Stackebrandtia endophytica sp. nov., an actinobacterium isolated from Tripterygium wilfordii

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A novel endophytic actinobacterium, designated strain YIM 64602T, was isolated from healthy stems of Tripterygium wilfordii. It grew at 15–40 °C, pH 6.0–9.0 and in the presence of 0–3% (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequence showed that strain YIM 64602T belongs to the genus Stackebrandtia. Whole-cell hydrolysates of strain YIM 64602T contained the amino acid meso-diaminopimelic acid with the sugars mannose, rhamnose and glucose, and a trace of ribose. The major polar lipids were diphosphatidylglycerol, phosphatidyldimethylethanolamine and phosphatidylethanolamine. MK-10(H6), MK-10(H4) and MK-11(H4) were the predominant components in the quinone system. The fatty-acid pattern was mainly composed of the saturated branched-chain acids iso-C16:0, anteiso-C17:0, iso-C15:0 and iso-C17:0. The DNA G+C content was 72.4 mol%. 16S rRNA gene sequence analysis showed the highest pairwise sequence identity (96.0–98.5%) with the members of the genus Stackebrandtia. Strain YIM 64602T displayed a DNA–DNA relatedness of 43.9 ± 0.4% with the type strain Stackebrandtia albiflava YIM 45751T. Based on evidence from this polyphasic study, strain YIM 64602T (=BCRC 16954T=DSM 45928T) is considered to represent a novel species of the genus Stackebrandtia, for which the name Stackebrandtia endophytica is proposed.

The family Glycomycetaceae contains three recognized genera: Glycomyces (Labeda et al., 1985; Labeda & Kroppenstedt, 2004), Stackebrandtia (Labeda & Kroppenstedt, 2005) and Haloglycomyces (Guan et al., 2009). At the time of writing, the family comprises 14 members, and the genus Stackebrandtia contains two members, Stackebrandtia nassauensis (Labeda & Kroppenstedt, 2005) and Stackebrandtia albiflava (Wang et al., 2009), which were isolated from different soil samples. The two species in the genus Stackebrandtia are Gram-positive, strictly aerobic, filamentous actinomycetes. The cell-wall peptidoglycan contains meso-diaminopimelic acid (DAP). The major polar lipids consist of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidymethylethanolamine (PME) and phosphatidylglycerol (PG). The predominant menaquinones are MK-10(H6), MK-10(H4) and MK-11(H4). The major fatty acids are saturated, iso- and anteiso-branched fatty acids. The G+C contents of the genomic DNA are 69–73 mol%. In the present study, we report another novel species of this genus, represented by strain YIM 64602T, which was isolated from healthy stems of Tripterygium wilfordii, a traditional Chinese medicinal plant.

The stems of Tripterygium wilfordii were collected in Yunnan Province, south-west China. The samples were firstly washed in running water to remove soil particles and sterilized by using 5% sodium hypochlorite and 70% ethanol according to an established procedure (Li et al., 2008), then sliced into pieces, followed by plating on cellulose-asparagine agar (2.5 g cellulose, 2.0 g sodium pyruvate, 1.0 g asparagine, 0.5 g CaCl2, 0.25 g KNO3, 0.2 g MgSO4·7H2O, 0.2 g K2HPO4, 10 mg FeSO4·7H2O and 15 g agar; pH 7.2) containing nalidixic acid (25 mg l−1), nystatin (75 mg l−1) and potassium dichromate (50 mg l−1) to inhibit the growth of...
bacteria and fungi. The plates were incubated at 28 °C for 4–6 weeks until the outgrowth of endophytic actinomycetes was discerned. Strain YIM 64602T was purified and maintained on ISP (International Streptomyces Project) 2 (Shirling & Gottlieb, 1966) agar slants at 4 °C and as 20 % (v/v) glycerol suspensions at −80 °C.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Li et al. (2007) and Cui et al. (2001). The values for sequence similarity among the closest strains were determined using the EzTaxon-e server database (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). Multiple alignments with sequences of the most closely related actinobacteria were carried out using the CLUSTAL X 1.8 program (Thompson et al., 1997). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likeness (Felsenstein, 1981) tree-making algorithms by using the software package MEGA version 5.05 (Tamura et al., 2011). The stability of relationships was assessed by performing bootstrap analyses with 1000 resamplings (Felsenstein, 1985). DNA–DNA hybridization was determined according to the fluorometric micro-well method (Ezaki et al., 1989; He et al., 2005).

