Bacillus beijingensis sp. nov. and *Bacillus ginsengi* sp. nov., isolated from ginseng root

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Four alkaligenous, moderately halotolerant strains, designated ge09, ge10^T, ge14^T and ge15, were isolated from the internal tissue of ginseng root and their taxonomic positions were investigated by using a polyphasic approach. Cells of the four strains were Gram-positive-staining, non-motile, short rods. Phylogenetic analysis based on 16S rRNA gene sequences showed that strains ge09 and ge10^T formed one cluster and strains ge14^T and ge15 formed another separate cluster within the genus *Bacillus*. 16S rRNA gene sequence similarities with type strains of other *Bacillus* species were less than 97 %. Levels of DNA–DNA relatedness among the four strains showed that strains ge09 and ge10^T and strains ge14^T and ge15 belonged to two separate species; the mean level of DNA–DNA relatedness between ge10^T and ge14^T was only 28.7 %. Their phenotypic and physiological properties supported the view that the two strains represent two different novel species of the genus *Bacillus*. The DNA G+C contents of strains ge10^T and ge14^T were 49.9 and 49.6 mol%, respectively. Strains ge10^T and ge14^T showed the peptidoglycan type A4 α L-Lys–D-Glu. The lipids present in strains ge10^T and ge14^T were diphosphatidylglycerol, phosphatidylglycerol, a minor amount of phosphatidylcholine and two unknown phospholipids. Their predominant respiratory quinone was MK-7. The fatty acid profiles of the four novel strains contained large quantities of branched and saturated fatty acids. The predominant cellular fatty acids were iso-C_{15:0} (42.5 %), anteiso-C_{15:0} (22.2 %), anteiso-C_{17:0} (7.3 %) and C_{16:1 ω 7c} alcohol (5.7 %) in ge10^T and iso-C_{15:0} (50.7 %) and anteiso-C_{15:0} (20.1 %) in ge14^T. On the basis of their phenotypic properties and phylogenetic distinctiveness, two novel species of the genus *Bacillus* are proposed, *Bacillus beijingensis* sp. nov. (type strain ge10^T = DSM 19037^T = CGMCC 1.6762^T) and *Bacillus ginsengi* sp. nov. (type strain ge14^T = DSM 19038^T = CGMCC 1.6763^T).

The genus *Bacillus* contains aerobic or facultatively anaerobic, Gram-positive, sporulating, rod-shaped bacteria that are ubiquitous in nature. *Bacillus* species have a wide

range of physiological adaptations that enable them to survive or thrive in harsh environments, ranging from desert sands and hot springs to Arctic soils and from freshwater to marine sediments. Some of them are alkaliphilic and halotolerant (Nielsen *et al.*, 1995; Muntyan *et al.*, 2002; Nogi *et al.*, 2005; Pollock *et al.*, 2007). In this paper, the taxonomic characterization of four novel bacterial strains (ge09, ge10^T, ge14^T and ge15) isolated from the internal tissue of ginseng roots is reported. 16S rRNA gene sequencing, DNA–DNA relatedness studies and phenotypic testing showed that these four bacterial isolates represent two novel species of the genus *Bacillus*.

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Abbreviations: ddH₂O, double-distilled water; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains ge09, ge10^T, ge14^T and ge15 are EF371373–EF371376, respectively.

Carbon source utilization data and fatty acid profiles of strains ge09, ge10^T, ge14^T and ge15 are available as supplementary material with the online version of this paper.

