Nocardioides ginsengagri sp. nov., isolated from the soil of a ginseng field

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A Gram-reaction-positive, rod-shaped, non-motile, non-spore-forming bacterium (strain BX5-10^T) was isolated from the soil of a ginseng field on Baekdu Mountain in Jilin district, China. The taxonomic position of this bacterium was determined in an investigation based on a polyphasic approach. On the basis of 16S rRNA gene sequence analysis, strain BX5-10^T was shown to belong to the family *Nocardioideaceae* and to be most closely related to *Nocardioides plantarum* NCIMB 12834^T (96.5 % sequence similarity), *Nocardioides dokdonensis* KCTC 19309^T (96.2 %) and *Nocardioides fonticola* NAA-13^T (95.1 %). Strain BX5-10^T was characterized chemotaxonomically as having LL-2,6-diaminopimelic acid in its cell-wall peptidoglycan, MK-8(H₄) as the predominant menaquinone and C_{18:1}ω9c, C_{16:0} and C_{17:1}ω8c as its major fatty acids. The G + C content of the genomic DNA was 70.3 mol%. The novel strain could be differentiated genotypically and phenotypically from all recognized species of the genus *Nocardioides*. Based on the results of the phylogenetic analyses and the genotypic and phenotypic data, a novel species, *Nocardioides ginsengagri* sp. nov., is proposed. The type strain is BX5-10^T (=KCTC 19467^T=DSM 21362^T).

The genus *Nocardioides* was proposed by Prauser (1976). At the time of writing, the genus comprises 51 recognized species (Euzéby, 1997), including the recently described species *Nocardioides panacisoli* (Cho *et al.*, 2010), *Nocardioides daedukensis* (Yoon *et al.*, 2010) and *Nocardioides hungaricus* (Tóth *et al.*, 2011). The genus *Nocardioides* includes Gram-reaction-positive, coccus-to-rod-shaped bacteria with LL-diaminopimelic acid as the diagnostic diamino acid of their cell-wall peptidoglycan, MK-8(H₄) as the predominant menaquinone and C_{18:1}ω9c, iso-C_{16:0}, iso-C_{17:0} and 10-methyl C_{18:0} as the major fatty acids. Phylogenetically, the genus belongs to the family *Nocardioideaceae*, in the suborder *Propionibacterineae* (Zhi *et al.*, 2009).

During the course of a study on the cultivable aerobic bacterial community in the soil of a ginseng field on Baekdu Mountain, in the Jilin district of China, a large number of novel bacterial strains were isolated. Here, we report on the taxonomic characterization of one of these strains, designated BX5-10^T, which appeared to be a member of the genus *Nocardioides*.

A soil sample from the ginseng field (10 g) was thoroughly suspended in 50 mM phosphate buffer (100 ml, pH 7.0)

and spread on modified xylan-nutrient agar (containing per litre distilled water: 0.02 g tryptone, 0.02 g yeast extract, 0.02 g malt extract, 0.02 g beef extract, 0.02 g Casamino acids, 0.02 g Soytone, 1.0 g xylan, 0.1 g sodium pyruvate, 0.3 g K₂HPO₄, 0.05 g MgSO₄·7H₂O, 0.05 g CaCl₂ and 15 g agar) plates, after serial dilution with 50 mM phosphate buffer (pH 7.0). The plates were incubated at 30 °C for 1 month. Single colonies were transferred first onto new modified xylan-nutrient agar plates and then onto conventional R2A agar (Reasoner & Geldreich, 1985) or half-strength R2A agar plates. Strain BX5-10^T was cultured routinely on full- or half-strength R2A agar at 25 °C or 30 °C and preserved, as a suspension in R2A broth with 20 % (w/v) glycerol, at –70 °C.

The genomic DNA of strain BX5-10^T, extracted using a commercial DNA-extraction kit (Solgent), was used in a PCR with the universal bacterial primer pair 9F and 1512R to amplify the 16S rRNA gene (Weisburg *et al.*, 1991). The purified PCR products were sequenced by Solgent Co. Ltd (Im *et al.*, 2010). The almost complete (1424 nt) sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR) and compared with the 16S rRNA gene sequences of related taxa, which were obtained from the GenBank database or the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Multiple alignments were made using CLUSTAL_X (Thompson *et al.*, 1997), with gaps edited using the BioEdit program (Hall, 1999).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BX5-10^T is GQ339904.

