Bacillus endoradicis sp. nov., an endophytic bacterium isolated from soybean root

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A Gram-positive, aerobic, motile rod, designated strain CCBAU 05776^T, was isolated from the inner tissues of a healthy soybean (*Glycine max* L.) root collected from an agricultural field in the countryside of Shijiazhuang city, Hebei Province, China. Phylogenetic analysis of the 16S rRNA gene indicated that this strain was most closely related to *Bacillus muralis* LMG 20238^T and *Bacillus simplex* NBRC 15720^T with similarity of 96.5 % and 96.3 %, respectively, lower than the suggested threshold (97.0 %) for separating bacterial species. In phenotypic characterization, the novel strain differed from the two most related species in that it did not hydrolyse casein or starch but could grow on MacConkey agar. It grew between 15 and 45 °C and tolerated up to 7 % NaCl (w/v). Strain CCBAU 05776^T grew in media with pH 5.5 to 10 (optimal growth at pH 7.0–8.0). The predominant cellular fatty acids were iso-$C_{15:0}$ (40.81 %) and $C_{16:1}\omega7c$ alcohol (10.61 %). The predominant isoprenoid quinone was menaquinone 7 (MK-7). The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. The major polar lipids were diphosphatidylglycerol and phosphatidylglycerol. The DNA G+C was 40.8 mol% (T_m). DNA–DNA relatedness of the novel isolate with *B. muralis* and *B. simplex* was 42.4 % and 32.7 %, respectively. Based upon the consensus of phylogenetic and phenotypic analyses, strain CCBAU 05776^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus endoradicis* sp. nov. is proposed. The type strain is CCBAU 05776^T (=LMG 25492^T =HAMBI 3097^T).

Many species of the genus *Bacillus* are endophytes frequently isolated from the inner tissues of different plants, such as *Bacillus subtilis*, *Bacillus thuringiensis* and *Bacillus pumilus* strains in soybean nodules (Bai *et al.*, 2002; Li *et al.*, 2008), *B. subtilis* in wheat (Liu *et al.*, 2009), *B. subtilis*, *B. pumilus* and *Bacillus* sp. in maize (Rai *et al.*, 2007; Rijavec *et al.*, 2007) and *Bacillus endophyticus* and *Bacillus* sp. in cotton (Rajendran & Samiyappan, 2008; Rajendran *et al.*, 2007; Reva *et al.*, 2002). These endophytic bacteria can be pertinent to plant growth by suppressing pathogens (Liu *et al.*, 2009; Senthilkumar *et al.*, 2009) or producing phytohormones (Arkhipova *et al.*, 2005). Some endophytic species of the genus *Bacillus* can enhance the nodulation of rhizobia on legumes when they are co-inoculated (Bai *et al.*, 2002; Rajendran *et al.*, 2008).

In our investigation of endophytic bacterial diversity in soybean roots, an aerobic Gram-positive bacterium designated strain CCBAU 05776^T was isolated and identified as a distinct lineage in the genus *Bacillus* by phylogenetic screening. To clarify its taxonomic position, this strain was characterized phenotypically, genomically and phylogenetically. The results suggested that strain CCBAU 05776^T represents a novel species of the genus *Bacillus*.

Soybean roots [*Glycine max* (L.) Merr. 'Jidou no.17'] were collected from an agricultural field in the countryside of Shijiazhuang city (38° 03′ N 114° 29′ E), Hebei Province, China. The roots were pulled out from soil and transported on ice to the laboratory on the day of collection. In the laboratory, attached soil was removed from the roots by washing under running tap water and epiphytes were eliminated by surface disinfection through immersion in 95 % (v/v) ethanol for 30s and in 0.2 % (w/v) $HgCl_2$ solution for 4 min, followed by six rinses in sterilized distilled water. The disinfection efficiency was checked by plating aliquots of the sterile distilled water used in the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCBAU 05776^T is GU434676.

Three supplementary figures are available with the online version of this paper.

final rinse onto nutrient agar [NA; containing 0.5 % peptone, 0.3 % beef extract, 0.5 % NaCl and 1.5 % agar (all w/v), pH adjusted to 6.8 at 25 °C], which were then incubated at 28 °C for 3 days. To isolate the endophytes, one gram of surface-sterilized roots was ground vigorously in 9 ml 0.85 % NaCl solution for 5 min and decimal dilutions up to 10^{-4} were prepared. Aliquots of 0.1 ml, in duplicate, from each of the last three dilutions were spread on NA medium and incubated for 3 days at 28 °C. Single colonies were picked up and purified by streaking on the same medium three times.

