Streptomyces artemisiae sp. nov., isolated from surface-sterilized tissue of *Artemisia annua* L.

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The taxonomic position of an actinomycete strain YIM 63135^T, which was isolated from the surface-sterilized tissue of *Artemisia annua* L. collected from Yunnan province, south-west China, was determined by using a polyphasic approach. Morphological and chemical characteristics of the novel strain were consistent with those of the genus *Streptomyces*. It developed a pinkish aerial mycelium and pinkish-brown substrate mycelium on oatmeal agar. The cell wall of the strain contained LL-diaminopimelic acid. The menaquinones comprised MK-9(H_6) (62.8 %), MK-9(H_8) (31.4 %) and MK-9(H_4) (5.9 %). The phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, an unknown glucosamine-containing phospholipid (GluNu), phosphatidylinositol mannosides and four unknown ninhydrin-negative phospholipids. The major fatty acids were iso-$C_{16:0}$ (30.0 %), anteiso-$C_{17:0}$ (27.3 %) and anteiso-$C_{15:0}$ (17.0 %). The DNA G+C content of strain YIM 63135^T was 72.6 mol%. Phylogenetic analysis of 16S rRNA gene sequences revealed that strain YIM 63135^T is a member of the genus *Streptomyces* and exhibited 99.9 % gene sequence similarity to *Streptomyces armeniacus* NBRC 12555^T, while low sequence similarity values (<97.0 %) distinguished strain YIM 63135^T from all other *Streptomyces* species. DNA–DNA hybridization studies suggested that strain YIM 63135^T represents a different genomic species. On the basis of phenotypic and phylogenetic characteristics, strain YIM 63135^T was considered to represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces artemisiae* sp. nov. is proposed, with YIM 63135^T (=CCTCC AA 208059^T =DSM 41953^T) as the type strain.

The genus *Streptomyces* was initially introduced by Waksman & Henrici (1943) to encompass aerobic, spore-forming actinomycetes. *Streptomyces* species have distinct features, such as Gram-positive cell walls, production of extensively branched substrate and aerial mycelia, high DNA G+C contents and the presence of LL-diaminopimelic acid and absence of characteristic sugars in the cell wall (Locci, 1989; Anderson & Wellington, 2001). At the time of writing, the genus *Streptomyces* encompasses nearly 600 species and subspecies with validly published names. *Streptomyces* species are abundant in soil and are well known for their ability to produce biologically active secondary metabolites. Streptomycetes are used extensively in industry because of their ability to generate a number of chemical compounds, including antibiotics, enzymes, enzyme inhibitors, antitumour agents and antifungal compounds (Chun *et al.*, 1997; Kim & Hwang, 2003). In this study, strain YIM 63135^T was isolated from surface-sterilized tissue of *Artemisia annua* L., which was collected from Yunnan province, south-west China. Our polyphasic taxonomic analysis demonstrated that this isolate represents a novel species of the genus *Streptomyces*.

Plant samples were thoroughly washed in running water to remove all soil and sterilized by an established procedure (Coombs & Franco, 2003). After being surface-sterilized, the plant samples were sliced and plated on HV agar (Hayakawa & Nonomura, 1987) plates supplemented with cycloheximide (50 mg l^{-1}) to suppress fungal growth. Plates were incubated at 28 °C until the outgrowth of endophytic bacteria was discerned. Colonies originating from plant segments were picked up and pure cultures were obtained by repeated streaking on plates containing TWYE

Abbreviations: DPG, Diphosphatidylglycerol; GluNu, unknown glucosamine-containing phospholipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 63135^T is EU200685.

A supplementary table comparing fatty acid profiles of strain YIM 63135^T and *S. armeniacus* NBRC 12555^T is available with the online version of this paper.

medium (0.25 g yeast extract, 0.5 g K_2HPO_4 and 18 g agar per litre tap water, pH 7.2). The purified strain YIM 63135^T was picked and maintained on tryptic soy agar slants at 4 °C and as 20 % (w/v) glycerol suspensions at −20 °C.

