Phycococcus ginsenosidimutans sp. nov., isolated from soil of a ginseng field

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A Gram-positive, non-motile, non-spore-forming, aerobic, coccoid-shaped bacterium, designated BXN5-13^T, was isolated from the soil of a ginseng field from Baekdu Mountain in Jilin district, China. Strain BXN5-13^T grew optimally at 30 °C and pH 6.5–7.5 with 0–2 % (w/v) NaCl. Strain BXN5-13^T had β -glucosidase activity that was connected with ginsenoside-converting ability, so that it was able to convert ginsenoside Rb₁ to ginsenoside F2. On the basis of 16S rRNA gene sequence analysis, the closest phylogenetic relatives of strain BXN5-13^T were *Phycococcus aerophilus* 5516T-20^T (98.4 % 16S rRNA gene sequence similarity), *P. bigeumensis* MSL-03^T (98.3 %), *P. dokdonensis* DS-8^T (97.9 %) and *P. jejuensis* KSW2-15^T (96.9 %). Lower sequence similarity (<97.0 %) was found with the type strains of other recognized species of the family *Intrasporangiaceae*. The predominant quinone was MK-8(H₄). The major fatty acids (>10 %) were iso-C_{15:0}, C_{17:0}, anteiso-C_{15:0} and iso-C_{16:0}. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol. The cell-wall peptidoglycan contained meso-diaminopimelic acid. The chemotaxonomic data and the high genomic DNA G + C content of strain BXN5-13^T (70.8 mol%) supported its affiliation with the genus *Phycococcus*. DNA–DNA relatedness between strain BXN5-13^T and its closest phylogenetic neighbours was below 16 %. Strain BXN5-13^T represents a novel species within the genus *Phycococcus*, for which the name *Phycococcus ginsenosidimutans* sp. nov. is proposed. The type strain is BXN5-13^T (=KCTC 19419^T=DSM 21006^T=LMG 24462^T).

The genus *Phycococcus* was established by Lee (2006) with a single species, *Phycococcus jejuensis*, which was isolated from dried seaweed collected at Jeju Island, Korea. Subsequently, three more species have been added to the genus: *Phycococcus dokdonensis*, from soil, *Phycococcus aerophilus*, from air, and *Phycococcus bigeumensis*, from farmed soil (Yoon *et al.*, 2008; Weon *et al.*, 2008; Dastager *et al.*, 2008). The genus *Phycococcus* includes Gram-positive, coccus-shaped bacteria that contain meso-diaminopimelic acid as the diagnostic diamino acid of the peptidoglycan, MK-8(H₄) as the predominant menaquinone and C_{17:1}ω8c, iso-C_{16:0}, iso-C_{15:0}, C_{15:0} and C_{17:0} as the major fatty acids. Phylogenetically, the genus belongs to the

family *Intrasporangiaceae*, suborder *Micrococcineae* (Stackebrandt *et al.*, 1997).

Ginseng (the root of *Panax ginseng* C. A. Meyer) is a popular medicine and has been used to strengthen immunity, provide nutrition and aid recovery from fatigue. Ginseng saponins (ginsenosides) have been recognized as being responsible for the biological and pharmacological activities of ginseng (Choi, 2008). More than 40 ginsenosides have been isolated from ginseng roots and six major ginsenosides (Rb₁, Rb₂, Rc, Rd, Re and Rg₁) constitute more than 90 % of the total ginsenoside content (Park, 2004). Biotransformation of the major ginsenosides can be achieved by hydrolysing and removing sugar moieties with bacterial and fungal strains (Kim *et al.*, 2005; Son *et al.*, 2008; Zhao *et al.*, 2009).

During the course of a study on the cultivable aerobic bacterial community in the soil of a ginseng field of Baekdu

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BXN5-13^T is EU332824.

A supplementary table and figure are available with the online version of this paper.

Mountain, Jilin district, China, a large number of novel bacterial strains were isolated. One of these isolates, strain BXN5-13^T, appeared to be a member of genus *Phycococcus* in the family *Intrasporangiaceae* and could convert ginsenoside Rb₁ to ginsenoside F2 via Rd.

