Tenggerimyces flavus sp. nov., isolated from soil in a karst cave, and emended description of the genus *Tenggerimyces*

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A novel actinomycete, designated strain S6R2A4-9^T, was isolated from a soil sample collected from a karst cave in Henan Province, China, and subjected to a polyphasic taxonomic study. This isolate grew optimally at 25–28 °C, pH 6.5–8.0 and in the absence of NaCl. The substrate mycelium of the isolate was well developed with irregular branches. Aerial mycelium fragmented into long, rod-shaped elements. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain S6R2A4-9^T resided in the cluster of the genus *Tenggerimyces* within the family *Nocardioidaceae* and shared the highest 16S rRNA gene sequence similarity (98.98 %) with *Tenggerimyces mesophilus* I12A-02601^T. The G+C content of the genomic DNA was 67.0 mol%. The strain contained glucose, ribose and xylose in its whole-cell hydrolysates. Strain S6R2A4-9^T possessed a novel variation of peptidoglycan derived from the type A1γ *meso*-Dpm-direct. The polar lipids consisted of diphosphatidylglycerol, *N*-acetylglucosamine-containing phospholipid, phosphatidylinositol mannoside, phosphatidylglycerol, phosphoglycolipids and glycolipids. The predominant menaquinones were MK-10(H_6) and MK-10(H_8). The major fatty acids were $C_{16:0}$, iso-$C_{16:0}$ and 10-methyl $C_{17:0}$. The level of DNA–DNA relatedness between strain S6R2A4-9^T and *T. mesophilus* I12A-02601^T was 27.6±3.0 %, which was low enough to indicate that the strain represents a distinct species of the genus *Tenggerimyces*. On the basis of the polyphasic taxonomic evidence, a novel species, *Tenggerimyces flavus* sp. nov., is proposed. The type strain of the novel species is S6R2A4-9^T (=DSM 28944^T=CGMCC 4.7241^T).

The genus *Tenggerimyces* was first proposed by Sun *et al.* (2015) and belongs to the family *Nocardioidaceae*, which includes eight other genera: *Nocardioides* (Prauser, 1976), *Aeromicrobium* (Miller *et al.*, 1991), *Kribbella* (Park *et al.*, 1999; Sohn *et al.*, 2003), *Marmoricola* (Urzì *et al.*, 2000), *Actinopolymorpha* (Wang *et al.*, 2001), *Thermasporomyces* (Yabe *et al.*, 2011), *Flindersiella* (Kaewkla & Franco, 2011) and *Mumia* (Lee *et al.*, 2014). At the time of writing, the genus *Tenggerimyces* comprised only one species with a validly published name, *Tenggerimyces mesophilus* (Sun *et al.*, 2015), which was isolated from a desert soil crusts sample collected from the Shapotou region of Tengger Desert, north-west China.

During our previous investigation of the cultivable actinobacterial diversity in karst caves, a *Tenggerimyces*-like strain, designated S6R2A4-9^T, was isolated from a soil sample collected from the surface of limestone of Shenxian Cave in Henan Province, China. In this paper, the taxonomic characterization of this new isolate is described and a novel species of the genus *Tenggerimyces* is proposed.

Strain S6R2A4-9^T was isolated by the dilution plating method using R2A agar (BD) plates supplemented with cycloheximide (45 mg l^{-1}), nalidixic acid (25 mg l^{-1}) and potassium dichromate (45 mg l^{-1}). A colony of strain S6R2A4-9^T appeared on the agar after incubation for 6 weeks at 28 °C and was transferred onto International

Abbreviations: DAP, diaminopimelic acid; DPG, diphosphatidylglycerol; GL, unidentified glycolipid; GluNu, *N*-acetylglucosamine-containing phospholipid; PG, phosphatidylglycerol; PIM, phosphatidylinositol mannoside.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S6R2A4-9^T is KP405230.

Three supplementary tables and three supplementary figures are available with the online Supplementary Material.