Two supplementary figures and a supplementary table are available with the online version of this paper.

Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) phylogenetic trees were constructed using the MEGA4 program (Kumar *et al.*, 2008), with bootstrap values based on 1000 replications (Felsenstein, 1985).

The results of these comparative 16S rRNA gene sequence analyses indicated that strain BX5-10^T was phylogenetically affiliated to the genus *Nocardioide*s. The phylogenetic tree based on the neighbour-joining algorithm placed strain BX5-10^T within the genus *Nocardioide*s, clustering with *Nocardioide*s *plantarum* NCIMB 12834^T (Fig. 1; a neighbour-joining phylogenetic tree showing all recognized species of the genus *Nocardioide*s is available as Supplementary Fig. S1 in IJSEM Online). Strain BX5-10^T, *N. plantarum* NCIMB 12834^T and *Nocardioide*s *fonticola* NAA-13^T formed a monophyletic group in both the neighbour-joining and maximum-parsimony trees. Strain BX5-10^T exhibited the highest levels of 16S rRNA gene sequence similarity with *Nocardioide*s *salaris* CL-Z59^T (96.9%), *Nocardioide*s *basaltis* J112^T (96.7%), *Nocardioide*s *marinisabuli* SBS-12^T (96.6%), *N. plantarum* NCIMB 12834^T (96.5%), *Nocardioide*s *marinus* CL-DD14^T (96.3%) and *Nocardioide*s *dokdonensis* KCTC 19309^T (96.2%). The 16S rRNA gene sequence similarities between strain BX5-10^T and other recognized species within the family

Nocardioideaceae were <96.3%. On the basis of these phylogenetic results, *N. plantarum*, *N. dokdonensis* and *N. fonticola* were selected as the closest recognized neighbours of strain BX5-10^T. The type strains of these three species (NCIMB 12834^T, KCTC 19309^T and NAA-13^T, respectively) were obtained from culture collections, grown under the same conditions and used as reference strains in most of the subsequent phenotypic tests.

The Gram reaction was tested by the non-staining method, as described by Buck (1982). Cell morphology was observed under a light microscope (E600; Nikon), using cells grown for 2 days at 30 °C on R2A agar. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂ and oxidase activity was determined using 1% (w/v) tetramethyl *p*-phenylenediamine (Cappuccino & Sherman, 2002). Carbon-source utilization and enzyme activities were tested using API 20 NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Tests for the hydrolysis of casein, starch, xanthine and hypoxanthine (Atlas, 1993) were carried out over 7 days at 30 °C. Growth at different temperatures (4, 15, 20, 25, 30, 37 and 42 °C) was tested on R2A agar over 7 days and growth at various pH values (pH 5.0–10.0, at intervals of 0.5 pH units) was assessed after 7 days' incubation at 30 °C in R2A broth. For the pH experiments, three different buffers were used (each at a final concentration of 50 mM):

Fig. 1. Neighbour-joining phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences (>1350 nt) showing the relationship of strain BX5-10^T with related species of the genus *Nocardioide*s. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony algorithm. Bootstrap values (>65%) based on 1000 replications are shown at the branch nodes. Bar, 0.01 substitution per nucleotide position.

acetate for pH 5.0–5.5; phosphate for pH 6.0–8.0; and Tris for pH 8.5–10.0. Salt tolerance was tested in R2A broth supplemented with 1–10 % (w/v) NaCl (at intervals of 1 %) after 7 days' incubation at 30 °C. Anaerobic growth was tested in serum bottles, using R2A broth supplemented with thioglycolate (1 g l⁻¹) and replacing the air in the bottles with nitrogen gas. Growth on nutrient agar (BD), trypticase soy agar (TSA; BD) and MacConkey agar (Difco)

was also evaluated at 30 °C. All growth in broths was estimated by monitoring optical density at 600 nm.

