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ORIGINAL PAPER

Amycolatopsis endophytica sp. nov., a novel endophytic actinomycete isolated from oil-seed plant *Jatropha curcas* L.

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Abstract A novel actinomycete, designated KLBMP 1221^T, was isolated from the surface-sterilized seeds of an oil-seed plant *Jatropha curcas* L. collected from Sichuan Province, south-west China and was characterized taxonomically by using a polyphasic approach. Phylogenetic analyses based on 16S rRNA gene sequence showed that this strain formed a distinct phyletic line within the radiation of the genus *Amycolatopsis*. The 16S rRNA gene sequence similarity indicated that strain KLBMP 1221^T was most closely related to *Amycolatopsis eurytherma* NT202^T (98.9%), *Amycolatopsis tucumanensis* ABO^T (98.8%), *Amycolatopsis thermoflava* N1165^T (98.6%) and *Amycolatopsis methanolica* IMSNU 20055^T (98.5%). Strain KLBMP 1221^T had morphological and chemotaxonomic properties that were consistent with its classification in the genus *Amycolatopsis*. However, DNA–DNA relatedness data and phenotypic differences clearly distinguished the isolate from its closest relatives. Based on the combined genotypic and phenotypic evidence, it is proposed that strain KLBMP 1221^T be classified as representative of a novel species for which the name *Amycolatopsis endophytica* sp. nov. is proposed. The type strain is KLBMP 1221^T (= KCTC 19776^T = CCTCC AA 2010003^T).

Keywords *Amycolatopsis endophytica* sp. nov. · Polyphasic taxonomy · 16S rRNA

Qian Miao and Sheng Qin contributed equally to this work.

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Introduction

The genus *Amycolatopsis* was proposed by Lechevalier et al. (1986) and its description has been emended recently by Lee (2009) and Tang et al. (2010). At the time of writing, the genus *Amycolatopsis* comprises 45 species with validly published names. The species of the genus have been isolated from various environments such as clinical material, soils, catacomb, ocean sediment, polluted environments and surface-sterilzed roots of plant (Labeda et al. 2003; Huang et al. 2004;

Tseng et al. 2006; Groth et al. 2007; Bian et al. 2009; Tamura et al. 2010; Albarracín et al. 2010; Duangmal et al. 2011). At present, there is only one record of the isolation of a new species belonging to the genus *Amycolatopsis* from the endophytic environment (Duangmal et al. 2011). During an investigation of the endophytic actinobacterial community in the oil-seed medicinal plant *Jatropha curcas* L., an *Amycolatopsis*-like strain was isolated. The aim of the present study was to determine the taxonomic position of another endophytic actinobacterium using a polyphasic taxonomic approach.

Materials and methods

Isolation and maintenance of organism

Strain KLBMP 1221^T was isolated from healthy oil-seed plant *Jatropha curcas* L. collected from the city of Panzhihua, Sichuan Province, south-west China. Healthy seed samples of *J. curcas* L. were washed in running tap water to remove adhered epiphytes and surface-sterilized according to the five-step sterilization procedure (Qin et al. 2008). After that, the surface sterilized seeds were aseptically crumbled into smaller fragments using a commercial Joyoung blender, spread onto sodium propionate agar (Qin et al. 2009), and incubated at 28°C for 2–6 weeks. The purified strain was maintained on yeast extract-malt extract agar [International *Streptomyces* Project medium 2 (ISP 2); Shirling and Gottlieb 1966] slants at 4°C and as 20% (v/v) glycerol suspensions at 28°C.

Phenotypic characterization

Cell morphology was observed using light microscopy (SA3300-PL) and scanning electron microscopy (Hitachi; S-3400 N) after 2 weeks growth on ISP 2 medium agar. Growth at different temperatures (4, 10, 15, 20, 25, 28, 30, 37, 40, 45, 50°C), different NaCl concentrations (0–15%, w/v) (at intervals of 0.5%) and at various pH values (pH 5.0–12.0 at intervals of 0.5 pH unit with different pH buffers) at 28°C was tested on ISP 2 basal medium after 14 days of incubation. Cultural and morphological characteristics were observed in the organisms grown on ISP 2, oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5) (Shirling and Gottlieb 1966), as well as potato-dextrose agar (PDA; Difco), Czapek's agar and nutrient agar (Waksman 1967) for three weeks at 28°C. Colours of colonies were determined by using colour chips from the ISCC–NBS colour charts standard (Kelly 1964). The ability to use a range of carbon and nitrogen sources for energy and growth and other biochemical features was tested at 28°C for 14 days according to Kurup and Schmitt (1973). Acid production from carbohydrates was determined using media and methods described by Gordon et al. (1974). Four phylogenetic nearest reference *Amycolatopsis* strains were used for comparison under the same condition.

