Actinomadura flavalba sp. nov., an endophytic actinomycete isolated from leaves of *Maytenus austroyunnanensis*

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An actinomycete strain, designated YIM 61435^T, was isolated from leaves of *Maytenus austroyunnanensis* collected from a tropical rainforest in Xishuangbanna, Yunnan Province, south-west China. The isolate produced aerial mycelium with long, curved to hooked spore chains. The chemotaxonomic characteristics of the isolate were consistent with its assignment to the genus *Actinomadura*. Phylogenetic analysis using 16S rRNA gene sequences also indicated that this strain should be classified in the genus *Actinomadura*; however, it could be separated clearly from its closest neighbour, *Actinomadura atramentaria* DSM 43919^T. Furthermore, a combination of DNA–DNA hybridization results and significant differences in morphological and physiological characteristics indicate that strain YIM 61435^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura flavalba* sp. nov. is proposed. The type strain is YIM 61435^T (=DSM 45200^T =CCTCC AA 208017^T).

The genus *Actinomadura* belongs to the family *Thermomonosporaceae*, which also includes the genera *Actinocorallia*, *Spirillospora* and *Thermomonospora* (Zhang *et al.*, 2001). Members of the genus *Actinomadura* are aerobic, Gram-positive, non-acid-fast, non-motile actinomycetes that typically form non-fragmenting, extensively branched substrate mycelium and aerial hyphae that differentiate into short to long, straight, hooked or spiral spore chains with either folded, irregular, smooth, spiny or warty spores. The genus is characterized chemotaxonomically by the presence of *meso*-diaminopimelic acid in the cell wall, peptidoglycan structures of the acetyl type, madurose as the characteristic sugar in whole-cell hydrolysates (Goodfellow, 1989; Lechevalier & Lechevalier, 1970) and hexahydrogenated menaquinones with nine isoprene units as the major menaquinones. The phospholipid pattern is type PI (diphosphatidylglycerol and phosphatidylinositol are present as major phospholipids) and the complex fatty acid profiles include hexadecanoic, 14-methylpentadecanoic and 10-methyloctadecanoic acids as the predominant components. At the time of writing, the genus comprised 39 species, including two subspecies, with validly published names (<http://www.bacterio.cict.fr/>).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 61435^T is FJ157185.

An extended neighbour-joining tree based on nearly complete 16S rRNA gene sequences is available as supplementary material with the online version of this paper.

As part of our long-term study on endophytic actinomycete diversity and bioactive metabolites isolated from tropical rainforest medicinal plants of Xishuangbanna, several novel species have been characterized: *Dietzia schimae*, *Dietzia cercidiphylli*, *Pseudonocardia endophytica*, *Glycomyces scopariae*, *Glycomyces mayteni* and *Saccharopolyspora endophytica* (Chen *et al.*, 2009; Li *et al.*, 2008; Qin *et al.*, 2008b, 2009). Here, the characterization and classification of an *Actinomadura*-like isolate is described. It is proposed that this isolate represents a novel species of the genus *Actinomadura*.

Strain YIM 61435^T was isolated from healthy leaf samples of a traditional Chinese medicinal plant, *Maytenus austroyunnanensis*, collected in November 2007 from a tropical rainforest in Xishuangbanna, Yunnan Province, south-west China. Samples were placed in sterile plastic bags, taken to the laboratory and stored at 4 °C before isolation. Plant samples were air-dried at room temperature after being thoroughly washed under tap water and surface-sterilized according to the five-step sterilization procedure described by Qin *et al.* (2008a). After that, samples were processed by the calcium carbonate enrichment method (Otoguro *et al.*, 2001), serially diluted in sterile distilled water, spread-plated onto cellulose-proline agar [2.0 g cellulose, 0.25 g KNO₃, 1.0 g proline, 0.2 g MgSO₄·7H₂O, 0.2 g K₂HPO₄, 0.5 g CaCl₂, 10 mg FeSO₄·7H₂O and 15 g agar in 1000 ml tap water (pH 7.2–7.4)] and incubated at 28 °C for 2 weeks.

Extraction of genomic DNA and amplification of the 16S rRNA gene from strain YIM 61435^T were done as described by Li *et al.* (2007). Phylogenetic analysis was performed using the software package MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of data using the program CLUSTAL_X 1.8 (Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) methods. The stability of relationships was assessed by performing bootstrap analyses (Felsenstein, 1985) of the neighbour-joining data based on 1000 resamplings. The almost-complete sequence (1451 bp) of the 16S rRNA gene of strain YIM 61435^T was used for phylogenetic analysis. The phylogenetic tree (Fig. 1) confirmed that this isolate was a member of the genus *Actinomadura* and it formed a distinct clade with *Actinomadura atramentaria* DSM 43919^T (97.4% 16S rRNA gene sequence similarity) with a high bootstrap value.

