Bacillus koreensis sp. nov., a spore-forming bacterium, isolated from the rhizosphere of willow roots in Korea

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An endospore-forming, rod-shaped bacterium was isolated from the rhizosphere of willow roots in Korea. The bacterium, designated strain BR030^T, was a strictly aerobic, motile rod. The cell wall contained type A1 γ peptidoglycan with *meso*-diaminopimelic acid as the diagnostic diamino acid. The major cellular fatty acids were anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{14:0} and iso-C_{16:0}. The major cellular phospholipids were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and unknown phospholipids (PL1, PL2). The genomic DNA G + C content was 36 mol%. Comparative 16S rRNA gene sequence analyses showed that strain BR030^T formed a distinct phyletic line within the genus *Bacillus* and was most closely related to *Bacillus flexus* DSM 1320^T, with 16S rRNA gene sequence similarity of 96.8%. Sequence similarities to other type strains were lower than 96.2%. On the basis of physiological and molecular properties, the isolate represents a novel species within the genus *Bacillus*, for which the name *Bacillus koreensis* sp. nov. is proposed. The type strain is BR030^T (=KCTC 3914^T=DSM 16467^T).

Micro-organisms near plant roots have many effects on plant growth via plant–microbe interactions. The net effect of such interactions on plant growth can be positive, neutral or negative. Bacteria inhabiting areas near plant roots and positively influencing plant growth, referred to as plant growth-promoting rhizobacteria (PGPR), affect growth by increasing nutrient cycling and suppressing pathogens by producing bacterial and fungal antagonistic substances or biologically active substances, such as auxins and other plant hormones (Bashan *et al.*, 2004; Somers *et al.*, 2004). Due to their potential for increasing agricultural yields and controlling plant diseases, bacteria in the rhizosphere and rhizoplane of grasses and crop plants have attracted a great deal of attention (Berge *et al.*, 2002; Penrose & Glick, 2003;

Sessitsch *et al.*, 2004). In the course of an investigation of rhizosphere micro-organisms, a novel aerobic Gram-positive bacterium, designated strain BR030^T, was isolated from the rhizosphere of willow roots (*Salix koreensis* Andersson) in Korea. On the basis of phylogenetic and phenotypic characteristics, strain BR030^T was classified as a novel species of the genus *Bacillus*.

Strain BR030^T was isolated from the roots of a willow tree, more than 3 m tall, growing on hillside silt soil of the Daejeon area in Korea. We sampled root branches from the willow tree and removed soil debris from the roots by tapping. The root branches were washed and the resulting solution was serially diluted using 0.9% (w/v) saline. The diluted solution was spread on nutrient agar (NA; Difco) and incubated for 3 days at 30 °C. The isolated strain was routinely grown aerobically on Luria–Bertani (LB; Difco) agar for 2 days at 35 °C, except where indicated otherwise. Requirement for and tolerance of NaCl were determined in LB broth with different amounts of NaCl after 2 days incubation at 35 °C. Anaerobic growth was determined by incubation in an anaerobic chamber at 35 °C for 5 days on LB agar. Optimum growth conditions were established by incubation at temperatures between 4 and 55 °C on LB agar and at pH values between 5.0 and 10.0 in LB broth.

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Abbreviations: *m*-DAP, *meso*-diaminopimelic acid; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Bacillus koreensis* sp. nov. BR030^T is AY667496.

A transmission electron micrograph showing the spore morphology of *Bacillus koreensis* sp. nov. and tables detailing fatty acid profiles and the results of DNA hybridization studies are available as supplementary material in IJSEM Online.

Cell morphology was studied using light microscopy and transmission electron microscopy (TEM). Motility was observed at 12 and 36 h on agar-coated wet mounts with a light microscope (E600; Nikon). The agar-coated wet mounts were prepared by placing 10 µl culture under a cover slip on a glass slide that had been previously coated with a film of 0.5 % (w/v) agarose (Cambrex) in distilled water and then air-dried. The flagellum type was examined by TEM using cells from the exponential growth phase as described by Lee *et al.* (2005). For investigation of the spore shape of the isolate, specimens were prepared according to Jeon *et al.* (2003) and then subjected to TEM (JEM-1010; JEOL). Endospores were stained according to the method of Schaeffer-Fulton (Smibert & Krieg, 1981).

Gram staining was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Catalase activity was determined by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck). Hydrolysis of aesculin, casein, starch, Tween 80, urea, hypoxanthine, tyrosine, gelatin and xanthine was determined on LB agar according to methods described previously (Cowan & Steel, 1965; Lanyi, 1987; Smibert & Krieg, 1994). Nitrate reduction tests were performed as described by Lanyi (1987). Acid production from carbohydrates was tested according to Leifson (1963).

