Amycolatopsis australiensis sp. nov., an actinomycete isolated from arid soils

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The taxonomic position of a group of mesophilic actinomycetes isolated from arid Australian soils was determined using a polyphasic approach. The organisms shared chemical and morphological markers typical of members of the genus *Amycolatopsis*. They had identical 16S rRNA gene sequences and formed a distinct phyletic line in the *Amycolatopsis mediterranei* clade, being most closely related to *A. mediterranei*. In addition, they shared a range of phenotypic properties that distinguished them from representatives of all of the species classified in this clade. The combined genotypic and phenotypic data indicate that the strains merit species status within the genus *Amycolatopsis*. The name proposed for the novel species is *Amycolatopsis australiensis* sp. nov.; the type strain is GY048^T (=DSM 44671^T=NCIMB 14142^T).

The genus *Amycolatopsis* was proposed by Lechevalier *et al.* (1986) for aerobic, filamentous actinomycetes that contained *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), phosphatidylethanolamine as the diagnostic polar lipid (phospholipid type 2 *sensu* Lechevalier *et al.*, 1977) and di- and tetra-hydrogenated menaquinones with nine isoprene units as the predominant isoprenologues, but lacked mycolic acids. The genus currently encompasses 31 species with validly published names, which form a distinct phyletic line within the 16S rRNA gene tree for the *Pseudonocardiaceae* (Lee, 2006). *Amycolatopsis* species fall into several multi-membered 16S rRNA gene groups, such as the *Amycolatopsis methanolica* and *Amycolatopsis orientalis* clades (Kim *et al.*, 2002; Saintpierre-Bonaccio *et al.*, 2005); the type strain of *Amycolatopsis fastidiosa* forms a single-membered phyletic line at the foot of the *Amycolatopsis* tree (Goodfellow *et al.*, 2001; Huang *et al.*, 2001, 2004).

Amycolatopsis strains can be distinguished from members of the other genera classified in the family *Pseudonocardiaceae* by using genus-specific oligonucleotide primers (Tan *et al.*, 2006) and chemical and morphological markers (Kim & Goodfellow, 1999). Similarly, a range of phenotypic properties are available for the identification of

Amycolatopsis strains (Goodfellow *et al.*, 2001; Wink *et al.*, 2003; Saintpierre-Bonaccio *et al.*, 2005). Members of the genus have been isolated from vegetable matter (Lechevalier *et al.*, 1986) and clinical material (Labeda *et al.*, 2003; Huang *et al.*, 2004) but it is evident from the application of improved selective isolation and characterization procedures that they are common in soil (Tan *et al.*, 2006). *Amycolatopsis* strains are a rich source of secondary metabolites, including ansamycins (rifamycins and tolypomycins) and glycopeptides (balhimycins and vancomycins). Their commercial significance has led to an intensive search of *Amycolatopsis* strains for sources of novel pharmaceutical activities – an activity that has promoted a better understanding of the taxonomic structure of the genus (Wink *et al.*, 2003).

The present polyphasic study was designed to establish the taxonomic status of seven *Amycolatopsis* isolates, of which three, strains GY048^T, GY056 and GY064, form a distinct lineage in the *Amycolatopsis* 16S rRNA gene tree (Tan *et al.*, 2006). The resultant data show that all of the isolates represent a novel species of the genus *Amycolatopsis*.

Strains GY048^T, GY049, GY056, GY064, GY269, GY276 and GY286 were isolated on SM2 agar plates that had been inoculated with suspensions of a composite arid Australian soil sample and incubated at 28 °C for 3 weeks, as described by Tan *et al.* (2006). The organisms were maintained on modified Bennett's agar slants (Jones, 1949) at room temperature and as suspensions of mycelial fragments in glycerol (20 %, v/v) at –20 °C. Biomass samples for the molecular systematic studies were prepared by growing the

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains GY048^T, GY049, GY056, GY064, GY269, GY276 and GY286 are AY129753, AY129754, AY129757, AY129759, AY129770, AY129771 and AY129772, respectively.

isolates in glucose/yeast extract broth shake flasks for 3 days at 28 °C. Cultures were checked for purity, killed by shaking with formalin (1 %, v/v) and harvested by centrifugation. Cells were washed in NaCl/EDTA buffer (0.1 M EDTA, pH 8.0, 0.1 M NaCl) and stored at -20 °C until needed.

