Rhodanobacter panaciterrae sp. nov., a bacterium with ginsenoside-converting activity isolated from soil of a ginseng field

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A novel gammaproteobacterium, designated LnR5-47^T, was isolated from soil of a ginseng field in Liaoning province, China. The isolate was a Gram-negative, aerobic, non-motile, non-spore-forming rod. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain LnR5-47^T belonged to the genus *Rhodanobacter*. The isolate was most closely related to *Rhodanobacter ginsengisoli* GR17-7^T, *Rhodanobacter terrae* GP18-1^T, *Dyella ginsengisoli* Gsoil 3046^T, *Rhodanobacter soli* DCY45^T, *Dyella soli* JS12-10^T and *Dyella japonica* IAM 15069^T (98.0, 97.9, 97.7, 97.3, 97.2 and 97.1 % 16S rRNA gene sequence similarity, respectively). Chemotaxonomic data (Q-8 as the predominant ubiquinone, and iso-C_{16:0}, iso-C_{17:1ω9c} and iso-C_{15:0} as the major fatty acids) also supported the affiliation of strain LnR5-47^T with the genus *Rhodanobacter*. However, DNA–DNA relatedness between strain LnR5-47^T and its closest phylogenetic neighbours was <25.8 %. Moreover, physiological and biochemical tests phenotypically differentiated the isolate from other members of the genus *Rhodanobacter*. Therefore, strain LnR5-47^T represents a novel species, for which the name *Rhodanobacter panaciterrae* sp. nov. is proposed; the type strain is LnR5-47^T (=KACC 12826^T=KCTC 22232^T=LMG 24460^T).

The genus *Rhodanobacter*, in the family *Xanthomonadaceae*, was originally described by Nalin *et al.* (1999) on the basis of a lindane-degrading bacterium isolated from soil of a wood-treatment site. Subsequently, several novel species have been classified as members of the genus *Rhodanobacter*, which at the time of writing comprised *Rhodanobacter lindaniclasticus* (Mergaert *et al.*, 2002) and seven other species with validly published names: *Rhodanobacter fulvus* (Im *et al.*, 2004), *R. spathiphylli* (De Clercq *et al.*, 2006), *R. thiooxydans* (Lee *et al.*, 2007), *R. ginsengisoli* and *R. terrae* (Weon *et al.*, 2007), *R. ginsenosidimutans* (An *et al.*, 2009) and *R. soli* (Bui *et al.*, 2010).

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Abbreviations: Rb₁, 3-O-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl-(1-6)-β-D-glucopyranosyl]-20(S)-protopanaxadiol; Rd, 3-O-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LnR5-47^T is EU332829.

During a study on the cultivable aerobic bacterial community in the soil of a ginseng field in Liaoning province, China, many novel bacterial strains were isolated. One of them, designated LnR5-47^T, which could convert 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 Rd {3-O-[β-D-glucopyranosyl-(1-2)-β-D-glucopyranosyl]-20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol}, appeared to be a member of the genus *Rhodanobacter*.

Strain LnR5-47^T was isolated using modified R2A agar, as described by Liu *et al.* (2006). Plates were incubated at room temperature for 1 month and single colonies were purified by subcultivation on full- or half-strength modified R2A agar or R2A agar (Difco). Strain LnR5-47^T was cultured routinely on R2A agar at 25 °C and maintained as a glycerol suspension (20 %, w/v) at –70 °C.

The Gram-reaction was performed by the non-staining method, as described by Buck (1982). Morphology of cells

