Micrococcus yunnanensis sp. nov., a novel actinobacterium isolated from surface-sterilized *Polyspora axillaris* roots

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In this study, strain YIM 65004^T, isolated from roots of *Polyspora axillaris*, was shown to represent a novel species of the genus *Micrococcus* by means of a polyphasic approach. Chemotaxonomic data gathered for peptidoglycan type, menaquinones, phospholipids and fatty acids strongly supported the classification of this strain within the genus *Micrococcus*: the cell-wall peptidoglycan contained lysine, glutamic acid, alanine, glycine and aspartic acid, the predominant menaquinones were MK-8(H₂) (66.97 %) and MK-7(H₂) (23.26 %), the phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unknown ninhydrin-negative phospholipid, and the major cellular fatty acids were anteiso-C_{15:0} (61.98 %), iso-C_{16:0} (14.25 %) and iso-C_{15:0} (13.04 %). The G + C content of the genomic DNA was 71.7 mol%. A number of physiological features were found that clearly distinguished strain YIM 65004^T from recognized *Micrococcus* species. DNA–DNA hybridization studies suggested that the novel strain represents a separate genomic species. Based on the above data, a novel species of the genus *Micrococcus*, *Micrococcus yunnanensis* sp. nov., is proposed, with the type strain YIM 65004^T (=CCTCC AA 208060^T=DSM 21948^T).

Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host (Holliday, 1989; Schulz & Boyle, 2006). There are more than 300 000 plant species on the earth, and each individual plant is host to one or more endophytes (Strobel *et al.*, 2004). However, only a few of these plants have ever been studied completely relative to their endophytic biology. Consequently, the opportunity to find novel and beneficial endophytic micro-organisms among the diversity of plants in different ecosystems is considerable (Ryan *et al.*, 2008).

The genus *Micrococcus* was first described by Cohn (1872), and since then the description of the genus has been revised repeatedly (Stackebrandt *et al.*, 1995; Wieser *et al.*, 2002).

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 65004^T is FJ214355.

A transmission electron micrograph of cells of strain YIM 65004^T and a table comparing the fatty acid compositions of strain YIM 65004^T and recognized *Micrococcus* species are available as supplementary material with the online version of this paper.

At the time of writing, the genus *Micrococcus* comprised five species, *Micrococcus luteus* (Cohn, 1872), *Micrococcus lylae* (Kloos *et al.*, 1974), *Micrococcus antarcticus* (Liu *et al.*, 2000), *Micrococcus flavus* (Liu *et al.*, 2007) and *Micrococcus endophyticus* (Chen *et al.*, 2009). Members of the genus *Micrococcus* are aerobic, Gram-positive and catalase-positive. Cells are spherical and non-motile, and endospores are not formed. The G + C content of the DNA ranges from 66.3 to 73.3 mol%.

In the course of our research on new actinobacterial sources, we obtained a novel isolate, designated strain YIM 65004^T. Based on phenotypic and genotypic evidence, it is proposed that this isolate represents a novel species of the genus *Micrococcus*.

Strain YIM 65004^T was isolated from roots of *Polyspora axillaris*, collected in Yunnan Province, south-west China. Samples were washed in running water to remove soil particles and sterilized by using an established procedure (Coombs & Franco, 2003). After being surface-sterilized, the root samples were sliced into pieces, followed by plating on TWYE agar plates (containing 1⁻¹ tap water: 0.25 g yeast extract, 0.5 g K₂HPO₄ and 18 g agar, pH 7.2).

