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ORIGINAL PAPER

Streptomyces phytohabitans sp. nov., a novel endophytic actinomycete isolated from medicinal plant *Curcuma phaeocaulis*

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Abstract A novel actinomycete, designated strain KLBMP 4601^T, was isolated from the root of the medicinal plant *Curcuma phaeocaulis* collected from Sichuan Province, south-west China. The strain produced extensively branched substrate and aerial hyphae that carried straight to flexuous spore chains. Chemotaxonomic properties of this strain were consistent with those of members of the genus *Streptomyces*. The cell wall of strain KLBMP 4601^T contained LL-diaminopimelic acid as the characteristic diamino acid. The major menaquinone was MK-9(H_4), with minor amounts of MK-9(H_6), MK-9(H_8) and MK-10(H_2). The major fatty acids were $C_{16:0}$, iso-$C_{16:0}$, $C_{18:1}\omega 9c$ and $C_{16:1}$, iso G. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain KLBMP 4601^T belongs to the genus *Streptomyces* and is most closely related to *Streptomyces armeniacus* JCM 3070^T (97.9 %), *Streptomyces pharmamarensis* PM267^T (97.6 %) and *Streptomyces artemisiae* YIM 63135^T (97.5 %). The 16S rRNA gene sequence similarity between strain KLBMP 4601^T and other members of this genus were lower than 97.5 %. DNA–DNA hybridization studies of strain KLBMP 4601^T with the three closest species showed relatedness values of 36.3 ± 4.2 %, 27.3 ± 0.6 %, and 30.9 ± 2.5 %, respectively. On the basis of chemotaxonomic, phenotypic and genotypic characteristics, it is evident that strain KLBMP 4601^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces phytohabitans* sp. nov. is proposed. The type strain is KLBMP 4601^T (=KCTC 19892^T = NBRC 108772^T).

Keywords *Streptomyces phytohabitans* sp. nov · 16S rRNA gene · Polyphasic taxonomy

Guang-Kai Bian and Sheng Qin are contributed equally to this work.

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Introduction

The genus *Streptomyces* was proposed by Waksman and Henrici (1943) and is a unique source of novel antibiotics (Bérdy 2005; Goodfellow and Fiedler

2010). At present, the genus *Streptomyces* contains the large number of described species and nearly 600 were validly published (Euzéby 2012). Endophytes that live within the healthy plants are now considered as an important component of biodiversity. In recent years, endophytic actinomycetes have attracted significant interest for their capacity to produce a vast array of secondary metabolites exhibiting a wide variety of biological activities (Qin et al. 2011). In the course of investigating endophytic actinomycetes associated with medicinal plants in Sichuan Province, China, one streptomycete strain KLBMP 4601^T was isolated. This strain showed characteristics different from other members of the *Streptomyces* genus by the polyphasic characterization. Here we report a polyphasic taxonomic study and showed that isolate KLBMP 4601^T belonged to a new *Streptomyces* species, *Streptomyces phytohabitans* sp. nov.

Materials and methods

Isolation and maintenance of isolate

Strain KLBMP 4601^T was isolated from the healthy roots of a medicinal plant *Curcuma phaeocaulis* collected from Sichuan Province, south-west China, in 2009. The root samples were firstly surface sterilized according to the procedure described previously (Qin et al. 2009). Subsequently, the surface sterilized samples were aseptically crumbled into smaller fragments using a commercial blender (Joyoung), spread onto tap water-yeast extract agar (TWYE, Crawford et al. 1993) and colonies were picked up after incubation for 4 weeks at 28 °C. The purified isolate was routinely cultured on yeast extract-malt extract agar (ISP 2) (Shirling and Gottlieb 1966) and maintained as a glycerol suspension (20 %, w/v) at −80 °C.

