Actinomadura alba sp. nov., isolated from soil in Yunnan, China

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A novel actinomycete, strain YIM 45681^T, which was isolated from soil in a suburb of Kunming, Yunnan Province, China, was subjected to phenotypic and genotypic characterization. The micro-organism, which produces short spore chains arranged in spirals on the aerial mycelium, was shown to have *meso*-diaminopimelic acid in the cell wall. The sugars present in whole-cell hydrolysates were ribose, xylose, galactose, madurose and glucose. The predominant menaquinones were MK-9(H₄), MK-9(H₆) and MK-9(H₂). The phospholipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannoside. The major fatty acids were iso-C_{16:1} H, C_{17:1} ω8c, 10-methyl C_{17:0} and C_{16:0}. A comparative analysis of 16S rRNA gene sequences indicated that the organism formed a distinct clade within the evolutionary radiation of the family *Thermomonosporaceae*, and that it was closely associated with members of the genus *Actinomadura*. A broad range of phenotypic and genetic data supported the suggestion that this organism represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura alba* sp. nov. is proposed, with YIM 45681^T (=DSM 45045^T =CCTCC AA206005^T) as the type strain.

The genus *Actinomadura* was established by Lechevalier & Lechevalier (1970b) and its description has been emended by Zhang *et al.* (1998, 2001) and Miyadoh & Miyara (2001). Currently, the genus comprises 35 recognized species and two subspecies with validly published names. The genus *Actinomadura* accommodates aerobic, Gram-positive, non-acid-fast, non-motile actinomycetes that produce well-developed, non-fragmenting vegetative mycelia and aerial hyphae that differentiate into surface-ornamented spore chains. These chains are of various lengths and can be straight, hooked or spiral in form, with irregular, smooth, spiny or warty spores. Members of the genus *Actinomadura* are characterized chemotaxonomically by the presence of type III/B cell walls (i.e. containing *meso*-diaminopimelic acid and madurose) with peptidoglycan structures of the acetyl type, major proportions of hexahydrogenated menaquinones with nine isoprene units, complex fatty acid profiles, including hexadecanoic, 14-methylpentadecanoic and 10-methyloctadecanoic acids as predominant components, and diphosphatidylglycerol and phosphatidylinositol as major phospholipids (Kroppenstedt *et al.*, 1990).

Strain YIM 45681^T was isolated by using the following procedure. Soil samples were collected from the rhizosphere of *Catharanthus roseus*, at the surface and down to a depth of about 10 cm. Soil samples were air-dried for about 7 days. One gram of the soil sample was first suspended in 9 ml MOPS (10 mM) containing 1 % keratin and incubated for 2 h at 45 °C with vigorous shaking, in order to kill fast-growing bacteria and promote actinomycete spore germination. This culture was centrifuged and the supernatant was serially diluted to 10⁻³ and spread on a humic acid/vitamin/gellan gum (HVG; Suzuki *et al.*, 1999) medium and incubated at 28 °C for 30 days.

Strain YIM 45681^T was grown on ISP 2, ISP 3, ISP 4, ISP 5 (Shirling & Gottlieb, 1966) and Czapek agar plates (Pridham & Lyons, 1980) at 28 °C. Colour determination was performed with colour chips from the Inter-Society Color Council–National Bureau of Standards Color Name Charts (Standard Samples, no. 2106; Kelly, 1964). Spore chains were observed using the cover-slip technique of Kawato & Shinobu (1959). Morphological characteristics were examined by light microscopy with a model BH-2 microscope (Olympus) and scanning electron microscopy (XL30, ESEM-TMP; Philips). Morphological features were observed on ISP 4 medium at 28 °C. Growth was tested over a range of temperatures (4–45 °C) and pH

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 45681^T is DQ985164.

Fig. 1. Scanning electron micrograph of strain YIM 45681^T grown on ISP 4 for 21 days at 28 °C. Bar, 5 μm.

(pH 6.0–12.0) on ISP 2 medium. The phenotypic properties of the isolate are also consistent with its classification in the genus *Actinomadura*. Strain YIM 45681^T grew well on ISP 2, ISP 3 and ISP 4 media, but grew only moderately well on ISP 5 and Czapek agar plates. Vegetative hyphae were well developed on all of the media tested. The whitish aerial mycelium, which was produced only on ISP 3, ISP 4 and Czapek agar media, formed short, spiral chains of irregular-surfaced spores (Fig. 1). No diffusion pigments were produced on any of the media tested. The optimal temperature and pH for growth were 28 °C and pH 7.0–8.0.