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Streptomyces Project 2 (ISP 2; Shirling & Gottlieb, 1966) agar plates using the serial streaking technique until pure isolates were obtained. The purified isolates were maintained on ISP 2 agar slants at 4 °C and stored as aqueous glycerol suspensions (20 %, v/v) at −80 °C.

Gram staining was performed as described by Magee *et al.* (1975). Cultural characteristics of strain S6R2A4-9^T were observed following growth on yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7) (Shirling & Gottlieb, 1966), R2A (BD), nutrient agar (BD), tomato paste–oatmeal agar (Waksman, 1961), PYG agar (Sun *et al.*, 2015) and Bennett's agar (Atlas, 1993) plates at 28 °C for 7–28 days. The colour of colonies and diffusible pigments was determined with chips from the ISCC-NBS colour charts (Kelly, 1964). Cell morphology was observed on ISP 2 by light microscopy (BH2; Olympus) and scanning electron microscopy (Quanta 200; FEI) after incubation at 28 °C for 7, 14, 21 and 28 days. Growth at different temperatures (4, 10, 15, 20, 25, 28, 32, 35, 37 and 42 °C) and NaCl concentrations (0, 1, 2, 3, 4, 5, 7 and 8 %, w/v) were determined on ISP 2 after 3 weeks. The pH range for growth was tested in ISP 2 broth between pH 4.0 and 11.0 at intervals of 0.5 pH unit using the buffer system described by Xu *et al.* (2005). Carbon source utilization and acid production from carbohydrates were determined by using Biolog GEN III MicroPlates and the API 50CH system (bioMeriéux), respectively. Enzyme activities were examined using API ZYM strips (bioMeriéux) according to the manufacturer's instructions. Oxidase activity was determined by the oxidation of tetramethyl-*p*-phenylenediamine. Catalase activity was determined using 3 % H_2O_2, and bubble production was classified as a positive reaction. Hydrolysis of cellulose, urea, starch, Tween 20, Tween 40 and Tween 80, gelatin liquefaction, H_2S production and nitrate reduction were determined as described by Williams *et al.* (1983).

The colonies of S6R2A4-9^T were wrinkled. No diffusible pigment was produced on any media tested. Well-developed substrate mycelium occurred on ISP 2, PYG agar, ISP 3, ISP 6, Bennett's agar and tomato paste–oatmeal agar, and aerial mycelium was observed on ISP 2, ISP 4, ISP 7 and R2A agar (Table S1, available in the online Supplementary Material). The substrate mycelium exhibited irregular branches and lateral buds were observed on the hyphae. Aerial mycelium fragmented into long, rod-shaped elements (Fig. 1). Optimum growth was observed at 25–28 °C and pH 6.5–8.0. Details of the physiological characteristics of strain S6R2A4-9^T are given in Table 1 and species description.

The whole-cell sugars were prepared and determined by TLC on cellulose sheets (Merck) as described by Hasegawa *et al.* (1983). Polar lipids were extracted, examined by two-dimensional TLC on silica gel 60 F_{254} plates (Merck) and identified using the procedures of Minnikin *et al.* (1984). The solvent systems chloroform/methanol/water (64 : 27 : 5, by vol.) and chloroform/methanol/acetic acid/water (80 : 18 : 12 : 5, by vol.) were used in the first and second dimensions, respectively. Menaquinones were extracted using the method of Collins *et al.* (1977), then analysed and confirmed by HPLC with a single quadrupole mass spectrometer as described by Guo *et al.* (2015). For the analysis of cellular fatty acids, cells of strain S6R2A4-9^T and reference strain *T. mesophilus* I12A-02601^T were harvested after cultivation on tryptic soy agar (BD) at 28 °C for 5 days, when the bacterial communities reached the late-exponential stage of growth. Cellular fatty acids were extracted according to the standard protocol of Sasser (1990), and the fatty acid methyl esters were analysed on an Agilent 7890A gas chromatograph coupled with an Agilent 5975C single quadrupole mass spectrometer equipped with the Nist08 Library software database as described by Liu *et al.* (2015). The analysis of the peptidoglycan structure and polar lipids for strain S6R2A4-9^T was carried out by the Identification Service, DSMZ, Braunschweig, Germany. The cell-wall peptidoglycan was prepared and its structure was analysed following the protocols described by Schumann (2011).

