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Streptomyces yunnanensis sp. nov., a mesophile from soils in Yunnan, China

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A strain was isolated from red soil from the suburb of Kunming in Yunnan, China, during the screening of agricultural antibiotics which prevented and cured wheat-stem rust. This isolate, designated YIM 41004^T (=CGMCC 4.1004^T =DSM 41793^T), was identified by a polyphasic approach. The test results suggested that this strain was clearly assigned to the genus *Streptomyces* and found to be marginally close to Williams cluster 32 based on the morphological and physiological data. The almost-complete 16S rRNA gene sequence of the strain was determined and compared with those of representative streptomycetes. The phylogenetic tree confirmed its membership in the genus *Streptomyces* and demonstrated that this strain represented a separate phyletic line in a clade encompassed by streptomycetes within cluster 32. Based on the polyphasic evidence, it is therefore proposed that strain YIM 41004^T should be classified as *Streptomyces yunnanensis* sp. nov.

INTRODUCTION

The numerical taxonomic study of the genus *Streptomyces* by Williams *et al.* (1983) and the *Streptomyces* chapter in vol. 4 of *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1989) delineated the *Streptomyces violaceusniger* cluster in terms of morphology for strains having grey, rough-surfaced spores in spiral chains, which encompassed *S. violaceusniger*, several *Streptomyces hygroscopicus* strains, *Streptomyces sparsogenes* and *Streptomyces melanosporofaciens*. A study of levels of DNA relatedness among strains of *S. violaceusniger* and other related taxa belonging to the *S. violaceusniger* cluster by Williams *et al.* (1983, 1989) demonstrated that the *Streptomyces violaceusniger* phenotypic cluster is heterogeneous in DNA relatedness among strains at a level of >70 %, and strains in this cluster should be considered to be four distinct species, *S. melanosporofaciens*, *S. sparsogenes*, *S. violaceusniger* and *S. hygroscopicus* (*Streptomyces endus* was assigned as a subjective synonym in the revised description of *S. hygroscopicus*) (Labeda & Lyons, 1991). A numerical classification, using miniaturized physiological tests, of the genera *Streptomyces* and *Streptoverticillium* by Kämpfer *et al.* (1991) showed that the distinct species described by Labeda & Lyons (1991) in Williams cluster 32 were distributed into clusters 10, 41, 51, 53, 54 and 85.

In the course of screening agricultural antibiotics that prevent and cure wheat-stem rust, strain YIM 41004^T was isolated from soil samples in Yunnan. It produces the antifungal agent cycloheximide. It is morphologically and physiologically similar to strains assigned to the *Streptomyces violaceusniger* cluster (Williams *et al.*, 1983, 1989). The taxonomic results are reported in this paper.

Abbreviation: ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the partial 16S rDNA sequence of strain YIM 41004^T is AF346818.

METHODS

Organism. Strain YIM 41004^T was isolated from the red soil of suburb of Kunming of Yunnan, China. The strain was maintained by cultivation on 38$^{\#}$ agar medium that contained (per litre) 4 g glucose, 4 g yeast extract, 5 g malt extract and vitamin/amino acid mixture (1 mg each of vitamin B_1, vitamin B_2, vitamin B_6, biotin, nicotinic acid and phenylalanine, and 0·3 g alanine), with pH adjusted to 7·2, and incubated at 25–30 °C for 7–15 days. The effect of temperature on growth rate was determined on 38$^{\#}$ agar at 24–32 °C at intervals of 2 °C; optimum growth was at 28 °C.

Phenotypic characterization. The medium used for morphological studies was yeast extract-malt extract agar (International *Streptomyces* Project medium no. 2, ISP 2) (Shirling & Gottlieb, 1966) and the incubation time of the pure culture was 7–15 days at 28–30 °C. Morphological observations were made by using optical and electron microscopy (model EPMA-8705). Cultural and physiological characteristics of strain YIM 41004^T were determined according to the methods proposed by Shirling & Gottlieb (1966) and Williams *et al.* (1983). Colour determinations were made by comparing the cultures with colour chips from the ISCC–NBS Color Charts Standard Sample No. 2106 (Kelly, 1964).

Chemotaxonomy. Cell wall was purified and analysed by the methods of Lechevalier & Lechevalier (1980). The procedures of Becker *et al.* (1964) and Lechevalier & Lechevalier (1980) were used for analyses of whole-cell chemical compositions.