grown for 3 days at 30 °C on R2A agar was observed under a Nikon light microscope at magnification $\times 1000$. Motility was determined using the hanging-drop technique (Skerman, 1967). 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 20NE, API ID 32GN and API ZYM systems (bioMérieux) at 30 °C. Tests for the degradation of DNA (DNase agar; Scharlau; DNase activity detected by flooding plates with 1 M HCl), casein, chitin, starch (Atlas, 1993), xylan and cellulose (Ten *et al.*, 2004) were evaluated after 5 days. Anaerobic growth was performed in serum bottles containing R2A broth and sodium thioglycolate (1 g l⁻¹) with the upper air layer replaced with nitrogen gas. Conversion of ginsenoside Rb₁ was determined using TLC analysis as described by Kim *et al.* (2005). Growth at 4, 15, 20, 25, 30, 37 and 42 °C and pH 5.0–10.0 (at intervals of 0.5 pH units) was assessed after 10 days in R2A broth. For the pH experiments, three buffers were used (final concentration, 50 mM): acetate buffer (pH 5.0–5.5), phosphate buffer (pH 6.0–8.0) and Tris buffer (pH 8.5–10.0). Growth with 1–10 % (w/v) NaCl was determined on R2A agar after 5 days at 30 °C. Growth on nutrient agar, trypticase soy agar (TSA), MRS agar and LB agar (all Difco) was also evaluated at 30 °C.

An almost-complete 16S rRNA gene sequence for strain LnR5-47^T was determined. Genomic DNA was extracted using a commercial kit (Solgent). The 16S rRNA gene was amplified using the universal bacterial primer set comprising 9F and 1512R and the purified PCR products were sequenced by Solgent (Ten *et al.*, 2008). The 16S rRNA gene sequence was compiled using SeqMan software (DNASTAR). 16S rRNA gene sequences of related taxa were obtained from GenBank using the EzTaxon server (Chun *et al.*, 2007). Multiple alignments were performed using CLUSTAL X (Thompson *et al.*, 1997). Gaps were edited in the program BioEdit (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), with bootstrap values based on 1000 replications (Felsenstein, 1985).

For measurement of the G + C content, the genomic DNA of strain LnR5-47^T was extracted and purified as described by Moore & Dowhan (1995) and enzymically degraded into nucleosides. The G + C content was then determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum and re-extracted in *n*-hexane/water (1:1, v/v). The crude quinone in *n*-hexane solution was purified using Sep-Pak Vac Cartridges Silica (Waters) and subsequently analysed by HPLC, as described by Hiraishi *et al.* (1996). 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) and identified using capillary GLC (6890; Hewlett Packard) with Sherlock software (version 6.0) and the Sherlock aerobic bacterial database (TSBA60) (Sasser, 1990). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for

Table 1. Phenotypic characteristics of strain LnR5-47^T and closely related members of the genus *Rhodanobacter*

Strains: 1, *R. panaciterrae* sp. nov. LnR5-47^T; 2, *R. ginsenosidimutans* KCTC 22231^T; 3, *R. fulvus* KCTC 12098^T; 4, *R. thiooxydans* KCTC 12771^T; 5, *R. spathiphylli* KACC 12210^T; 6, *R. terrae* KACC 11761^T; 7, *R. ginsengisoli* KACC 11762^T. All data were taken from this study unless otherwise stated. All strains are positive for catalase, oxidase, and assimilation of D-glucose and N-acetylglucosamine. All strains are negative for indole production, arginine dihydrolase, urease, and assimilation of L-arabinose, L-rhamnose, malonate, potassium 5-ketogluconate and phenylacetic acid. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6	7
Motility*	–	–	+	–	–	–	+
API 20NE							
Nitrate reduction	–	–	–	+	–	–	–
Gelatin hydrolysis	–	–	–	+	–	–	+
API ZYM							
Esterase (C4)	+	+	–	+	–†	+	+
Esterase lipase (C8)	+	+	+	–	+	+	+
α -Galactosidase	–	+	+	–	–	–	–
β -Galactosidase	–	+	+	+	–	+	–
β -Glucuronidase	–	+	+	–	–	–	–
N-Acetyl- β -glucosaminidase	+	+	+	–	+	–	+
Utilization of (API ID 32GN):							
Inositol	–	+	–	–	–	–	–
Acetate	–	–	–	–	+	–	+
Melibiose	–	+	+	–	–	–	–
L-Fucose	–	–	–	–	–	+	–
D-Ribose	+	–	–	+	–	–	–
Sucrose	–	+	+	–	–	–	–
Glycogen	–	–	–	+	–†	–	–
Salicin	–	+	+	–	–	–	–
Valerate	–	+	–	+	+	–	+
Utilization of (API 20NE):							
D-Mannose	w	+	+	–	–	+	–
DNA G + C content (mol%)*	63.2	65.5	65.3	64.6	67.6	62.5	61

*Data for columns 2–7 were taken from An *et al.* (2009), Im *et al.* (2004), Lee *et al.* (2007), De Clercq *et al.* (2006) and Weon *et al.* (2007).