The whole-cell hydrolysates of strain S6R2A4-9^T contained glucose and traces of ribose and xylose. Polar lipids of strain S6R2A4-9^T comprised diphosphatidylglycerol (DPG), *N*-acetylglucosamine-containing phospholipid (GluNu), phosphatidylinositol mannoside (PIM), phosphatidylglycerol (PG), two unidentified phosphoglycolipids, two unidentified glycolipids (GLs) and two unidentified lipids, which were quite similar to those of strain *T. mesophilus* I12A-02601^T as shown in Fig. S1. The menaquinone profile of strain S6R2A4-9^T was composed of MK-10(H_6) (37.1 %), MK-10(H_8) (21.0 %), MK-10(H_4) (14.4 %), MK-11(H_6) (14.1 %), MK-11(H_4) (7.0 %), MK-11 (H_8) (4.6 %) and MK-10 (H_2) (1.8 %). Major cellular fatty acids of strain S6R2A4-9^T were $C_{16:0}$ (36.17 %), iso-$C_{16:0}$ (14.72 %) and 10-methyl $C_{17:0}$ (11.45 %). Detailed menaquinone and fatty acid profiles for strain S6R2A4-9^T and *T. mesophilus* I12A-02601^T are given in Tables S2 and S3. The major polar lipids, menaquinones and fatty acids of the reference strain *T. mesophilus* I12A-02601^T detected in this study were similar to those previously reported (Sun *et al.*, 2015), but some differences in the types and proportions from those previously reported (Sun *et al.*, 2015) may be due to the different experimental conditions used. The total hydrolysates of the peptidoglycan contained *meso*-diaminopimelic acid (*meso*-DAP), LL-DAP and 2,6-diamino-3-hydroxypimelic acid as well as glycine (Gly), alanine (Ala) and glutamic acid (Glu). The approximate molar ratio was 1.0 Glu : 0.9 Gly : 0.2 Ala : 0.1 DAP (sum of *meso*- and LL-DAP). Gly–D-Glu and DAP–D-Ala were detected in the partial hydrolysates. These data suggested that the peptidoglycan type of strain S6R2A4-9^T was a novel variation derived from the type A1γ *meso*-Dpm-direct

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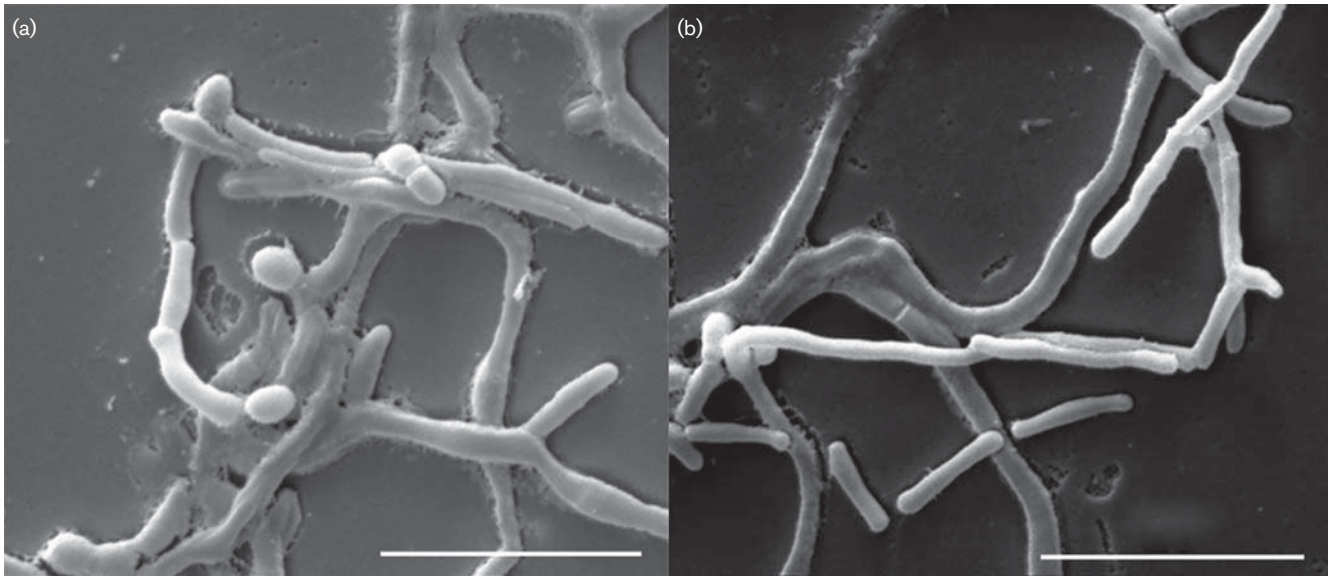