Chemotaxonomy

Biomass for quantitative fatty acid analysis was obtained from cultures grown in tryptic soy broth (TSB) at 150 rpm for 7 days at 28°C. Biomass for other chemotaxonomic studies was obtained after cultivation at 28°C for 7–10 days in shaken cultures with yeast extract-malt extract broth. Cells were harvested and then freeze-dried. The isomer of diaminopimelic acid and sugar analysis of whole-cell hydrolysates were performed according to the procedures described by Hasegawa et al. (1983) and Lechevalier and Lechevalier (1970). Analysis of mycolic acids was performed using the previously described method by Minnikin et al. (1980). Polar lipids were extracted and identified by two-dimensional TLC (Minnikin et al. 1979; Collins and Jones 1980). Menaquinones were extracted and purified as described by Collins et al. (1977) and analysed by HPLC (Groth et al. 1997). The fatty acids were extracted, purified, methylated and quantified by GC using the standard Microbial Identification System (version 6; MIDI) (Sasser 1990; Kämpfer and Kroppenstedt 1996).

Molecular analysis

Extraction of chromosomal DNA and the amplification of the 16S rRNA gene by PCR were performed as described previously (Li et al. 2007). The resultant sequences were first aligned via the BLAST search program in NCBI. Then the identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using

the EzTaxon server (http://www.eztaxon.org/; Chun et al. 2007). Multiple alignments were performed using the CLUSTAL_X program (Thompson et al. 1997). The phylogenetic tree was constructed with the neighbour-joining (Saitou and Nei 1987) and maximum-parsimony (Kluge and Farris 1969) methods using the software package MEGA version 4.0 (Tamura et al. 2007) and distances were calculated according to Kimura's two-parameter model (Kimura 1980). The reliability of the neighbour-joining phylogenetic tree was evaluated using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. The G+C content of the DNA was determined by the method of Mesbah et al. (1989). DNA–DNA hybridization was determined using the photobiotin-labelled probes in microplate wells as described by Ezaki et al. (1989) and He et al. (2005), the hybridization temperature was 47°C.

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain KLBMP 1221^T determined in this study has been deposited in GenBank under the accession number HM153799.

Results and discussion

Strain KLBMP 1221^T showed good growth on ISP 2, ISP 3, ISP 5, nutrient agar and PDA media, and poor growth on ISP 4 and Czapek's medium agar. It formed extensively branched substrate mycelia with white (on ISP 4 and ISP 5 agar), yellowish white (on Potato dextrose, Czapek's and Nutrient agar) to yellow colour (on ISP 2 and ISP 3 agar). White aerial mycelia were observed on all media tested. Straight or flexuous chains of fragmented rod-shaped elements of aerial mycelium were observed (Fig. 1). The surface of spore-like structures was smooth. No diffusible pigments were produced on the tested media. Strain KLBMP 1221^T had morphological properties typical of members of the genus *Amycolatopsis*. Growth was observed at 15–45°C, 0–7% NaCl (w/v) and pH 6.0–8.0. Detailed physiological and biochemical properties of strain KLBMP 1221^T were listed in Table 1 and the species description. Based on the phenotypic characteristics observed, differences between strain KLBMP 1221^T and the type strains of the four related species of the genus *Amycolatopsis* were evident. The new isolate can assimilate more and different carbon and nitrogen sources than the recognized *Amycolatopsis* species. Moreover, the requirement for NaCl and temperature range for growth were also different. These characteristics could differentiate the new isolate and other type strains of the genus *Amycolatopsis*.

