Brevibacillus panacihumi sp. nov., a β -glucosidase-producing bacterium

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Two Gram-positive-staining, endospore-forming, rod-shaped, motile bacteria, strains DCY35^T and C17, were isolated from soil of a ginseng field in South Korea and were characterized in order to determine their taxonomic positions. 16S rRNA gene sequence analysis revealed that the two strains belonged to the family *Paenibacillaceae*; strain DCY35^T showed highest levels of similarity to strain C17 (99.9%), *Brevibacillus invocatus* LMG 18962^T (98.9%), *B. centrosporus* DSM 8445^T (98.0%), *B. borstelensis* DSM 6347^T (97.6%), *B. formosus* DSM 9885^T (97.4%), *B. agri* DSM 6348^T (97.3%), *B. brevis* DSM 30^T (97.3%) and *B. levickii* LMG 22481^T (97.0%). Chemotaxonomic analyses revealed that strains DCY35^T and C17 possess menaquinone MK-7, common to members of the genus *Brevibacillus*, and that the predominant fatty acids were iso-C_{15:0} (37.3% of the total), anteiso-C_{15:0} (32.9%), iso-C_{14:0} (11.8%) and iso-C_{16:0} (6.5%). The results of physiological and biochemical tests clearly demonstrated that strains DCY35^T and C17 represent a distinct species. Based on these data, the two strains are considered to represent a novel species of the genus *Brevibacillus*, for which the name *Brevibacillus panacihumi* sp. nov. is proposed. The type strain is DCY35^T (=KCTC 13206^T =JCM 15085^T).

The genus *Brevibacillus* was created by Shida *et al.* (1996) with the reclassification of ten species of the genus *Bacillus*. Additional *Brevibacillus* species have subsequently been described: *Brevibacillus invocatus* (Logan *et al.*, 2002) was isolated from contaminants of an industrial fermentation process, *Brevibacillus limnophilus* was proposed based on the reclassification of a strain of *Brevibacillus brevis* (Goto *et al.*, 2004), *Brevibacillus levickii* was isolated from the slope of Mount Melbourne (Allan *et al.*, 2005) and *Brevibacillus ginsengisoli* was isolated from soil of a ginseng field (Baek *et al.*, 2006). At the time of writing, the genus comprises 14 recognized species. Members of the genus contain MK-7 as the major respiratory quinone, produce oval endospores in swollen sporangia, possess anteiso-C_{15:0} and iso-C_{15:0} as the major cellular fatty acids and have DNA G+C contents in the range 40.4–57.3 mol% (Shida *et al.*, 1996).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains DCY35^T and C17 are EU383033 and EU383032, respectively.

A table giving levels of DNA–DNA relatedness among strains DCY35^T and C17 and the type strains of eight closely related *Brevibacillus* species is available with the online version of this paper.

In a series of studies, we attempted to isolate micro-organisms from soil in order to investigate the community structure based on a culture-dependent method. In the present study, two strains (DCY35^T and C17) were isolated from soil in a ginseng field and were characterized based on data from a polyphasic approach, including 16S rRNA gene sequence analysis, DNA–DNA hybridizations and chemotaxonomic and phenotypic properties. The results indicated that strains DCY35^T and C17 represent a novel species of the genus *Brevibacillus*.

Strains DCY35^T and C17 were isolated from surface soil of an agricultural field where ginseng was planted. One gram of soil was immersed in 50 ml saline solution and vortexed; the suspension was then serially diluted and 100 μ l aliquots were inoculated on tenfold-diluted R2A agar (Difco). Purified colonies were tentatively identified by using partial 16S rRNA gene sequences. Cell morphology and motility were observed with a Nikon light microscope ($\times 1000$ magnification) after incubation on diluted Luria–Bertani (LB) agar (0.5% agar) for 1 day. The Gram reaction was conducted according to the non-staining method as described by Buck (1982). Oxidase activity was evaluated via the oxidation of 1% *p*-aminodimethylaniline oxalate and catalase activity was determined by measurements of

bubble production after the application of 3% (v/v) hydrogen peroxide solution (Cappuccino & Sherman, 2002). Growth at various temperatures (4, 15, 25, 30, 37 and 42 °C) was assessed on R2A agar and growth at different pH values (pH 5.0–11.0 at intervals of 0.5 pH units) was assessed in R2A broth. Growth on nutrient agar (NA), LB agar and trypticase soy agar (TSA) was also evaluated at 30 °C. The API 20NE, API ID32 GN, API 50CH and API ZYM microtest systems were employed in these tests according to the recommendations of the manufacturer (bioMérieux).