Fig. 1. Scanning electron micrographs of the aerial mycelium of strain S6R2A4-9^T grown on ISP 2 for 21 days at 28 °C. Bars, 5 μm.

(www.peptidoglycan-types.info), but contained Gly instead of L-Ala at position 1 of the peptide subunit (A1γ') and *meso*-DAP was partially replaced by LL-DAP and 2,6-diamino-3-hydroxypimelic acid.

The genomic DNA of strain S6R2A4-9^T was extracted following the procedure described by Li *et al.* (2007). The 16S rRNA gene was amplified by PCR using forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Miyoshi *et al.*, 2005). The PCR product was cloned into a pEASY-T1 cloning vector (TransGen Biotech) according to the manufacturer's instructions and sequenced using an ABI PRISM 3730XL DNA Analyser. The EzTaxon-e server (Kim *et al.*, 2012) and nucleotide–nucleotide BLAST search program (Altschul *et al.*, 1997) were employed to identify phylogenetic neighbours and calculate 16S rRNA gene sequence similarities. Multiple alignments with the corresponding sequences obtained from the GenBank/EMBL/DDBJ databases were performed using BioEdit (version 7.0.9.0) (Hall, 1999). Phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms using the software package MEGA version 5.0 (Tamura *et al.*, 2011). Evolutionary distance matrices for neighbour-joining and maximum-likelihood algorithms were generated according to Kimura's two-parameter model (Kimura, 1980, 1983), and close-neighbour interchange (search level=2, random addition=100) was applied in maximum-parsimony analysis. The topologies of the evolutionary trees were assessed by bootstrap analysis (Felsenstein, 1985) based on 1000 replications.

For the determination of DNA G+C content and level of DNA–DNA relatedness, the genomic DNA of strain S6R2A4-9^T was prepared according to the method described by Marmur (1961). The DNA G+C content of strain S6R2A4-9^T was determined using the thermal denaturation (T_m) method (Marmur & Doty, 1962) with *T. mesophilus* I12A-02601^T as a reference. DNA–DNA hybridization was performed with *T. mesophilus* I12A-02601^T by the thermal denaturation and renaturation method of De Ley *et al.* (1970) using a PharmaSpec UV/VIS spectrophotometer (UV-2550; Shimadzu) equipped with a Peltier-thermostated multicell changer and a temperature controller (S-1700; Shimadzu) with *in-situ* temperature probe. The DNA–DNA hybridization was conducted three times in each case with three replicates.

A nearly full-length 16S rRNA gene sequence (1476 bp) was determined for strain S6R2A4-9^T. Phylogenetic trees reconstructed with all three tree-making methods clearly showed that strain S6R2A4-9^T resided in the clade of the genus *Tenggerimyces*, which formed a large cluster together with the species of the genera *Flindersiella*, *Actinopolymorpha*, *Thermasporomyces* and *Kribbella* within the family *Nocardioidaceae* (Figs 2, S2 and S3). Comparative analyses of the 16S rRNA gene sequences revealed that strain S6R2A4-9^T had the highest 16S rRNA gene sequence similarity (98.98 %) with *T. mesophilus* I12A-02601^T and lower similarities (<94 %) with all the type strains of species of the genera *Flindersiella*, *Actinopolymorpha*, *Thermasporomyces* and *Kribbella* with validly published names. The G+C content of the genomic DNA of strain S6R2A4-9^T was 67.0 mol%. The level of DNA–DNA