16S rDNA sequencing. The chromosomal DNA of strain YIM 41004^T was isolated according to the procedure described by Hopwood *et al.* (1985). 16S rDNA was amplified by PCR using a PCR kit (Sino-American Biotechnology, Beijing), primer A 8-27f (5′-CCGTCGACGAGCTC **AGAGTTTGATCCTGGCTCAG**-3′) and primer B 1523-1504r (5′-CCCGGGTACCAAGCTT **AAGGAGGTG-ATCCAGCCGCA**-3′) (primers are in bold according to the *Escherichia coli* numbering system of Brosius *et al.*, 1978). The conditions used for thermal cycling were as the follows: denaturation at 95 °C for 5 min followed by 35 cycles consisting of denaturation at 95 °C for 1 min, primer annealing at 56 °C for 1 min, and primer extention at 72 °C for 3 min. At the end of the cycles, the reaction mixture was kept at 72 °C for 5 min and then cooled to 4 °C. The amplified 1·5 kb 16S rDNA (rDNA) fragment was separated by agarose gel electrophoresis. The purified fragment was directly sequenced by using a *Taq* DyeDeoxy terminator Cycle Sequencing kit (Applied Biosystems) and analysed with an ABI PRISM 377 DNA sequencer (Applied Biosystems). Sequencing primers used included KMSO98PB1r (5′-TAAGGAGGTGATCCAGCC-3′), KMS584P1r (5′-TGCTGGCAACACAG AACAAG-3′) and KMS584P2r (5′-ACTCTG CCTGCCCGTATCG-3′).

Sequence alignment and phylogenetic analysis. The partial 16S rDNA sequence of strain YIM 41004^T was aligned manually with representative sequences of related streptomycetes from the GenBank database. The evolutionary tree, rooted with *Streptomyces megasporus* as the outgroup, was inferred by using the neighbour-joining method (Saitou & Nei, 1987) from the evolutionary distance data corrected by Kimura's two-parameter model (Kimura, 1980). The topology of the resultant tree was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. The CLUSTAL X program (Thompson *et al.*, 1997) was used for multiple alignment and phylogenetic analysis. The TreeView program (Page, 1996) was used to display, edit and print phylogenetic trees.

RESULTS AND DISCUSSION

Morphological observation of the 7–15-day-old culture of strain YIM 41004^T grown on yeast extract-malt extract agar (ISP 2) (Shirling & Gottlieb, 1966) revealed that both aerial and vegetative hyphea were abundant, well-developed and not fragmented; spore chains with many spores were spiral; spores (0·5–1·0 μm in diameter) were rugose with short spines and were short pillar-shaped and non-motile (Fig. 1).

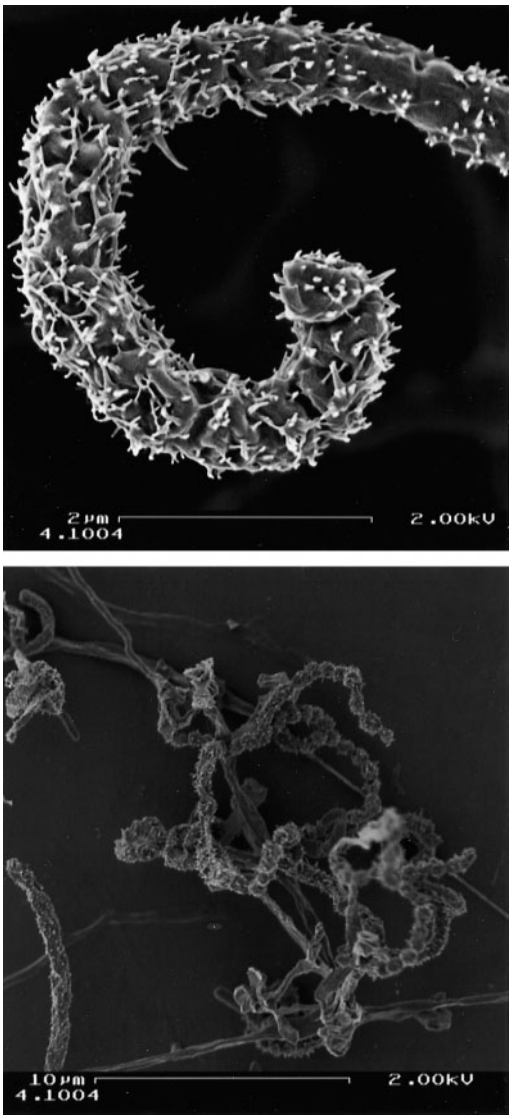

Fig. 1. Scanning electron micrographs showing strain YIM 41004^T rugose spores and spiral spore chains (top and bottom) after growth on yeast extract-malt extract agar (ISP 2) at 28 °C for 15 days.