Phenotypic characterization

Cultural characteristics of strain KLBMP 4601^T were determined using various agar media: ISP 2, oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5) (Shirling and Gottlieb 1966), potato-dextrose agar, Czapek's agar and nutrient agar (Waksman 1967) for 14 days at 28 °C. The ISCC-NBS color charts were used to determine the designations of colony colors (Kelly 1964). Morphological characteristics were observed by light microscopy (SA3300-PL) and scanning electron microscopy (Hitachi; S-3400N) using cultures grown on ISP 2 medium at 28 °C for 14 days. The growth temperature (4, 10, 15, 20, 28, 37, 45 and 55 °C) and NaCl tolerance (0–15 %, at intervals of 1 %) was determined on ISP 2 agar at 28 °C for 14 days. The pH range (pH 4.0–12.0, at intervals of 1.0 pH units) for growth was determined using both solid and liquid ISP 2 medium at 28 °C for 14 days. Carbon-source utilization was tested by using ISP 9 medium (Shirling and Gottlieb 1966) supplemented with 1 % (final concentration) carbon sources. The utilization of amino acids as sole nitrogen sources was tested as described by Williams et al. (1983). Production of acid and other physiological and biochemical characteristics were tested by using the well established procedures (Gordon et al. 1974). The reference strains *Streptomyces armeniacus* JCM 3070^T, *Streptomyces pharmamarensis* PM267^T and *Streptomyces artemisiae* YIM 63135^T were tested at the same condition in this study.

Chemotaxonomy

Biomass used for chemotaxonomic analyses was obtained from cultures grown in ISP 2 broth on a rotary shaker (about 150 rpm) for 8 days at 28 °C until good growth was obtained. Amino acids and sugars in whole-cell hydrolysates were analyzed according to the standard procedures (Lechevalier and Lechevalier 1980). Menaquinones were extracted and purified according to the method of Collins et al. (1977) and then analyzed by HPLC (Kroppenstedt 1985). Polar lipids were extracted and identified by two-dimensional TLC according to the method described by Minnikin et al. (1984). Fatty acids were analyzed using the standard MIDI (Microbial Identification, Sherlock version 6.0) procedure (Sasser 1990) and the gas chromatograph Agilent GC 6850. The resulting profiles were identified using the database library TSBA6 version 6.0.

Molecular analysis

Extraction of genomic DNA, PCR amplification and sequencing of 16S rRNA gene were carried out using

the method of Li et al. (2007). The 1,422 bp sequence of strain KLBMP 4601^T was aligned with those most closely related species by using CLUSTAL_X (Thompson et al. 1997). The 16S rRNA gene sequence similarity values were calculated by using the EzTaxon server (http://www.Eztaxon.org) (Chun et al. 2007). Phylogenetic trees were constructed using the neighbour-joining (Saitou and Nei 1987), maximum-parsimony (Fitch 1971) and maximum-likelihood (Felsenstein 1981) methods using MEGA version 5.0 (Tamura et al. 2011). The topologies of the phylogenetic trees were evaluated by the bootstrap resampling method of Felsenstein (1985) with 1,000 replicates. Determination of DNA G+C content was performed according to Mesbah et al. (1989). Levels of DNA–DNA relatedness were determined according to the fluorometric micro-well method (Ezaki et al. 1989; He et al. 2005).

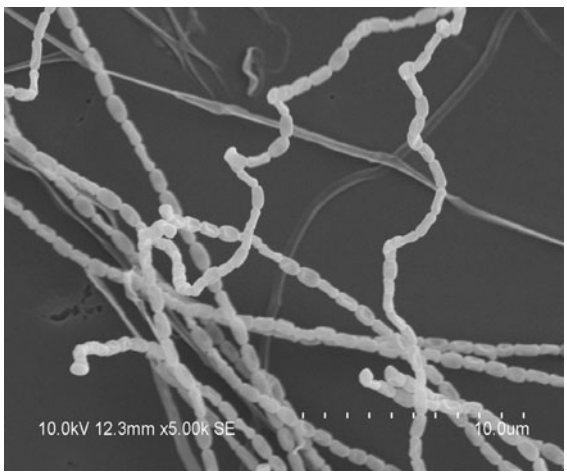

Fig. 1 Scanning electron micrograph of strain KLBMP 4601^T, showing aerial mycelium and spore chains after growth on ISP 2 medium agar at 28 °C for 2 weeks. *Bar* 10.0 μm

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain KLBMP 4601^T determined in this study has been deposited in GenBank under the accession number JQ345722.