Strain BXN5-13^T was isolated by serial dilution-plating from a soil suspension (50 mM phosphate buffer; pH 7.0) on modified xylan-nutrient agar (l⁻¹: 0.02 g tryptone, 0.02 g yeast extract, 0.02 g malt extract, 0.02 g beef extract, 0.02 g Casamino acid, 0.02 g soytone, 1.0 g xylan, 0.1 g sodium pyruvate, 0.3 g K₂HPO₄, 0.05 g MgSO₄, 0.05 g CaCl₂, 15 g agar). The plates were incubated at 30 °C for 1 month and single colonies were subcultivated on R2A (Difco) or half-strength R2A agar. Strain BXN5-13^T was maintained on R2A agar at 25 °C and preserved in 20 % (w/v) glycerol at -70 °C. The type strains of the four recognized species of the genus *Phycococcus* were obtained from the Korean Agricultural Culture Collection or the Korean Collection for Type Cultures.

Gram-reaction was tested by the non-staining method as described by Buck (1982). Cell morphology was observed at ×1000 magnification with a light microscope (Nikon) using cells grown on trypticase soy agar at 30 °C for 24 h. Catalase activity was determined by bubble production in 3 % (v/v) H₂O₂ and oxidase activity was determined using 1 % (w/v) tetramethyl *p*-phenylenediamine. Carbon-source utilization and enzyme activities were tested using the API 20 NE, ID 32 GN and API ZYM systems (bioMérieux), according to the instructions of the manufacturer. Growth at 4, 15, 20, 25, 30, 37 and 42 °C was determined on R2A agar and growth at pH 5.0–10.0 (at intervals of 0.5 pH unit) was assessed after 5 days in R2A broth with the pH modified using acetate buffer (pH 5.0–5.5), phosphate buffer (pH 6.0–8.0) or Tris buffer (pH 8.5–10.0; all at 50 mM). Growth with 1–10 % (w/v) NaCl was estimated by measuring OD₆₀₀ in R2A broth after 5 days. Growth on nutrient agar and MacConkey agar was also evaluated at 30 °C.

For phylogenetic analysis of the 16S rRNA gene sequence of strain BXN5-13^T, genomic DNA was extracted using a commercial kit (Solgent). The 16S rRNA gene was amplified using the universal bacterial primer set 9F and 1512R and the purified amplification products were sequenced by Solgent (Ten *et al.*, 2008). The full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR) and was aligned with sequences of related taxa obtained from public databases using CLUSTAL_X (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in MEGA4 (Kumar *et al.*, 2008) and bootstrap values (Felsenstein, 1985) were calculated using 1000 replications. Sequence similarities were calculated using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007).

For the measurement of G + C content of the chromosomal DNA of strain BXN5-13^T, genomic DNA was extracted and purified as described by Moore & Dowhan (1995) and analysed as described by Mesbah *et al.* (1989) using reversed-phase HPLC. The cellular fatty acid profiles of strain BXN5-13^T, *P. aerophilus* KACC 20658^T, *P. dokdonensis* KCTC 19248^T and *P. jejuensis* KACC 11764^T were determined from cells grown on R2A agar at 30 °C for 3 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI) and analysed by GC (model 6890; Hewlett Packard) and the Microbial Identification software package (Sasser, 1990). For analysis of isoprenoid quinones, the cell-wall peptidoglycan and polar lipids, strain BXN5-13^T was grown in R2A broth for 5 days at 30 °C and then harvested cells were lyophilized for 24 h. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum conditions and re-extracted in *n*-hexane/water (1:1, v/v). The crude quinone solution was purified using a Sep-Pak Vac silica cartridge (Waters) and subsequently analysed by HPLC as described by Hiraishi *et al.* (1996). The isomer type of the diamino acid of the cell-wall peptidoglycan was determined by TLC after hydrolysis with 6 M HCl at 100 °C for 18 h, as described by Komagata & Suzuki (1987). Polar lipids were extracted, separated by two-dimensional TLC and identified as described by Minnikin *et al.* (1977). DNA–DNA hybridization was performed between strain BXN5-13^T and *P. aerophilus* KACC 20658^T, *P. bigeumensis* KCTC 19266^T, *Phycococcus jejuensis* KACC 11764^T and *P. dokdonensis* KCTC 19248^T as described by Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were used to calculate DNA–DNA relatedness.