To determine the taxonomic status of the isolates rapidly, amplification and sequencing of the 16S rRNA gene were carried out using primers 27F and 1492R (Ritchie *et al.*, 1997). A partial 16S rRNA gene sequence (1398 bp) was obtained from strain CCBAU 05776^T, which showed close relationships to species of the genus *Bacillus* in MEGABLAST searches using the NCBI and RDP databases. Sequence similarities of strain CCBAU 05776^T with the most closely related species were calculated using the EzTaxon server (Chun *et al.*, 2007) as 96.5 % with *B. muralis* LMG 20238^T, followed by 96.3 % with *B. simplex* NBRC 15720^T, 96.2 % with *Bacillus butanolivorans* K9^T, 96.1 % with *Bacillus pocheonensis* Gsoil 420^T and 96.0 % with *Bacillus asahii* MA001^T. The sequences were aligned with sequences of related strains extracted from the RDP database using CLUSTAL X version 2 software (Larkin *et al.*, 2007). The phylogenetic tree reconstructed by MEGA 4 software (Tamura *et al.*, 2007) using the neighbour-joining method (Saitou & Nei, 1987) with the model of Jukes & Cantor (1969), and bootstrapped with 1000 pseudosamples (Felsenstein, 1985) revealed that strain CCBAU 05776^T occupied a distinctive position within the group of species mentioned above, except for *B. pocheonensis* (Fig. 1). A phylogenetic tree based on the maximum-likelihood algorithm was also reconstructed (Supplementary Fig. S1 available in IJSEM Online) using the PhyML program, version 3.0 (Guindon & Gascuel, 2003) and showed phylogenetic relationships similar to those in the neighbour-joining tree. Since strain CCBAU 05776^T had 16S rRNA gene sequence similarities lower than the threshold (97 %) for delineating bacterial species (Stackebrandt & Goebel, 1994), this strain was further characterized to clarify its species identity.

Cell morphology of strain CCBAU 05776^T was investigated with scanning electron microscopy after cultivation on NA plates at 28 °C for 24 h. Cell motility and aerotactic ability were determined by observing the growth spread of cells in a test tube containing semi-solid NA medium. To detect the endospore morphology, the strain was grown on PCA medium (containing 0.5 % peptone, 0.25 % yeast extract, 0.1 % glucose, 1.5 % agar) at 28 °C for 36 h, and was then stained as described by Bartholomew & Mittwer (1950). Cells of this strain were rods (0.7–0.9 μm wide and 1.9–2.7 μm long) (Supplementary Fig. S2). The strain is motile and aerobic as determined by observing the growth extending from an inoculating stab line in semi-solid NA medium and gathering at the top of the test tube in order to absorb maximal oxygen. Cells produced ellipsoidal spores located centrally or subterminally in swollen sporangia after being cultivated for more than 24 h.

Phenotypic and biochemical properties of strains CCBAU 05776^T, *B. muralis* LMG 20238^T, *B. simplex* LMG 11160^T and *B. asahii* JCM 12112^T were determined using the methods described by Dong & Cai (2001). Salt tolerance

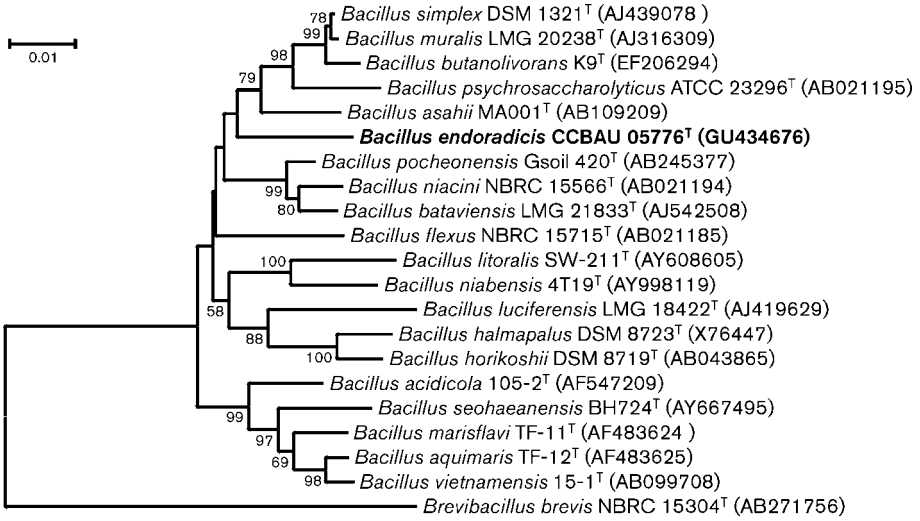

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences of strain CCBAU 05776^T and other members of the genus *Bacillus* using the neighbour-joining method. Numbers at branching points represent percentage bootstrap values obtained (based on 1000 pseudosamples; only those greater than 50 % are shown). Bar, 0.01 K_{nuc} unit, representing 0.01 inferred substitutions per nucleotide position.

