

Amycolatopsis dongchuanensis sp. nov., an actinobacterium isolated from soil

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A novel actinomycete strain, designated YIM 75904^T, was isolated from a soil sample that had been collected from a dry and hot river valley in Dongchuan county, Yunnan province, south-western China. The taxonomic position of the novel strain was investigated by a polyphasic approach. In phylogenetic analyses based on 16S rRNA gene sequences, strain YIM 75904^T formed a distinct clade within the genus *Amycolatopsis* and appeared to be closely related to *Amycolatopsis sacchari* K24^T (99.3 % sequence similarity). Strain YIM 75904^T had a type-IV cell wall, with no detectable mycolic acids, and had MK-9(H₄) as its predominant menaquinone. Its cell wall contained meso-diaminopimelic acid, galactose, glucose and arabinose, and its major cellular fatty acids were iso-C_{16:0}, iso-C_{15:0}, anteiso-C_{17:0} and anteiso-C_{15:0}. The genomic DNA G + C content of the novel strain was 68.5 mol%. Based on the results of physiological and biochemical tests and DNA–DNA hybridizations, strain YIM 75904^T represents a novel species of the genus *Amycolatopsis* for which the name *Amycolatopsis dongchuanensis* sp. nov. is proposed. The type strain is YIM 75904^T (=CCTCC AA 2011016^T =JCM 18054^T).

Actinomycetes are Gram-staining-positive bacteria, with filamentous growth, high genomic DNA G + C contents and considerable functional diversity, that have been found to be widely distributed in natural and man-made environments (Mann, 2001; Nascimento *et al.*, 2002; Maskey *et al.*, 2003; Qin *et al.*, 2009a). The genus *Amycolatopsis*, which was first proposed by Lechevalier *et al.* (1986), was considered to belong to one actinomycete family: the *Pseudonocardiaceae* (Embley *et al.*, 1988; Warwick *et al.*, 1994). The genus *Amycolatopsis* has been the subject of considerable chemotaxonomic characterization (Henssen *et al.*, 1987; Mertz & Yao, 1993; Yassin *et al.*, 1993; Kim & Goodfellow, 1999). The description of the genus was emended first by Groth *et al.* (2007) and then by Lee (2009). The current members of the genus *Amycolatopsis* have cell walls of type IV and contain complex mixtures of saturated and branched-chain fatty acids. They have no detectable mycolic acids. Their polar

lipid patterns are of type II [*sensu* Lechevalier *et al.* (1986)], with phosphatidylethanolamine as the predominant phospholipid. The predominant menaquinone is generally MK-9(H₄) or MK-11(H₄), although Tang *et al.* (2010) recently identified MK-8(H₄) as the predominant menaquinone of *Amycolatopsis halophila* YIM 93223^T. The genomic DNA G + C contents of the current members of the genus *Amycolatopsis* range from 66 to 73 mol%. Although most *Amycolatopsis* species have been isolated from soil samples (Lee *et al.*, 2006; Chen *et al.*, 2010; Otoguro *et al.*, 2011), some have been isolated from plants (Miao *et al.*, 2011), salt lakes (Tang *et al.*, 2010; Guan *et al.*, 2012), ocean sediments (Bian *et al.*, 2009), clinical samples from humans and horses (Labeda *et al.*, 2003; Huang *et al.*, 2004), sugar cane bagasse (Goodfellow *et al.*, 2001) and a cave, a mine and a catacomb (Lee, 2006; Groth *et al.*, 2007; Carlsohn *et al.*, 2007). At the time of writing, the genus *Amycolatopsis* comprised 53 recognized species.

In the course of research on actinobacterial sources, we obtained many novel isolates from a dry and hot river valley in Dongchuan county, which lies in Yunnan province, in south-western China. The aim of the present study was to determine the taxonomic status of one of these isolates,

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM 75904^T is JN656710.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

designated strain YIM 75904^T, by using a polyphasic approach. The results of phenotypic, chemotaxonomic and phylogenetic analyses indicated that the isolate represents a novel species in the genus *Amycolatopsis*.