The morphological, physiological and biochemical characteristics of strain BXN5-13^T are given in the species description and Table 1. A comparison of some physiological characteristics for strain BXN5-13^T and the type strains of species of the genus *Phycococcus* is also given in Table 1. Strain BXN5-13^T had β-glucosidase activity that was connected with ginsenoside-converting ability so that it was able to transform ginsenoside Rb₁ {3-O-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol} to F2 [3-O-(β-D-glucopyranosyl)-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol] via Rd {3-O-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol}.

An almost-complete 16S rRNA gene sequence for strain BXN5-13^T was determined in this study (continuous stretch of 1405 bp, base positions 46–1464, *Escherichia coli* numbering). The closest relatives of strain BXN5-13^T were *P. aerophilus* 5516T-20^T (98.4 % 16S rRNA gene sequence similarity), *P. bigeumensis* MSL-03^T (98.3 %), *P. dokdonensis* DS-8^T (97.9 %) and *P. jejuensis* KSW2-15^T (96.9 %).

Table 1. Physiological characteristics of strain BXN5-13^T and the type strains of species of the genus *Phycococcus*

Strains: 1, BXN5-13^T (data from this study); 2, *P. aerophilus* 5516T-20^T (Weon *et al.*, 2008); 3, *P. bigeumensis* MSL-03^T (Dastager *et al.*, 2008); 4, *P. dokdonensis* DS-8^T (Yoon *et al.*, 2008); 5, *P. jejuensis* KSW2-15^T (Lee, 2006). All strains are positive for assimilation of melibiose, glycogen and sucrose and β -galactosidase, β -glucosidase, arginine dihydrolase and urease. All strains are negative for indole production, acid production from glucose, assimilation of L-arabinose, fucose, rhamnose, adipate, caprate, citrate, itaconate, lactate, phenylacetate, malonate, suberate, 2-ketogluconate, 5-ketogluconate, 3-hydroxybenzoate, 4-hydroxybenzoate, inositol and L-serine and lipase (C14), valine arylamidase, trypsin, β -glucuronidase, N-acetyl- β -glucosaminidase and α -fucosidase. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Cell shape	Coccus	Short rod	Coccus	Coccus	Coccus
Colony colour	Greyish yellow	White	Yellow	Yellow	Greyish
Temperature for growth (°C)					
Range	10–37	5–37	20–37	10–36	4–37
Optimum	30	30	28	30	30
pH for growth					
Range	5–10	5–9	7–12	5–10	5–10
Optimum	7	6–7	7.4	5.0–8.5	7.1
NaCl range for growth (%)	0–5	0–7	0–5	0–5	0–7
API 20 NE & ID 32 GN*					
Acetate	–	w	–	w	–
N-Acetylglucosamine	–	+	–	+	+
L-Alanine	–	+	+	w	–
Gelatin hydrolysis	–	–	–	+	+
Gluconate	+	+	–	+	+
D-Glucose	+	+	–	+	+
L-Histidine	–	–	–	+	+
3-Hydroxybutyrate	–	+	w	+	+
Malate	+	+	–	+	+
Maltose	–	+	+	+	+
D-Mannitol	+	+	–	+	+
D-Mannose	–	w	–	+	+
Nitrate reduction	+	–	+	–	+
L-Proline	–	–	–	+	w
Propionate	–	+	w	+	+
D-Ribose	–	–	–	–	+
Salicin	–	–	–	+	–
D-Sorbitol	–	+	+	–	+
Valerate	–	+	+	+	+
API ZYM*					
Acid phosphatase	–	+	+	+	+
Alkaline phosphatase	–	+	+	+	–
Esterase (C4)	–	+	+	+	+
Esterase lipase (C8)	–	+	+	+	+
Cystine arylamidase	–	–	–	+	–
Leucine arylamidase	–	–	w	+	+
α -Chymotrypsin	–	+	w	–	w
α -Galactosidase	–	–	–	+	+
α -Glucosidase	–	–	+	+	+
α -Mannosidase	–	–	–	+	w
Naphthol-AS-BI-phosphohydrolase	w	+	+	+	–
DNA G + C content (mol%)	70.8	70.5	73.4	70.7	74.0

*Data were taken from this study using the following strains: column 2, *P. aerophilus* KACC 20658^T; 3, *P. bigeumensis* KCTC 19266^T; 4, *P. dokdonensis* KCTC 19248^T; 5, *P. jejuensis* KACC 11764^T.