Results and discussion

Strain KLBMP 4601^T showed abundant growth on ISP 2, ISP 3, Czapek's agar and nutrient agar, moderate on ISP 4 and ISP 5 media and poor growth on PDA agar. White to gray-white aerial mycelium was present on these media. Substrate mycelium of strain KLBMP 4601^T was yellowish-white to dark green on all media tested. Pink soluble pigment was formed on ISP 4 agar plate. The strain produced extensively branched substrate and aerial hyphae that carried straight to flexuous spore chains. The surface of the spores was smooth (Fig. 1). Differences in cultural characteristics from its closest related type strains are given in Table 1. Growth of strain KLBMP 4601^T occurred in the pH range 6.0–8.0 and 0–7 % NaCl (w/v), with optimum growth at pH 7.0 and 3 % NaCl (w/v). The temperature range for growth was 4–45 °C, with the optimum temperature being 28 °C. Other physiological characteristics are given in the type strain description and Table 2.

Table 1 Differential cultural characteristics of strain KLBMP 4601^T and its closest phylogenetic neighbours

Characteristics	KLBMP 4601^T	*S. armeniacus* JCM 3070^T	*S. pharmamarensis* PM 267^T	*S. artemisiae* YIM 63135^T
Growth on ISP 2 medium:	Good	Good	Moderate	Good
Aerial mycelium	Abundant, white	Sparse, yellow-white	Sparse, white	Abundant, white
Substrate mycelium	Yellowish-white	Brown	Cream	Yellow-white
Growth on ISP 3 medium	Good	Poor	Moderate	Moderate
Aerial mycelium	Abundant, white	Absent	Absent	Pink
Substrate mycelium	Yellow-white	Cream	White	Pink-brown
Aerial mycelium on ISP 4 medium	Abundant, gray-white	Sparse, white	Absent	Abundant, white
Spore chain morphology	Straight to flexuous	Spiral	Spiral	Spiral
Production of diffusible pigment	Pink	Absent	Absent	Pink, red

Table 2 Features that distinguish strain KLBMP 4601^T from the closely related *Streptomyces* species

Characteristic	KLBMP 4601^T	*S. armeniacus* JCM 3070^T	*S. pharmamarensis* PM 267^T	*S. artemisiae* YIM 63135^T
Growth at 4 °C	+	+	−	−
Growth at 45 °C	+	−	−	+
Growth at pH 9.0	−	+	−	+
NaCl tolerance (%)	0–7 %	0–9 %	0–9 %	0–5 %
Optimum NaCl for growth	3 %	3 %	2 %	1 %
Utilization of:				
D-Arabinose	+	−	+	−
Cellobiose	+	−	+	+
Cellulose	+	−	−	−
Glucose	+	−	+	+
D-Raffinose	+	−	+	−
L-Rhamnose	+	+	+	−
Sucrose	+	−	+	+
Trehalose	+	−	+	+
L-Asparagine	−	−	−	+
L-Valine	−	−	+	+
Menaquinone composition	MK-9(H_4), MK-9(H_6), MK-9(H_8), MK-10(H_2)	MK-9(H_4), MK-9(H_6)[a]	MK-9(H_2), MK-9(H_4), MK-9(H_6), MK-9(H_8), MK-10, MK-10(H_2)[b]	MK-9(H_4), MK-9(H_6), MK-9(H_8)[a]
Polar lipids composition	DPG, PG, PE, PI, PIM, 1PL, 2GL	ND	DPG, PE, PI, PIM, 1PL[b]	DPG, PG, PI, PE, PIM, 4PL, GluNu[a],
DNA G+C content (mol%)	69.0	ND	71.2[b]	72.6[a]

+ Positive or present, *w* weakly positive, − negative or absent, *ND* not detected. *DPG* diphosphatidylglycerol, *PG* phosphatidylglycerol, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PIM* methyl-phosphatidylinositol, *PL* unknown phospholipid, *GL* unknown glycolipid, *GluNu* unknown glucosamine-containing phospholipid

