Amycolatopsis thermophila sp. nov. and *Amycolatopsis viridis* sp. nov., thermophilic actinomycetes isolated from arid soil

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The taxonomic positions of two thermophilic actinomycetes isolated from an arid Australian soil sample were established based on an investigation using a polyphasic taxonomic approach. The organisms had chemical and morphological properties typical of members of the genus *Amycolatopsis* and formed distinct phyletic lines in the *Amycolatopsis methanolica* 16S rRNA subclade. The two organisms were distinguished from one another and from the type strains of related species of the genus *Amycolatopsis* using a range of phenotypic properties. Based on the combined genotypic and phenotypic data, it is proposed that the two isolates be classified in the genus *Amycolatopsis* as *Amycolatopsis thermophila* sp. nov. (type strain GY088^T=NCIMB 14699^T=NRRL B-24836^T) and *Amycolatopsis viridis* sp. nov. (type strain GY115^T=NCIMB 14700^T=NRRL B-24837^T).

The genus *Amycolatopsis* Lechevalier *et al.* 1986, a member of the family *Pseudonocardiaceae*, can be distinguished from other genera classified in this taxon by using genus-specific oligonucleotide primers based on 16S rRNA gene sequences (Tan *et al.*, 2006a) and by a combination of chemotaxonomic and morphological properties (Labeda *et al.*, 2011). At the time of writing, the genus contains 45 species with validly published names, although there are strong grounds for believing that it is grossly underspeciated (Tan *et al.*, 2006a). The genus encompasses alkaliphilic, halophilic, mesophilic and thermophilic species that can be distinguished using a combination of phenotypic properties (Goodfellow *et al.*, 2001; Labeda *et al.*, 2003; Tan *et al.*, 2007). These taxa fall into several multi- and single-membered subclades in the *Amycolatopsis* 16S rRNA gene tree (Tseng *et al.*, 2006; Bian *et al.*, 2009; Lee, 2009). The thermophilic species form a distinct phyletic line, the *Amycolatopsis methanolica* subclade, which also encompasses *Amycolatopsis eurytherma*, *Amycolatopsis thermoflava* and *Amycolatopsis tucumanensis* (Chun *et al.*, 1999; Kim *et al.*, 2002; Albarracín *et al.*, 2010).

The current classification of the genus *Amycolatopsis* provides a sound basis for the delineation of additional

species, as shown by the description of novel species of clinical (Labeda *et al.*, 2003; Huang *et al.*, 2004), ecological (Albarracín *et al.*, 2010; Bian *et al.*, 2009) and biotechnological (Wink *et al.*, 2003; Tan *et al.*, 2007) interest. The present study, conducted using a polyphasic approach, was designed to establish the taxonomic status of two strains that were isolated from a composite arid Australian soil sample and found to form distinct phyletic lines in the 16S rRNA *A. methanolica* subclade (Tan *et al.*, 2006a). The strains, isolates GY088^T and GY115^T, exhibited a range of properties consistent with their recognition as two separate novel species of the genus *Amycolatopsis*.

Isolates GY088^T and GY115^T were isolated from SM1 and SM2 agar plates (Tan *et al.*, 2006a), respectively, which had been inoculated with a suspension of an arid composite Australian soil sample and incubated at 28 °C for 21 days. The strains were maintained on plates of modified Bennett's agar (Jones, 1949) at room temperature and as suspensions of mycelia fragments in glycerol (20%, v/v) at –20 °C. Biomass for the chemotaxonomic and molecular systematic studies was prepared by harvesting from shake flasks of glucose-yeast extract (GYE) broth (Gordon & Mihm, 1962), as described earlier (Tan *et al.*, 2006b).

The almost complete 16S rRNA gene sequences (1391–1465 nt) generated by Tan *et al.* (2006a) were aligned manually against corresponding sequences of the type

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of *Amycolatopsis thermophila* GY088^T and *Amycolatopsis viridis* GY115^T are AY129774 and AF466095, respectively.

strains of species of the genus *Amycolatopsis* retrieved from GenBank/EMBL/DBJ databases using the PHYDIT program (<http://plaza.snu.ac.kr/~jchun/phydit>). Phylogenetic trees were inferred by using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms drawn from the MEGA4 (Tamura *et al.*, 2007) and PHYML (Guindon & Gascuel, 2003) packages; an evolutionary distance matrix for the neighbour-joining algorithm was generated using the Jukes & Cantor (1969) model. The topologies of the evolutionary trees were evaluated by a bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based upon 1000 replicates using the MEGA4 program. The root position of the *Amycolatopsis* tree based on the neighbour-joining algorithm was inferred, using *Prauserella rugosa* DSM 43194^T (GenBank accession no. AF051342) as the outgroup.