For the initial isolation of strain YIM 75904^T, a soil sample was air-dried at room temperature. A subsample (2 g) of the dry soil was placed in a flask with 18 ml sterile water and several glass beads and then shaken (at 200 r.p.m.) for 1 h at 30 °C. A sample (1 ml) of the resultant suspension was diluted 1000-fold with sterile water and then 0.2 ml of the diluted sample was spread on modified ISP (International *Streptomyces* Project; Shirling & Gottlieb, 1966) 5 medium that had been supplemented with nalidixic acid (25 mg l⁻¹) and nystatin (50 mg l⁻¹). After incubation at 28 °C for 1 week, strain YIM 75904^T was obtained. The novel strain was routinely cultured on ISP 2 medium, and stored as a glycerol suspension (20 %, w/v) at -80 °C. The biomass used for the chemical and molecular studies was obtained by cultivation, at 28 °C, in flasks of either ISP 2 liquid medium (Shirling & Gottlieb, 1966) or tryptic soy broth (TSB; at pH 7.2) containing (l⁻¹) 15 g tryptone, 5 g soya peptone and 5 g NaCl. The flasks were shaken at 200 r.p.m. for 1 week before the cells were harvested and freeze-dried.

Genomic DNA was extracted, and the 16S rRNA gene was amplified and sequenced, as described by Li *et al.* (2007). The amplicons were purified using a PCR purification kit (Sangon Biotech). The 16S rRNA gene sequence of the novel strain was then compared with the corresponding sequences of cultured species by using a BLAST search (Altschul *et al.*, 1990) and EzTaxon-e server (Kim *et al.*, 2012). Multiple alignments were performed using the CLUSTAL_X software package (Thompson *et al.*, 1997). Evolutionary distances were calculated by use of the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms. Phylogenetic and molecular evolutionary analyses were conducted using version 5.0 of the MEGA software package (Tamura *et al.*, 2011) and the PHYML package (Guindon & Gascuel, 2003). The topology of each tree was evaluated by bootstrap analysis (Felsenstein, 1985) with 1000 replications, using the sequence from *Prauserella rugosa* DSM 43194^T as the outgroup.

The almost-complete 16S rRNA gene sequence (1488 nt) of strain YIM 75904^T was determined. Comparison of the sequence with the corresponding 16S rRNA gene sequences retrieved from GenBank/EMBL/DDBJ clearly demonstrated that strain YIM 75904^T belongs to the genus *Amycolatopsis*. Phylogenetic analyses showed that strain YIM 75904^T formed a monophyletic clade with the type strain of *A. sacchari* K24^T, which shares 99.3 % 16S rRNA gene sequence similarity. Sequence similarities between the isolate and all other members of the genus *Amycolatopsis* were 95.4–97.1 % (Fig. 1). Strain YIM 75904^T formed a

stable clade with its closest neighbour (*A. sacchari* K24^T), which was supported by a bootstrap value of 100 % and separate from the other species in the genus *Amycolatopsis*. Moreover, an apparent distinct subclade was also displayed in other phylogenetic trees reconstructed using minimum-evolution phylogenetic, maximum-parsimony phylogenetic and maximum-likelihood algorithms, with bootstrap support values of 100, 100 and 99 %, respectively (data not shown).

Morphological, cultural, physiological and biochemical characterizations of strain YIM 75904^T were carried out following the guidelines of the ISP (Shirling & Gottlieb, 1966). Cell motility was confirmed by the development of turbidity throughout a tube of semi-solid medium (Leifson, 1960). Cultural characteristics were tested on ISP 2, oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), potato-glucose agar, Czapek's agar and nutrient agar (Shirling & Gottlieb, 1966; Waksman, 1967). The colours of the aerial and substrate mycelia and any diffusible pigments produced on these media were recorded as described by Dong & Cai (2001), with chips from the ISCC-NBS colour charts (Kelly, 1964) used for reference. Morphological characteristics were investigated under a light microscope and in a scanning electron microscope (XL30 ESEM-TMP, Philips) using cells that had been incubated on ISP 2 medium, at 28 °C, for 7–21 days. Growth at various temperatures (4, 10, 15, 20, 28, 37, 45, 50 and 55 °C), at various pH values (pH 4.0–10.0, at intervals of 1.0 pH unit and at 28 °C) and with 0–20 % (w/v) NaCl (at intervals of 1 % and at 28 °C) was tested on ISP 2 plates. The buffers used (each at 0.1 M) to alter the initial pH of the medium were citric acid/sodium citrate (pH 4.0–5.0), KH₂PO₄/NaOH (pH 6.0–8.0) and NaHCO₃/Na₂CO₃ (pH 9.0–10.0). Utilization of compounds as sole carbon sources was observed in basal ISP 9 medium (Shirling & Gottlieb, 1966) supplemented with the test compound. Nitrogen source utilization was investigated by using a basal liquid medium containing (l⁻¹) 1 g D-glucose, 0.05 g MgSO₄·7H₂O, 0.05 g NaCl, 0.001 g FeSO₄·7H₂O and 0.01 g K₂HPO₄, at pH 7.2. The methods described by Williams *et al.* (1989) and Gordon *et al.* (1974) were used to test the novel strain for the degradation of Tweens, starch and cellulose, gelatin liquefaction, nitrate reduction, urease, oxidase and catalase activities, and milk peptonization and coagulation.