Extraction of chromosomal DNA, PCR amplification and sequencing of 16S rRNA genes from isolates GY049, GY269, GY276 and GY286 were achieved using procedures described previously (Kim *et al.*, 2002; Tan *et al.*, 2006). The resultant 16S rRNA gene sequences were aligned manually, using the PHYDIT program (available at <http://plaza.snu.ac.kr/~jchun/phydit/>), against corresponding sequences of representatives of genera classified in the family *Pseudonocardiaceae* (data not shown) and of type species of the genus *Amycolatopsis* (as retrieved from the GenBank database). Unrooted trees were inferred by using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms. Evolutionary distance matrices were generated for the least-squares and neighbour-joining algorithms after Jukes & Cantor (1969). The analyses were carried out using the PHYLIP suite of programs (Felsenstein, 1993) and the topologies of the resultant trees were

evaluated by using a bootstrap analysis (Felsenstein, 1985) of the neighbour-joining dataset with the CONSENSE and SEQBOOT programs from the PHYLIP package. The root position of the unrooted *Amycolatopsis* tree based on the neighbour-joining method was estimated by using *Pseudonocardia thermophila* ATCC 19285^T (GenBank accession no. X53195) as the outgroup.

It is evident from Fig. 1 that the strains tested share identical (100 % similarity) 16S rRNA gene sequences both with each other and with isolates GY048^T, GY056 and GY064. These strains form a distinct phyletic line within the *Amycolatopsis mediterranei* clade, a taxon that encompasses the type strains of nine *Amycolatopsis* species with validly published names; the taxonomic integrity of this clade was supported by the results from all of the tree-making algorithms and by a bootstrap value of 86 % in the neighbour-joining analysis. The isolates showed the highest level of 16S rRNA gene similarity with the type strain of *A. mediterranei* (98.6 %, a value that corresponded to 20 nucleotide differences at 1411 locations). Relatively high similarity values were shown with the type strains of *Amycolatopsis balhimycina* (97.8 %), *Amycolatopsis kentuckyensis* (98.2 %), *Amycolatopsis lexingtonensis* (98.0 %), *Amycolatopsis plumensis*

Fig. 1. Neighbour-joining (Saitou & Nei, 1987) phylogenetic tree, based on almost-complete 16S rRNA gene sequences (≥ 1460 nt), showing the relationships between the novel strains and representatives of the genus *Amycolatopsis*. Asterisks indicate branches of the tree that were also found using the least-squares (Fitch & Margoliash, 1967) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 0.02 substitutions per nucleotide position.

(98.0 %), *Amycolatopsis pretoriensis* (98.2 %), *Amycolatopsis rifamycinica* (98.2 %), *Amycolatopsis tolypomycina* (98.5 %) and *Amycolatopsis vancoremsycina* (98.1 %).

DNA–DNA relatedness studies were not carried out between isolate GY048^T and its closest phylogenetic neighbours, as some of the latter have almost identical 16S rRNA gene similarity values but show DNA–DNA hybridization scores well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the circumscription of strains that belong to the same genomic species. The type strains of *A. lexingtonensis* and *A. pretoriensis*, for example, share a 16S rRNA gene similarity of 99.9 % and a DNA–DNA relatedness value of 54.1 % (Labeda *et al.*, 2003). Similarly, the type strain of *A. rifamycinica* has only six mismatches (99.6 % similarity) with respect to its closest neighbour, *A. kentuckyensis*, but shows a DNA–DNA hybridization value of only 67 % with the latter (Bala *et al.*, 2004).

Isolate GY048^T was examined for key chemical markers to determine whether it had a chemical profile consistent with its classification in the genus *Amycolatopsis*. Biomass for the chemotaxonomic studies was prepared by growing the isolate in glucose/yeast extract broth shake flasks (150 r.p.m.) for 3 days at 28 °C; the cells were washed in distilled water and then freeze-dried. Standard HPLC and TLC procedures were used to determine the predominant menaquinones (Collins, 1994), the type of muramic acid residue (Uchida *et al.*, 1999), the major polar lipids (Minnikin *et al.*, 1984) and the predominant whole-organism sugars (Schaal, 1985) of strain GY048^T. The control organisms were *A. orientalis* KCTC 9412^T, *Gordonia bronchialis* N654^T and *Nocardia brasiliensis* N318^T.

