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 S6R2A4-9T, 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 S6R2A4-9T 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-02601T. 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 S6R2A4-9T 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(H6) and MK-10(H8). The major fatty acids were C16 : 0, iso-C16 : 0 and 10-methyl C17 : 0. The level of DNA–DNA relatedness between strain S6R2A4-9T and T. mesophilus I12A-02601T 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 S6R2A4-9T (=DSM 28944T = CGMCC 4.7241T).

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 (Urzi 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 S6R2A4-9T, 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 S6R2A4-9T 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 S6R2A4-9T appeared on the agar after incubation for 6 weeks at 28 °C and was transferred onto International...
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 S6R2A4-9T 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 (bioMérieux), respectively. Enzyme activities were examined using API ZYM strips (bioMérieux) according to the manufacturer’s instructions. Oxidase activity was determined by the oxidation of tetramethyl-p-phenylenediamine. Catalase activity was determined using 3% H2O2, and bubble production was classified as a positive reaction. Hydrolysis of cellulose, urea, starch, Tween 20, Tween 40 and Tween 80, gelatin liquefaction, H2S production and nitrate reduction were determined as described by Williams et al. (1983).

The colonies of S6R2A4-9T 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 S6R2A4-9T 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 F254 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 S6R2A4-9T and reference strain T. mesophilus I12A-02601T 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 S6R2A4-9T 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 S6R2A4-9T contained glucose and traces of ribose and xylose. Polar lipids of strain S6R2A4-9T 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-02601T as shown in Fig. S1. The menaquinone profile of strain S6R2A4-9T was composed of MK-10(H8) (37.1%), MK-10(H6) (21.0%), MK-10(H4) (14.4%), MK-11(H6) (14.1%), MK-11(H4) (7.0%), MK-11 (H6) (4.6%) and MK-10 (H4) (1.8%). Major cellular fatty acids of strain S6R2A4-9T were C16:0 (36.17%), iso-C16:0 (14.72%) and 10-methyl C17:0 (11.45%). Detailed menaquinone and fatty acid profiles for strain S6R2A4-9T and T. mesophilus I12A-02601T are given in Tables S2 and S3. The major polar lipids, menaquinones and fatty acids of the reference strain T. mesophilus I12A-02601T 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-hydroxyptimelic 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 S6R2A4-9T was a novel variation derived from the type A1γ meso-Dpm-direct
but contained Gly instead of L-Ala at position 1 of the peptide subunit (A1c) and meso-DAP was partially replaced by LL-DAP and 2,6-diamino-3-hydroxypimelic acid.

The genomic DNA of strain S6R2A4-9T 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 S6R2A4-9T was prepared according to the method described by Marmur (1961). The DNA G+C content of strain S6R2A4-9T was determined using the thermal denaturation ($T_m$) method (Marmur & Doty, 1962) with T. mesophilus I12A-02601T as a reference. DNA–DNA hybridization was performed with T. mesophilus I12A-02601T 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 S6R2A4-9T. Phylogenetic trees reconstructed with all three tree-making methods clearly showed that strain S6R2A4-9T 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 S6R2A4-9T had the highest 16S rRNA gene sequence similarity (98.98%) with T. mesophilus I12A-02601T 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 S6R2A4-9T was 67.0 mol%. The level of DNA–DNA

Fig. 1. Scanning electron micrographs of the aerial mycelium of strain S6R2A4-9T grown on ISP 2 for 21 days at 28°C. Bars, 5 μm.
relatedness between strain S6R2A4-9<sup>T</sup> and <i>T. mesophilus</i> I12A-02601<sup>T</sup> was determined to be 27.6 ± 3.0 % (mean ± SD).