Cells of strain BX5-10^T were Gram-reaction-positive, aerobic, non-spore-forming, and non-motile. Young cultures were composed of oval cells or short rods, whereas older cultures were composed entirely of rods (measuring 0.4–0.7 µm × 1.5–2.8 µm). Tests for oxidase and catalase

Table 1. Physiological characteristics of strain BX5-10^T and related type strains of the genus *Nocardioides*

Strains: 1, BX5-10^T; 2, *N. plantarum* NCIMB 12834^T; 3, *N. fonticola* NAA-13^T; 4, *N. dokdonensis* KCTC 19309^T. All data are from this study, except the DNA G + C contents of the reference strains (taken from Collins *et al.*, 1994; Chou *et al.*, 2008; Park *et al.*, 2008). All strains were Gram-reaction-positive and positive for catalase, but negative for hydrolysis of xanthine and hypoxanthine. None of the strains were motile. In API 20 NE kits, all strains gave a negative result in tests for indole production, acid production from glucose and assimilation of adipate and citrate. In API ZYM kits, all the strains were positive for esterase lipase (C8), leucine arylamidase and α-glucosidase activities, but negative for lipase (C14), α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities. +, Positive; –, negative.

Characteristic	1	2	3	4
Cell morphology	Short rods	Short rods, cocci	Rods	Short rods
Cell size (µm)	0.4–0.7 × 1.5–2.8	0.5–0.7 × 1.0–1.6	0.8 × 2.0–9.0	0.6–0.9 × 1.2–1.8
Colony colour	Light brown	Light brown	Yellow	Light brown
Growth temperature (°C)				
Range	10–37	5–30	25–37	4–30
Optimum	30	25	30	25
Growth pH range	5.5–9.5	5.0–9.0	5.0–9.0	5.0–10.0
Oxidase	+	–	–	–
Arginine dihydrolase	–	+	–	–
Urease	–	+	–	+
Nitrate reduction	–	–	–	+
Hydrolysis of:				
Casein	+	–	+	–
Starch	–	–	+	–
Tween 80	–	+	+	+
Gelatin	–	–	+	–
Carbon source utilization:				
D-Glucose	–	–	+	–
L-Arabinose	+	–	+	–
D-Mannose	–	–	+	–
N-Acetyl-D-glucosamine	–	+	–	–
Maltose	–	+	–	–
Gluconate	–	+	–	+
Caprate	–	+	–	–
Malate	–	+	–	–
Phenylacetate	–	–	–	–
API ZYM results:				
Alkaline phosphatase	–	+	+	+
Esterase (C4)	–	+	+	+
Valine arylamidase	+	+	–	–
Cystine arylamidase	–	+	–	–
Trypsin	–	+	–	+
α-Chymotrypsin	–	–	–	+
Acid phosphatase	+	+	+	–
Naphthol-AS-BI-phosphohydrolase	–	+	+	–
β-Galactosidase	–	+	+	–
β-Glucosidase	–	+	+	–
Isolation source	Ginseng-field soil	Herbage	Spring water	Sandy sediment
DNA G + C content (mol%)	70.3	69.0	71.8	69.1

gave positive results. Colonies grown on the R2A agar plates for 5 days at 30 °C were smooth, translucent, circular, light brown in colour, convex and 1.0–1.5 mm in diameter. Strain BX5-10^T grew much more slowly than any of the three type strains used for reference. The morphological, physiological, and biochemical characteristics of strain BX5-10^T are given in the species description and Table 1. Some physiological characteristics of strain BX5-10^T are compared with those of the three reference type strains in Table 1.

Genomic DNA of the novel strain and three reference strains was extracted and purified, as described by Moore & Dowhan (1995), so that G + C content could be determined by reverse-phase HPLC (Mesbah *et al.*, 1989). Cellular fatty acid profiles were determined using cells grown on R2A agar for 2 days at 30 °C. The cellular fatty acids were saponified, methylated, and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). They were then identified by capillary GLC, using version 6.0 of the Microbial Identification software package (MIDI), the TSBA60 database (Sasser, 1990) and a 6890 chromatograph (Hewlett Packard). For the chemotaxonomic analyses (of isoprenoid quinones and cell-wall peptidoglycan type), strain BX5-10^T was grown in R2A broth for 5 days at 30 °C. Collected cells were lyophilized for 24 h before the isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum, and re-extracted in n-hexane/water (1:1, v/v). The crude n-hexane-quinone solution was purified using a Sep-Pak Vac silica cartridge (Waters) and subsequently analysed by HPLC, as previously described (Hiraishi *et al.*, 1996). The isomer of the diamino acid of the cell-wall peptidoglycan was determined by using TLC, after hydrolysis of whole cells with 6 M HCl at 100 °C for 18 h, as described by Komagata & Suzuki (1987). Polar lipids, extracted from freeze-dried cells that had been grown in R2A broth for 96 h at 30 °C, were examined by two-dimensional TLC and identified as described by Minnikin *et al.* (1984).