Strain GY048^T, which is known to contain *meso*-diaminopimelic acid but which lacks mycolic acids (Tan *et al.*, 2006), contained arabinose and galactose in whole-organism hydrolysates, contained *N*-acetylated muramic acid, possessed diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylmethylethanolamine as the major polar lipids (phospholipid pattern type 2 *sensu* Lechevalier *et al.*, 1977) and contained tetrahydrogenated menaquinone with nine isoprene units [MK-9(H₄)] as the predominant isoprenologue (66 % of the total menaquinone composition) and smaller proportions of MK-8(H₂) (26 % of the total) and MK-9(H₆) (8 % of the total). These chemical markers serve to distinguish strain GY048^T from all other actinomycetes with wall chemotype IV, apart from those classified in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Embley *et al.*, 1988; Yassin *et al.*, 1991, 1993; Kim & Goodfellow, 1999).

Crude extracts of the isolates were prepared by methanol extraction of agar cultures grown on modified Bennett's agar (Jones, 1949) and yeast extract-malt extract agar (ISP 2; Shirling & Gottlieb, 1966) at 28 °C for 7 days. Antibacterial and anti-yeast activities were assessed using *Bacillus subtilis* ATCC 6633 and *Saccharomyces cerevisiae* ATCC 9763, respectively. All strains produced extracts that exhibited low

titres of antibacterial or anti-yeast activity when grown on either medium.

The morphological properties of the isolates were observed, using a Nikon Optiphot light microscope fitted with a long-working-distance objective, after growth on modified Bennett's agar (Jones, 1949) supplemented with mannitol (0.5 %, w/v) and soybean flour (0.5 %, w/v), and after growth on tyrosine agar (ISP 7; Shirling & Gottlieb, 1966) for 14 days at 28 °C. In addition, a range of phenotypic tests were carried out using media and methods described previously (de Boer *et al.*, 1990; Goodfellow *et al.*, 1997, 2001). The strains shared a combination of phenotypic properties that serve to distinguish them from the type strains of *Amycolatopsis* species classified in the *A. mediterranei* clade (Table 1).

It is evident from the genotypic and phenotypic data that the seven isolates form a homogeneous taxon that can be distinguished from representatives of all of the *Amycolatopsis* species with validly published names. This taxon should be recognized as a novel *Amycolatopsis* species, for which the name *Amycolatopsis australiensis* sp. nov. is proposed.

Description of *Amycolatopsis australiensis* sp. nov.

Amycolatopsis australiensis (aus.tra.li.en'sis. N.L. fem. adj. *australiensis* pertaining to Australia, the source of the soil from which the first strains were isolated).

Aerobic, Gram-positive, non-acid–alcohol-fast, non-motile actinobacteria that form extensively branched substrate mycelia that fragment into squarish, rod-shaped elements. Abundant, white aerial hyphae and pale-yellow substrate mycelia are formed on modified Bennett's agar supplemented with mannitol and soybean flour; a medium-yellow diffusible pigment is evident on this medium. Melanin pigments are not produced on peptone-yeast extract-iron or tyrosine agars. Good growth occurs between 28 and 45 °C, and from pH 5 to 7. Nitrate is reduced to nitrite; elastin, Tween 40 and uric acid are degraded. D(+)-Arabitol, D(+)-cellobiose, dextrin, D(+)-galactose, D(+)-glucose, glycerol, glycogen, *myo*-inositol, α -lactose, D(+)-maltose, D(+)-mannitol, D(+)-melezitose, D(+)-raffinose, sucrose, D(+)-trehalose, xylitol, D(+)-xylose (all at 1 %, w/v) and citric acid, oxalic acid and propionic acid (all at 0.1 %, w/v) are used as sole carbon sources, but adonitol, *meso*-erythritol, D(+)-melibiose, methyl α -D-glucoside, D(+)-ribose, D(–)-sorbitol (all at 1 %, w/v) and sodium benzoate, sodium lactate and sodium mucate (at 0.1 %, w/v) are not. Acid is formed from D(+)-arabitol, D(+)-glucose, glycerol, glycogen, D(+)-mannose and D(+)-turanose, but not from dulcitol, D(+)-ribose or xylitol. The organism is sensitive to novobiocin (10 μ g ml^{–1}), penicillin G (20 μ g ml^{–1}), rifampicin (10 μ g ml^{–1}) and streptomycin sulphate (16 μ g ml^{–1}), but resistant to gentamicin sulphate (5 μ g ml^{–1}), neomycin