Data were obtained from this study under identical growth conditions, except those as labeled

[a] Data from Zhao et al. (2010)

[b] Data from Carro et al. (2011)

The cell wall of the novel isolate contained LL-diaminopimelic acid, which is characteristic for the genus *Streptomyces*. The whole-cell sugars were detected as mannose and glucose. The diagnostic phospholipids are diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylinositol-mannoside (PIM), two unidentified glycolipids and an unknown phospholipid (Supplementary Fig. S1). The phospholipid type, PII (Lechevalier et al. 1977), agrees with that reported for the genus *Streptomyces*. The major menaquinone found was MK-9(H_4) (89.4 %), with minor amounts of MK-9(H_8) (6.5 %), MK-9(H_6) (1.9 %) and MK-10(H_2) (2.2 %). The major fatty acids (>5.0 %) were $C_{16:0}$ (15.8 %), iso-$C_{16:0}$ (9.3 %), $C_{18:1}\omega 9c$ (8.9 %), $C_{16:1}$ iso G (8.2 %), $C_{17:1}\omega 8c$ (8.0 %), $C_{17:0}$ 10-methyl (7.7 %), $C_{16:1}\omega 7c$/$C_{16:1}\omega 6c$ (7.4 %), $C_{17:1}$ iso w9c/$C_{16:0}$ 10-methyl (7.3 %) and anteiso-$C_{17:1}$ A (5.4 %). A detailed fatty acid profile comparison with its nearest neighbour species is given in Table 3. The chemical properties of strain KLBMP 4601^T are consistent with its classification as a member of the genus *Streptomyces*. The G+C content of the DNA was 69.0 mol%.

Table 3 Fatty acid profiles (%) of strain KLBMP 4601^T and its nearest neighbour *S. armeniacus* JCM 3070^T

Fatty acid	KLBMP 4601^T	*S. armeniacus* JCM3070^T
$C_{14:0}$	1.4	0.9
Iso-$C_{14:0}$	–	0.5
$C_{15:1}$ iso G	0.6	–
Iso-$C_{15:0}$	–	1.2
Anteiso-$C_{15:0}$	0.9	5.5
Anteiso-$C_{15:1}$ A	–	0.4
Iso-$C_{16:0}$	9.3	15.3
$C_{16:0}$	15.8	9.6
$C_{16:1}$ iso G	8.2	5.5
Iso-$C_{17:0}$	2.0	0.9
Anteiso-$C_{17:0}$	4.4	10.8
$C_{17:0}$	1.7	0.4
Anteiso-$C_{17:1}$ A	5.4	–
$C_{17:0}$ 10-methyl	7.7	0.6
Anteiso-$C_{17:1}\omega 9c$	–	5.7
$C_{17:1}\omega 8c$	8.0	2.0
Iso-$C_{18:0}$	2.9	–
Iso-$C_{18:1}$ H	–	0.5
$C_{18:1}\omega 9c$	8.9	25.5
$C_{18:0}$	4.0	2.1
Sum in feature 3[a]	7.4	9.7
Sum in feature 5[a]	4.4	2.4
Sum in feature 9[a]	7.3	0.9

All the data are from this study. Values are percentages of total fatty acids; – not detected

[a] Summed features represent groups of two or three fatty acids that cannot be separated by GC with the MIDI system. Summed features 3, 5, 9 comprised 16:1 w7*c*/16:1 w6*c*, 18:2 w6, 9*c*/18:0 ante, 17:1 iso w9*c*/16:0 10-methyl, respectively