The morphological characteristics of strain YIM 63135^T, including spore-chain morphology, spore size and surface ornamentation, were assessed by light and scanning electron microscopy (Philips XL30; ESEM-TMP) of 14-day-old cultures on YIM 38 medium [5 g malt extract, 4 g yeast extract, 4 g glucose, vitamin mixture (0.5 mg each of thiamine-hydrochloride, riboflavin, niacin, pyridoxine-hydrochloride, inositol, calcium pantothenate and *p*-aminobenzoic acid and 0.25 mg biotin), 20 g agar; pH 7.2]. Aerial spore-mass colour, substrate mycelium pigmentation and coloration of the diffusible pigments of strain YIM 63135^T were recorded on ISP (International Streptomyces Project) media (Shirling & Gottlieb, 1966), Czapek's agar and nutrient agar prepared as described by Dong & Cai (2001). Colours were determined by using colour chips from the ISCC–NBS colour charts (standard samples, no. 2106) (Kelly, 1964).

Strain YIM 63135^T formed an extensively branched substrate mycelium, and aerial hyphae that carried smooth-surfaced spores in spiral spore chains (Fig. 1). The strain is characterized by long spore chains each containing more than 10 spores. Spores were elliptical and around 1.0 μm long. Strain YIM 63135^T was observed to grow well on a variety of ISP agar media, including yeast extract–malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts starch agar (ISP 4) and glycerol asparagine agar (ISP 5), Czapek's agar, potato extract agar and nutrient agar. Cultural characteristics of strain YIM 63135^T are shown in Table 1. The aerial mycelium with spores was abundant, well-developed and varied from white to pink on different test media. The substrate mycelium was white to pink and orange–brown. Soluble pigments were produced only on ISP 2 agar (pink) and ISP 3 agar (red).

Carbon source utilization was determined according to the methods of Shirling & Gottlieb (1966) and Locci (1989). Acid production from carbohydrates was assessed as described by Gordon *et al.* (1974). Growth at various temperatures, pH and NaCl concentrations was examined according to Xu *et al.* (2005) using tryptic soy broth (TSB) medium as the basal medium. Oxidase activity was determined from the oxidation of tetramethyl-*p*-phenylenediamine. Catalase activity was determined with 3 % H_2O_2 according to standard methods. Other phenotypic characteristics were tested using standard procedures (Goodfellow, 1971; Williams *et al.*, 1983). The physiological and biochemical characteristics of strain YIM 63135^T are given in Table 2 and in the species description.

For chemical analysis, biomass from strain YIM 63135^T was prepared by culturing in TSB for 7 days at 28 °C in a rotary shaker (200 r.p.m.), harvesting cells by centrifugation and washing with sterilized water. Isomers of diaminopimelic acid and sugars of whole-cell hydrolysates were analysed according to the procedures developed by Hasegawa *et al.* (1983) and Lechevalier & Lechevalier (1970). Phospholipids were examined and identified using the method of Minnikin *et al.* (1979). Menaquinones were extracted and purified following Collins *et al.* (1977) and then analysed by HPLC (Tamaoka *et al.*, 1983). Cellular fatty acid analysis was performed using the Sherlock Microbial Identification System (MIDI), according to the manufacturer's instructions. The G+C content of genomic DNA was determined by the HPLC method according to Mesbah *et al.* (1989), with *Escherichia coli* JM-109 as a control.