Lower sequence similarity (<97.0 %) was found with other recognized members of the family *Intrasporangiaceae*. The affiliation of strain BXN5-13^T with the genus *Phycococcus*

was also evident in the neighbour-joining phylogenetic tree (Fig. 1). Strain BXN5-13^T and the four type strains of species of the genus *Phycococcus* formed a monophyletic

group with a high bootstrap value (99%), which was also recovered in the tree generated with the maximum-parsimony method. DNA–DNA relatedness values between strain BXN5-13^T and *P. aerophilus* KACC 20658^T, *P. bigeumensis* KCTC 19266^T and *P. dokdonensis* KCTC 19248^T were in the range of 6–16% (11, 16 and 6%, respectively). These values were low enough to assign strain BXN5-13^T to a novel species of the genus *Phycoccus* (Wayne *et al.*, 1987).

The DNA G+C content of strain BXN5-13^T was 70.8 mol%, which was similar to those of *P. aerophilus* 5516T-20^T and *P. dokdonensis* DS-8^T (70.5 and 70.7 mol%, respectively). The respiratory quinone system of strain BXN5-13^T was MK-8(H₄), which was consistent with the quinone system of the genus *Phycoccus*. The presence of *meso*-diaminopimelic acid in the cell-wall peptidoglycan also supported the placement of strain BXN5-13^T in the genus *Phycoccus*. The phospholipid profile contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol (see Supplementary Fig. S1, available in IJSEM Online). The fatty acid profile of strain BXN5-13^T was mainly composed of iso-C_{15:0}, C_{17:0}, anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{14:0}, which was typical for the genus *Phycoccus* (Supplementary Table S1). However, some minor qualitative and quantitative differences in fatty acid content could be observed between strain BXN5-13^T and its closest phylogenetic relatives.

In summary, the morphological, biochemical and chemotaxonomic characteristics of strain BXN5-13^T were consistent with those described for the genus *Phycoccus*. However, on the basis of 16S rRNA gene sequence phylogenetic analysis, the combination of phenotypic

characteristics and low DNA–DNA relatedness between strain BXN5-13^T and recognized members of the genus *Phycoccus*, strain BXN5-13^T should be assigned to the genus *Phycoccus* as representing a novel species, for which the name *Phycoccus ginsenoidimutans* sp. nov. is proposed.

Description of *Phycoccus ginsenoidimutans* sp. nov.

Phycoccus ginsenoidimutans (gin.se.no.si.di.mu'tans. N.L. n. *ginsenoidimutans* ginsenoidimutans; L. part. adj. *mutans* transforming, converting; N.L. part. adj. *ginsenoidimutans* ginsenoidimutans; N.L. part. adj. *ginsenoidimutans* ginsenoidimutans).

Cells are Gram-positive, non-motile, non-spore-forming, aerobic cocci (0.3–0.7 µm in diameter). Colonies after 3 days on R2A agar are smooth, circular, transparent, convex and greyish yellow (0.6–1.5 mm in diameter). Grows at 10–37 °C, but not at 4, 42 or 45 °C, at pH 5.0–10.0 and with 0–5.0% (w/v) NaCl, but not with 6.0% (w/v) NaCl. Optimum growth occurs at 30 °C and pH 7.0 with 0–2% (w/v) NaCl. Catalase- and oxidase-positive. Grows on nutrient agar and trypticase soy agar, but not on MacConkey agar. Assimilation of sole carbon sources and enzyme activities are given in Table 1. The predominant menaquinone is MK-8(H₄). The major fatty acids (>10%) are iso-C_{15:0}, C_{17:0}, anteiso-C_{15:0} and iso-C_{16:0}. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid as the diagnostic diamino acid. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol.

The type strain, BXN5-13^T (=KCTC 19419^T=DSM 21006^T=LMG 24462^T), was isolated from soil of a ginseng field of Baekdu Mountain, Jinlin district, China. The DNA G+C content of the type strain is 70.8 mol%.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain BXN5-13^T and the type strains of related species in the family *Intrasporangiaceae*. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bootstrap values ($\geq 70\%$) based on 1000 replications are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

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