†Discrepancies were found between this study and De Clercq *et al.* (2006).

each sample. The highest and lowest values obtained for each sample were excluded and means of the remaining three values were quoted as DNA–DNA relatedness values.

Cells of strain LnR5-47^T were aerobic, Gram-negative, non-motile, non-spore-forming rods. Colonies grown on R2A agar for 3 days were smooth, circular, convex, transparent and yellowish. The isolate grew well on nutrient agar and TSA and grew slightly on LB agar, but did not grow on MRS agar. The isolate hydrolysed skimmed milk, but not DNA, cellulose, chitin, xylan or starch. β -Glucosidase activity was detected; this activity was responsible for the conversion of ginsenoside Rb₁ to Rd (data not shown). Other physiological characteristics of strain LnR5-47^T are summarized in the species description and a comparison of characteristics of strain LnR5-47^T with those of closely related type strains is shown in Table 1.

A nearly complete 16S rRNA gene sequence (1464 bp, base positions 30–1489 with respect to the *Escherichia coli* numbering system) was obtained from strain LnR5-47^T. The closest relatives of the isolate were *R. ginsengisoli* GR17-7^T (98.0 % 16S rRNA gene sequence similarity), *R. terrae* GP18-1^T (97.9 %), *Dyella ginsengisoli* Gsoil 3046^T (97.7 %), *R. soli* DCY45^T (97.3 %), *Dyella soli* JS12-10^T (97.2 %) and *Dyella japonica* IAM 15069^T (97.1 %). Lower sequence similarity

(<97.0 %) was found with all other recognized taxa. The close affiliation of strain LnR5-47^T with the genus *Rhodanobacter* was also evident in the neighbour-joining tree and supported by the maximum-parsimony analysis (Fig. 1). DNA–DNA relatedness values between strain LnR5-47^T and *R. ginsengisoli* GR17-7^T, *R. terrae* GP18-1^T, *D. ginsengisoli* Gsoil 3046^T, *R. soli* DCY45^T, *D. soli* JS12-10^T and *D. japonica* IAM 15069^T were 7.1, 11.8, 25.6, 12.7, 8.9 and 25.8 %, respectively. According to Wayne *et al.* (1987) and Stackebrandt & Goebel (1994), the combined results from the DNA–DNA relatedness and 16S rRNA gene sequence studies suggested that strain LnR5-47^T represents a novel species in the genus *Rhodanobacter*.

The genomic DNA G + C content of strain LnR5-47^T was 63.2 mol%. Q-8 was the major ubiquinone. As shown in Table 2, the major fatty acids of strain LnR5-47^T were iso-C_{16:0} (20.6 %), iso-C_{17:1} ω 9c (20 %) and iso-C_{15:0} (12.5 %). These were consistent with the major fatty acids (>5 %) of *R. spathiphylli* KACC 12210^T, *R. terrae* GP18-1^T and *R. ginsengisoli* GR17-7^T; however, strain LnR5-47^T could be distinguished on the basis of qualitative and quantitative differences for these and other fatty acids (Table 2).

In conclusion, phylogenetic analysis based on 16S rRNA gene sequences indicated that strain LnR5-47^T belongs to the genus *Rhodanobacter*; this was supported by chemotaxonomic data,

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationship of strain LnR5-47^T with related taxa. Bootstrap values (>60 %) based on 1000 replications are shown at branch nodes. Filled circles indicate that nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 0.01 substitutions per nucleotide.

Table 2. Cellular fatty acid compositions of strain LnR5-47^T and related taxa

Strains: 1, *R. panaciterrae* sp. nov. LnR5-47^T; 2, *R. spathiphylli* B39^T; 3, *R. terrae* GP18-1^T; 4, *R. ginsengisoli* GR17-7^T; 5, *Dyella ginsengisoli* Gsoil 3046^T; 6, *Dyella soli* JS12-10^T. Data for columns 1, 2, 5 and 6 were taken from this study; data for columns 3 and 4 were from Weon *et al.* (2007). All strains were analysed under the same conditions. Fatty acids that accounted for <0.5 % of the total are not shown. Major fatty acids are shown in bold.