At 70.3 mol%, the genomic DNA G + C content of strain BX5-10^T was similar to those of *N. plantarum* NCIMB 12834^T, *N. dokdonensis* KCTC 19309^T and *N. fonticola* NAA-13^T, which all fell between 69.0 and 71.8 mol% (Table 1). The major menaquinone of strain BX5-10^T, like that previously reported for members of the genus *Nocardioide*s, was MK-8(H₄). The presence of LL-diaminopimelic acid in the cell-wall peptidoglycan also supported the placement of strain BX5-10^T in the genus *Nocardioide*s. The fatty acid profile of strain BX5-10^T was mainly composed of C_{18:1}ω9c (33.2%), C_{16:0} (17.7%) and C_{17:1}ω8c (8.7%), and therefore quite different from those of the three reference strains (Supplementary Table S1). Strain BX5-10^T did, however, have several common fatty acids that are detected in most members of the genus *Nocardioide*s, such as C_{17:0}, iso-C_{15:0}, iso-C_{16:0} and 10-methyl C_{18:0}. The polar lipids detected (Supplementary Fig. S2) were phosphatidylglycerol, unknown phospholipids, an unknown amino lipid and an unknown lipid.

The morphological, biochemical and chemotaxonomic properties of strain BX5-10^T were consistent with descriptions of the genus *Nocardioide*s. On the basis of the phylogenetic differences seen between strain BX5-10^T and recognized species of the genus *Nocardioide*s (in terms of 16S rRNA gene sequences) and some phenotypic characteristics of strain BX5-10^T that have not been seen in any recognized species of this genus (Table 1), a novel species, *Nocardioide*s *ginsengagri* sp. nov., is proposed, with BX5-10^T as the type strain.

Description of *Nocardioide*s *ginsengagri* sp. nov.

*Nocardioide*s *ginsengagri* [gin.seng.a'gri. N.L. n. *ginsengum* ginseng; L. n. *ager*, *agri* a field; N.L. gen. n. *ginsengagri* of a ginseng field].

Cells are Gram-reaction-positive, strictly aerobic, non-spore-forming and non-motile. Young cultures are composed of oval cells or short rods, whereas older cultures are composed entirely of rods (measuring 0.4–0.7 µm × 1.5–2.8 µm). Substrate and aerial mycelia are not observed. Colonies grown on R2A agar for 5 days at 30 °C are smooth, circular, translucent, light brown and convex. Grows at 10–37 °C (optimum, 30 °C) and at pH 5.5–9.5 (optimum, pH 7.0), but not at 4 °C or 42 °C. Growth occurs in the absence of NaCl and in the presence of 1.0% (w/v) NaCl, but not with NaCl at ≥2.0% (w/v). No growth on nutrient agar, TSA or MacConkey agar. Catalase- and oxidase-positive. Nitrate is not reduced. The data on carbon assimilation (API 20 NE) and enzyme activities (API ZYM) are given in Table 1. MK-8(H₄) is the predominant menaquinone, and C_{18:1}ω9c, C_{16:0}, and C_{17:1}ω8c are the major cellular fatty acids (>8%). The cell-wall peptidoglycan contains LL-diaminopimelic acid as the diagnostic diamino acid. The polar lipids are phosphatidylglycerol, unknown phospholipids, an unknown amino lipid and an unknown lipid.

The type strain, isolated from the soil in a ginseng field on Baekdu Mountain, China, is BX5-10^T (=KCTC 19467^T=DSM 21362^T). The genomic DNA G + C content of the type strain is 70.3 mol%.

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