Physiological and biochemical characteristics of strain YIM 45681^T are given in Table 1 and the species description. ISP

9 (Shirling & Gottlieb, 1966) was used as the basic medium for testing carbohydrate utilization; each filter-sterilized compound was tested at a final concentration of 1 % (w/v). Urease activity was determined by assessing a colour change in Bacto urea broth (Difco). The production of H₂S was tested on peptone iron agar (Difco). Nitrate reduction, gelatin liquefaction and the degradation of elastin and starch were investigated by using previously described methods (MacFaddin, 1980). The decomposition of adenine, hypoxanthine, casein, DL-tyrosine and xanthine was examined by using the methods of Gordon *et al.* (1974).

The procedures used to identify the cell-wall amino acids and sugars in whole-cell hydrolysates were as described by Stanek & Roberts (1974). Menaquinones were extracted by using the method of Collins *et al.* (1977) and were analysed by HPLC, as described by Tamaoka *et al.* (1983). 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). Biomass for a quantitative fatty acid analysis of strain YIM 45681^T was prepared by scraping growth from TSB agar plates that had been incubated for 7 days at 28 °C. Fatty acids were extracted, methylated and analysed using the MIDI (Microbial Identification) system. The wall diamino acid in the peptidoglycan layer of strain YIM 45681^T was *meso*-diaminopimelic acid and the whole-cell sugars were ribose, madurose, xylose, galactose and glucose (cell-wall chemotype III, according to Lechevalier & Lechevalier, 1970a). The predominant menaquinones were MK-9(H₄), MK-9(H₆) and MK-9(H₂) (ratio of peak areas, 64.6:18.2:11.5). The phospholipids included diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol

Table 1. Physiological and biochemical characteristics that distinguish strain YIM 45681^T from the type strains of closely related *Actinomadura* species

Strains: 1, YIM 45681^T; 2, *A. spadix* JCM 3146^T; 3, *A. echinospora* DSM 43163^T. Data were taken from the present study and from Holt *et al.* (1994). +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3
Spore-chain arrangement	Spirals	Hooks	Longitudinally paired spores
Spore-surface ornamentation	Irregular	Smooth	Spiny
Colour of growth on ISP 4			
Aerial mycelium	White	Greyish yellow	Yellow–pink
Substrate mycelium	Greyish yellow	Dust	Greyish yellow
Diffusible pigment	None	Dark	Tan
Utilization of:			
Glycerol	–	–	w
<i>myo</i> -Inositol	–	–	w
D-Raffinose	–	+	–
L-Rhamnose	–	+	ND
Liquefaction of gelatin	+	–	+
Hydrolysis of starch	+	+	–
Coagulation of milk	–	+	+
Nitrate reduction	+	+	–

Fig. 2. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequences, showing the position of strain YIM 45681^T and related phylogenetic neighbours. Numbers on branch nodes are bootstrap percentages (of 1000 resamplings); only values above 50 % are shown. The sequence of *Streptomyces glauciniger* FXJ14^T was used as the outgroup. Bar, 1 % sequence divergence.

and phosphatidylinositol mannoside as the major polar lipids (phospholipid type I; Lechevalier *et al.*, 1977), and the fatty acid profile is described in more detail in the species description.

Genomic DNA for PCR amplification was prepared from cells lysed by microwave: a small amount of biomass was transferred from solid medium to a new Eppendorf tube. After washing of the cells with 1 ml PBS (pH 8.0) and 1 ml washing buffer (50 mM Tris/HCl, pH 7.7; 25 mM EDTA; 0.1 % SDS, 0.1 % polyvinylpyrrolidone), 50 µl lysis buffer (50 mM Tris/HCl, pH 8.0; 25 mM EDTA; 3 % SDS; 1.2 % polyvinylpyrrolidone) was added to resuspend the cells, which were then heated in a microwave oven at 700 W for 45 s. Four hundred microlitres warm extraction buffer (10 mM Tris/HCl, pH 8.0; 1 mM EDTA; 0.5 M sodium acetate; 1.2 % polyvinylpyrrolidone, pre-warmed at 65 °C) was then added immediately; after mixing, extraction was performed using the same volume of phenol/chloroform. The DNA was precipitated with isopropanol. After washing

with 70 % ethanol, the DNA was dissolved using 20 µl TE solution.

