Microlunatus aurantiacus sp. nov., a novel actinobacterium isolated from a rhizosphere soil sample

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A Gram-positive, aerobic, non-motile, mesophilic strain, designated YIM 45721^T, was isolated from a rhizosphere soil sample in Yunnan Province, China. On the basis of 16S rRNA gene sequence similarity analysis, strain YIM 45721^T was assigned to the genus *Microlunatus* and was most closely related to the type strain of *Microlunatus phosphovorus* (98.2 % 16S rRNA gene sequence similarity). Chemotaxonomic data, including cell-wall peptidoglycan structure, menaquinones and fatty acid profile, supported affiliation of strain YIM 45721^T to the genus *Microlunatus.* Results of DNA–DNA hybridization and physiological and biochemical tests enabled strain YIM 45721^T to be differentiated genotypically and phenotypically from *M. phosphovorus*. Therefore, strain YIM 45721^T represents a novel species of the genus *Microlunatus*, for which the name *Microlunatus aurantiacus* sp. nov. is proposed. The type strain is YIM 45721^T (=CCTCC AB 206067^T=DSM 18424^T).

The genus *Microlunatus* was established by Nakamura *et al.* (1995) and has not undergone expansion in the past few years. At the time of writing, the genus contained two species with validly published names: *Microlunatus phosphovorus* (Nakamura *et al.*, 1995) and *Microlunatus ginsengisoli* (Cui *et al.*, 2007). The genus *Microlunatus* accommodates aerobic, Gram-positive, non-motile, coccus-shaped actinobacteria. The cell-wall peptidoglycan contains LL-diaminopimelic acid (LL-DAP), MK-9(H_4) is the predominant menaquinone, and anteiso-$C_{15:0}$, iso-$C_{15:0}$ and iso-$C_{16:0}$ are the major cellular fatty acids.

In this study, a *Microlunatus*-like actinobacterium, designated strain YIM 45721^T, was characterized by using a polyphasic approach. The strain was isolated from a soil sample, which was collected from a rhizosphere of *Taxus chinensis*. For isolation of actinobacteria, soil samples were dried at room temperature for about 7 days. Then, 1 g soil sample was suspended in 9 ml 50 mM phosphate buffer (pH 7.0) containing 0.1 % sodium cholate and incubated for 1 h at 45 °C with vigorous shaking in order to disperse soil aggregates and kill fast-growing bacteria. The soil/water suspension was centrifuged and 1 ml supernatant was resuspended in 9 ml of the same sterile buffer before being spread onto glycerol–asparagine agar (ISP5 medium; Shirling & Gottlieb, 1966). Plates were incubated at 28 °C for 21–30 days. The strain was picked and cultivated on modified ISP2 agar medium (Shirling & Gottlieb, 1966), pH 7.2, that contained (l^{-1}): 4 g glucose, 4 g yeast extract, 5 g malt extract and a vitamin/amino acid mixture (1 mg vitamin B1, 1 mg vitamin B2, 1 mg vitamin B6, 1 mg biotin, 1 mg nicotinic acid, 1 mg phenylalanine, 0.3 g alanine). The strain was maintained as a glycerol suspension (20 %, w/v) at −70 °C.

Genomic DNA extraction, PCR amplification and sequencing of the 16S rRNA gene were performed according to Li *et al.* (2007). An almost-complete 16S rRNA gene sequence (1517 bp) of strain YIM 45721^T was obtained and aligned with related sequences of type strains in the suborder *Propionibacterineae* (downloaded from the GenBank/EMBL/DDBJ database). Phylogenetic analysis was performed using the software package MEGA 3.1 (Kumar *et al.*, 2004) after multiple alignments of the data using CLUSTAL_X (Thompson *et al.*, 1997). Distances (using distance options according to the Kimura two-parameter

Abbreviation: LL-DAP, LL-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 45721^T is EF601828.

model; Kimura, 1980, 1983) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis (1000 resamplings) was used to evaluate tree topology of the neighbour-joining data (Felsenstein, 1985).

Phylogenetic analysis showed that strain YIM 45721^T had the highest 16S rRNA gene sequence similarity (98.2 %) with *M. phosphovorus* DSM 10555^T and both strains formed a distinct cluster in the genus *Microlunatus* (Fig. 1). The 16S rRNA gene sequence similarity between strain YIM 45721^T and the type strain of *M. ginsengisoli* was below 97.0 % (96.4 %). To establish whether strain YIM 45721^T and *M. phosphovorus* DSM 10555^T represent distinct species, DNA–DNA hybridization was performed as described by He *et al.* (2005). Strain YIM 45721^T displayed relatively low DNA–DNA hybridization (mean value, 53.3 %; SD, 4.7 %) with *M. phosphovorus* DSM 10555^T; these results are below the cut-off point recommended by Wayne *et al.* (1987) for the recognition of bacterial genomic species.