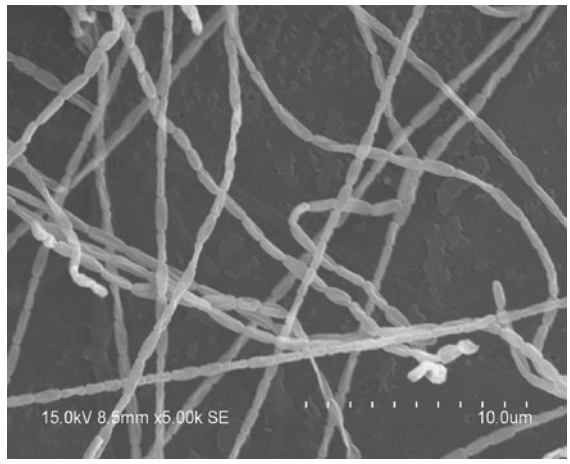

Fig. 1 Scanning electron micrograph of strain KLBMP 1221^T grown on ISP 2 medium for 14 days at 28°C. *Bar* 10 μm

The cell wall hydrolysates contained *meso*-diaminopimelic acid (*meso*-DAP) and arabinose and galatose as major cell-wall sugars (cell wall type IV) (Lechevalier and Lechevalier 1970). The predominant menaquinones was MK-9(H_4), but MK-9(H_2) and MK-8(H_4) were also found in minor amounts. Mycolic acids were not present. Polar lipid analysis showed that this organism contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol and two unidentified phospholipids (see Online Supplementary Fig. S1). The major fatty acids were iso-$C_{16:0}$ (33.47%), anteiso-$C_{17:0}$ (9.32%), iso-$C_{15:0}$ (8.97%), $C_{17:1}\omega 6c$ (7.84%), 16:1 w7c/16:1 w6c (7.53%), iso-$C_{17:0}$ (6.22%), $C_{17:1}\omega 8c$ (5.52%) and $C_{16:0}$ (5.04%). Detailed fatty acid data for the new strain and related *Amycolatopsis* species are given in Table 2. The results of chemical analysis indicated that the organism has chemotaxonomic markers typical of the genus *Amycolatopsis*.

The almost complete sequence of the 16S rRNA gene of strain KLBMP 1221^T (1443 bp) was used for

Table 1 Different characteristics of strain KLBMP 1221^T and its closely related *Amycolatopsis* species

Characteristic	1	2	3	4	5
Growth on ISP 4 medium	Poor	Moderate	Good	Good	Good
Growth on Czapek's medium	Poor	Good	Good	Good	Good
Production of soluble pigment	–	–	–	–	+
Assimilation of sole carbon sources					
D-Arabinose	+	+	+	–	–
D-Galactose	+	–	+	–	–
D-Lactose	+	+	+	–	+
D-Mannose	+	+	+	–	+
D-Raffinose	+	–	w	+	+
D-Rhamnose	+	+	+	–	+
D-Ribose	+	w	+	+	+
Sucrose	w	–	+	–	–
D-Xylose	+	+	w	+	+
Acid produced from					
D-Cellobiose	–	+	–	–	–
D-Raffinose	–	–	–	–	+
D-Rhamnose	+	+	+	–	–
Trehalose	–	–	–	+	+
Assimilation of sole nitrogen sources					
L-Arginine	+	–	+	+	+
L-Asparagine	+	w	+	+	–
L-Histidine	+	+	w	+	+
L-Lysine	+	+	+	–	–
L-Glutamic acid	–	–	–	+	–
Reduction of nitrate	–	–	–	+	–
NaCl tolerance (%, w/v)	0–7%	0–3%	0–6%	0–3%	0–8%
Temperature range (°C)	15–45	15–50	15–50	20–45	15–45
DNA G+C content (mol%)	73.3	ND	73.1*	ND	75*

Strains 1, KLBMP 1221^T; 2, *A. tucumanensis* ABO^T; 3, *A. eurytherma* NT202^T; 4, *A. methanolica* IMSNU 20055^T; 5, *A. thermoflava* N1165^T. + Positive or present; *w* weakly positive; − negative or absent; *ND* not done. All the data obtained during this study were carried out under identical growth conditions. * Data were taken from Kim et al. (2002) and Chun et al. (1999)