Table 1. Cultural characteristics of strain YIM 41004^T

Colours are according to the ISCC–NBS Color Charts Standard Sample No. 2106 (Kelly, 1964).

Agar medium	Colour of mycelium:		Soluble pigment
	Aerial	Substrate	
Czapek's	Light brown-grey	Brown-pink	Absent
Glycerol asparagine (ISP 5)	Pale-yellow	Light orange-yellow	Light yellow
Inorganic salt-starch (ISP 4)	Light grey-brown	Light grey-yellow	Absent
Oatmeal (ISP 3)	Light brown-grey	Light grey-yellow	Absent
Yeast extract-malt extract (ISP 2)	Light brown-grey	Light yellow	Absent
Glucose asparagine	Light grey-brown	Deep grey-yellow	Absent
Potato extract	Brown-grey	Deep grey-yellow	Light yellow

Table 2. Physiological characteristics of strain YIM 41004^T and related species in the *Streptomyces violaceusniger* phenotypic cluster

Strains: 1, strain YIM 41004^T; 2, *Streptomyces endus* NRRL ISP-5187^T (=DSM 40187^T); 3, *Streptomyces hygroscopicus* NRRL-ISP 5578^T (=DSM 40578^T); 4, *Streptomyces melanosporofaciens* NRRL B-12234^T (DSM 40318^T); 5, *Streptomyces sparsogenes* ISP 5356^T (=NRRL 2940^T =DSM 40356^T); 6, *Streptomyces violaceusniger* NRRL B-1476^T (=DSM4 1600^T). +, Positive; −, negative. All strains were negative for milk coagulation and positive for gelatin liquefaction.

Characteristic	1	2	3	4	5	6
Milk peptonization	+	+	−	−	+	−
Starch hydrolysis	+	+	−	+	−	+
Nitrate reduction	−	−	−	−	+	−
Urea utilization	+	−	+	−	+	+
Carbon source utilization:						
D-Sucrose	−	+	+	−	+	+
D-Xylose	−	+	+	+	+	+
D-Raffinose	+	+	+	+	−	+
Antimicrobial activity against:						
Bacillus subtilis	−	−	+	+	+	−
Aspergillus niger	+	−	−	+	−	−

Cultural characteristics of strain YIM 41004^T are shown in Table 1. Aerial mycelium of strain YIM 41004^T was abundant, well-developed and varied from light brown-grey to brown-grey on different test media. The substrate hyphae from light yellow to light brown-yellow. Diffusible pigments were not produced on most test media, and melanin was not produced. The cell-wall peptidoglycan of strain YIM 41004^T contained only LL-diaminopimelic acid and glycine, indicating that strain YIM 41004^T has a chemotype cell-wall type I (Lechevalier & Lechevalier, 1970a, b). The whole-cell hydrolysates contained galactose.

On the basis of morphological, cultural and chemotaxonomic properties above, together with the physiological properties of strain YIM 41004^T and five other related species in *Streptomyces violaceusniger* phenotypic cluster (Williams *et al.*, 1983, 1989; Labeda & Lyons, 1991) shown in Table 2, it is evident that strain YIM 41004^T not only belongs to the genus *Streptomyces* but also should be assigned to the *Streptomyces violaceusniger* cluster (Williams *et al.*, 1983, 1989). Although strain YIM 41004^T is similar to members of the *Streptomyces violaceusniger* cluster and clusters 10, 41, 51, 53, 54 and 85 (Kämpfer *et al.*, 1991) on the basis of phenotypic data, this organism cannot be exactly assigned to any of the known streptomycete species of these clusters on the basis of its phenotypic characteristics. Therefore, it is concluded from phenotypic data that strain YIM 41004^T shows no apparent relationship with the validly described species of these clusters (Williams *et al.*, 1983, 1989; Kämpfer *et al.*, 1991). Similarly, strain YIM 41004^T is differentiated primarily from four other cycloheximide-producing species based on the surface of spore and carbon-source utilization from Table 3.

The phylogenetic analysis of strain YIM 41004^T with members of the *Streptomyces violaceusniger* cluster (Williams *et al.*, 1983, 1989; Labeda & Lyons, 1991) reveals that strain YIM 41004^T is distinct from species in this cluster, as showed in Fig. 2. The sequence divergence values between strain YIM 41004^T and members of the *Streptomyces violaceusniger* cluster (Williams *et al.*, 1983, 1989; Labeda & Lyons, 1991) are 2·91 % (*S. hygroscopicus*), 2·70 % (*S. melanosporofaciens*), 2·70 % (*S. violaceusniger*), 3·27 % (*S. sparsogenes*), and these indicate that strain YIM 41004^T represents a hitherto unpublished species.

