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# Stackebrandtia endophytica sp. nov., a novel actinobacterium isolated from Tripterygium wilfordii

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| **Abstract:** | 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 hydrolyzates of strain YIM 64602T contained amino acid of meso-diaminopimelic acid with sugars mannose, rhamnose and glucose, a trace of ribose. The major polar lipids were diphosphatidylglycerol, phosphatidylmethylethanolamine 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 %. The 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 polyphasic evidence from this study, strain YIM 64602T (= BCRC 16954T = DSM 45928T) was considered to represent a novel species of the genus Stackebrandtia, for which the name Stackebrandtia endophytica is proposed. |

# *Stackebrandtia endophytica* sp. nov., a novel actinobacterium isolated from *Tripterygium wilfordii*

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The 16S rRNA gene sequence of strain YIM 64602[T] has been deposited in GenBank under the accession number KJ781245.

**A novel endophytic actinobacterium, designated strain YIM 64602$^T$, 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 64602$^T$ belongs to the genus *Stackebrandtia*. Whole-cell hydrolyzates of strain YIM 64602$^T$ contained amino acid of *meso*-diaminopimelic acid with sugars mannose, rhamnose and glucose, a trace of ribose. The major polar lipids were diphosphatidylglycerol, phosphatidylmethylethanolamine and phosphatidylethanolamine. MK-10($H_6$), MK-10($H_4$) and MK-11($H_4$) were the predominant components in the quinone system. The fatty-acid pattern was mainly composed of the saturated branched-chain acids iso-$C_{16:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$ and iso-$C_{17:0}$. The DNA G+C content was 72.4 mol %. The 16S rRNA gene sequence analysis showed the highest pairwise sequence identity (96.0-98.5 %) with the members of the genus *Stackebrandtia*. Strain YIM 64602$^T$ displayed a DNA-DNA relatedness of 43.9 ± 0.4 % with the type strain *Stackebrandtia albiflava* YIM 45751$^T$. Based on polyphasic evidence from this study, strain YIM 64602$^T$ (= BCRC 16954$^T$ = DSM 45928$^T$) was 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). Currently, the family comprises 14 members, and the genus *Stackebrandtia* contains 2 members, *Stackebrandtia nassauensis* (Labeda & Kroppenstedt, 2005) and *Stackebrandtia albiflava* (Wang *et al.*, 2009) which were isolated from different soil samples. The two species in *Stackebrandtia* are Gram-positive, strictly aerobic, filamentous actinomycetes. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major polar lipids consist of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylmethylethanolamine (PME) and phosphatedylglycerol (PG). The predominant menaquinones are MK-10($H_4$), MK-10($H_6$), MK-11($H_4$) and MK-11($H_6$).

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, strain YIM 64602$^{T}$, 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 5% sodium hypochlorite and 70% ethanol according to the established procedure (Li *et al.*, 2008), then sliced into pieces, followed by plating on the cellulose-asparagine agar [ 2.5 g cellulose, 2.0 g sodium pyruvate, 1.0 g asparagine, 0.5 g $CaCl_2$, 0.25 g $KNO_3$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g $K_2HPO_4$, 10 mg $FeSO_4 \cdot 7H_2O$ 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 were discerned. Strain YIM 64602 $^{T}$ 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 constructed by the neighbour-joining (Saitou &Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) tree-making algorithms by using the software packages MEGA version 5.05 (Tamura *et al.*, 2011). The stability of relationships was assessed by performing bootstrap analyses with 1000 resamplings (Felsenstein, 1985). The 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 64602$^T$ was determined and deposited in GenBank as KJ781245. The 16S rRNA gene sequence showed the highest similarity with the members of the genus *Stackebrandtia*, family *Glycomycetaceae*, especially with the type strains of *S. albiflava* YIM45751$^T$ (98.5 %) and *S. nassauensis*a DSM 44728$^T$ (96.0 %). Genomic relatedness of strain YIM 64602$^T$ to *S. albiflava* YIM 45751$^T$ was 43.9 ± 0.4%. A comparison of the sequences with the type species of the related genera showed that the organism fell within the evolutionary radiation occupied by the genus *Stackebrandtia* (Fig. 1). In the tree based on the neighbour-joining algorithm, strain YIM 64602$^T$ formed a coherent cluster with *S. albiflava* YIM 45751$^T$ and *S. nassauensis*a DSM 44728$^T$; the branching order was supported further by the bootstrap value 100% and 98%. The similar tree topology was also obtained with the phylogenetic trees generated using maximum-parsimony and maximum-likelihood algorithms (Figs. S1-2). These data supported the finding that strain YIM 64602$^T$ represents a different genomic species.

Biomass for chemical studies of strain YIM 64602$^T$ was grown on ISP 2 agar plates for 7 days at 28 °C. The isomer of diaminopimelic acid 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 Collins *et al.* (1977) and separated by HPLC (Tamaoka *et al.*, 1983). Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). Biomass for fatty acid analysis was obtained by cultivation on tryptic soya agar (TSA) at 28 ºC for 3 days. Cellular fatty acid analysis was performed by using the Microbial Identification System (Sherlock Version 6.1; MIDI database: TSBA6). The G + C DNA content of the strain YIM 64602$^T$ was determined by using the HPLC method (Mesbah *et al*., 1989).