The plates were incubated at 28 °C until the outgrowth of endophytic bacteria was discerned. Colonies originating from plant segments were picked and pure cultures were obtained by repeated streaking on TWYE plates. The purified strain (YIM 65004^T) was picked and maintained on tryptic soy agar (TSA; BD) slants at 4 °C and as 20 % (w/v) glycerol suspensions at -20 °C. Biomass for chemical and molecular studies was obtained by cultivation in shaken flasks (about 200 r.p.m.) using tryptic soy broth (TSB; containing 1⁻¹ tap water: 15 g tryptone, 5 g soya peptone and 5 g NaCl, pH 7.2) at 28 °C for 1 week.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were performed as described by Li *et al.* (2007). Multiple alignments with sequences of the most closely related actinobacteria and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980), by using MEGA version 3.1 (Kumar *et al.*, 2004). The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The almost-complete 16S rRNA gene sequence (1426 bp) of strain YIM 65004^T was determined. Phylogenetic analysis revealed that the closest relatives of strain YIM 65004^T were *M. luteus* DSM 20030^T, *M. endophyticus* YIM 56238^T, *M. antarcticus* T2^T, *M. lylae* DSM 20315^T and *M. flavus* LW4^T, with 16S rRNA gene sequence similarities of 99.71, 99.49, 98.53, 98.31 and 98.23 %, respectively. The phylogenetic tree (Fig. 1) also showed that strain YIM 65004^T clustered with *Micrococcus* species and that this cluster was strongly supported with a bootstrap value of 99 %. The results of the comparative 16S rRNA gene sequence analysis clearly demonstrated that strain YIM 65004^T represents a member of the genus *Micrococcus*.

DNA–DNA relatedness was studied using the fluorometric micro-well method (Ezaki *et al.*, 1989; Christensen *et al.*,

2000; He *et al.*, 2005). Fluorescence intensities were measured using a fluorescence microplate reader (SpectraMax Gemini XPS; Molecular Devices). Hybridizations were performed with four replications for each sample. The DNA–DNA hybridization rate was calculated according to the method of He *et al.* (2005). The DNA–DNA hybridization values for strain YIM 65004^T with respect to *M. luteus* DSM 20030^T, *M. endophyticus* YIM 56238^T, *M. antarcticus* T2^T, *M. lylae* DSM 20315^T and *M. flavus* LW4^T were 65.4 % (SD, 4.6 %), 58.9 % (SD, 1.0 %), 32.0 % (SD, 3.2 %), 43.3 % (SD, 3.6 %) and 27.1 % (SD, 2.7 %), respectively. All of the DNA–DNA hybridization values determined were less than 70 %, the recommended threshold value for the delineation of genomic species (Stackebrandt & Goebel, 1994). The levels of DNA–DNA relatedness supported the proposal that strain YIM 65004^T represents a novel genomic species of the genus *Micrococcus*.

The G + C content of the genomic DNA was determined by using the HPLC method (Mesbah *et al.*, 1989) with *Escherichia coli* JM-109 as the reference strain. The G + C content of the DNA of strain YIM 65004^T was 71.7 mol%, which is in accordance with values for members of the genus *Micrococcus* (66.3–73.3 mol%).

Gram staining was carried out by using the standard Gram reaction and was confirmed by using the KOH test (Moaledj, 1986). Morphology and motility were examined by using light microscopy (BH-2; Olympus) and transmission electron microscopy (H-800) with cells from exponentially growing cultures. Colony morphology was observed on TSA after incubation at 28 °C for 3 days. Physiological tests, such as growth at various temperatures (0, 4, 15, 20, 28, 37, 45, 50 and 55 °C), pH (pH 4, 5, 6, 7, 8, 9, 10) and NaCl concentrations (0, 1, 3, 5, 7, 10, 15 and 20 %), were performed in TSB. Catalase activity, Methyl red and Voges–Proskauer tests, oxidase and gelatinase activities, starch hydrolysis, nitrate reduction and urease were assessed as described by Smibert & Krieg (1994).

Fig. 1. Neighbour-joining phylogenetic dendrogram based on 16S rRNA sequences showing the relationships between strain YIM 65004^T and related taxa. *Kytococcus sedentarius* DSM 20547^T (X87755) was used as an outgroup. Numbers at nodes are bootstrap percentages (based on 1000 resamplings). Only bootstrap values greater than 50 % are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

Table 1. Differential phenotypic characteristics of strain YIM 65004^T and related *Micrococcus* species

Strains: 1, YIM 65004^T (*M. yunnanensis* sp. nov.); 2, *M. endophyticus* YIM 56238^T; 3, *M. luteus* DSM 20030^T; 4, *M. antarcticus* T2^T; 5, *M. flavus* LW4^T; 6, *M. lylae* DSM 20315^T. +, Positive or present; –, negative. Data were obtained during this study under identical growth conditions, except where indicated otherwise.