phylogenetic analysis. Phylogenetic analyses of 16S rRNA gene sequences showed that strain KLBMP 1221^T was a member of the genus *Amycolatopsis* (Fig. 2; an extended phylogenetic tree is also available as Supplementary Fig. S2). It is evident from 16S rRNA gene neighbour-joining tree that strain KLBMP 1221^T formed a distinct clade with 100% bootstrap support. Strain KLBMP 1221^T exhibited 16S rRNA gene sequence similarity values of 98.9, 98.8, 98.6 and 98.5% with its nearest neighbours *A. eurytherma* NT202^T, *A. tucumanensis* ABO^T, *A. thermoflava* N1165^T and *A. methanolica* IMSNU 20055^T. Levels of 16S rRNA gene sequence similarity between strain KLBMP 1221^T and the other *Amycolatopsis* species were less than 97%. However, DNA–DNA relatedness between strain KLBMP 1221^T and the type strains *A. eurytherma* NT202^T, *A. tucumanensis* ABO^T, *A. thermoflava* N1165^T and *A. methanolica* IMSNU 20055^T were 29.3 ± 4.5, 36.5 ± 1.7, 34.5 ± 2.6 and 49.5 ± 1.3%, respectively. These hybridization values were significantly less than 70% cut-off point according to the criterion recommended for the delineation of bacterial species by Stackebrandt and Goebel (1994), The DNA G+C content of strain KLBMP 1221^T was 73.3 mol%.

On the basis of the phylogenetic, physiological and chemotaxonomic data, Strain KLBMP 1221^T could be easily distinguished from its closest phylogenetic neighbours, *A. eurytherma* NT202^T, *A. tucumanensis* ABO^T, *A. thermoflava* N1165^T and *A. methanolica*

Table 2 Fatty acid profiles (%) of strain KLBMP 1221^T and related *Amycolatopsis* species

Fatty acid	1	2	3	4	5
iso-$C_{10:0}$	0.13	–	–	–	–
iso-$C_{11:0}$	0.15	–	–	–	–
anteiso-$C_{11:0}$	0.17	–	–	–	–
iso-$C_{12:0}$	0.44	–	–	0.09	0.05
$C_{12:0}$	0.23	–	–	0.17	–
iso-$C_{13:0}$	0.28	–	0.05	0.06	–
anteiso-$C_{13:0}$	0.18	–	–	0.06	–
$C_{13:0}$	0.17	–	–	–	–
iso-$C_{14:0}$	2.51	0.31	0.44	1.11	0.16
$C_{14:1}\omega5c$	0.08	0.07	0.06	–	0.03
$C_{14:0}$	0.77	0.10	0.10	0.24	0.38
iso-$C_{15:0}$	8.97	2.58	2.81	2.33	1.48
anteiso-$C_{15:0}$	1.87	0.76	9.14	1.15	0.39
$C_{15:1}\omega6c$	1.38	0.59	0.75	0.78	0.45
$C_{15:0}$ 2 OH	0.09	0.08	–	–	0.06
iso-$C_{16:1}$ H	2.04	4.30	6.63	7.40	5.69
iso-$C_{16:0}$	33.47	36.68	28.65	36.74	21.35
anteiso-$C_{16:0}$	0.26	0.48	0.45	0.47	0.57
$C_{16:0}$	5.04	3.36	3.18	2.39	3.02
$C_{16:1}\omega5c$	–	–	–	–	2.22
iso-$C_{17:0}$	6.22	7.65	5.83	3.62	5.46
anteiso-$C_{17:0}$	9.32	11.32	10.21	9.35	5.56
anteiso-$C_{17:1}\omega9c$	–	0.37	0.69	0.41	0.41
$C_{17:1}\omega8c$	5.52	5.53	5.92	8.10	4.00
$C_{17:1}\omega6c$	7.84	11.56	11.19	12.94	11.45
$C_{17:0}$	2.40	1.79	2.16	2.31	0.89
$C_{17:0}$ 10-methyl	–	0.49	0.98	0.92	0.75
$C_{17:0}$ 2 OH	0.09	–	–	0.10	–
iso-$C_{18:0}$	0.20	0.52	0.60	0.85	0.51
iso-$C_{18:1}$ H	–	–	0.21	–	0.25
$C_{18:1}\omega9c$	1.51	3.16	2.33	2.85	1.40
$C_{18:1}\omega5c$	–	–	–	–	0.73
$C_{18:1}$ 2 OH	–	–	–	–	1.33
Sum in feature 1	–	–	0.03	–	–
Sum in feature 3	7.53	6.76	5.68	4.19	15.79
Sum in feature 4	–	–	0.08	–	–
Sum in feature 5	0.71	0.17	0.25	0.19	0.3
Sum in feature 7	–	0.13	–	0.21	–
Sum in feature 8	–	0.15	0.13	–	–
Sum in feature 9	0.46	1.08	1.44	0.94	1.61