An almost-complete 16S rRNA gene sequence (1,422 bp) was obtained for the isolate. The sequence similarities between strain KLBMP 4601^T and its closest relatives, *S. armeniacus* JCM 3070^T, *S. pharmamarensis* PM267^T and *S. artemisiae* YIM 63135^T, were 97.9, 97.6 and 97.5 %, respectively. The 16S rRNA gene sequence similarities between strain KLBMP 4601^T and the other type strains in this genus were <97.5 %. It was evident from the neighbor-joining dendrogram shown in Fig. 2 that the new isolate formed a distinct branch with *S. armeniacus* JCM 3070^T, *S. pharmamarensis* PM267^T and *S. artemisiae* YIM 63135^T by a high bootstrap value of 99 %. This distinct branch was also recovered from maximum-parsimony and maximum-likelihood trees (Supplementary Fig. S2–S3). The mean DNA–DNA hybridization values found between the isolate and type strains of *S. armeniacus* JCM 3070^T, *S. pharmamarensis* PM267^T and *S. artemisiae* YIM 63135^T were 36.3 ± 4.2 %, 27.3 ± 0.6 % and 30.9 ± 2.5 %, respectively, all of which are below the 70 % threshold value proposed by Wayne et al. (1987), indicating that strain KLBMP 4601^T should be identified as a novel species.

The characteristics shown in Table 1, 2 clearly indicate that strain KLBMP 4601^T possesses obvious distinct phenotypic and chemotaxonomic profiles that distinguish it from its closest phylogenetic relatives. For example, the different growth characteristics on ISP 2 and ISP 3 media and only strain KLBMP 4601^T produced soluble pink pigment on ISP 4 medium, differences in utilization of carbon sources and the menaquinones composition. For another, the major fatty acids of strain KLBMP 4601^T are clearly different from the nearest neighbour *S. armeniacus* JCM 3070^T (Table 3). Moreover, the differences in DNA G+C content, low level of DNA–DNA relatedness can be used to distinguish strain KLBMP 4601^T from their closely related phylogenetic neighbours. Therefore, strain KLBMP 4601^T represents a novel species of the genus *Streptomyces*, for which the name *S. phytohabitans* sp. nov. is proposed.

Description of *S. phytohabitans* sp. nov

Streptomyces phytohabitans (Phy.to.ha'bi.tans. Gr. n. phyton, plant; L. part. adj. habitans, inhabiting; N. L. part. adj. used as a masc. n. *phytohabitans*, plant inhabiting, isolated from a plant).

Aerobic, Gram-positive, catalase-positive actinomycete that forms white aerial mycelia and yellowish-white substrate mycelia on ISP 2 medium. The substrate mycelium does not fragment. Pink diffusible pigments are produced on ISP 4 agar. Produces straight to flexuous spore chains with smooth-surfaced spores (about 0.8–1.2 × 0.5–0.7 μm). Develops well on ISP 2, ISP 3, Czapek's and nutrient agar. Moderate growth on ISP 4 and ISP 5. Growth occurs at 4–45 °C, at pH 6.0–8.0 and in the presence of 0–7 % (w/v) NaCl. Uses D-arabinose, cellobiose, cellulose, D-fructose, D-galactose, D-glucose, mannose, D-raffinose, D-ribose, L-rhamnose, sucrose, trehalose, xylitol and D-xylose as sole carbon and energy sources. Uses