Characteristic	1	2	3	4	5	6
Colony colour	Yellow	Yellow	Creamy yellow	Creamy white to pale yellow	Yellow	Orange
Growth at:						
4 °C	+	–	–	+	–	–
45 °C	+	–	–	–	–	+
7 % NaCl (w/v)	+	+	+	+	–	–
Nitrate reduction	–	+	–	+	–	–
Voges–Proskauer reaction	–	–	–	+	–	–
Hydrolysis of:						
Tween 80	+	–	–	+	–	–
Starch	–	–	–	+	+	–
Acid production from:						
Adonitol	–	–	–	–	–	+
Amylum	–	–	–	+	+	+
D-Arabinose	–	+	+	–	–	–
L-Arabinose	–	+	–	+	–	–
Arbutin	–	+	+	+	–	–
Cellobiose	–	+	–	+	+	–
Dulcitol	–	–	–	–	+	+
Erythritol	–	–	–	–	+	–
Aesculin	–	+	–	+	+	–
Fructose	–	+	+	+	–	+
D-Fucose	–	–	–	–	–	+
Galactose	–	+	–	+	–	–
β-Gentiobiose	–	–	–	+	–	+
Glycogen	–	–	–	+	–	+
Gluconate	–	–	–	–	+	–
Inositol	–	–	–	+	–	–
Inulin	–	–	–	–	–	+
Lactose	–	–	–	+	+	+
D-Lyxose	–	+	–	+	–	+
Mannitol	–	–	–	–	–	+
Melibiose	+	–	–	+	–	–
Melezitose	–	–	–	–	–	+
Methyl α-D-mannoside	–	–	–	–	+	–
Methyl α-D-glucoside	–	+	+	–	–	–
N-Acetylglucosamine	–	+	–	+	–	–
Raffinose	–	+	–	–	–	–
Rhamnose	–	–	–	+	–	+
Ribose	–	+	–	+	+	–
Salicin	–	–	+	+	+	+
Sorbitol	–	+	–	–	–	+
Sorbose	–	–	–	–	–	+
D-Tagatose	–	–	–	+	–	–
Trehalose	+	+	+	–	+	+
D-Xylose	–	–	–	+	–	–
Major quinone(s)	MK-8(H ₂), MK-7(H ₂)	MK-8(H ₂), MK-7(H ₂)	MK-8, MK-8(H ₂)*	MK-8, MK-8(H ₂)*	MK-8(H ₂), MK-7(H ₂)*	MK-8(H ₂)*
DNA G + C content (mol%)	71.7	72.9	70.0*	66.4*	71.4*	69*

*Data for the type strains were taken from Liu *et al.* (2007).

Metabolic properties were determined using API 50 CH test kits according to the instructions of the manufacturer (bioMérieux). Other physiological and biochemical tests were performed as described by Barrow & Feltham (1993) and Wieser *et al.* (2002).

Cells of strain YIM 65004^T were Gram-positive, aerobic, non-motile, non-endospore-forming and coccoid (0.8–1 µm in diameter; see Supplementary Fig S1 available in IJSEM Online). The colonies were yellow, circular and opaque, and had a maximum diameter of about 2 mm after incubation at 28 °C for 48 h on TSA. The novel isolate could grow at temperatures in the range 4–45 °C, and at pH 6–8 and in NaCl concentrations of 0–15 % (w/v). Optimal growth was observed at 28 °C and around pH 7.0–8.0. Strain YIM 65004^T was catalase-positive and gave a negative oxidase reaction. Detailed physiological and biochemical properties are given in Table 1 and in the species description. It is evident from Table 1 that there were some phenotypic differences between YIM 65004^T and recognized *Micrococcus* species.