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), purified by TLC and subsequently analysed by HPLC, as described by Collins & Jones (1981) and Shin *et al.* (1996). In order to perform fatty acid methyl ester analysis, cells were allowed to grow on TSA for 48 h at 30 °C and two loops of well-grown cells were then harvested. Fatty acid methyl esters were prepared, separated and identified with the Sherlock Microbial Identification System (MIDI, Inc.) (Sasser, 1990).

For determination of the DNA G+C content, genomic DNA was extracted and purified with the Qiagen Genomic-tip system 100/G and then enzymically degraded into nucleosides. The nucleosides were analysed by using HPLC as described by Tamaoka & Komagata (1984) and Mesbah *et al.* (1989). DNA–DNA hybridizations were performed fluorometrically, according to the method developed by Ezaki *et al.* (1989), by using photobiotin-labelled DNA probes and microdilution wells. Hybridizations were conducted in five replications for each sample. The highest and lowest values obtained for each sample were excluded: means of the remaining three values are quoted as DNA–DNA relatedness values.

The 16S rRNA gene was amplified from chromosomal DNA by using the universal bacterial primer set fd1 and rP1 (Weisburg *et al.*, 1991) and the purified PCR product was sequenced by Genotec (Kim *et al.*, 2005). The full-length 16S rRNA gene sequence was compiled with SeqMan software (DNASTAR Inc.). The 16S rRNA gene sequences of related taxa were obtained from GenBank and edited by using the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL X program (Thompson *et al.*, 1997). Evolutionary distances were calculated by using Kimura's two-parameter model (Kimura, 1983). A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) in the MEGA 3 program (Kumar *et al.*, 2001). Bootstrap analysis based on 1000 replicates was also conducted in order to obtain confidence levels for the branches (Felsenstein, 1985). The closest relatives of strain DCY35^T, i.e. the type strains of all recognized *Brevibacillus* species, were included in the phylogenetic tree.

Strains DCY35^T and C17 were cultured on R2A agar and LB agar at 30 °C, yielding pale-yellow, circular colonies. The two strains were aerobic, Gram-positive-staining,

motile, rod-shaped bacteria that were able to grow at 15–42 °C and at pH 5–9. No growth was observed at 4 °C. Physiological characteristics of strains DCY35^T and C17 are summarized in the species description below, and differential characteristics between the novel strains and the type strains of recognized *Brevibacillus* species are shown in Table 1.

The cellular fatty acid profiles of strain DCY35^T and related taxa are given in Table 2. The major cellular fatty acids of strain DCY35^T were iso-C_{15:0} (37.3% of the total), anteiso-C_{15:0} (32.9%), iso-C_{14:0} (11.8%) and iso-C_{16:0} (6.5%). The fatty acid profile of strain DCY35^T was very similar to those of recognized *Brevibacillus* species (Baek *et*

Table 1. Differential characteristics between strain DCY35^T and closely related *Brevibacillus* species

Taxa: 1, strain DCY35^T (identical results for strain C17); 2, *B. agri*; 3, *B. borstelensis*; 4, *B. brevis*; 5, *B. centrosporus*; 6, *B. formosus*; 7, *B. invocatus*; 8, *B. levickii* (unless indicated, data from Allan *et al.*, 2005); 9, *B. parabrevis*. Unless indicated, data for reference species were taken from Logan *et al.* (2002). +, Positive; –, negative; v, variable; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Nitrate reduction	–	–	v	+	+	–	–	v	+
Enzyme activity									
<i>N</i> -Acetyl-β-glucosaminidase	–	ND	+	+	+	ND	+	+	ND
α-Glucosidase (starch hydrolysis)	–	v	–	+	–	–	–	–	–
Protease (gelatin hydrolysis)	–	+	+	+	–	+	–	v	+
Acid production from:*									
5-Ketogluconate	–	–	–	–	–	–	w	+	–
Gluconate	–	–	–	–	w	–	–	–	–
L-Fucose	–	–	–	–	w	–	–	–	–
D-Galactose	–	–	–	w	–	–	+	+	–
L-Rhamnose	+	–	–	–	+	–	w	–	–
D-Adonitol (ribitol)	–	–	–	–	+	–	–	–	w
L-Arabitol	–	–	–	–	–	–	w	–	–
Dulcitol (galactitol)	–	–	–	–	–	–	w	–	–
Erythritol	–	–	w	–	–	–	–	+	–
Glycerol	–	+	w	–	–	+	–	–	+
Inositol	–	–	–	–	+	–	w	–	–
Mannitol	+	+	–	+	+	+	+	+	+
Amygdalin	–	–	–	w	–	–	–	–	–
Aesculin	+	w	–	+	+	+	+	+	+
Starch	–	–	–	w	–	–	–	–	–
Assimilation of:									
2-Ketogluconate	–	ND	+	+	v	ND	+	v	ND
Gluconate	+	+	+	+	+	+	–	+	+
DL-Lactate	–	+	–	–	+	+	+	+	–
Phenylacetate	+	+	–	+	+	+	+	+	–
Maltose	–	+	–	+	+	+	+	+	+
Sucrose	–	+	–	+	–	+	–	–	+
L-Alanine	+	+	+	+	–	+	+	+	+
<i>N</i> -Acetyl-D-glucosamine	+	+	–	+	+	+	–	+	+

*Results for acid production tests were from the present study.