Strains 1, KLBMP 1221^T; 2, *A. tucumanensis* ABO^T; 3, *A. eurytherma* NT202^T; 4, *A. methanolica* IMSNU 20055^T; 5, *A. thermoflava* N1165^T. All the data are from this study. Values are percentages of total fatty acids; – not detected

IMSNU 20055^T. Therefore, we propose that strain KLBMP 1221^T should be classified as a novel species, for which the name *Amycolatopsis endophytica* sp. nov. is proposed.

Description of *Amycolatopsis endophytica* sp. nov

Amycolatopsis 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 original isolation from plant tissues).

Gram positive, aerobic, non-motile actinomycete. The aerial mycelium is white and the vegetative mycelium is white to yellow. This strain forms extensively branched substrate mycelia. Aerial hyphae fragment into rod-shaped elements. The spore-like structures presents smooth surface and displays in long straight to flexuous chains. No diffusible pigment is produced. The pH range for growth is 6.0–8.0, with an optimum pH 7.0. The temperature range for growth is 15–45°C, with optimal growth temperature of 25–37°C. The NaCl tolerance range is up to 7%. D-Arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, D-lactose, D-mannitol, D-mannose, D-raffinose, D-rhamnose, D-ribose, sucrose, trehalose and D-xylose are utilized as sole carbon sources. Positive for starch hydrolysis, but negative for nitrate reduction, gelatin liquefaction and H_2S production. Acid is produced from D-arabinose, D-fructose, D-galactose, D-glucose, D-mannose, D-rhamnose and D-ribose. Cell wall hydrolysates contain *meso*-DAP, arabinose and galatose. The major menaquinone is MK-9(H_4). The phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol and two unidentified phospholipids. Major fatty acids (>5%) are iso-$C_{16:0}$ (33.47%), anteiso-$C_{17:0}$ (9.32%), iso-$C_{15:0}$ (8.97%), $C_{17:1}\omega6c$ (7.84%), 16:1 w7c/16:1 w6c (7.53%), iso-$C_{17:0}$ (6.22%), $C_{17:1}\omega8c$ (5.52%) and $C_{16:0}$ (5.04%). The G+C content of the DNA is 73.3 mol%.

The type strain, KLBMP 1221^T (=KCTC 19776^T = CCTCC AA 2010003^T) was isolated from surface-sterilized seeds of *Jatropha curcas* L. collected from the city of Panzhihua, Sichuan Province, south-west China.

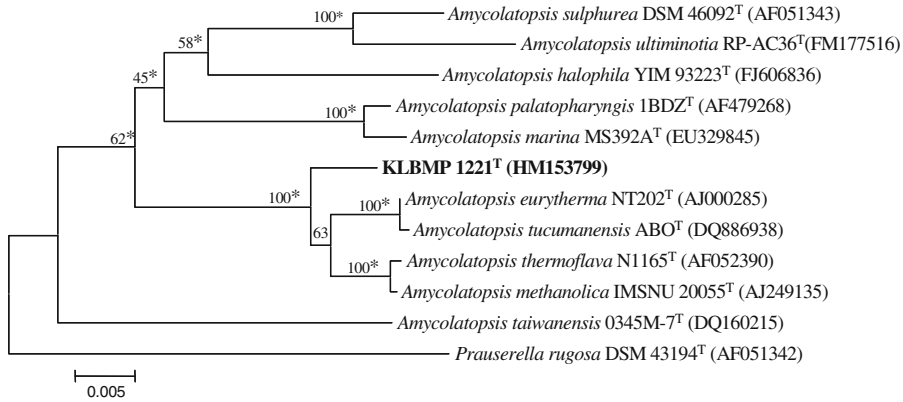

Fig. 2 A neighbour-joining phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain KLBMP 1221^T among members of the genus *Amycolatopsis* species. Numbers on branch nodes are percentage bootstrap values (1000 resamplings). Asterisks indicate the clades that were conserved when neighbour-joining and maximum-parsimony methods were used to construct phylogenetic trees. Outgroup sequence used for analysis was from *Prauserella rugosa*. *Bar* 0.005 substitutions per nucleotide position

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