Strains ge09, ge10^T, ge14^T and ge15 were isolated originally on Luria–Bertani (LB) agar plates that had been seeded with a tissue suspension of ginseng roots and incubated at 28 °C for 3 days. Healthy 3-year-old ginseng roots were sampled by the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences in Beijing, China. The root tissue suspension was prepared according to the following procedure (Qiu *et al.*, 2007). Roots were separated from the soil, washed with tap water and surface-sterilized with 75 % ethanol for 3 min and 2.6 % sodium hypochlorite solution for 3–5 min followed by rinsing with sterile double-distilled water (ddH₂O). The surface-sterilized root mass was pulverized in a ceramic mortar and diluted with sterile ddH₂O using the standard dilution plating technique. Twenty-eight colonies formed on one of the plates. 16S rRNA gene sequence analysis revealed that four of them (strains ge09, ge10, ge14 and ge15) probably represented novel species of the genus *Bacillus*. The four strains were maintained on LB agar slants at 4 °C and as a glycerol suspensions (20 %, v/v) at –20 °C. Biomass for chemotaxonomic and molecular systematic studies was prepared by growing the strains in shake flasks of LB broth [containing (1⁻¹) 10 g casein peptone, 5 g yeast extract and 10 g NaCl; pH 7.0] at 28 °C for 3–5 days. Cells were harvested by centrifugation, washed with ddH₂O and freeze-dried before use in chemical studies.

Colonial properties of the isolates were observed on LB agar. Cell morphology was examined by light microscopy and transmission electron microscopy (TEM; Hitachi H-600). For visualization of flagella, cells were mounted on copper grids, negatively stained with 1 % (w/v) uranyl acetate for 15 min and then subjected to TEM at 100 kV. To facilitate sporulation, MnSO₄ (50 mg l⁻¹) was added to the medium. Spore formation was determined by staining with malachite green as described previously by Gerhardt *et al.* (1994). Strains were also observed by phase-contrast microscopy (DMRE; Leica) using an oil-immersion objective (×100) to ascertain shape and motility. Gram staining was carried out using a standard procedure (Hucker & Conn, 1923).

The catalase reaction, anaerobic growth, Voges–Proskauer test, resistance to lysozyme, growth in NaCl, growth at pH 5.7, hydrolysis of starch, utilization of citrate and propionate, production of indole, deamination of phenylalanine, decomposition of casein and liquefaction of gelatin were examined by the methods of Gordon *et al.* (1973). Growth was tested at different temperatures (4–50 °C) and pH (5.0–12.0) in LB medium. Utilization of a variety of substrates as sole carbon sources was tested using the Biolog system (Gram Positive Identification Test Panel; GP2 MicroPlate).

Purified peptidoglycan preparations were obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion according to the method of Schleifer (1985). Amino acids and peptides in cell-wall

hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates by using previously described solvent systems (Schleifer, 1985). The molar ratios of the amino acids were determined by GC and GC-MS of *N*-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987). Cellular menaquinones were extracted and purified as described by Collins (1985) and analysed by HPLC (Wu *et al.*, 1989). Polar lipids were extracted and analysed by two-dimensional TLC according to Tindall (1990). For determination of fatty acid composition, the four novel strains were cultivated on trypticase soy broth agar [containing (1⁻¹) 30 g trypticase soy broth and 15 g agar (Difco)] at 28 °C for 24 h. Fatty acids were extracted, purified, methylated, identified and quantified by GC with the standard Microbial Identification system (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

A loop of biomass was scraped off the agar plate, suspended in 20 µl ddH₂O and lysed by boiling for 10 min and freezing for 5 min. Following centrifugation, the supernatant was used as the template for PCR. The 16S rRNA gene was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991). The amplified products were purified and cloned into vector Top10 (Tiagen) for sequence determination. Automated sequencing was performed by using an ABI BigDye Primer cycle sequencing ready reaction kit and an Applied Biosystems 3730 DNA sequencer. The sequencing primers were SP6 (5'-ATTTAGGTGACACTATAGAATAC-3') and T7 (5'-TAATACGACTCACTATAGGG-3'). The 16S rRNA gene sequences of the four novel bacterial strains (ge09, 1551 bp; ge10^T, 1512 bp; ge14^T, 1512 bp; ge15, 1552 bp) and those of other *Bacillus* species retrieved from GenBank were aligned using the program CLUSTAL_X v. 1.8 (Thompson *et al.*, 1997). Three tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Eck & Dayhoff, 1966; Fitch, 1971) and minimum evolution (Rzhetsky & Nei, 1992) methods from the MEGA4 package (Tamura, *et al.*, 2007), were used to infer phylogenetic evolutionary trees.