The morphological properties of strain YIM 45721^T were examined by scanning electron microscopy (Philips XL30 ESEM-TMP) of cells grown for 4 days at 28 °C on ISP2 agar. Growth on ISP2, ISP5 (Shirling & Gottlieb, 1966), trypticase soy agar (TSA; Difco) and nutrient agar was also evaluated. Colony colour was determined by comparison with colour chips from the ISCC-NBS colour chart standard samples (Kelly, 1964). The Gram reaction was tested using the non-staining method as described by Buck (1982). Determination of phosphate accumulation of strain YIM 45721^T was performed as described by Nakamura *et al.* (1995), with *M. phosphovorus* DSM 10555^T as the reference strain.

Strain YIM 45721^T was a Gram-positive, non-motile, non-endospore-forming, coccus-shaped actinobacterium. Cells were 0.9–1.3 μm in diameter and occurred singly or in pairs after incubation at 28 °C for 4 days. Cell size depended on the growth stage. Rod-shaped cells were not observed at any stage of growth. Growth occurred on ISP2, ISP5 and TSA, but not on nutrient agar. On ISP2 agar medium, colonies were very small (approx. 0.5–2.0 mm in diameter after 4 days incubation at 28 °C), orange–yellow, circular and convex with a smooth surface. Strain YIM 45721^T accumulated phosphate weakly. As shown in Fig. 2, cells of YIM 45721^T exhibited weak phosphate-accumulating activity after incubation for 4 h in the absence of any carbon substrate in the medium; after 12 h, the phosphate-accumulating activity of YIM 45721^T became more stable.

Carbohydrate and organic acid utilization was determined as described by Cui *et al.* (2007); a basal liquid medium described by Cui *et al.* (2007) was used as basal medium. Sugar fermentation and enzyme activity tests were performed using API 50CH and API ZYM test kits (bioMérieux), respectively. Results were evaluated after incubation at 28 °C for 48 h. Growth at different temperatures (4, 15, 20, 28, 30, 37 and 45 °C), NaCl concentrations (1, 3, 5, 7 and 10 %) and pH values (4.0–10.0) was assessed after incubation for 7 days on TSA medium. Nitrate reduction and gelatin liquefaction were examined by using previously described methods (MacFaddin, 1980). Catalase activity was determined by bubble production after the addition of a drop of 3 % H_2O_2 and oxidase activity was determined by using a 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine (Kovacs, 1956).

The isolate was positive for the enzymes alkaline phosphatase, esterase C4, *N*-acetyl-β-glucosaminidase, lipase C14 and β-glucuronidase, but negative for acid phosphatase, esterase lipase C8, naphthol-AS-BI-phosphohydrolase, leucine arylamidase, cystine arylamidase, valine arylamidase, α-glucosidase, β-glucosidase, α-galactosidase,

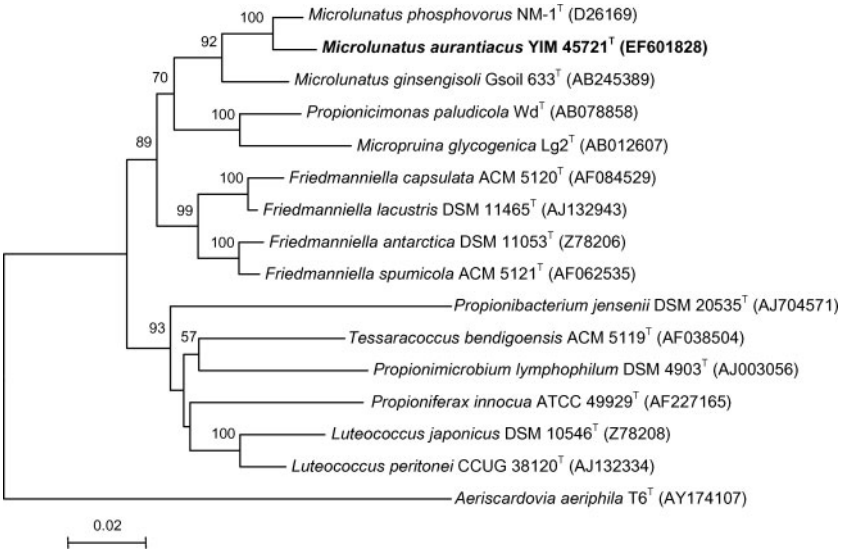

Fig. 1. Phylogenetic tree based on a comparative analysis of 16S rRNA gene sequences showing the relationship between strain YIM 45721^T and related species. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the Kimura two-parameter distance matrix (Kimura, 1980, 1983) and pairwise deletion. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. *Aeriscardovia aeriphila* T6^T was used as the outgroup. Bar, 2 substitutions per 100 nucleotide positions.