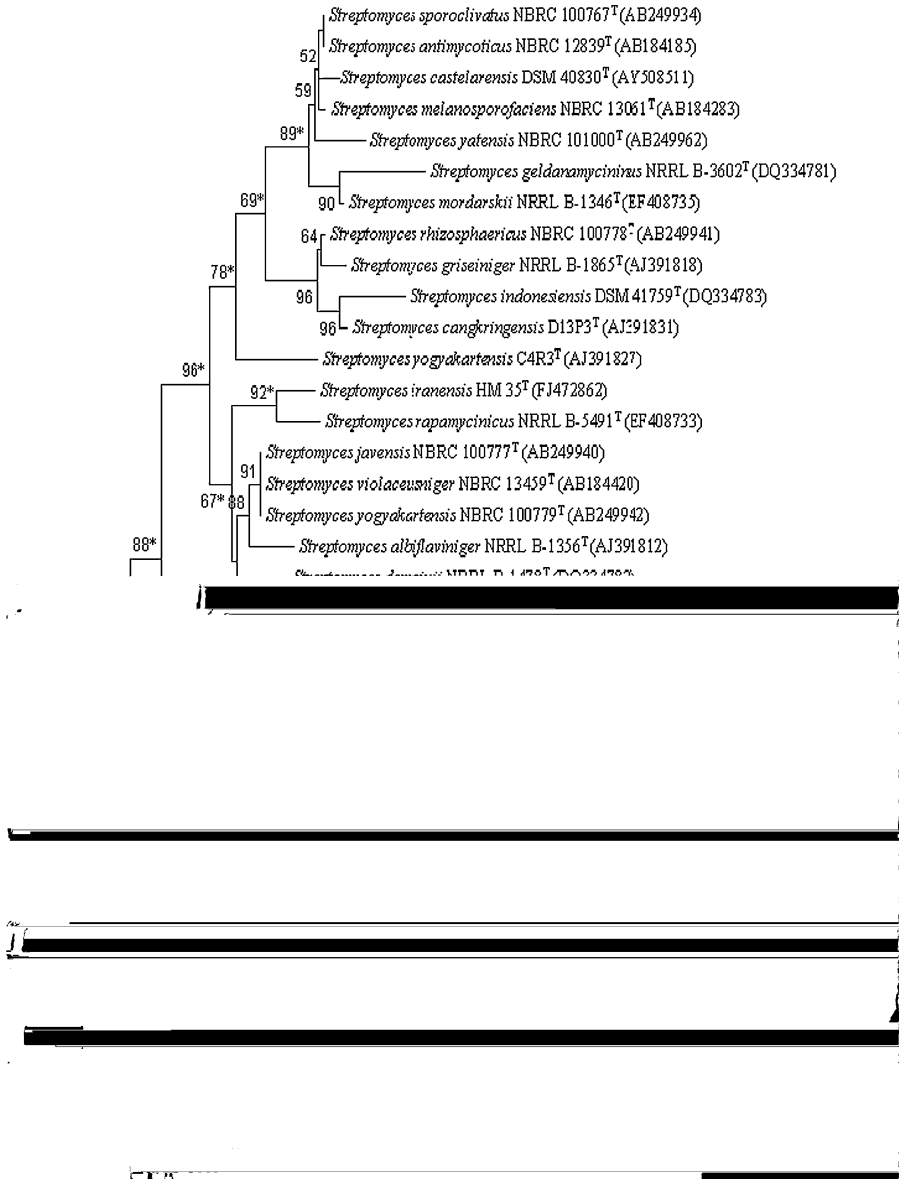

Fig. 2 Neighbour-joining tree based on almost complete 16S rRNA gene sequences (1,422 nt), showing the relationship between strain KLBMP 4601^T and its phylogenetic neighbours. Only bootstrap values above 50 %, expressed as percentages of 1,000 replications, are shown at the *branch points*. Kitasatospora setae KM-6054^T was used as the outgroup. *Asterisks* indicate that the corresponding nodes were also recovered in the maximum-parsimony and maximum-likelihood trees. *Bar* 0.005 substitutions per nucleotide position

L-arginine, L-glutamic acid, L-glycine, L-histidine, L-lysine and L-proline as sole nitrogen sources. Acid is produced from arabinose, D-fructose, D-glucose, mannose and D-xylose. Positive for urease production, milk peptonization and coagulation, but negative for gelatin liquefaction and H_2S production. Cell wall contains LL-diaminopimelic acid. The whole-cell hydrolysates contain mannose and glucose. The phospholipid composition includes DPG, PE, PG, PI, PIM, two unidentified glycolipids and an unknown phospholipid. Menaquinones found are MK-9(H_4), MK-9(H_6), MK-9(H_8) and MK-10(H_2). The major cellular fatty acids are $C_{16:0}$, iso-$C_{16:0}$, $C_{18:1}\omega9c$, $C_{16:1}$ iso G, $C_{17:1}\omega8c$, $C_{17:0}$ 10-methyl, $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{17:1}$ iso w9c/$C_{16:0}$ 10-methyl and anteiso-$C_{17:1}$ A. The G+C content of the DNA is 69.0 mol%.

The type strain, KLBMP 4601^T (=KCTC 19892^T = NBRC 108772^T) was isolated from surface-sterilized roots of *Curcuma phaeocaulis* collected from the city of Panzhihua, Sichuan Province, south-west China.

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