Strain YIM 64602$^T$ shared consistent chemotaxonomic characteristics with *S. albiflava* YIM 45751$^T$. The strain YIM 64602$^T$ contained *meso*-diaminopimelic acid (*meso*-DAP) as the diagnostic diamino acid in the peptidoglycan and sugars in whole-cell

hydrolysates contained mannose, rhamnose, glucose, and with a trace of ribose. Strain YIM 64602$^T$ is distinguished from the type strain of *S. albiflava* YIM 45751$^T$ by the absence of galactose and xylose (Wang *et al.*, 2009). In this study, *S. albiflava* YIM 45751$^T$ , *S. nassauensisa* DSM 44728$^T$ and *Glycomyces harbinensis* DSM 46494$^T$ were reanalyzed as described by Tang *et al.* (2009). All of them were found to contain mannose, galactose, rhamnose, glucose and ribose(Fig. S5). The predominant menaquinones of YIM 64602$^T$ were MK-10($H_6$), MK-10($H_4$) and MK-11($H_4$). The polar lipids consisted of DPG, PE and PME, and with some PG, phosphatidylinositol (PI); phosphatidylinositol mannosides (PIM), unknown phospholipids (PL) and unidentified polar lipid (UL) as minor components (Fig. S3). The major cellular fatty acid compositions (>10 %) of strain YIM 64602$^T$ showed the presence of iso-$C_{16:0}$ (20.29 %), anteiso-$C_{17:0}$ (18.48 %), iso-$C_{15:0}$ (11.37 %) and iso-$C_{17:0}$ (10.87 %). Detailed cellular fatty acid composition of stains YIM 64602$^T$ and *S. albiflava* YIM 45751$^T$ were presented in Table S1. The DNA G+C content was 72.4 mol %. The chemotaxonomic data for the new isolate matched genus *Stackebrandtia*, and also differentiated from them by absence of galactose in the whole-cell hyrolysate, absence of MK-10($H_6$) in the predominant menaquinone (Table 1).

Aerial spore-mass colour, substrate mycelium pigmentation and coloration of the diffusible pigments of strain YIM 64602$^T$ were recorded on ISP 2, 3, 4 and 5 media and Czapek's agar. Colours were determined by using colour chips from the ISCC-NBS colour charts (standard samples, no. 2106) (Kelly, 1964). Morphological properties were examined using a light microscopy (BH 2; Olympus) and scanning electron microscopy (Quanta 200; FEI) after 14-21 days 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 *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 OxoidAnaero*Gen* system (Miller *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 $H_2S$ production were determined using standard methods (Gerhardt *et al*., 1994; Lanyi, 1987; MacFaddin, 2000).

Strain YIM 64602$^T$ was a Gram- positive actinobacterium and it can grew well on ISP 2 and ISP 4 media, formed yellow-white to white substrate mycelia and yellow to white aerial mycelia. Yellow diffusible pigments were only produced on the ISP 2 medium. Substrate mycelia showed extensive branching without fragmenting (Fig. S4). It could not grow under anaerobic conditions. The isolates grew over the temperature range 15-40°C, pH range 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 Gram-positive, strictly aerobic, filamentous characters, containing *meso*-DAP, the major polar lipids are DPG, PE and PME, the predominant menaquinones are MK-10($H_6$), MK-10($H_4$) and MK-11($H_4$), the fatty-acid pattern was mainly composed of the saturated branched-chain acids, it is evident that strain YIM 64602$^T$ belongs to the genus *Stackebrandtia*. However, a few characteristics that are unique to strain YIM 64602$^T$ differentiate it from *S. albiflava* YIM 45751$^T$ and *S. nassauensisa* DSM 44728$^T$ (Table 1). YIM 64602$^T$ and *S. nassauensisa* DSM 44728$^T$ can utilize trehalose, while *S. albiflava* YIM 45751$^T$ cann't; *S. nassauensisa* DSM 44728$^T$ and *S. albiflava* YIM 45751$^T$ can utilize raffinose, fructose and glucose, hydrolyse gelatin, while YIM 64602$^T$ cann't. They can also be differentiated based on the growth temperature, pH and NaCl tolerance. Based on the phenotypic, chemotaxonomic and genotypic data presented above, we propose that strain YIM 64602$^T$ represents a novel species within the genus *Stackebrandtia*, and the name *Stackebrandtia endophytica* sp. nov. is proposed.