Preparation of genomic DNA was carried out according to the method of Marmur (1961). The DNA G+C content was determined using the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* K-12 as a control. Levels of DNA–DNA relatedness among the four strains were determined by using the reassociation rate method at a hybridization temperature of 60 °C (Dong *et al.*, 2000).

Comparisons with GenBank sequences revealed that the 16S rRNA gene sequences of the four isolates did not match those of any known bacterium. The bacterium with the greatest pairwise similarity to strain ge10^T was the type strain of *Bacillus pocheonensis* (96.1 % 16S rRNA gene sequence similarity), whereas the type strain of *Bacillus fumarioli* most closely matched strain ge14^T (96.0 % similarity). A comparative 16S rRNA gene sequence

similarity analysis revealed that the similarities among strains ge09, ge10^T, ge14^T and ge15 were more than 98 %. The level of DNA–DNA relatedness between ge09 and ge10^T was 86.4 % and that between ge14^T and ge15 was 70.7 %. The mean level of DNA–DNA relatedness between strains ge10^T and ge14^T (28.7 %) was low enough to distinguish these two strains at the species level. A phylogenetic tree (Fig. 1) constructed using the neighbour-joining method suggested that strains ge09, ge10^T, ge14^T and ge15 are members of the genus *Bacillus*, representing two distinct species. The phylogenetic trees inferred by maximum-parsimony and the minimum evolution method showed relationships similar to those presented in Fig. 1.

Morphological, cultural, physiological and biochemical characteristics of strains ge09, ge10^T, ge14^T and ge15 are given in the species descriptions and in Table 1 and Supplementary Table S1 (available in IJSEM Online). Endospores were not observed in the novel isolates; this has also been found in some other bacilli, e.g. endospore formation has not been detected in *Bacillus foraminis* (Tiago *et al.*, 2006) or *Bacillus thermoamylovorans* (Combet-Blanc *et al.*, 1995). Strains ge10^T and ge14^T both showed peptidoglycan type A4α L-Lys–D-Glu (A11.33 according to http://www.dsmz.de/microorganisms/main.php?content_id=35). In the case of strain ge14^T, a small portion of D-Glu in the interpeptide bridge was replaced by D-Asp. The lipids present in strains ge10^T and ge14^T were diphosphatidylglycerol, phosphatidylglycerol, a minor amount of phosphatidylcholine and two unknown phospholipids.

Strain ge14^T grew only slowly on LB agar when compared with the other strains. Capsules around the cells of strain ge14^T that could not be detected in the other strains were observed by light and electron microscopy. In addition, the four novel strains were alkaligenous when cultivated in LB medium. The pH of medium increased to more than 8.0 after inoculation with the novel strains. MK-7 was the major menaquinone component, which is in agreement

with the description of members of the genus *Bacillus*. The bacterial isolates were tested with the Biolog system (Gram Positive Identification Test Panel: GP2 MicroPlate) to assay their utilization of carbon sources (see Supplementary Table S1). The fatty acid profiles of the isolates were also compared (Supplementary Table S2). As with related *Bacillus* species, the fatty acid profiles of the four novel strains contained large quantities of branched and saturated fatty acids, especially iso-C_{15:0} and anteiso-C_{15:0}.

According to their biochemical characteristics and cellular fatty acid profiles, it was demonstrated that the four isolates represent two different species; this was confirmed by the results of 16S rRNA gene sequence comparisons and DNA–DNA hybridization data. Although a comparative 16S rRNA gene similarity analysis revealed that strain ge10^T was most closely related to strain ge14^T (98.49 %), DNA–DNA hybridization results supported species differentiation of the two strains. There are many examples of *Bacillus* species having pairwise sequence similarities of >98.5 % (Ko *et al.*, 2006), but with DNA–DNA relatedness values far below the 70 % threshold for delineation of species (Wayne *et al.*, 1987).