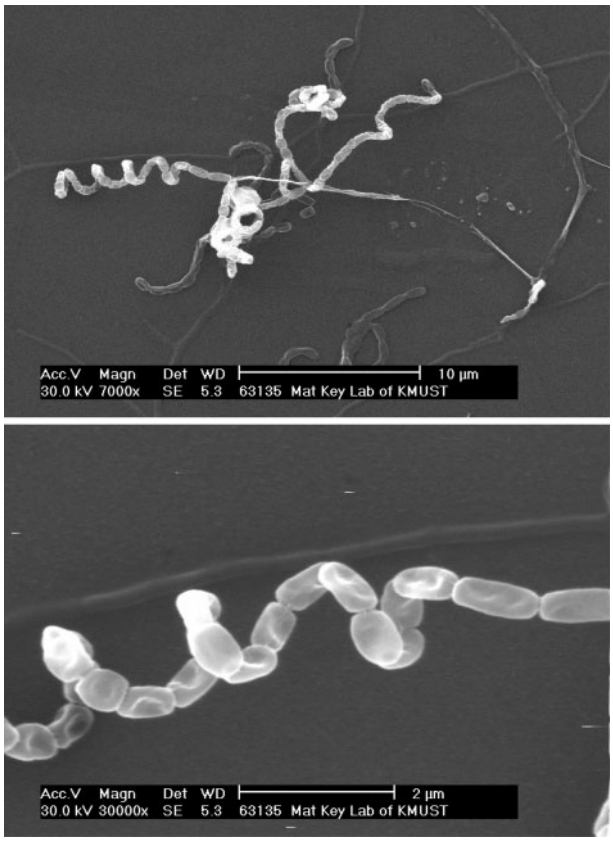

Fig. 1. Scanning electron micrographs showing spiral spore chains and smooth spores of strain YIM 63135^T after growth on YIM 38 medium at 28 °C for 14 days. Bars, 10 μm top, 2 μm bottom.

Strain YIM 63135^T contained LL-diaminopimelic acid as the major diamino acid. Whole-cell hydrolysates contained glucose, galactose, ribose and mannose. The menaquinones were MK-9(H_6) (62.8 %), MK-9(H_8) (31.4 %) and MK-9(H_4) (5.9 %) and the phospholipids were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), an unknown glucosamine-containing phospholipid (GluNu), phosphatidylinositol mannosides (PIM) and four unknown ninhydrin-negative phospholipids. The major fatty acids of strain YIM 63135^T were iso-$C_{16:0}$ (30.0 %),

Table 1. Cultural characteristics of strain YIM 63135^T

Colours are according to Kelly (1964).

Agar medium	Colour of mycelium		Soluble pigment
	Spore mass	Substrate	
Czapek's	Pink	White	Absent
Potato extract	Yellow–white	Pink	Absent
Nutrient	White	Yellow–white	Absent
Yeast extract–malt extract (ISP 2)	White	Yellow–white	Pink
Oatmeal (ISP 3)	Pink	Pink–brown	Red
Inorganic salts starch (ISP 4)	White	Yellow–white	Absent
Glycerol asparagine (ISP 5)	White	Yellow–white	Absent

anteiso-$C_{17:0}$ (27.3 %) and anteiso-$C_{15:0}$ (17.0 %); this profile was very similar to that of the type strain of *Streptomyces armeniacus* (Supplementary Table S1). The DNA G+C content of strain YIM 63135^T was 72.6 mol%. All morphological and chemical features of strain YIM 63135^T were consistent with its assignment to the genus *Streptomyces* (Williams *et al.*, 1989; Manfio *et al.*, 1995).

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 corresponding sequences of representatives of the genus *Streptomyces* (retrieved from the GenBank/EMBL/DDBJ database) and calculations of levels of sequence similarity were carried out using CLUSTAL_X (Thompson *et al.*, 1997).

Table 2. Phenotypic properties of strains YIM 63135^T and *S. armeniacus* NBRC 12555^T

Data from this study under identical growth conditions.