**Description of *Stackebrandtia endophytica* sp. nov.**

*Stackebrandtia endophytica*(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 mycelia and aerial mycelia). Weakly grow is observed on ISP 3, ISP 4, ISP 5 and Czapek's sucrose agar. Yellow soluble pigments are produced on the ISP 2 media. Grows over the temperature range 15-40°C, pH range 6.0-9.0 and NaCl concentration range 0-3% (w/v). Catalase-positive and oxidase-negative, nitrate is reducedto nitrite. $H_2S$ is not produced. The strain can degrade starch, Tweens 20, 60, 80 and urea, but not gelatin or Tweens 40. Utilizes trehalose, D-galactose, sucrose and xylose as sole carbon; arabinose, D-mannose,L-sorbose, succinic acid, inositol, raffinose, cellobiose, D-fructose, maltose or glucoseare not utilized.

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

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**Fig. 1.** Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships of strain YIM 64602$^{T}$ and the type species of the 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 35013$^{T}$ (X79290) was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

**Table 1. Differential characteristics of strain YIM 64602$^T$ and related species**

| Character | 1 | 2 | 3† |
|---|---|---|---|
| Temperature (°C) | 15-40 | 20-37 | 15-37 |
| Growth on ISP 4 | + | - | ND |
| Soluble pigment | + | - | ND |
| NaCl(%) | 3 | - | 4-9 |
| pH | 6-9 | 6-8 | ND |
| Gelatinase | - | + | + |
| Utilization of: | | | |
| Trehalose | + | - | + |
| Raffinose | - | + | + |
| Fructose | - | + | + |
| Glucose | - | + | + |
| Predominant menaquinones | MK-10($H_6$)、MK-10($H_4$) and MK-11($H_4$) | MK-10($H_6$)、MK-10($H_4$)、MK-11($H_6$) and MK-11($H_4$) | MK-10($H_6$)、MK-10($H_4$)、MK-11($H_6$) and MK-11($H_4$) |
| major fatty acids | iso -$C_{16:0}$、anteiso -$C_{17:0}$、iso -$C_{15:0}$ and iso -$C_{17:0}$ | anteiso -$C_{17:0}$、iso -$C_{15:0}$ and iso -$C_{17:0}$ | anteiso -$C_{17:0}$、2-hydroxy-anteiso -$C_{17:0}$、iso -$C_{17:0}$、iso -$C_{16:0}$ and iso -$C_{15:0}$ |
| G + C content (mol %) | 72.4 | 69.4* | 72.4 |

Taxa: 1, YIM 67072$^T$; 2, *S. albiflava* YIM 45751$^T$; 3, *S. nassauensisa* DSM 44728$^T$. *Data from Wang *et al.* (2009). †Data from Wang *et al.* (2009) and Labeda & Kroppenstedt,( 2005). +, Positive; -, negative; ND, no data.

Figure
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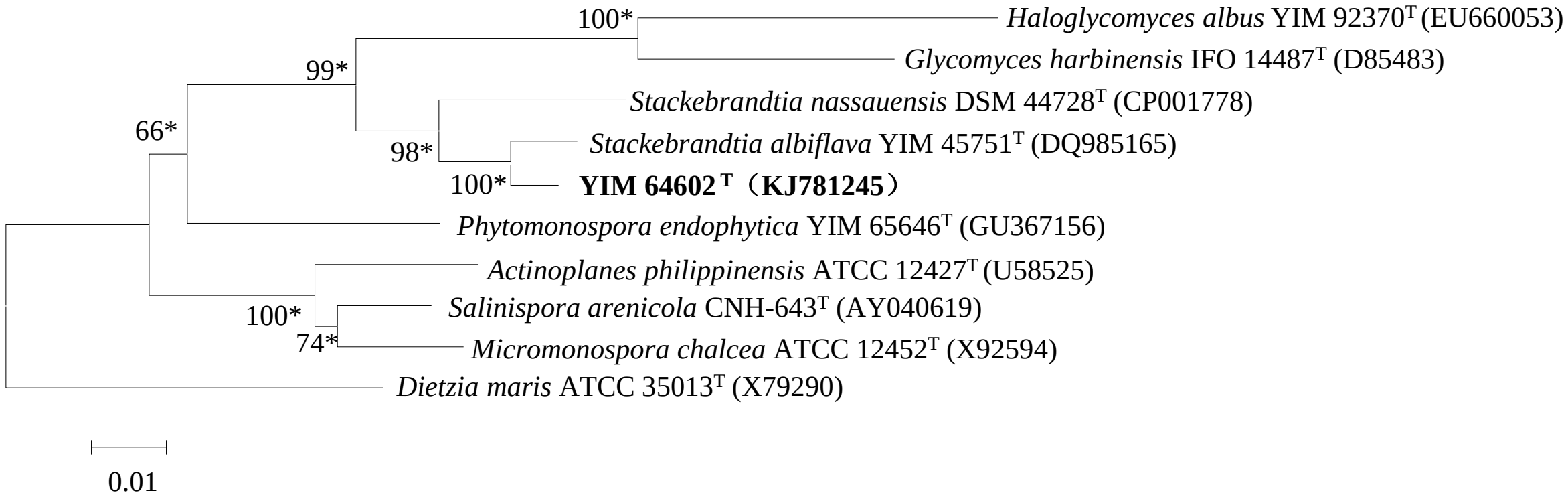


As is on our Paper ahead of Press page 11: **Fig. 1.**

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