Phylogenetic analysis revealed that the two isolates formed a group within the *A. methanolica* 16S rRNA subclade, and this was supported by all of the tree-making algorithms and by a bootstrap value of 72 % (Fig. 1). This taxon is most closely related to a corresponding group composed of the type strains of *A. methanolica* and *A. thermoflava*. The isolates shared a 16S rRNA gene similarity of 98.2 %, a value which corresponded to 25 nt differences at 1385 locations. Isolate GY088^T appears most closely related to the type strain of *A. thermoflava*; the two organisms share a 16S rRNA gene similarity of 98.3 %, a value equivalent to 24 nt differences at 1429 locations. Similarly, strain GY115^T appears closely related to the type strains of *A. methanolica* and *A. thermoflava*, sharing 98.6 % similarity with them, a value corresponding to 20 nt differences at 1389 and 1388

locations, respectively. DNA–DNA relatedness studies of these strains were not carried out because the levels of 16S rRNA gene sequence similarity between the strains were lower than those between the other organisms assigned to the *A. methanolica* subclade. The type strains of *A. methanolica* and *A. thermoflava*, for instance, share a 16S rRNA gene sequence similarity of 99.8 %, with a corresponding DNA–DNA relatedness score of only 21 % (Chun *et al.*, 1999) – a value well below the 70 % cut-off point recommended for assigning strains to the same genomic species (Wayne *et al.*, 1987). The corresponding DNA–DNA relatedness score between the type strains of *Amycolatopsis eurytherma* and *Amycolatopsis tucumanensis* is 39.5 %, and these organisms share a 16S rRNA gene sequence similarity of 99.9 % (Albarracín *et al.*, 2010).

The isolates were examined for chemical markers to establish whether they had a chemotaxonomic profile typical of members of the genus *Amycolatopsis*. Standard procedures were used to determine the isomers of diaminopimelic acid (A₂pm) (Staneck & Roberts, 1974), menaquinones (Minnikin *et al.*, 1984), muramic acid type (Uchida *et al.*, 1999), sugars (Schaal, 1985) and polar lipids (Minnikin *et al.*, 1984), using the type strains of *A. methanolica* and *A. thermoflava* as controls. Each of the isolates contained *meso*-A₂pm, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV, *sensu* Lechevalier & Lechevalier, 1970); *N*-acetylated muramic acid, tetrahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue (>96 %) and major amounts of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol and phosphatidylinositol (phospholipid pattern II *sensu* Lechevalier *et al.*, 1977). These data are in line with the

Fig. 1. Neighbour-joining (Saitou & Nei, 1987) phylogenetic tree based on almost complete 16S rRNA gene sequences (>1385 nt), showing relationships between isolates GY088^T and GY115^T and between them and representatives of the closest related species of the genus *Amycolatopsis*. Asterisks indicate branches of the tree that were also found using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) tree-making algorithms. Numbers at the nodes indicate the levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values above 50 % are given. The arrow indicates the inferred root position, using *Prauserella rugosa* DSM 43194^T (GenBank accession no. AF051342) as outgroup. Bar, 0.005 substitutions per nucleotide position.

classification of the isolates in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Lee, 2009).

The isolates, together with the type strains of *A. eurytherma*, *A. methanolica*, *A. thermoflava* and *A. tucumanensis*, were examined in a range of phenotypic tests known to be of value in the systematics of the genus *Amycolatopsis* (De Boer *et al.*, 1990; Goodfellow *et al.*, 2001; Tan *et al.*, 2006b, 2007). All of the strains were Gram-positive-staining, non-acid-alcohol-fast, formed a branched substrate mycelium that fragmented into coccoid and rod-like elements on oatmeal agar, grew well at 50 °C and produced acid from cellobiose and xylose. Nevertheless, all of the strains can be distinguished using a combination of phenotypic features (Table 1). Isolate GY115^T can readily be distinguished from isolate GY088^T as it forms white aerial hyphae on oatmeal agar, produces acid

from L-arabinose, galactose, lactose, salicin and trehalose, and degrades tyrosine but is unable to reduce nitrate or degrade casein, elastin or gelatin.

It is evident from the genotypic and phenotypic data that the two isolates can be readily distinguished from one another and from the other thermophilic strains classified in the *A. methanolica* 16S rRNA subclade. It is, therefore, proposed that isolates GY088^T and GY115^T be classified in the genus *Amycolatopsis*, as *Amycolatopsis thermophila* sp. nov. and *Amycolatopsis viridis* sp. nov., respectively.