For quantitative analyses of whole-cell fatty acids, strain BR030^T was cultivated on LB agar at 35 °C for 2 days. Preparation and analysis of fatty acid methyl esters were conducted according to the instructions of the MIDI microbial identification system (Microbial ID). Preparation of cell walls from strain BR030^T and analysis of peptidoglycan were carried out using methods described by Schleifer (1985), with the modification that TLC was performed on cellulose sheets rather than by paper chromatography. Isoprenoid quinones and polar lipids were analysed according to Komagata & Suzuki (1987). The DNA G + C content of strain BR030^T was determined by reverse-phase HPLC using the method of Tamaoka & Komagata (1984). DNA–DNA hybridization was performed to evaluate the genomic DNA–DNA relatedness between strain BR030^T and *Bacillus flexus* DSM 1320^T, obtained from the DSMZ. Both strains were grown aerobically on NA for 2 days at 35 °C and chromosomal DNA was isolated and purified according to Yoon *et al.* (1996). Randomly primed DNA labelling with digoxigenin (DIG)-dUTP and detection of hybrids by enzyme immunoassay on nylon membranes were performed using a DIG High Prime DNA labelling and detection starter kit II (Roche Applied Science) according to the manufacturer's instructions and Lim *et al.*, 2005.

Sequencing and assembly of 16S rRNA genes were carried out as described by Bakermans & Madsen (2002). The 16S rRNA gene sequence of strain BR030^T was compared with available 16S rRNA gene sequences from GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) to determine the approximate phylogenetic affiliation and was

aligned with closely related members using CLUSTAL W software (Thompson *et al.*, 1994). Unmatched regions of the 5'- and 3'-ends from the alignment, which were caused by different lengths of the 16S rRNA gene sequence data, were deleted. The resulting 16S rRNA gene sequence of strain BR030^T that was used for phylogenetic analysis comprised 1348 nucleotides. Phylogenetic trees were constructed by three different methods, neighbour-joining, maximum-likelihood and maximum-parsimony, using algorithms available in the PHYLIP software, version 3.6 (Felsenstein, 2002). Using the FASTA3 program in EBI, 16S rRNA gene sequence comparisons for similarity calculations were made between strain BR030^T and other members of the *Bacillaceae*. A bootstrap analysis was performed according to the Kimura two-parameter model (Kimura, 1980) of the neighbour-joining method in the PHYLIP package (Jeon *et al.*, 2005).

Strain BR030^T grew in LB broth containing 0–9 % (w/v) NaCl; optimum growth occurred on media with 0–3 % (w/v) NaCl. Colonies of the strain were yellow–cream, smooth, convex and circular/slightly irregular on LB agar medium. Strain BR030^T grew at a wide range of pH values, 4.5–9.0 in LB broth; optimal growth was observed at pH 7.0. Growth was observed at temperatures between 15 and 48 °C with an optimum growth temperature of 35–40 °C. Cells of the isolate were motile short rods (0.8–1.6 µm wide and 1.6–2.8 µm long) with peritrichous flagella (data not shown). The isolate produced an oval-type central endospore (see Supplementary Fig. S1 in IJSEM Online). Growth was not observed under anaerobic conditions.

Strain BR030^T showed obviously Gram-positive, catalase-positive and oxidase-negative reactions. The strain did not reduce nitrate to nitrite. Analysis of cell-wall peptidoglycan showed that strain BR030^T possessed A1γ type peptidoglycan with *meso*-diaminopimelic acid (*m*-DAP) as the diagnostic diamino acid, in common with the great majority of the genus *Bacillus* (Priest *et al.*, 1988). The predominant isoprenoid quinone of strain BR030^T was menaquinone-7 (MK-7) and the genomic DNA G + C content was 36 mol%. The strain contained phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and unknown phospholipids (PL1, PL2) as the major polar lipids. The cellular fatty acid profile of strain BR030^T was characterized by saturated branched fatty acids such as anteiso-C_{15:0} (27.25 %), iso-C_{15:0} (25.87 %), iso-C_{14:0} (16.76 %) and iso-C_{16:0} (14.16 %) as the major fatty acids. The fatty acid profile of strain BR030^T was similar to the profiles of its close relatives, *B. flexus* DSM 1320^T and *Bacillus megaterium* DSM 32^T, but the fatty acid compositions are somewhat different (see Supplementary Table S1 in IJSEM Online). The major fatty acid profiles, major lipoquinones and DNA G + C content of strain BR030^T were typical of those of members of the genus *Bacillus* (Priest *et al.*, 1988; Nielsen *et al.*, 1995; Heyrman *et al.*, 2004, 2005). Typical phenotypic and chemotaxonomic properties

Table 1. Characteristics of *Bacillus koreensis* sp. nov. BR030^T and related *Bacillus* species

Species: 1, *B. koreensis* sp. nov. BR030^T; 2, *B. flexus* DSM 1320^T; 3, *B. megaterium* DSM 32^T; 4, *B. asahii* JCM 12112^T; 5, *B. simplex* DSM 1321^T; 6, *B. sporothermodurans* DSM 10599^T; 7, *B. niacini* DSM 2923^T; 8, *B. subtilis* NCDO 1769^T. Data for reference species from Suresh *et al.* (2004), Priest *et al.* (1988), Pettersson *et al.* (1996), Yumoto *et al.* (2004), Heyrman *et al.* (2005) and Nagel & Andreesen (1991). O, Oval; s, spherical; E, ellipsoidal; T, terminal; C, central; ST, subterminal; +, positive; –, negative; W, weak; V, variable; ND, no data.