was tested at 0, 0.5, 1, 2, 3, 4, 5, 7 and 10 % (w/v) NaCl in nutrient broth (NB; NA medium omitting the agar). The temperature range for growth was tested at 4, 10, 15, 20, 28, 37, 45 and 55 °C. The pH range for growth in NB was tested from pH 5.5 to 10.0 in increments of 0.5 pH units. Catalase activity was determined by assessing bubble production in 3 % (v/v) H_2O_2. Growth on MacConkey agar (MAC) (20.0 g peptone, 10.0 g lactose, 5.0 g sodium chloride, 16.5 g agar, 0.03 g neutral red, 1.0 mg crystal violet, 1 l water) was also tested. Carbon metabolic characteristics were characterized with a Biolog GP2 MicroPlate according to the manufacturer's instructions (Biolog). All assays were performed in triplicate.

Strain CCBAU 05776^T grew in NB supplemented with 0–7 % (w/v) NaCl and optimum growth occurred in media with 0–0.5 % NaCl. Optimal growth was observed at pH 7.0–8.0. Weak growth occurred at pH 10.0 and no growth occurred at pH 5.5. Growth was observed at temperatures between 15 and 45 °C, with optimal growth between 28 and 37 °C. The physiological and biochemical properties useful for differentiation of strain CCBAU 05776^T from its closest phylogenetic neighbours are listed in Table 1. In all tests, *B. muralis*, *B. simplex* and *B. asahii* showed almost the same characteristics as in previous reports (Heyrman *et al.*, 2005; Yumoto *et al.*, 2004).

Analysis of whole-cell fatty acids was carried out with strain CCBAU 05776^T cultivated on tryptic soy agar (TSA) (containing 15 g tryptone, 5 g soytone, 5 g NaCl, 15 g agar, 1 l distilled water) at 28 °C for 24 h. Fatty acids were prepared and identified using the standard method described by Sasser (1990). The Microbial Identification System (MIDI) with the HP 6890 GC and database TSBA6 were used for identification of peaks. Differentiating characteristics of the fatty acid profiles of strain CCBAU 05776^T and related species are indicated in Table 2. Strain CCBAU 05776^T had larger proportions of iso-$C_{13:0}$, iso-$C_{15:0}$ and iso-$C_{17:1}\omega10c$ compared with the other type strains. Strain CCBAU 05776^T contained only 4.44 % anteiso-$C_{15:0}$, which was greatly different from its close relatives *B. muralis* LMG 20238^T and *B. simplex* LMG 11160^T.

Cellular menaquinones were isolated using the method of Collins (1985) and separated by HPLC (Kroppenstedt, 1982), and the results showed that MK-7 was the major menaquinone component of strain CCBAU 05776^T in agreement with the description of the genus *Bacillus*. Polar lipids were extracted as described by Minnikin *et al.* (1979) and were then identified by two-dimensional TLC. The major polar lipids presented in strain CCBAU 05776^T were diphosphatidylglycerol and phosphatidylglycerol (Supplementary Fig. S3). The isomer type of the diamino acid of the peptidoglycan was determined using the method described by Schleifer & Kandler (1972). Strain CCBAU 05776^T contained *meso*-diaminopimelic acid as its diagnostic diamino acid.

Total DNA of strain CCBAU 05776^T was extracted and purified according to the method of Marmur (1961). The DNA G+C content of strain CCBAU 05776^T was 40.8 mol% (T_m) determined as described by De Ley

Table 1. Phenotypic characteristics of strain CCBAU 05776^T and related species

Strains: 1, *Bacillus endoradicis* sp. nov. CCBAU 05776^T; 2, *B. muralis* LMG 20238^T; 3, *B. simplex* LMG 11160^T; 4, *B. asahii* JCM 12112^T; 5, *B. butanolivorans* (data from Kuisiene *et al.*, 2008); 6, *B. psychrosaccharolyticus* (Priest *et al.*, 1988). Data from the present study except where indicated. +, Positive; −, negative; NG, no growth; ND, not determined; V, variable.

Characteristic	1	2	3	4	5	6
Swollen sporangia	+	+	−	−	−	+
Growth on MacConkey agar	+	−	−	−*	−	+
Growth at:						
10 °C	−	+	+	−	+	+
45 °C	+	−	−	+	+	ND
Growth in medium with 7 % (w/v) NaCl	+	+	−	−	−	−
Hydrolysis of:						
Casein	−	+	+	+	−	+
Starch	−	+	+	+	−	+
Gelatin	+	+†	+	−	−	+
Acid produced from:						
Lactose	−	+	−	−	NG	−
D-Mannitol	NG	+	+	−	−	V
D-Mannose	+	+	−	−	−	+
D-Ribose	−	+	+	ND	−	ND
D-Glucose	+	+	+	−	−	+
L-Arabinose	NG	+	−	−	NG	−

*Data from Yumoto *et al.* (2004).
†Different from data described by Heyrman *et al.* (2005).