Characteristic	YIM 63135^T	*S. armeniacus* NBRC 12555^T
Urea hydrolysis	−	+
Utilization as sole carbon sources		
Dulcitol	+	−
Erythritol	−	+
Aesculin	−	+
D-Galactose	+	−
Glucose	+	−
Glycerol	+	−
Inositol	−	+
Maltose	−	+
D-Mannose	+	−
L-Rhamnose	+	−
D-Sorbitol	+	−
Utilization as sole nitrogen sources		
Adenine	−	+
L-Asparagine	+	−
L-Phenylalanine	+	−
L-Serine	+	−
L-Tyrosine	+	−
Acid production from D-xylose	+	−
Growth at:		
4 °C	−	+
45 °C	+	−
pH 5.0	+	−
pH 9.0	+	−
7 % NaCl	−	+
Menaquinones	MK-9(H_4), MK-9(H_6), MK-9(H_8)	MK-9(H_4), MK-9(H_6)

A phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) from K_{nuc} values (Kimura, 1980) by using MEGA version 4.0 (Tamura *et al.*, 2007). 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 (1419 bp) for strain YIM 63135^T was obtained. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the organism is phylogenetically related to members of the genus *Streptomyces*, with *Streptomyces armeniacus* NBRC 12555^T as the closest related type strain (99.9 %) (Fig. 2). All other representatives of the genus *Streptomyces* exhibited lower sequence similarity to strain YIM 63135^T (<97.0 %). In view of the high level of 16S rRNA gene sequence similarity between isolate YIM 63135^T and *S. armeniacus* NBRC 12555^T, DNA–DNA relatedness between them was studied according to the fluorometric microwell method (Ezaki *et al.*, 1989; Christensen *et al.*, 2000; He *et al.*, 2005). Fluorescence intensities were measured using a fluorescence microplate reader (Spectra Max Gemini xps). Hybridizations were performed with six replications. The DNA–DNA hybridization rate was calculated following the method of He *et al.* (2005). The DNA–DNA relatedness between strain YIM 63135^T and *S. armeniacus* NBRC 12555^T was 41.3 %, which is well below the 70 % cut-off point for recognition of genomic species (Stackebrandt & Goebel, 1994), suggesting that strain YIM 63135^T should be considered to represent a different genomic species of the genus *Streptomyces*.

Besides the genotypic evidence, strain YIM 63135^T can also be distinguished from its closest relative by additional phenotypic characteristics. According to results obtained in this study, there are many phenotypic differences between strain YIM 63135^T and *S. armeniacus* NBRC 12555^T, including differences in utilization of sole carbon and nitrogen sources, degradation activity, temperature and pH for growth, tolerance of NaCl and menaquinone profiles (Table 2).

Strain YIM 63135^T did not hydrolyse urea. It utilized dulcitol, D-galactose, glucose, glycerol, D-mannose, L-rhamnose, D-sorbitol, L-asparagine, L-phenylalanine, L-serine and L-tyrosine, but none of these were used by *S. armeniacus* NBRC 12555^T. In contrast, *S. armeniacus* NBRC 12555^T utilized erythritol, aesculin, inositol, maltose and adenine. Strain YIM 63135^T could grow at 45 °C and pH 5.0 and 9.0, but *S. armeniacus* NBRC 12555^T could not grow under these culture conditions. *S. armeniacus* NBRC 12555^T tolerated up to 7 % NaCl, while this ability was not shown by strain YIM 63135^T. MK-9(H_8) was present in the menaquinone profile of strain YIM 63135^T, whereas it was absent from *S. armeniacus* NBRC 12555^T. Based on this genotypic and phenotypic evidence, strain YIM 63135^T warrants classification as the type strain of a novel species of the genus *Streptomyces*, for which the name *Streptomyces artemisiae* sp. nov. is proposed.

Description of *Streptomyces artemisiae* sp. nov.

Streptomyces artemisiae (ar.te.mi′si.ae. L. n. *artemisia* mugwort, and also a plant genus; L. gen. n. *artemisiae* of *Artemisia*, referring to isolation of the type strain from *Artemisia annua* L.).