On the basis of the above biochemical data, cellular fatty acid compositions and molecular phylogenetic results, strains ge10^T and ge14^T represent two novel species of *Bacillus*, for which the names *Bacillus beijingsensis* sp. nov. and *Bacillus ginsengi* sp. nov., respectively, are proposed.

Description of *Bacillus beijingsensis* sp. nov.

Bacillus beijingsensis (bei.jing.en'sis. N.L. masc. adj. *beijingsensis* of Beijing, where the type strain was isolated).

Cells are alkaligenous, Gram-positive-staining, strictly aerobic, non-motile (non-flagellated), short rods to almost spherical in shape (1.0–1.5 × 0.5–0.8 µm). Endospores are not observed on LB agar supplemented with MnSO₄. Cells are not killed by heating at 80 °C for 10 min. After 24–48 h growth on LB agar, colonies are up to 3–7 mm in diameter, circular and raised, with entire edges and an off-white to

Fig. 1. Phylogenetic tree showing the relationships of the novel strains to related species within the genus *Bacillus*. The tree, based on an alignment of almost-complete 16S rRNA gene sequences, was constructed using the neighbour-joining method (Saitou & Nei, 1987). The sequence of *Paenibacillus polymyxa* DSM 36^T was used as an outgroup. Numbers at nodes indicate bootstrap support (%) based on neighbour-joining analysis of 1000 resampled datasets (Felsenstein, 1985). Bar, 0.01 substitutions per nucleotide position.

Table 1. Differential characteristic of strains ge09, ge10^T, ge14^T and ge15 and phylogenetically related species of the genus *Bacillus*

Taxa: 1, strain ge09; 2, strain ge10^T; 3, strain ge14^T; 4, strain ge15; 5, *B. pocheonensis* (data from Ten *et al.*, 2007); 6, *B. niacini* (Nagel & Andreessen, 1991); 7, *B. fumarioli* (Logan *et al.*, 2000). +, Positive or present; (+), weakly positive; v, variable; −, negative or absent; NR, not reported.

Characteristic	1	2	3	4	5	6	7
Gram stain	+	+	+	+	+	v	+
Motility	−	−	−	−	−	−	+
Anaerobic growth	−	−	−	−	−	(+)	−
Optimum growth temperature (°C)	30	30	30	30	25	30	50
Growth at 50 °C	−	−	−	−	−	−	+
pH 5.7	(+)	+	−	−	+	NR	+
Voges–Proskauer test	−	−	−	−	(+)	−	+
Hydrolysis of:							
Casein	+	+	−	+	−	−	−
Gelatin	+	+	(+)	+	−	+	+
Aesculin	+	+	−	+	+	+	−
Arginine dihydrolase	−	+	−	+	−	NR	−
NaCl tolerance (% w/v)	0–12	0–12	0–10	0–12	1–15	NR	NR
Resistance to lysozyme	−	−	+	−	NR	NR	NR
DNA G + C content (mol%)	50.6	49.9	49.6	50.6	44.9	37.0–39.0	40.7