The almost-complete (1521 bp) 16S rRNA gene sequence of strain YIM 64602T was determined. The 16S rRNA gene sequence showed the highest similarity with the members of the genus Stackebrandtia, family Glycomycetaceae, especially with the type strains S. albiflava YIM 45751T (98.5 %) and S. nassauensis DSM 44728T (96.0 %). Genomic relatedness of strain YIM 64602T to S. albiflava YIM 45751T was 43.9 ± 0.4 %. A comparison of the 16S rRNA gene sequence with those of the type species of related genera showed that the novel organism fell within the evolutionary radiation occupied by the genus Stackebrandtia (Fig. 1). In the tree based on the neighbour-joining algorithm, strain YIM 64602T formed a coherent cluster with S. albiflava YIM 45751T and S. nassauensis DSM 44728T; the branching order was supported further by the bootstrap values of 100 and 98 %. A similar tree topology was also obtained in the phylogenetic trees generated using the maximum-parsimony and maximum-likelihood algorithms (Figs S1 and S2, available in the online Supplementary Material). These data supported the finding that strain YIM 64602T represents a different genomic species.

Biomass for chemical studies of strain YIM 64602T was grown on ISP 2 agar plates for 7 days at 28 °C. The isomer of DAP and whole-cell sugars were analysed according to the procedures developed by Hasegawa et al. (1983) and Tang et al. (2009). Menaquinones were isolated according to the method of Collins et al. (1977) and separated by HPLC (Tamaoka et al., 1983). Polar lipids were extracted and analysed by two-dimensional TLC according to the protocol of Embley & Wait (1994). Biomass for fatty acid analysis was obtained by cultivation on tryptic soya agar (TSA; Tryptone 15 g, Soytone 5 g, NaCl 5 g, Agar 15 g, pH 7.2) at 28 °C for 3 days. Cellular fatty acid analysis was performed by using the Microbial Identification System (Sherlock version 6.1; database TSBA6; MIDI). The DNA G+C content of strain YIM 64602T was determined by using the HPLC method (Mesbah et al., 1989).

Strain YIM 64602T shared consistent chemotaxonomic characteristics with S. albiflava YIM 45751T. Strain YIM 64602T contained meso-DAP as the diagnostic diamino acid in the peptidoglycan and sugars in whole-cell hydrolysates contained mannose, rhamnose, glucose and a trace of ribose. Strain YIM 64602T could be distinguished from the type strain S. albiflava YIM 45751T by the absence of galactose and xylose (Wang et al., 2009). In this study, S. albiflava YIM 45751T, S. nassauensis DSM 44728T and Glycomyces harbinensis DSM 46494T were reanalysed as described by Tang et al. (2009). All of them were found to contain mannose, galactose, rhamnose, glucose and ribose (Fig. S3). The predominant menaquinones of YIM 64602T were MK-10(H4), MK-10(H4) and MK-11(H4). The polar lipids consisted of DPG, PE and PME, with some PG and

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**Fig. 1.** Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships of strain YIM 64602T, S. albiflava and the type species of related genera. Bootstrap values (>50 %) based on 1000 replicates are shown at the branch nodes. Asterisks indicate that the corresponding branches were also recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. *Dietzia maris* ATCC 35013T (X79290) was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.
phosphatidylinositol; phosphatidylinositol mannosides, unknown phospholipids and unidentified polar lipids as minor components (Fig. S4). The major cellular fatty acids (>10 %) of strain YIM 64602\textsuperscript{T} showed the presence of iso-C\textsubscript{16:0} (20.29 %), anteiso-C\textsubscript{17:0} (18.48 %), iso-C\textsubscript{15:0} (11.37 %) and iso-C\textsubscript{17:0} (10.87 %). Detailed cellular fatty acid composition of strains YIM 64602\textsuperscript{T} and \textit{S. albiflava} YIM 45751\textsuperscript{T} are presented in Table S1. The DNA G + C content of strain YIM 64602\textsuperscript{T} was 72.4 mol%. The chemotaxonomic data for the new isolate matched those given for the genus \textit{Stackebrandtia}, and also differentiated the strain from members of this genus by the absence of galactose in the whole-cell hydrolysate and absence of MK-10(H\textsubscript{6}) as a predominant menaquinone (Table 1).