Table 2. Cellular fatty acid profiles of strain DCY35^T and the type strains of recognized *Brevibacillus* species

Strains: 1, DCY35^T; 2, *B. agri* DSM 6348^T; 3, *B. borstelensis* DSM 6347^T; 4, *B. brevis* DSM 30^T; 5, *B. centrosporus* DSM 8445^T; 6, *B. choshinensis* DSM 8552^T; 7, *B. formosus* DSM 9885^T; 8, *B. ginsengisoli* Gsoil 3088^T (data from Baek *et al.*, 2006); 9, *B. invocatus* LMG 18962^T; 10, *B. laterosporus* DSM 25^T; 11, *B. levickii* LMG 22481^T (Allan *et al.*, 2005); 12, *B. limnophilus* DSM 6472^T (Goto *et al.*, 2004); 13, *B. parabrevis* IFO 12334^T; 14, *B. reuszeri* DSM 9887^T; 15, *B. thermoruber* DSM 7064^T. Unless indicated, data for reference strains were taken from Logan *et al.* (2002). Results for reference strains are mean percentages of the total fatty acids (standard deviations are not given). For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. –, Not detected/not reported.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Saturated															
iso-C _{13:0}	–	0.5	–	0.2	–	0.3	0.6	–	0.2	3.3	–	–	1.1	0.8	–
anteiso-C _{13:0}	–	–	–	0.1	–	0.1	0.3	–	0.2	0.3	–	–	0.6	–	–
C _{14:0}	–	0.5	0.2	0.6	0.7	1.0	–	0.6	0.2	2.7	1.0	–	0.7	0.3	3.1
iso-C _{14:0}	11.8	4.2	2.3	1.7	8.5	9.9	2.8	15.0	12.5	11.2	2.7	4.5	3.9	15.0	1.7
C _{15:0}	–	–	–	–	–	1.1	–	–	0.8	0.2	–	2.3	–	–	–
iso-C _{15:0}	37.3	40.7	43.9	18.8	15.8	16.4	38.2	36.3	17.9	39.0	9.6	21.3	39.8	27.9	51.7
anteiso-C _{15:0}	32.9	37.6	34.7	53.8	59.8	53.3	41.3	14.8	55.2	36.1	74.5	41.7	41.9	38.3	20.6
C _{16:0}	4.7	0.3	0.6	1.0	0.6	0.9	0.2	2.4	0.3	0.6	1.5	1.6	0.6	2.0	7.1
iso-C _{16:0}	6.5	2.8	1.5	2.4	4.7	6.5	2.0	6.8	2.5	2.9	3.0	12.4	2.8	5.9	4.4
iso-C _{17:0}	1.5	1.1	1.7	2.3	0.4	–	2.0	3.6	–	0.6	–	2.9	1.1	0.3	8.1
anteiso-C _{17:0}	1.0	1.4	1.2	2.4	2.2	1.6	0.9	1.0	0.5	0.3	4.8	5.4	1.3	0.7	3.4
C _{18:0}	–	–	0.5	–	–	–	–	–	–	–	–	–	–	2.8	–
Unsaturated															
C _{16:1} ω 7c alcohol	2.3	3.8	3.0	4.7	4.1	4.9	3.7	7.0	5.5	0.9	1.0	3.7	2.0	4.2	–
C _{16:1} ω 11c	–	1.6	2.8	2.7	1.4	1.7	1.2	2.1	1.0	1.1	1.0	1.1	1.2	0.8	–
iso-C _{17:1} ω 10c	0.8	3.1	5.4	3.3	0.3	0.1	4.1	7.2	1.2	0.2	1.0	1.3	1.7	0.7	–
Summed features*															
4	1.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	1.4	1.2	–	–	–

*Summed feature 4 comprises C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH. Summed feature 5 comprises iso-C_{17:1} I and/or anteiso-C_{17:1} B.

al., 2006). Strains DCY35^T and C17 contained a menaquinone with seven isoprene units (MK-7) as the predominant isoprenoid quinone, a feature common to species of the genus *Brevibacillus* (Shida *et al.*, 1996).