Cells of strain YIM 75904^T were Gram-reaction-positive, aerobic and non-motile. Substrate mycelia were well-developed and branched, fragmenting into long, rod-like elements. Aerial mycelia comprised abundant, long rod-like elements that differentiated into ovoid spore-like structures at maturity (Fig. S1, available in IJSEM Online). On all but Czapek's agar, the substrate and aerial mycelia had the same colour (Table S1). Such mycelia developed well on nutrient agar, potato-glucose agar and ISP 2 medium, moderately well on Czapek's, ISP 3 and ISP 5 agars, and poorly on ISP 4 agar. Strain YIM 75904^T grew well at 10–45 °C (optimum 28 °C), at pH 6.0–8.0

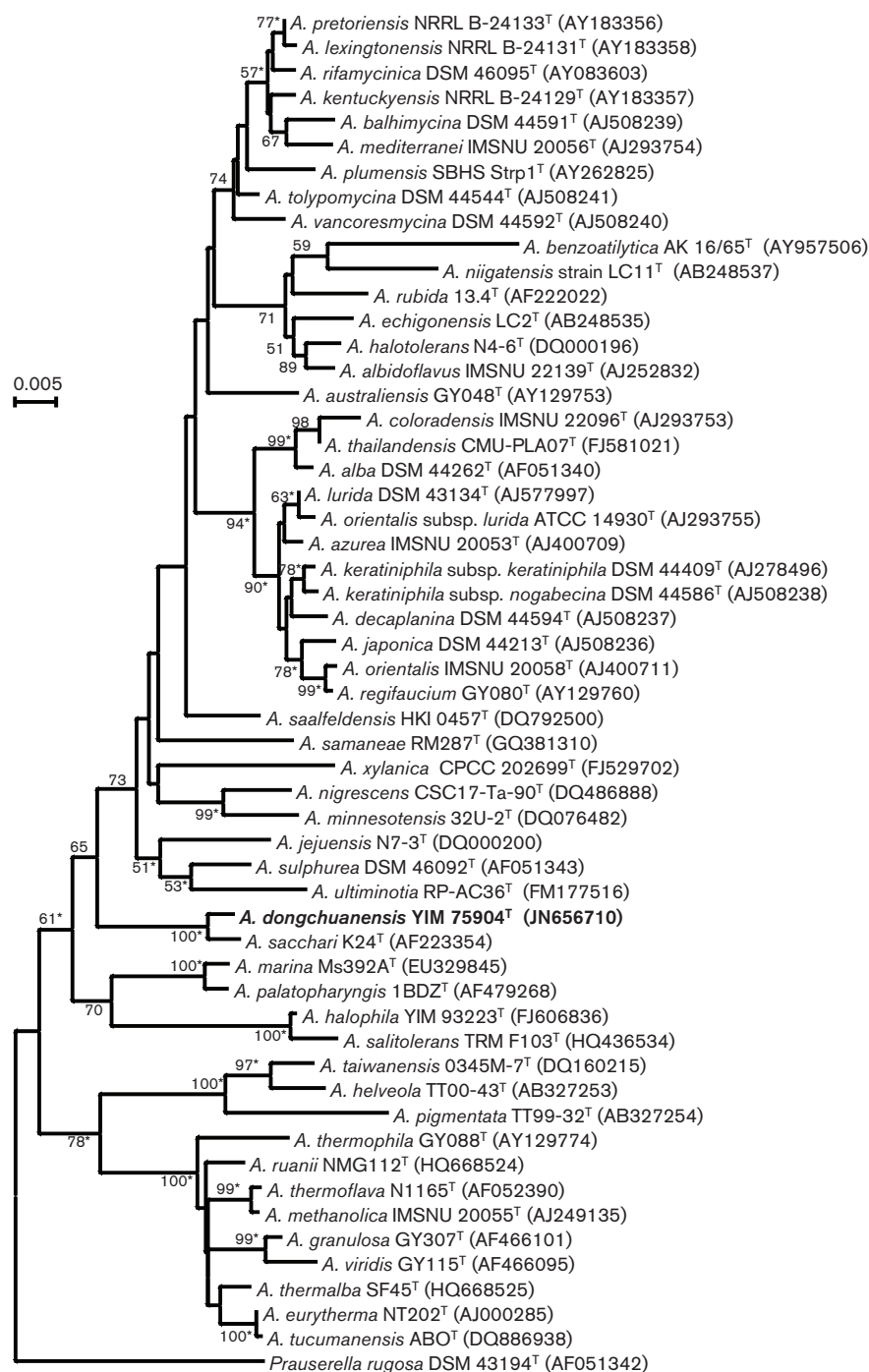


Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain YIM 75904^T and members of the genus *Amycolatopsis*. Asterisks indicate branches that were also recovered using both the maximum-parsimony and maximum-likelihood methods. Bootstrap values >50% (based on 1000 resamplings) are shown at branch points. *Prauserella rugosa* DSM 43194^T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.

(optimum between pH 7.0 and pH 8.0) and with 0–7% (w/v) NaCl (optimum between 0% and 5%). The strain was positive for urease and catalase activities, milk coagulation, milk peptonization, reduction of nitrate and the hydrolysis

of starch and Tweens 20, 40, 60 and 80 but negative for oxidase activity, the degradation of cellulose and gelatin, H₂S production and melanin formation. Although strain YIM 75904^T had morphological characteristics that are

Table 1. Characteristics that can be used to distinguish strain YIM 75904^T from *Amycolatopsis sacchari* DSM 44467^T

Strains: 1, YIM 75904^T; 2, *A. sacchari* DSM 44467^T. All data are from this study and obtained using cells from the two strains that were produced under identical growth conditions. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2
Utilization as sole carbon source:		
Cellulose	–	+
Glycerol	–	+
Maltose	w	+
D-Mannitol	–	+
Sodium acetate	–	w
Sucrose	+	–
Trehalose	–	+
D-Xylitol	–	+
Acid produced from:		
L-Arabinose	–	+
Cellulose	–	+
D-Galactose	–	+
Lactose	–	+
Maltose	–	+
D-Mannitol	–	+