Aerial spore-mass colour, substrate mycelium pigmentation and coloration of the diffusible pigments of strain YIM 64602\textsuperscript{T} were recorded on ISP 2, 3, 4 and 5 media (Shirling & Gottlieb, 1966) and Czapek’s agar (sucrose 30.0 g, NaNO\textsubscript{3} 2.0 g, K\textsubscript{2}HPO\textsubscript{4} 1.0 g, MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O 0.5 g, KCl 0.5 g, FeSO\textsubscript{4} 0.01 g; pH 7.2). Colours were determined by using colour chips from the ISCC-NBS colour charts (standard samples, no. 2106) (Kelly, 1964). Morphological properties were examined using light microscopy (BH 2; Olympus) and scanning electron microscopy (Quanta 200; FEI) after 14–21 days of incubation on ISP 2 medium at 28 °C. Growth was tested at 4, 10, 15, 20, 28, 30, 35, 40, 45 and 50 °C on ISP 2 medium by incubating the cultures for 14 days. The ability of the strain to grow at different pH (pH 4, 5, 6, 7, 8, 9, 10 and 11, using the buffer system described by Xu \textit{et al.}, 2005) and NaCl concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 %, w/v) was examined at 28 °C after 14 days. Anaerobic cultivation was performed on ISP 2 using the Oxoid AnaeroGen system (Miller \textit{et al.}, 1995). Carbon source utilization, catalase, oxidase and gelatinase activities, hydrolysis of starch, Tween 20, Tween 40, Tween 60 and Tween 80, nitrate reduction, urease and H\textsubscript{2}S production were determined using standard methods (Gerhardt \textit{et al.}, 1994; Lányi, 1987; MacFaddin, 2000).

Strain YIM 64602\textsuperscript{T} was a Gram-reaction-positive actinobacterium and it grew well on ISP 2 and ISP 4 media, formed yellow–white to white substrate mycelium and yellow to white aerial mycelium. Yellow diffusible pigments were only produced on ISP 2 medium. The substrate mycelium showed extensive branching without fragmentation (Fig. S5). Strain YIM 64602\textsuperscript{T} could not grow under anaerobic conditions. The isolate grew over the temperature range 15–40 °C, pH range pH 6.0–9.0 and NaCl concentration range 0–3 % (w/v). Optimal growth was observed at 28 °C and at pH 7.0 without NaCl. Other physiological characteristics are given in Table 1 and in the species description.

In view of the combination of morphological, physiological, chemotaxonomic and genotypic data (Table 1) discussed here, such as being Gram-reaction-positive, strictly aerobic and filamentous, containing \textit{meso}\textsubscript{-}DAP, the major polar lipids DPG, PE and PME, and the predominant menaquinones MK-10(H\textsubscript{6}), MK-10(H\textsubscript{4}) and MK-11(H\textsubscript{4}), and having a fatty-acid pattern mainly composed of saturated branched-chain acids, it is evident that strain YIM 64602\textsuperscript{T} belongs to the genus \textit{Stackebrandtia}.