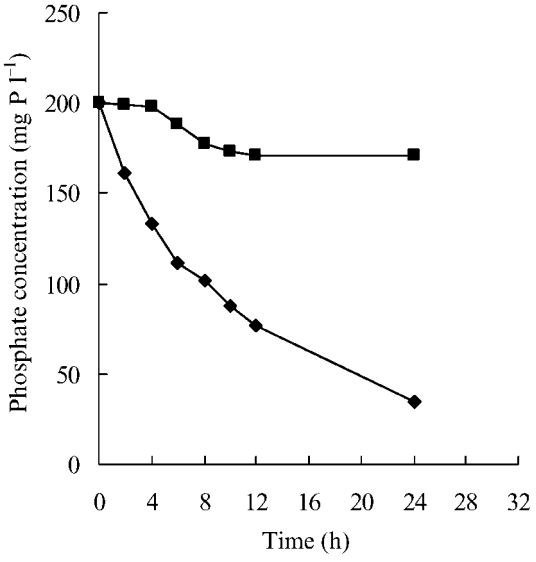

Fig. 2. Phosphate uptake profiles of strain YIM 45721^T and *M. phosphovorus* DSM 10555^T under aerobic conditions. Both strains were grown on ISP2 medium and harvested in the late exponential phase. The amount of phosphate was determined by the molybdenum blue method. ◆, *M. phosphovorus* DSM 10555^T (reference strain); ■, YIM 45721^T.

β-galactosidase, α-mannosidase, α-fucosidase, chymotrypsin and trypsin. Other phenotypic characteristics are presented in Table 1, which summarizes the physiological differences between strain YIM 45721^T and the most closely related species (*M. phosphovorus* DSM 10555^T and *M. ginsengisoli* DSM 17942^T), and in the species description.

The amino acid and sugar contents of the cell wall were determined according to procedures described by Staneck & Roberts (1974). Polar lipids were extracted as described by Minnikin *et al.* (1979) and identified by two-dimensional TLC and spraying with specific reagents (Collins & Jones, 1980). Menaquinones were extracted by using the method of Collins *et al.* (1977) and analysed by HPLC as described by Tamaoka *et al.* (1983). Biomass for quantitative fatty acid analysis of strain YIM 45721^T was prepared by scraping growth from a TSA plate that had been incubated for 4 days at 28 °C. Analysis of the whole-cell fatty acid pattern followed the described methods using the MIDI system (Microbial ID) (Kroppenstedt, 1985; Meier *et al.*, 1993). The G+C content of the genomic DNA was determined by HPLC according to Mesbah *et al.* (1989).

Strain YIM 45721^T contained LL-DAP as the diagnostic amino acid and the cell wall contained arabinose and glucose. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol (phospholipid type II *sensu* Lechevalier *et al.*, 1977). The menaquinone was MK-9(H_4) (100 %). The major fatty acids (>10 %) were anteiso-$C_{15:0}$ (54.53 %) and iso-$C_{16:0}$ (12.43 %). The DNA G+C content of strain YIM 45721^T was 70.9 mol%.

Table 1. Differential characteristics between strain YIM 45721^T and its two phylogenetic neighbours, *M. ginsengisoli* and *M. phosphovorus*

Strains: 1, *M. aurantiacus* YIM 45721^T; 2, *M. ginsengisoli* DSM 17942^T; 3, *M. phosphovorus* DSM 10555^T. Cells of all three strains are Gram-positive, non-endospore-forming, non-motile cocci. All strains can reduce nitrate to nitrite under aerobic conditions and are positive for catalase activity and utilization of D-glucose, D-mannose, galactose, DL-xylose, maltose, fructose, sorbitol, sucrose, trehalose, raffinose, xylitol, salicin, lactate and pyruvate as carbon sources. All strains are negative for utilization of methanol and ethanol as carbon sources. Data for *M. ginsengisoli* were taken from Cui *et al.* (2007); other data are from this study. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3
Temperature range for growth (°C)	20–37	20–30	5–35
Oxidase	−	−	+
Nitrate reduction	+	−	+
Utilization as sole carbon source:			
Starch	+	−	+
Acetate	+	ND	+
Alanine	+	+	−
Amygdalin	+	+	−
Asparagine	+	+	−
D-Arabinose	+	−	+
Glutamine	+	−	−
Glycerol	+	−	+
Glycogen	−	+	−
Inositol	+	−	−
Inulin	−	+	−
Lactose	+	−	−
Mannitol	+	−	+
Malate	+	+	−
Melibiose	+	−	+
Propionate	−	+	−
Serine	−	−	+
L-Sorbose	+	−	+
DNA G+C content (mol%)	70.9	69.8	67.9