Characteristic	1	2	3	4	5	6	7	8
Colony colour	Yellow–cream	Cream	Cream	White	Cream	White	Blue	ND
Flagellation	+	ND	ND	+	+	+	–	+
Gram reaction	+	ND	ND	+	V	+	V	ND
Spore shape	O	ND	O	E	E/S	E	O	O
Spore position	C	ND	C	T/C	C/ST	T	C/ST	C
Growth in NaCl at:								
5 % (w/v)	+	+	+	–	V	–	–	+
8 % (w/v)	+	+	+	–	–	–	–	V
Growth at:								
40 °C	+	+	+	+	ND	+	+	+
50 °C	–	–	–	–	–	+	–	–
Catalase	+	ND	ND	+	+	+	ND	ND
Oxidase	–	ND	ND	+	–	+	+	+
Nitrate reduction	+	–	–	W	+	–	+	+
Hydrolysis of:								
Aesculin	+	–	+	ND	V	+	+	ND
Casein	+	+	+	+	V	–	–	+
Starch	+	+	ND	W	+	–	ND	+
Urea	+	–	+	ND	–	–	–	–
Acid production from:								
D-Fructose	+	+	ND	–	+	–	+	ND
D-Glucose	+	+	+	–	+	–	+	+
Glycerol	+	+	ND	–	+	ND	–	ND
D-Lactose	–	+	–	–	–	–	+	–
Maltose	–	+	ND	–	+	ND	+	+
D-Mannitol	–	+	–	–	+	–	–	+
D-Raffinose	+	+	ND	–	–	–	+	+
Sucrose	+	+	ND	–	+	ND	+	ND
D-Trehalose	–	+	ND	–	+	ND	ND	+
D-Xylose	–	–	+	–	–	–	+	V
DNA G + C content (mol%)	36	37–39	37	39·4	39·5–41·6	36	37	43

of strain BR030^T are compared with those of phylogenetically related species in Table 1.

Phylogenetic analysis of 16S rRNA gene sequences showed that strain BR030^T formed a cluster with a relatively low bootstrap resampling value (37 %) with *B. flexus* DSM 1320^T and *B. megaterium* DSM 32^T within the genus *Bacillus*. Strain BR030^T was associated with ‘*Bacillus* group 1’ as defined by Ash *et al.* (1991) (Stackebrandt & Liesack, 1993; Nielsen *et al.*, 1994; Schlesner *et al.*, 2001) (Fig. 1). The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms also supported the conclusion that BR030^T belongs to the genus *Bacillus* (data not shown). Comparative 16S rRNA gene sequence analyses showed that strain BR030^T was most closely related to *B. flexus* DSM 1320^T, with a sequence

similarity of 96·8 %. Sequence similarities to other members used in the phylogenetic analysis were lower than 96·2 %. DNA hybridization was carried out to evaluate the genomic DNA–DNA relatedness between strain BR030^T and the most closely related type strain, *B. flexus* DSM 1320^T. A DNA–DNA relatedness value of 57 % was obtained (see Supplementary Table S2 in IJSEM Online). On the basis of its distinctive physiological, morphological and other key traits, it is proposed that strain BR030^T represents a novel species within the genus *Bacillus*, for which we propose the name *Bacillus koreensis* sp. nov.

Description of *Bacillus koreensis* sp. nov.

Bacillus koreensis (ko.re.en'sis. N.L. masc. adj. *koreensis* pertaining to Korea).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain BR030^T and related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Colonies are yellow-cream, smooth, convex and circular/slightly irregular. Cells are approximately 0.8–1.6 µm wide and 1.6–2.8 µm long. Strictly aerobic, spore-forming motile rods. Gram-positive and KOH test-negative. Catalase-positive and oxidase-negative. Nitrate is not reduced to nitrite. Growth occurs at 15–48 °C (optimum 35–40 °C), pH 4.5–9.0 (optimum pH 7.0) and 0–9% (w/v) NaCl (optimum 0–3%). Aesculin, casein, starch and urea are hydrolysed. Hydrolysis of Tween 80, L-tyrosine, hypoxanthine and xanthine is not observed. Acids are produced from D-glucose, D-fructose, D-ribose, α-D-lactose, maltose, D-trehalose, D-raffinose, glycerol, sucrose and D-melibiose, but not from D-mannitol, D-xylose, L-arabinose, L-rhamnose, adonitol or D-mannose. The cell wall contains A1γ type peptidoglycan with *m*-DAP as the diagnostic diamino acid. Predominant polar lipids are PG, DPG, PE and unknown PL1 and PL2. Major isoprenoid quinone is MK-7. DNA G+C content is 36 mol% (HPLC). Predominant cellular fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{14:0} and iso-C_{16:0}.

The type strain, BR030^T (= KCTC 3914^T = DSM 16467^T), was isolated from the rhizosphere of willow roots in Korea.

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