### Table 1. Differential characteristics of strain YIM 64602\textsuperscript{T} and the type strains of related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature for growth (°C)</td>
<td>15–40</td>
<td>20–37</td>
<td>15–37</td>
</tr>
<tr>
<td>Growth on ISP 4</td>
<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Soluble pigment</td>
<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>NaCl for growth (%)</td>
<td>3</td>
<td>–</td>
<td>4–9</td>
</tr>
<tr>
<td>pH for growth</td>
<td>6–9</td>
<td>6–8</td>
<td>ND</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Trehalose</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Raffinose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Predominant menaquinones</td>
<td>MK-10(H\textsubscript{6}), MK-10(H\textsubscript{4}), MK-11(H\textsubscript{6})</td>
<td>MK-10(H\textsubscript{6}), MK-10(H\textsubscript{4}), MK-11(H\textsubscript{6}), MK-11(H\textsubscript{4})</td>
<td>MK-10(H\textsubscript{6}), MK-10(H\textsubscript{4}), MK-11(H\textsubscript{6}), MK-11(H\textsubscript{4}), MK-11(H\textsubscript{6}), MK-11(H\textsubscript{4})</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>iso-C\textsubscript{16:0}, anteiso-C\textsubscript{17:0}, iso-C\textsubscript{15:0}, iso-C\textsubscript{17:0}</td>
<td>anteiso-C\textsubscript{17:0}, iso-C\textsubscript{15:0}, iso-C\textsubscript{17:0}</td>
<td>anteiso-C\textsubscript{17:0}, anteiso-C\textsubscript{17:0}</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>72.4</td>
<td>69.4*</td>
<td>72.4</td>
</tr>
</tbody>
</table>

*Data from Wang \textit{et al.} (2009).
However, a few characteristics that are unique to strain YIM 64602<sup>T</sup> differentiate it from <i>S. albiflava</i> YIM 45751<sup>T</sup> and <i>S. nassauensis</i> DSM 44728<sup>T</sup> (Table 1). YIM 64602<sup>T</sup> and <i>S. nassauensis</i> DSM 44728<sup>T</sup> can utilize trehalose, while <i>S. albiflava</i> YIM 45751<sup>T</sup> cannot; <i>S. nassauensis</i> DSM 44728<sup>T</sup> and <i>S. albiflava</i> YIM 45751<sup>T</sup> can utilize raffinose, fructose and glucose, and hydrolyse gelatin, while YIM 64602<sup>T</sup> cannot. The strains can also be differentiated on the basis of growth temperature, pH and NaCl tolerance. Based on the phenotypic, chemotaxonomic and genotypic data presented above, we propose that strain YIM 64602<sup>T</sup> represents a novel species within the genus <i>Stackebrandtia</i>, and the name <i>Stackebrandtia endophytica</i> sp. nov. is proposed.

### Description of <i>Stackebrandtia endophytica</i> sp. nov.

<i>Stackebrandtia endophytica</i> (en.do.phy’ti.ca. Gr. pref. endo within; Gr. n. phyton plant; L. fem. suff. -ica adjectival suffix used with the sense of belonging to; N.L. fem. adj. endophytica within plant, endophytic, pertaining to the isolation from plant tissues).

Good growth occurs on ISP 2 and ISP 4 (produces white to yellowish—white substrate and aerial mycelia). Weak growth is observed on ISP 3, ISP 4, ISP 5 and Czapek’s sucrose agar. Yellow soluble pigments are produced on ISP 2 media. Grows over the temperature range 15–40 °C, pH range pH 6.0–9.0 and NaCl concentration range 0–3 % (w/v). Catalase-positive and oxidase-negative; nitrate is reduced to nitrite. H<sub>2</sub>S is not produced. Can degrade starch, Tweens 20, 60 and 80 urea, but not gelatin or Tween 40. Utilizes trehalose, <i>D</i>-galactose, sucrose and xylose as sole carbon sources; arabinose, <i>D</i>-mannose, <i>L</i>-sorbose, succinic acid, inositol, raffinose, cellobiose, <i>D</i>-fructose, maltose and glucose are not utilized.

The type strain is YIM 64602<sup>T</sup> (=BCRC 16954<sup>T</sup> =DSM 45928<sup>T</sup>), isolated from surface-sterilized stems of <i>Tripterygium wilfordii</i>, collected in Yunnan Province, south-west China.

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### References