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Table 1. Differential characteristics of strain S6R2A4-9^T and *T. mesophilus* I12A-02601^T

Characteristic	S6R2A4-9^T	*T. mesophilus* I12A-02601^T
Isolation source	Cave soil	Desert soil
Growth at:		
37 °C	−	+
4 % (w/v) NaCl	−	+
Catalase	(+)	+
Nitrate reduction	(+)	+
Hydrolysis of:		
Starch	−	+
Cellulose	+	−
Carbon source utilization		
D-Mannitol	+	−
Inosine	+	−
D-Fructose	−	+
Pectin	−	+
D-Sorbitol	−	+
Salicin	+	−
Acid produced from:		
Xylitol	+	−
Potassium 5-ketogluconate	+	−
L-Rhamnose	−	+
D-Ribose	−	+
Sucrose	−	+
L-Arabinose	−	+
Enzyme activity		
Leucine arylamidase	+	−
N-Acetyl-β-glucosaminidase	+	−
Valine arylamidase	(+)	−
DNA G+C content (mol%)	67.0	71.5
Predominant menaquinones	MK-10(H_6), MK-10(H_8)	MK-10(H_6), MK-10(H_8)
Major cellular fatty acids (>10 %)	$C_{16:0}$, iso-$C_{16:0}$, 10-methyl $C_{17:0}$	$C_{16:0}$, iso-$C_{16:0}$

Data for both strains were obtained in this study. +, Positive reaction; (+), weakly positive reaction; −, negative reaction. Both strains were negative for oxidase activity, urease and H_2S production. Both strains were positive for catalase, liquefaction of gelatin, nitrate reduction and hydrolysis of Tween 20, Tween 40 and Tween 80. In the Biolog GEN III system, both strains were positive for assimilation of D-arabitol, cellobiose, dextrin, L-fucose, D-fucose, D-galactose, gelatin, gentiobiose, α-D-glucose, α-lactose, maltose, D-mannose, melibiose, sucrose, trehalose, turanose, L-rhamnose and Tween 40, and negative for assimilation of raffinose and *myo*-inositol. In the API ZYM strips, both strains were positive for acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C 4), esterase lipase (C 8), α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase and trypsin, and negative for cystine arylamidase, β-glucuronidase and lipase (C 14). In the API 50CH strips, both strains were positive for acid production from D-arabinose, aesculin ferric citrate, D-fucose, L-fucose, D-glucose, melibiose, trehalose and D-xylose.

relatedness between strain S6R2A4-9^T and *T. mesophilus* I12A-02601^T was determined to be 27.6±3.0 % (mean±SD).

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain S6R2A4-9^T should represent a novel species affiliated with the genus *Tenggerimyces*. This conclusion was also supported by the common chemotaxonomic characteristics, including DPG, GluNu, PIM, PGL and GL as the main polar lipids, MK-10(H_6) and MK-10(H_8) as predominant menaquinones, $C_{16:0}$ and iso-$C_{16:0}$ as major fatty acids, shared by strain S6R2A4-9^T and *T. mesophilus* I12A-02601^T (Tables 1, S2 and S3, Fig. S1). Meanwhile, strain S6R2A4-9^T could be distinguished from *T. mesophilus* I12A-02601^T by some other chemotaxonomic characteristics. The peptidoglycan type of strain S6R2A4-9^T represented a novel variation of peptidoglycan, containing *meso*-DAP, LL-DAP and 2,6-diamino-3-hydroxypimelic acid in the total hydrolysates of the peptidoglycan, while *T. mesophilus* I12A-02601^T contained LL-DAP and DD-DAP as the diagnostic diamino acids in whole-cell hydrolysates. For polar lipids, an unidentified phospholipid and one more PIM, detected in *T. mesophilus* I12A-02601^T, were not found in strain S6R2A4-9^T. Physiological characteristics that differentiate strain S6R2A4-9^T from *T. mesophilus* I12A-02601^T are summarized in Table 1. Furthermore, the validity of a novel species status for strain S6R2A4-9^T was also fully supported by the DNA–DNA hybridization result; the level of DNA–DNA