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain S6R2A4-9<sup>T</sup> should represent a novel species affiliated with the genus <i>Tenggerimyces</i>. 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<sub>6</sub>) and MK-10(H<sub>8</sub>) as predominant menaquinones, C<sub>16:0</sub> and iso-C<sub>16:0</sub> as major fatty acids, shared by strain S6R2A4-9<sup>T</sup> and <i>T. mesophilus</i> I12A-02601<sup>T</sup> (Tables 1, S2 and S3, Fig. S1). Meanwhile, strain S6R2A4-9<sup>T</sup> could be distinguished from <i>T. mesophilus</i> I12A-02601<sup>T</sup> by some other chemotaxonomic characteristics. The peptidoglycan type of strain S6R2A4-9<sup>T</sup> represented a novel variation of peptidoglycan, containing meso-DAP, lL-DAP and 2,6-diamino-3-hydroxyxymelic acid in the total hydrolysates of the peptidoglycan, while <i>T. mesophilus</i> I12A-02601<sup>T</sup> 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 <i>T. mesophilus</i> I12A-02601<sup>T</sup>, were not found in strain S6R2A4-9<sup>T</sup>. Physiological characteristics that differentiate strain S6R2A4-9<sup>T</sup> from <i>T. mesophilus</i> I12A-02601<sup>T</sup> are summarized in Table 1. Furthermore, the validity of a novel species status for strain S6R2A4-9<sup>T</sup> was also fully supported by the DNA–DNA hybridization result; the level of DNA–DNA

### Table 1. Differential characteristics of strain S6R2A4-9<sup>T</sup> and <i>T. mesophilus</i> I12A-02601<sup>T</sup>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>S6R2A4-9&lt;sup&gt;T&lt;/sup&gt;</th>
<th>&lt;i&gt;T. mesophilus&lt;/i&gt; I12A-02601&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Cave soil</td>
<td>Desert soil</td>
</tr>
<tr>
<td>Growth at:</td>
<td>37 °C</td>
<td></td>
</tr>
<tr>
<td>4 % (w/v) NaCl</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>( + )</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>( + )</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cellulose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Carbon source utilization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Inosine</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Pectin</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid produced from:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylitol</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Potassium 5-ketogluconate</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Ribose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine arylamidase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>( + )</td>
<td>–</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>67.0</td>
<td>71.5</td>
</tr>
<tr>
<td>Predominant menaquinones</td>
<td>MK-10(H&lt;sub&gt;6&lt;/sub&gt;), MK-10(H&lt;sub&gt;8&lt;/sub&gt;)</td>
<td>MK-10(H&lt;sub&gt;6&lt;/sub&gt;), MK-10(H&lt;sub&gt;8&lt;/sub&gt;)</td>
</tr>
<tr>
<td>Major cellular fatty acids (&gt;10 %)</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;, iso-C&lt;sub&gt;16:0&lt;/sub&gt;, 10-methyl C&lt;sub&gt;17:0&lt;/sub&gt;</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;, iso-C&lt;sub&gt;16:0&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

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<sub>2</sub>S production. Both strains were positive for catalase, liquefaction of gelatin, nitrate reduction and hydroylsis 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-L-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 cysteine 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\textsuperscript{T} and the phylogenetically related strain \textit{T. mesophilus} I12A-02601\textsuperscript{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 \textit{et al.}, 1987).

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

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**Emended description of the genus \textit{Tenggerimyces Sun et al. 2015}**

The genus description is as given by Sun \textit{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\textsubscript{16:0} and iso-C\textsubscript{16:0}. The DNA G + C content ranges from 67.0 to 72.2 mol\%.

**Description of \textit{Tenggerimyces flavus} sp. nov.**

\textit{Tenggerimyces flavus} (fla\'vus. L. masc. adj. flavus yellow, referring to the colour of the substrate mycelia).
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 H₂S 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-Arabinose, cellobiose, dextrin, L-fucose, D-fucose, gelatin, gentiobiose, 2,6-diamino-3-hydroxypimelic acid, D-glucosamine, meso-glucosamine, D-glucosidase, L-glucosidase, -galactosidase, -glucosaminidase, trehalose, xylitol and D-xylose. The peptidoglycan type A1 is a novel variation of peptidoglycan derived from the surface of limestone of Shenxian Cave in Henan and Technology of China (grant no. 2012ZX09301-002-001-018). The genomic DNA G+C content of the type strain is 67.0 mol%.

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References


Tenggerimyces flavus sp. nov.