PCR amplification and 16S rRNA gene sequencing were carried out as described previously (Cui *et al.*, 2001). An almost-complete 16S rRNA gene sequence for strain YIM 45681^T was determined by direct sequencing of the PCR-amplified gene. The G + C content of the DNA, determined by using the HPLC method (Mesbah *et al.*, 1989), was found to be 66.5 mol%.

A preliminary comparison between the almost-complete 16S rRNA gene sequence of strain YIM 45681^T (1479 bp) and sequences in the GenBank database indicated that the novel isolate was closely related to the members of the genus *Actinomadura*. A phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA, version 2.1 (Kumar *et al.*, 2001) after multiple alignment 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). A bootstrap analysis (1000 resamplings) was used to evaluate the tree topology of the neighbour-joining data (Felsenstein, 1985). The phylogenetic analysis (Fig. 2) indicated that isolate YIM 45681^T formed a distinct clade within the radiation encompassing members of the family *Thermomonosporaceae*, and that it was closely associated with members of the genus *Actinomadura*. The levels of 16S rRNA gene sequence similarity between strain 45681^T and its two closest neighbours, *Actinomadura echinospora* DSM 43163^T and *Actinomadura spadix* JCM 3146^T, were 96.6 and 95.9 %, respectively (see Fig. 2).

It is clear from the phylogenetic analyses based on almost-complete 16S rRNA gene sequences that our isolate belongs to the genus *Actinomadura* and represents a distinct genomic species (Stackebrandt & Goebel, 1994). Support for the novel species status of the organism also comes from the phenotypic evidence shown in Table 1. Therefore, on the basis of the phenotypic and molecular genetic data, it is evident that strain YIM 45681^T represents a novel species of the genus *Actinomadura*, for which we propose the name *Actinomadura alba* sp. nov.

Description of *Actinomadura alba* sp. nov.

Actinomadura alba (al'ba. L. fem. adj. *alba* white, referring to the white aerial mycelium).

Aerobic, Gram-positive. Cells grow well on ISP 2, ISP 3 and ISP 4 media and show moderate growth on ISP 5 and Czapek agar plates, forming a well-developed, non-fragmenting substrate mycelium. No diffusion pigment is produced on any of the media tested. On ISP 3 and ISP 4 media, a moderate amount of white aerial mycelium is formed; this matures into short spiral spore chains, and the spore surface is irregular. The optimal temperature and pH for growth are 28 °C and pH 7.0–8.0. Catalase- and urease-positive. Nitrate is reduced to nitrite. H₂S is not produced. Gelatin liquefaction is observed. D-Mannitol, D-xylose, adonitol, D-ribose, D-fructose, D-trehalose, methyl α -D-glucoside, maltose, D-lactose, sucrose and D-arabitol can be utilized as carbon sources, but glycerol, D-galactose, D-mannose, D-mannitol, L-rhamnose, D-cellobiose, myo-inositol, D-arabinose, D-melibiose and D-raffinose are not utilized. Decomposes hypoxanthine, casein, aesculin, gelatin and starch. Does not decompose xanthine, DL-tyrosine or Tween 80. *meso*-Diaminopimelic is the diagnostic diamino acid, and the cell-wall sugars are ribose, xylose, madurose, galactose and glucose. The predominant menaquinones are MK-9(H₄), MK-9(H₆) and MK-9(H₂). The phospholipids are diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol and phosphatidylinositol mannoside. The major fatty acids (>10 %) are iso-C_{16:1} H (19.77 %), C_{17:1} ω 8c (18.51 %), 10-methyl C_{17:0} (13.31 %) and C_{16:0} (11.03 %). The G+C content of the DNA of the type strain is 66.5 mol%.

The type strain, YIM 45681^T (=DSM 45045^T=CCTCC AA206005^T), was isolated from a soil sample collected in a suburb of Kunming, Yunnan Province, south-west China.

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