L-Rhamnose	–	+
Sorbitol	–	+
Sucrose	–	+
Trehalose	–	+
D-Xylose	–	+
Utilization as sole nitrogen source:		
Adenine	–	+
L-Lysine	–	+
Degradation of starch	+	–
Growth at/in:		
pH 9.0	+	–
12 °C	+	w
7 % (w/v) NaCl	+	–

typical of members of the genus *Amycolatopsis*, it also displayed many phenotypic properties that distinguished it from *A. sacchari*, its closest known relative (see Table 1 and the species description).

The genomic DNA G+C content of the novel strain was determined by HPLC (Mesbah *et al.*, 1989), with *E. coli* JM-109 as the reference strain. Analyses of the isomer of diaminopimelic acid and the sugars in whole-cell hydrolysates were performed as described by Stanek & Roberts (1974), Saddler *et al.* (1991) and Tang *et al.* (2009a, b). Biomass for the cellular fatty acid analysis was produced by incubating the novel strain in TSB medium at 28 °C for 3 days. The cellular fatty acids were extracted, methylated and analysed by using the protocol of version 6.1 of the Sherlock Microbial Identification System (MIDI) and the TSBA6 database (Sasser, 1990). Menaquinones, extracted from lyophilized cells as described by Minnikin *et al.*

(1984), were purified and analysed by HPLC (Kroppenstedt, 1982). Polar lipids were extracted, and examined and identified by two-dimensional TLC, using the procedures of Minnikin *et al.* (1979, 1984). Mycolic acids were investigated by one-dimensional TLC, as described by Minnikin *et al.* (1980).

Whole-cell hydrolysates of strain YIM 75904^T contained *meso*-diaminopimelic acid, galactose, glucose, arabinose and small amounts of mannose. The major cellular fatty acids were iso-C_{16:0} (19.30 %), iso-C_{15:0} (19.02 %), anteiso-C_{17:0} (14.58 %) and anteiso-C_{15:0} (11.55 %). Detailed fatty acid profiles of strain YIM 75904^T and *A. sacchari* K24^T are given in Table S2. The predominant menaquinone was MK-9(H₄). The polar lipid profile comprised diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, two unidentified glycolipids and three unidentified phospholipids (Fig. S2). No mycolic acids were detected. The genomic DNA G+C content of strain YIM 75904^T was 68.5 mol%.

