.

The type strain, MS5^T (=DSM25506^T =KCTC13895^T), was isolated from a soil sample collected on Jeju Island, Republic of Korea.

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