Aerobic, Gram-stain-positive actinomycete that forms an extensively branched substrate mycelium and an aerial mycelium that differentiates into spiral spore chains. Spores are elliptical or short rods, and the spore surface is smooth. Aerial mycelium is white to pink, substrate mycelium is white to pink and orange–brown. Soluble pigments are produced on ISP 2 agar (pink) and on ISP 3 agar (red). Additional cultural characteristics on various agar media are given in Table 1. Growth occurs at 10–45 °C

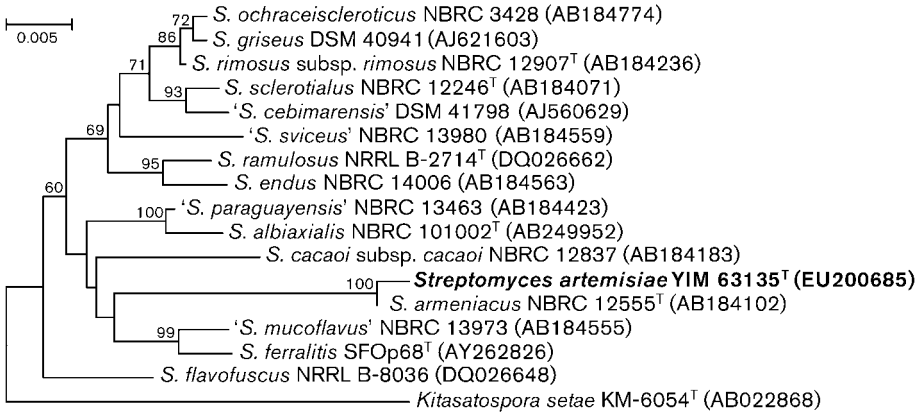

Fig. 2. Neighbour-joining phylogenetic dendrogram based on 16S rRNA gene sequences showing relationships between strain YIM 63135^T and related taxa. The sequence of *Kitasatospora setae* KM-6054^T (AB022868) was used as an outgroup. Numbers represent confidence levels from 1000 replicate bootstrap samplings as percentages; values >50 % are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

and pH 5.0–9.0. Optimal growth at 28 °C and pH 7.0–8.0. Tolerates NaCl up to 5 %. Catalase is produced. Negative for Voges–Proskauer and methyl red tests, oxidase reaction, production of H_2S, nitrate reduction and milk coagulation and peptonization. Starch and Tweens 20, 40 and 80 are hydrolysed, but cellulose, gelatin and urea are not hydrolysed. Utilizes dulcitol, D-galactose, glucose, glycerol, D-mannose, raffinose, L-rhamnose and D-sorbitol as sole carbon sources. Erythritol, aesculin, inositol, maltose, sodium acetate and sucrose are not utilized. L-Alanine, L-arginine, L-asparagine, glycine, hydroxy-L-proline, hypoxanthine, L-lysine, L-phenylalanine, L-serine, L-tyrosine, L-valine and xanthine, but not adenine, can be used as sole nitrogen sources. Acid is produced from D-arabinose, L-arabinose, cellobiose, fructose, D-fucose, lactose, mannitol, ribose, starch, trehalose and D-xylose. The diagnostic amino acid in the peptidoglycan is LL-diaminopimelic acid, and glucose, galactose, ribose and mannose are present in whole-cell hydrolysates (cell wall type I). Phospholipids are DPG, PG, PI, PE and GluNu, with trace amounts of PIM and four unknown phospholipids. The quinone system is composed of the major compounds MK-9(H_6) and MK-9(H_8) and minor amounts of MK-9(H_4). The major cellular fatty acids are iso-$C_{16:0}$, anteiso-$C_{17:0}$ and anteiso-$C_{15:0}$. The G+C content of genomic DNA from the type strain is 72.6 mol%.

The type strain is YIM 63135^T (=CCTCC AA 208059^T =DSM 41953^T), isolated from surface-sterilized tissue of *Artemisia annua* L. collected in Yunnan Province, south-west China.

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