The 16S rRNA gene sequences of strains DCY35^T and C17 were found to be continuous stretches of 1473 nt. The sequences of the two strains differed at only one position. The two strains were determined to belong to the class *Bacilli*, order *Bacillales*, family *Paenibacillaceae*. Strain DCY35^T showed highest levels of 16S rRNA gene sequence similarity to strain C17 (99.9%), *B. invocatus* LMG 18962^T (98.9%), *Brevibacillus centrosporus* DSM 8445^T (98.0%), *Brevibacillus borstelensis* DSM 6347^T (97.6%), *Brevibacillus formosus* DSM 9885^T (97.4%), *Brevibacillus agri* DSM 6348^T (97.3%), *B. brevis* DSM 30^T (97.3%) and *B. levickii* LMG 22481^T (97.0%). Levels of similarity between strain C17 and the type strains of recognized *Brevibacillus* species were almost the same as those for strain DCY35^T because the 16S rRNA gene sequences of the two novel strains were almost identical. In the phylogenetic tree (Fig. 1), strains DCY35^T and C17 clearly belonged to lineage encompassing the genus *Brevibacillus*.

The mean G+C content of the genomic DNA of strains DCY35^T and C17 was 50.1 and 50.5 mol%, respectively,

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strains DCY35^T and C17 and the type strains of recognized *Brevibacillus* species. Accession numbers are given in parentheses. *Aneurinibacillus aneurinilyticus* DSM 5562^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

within the range reported for recognized *Brevibacillus* species (40.4–57.3 mol%). Strain DCY35^T exhibited a high level of DNA–DNA relatedness with strain C17 (75 %) but relatively low levels with the type strains of its other phylogenetic neighbours, namely *B. agri* DSM 6348^T (17 %), *B. borstelensis* DSM 6347^T (26 %), *B. brevis* DSM 30^T (4 %), *B. centrosporus* DSM 8445^T (1 %), *B. formosus* DSM 9885^T (42 %), *B. invocatus* LMG 18962^T (15 %), *B. levickii* LMG 22481^T (16 %) and *B. parabrevis* NBRC 12334^T (0 %). These values were clearly less than the recommended threshold of 70 % for the delineation of a genomic species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Thus, our results support the placement of strains DCY35^T and C17 within a separate and previously unrecognized species. The results of the present polyphasic analyses clearly show that strains DCY35^T and C17 represent a novel species of the genus *Brevibacillus*, for which the name *Brevibacillus panacihumi* sp. nov. is proposed.

Description of *Brevibacillus panacihumi* sp. nov.

Brevibacillus panacihumi (pa.na.ci.hu'mi. N.L. n. *Panax-acis* scientific name of ginseng; L. n. *humus* soil; N.L. gen. n. *panacihumi* of soil of a ginseng field).

Gram-positive-staining, aerobic, motile rods, 0.3–0.6 µm in diameter and 4.0–10.0 µm long after growth on R2A agar or LB agar at 30 °C for 3 days. Colonies grown on R2A agar for 3 days are circular and pale yellow. Ellipsoidal endospores occur subterminally. Cells grow at 15–42 °C (optimum, 30 °C) but not at 4 °C and at pH 5–9 (optimum, pH 7) but not at pH 11. Growth is inhibited by more than 2 % NaCl. Oxidase-negative but catalase-positive. Negative for the methyl red reaction and the Voges–Proskauer reaction. Does not produce hydrogen sulfide, acid from D-glucose or indole from tryptophan. Does not reduce nitrate to nitrite or to nitrogen gas. Hydrolyses aesculin but not casein, gelatin or Tween 80. Positive for acid phosphatase, alkaline phosphatase, esterase (C4), esterase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and β-glucosidase (aesculin hydrolysis). Negative for N-acetyl-β-glucosaminidase, arginine dihydrolase, cystine arylamidase, DNase, lipase (C14), lysine decarboxylase, protease (gelatin hydrolysis), trypsin, urease, valine arylamidase, α-chymotrypsin, α-fucosidase, α-galactosidase, α-glucosidase (starch hydrolysis), α-mannosidase, β-galactosidase (PNPG) and β-glucuronidase. Assimilates acetate, adipate, DL-3-hydroxybutyrate, gluconate, L-malate, phenylacetate, n-valerate, D-mannitol, D-mannose, N-acetyl-D-glucosamine, L-alanine, L-proline and L-rhamnose, but not caprate, citrate, 3-hydroxybenzoate, 4-hydroxybenzoate, itaconate, 2-keto-gluconate, 5-ketogluconate, DL-lactate, malonate, propionate, suberate, L-arabinose, L-fucose, D-glucose, maltose, melibiose, D-ribose, sucrose, myo-inositol, D-sorbitol, L-histidine, L-serine, glycogen or salicin. The DNA G+C content of the two known strains is 50.1–50.5 mol%, as determined by HPLC. The predominant quinone is MK-7. The major cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{14:0} and iso-C_{16:0}.

The type strain, DCY35^T (=KCTC 13206^T =JCM 15085^T), was isolated from soil of a ginseng field in South Korea. C17, isolated from a similar source, is a second strain of the species.

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