A purified cell-wall preparation was obtained and hydrolysed as described by Schleifer & Kandler (1972). Amino acids and peptides in cell-wall hydrolysates were analysed by using precolumn derivatization with o-phthalaldehyde by HPLC (1100; Agilent). Phospholipids were extracted, examined by two-dimensional TLC and identified using previously described procedures (Minnikin *et al.*, 1984). Menaquinones were isolated according to Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt, 1982). Cellular fatty acid analysis was performed using the Sherlock Microbial Identification System (MIDI), according to the manufacturer's instructions.

The results indicated that the cell-wall peptidoglycan of strain YIM 65004^T contained lysine, glutamic acid, alanine, glycine and aspartic acid. The phospholipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unknown ninhydrin-negative phospholipid. The menaquinones were represented by MK-8(H₂) (66.97 %) and MK-7(H₂) (23.26 %). The major fatty acids were anteiso-C_{15:0} (61.98 %), iso-C_{16:0} (14.25 %) and iso-C_{15:0} (13.04 %) and its profile was very similar to those described for recognized *Micrococcus* species (Supplementary Table S1, in IJSEM Online). However, a significant amount of iso-C_{16:0} (14.25 %) was detected in strain YIM 65004^T, with only minor amounts in recognized *Micrococcus* species. The chemotaxonomic characteristics of strain YIM 65004^T, such as peptidoglycan type, menaquinones, major fatty acids, phospholipids and DNA G+C content, were consistent with its assignment to the genus *Micrococcus*.

Based on the phenotypic and phylogenetic studies, it is clear that strain YIM 65004^T represents a member of the genus *Micrococcus*. Strain YIM 65004^T could be clearly distinguished from recognized *Micrococcus* species on the basis of phenotypic traits: pH, temperature and NaCl ranges for growth, the ability to hydrolyse Tween 80 and acid

production from various carbon resources. The quinone system with the predominant MK-8(H₂) and MK-7(H₂) derivatives for strain YIM 65004^T is significantly different from those of *M. luteus* DSM 20030^T, *M. antarcticus* T2^T and *M. lylae* DSM 20315^T. Furthermore, the levels of DNA–DNA relatedness supported that strain YIM 65004^T represents a novel species of the genus *Micrococcus*. Thus, on the basis of the phenotypic and genotypic characteristics, strain YIM 65004^T is considered to represent a novel species of the genus *Micrococcus*, for which the name *Micrococcus yunnanensis* sp. nov. is proposed.

Description of *Micrococcus yunnanensis* sp. nov.

Micrococcus yunnanensis (yun.na.nen'sis. N.L. masc. adj. *yunnanensis* pertaining to Yunnan, a province of south-west China).

Cells are Gram-positive, aerobic, non-endospore-forming cocci (0.8–1 µm in diameter). Motility is not observed. Colonies on TSA are yellow, smooth and circular with entire margins. No pigment is produced. Temperature, pH and NaCl tolerance ranges for growth are 4–45 °C, pH 6–8 and 0–15 % (w/v), respectively. Optimal growth occurs at 28 °C and about pH 7.0–8.0. Catalase-positive and oxidase-negative. Gelatin and starch are not hydrolysed. Negative for Methyl red, Voges–Proskauer reaction, urease and the reduction of nitrate. Acid is produced from melibiose and trehalose. The cell-wall peptidoglycan contains lysine, glutamic acid, alanine, glycine and aspartic acid. The phospholipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unknown ninhydrin-negative phospholipid. The predominant menaquinones are MK-8(H₂) and MK-7(H₂). Major cellular fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and iso-C_{15:0}. The G+C content of the genomic DNA of the type strain is 71.7 mol%. Other characteristics of the species are indicated in Table 1 and Supplementary Table S1 (in IJSEM Online).

The type strain, YIM 65004^T (=CCTCC AA 208060^T=DSM 21948^T), was isolated from surface-sterilized roots of *Polyspora axillaris*, collected in Yunnan Province, south-west China.

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