**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 45721T, was isolated from a rhizosphere soil sample in Yunnan Province, China. On the basis of 16S rRNA gene sequence similarity analysis, strain YIM 45721T 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 45721T to the genus Microlunatus. Results of DNA–DNA hybridization and physiological and biochemical tests enabled strain YIM 45721T to be differentiated genotypically and phenotypically from *M. phosphovorus*. Therefore, strain YIM 45721T represents a novel species of the genus *Microlunatus*, for which the name *Microlunatus aurantiacus* sp. nov. is proposed. The type strain is YIM 45721T (=CCTCC AB 206067T=DSM 18424T).

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, cocccus-shaped actinobacteria. The cell-wall peptidoglycan contains L- diaminopimelic acid (L-L-DAP), MK-9(H4) is the predominant menaquinone, and anteiso-C15:0, iso-C15:0 and iso-C16:0 are the major cellular fatty acids.

In this study, a *Microlunatus*-like actinobacterium, designated strain YIM 45721T, 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 (100 ml): 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 45721T 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
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 45721T had the highest 16S rRNA gene sequence similarity (98.2%) with *M. phosphovorus* DSM 10555T and both strains formed a distinct cluster in the genus *Microlunatus* (Fig. 1). The 16S rRNA gene sequence similarity between strain YIM 45721T and the type strain of *M. ginsengisoli* was below 97.0% (96.4%). To establish whether strain YIM 45721T and *M. phosphovorus* DSM 10555T represent distinct species, DNA–DNA hybridization was performed as described by He et al. (2005). Strain YIM 45721T displayed relatively low DNA–DNA hybridization (mean value, 53.3%; SD, 4.7%) with *M. phosphovorus* DSM 10555T; 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 45721T 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 45721T was performed as described by Nakamura et al. (1995), with *M. phosphovorus* DSM 10555T as the reference strain.

Strain YIM 45721T 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 45721T accumulated phosphate weakly. As shown in Fig. 2, cells of YIM 45721T 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 45721T 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% H2O2 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, β-glucosidase, α-galactosidase, β-glucosidase, α-galactosidase.

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**Fig. 1.** Phylogenetic tree based on a comparative analysis of 16S rRNA gene sequences showing the relationship between strain YIM 45721T 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* T6T was used as the outgroup. Bar, 2 substitutions per 100 nucleotide positions.
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 45721T and the most closely related species (M. phosphovorus DSM 10555T and M. ginsengisoli DSM 17942T), 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 45721T 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 45721T contained LL-DAP as the diagnostic amino acid and the cell wall contained arabinose and glucose. The polar lipids were diphasphatidyglycerol, phosphatidylglycerol and phosphatidylinositol (phospholipid type II sensu Lechevalier et al., 1977). The menaquinone was MK9(H4) (100%). The major fatty acids (>10%) were anteiso-C15:0 (54.53%) and iso-C16:0 (12.43%). The DNA G+C content of strain YIM 45721T was 70.9 mol%.

It is therefore evident from phylogenetic data, DNA–DNA hybridization data and differences in phenotypic characteristics (Table 1) that strain YIM 45721T can be distinguished from its closest phylogenetic neighbours. Therefore, strain YIM 45721T 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. H2S is not produced. Nitrate is reduced. In addition to the data shown in Table 1, utilizes arbutin, fructose, D-adonitol, ascorbin, alanine, amygdalin, fumarate, 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, maltooltriose, methyl β-D-glucoside, L-rhamnose, serine, Tween 20, urea, xylitol, Dl-xylene, adenein, asparagine, arginine, glutamate, histidine, hypoxanthine, threonine, tyrosine and xanthine as sole carbon sources.

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

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