Cultural characteristics of the isolate were determined after 2–3 weeks at 28 °C by the methods described in the International *Streptomyces* Project (ISP; Shirling & Gottlieb, 1966), as well as by examining growth on potato dextrose agar (PDA), Czapek's agar and nutrient agar (Waksman, 1967). The colony colour was determined with the ISCC–

Fig. 1. Neighbour-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationship between strain YIM 61435^T and related members of the genus *Actinomadura*. Numbers at nodes are levels of bootstrap support for branch points, based on 1000 resamplings; only values ≥ 40 are shown. Asterisks indicate clades that were conserved when neighbour-joining and maximum-parsimony methods were used to construct phylogenetic trees. The sequence of *Streptosporangium album* DSM 43023^T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position. A more complete version of this tree is available as Supplementary Fig. S1 (available in IJSEM Online).

NBS colour charts (Kelly, 1964). Microscopic observation of spore chains and mycelium of strain YIM 61435^T grown on ISP 2 medium for 21 days was made by light microscopy (BH2; Olympus) and scanning electron microscopy (JSM5600LV; JEOL). Growth was tested at 0, 4, 10, 15, 20, 28, 37, 40, 45 and 55 °C, at pH 5.0–10.0 (at intervals of 0.5 pH units) and at 0–15% (w/v) NaCl (at intervals of 0.5%) using ISP 2 as the basal medium. Utilization of sole carbon sources for energy and growth and decomposition of test substances were determined according to Gordon *et al.* (1974). Enzyme activities were determined by using API 20E and API ZYM test strips (bioMérieux) according to the manufacturer's instructions. A comparison of the phenotypic characteristics of strain YIM 61435^T and its closest phylogenetic neighbour is shown in Table 1.

Strain YIM 61435^T grew well on ISP 2, ISP 3, PDA and nutrient agar, with moderate growth on ISP 4, ISP 5 and Czapek's medium. Well-developed, non-fragmenting, orange–yellow to moderate-brown substrate mycelium was formed on ISP 2, ISP 3 and Czapek's medium. No soluble pigments were observed on any of these media. Aerial mycelium was observed on all media tested. Yellowish-white aerial mycelium was formed on ISP 2, ISP 4 and ISP 5; white aerial mycelium was formed on ISP 3 and nutrient agar. Examination of spore chains on ISP 2 agar by scanning electron microscopy revealed smooth spores in long curved or hooked spore chains (Fig. 2). Optimal growth was observed at 28 °C and pH 7.0.

Amino acid and sugar analysis of whole-cell hydrolysates was performed according to the procedures described by Hasegawa *et al.* (1983). Polar lipids were extracted, examined by two-dimensional TLC and identified using published procedures (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were analysed by HPLC as described by Groth *et al.* (1997). Fatty acids were extracted, purified, methylated and quantified by GC using the standard Microbial Identification system (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). The cell wall diamino acid in the peptidoglycan layer of strain YIM 61435^T was *meso*-diaminopimelic acid and the whole-cell sugars were galactose, glucose, madurose and ribose. No mycolic acids were detected in the cell wall. Phospholipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The predominant menaquinones were MK-9(H₄) (63%) and MK-9(H₆) (25%); minor components were MK-9(H₂) (9%) and MK-9(H₈) (3%). The major fatty acids found in strain YIM 61435^T were iso-C_{16:0} (24.5%), 10-methyl C_{18:0} [tuberculostearic acid; 14.9%], 10-methyl C_{17:0} (9.0%), C_{18:1 cis9} (8.7%), C_{16:0} (8.4%) and iso-C_{18:0} (6.5%), with minor amounts of anteiso-C_{17:0} (4.5%), anteiso-C_{15:0} (3.7%), C_{17:1 cis9} (3.4%), C_{17:0} (3.2%), C_{18:0} (2.5%), iso-C_{17:0} (2.2%), C_{16:1 cis9} (2.2%), iso-C_{15:0} (2.1%), C_{15:0} (1.4%), C_{14:0} (1.0%) and iso-C_{14:0} (0.9%).

The DNA G+C content of strain YIM 61435^T was 71.1 mol%, determined according to Mesbah *et al.*

Table 1. Differential characteristics of strain YIM 61435^T and *A. atramentaria* DSM 43919^T

Data for *A. atramentaria* DSM 43919^T were taken from Miyadoh *et al.* (1987) and Wink *et al.* (2003) unless indicated. +, Positive; –, negative; w, weak.