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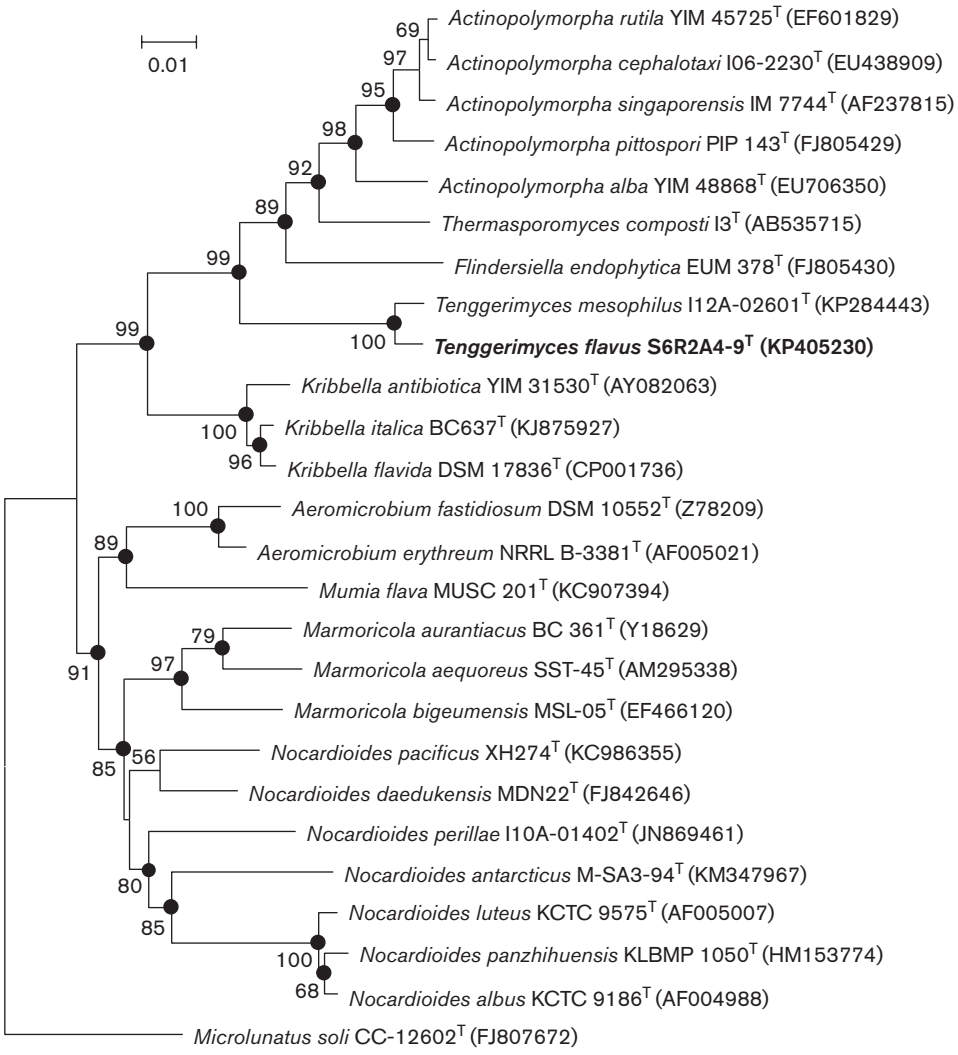

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences (1419 bp) showing the taxonomic position of strain S6R2A4-9^T relative to representatives of the family *Nocardioidaceae*. The sequence of *Microlunatus soli* CC-12602^T was used as the outgroup. Filled circles at nodes indicate corresponding branches that were also recovered by using the maximum-likelihood and maximum-parsimony algorithms. Numbers at branch nodes refer to bootstrap values of 1000 replications; only values >50 % are shown. Bar, 0.01 substitutions per nucleotide positions.

relatedness between strain S6R2A4-9^T and the phylogenetically related strain *T. mesophilus* I12A-02601^T was 27.6±3.0 %, clearly below the 70 % cut-off value considered to be the threshold for the definition of genomic species (Wayne *et al.*, 1987).