The phenotypic and genotypic data of strain YIM 41004^T demonstrated that strain YIM 41004^T should be given novel species status in the genus *Streptomyces* Waksman and Henrici 1943^{AL}. Therefore, we proposed this organism

Table 3. Partial features for differentiating strain YIM 41004^T from cycloheximide-producing species

Strains: 1, strain YIM 41004^T; 2, *Streptomyces albulus* ATCC 12757^T (*Streptomyces lydicus* cluster); 3, *Streptomyces noursei* ATCC 11455^T (*Streptomyces noursei* cluster); 4, *Streptomyces griseus* ATCC 23345^T (*Streptomyces anulatus* cluster); 5, *Streptomyces pulveraceus* ATCC 13875^T (*Streptomyces pulveraceus* cluster). Comparative data taken from previous studies (Williams *et al.*, 1983, 1989). +, Positive; −, negative.

Characteristic	1	2	3	4	5
Spore surface	Rugose with short spines	Hairy	Spiny	Smooth	Smooth
Carbon source utilization:					
Sucrose	+	−	−	−	−
D-Raffinose	+	−	−	−	+
L-Arabinose	+	−	+	−	−
L-Rhamnose	+	−	−	−	+
D-Xylose	−	−	−	+	+
D-Mannitol	+	+	+	+	−

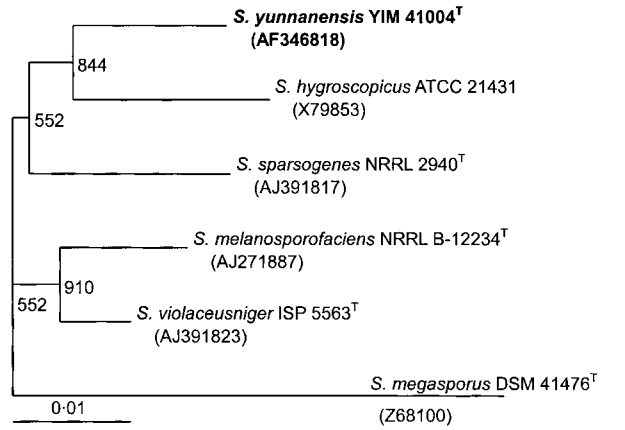

Fig. 2. Neighbour-joining tree (Saitou & Nei, 1987) showing the phylogenetic relationships among members of the *Streptomyces violaceusniger* cluster. The analysis included 1453 unambiguous nucleotide positions. *Streptomyces megasporus* was used as the outgroup. Bootstrap values from 1000 analyses were shown at the nodes of the tree. The scale bar represents one nucleotide substitution per 100 nucleotides of 16S rDNA sequence.

should be a new species with the name *Streptomyces yunnanensis* sp. nov.

Description of *Streptomyces yunnanensis* sp. nov.

Streptomyces yunnanensis (yun.nan.en′sis. N.L. masc. adj. *yunnanensis* pertaining to Yunnan, a province of south-west China).

Both vegetative and aerial hyphae are abundant and well-developed. The colour of aerial and substrate mycelium on various solid media is given in Table 1. Spore chains with many spores are spiral. The spores are rugose with short spines and are short pillar-shaped (0·5–1·0 μm in diameter) and non-motile. Diffusible pigments are not produced and melanin is not produced. Milk is not coagulated but peptonized, starch is hydrolysed and H_2S is not produced. Nitrate is not reduced and gelatin is liquefied. Does not hydrolyse cellulose. Utilizes glucose, fructose, rhamnose, inositol, mannitol, arabinose and raffinose for growth; does not utilize sucrose or xylose. It has antimicrobial activity against *Aspergillus niger* but not against *Bacillus subtilis*. Optimum growth is at 28 °C. The cell wall contains LL-diaminopimelic acid and glycine (cell-wall chemotype I). Whole-cell hydrolysates contain galactose. The type strain, YIM 41004^T, isolated from red soil of the suburb of Kunming in Yunnan, China, was deposited in the China General Microbiological Culture Collection Center (CGMCC) Beijing, China, as strain CGMCC 4.1004^T, and the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen (GmbH), Germany, as strain DSM 41793^T.

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