Table 1. Characteristics that separate the novel strains from representatives of *Amycolatopsis* species classified in the *A. mediterranei* 16S rRNA gene clade

Strains: 1, strains GY048^T, GY049, GY056, GY064, GY269, GY276 and GY286 (number of strains testing positive is given); 2, *A. balhimycina* DSM 44591^T; 3, *A. kentuckyensis* NRRL B-24129^T; 4, *A. lexingtonensis* NRRL B-24131^T; 5, *A. mediterranei* DSM 13685^T; 6, *A. orientalis* IMSNU 20058^T; 7, *A. plumensis* DSM 44776^T; 8, *A. pretoriensis* NRRL B-24133^T; 9, *A. rifamycinica* DSM 46095^T; 10, *A. tolypomycina* DSM 44544^T; 11, *A. vancoresmycina* DSM 44592^T. Data for type strains were taken from Bala *et al.* (2004), Kim *et al.* (2002), Labeda *et al.* (2003), Saintpierre-Bonaccio *et al.* (2005) and Wink *et al.* (2003). The novel strains gave variable responses to the following tests: acid from dextrin (4 positive), D(+)-maltose (5 positive), D(+)-raffinose (3 positive), sucrose (5 positive) and D(+)-trehalose (4 positive); decomposition of hypoxanthine (5 positive); allantoin hydrolysis (4 positive) and growth at 10 °C (4 positive).+, Positive; +w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Colour of aerial mycelium*	W (7)	W	YO/BO	OB/RB	W	W	PO	GY/OB	WP	W	W
Production of soluble pigment	7	–	+	+	–	–	+	+	+	–	+
Acid production from:											
Adonitol	0	ND	+	+	+	+	+	–	+	–	–
L(+)-Arabinose	7	+	+	+	–	+	+	+	+	+	+
D(+)-Cellobiose	7	ND	+	+	+	+	+	+	+	+	+
D(+)-Fructose	6	+	+	+	+	+	+	+	+	+	+
D(+)-Galactose	7	ND	+	+	+	+	–	+	+	+	+
myo-Inositol	6	+	+	+	–	+	ND	+	+	+	+
D(+)-Lactose	6	ND	+	+	–	+	–	+	–	–	–
D(+)-Mannitol	6	+	–	+W	+	+	+	–	–	+	+
D(+)-Melibiose	0	+	+	+	–	–	–	+	ND	–	–
D(–)-Sorbitol	0	–	+	–	+	–	–	+W	–	–	–
D(+)-Xylose	7	–	+	+	+	+	–	+	+	–	–
Decomposition of:											
Aesculin	7	ND	+	+	+	+	ND	+	+	ND	ND
Casein	7	ND	+	+	+	+	+	+	+	+	–
Gelatin	7	ND	+	+	+	+	+	+	+	+	–
Xanthine	0	–	–	–	–	+	–	–	–	+	–
Growth in/at:											
45 °C	7	–	+	+	–	–	–	–	–	+	–
5 % (w/v) NaCl	0	ND	+	+	+W	+W	–	+	+W	+	+
Production of:											
Amylase	7	–	–	–	–	+	+	–	–	–	–
Nitrate reductase	7	–	–	+	–	+	ND	–	ND	+	–
Urease	0	+	+	+	+	+	+	+W	+	+	+

*BO, Brownish orange; GY, greyish yellow; OB, orange–brown; PO, pale orange; RB, reddish brown; W, white; WO, white to olive buff; WP, white to pale pink; YO, yellow–orange.

sulphate (8 µg ml^{–1}), polymyxin B sulphate (50 µg ml^{–1}) and tobramycin sulphate (8 µg ml^{–1}). Activity is shown against *B. subtilis* ATCC 6633 and *S. cerevisiae* ATCC 9763 but not against *Candida albicans* ATCC 10231. Additional phenotypic properties are shown in Table 1.

The type strain, GY048^T (=DSM 44671^T=NCIMB 14142^T), was isolated from arid Australian soils.

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