In conclusion, phylogenetic analysis, phenotypic characteristics and chemotaxonomic data, especially the low level of DNA–DNA relatedness between strain S6R2A4-9^T and *T. mesophilus* I12A-02601^T, clearly support that the new isolate, strain S6R2A4-9^T, represents a novel species of the genus *Tenggerimyces*, for which the name *Tenggerimyces flavus* sp. nov. is proposed.

Emended description of the genus *Tenggerimyces* Sun *et al.* 2015

The genus description is as given by Sun *et al.* (2015) with the following changes. The diagnostic diamino acids in the peptidoglycan are LL-DAP and DD-DAP or LL-DAP, *meso*-DAP and 2,6-diamino-3-hydroxypimelic acid. The major fatty acids are $C_{16:0}$ and iso-$C_{16:0}$. The DNA G+C content ranges from 67.0 to 72.2 mol%.

Description of *Tenggerimyces flavus* sp. nov.

Tenggerimyces flavus (fla′vus. L. masc. adj. *flavus* yellow, referring to the colour of the substrate mycelia).

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Gram-staining-positive actinomycete that forms well-developed substrate mycelium and sparse aerial mycelium. The substrate mycelium exhibits irregular branches and lateral buds occur on the hyphae. Aerial mycelium fragments into long, rod-shaped elements. The colour of substrate mycelium is vivid yellow. Good growth occurs on ISP 2, PYG agar, ISP 3, ISP 6, tomato paste–oatmeal agar and Bennett's agar, and poor growth occurs on nutrient agar, ISP 7, R2A agar, ISP 4 and ISP 5. Growth occurs at 10–35 °C (optimum 25–28 °C), pH 5.5–9.0 (optimum pH 6.5–8.0) and in the presence of 0–2% (w/v) NaCl. Optimal growth is observed in the absence of NaCl. Oxidase-negative and catalase-positive (weakly). Negative for H_2S production and urease. Positive for liquefaction of gelatin and nitrate reduction (weakly). Tween 20, Tween 40, Tween 80 and cellulose are hydrolysed, but starch is not. D-Arabitol, cellobiose, dextrin, L-fucose, D-fucose, gelatin, gentiobiose, α-D-glucose, D-galactose, inosine, α-lactose, maltose, D-mannitol, D-mannose, melibiose, L-rhamnose, salicin, sucrose, trehalose, turanose and Tween 40 can be utilized as sole carbon sources, but D-fructose, *myo*-inositol, pectin, raffinose and D-sorbitol cannot be utilized. Acids are produced from D-arabinose, aesculin ferric citrate, D-fucose, L-fucose, D-glucose, melibiose, potassium 5-ketogluconate, trehalose, xylitol and D-xylose. Positive activities for acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C 4), esterase lipase (C 8), α-galactosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, α-glucosidase, β-glucosidase, leucine arylamidase, α-mannosidase, trypsin and valine arylamidase (weakly). The polar lipid profile comprises DPG, GluNu, PIM, PG, two phosphoglycolipids, two GLs and two unidentified lipids. The predominant menaquinones are MK-10(H_6) and MK-10(H_8). The major fatty acids are $C_{16:0}$, iso-$C_{16:0}$ and 10-methyl $C_{17:0}$. The whole-cell hydrolysates contain glucose, ribose and xylose as the diagnostic sugars. The peptidoglycan type is a novel variation of peptidoglycan derived from the type A1γ *meso*-Dpm-direct; Gly, Ala, Glu, *meso*-DAP, LL-DAP and 2,6-diamino-3-hydroxypimelic acid are present in the total hydrolysates of the peptidoglycan.

The type strain is S6R2A4-9^T (= DSM 28944^T=CGMCC 4.7241^T), which was isolated from a soil sample collected from the surface of limestone of Shenxian Cave in Henan Province, China. The genomic DNA G+C content of the type strain is 67.0 mol%.

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