Fatty acid (%)	1	2	3	4	5	6
Saturated						
C _{16:0}	2.3	4.3	4.1	1.3	5.7	6.6
C _{18:0}	—	—	1.0	1.0	—	—
Unsaturated						
iso-C _{16:1} H	0.6	—	—	0.6	—	—
C _{17:1} ω6c	5.3	—	2.7	7.2	—	—
Branched-chain						
iso-C _{11:0}	3.5	—	3.7	8.3	—	4.0
iso-C _{11:0} 3-OH	4.4	7.0	5.6	10.2	7.8	3.7
iso-C _{12:0} 3-OH	1.6	2.6	1.7	3.1	1.4	0.5
iso-C _{13:0} 3-OH	3.0	3.9	2.6	5.5	4.8	2.2
iso-C _{14:0}	2.6	5.1	1.9	2.2	1.1	1.1
iso-C _{15:0}	12.5	19.9	10.4	7.3	9.6	25
iso-C _{16:0}	20.6	20.8	19.1	14.8	16.7	14.4
iso-C _{17:0}	5.7	7.7	8.8	4.7	13	14.5
iso-C _{17:1} ω9c	20	14.2	19.3	16.2	17.1	10.0
anteiso-C _{15:0}	4.3	8.8	7.3	5.0	11.6	9.1
anteiso-C _{17:0}	0.5	0.5	0.7	—	2.0	2.2
C _{17:0} cyclo	—	0.7	—	—	0.8	—
Summed feature 4*	6.3	2.1	6.5	3.8	3.9	3.8

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 4 consisted of iso-C_{15:0} 2-OH and/or C_{16:1} ω7c.

i.e. Q-8 as the predominant ubiquinone, and iso-C_{16:0}, iso-C_{17:1} ω9c and iso-C_{15:0} as the major fatty acids. However, DNA–DNA relatedness and phenotypic characteristics differentiated strain LnR5-47^T from members of the other established species of *Rhodanobacter*. Therefore, strain LnR5-47^T should be assigned to the genus *Rhodanobacter* as the type strain of a novel species, for which the name *Rhodanobacter panaciterrae* sp. nov. is proposed.

Description of *Rhodanobacter panaciterrae* sp. nov.

Rhodanobacter panaciterrae (pa.na.ci.ter'ra.e. N.L. n. *Panax*, -acis scientific name of ginseng; L. n. *terra* soil; N.L. gen. n. *panaciterrae* of soil of a ginseng field).

Cells are Gram-negative, aerobic, non-motile rods after growth on R2A agar for 3 days. Colonies on R2A agar after 3 days are smooth, circular, convex, transparent and yellowish. Grows at 10–37 °C, but not at 4 or 42 °C, at pH 4–10 (optimum pH 7.0) and with 0–1 % NaCl (optimum 0 % NaCl). Using the API ID 32GN system,

utilizes D-glucose, maltose, L-proline, N-acetylglucosamine and D-ribose, but not L-arabinose, D-mannitol, caprate, L-histidine, L-alanine, citrate, salicin, 3-hydroxybenzoate, 4-hydroxybenzoate, 3-hydroxybutyrate, itaconate, 2-keto-gluconate, lactate, propionate, L-serine, suberate, gluconate, malate, adipate or phenylacetate as sole carbon sources. With the API 20NE system, utilizes maltose but not D-mannitol or citrate. With API ZYM, produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucosidase (linked with ginsenoside-converting activity), but not α- or β-galactosidase, α-glucosidase, β-glucuronidase, α-mannosidase, α-fucosidase, N-acetyl-β-glucosaminidase, lipase (C14) or trypsin. Q-8 is the predominant major ubiquinone and iso-C_{16:0}, iso-C_{17:1} ω9c and iso-C_{15:0} are the predominant cellular fatty acids.

The type strain is LnR5-47^T (=KACC 12826^T=KCTC 22232^T=LMG 24460^T), isolated from soil of a ginseng field in Liaoning province, China. The DNA G+C content of the type strain is 63.2 mol%.

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