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

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A novel actinomycete strain, designated YIM 75904T, 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 75904T formed a distinct clade within the genus Amycolatopsis and appeared to be closely related to Amycolatopsis sacchari K24T (99.3% sequence similarity). Strain YIM 75904T 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-C16:0, iso-C15:0, anteiso-C17:0 and anteiso-C15:0. 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 75904T represents a novel species of the genus Amycolatopsis for which the name Amycolatopsis dongchuanensis sp. nov. is proposed. The type strain is YIM 75904T (=CCTCC AA 2011016T =JCM 18054T).

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 Pseudonocardiaeae (Embley et al., 1988; Warwick et al., 1994). The genus Amycolatopsis has been the subject of considerable chemotaxonomic characterization (Hensen 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 93223T. 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 (Labeleda 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,
designated strain YIM 75904\textsuperscript{7}, 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\textsuperscript{7}, 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\textsuperscript{-1}) and nystatin (50 mg l\textsuperscript{-1}). After incubation at 28 °C for 1 week, strain YIM 75904\textsuperscript{7} 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\textsuperscript{-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\textsuperscript{T} as the outgroup.

The almost-complete 16S rRNA gene sequence (1488 nt) of strain YIM 75904\textsuperscript{7} was determined. Comparison of the sequence with the corresponding 16S rRNA gene sequences retrieved from GenBank/EMBL/DDBJ clearly demonstrated that strain YIM 75904\textsuperscript{7} belongs to the genus *Amycolatopsis*. Phylogenetic analyses showed that strain YIM 75904\textsuperscript{7} formed a monophyletic clade with the type strain of *A. sacchari* K24\textsuperscript{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\textsuperscript{7} formed a stable clade with its closest neighbour (*A. sacchari* K24\textsuperscript{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\textsuperscript{7} 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), KH\textsubscript{2}PO\textsubscript{4}/NaOH (pH 6.0–8.0) and NaHCO\textsubscript{3}/Na\textsubscript{2}CO\textsubscript{3} (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\textsuperscript{-1}) 1 g D-glucose, 0.05 g MgSO\textsubscript{4}.7H\textsubscript{2}O, 0.05 g NaCl, 0.001 g FeSO\textsubscript{4}.7H\textsubscript{2}O and 0.01 g K\textsubscript{2}HPO\textsubscript{4}, 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 Tweenes, starch and cellulose, gelatin liquefaction, nitrate reduction, urease, oxidase and catalase activities, and milk peptonization and coagulation.

Cells of strain YIM 75904\textsuperscript{7} 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 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 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 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 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 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 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 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).
(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 75904ᵀ had morphological characteristics that are

![Phylogenetic tree](image-url)

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain YIM 75904ᵀ 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 43194ᵀ was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.
Table 1. Characteristics that can be used to distinguish strain YIM 75904T from Amycolatopsis sacchari DSM 44467T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<tr>
<td>Utilization as sole carbon source:</td>
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<tr>
<td>Cellobiose</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Glycerol</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Maltose</td>
<td>w</td>
<td>+</td>
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<tr>
<td>d-Mannitol</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Sodium acetate</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Trehalose</td>
<td>–</td>
<td>+</td>
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<tr>
<td>d-Xyitol</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Acid produced from:</td>
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<tr>
<td>L-Arabinose</td>
<td>–</td>
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<tr>
<td>Cellobiose</td>
<td>–</td>
<td>+</td>
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<tr>
<td>d-Galactose</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Lactose</td>
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<td>Maltose</td>
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<tr>
<td>d-Mannitol</td>
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<td>+</td>
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<tr>
<td>L-Rhamnose</td>
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<tr>
<td>Sorbitol</td>
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<tr>
<td>Sucrose</td>
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<td>Trehalose</td>
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<tr>
<td>d-Xylose</td>
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<td>+</td>
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<tr>
<td>Utilization as sole nitrogen source:</td>
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<td></td>
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<tr>
<td>Adenine</td>
<td>–</td>
<td>+</td>
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<tr>
<td>L-Lysine</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Degradation of starch</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Growth at/in:</td>
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<tr>
<td>pH 9.0</td>
<td>+</td>
<td>–</td>
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<tr>
<td>12 °C</td>
<td>+</td>
<td>w</td>
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<tr>
<td>7 % (w/v) NaCl</td>
<td>+</td>
<td>–</td>
</tr>
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</table>

Strains: 1, YIM 75904T; 2, A. sacchari DSM 44467T. 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.

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 Staneck & 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 75904T contained meso-diaminopimelic acid, galactose, glucose, arabinose and small amounts of mannose. The major cellular fatty acids were iso-C16:0 (19.30 %), iso-C15:0 (19.02 %), anteiso-C17:0 (14.58 %) and anteiso-C15:0 (11.55 %). Detailed fatty acid profiles of strain YIM 75904T and A. sacchari K24T are given in Table S2. The predominant menaquinone was MK-9(H4). The polar lipid profile comprised diphasphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxy-phosphatidylethanolamine, 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 75904T was 68.5 mol%.

To determine the genomic relatedness between strain YIM 75904T and its closest known relative, A. sacchari K24T, 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 75904T and A. sacchari K24T (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 75904T grew well with 7 % (w/v) NaCl, at pH 9.0 and at 12 °C, no growth of A. sacchari K24T could be detected under any of these conditions. Strain YIM 75904T 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-C16:0, iso-C15:0, anteiso-C17:0 and anteiso-C15:0 as its major cellular fatty acids. In contrast, under the same conditions, A. sacchari K24T formed white aerial mycelia, had arabinose and galactose as its major whole-cell sugars and had iso-C16:0, C16:0 and C17:1ω9c as its major cellular fatty acids. Based on the phenotypic, phylogenetic and chemotaxonomic evidence and the results of the DNA–DNA hybridizations, strain YIM 75904T 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. Degradates Tween 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, succrose, trisodium citrate and D-xylene as sole carbon and energy sources but not cellulose, glycerol, D-mannitol, raffinose, sodium acetate, sorbitol, trehalose or D-xyritol. 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 xanithine 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-diaminopimelate, galactose, glucose, arabinose and a small amount of mannose. MK-8(H4) is the predominant menaquinone. The major fatty acids are iso-C16:0, iso-C15:0 anteiso-C17:0 and anteiso-C15:0. The polar lipids comprise diphosphatidyglycerol, phosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside, two unidentified glycolipids and three unidentified phospholipids. Mycolic acids appear to be absent.

The type strain, YIM 75904T (=CCTCC AA 2011016T =JCM 18054T), 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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