To determine the genomic relatedness between strain YIM 75904^T and its closest known relative, *A. sacchari* K24^T, DNA–DNA hybridization tests were carried out by the fluorometric micro-well method (Ezaki *et al.*, 1989; Christensen *et al.*, 2000; He *et al.*, 2005). The hybridizations were performed, with six replications, at the optimal hybridization temperature (47 °C). The mean level of DNA–DNA relatedness observed between strain YIM 75904^T and *A. sacchari* K24^T (45.7 %) fell well below the threshold value (70 %) that might indicate that the two strains belonged to the same species (Stackebrandt & Goebel, 1994).

Although strain YIM 75904^T grew well with 7 % (w/v) NaCl, at pH 9.0 and at 12 °C, no growth of *A. sacchari* K24^T could be detected under any of these conditions. Strain YIM 75904^T formed yellow, orange or yellow–brown aerial mycelia, had galactose, glucose and arabinose as its major whole-cell sugars, had a small amount of mannose in its cell wall, and had iso-C_{16:0}, iso-C_{15:0}, anteiso-C_{17:0} and anteiso-C_{15:0} as its major cellular fatty acids. In contrast, under the same conditions, *A. sacchari* K24^T formed white aerial mycelia, had arabinose and galactose as its major whole-cell sugars and had iso-C_{16:0}, C_{16:0} and C_{17:1ω6c} as its major cellular fatty acids. Based on the phenotypic, phylogenetic and chemotaxonomic evidence and the results of the DNA–DNA hybridizations, strain YIM 75904^T represents a novel species of the genus *Amycolatopsis* for which the name *Amycolatopsis dongchuanensis* sp. nov. is proposed.

Description of *Amycolatopsis dongchuanensis* sp. nov.

Amycolatopsis dongchuanensis (dong.chu.an.en'sis. N.L. fem. adj. *dongchuanensis* pertaining to the Dongchuan county of Yunnan province in south-western China, the source of the soil from which the type strain was isolated).

Cells are Gram-reaction-positive and aerobic and form well-developed aerial and substrate mycelia on several agars. Substrate mycelia are fragmented into long, rod-like elements. Aerial mycelia comprise long rod-like elements that produce ovoid, spore-like structures at maturity. Substrate and aerial mycelia may be yellow, orange or yellow-brown, depending on the medium used. No diffusible pigment is produced. Growth occurs at 10–45 °C (optimum 28 °C), at pH 6.0–8.0 (optimum between pH 7.0 and pH 8.0) and with 0–7 % (w/v) NaCl. Positive for urease and catalase activities but negative for oxidase activity. Positive for gelatin liquefaction, milk peptonization and coagulation and nitrate reduction but negative for melanin formation. Degrades Tweens 20, 40, 60, 80 and starch but not cellulose. H₂S is not produced. Acid is produced from D-fructose, D-glucose and D-mannose. Utilizes L-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, L-rhamnose, succinic acid, sucrose, trisodium citrate and D-xylose as sole carbon and energy sources but not cellobiose, glycerol, D-mannitol, raffinose, sodium acetate, sorbitol, trehalose or D-xylitol. Utilizes adenine, L-alanine, L-asparagine, L-cysteine, cystine, glycine, L-histidine, hypoxanthine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine and xanthine as sole nitrogen sources but not adenine, L-arginine, L-glutamic acid or L-lysine. Has a cell wall of type IV. Whole-cell hydrolysates contain meso-diaminopimelic acid, galactose, glucose, arabinose and a small amount of mannose. MK-8(H₄) is the predominant menaquinone. The major fatty acids are iso-C_{16:0}, iso-C_{15:0}, anteiso-C_{17:0} and anteiso-C_{15:0}. The polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, two unidentified glycolipids and three unidentified phospholipids. Mycolic acids appear to be absent.

The type strain, YIM 75904^T (=CCTCC AA 2011016^T =JCM 18054^T), was isolated from a soil sample collected from a dry and hot river valley in Dongchuan county, Yunnan province, south-western China. The genomic DNA G+C content of the type strain is 68.5 mol%.

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