Table 2. Comparison of the fatty acid profiles of strain CCBAU 05776^T and related species of the genus *Bacillus*

Strains: 1, *B. endoradicis* sp. nov. CCBAU 05776^T; 2, *B. muralis* LMG 20238^T; 3, *B. simplex* LMG 11160^T; 4, *B. asahii* JCM 12112^T; 5, *B. psychrosaccharolyticus* LMG 9580^T. ND, not detected.

Fatty acid	1	2	3	4	5
iso-$C_{13:0}$	2.59	0.11	0.07	0.92	0.55
iso-$C_{14:0}$	11.87	2.39	19.49	42.80	2.54
$C_{14:0}$	1.57	0.64	0.81	3.90	0.56
iso-$C_{15:0}$	40.81	15.24	10.25	18.24	20.45
anteiso-$C_{15:0}$	4.44	64.73	36.84	4.85	52.41
$C_{16:1}\omega7c$ alcohol	10.61	2.11	11.52	8.19	1.03
iso-$C_{16:0}$	7.81	0.97	9.93	1.99	0.27
$C_{16:1}\omega11c$	5.22	2.12	2.18	5.39	0.52
$C_{16:0}$	4.24	1.79	2.83	4.60	5.96
iso-$C_{17:0}$	1.40	0.78	0.73	ND	0.51
iso-$C_{17:1}\omega10c$	2.72	1.72	0.60	0.59	1.14

(1970). In DNA–DNA hybridization studies using the initial renaturation-rate technique (De Ley *et al.*, 1970), strain CCBAU 05776^T showed DNA–DNA relatedness much lower than the threshold (70 %) for differentiating species (Wayne *et al.*, 1987) with *B. muralis* (42.4 %) and *B. simplex* (32.7 %), respectively, confirming that strain CCBAU 05776^T represents a distinct genomic species within the genus *Bacillus*.

Based on the consensus of results mentioned above and the minimal standard for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.*, 2009), it can be firmly concluded that strain CCBAU 05776^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus endoradicis* sp. nov. is proposed.

Description of *Bacillus endoradicis* sp. nov.

Bacillus endoradicis (en.do.ra′di.cis. L. prep. *endo* in, within; L. n. *radix -icis* a root; N.L. gen. n. *endoradicis* of the inside of a root).

Cells are aerobic, Gram-positive, rod-shaped, 0.7–0.9 μm in diameter and 1.9–2.7 μm in length. They bear an ellipsoidal endospore which lies in central or subterminal position. After 2 days at 28 °C on NA, colonies are transparent and white with a slightly irregular edge. Cells usually occur singly or in pairs, occasionally in chains. Optimal growth at 28–37 °C, pH 7.0–8.0 and with 0–0.5 % NaCl in nutrient broth. Catalase-positive, and does not hydrolyse casein or starch. Citrate is not utilized. Indole production and Voges–Proskauer test are negative. Oxidase-negative. Nitrate reduction is positive. Assimilates (in Biolog GP2 MicroPlate) α-cyclodextrin, dextrin, *N*-acetyl-D-glucosamine, D-fructose, α-D-glucose, maltotriose, D-mannose, 3-methyl glucose, D-psicose, D-ribose, salicin, α-ketovaleric acid, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, adenosine, 2′-deoxyadenosine, inosine, thymidine, uridine, adenosine 5′-monophosphate and uridine 5′-monophosphate. Does not assimilate β-cyclodextrin, glycogen, inulin, mannan, Tween 40, Tween 80, *N*-acetyl-β-D-mannosamine, amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, *myo*-inositol, α-lactose, lactulose, maltose, D-mannitol, melezitose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, palatinose, raffinose, L-rhamnose, sedoheptulosan, D-sorbitol, stachyose, sucrose, D-tagatose, trehalose, turanose, xylitol, D-xylose, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, L-malic acid, succinic acid monomethyl ester, propionic acid, pyruvic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-serine, putrescine, 2,3-butanediol, glycerol, thymidine 5′-monophosphate, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate or DL-α-glycerol phosphate. The cellular fatty acids (≥1 %) are iso-$C_{13:0}$, $C_{14:0}$, iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:1}\omega7c$ alcohol, iso-$C_{16:0}$, $C_{16:1}\omega11c$, $C_{16:0}$, iso-$C_{17:1}\omega10c$ and iso-$C_{17:0}$. The predominant menaquinone is MK-7. The cell wall contains *meso*-diaminopimelic acid. The major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The DNA G+C content is 40.8 mol% (T_m).

The type strain is CCBAU 05776^T (=LMG 25492^T =HAMBI 3097^T), isolated from the inner tissues of soybean roots from Hebei Province, China.

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