Characteristic	YIM 61435 ^T	<i>A. atramentaria</i> DSM 43919 ^T
Spore chain arrangement	Curved/hooked	Tufted and straight
Growth on ISP 2 medium		
Aerial mycelium	Yellowish white	White
Substrate mycelium	Orange–yellow	Dark brown
Diffusible pigment	None	Inky brown
Growth on ISP 3 medium	Good	Poor
Growth at 45 °C	–	+*
Growth in 5 % NaCl	+	–*
Major menaquinones	9(H ₄) (63 %), 9(H ₆) (25 %)	9(H ₆) (69 %), 9(H ₄) (14 %)
Utilization of glucose, fructose, inositol and sucrose	+	–*
Hydrolysis of starch	+	–*
Liquefaction of gelatin	+	–*
API ZYM tests		
Alkaline phosphatase	+	–
Esterase (C4)	w	+
Lipase (C14)	+	–
Valine arylamidase	+	–
Cystine arylamidase	w	–
API 20E tests		
Arginine dihydrolase	+	–
Lysine decarboxylase	+	–
DNA G+C content (mol%)	71.1	72

*Data from this study.

(1989). Levels of DNA–DNA relatedness were determined according to the fluorometric microwell method (Ezaki *et al.*, 1989; He *et al.*, 2005). DNA–DNA hybridization studies revealed low relatedness values of 21.5 ± 1.8 % with its closest phylogenetic neighbour, *A. atramentaria* DSM 43919^T, which is much lower than the threshold value of 70 % recommended for the delineation of genomic species (Stackebrandt & Goebel, 1994). This clearly indicates that strain YIM 61435^T represents a different genomic species.

Fig. 2. Scanning electron micrograph of strain YIM 61435^T grown on ISP 2 medium at 28 °C for 3 weeks. Bar, 5 μm.

There were many obvious differences between strain YIM 61435^T and *A. atramentaria* DSM 43919^T, e.g. differences in growth characteristics on ISP 2 and ISP 3 media, spore chain ornamentation, utilization of carbon sources and the major menaquinones. Based on the broad range of genotypic and phenotypic data, strain YIM 61435^T is a member of the genus *Actinomadura* and represents a novel species, for which the name *Actinomadura flavalba* sp. nov. is proposed.

Description of *Actinomadura flavalba* sp. nov.

Actinomadura flavalba (fla.val'ba. L. adj. *flavus* -a -um yellow; L. adj. *albus* -a -um white; N.L. fem. adj. *flavalba* yellowish-white, referring to the colour of the aerial mycelium).

Aerobic, Gram-positive, non-acid–alcohol-fast, non-motile actinomycete. Forms well-developed, branched, non-fragmenting substrate mycelium. Abundant aerial mycelium is present, which differentiates into curved or hooked chains of spores (spore size $0.5\text{--}0.7 \times 0.8\text{--}1.2$ μm). The spore surface is smooth. Good growth occurs on ISP 2, ISP 3, PDA and nutrient agar. Moderate growth occurs on ISP 4, ISP 5 and Czapek's medium. Yellowish-white aerial mycelium is formed on ISP 2, ISP 4 and ISP 5 media. No diffusible pigments are produced on any of the media

tested. Temperature range for growth is 15–37 °C, with optimal growth at 28 °C. Grows in 0–10 % NaCl and at pH 6.0–8.0. Decomposes adenine, aesculin, casein, gelatin, hypoxanthine, starch and xanthine. Does not decompose Tween 80. Gelatin liquefaction is observed. H₂S is not produced. Nitrate reduction is negative. Dulcitol, erythritol, D-fructose, D-galactose, glucose, inositol, lactose, maltose, L-rhamnose, D-ribose, sorbitol, sucrose and trehalose are utilized as sole carbon sources, but D-arabinose, cellobiose, mannitol, D-mannose, raffinose, L-sorbose, xylitol and D-xylose are not utilized. Other physiological properties are indicated in Table 1. Type III/B cell wall (*meso*-diaminopimelic is the diagnostic diamino acid and madurose is the characteristic sugar). Predominant menaquinones are MK-9(H₄) and MK-9(H₆), with MK-9(H₂) and MK-9(H₈) as minor components. The phospholipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. Major fatty acids (>5.0 % of the total) are iso-C_{16:0}, 10-methyl C_{18:0}, 10-methyl C_{17:0}, C_{18:1 cis9}, C_{16:0} and iso-C_{18:0}.

The type strain is YIM 61435^T (=DSM 45200^T =CCTCC AA 208017^T), isolated from surface-sterilized leaves of *Maytenus austroyunnanensis* collected from the tropical rainforest in Xishuangbanna, Yunnan Province, south-west China. The DNA G+C content of strain YIM 61435^T is 71.1 mol%.

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