All of the tested strains grew well at 50 °C on GYE agar and can thereby be considered to be thermophilic actinomycetes (Cross, 1968; Brock, 1986). Further comparative studies between members of the *A. methanolica* subclade

Table 1. Diagnostic characteristics that differentiate strains GY088^T and GY115^T from one another and from their closest phylogenetic relatives in the genus *Amycolatopsis*

Strains: 1, GY088^T; 2, GY115^T; 3, *A. eurytherma* DSM 44348^T; 4, *A. methanolica* KCTC 9411^T; 5, *A. thermoflava* KCTC 9833^T; 6, *A. tucumanensis* ABO^T. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6
Morphological properties on oatmeal agar:						
Formation/colour of aerial hyphae	–	White	White	White	White	–
Colour of substrate mycelium	Yellow–brown	Yellow–green	Yellow	Yellow	Yellow	Yellow
Production of soluble pigment	–	–	–	–	+	–
Acid from:						
(+)-L-Arabinose	–	+	+	+	+	+
Dextrin	–	–	w	–	–	–
meso-Erythritol	–	–	+	–	+	+
(+)-D-Galactose	–	+	+	+	+	+
myo-Inositol	–	–	+	–	–	–
(+)-D-Lactose	–	+	w	+	+	+
(–)-D-Mannitol	–	–	+	–	+	+
(+)-D-Melibiose	–	–	–	–	+	–
Methyl α-D-glucoside	–	–	–	–	+	+
(+)-D-Raffinose	–	–	–	+	+	+
(+)-D-Ribose	–	–	–	+	+	–
(–)-D-Sorbitol	–	–	+	+	+	+
(–)-D-Sucrose	–	–	–	+	–	+
(+)-D-Trehalose	–	+	+	+	+	–
(+)-D-Xylose	–	–	+	+	+	+
Hydrolysis of:						
Aesculin	–	–	–	+	+	+
Allantoin	–	–	+	–	+	–
Nitrate	+	–	+	+	–	–
Urease	–	–	+	–	+	–
Degradation of:						
Casein	+	–	+	+	+	+
Elastin	+	–	+	+	+	+
Gelatin	+	–	+	+	+	+
Hypoxanthine	–	+	w	+	+	–
Starch	+	+	–	–	–	+
Tyrosine	–	+	+	+	+	–
Xanthine	+	+	–	+	–	–
Growth at 10 °C	+	+	–	+	–	–

and those of the other *Amycolatopsis* subclades are needed, to determine whether the former merits generic status.

Description of *Amycolatopsis thermophila* sp. nov.

Amycolatopsis thermophila (ther.mo'phi.la. Gr. n. *thermê* heat; Gr. fem. adj. *philê* loving; N.L. fem. adj. *thermophila* heat-loving organism).

Aerobic, Gram-positive-staining, non-acid–alcohol-fast, non-motile, catalase-positive actinomycete that forms a pale yellow to brown substrate mycelium that fragments into coccoid to rod-shaped elements when grown on modified Bennett's agar supplemented with mannitol and soybean flour. Neither aerial hyphae nor diffusible pigments are formed on this medium. Grows at 10 and 50 °C and from pH 5.0 to 10.0. Xylan is degraded, but not xanthine. Grows in the presence of ($\mu\text{g ml}^{-1}$) neomycin sulphate (4), penicillin G (20) and rifampicin (10), but is sensitive to gentamicin sulphate (5), neomycin sulphate (8), streptomycin sulphate (10), novobiocin (10) and vancomycin hydrochloride (10). Lysozyme (0.005 %, w/v) sensitive. Results of additional phenotypic tests are cited in the text and in Table 1. Chemotaxonomic properties are typical of the genus.

The type strain GY088^T (=NCIMB 14699^T=NRRL B-24836^T) was isolated from an arid composite Australian soil sample. The species description is based on a single strain, which hence serves as the type strain.

Description of *Amycolatopsis viridis* sp. nov.

Amycolatopsis viridis (vi'ri.dis. L. fem. adj. *viridis* green, referring to the production of a greenish substrate mycelium).

Aerobic, Gram-positive-staining, non-acid–alcohol-fast, non-motile, catalase-positive actinomycete that forms a medium yellow to green substrate mycelium that fragments into coccoid to rod-like elements, but which does not produce diffusible pigments on modified Bennett's agar supplemented with mannitol and soybean flour. Abundant white, sterile aerial hyphae are produced. Grows at 10 and 50 °C and from pH 5.0 to 10.0. Degrades hypoxanthine, uric acid and xylan, but not arbutin. Grows in the presence ($\mu\text{g ml}^{-1}$) of gentamicin sulphate (5), neomycin sulphate (8), novobiocin (10), rifampicin (10), tobramycin sulphate (8) and vancomycin hydrochloride (10), but is susceptible to penicillin G (20) and streptomycin sulphate (16). Lysozyme (0.005 %, w/v) sensitive. Additional phenotypic properties are cited in the text and in Table 1. Chemotaxonomic properties are typical of the genus.

The type strain GY115^T (=NCIMB 14700^T=NRRL B-24837^T) was isolated from an arid composite Australian soil sample. The species description is based on a single strain, which hence serves as the type strain.

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