yellow, opaque, glossy appearance. The optimum temperature for growth is 30 °C; grows at 7–45 °C. The pH range for growth is 5.5–11.0; optimum pH is 7.0–8.0. Can grow in 0–12 % NaCl. Casein, gelatin and aesculin are hydrolysed; tests for catalase, oxidase and nitrate reduction are positive. Negative for utilization of citrate and propionate, hydrolysis of starch and Tween 80, phenylalanine deaminase, indole production and Voges–Proskauer and methyl red tests. Arginine dihydrolase activity is variable. Sensitive to lysozyme. The medium pH changes gradually from 7.0 to 8.5 during cultivation. Acetic acid, α -ketovaleric acid, pyruvic acid methyl ester, pyruvic acid, L-alanine, L-alanyl glycine, L-glutamic acid, adenosine, 2'-deoxyadenosine, inosine, thymidine, L-malic acid, succinic acid mono-methyl ester, succinamic acid, succinic acid and L-serine are used as sole carbon sources for energy and growth. Utilization of β -cyclodextrin, dextrin, glycogen, Tween 40, N-acetyl-D-glucosamine, L-arabinose, D-arabitol, D-fructose, myo-inositol, maltose, 3-methyl glucose, methyl α -D-glucoside, methyl β -D-glucoside, methyl α -D-mannoside, stachyose, turanose, xylitol, β -hydroxybutyric acid, α -ketoglutaric acid, lactamide, D-lactic acid methyl ester, D-malic acid, N-acetyl-L-glutamic acid, L-alaninamide, D-alanine, glycyl L-glutamic acid, putrescine, glycerol and uridine as sole carbon sources is variable (Biolog). The major isoprenoid quinone is MK-7. The predominant cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, anteiso-C_{17:0}, summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B) and C_{16:1} ω 7c alcohol.

The type strain is ge10^T (=DSM 19037^T =CGMCC 1.6762^T), isolated from the internal tissue of ginseng roots cultivated in Beijing, China. The DNA G + C content of strain ge10^T is 49.9 mol%. Strain ge09 is a second strain of the species.

Description of *Bacillus ginsengi* sp. nov.

Bacillus ginsengi (gin.sen'gi. N.L. gen. n. *ginsengi* of ginseng, the source of the type strain).

Cells are alkaligenous, Gram-positive-staining, strictly aerobic, non-motile (non-flagellated), short rods to almost spherical in shape (1.1–1.5 $\times$ 0.9–1.3 μ m). Endospores are not observed on LB agar supplemented with MnSO₄. Capsules are observed by light and electron microscopy. Cells are not killed by heating at 80 °C for 10 min. After 24–48 h growth on LB agar, colonies are up to 2–3 mm in diameter, circular and raised, with entire edges and an off-white to yellow, opaque, glossy appearance. Cells grow slowly on LB agar and in LB medium. Usually, it is necessary to cultivate cells for more than 5 days in shake flasks of LB broth at 28 °C to reach the concentration required for chemotaxonomic studies. Optimum growth temperature is 30 °C; grows at 4–45 °C. The pH range for growth is 6.0–11.0; optimum pH is 7.0–8.0. Can grow in 0–10 % NaCl. Positive for catalase and oxidase activities, gelatin hydrolysis and nitrate reduction. Negative for hydrolysis of Tween 80 and starch, phenylalanine deaminase, indole production, Voges–Proskauer and methyl red tests and utilization of citrate and propionate. Variable for hydrolysis of casein and aesculin, arginine dihydrolase and lysozyme resistance. The medium pH changes gradually from 7.0 to 8.5 during cultivation. Acetic acid, α -ketovaleric acid, pyruvic acid methyl ester, pyruvic acid, L-alanine, L-alanyl glycine, L-glutamic acid, adenosine, 2'-deoxyadenosine, inosine, thymidine, glycogen, N-acetyl-D-glucosamine, N-acetyl- β -D-mannosamine, L-alaninamide, uridine, AMP, UMP and TMP are used as sole carbon sources for energy and growth. Variable (Biolog) for utilization of β -cyclodextrin, Tween 40, D-fructose,

D-mannose, D-psicose, sedoheptulosan, β -hydroxybutyric acid, γ -hydroxybutyric acid, α -ketoglutaric acid, D-lactic acid methyl ester, L-malic acid, succinic acid monomethyl ester, propionic acid, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, D-alanine, L-asparagine, glycyl L-glutamic acid, L-serine, 2,3-butanediol and glycerol as sole carbon sources. The major isoprenoid quinone is MK-7. The predominant cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, anteiso-C_{17:0} and summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B).

The type strain is ge14^T (=DSM 19038^T =CGMCC 1.6763^T), isolated from the internal tissue of ginseng roots cultivated in Beijing, China. The DNA G+C content of strain ge14^T is 49.6 mol%. Strain ge15 is a second strain of the species.

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