It is therefore evident from phylogenetic data, DNA–DNA hybridization data and differences in phenotypic characteristics (Table 1) that strain YIM 45721^T can be distinguished from its closest phylogenetic neighbours. Therefore, strain YIM 45721^T represents a novel species of the genus *Microlunatus*, for which the name *Microlunatus aurantiacus* sp. nov. is proposed.

Description of *Microlunatus aurantiacus* sp. nov.

Microlunatus aurantiacus (au.ran.ti.a′cus. N.L. masc. adj. *aurantiacus* orange-coloured, referring to the orange colour of the colonies).

Cells are Gram-positive, aerobic, coccus-shaped, non-motile, non-endospore-forming and 0.9–1.3 μm in diameter after 4 days culture on ISP2 agar. Colonies are very small (approx. 0.5–2.0 mm in diameter after incubation for 4 days on ISP2 agar medium at 28 °C), smooth, circular, convex and orange–yellow. Growth occurs at 15–37 °C and pH 7.0–7.5, but no growth occurs at or below 15 °C or above 37 °C. Growth occurs in the absence of NaCl. Catalase-positive, oxidase-negative. H_2S is not produced. Nitrate is reduced. In addition to the data shown in Table 1, utilizes arbutin, fructose, D-adonitol, aesculin, alanine, amygdalin, fumarate, inulin, D-mannose, methyl α-D-galactoside, methyl β-D-galactoside, methyl α-D-glucoside, raffinose, D-ribose, salicin, D-sorbitol, starch, sucrose, trehalose, Tween 20, urea, xylitol, DL-xylose, adenine, asparagine, arginine, glutamate, histidine, hypoxanthine, threonine, tyrosine and xanthine as sole carbon sources, but does not utilize L-arabinose, casein, cellobiose, chitin, DNA, lysine, maltotriose, methyl β-D-glucoside, L-rhamnose, serine, Tween 60, Tween 80, tryptophan or valine as sole carbon sources. Acid is produced from amygdalin, fructose, D-glucose, lactose, maltose, mannose, melibiose, raffinose, sucrose, trehalose, xylitol and L-xylose, but not from L-arabinose, L-rhamnose, ribose, D-sorbitol or D-xylose. MK-9(H_4) is the menaquinone. The phospholipid pattern consists of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The major fatty acids are anteiso-$C_{15:0}$ (54.53 %) and iso-$C_{16:0}$ (12.43 %); other minor components are iso-$C_{15:0}$ (5.46 %), $C_{16:0}$ (3.76 %), iso-$C_{14:0}$ (2.87 %), $C_{16:1}9c$ (2.76 %), anteiso-$C_{17:0}$ (2.76 %), anteiso-$C_{18:0}$/$C_{18:2}c$ (2.68 %), iso-$C_{14:0}$ 3-OH (1.98 %), $C_{14:0}$ (1.87 %), anteiso-$C_{15:0}$ (1.81 %), anteiso-$C_{13:0}$ (1.80 %), $C_{15:0}$ (1.46 %), iso-$C_{17:0}$ (1.02 %), $C_{14:0}$ (0.87 %), anteiso-$C_{17:1}$ (0.69 %), iso-$C_{16:1}$H (0.59 %) and iso-$C_{12:0}$ (0.28 %). The DNA G+C content is 70.9 mol%.

The type strain, YIM 45721^T (=CCTCC AB 206067^T= DSM 18424^T), was isolated from a rhizosphere soil sample in Yunnan Province, China.

Acknowledgements

This research was supported by the National Basic Research Program of China (no. 2004CB719601), the Key Project of the Chinese Ministry of Education (no. 206139), the National Natural Science Foundation of China (no. 30560001,30600001), the Yunnan Provincial International Cooperative Program (no. 2005GH21) and the Ministry of Science and Technology, PR China (2006DFA33550). W.-J. L. was also supported by the Program for New Century Excellent Talent in University (NCET).

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