# *Amycolatopsis granulosa* sp. nov., *Amycolatopsis ruanii* sp. nov. and *Amycolatopsis thermalba* sp. nov., thermophilic actinomycetes isolated from arid soils

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The taxonomic positions of three thermophilic actinomycetes isolated from arid soil samples were established by using a polyphasic approach. The organisms had chemical and morphological features that were consistent with their classification in the genus *Amycolatopsis*. 16S rRNA gene sequence data supported the classification of the isolates in the genus *Amycolatopsis* and showed that they formed distinct branches in the *Amycolatopsis methanolica* subclade. DNA–DNA relatedness studies between the isolates and their phylogenetic neighbours showed that they belonged to distinct genomic species. The three isolates were readily distinguished from one another and from the type strains of species classified in the *A. methanolica* subclade based on a combination of phenotypic properties and by genomic fingerprinting. Consequently, it is proposed that the three isolates be classified in the genus *Amycolatopsis* as representatives of *Amycolatopsis granulosa* sp. nov. (type strain GY307$^T$=NCIMB 14709$^T$=NRRL B-24844$^T$), *Amycolatopsis ruanii* sp. nov. (type strain NMG112$^T$=NCIMB 14711$^T$=NRRL B-24848$^T$) and *Amycolatopsis thermalba* sp. nov. (type strain SF45$^T$=NCIMB 14705$^T$=NRRL B-24845$^T$).

The genus *Amycolatopsis* Lechevalier *et al.* (1986) can be readily distinguished from other genera classified in the family *Pseudonocardiaceae* by using genus-specific oligonucleotide primers based on 16S rRNA gene sequences (Tan *et al.*, 2006) and by a combination of chemotaxonomic features and morphological markers (Kim & Goodfellow, 1999; Labeda *et al.*, 2011). At the time of writing, the genus comprised 45 recognized species which fall into several multi- and single-membered subclades within the *Amycolatopsis* 16S rRNA gene tree (Albarracín *et al.*, 2010; Tan & Goodfellow, 2012). *Amycolatopsis* strains that grow at 50–55 °C belong to a well-delineated phyletic line, the *Amycolatopsis methanolica* subclade, which also encompasses *Amycolatopsis eurytherma*, *A. thermoflava*, *A. thermophila*, *A. tucumanensis* and *A. viridis* (Kim *et al.*, 2002; Albarracín *et al.*, 2010; Zucchi *et al.*, 2012). Members of this subclade are of potential value in biotechnology, notably as vehicles for fermentative overproduction of aromatic amino acids (De Boer *et al.*, 1990; Abou-Zeid *et al.*, 1995) and as agents of bioremediation (Albarracín *et al.*, 2008, 2010).

The present polyphasic taxonomic study was designed to establish the taxonomic status of three strains isolated from arid soil samples and found to have morphological properties similar to members of several genera classified in the family *Pseudonocardiaceae*, notably the genus *Amycolatopsis*. The organisms, isolates GY307$^T$, NMG112$^T$ and SF45$^T$, were found to have properties resembling those of members of the *A. methanolica* subclade. It is proposed that these isolates be recognized as representatives of three novel species of the genus *Amycolatopsis*.

Strains GY307$^T$ and SF45$^T$ were isolated from SM2 and SM3 agar plates (Tan *et al.*, 2006), respectively. Strain SF45$^T$ was obtained from an arid soil sample collected at Marla in Australia and strain GY307$^T$ was from a composite Australian

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains GY307$^T$, NMG112$^T$ and SF45$^T$ are AF466101, HQ668524 and HQ668525, respectively.

One supplementary figure and one supplementary table are available with the online version of this paper.

soil sample (Tan *et al.*, 2006). Strain NMG112$^T$ was isolated from a starch-casein agar plate (Küster & Williams, 1964), which had been inoculated with a soil suspension of an arid soil sample and incubated at 28 °C for 21 days. The isolates were maintained on modified Bennett's agar plates (Jones, 1949) at room temperature and as suspensions of mycelial fragments in glycerol (20 %, v/v) at −20 °C. Biomass for chemotaxonomic and molecular systematic studies was grown in shake flasks of glucose-yeast extract broth (Gordon & Mihm, 1962) for 7 days at 28 °C, harvested by centrifugation and washed twice in distilled water; biomass for chemotaxonomic studies was freeze-dried.

Extraction of genomic DNA, PCR amplification and sequencing of 16S rRNA genes of the novel isolates were performed as described by Kim *et al.* (1996). The almost-complete 16S rRNA gene sequences (1392–1476 nt) were aligned against corresponding sequences of the type strains of *Amycolatopsis* species retrieved from the EzTaxon database (Chun *et al.*, 2007) by using MEGA4 software (Tamura *et al.*, 2007). 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 and PHYML packages (Guindon & Gascuel, 2003; Tamura *et al.*, 2007); an evolutionary distance matrix was generated for the neighbour-joining algorithm by using the Jukes & Cantor (1969) distance model. The topologies of the evolutionary trees were evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based on 1000 replicates by using the MEGA4 software package. The root position of the neighbour-joining tree was inferred by using *Prauserella rugosa* DSM 43194$^T$ (GenBank accession no. AF051342) and *Saccharomonospora viridis* NCIB 9602$^T$ (GenBank accession no. Z38007) as outgroups.

Phylogenetic analysis showed that each of the novel isolates formed a distinct phyletic line within the *A. methanolica* subclade (Fig. 1). They shared 99.0–99.2 % 16S rRNA gene similarity, which corresponded to 14–23 nt differences at 1390–1415 locations. The relationship between strain GY307$^T$ and its closest neighbour, the type strain of *A. viridis*, was supported by the maximum-parsimony algorithm and a 98 % bootstrap value; the two organisms shared 99.0 % 16S rRNA gene sequence similarity, a value corresponding to 14 nt differences at 1387 sites. The loose relationship between strain NMG112$^T$ and the type strains of *A. methanolica* and *A. thermoflava* was not supported by the other tree-making algorithms or by a high bootstrap value. Indeed, this isolate was recovered at the periphery of the *A. methanolica* subclade in the analyses based on the maximum-likelihood and maximum-parsimony algorithms. Strain NMG112$^T$ was related most closely to the type strains of *A. methanolica* and *A. thermoflava*, with which it shared 98.8 and 98.9 % 16S rRNA gene sequence similarity, respectively, values equivalent to 17 and 16 nt differences. In contrast, strain SF45$^T$ and the type strains of *A. eurytherma* and *A. tucumanensis* formed a phyletic branch that was supported by all of the tree-making algorithms but not by a high bootstrap value. Strain SF45$^T$ was related most closely to the type strain of *A. eurytherma*, with which it shared 99.2 % 16S rRNA gene sequence similarity, a value which corresponded to 11 nt differences at 1389 sites.

DNA–DNA hybridization experiments were carried out between strain GY307$^T$ and *A. viridis* GY115$^T$, strain NMG112$^T$ and *A. methanolica* IMSNU 20055$^T$, and strain

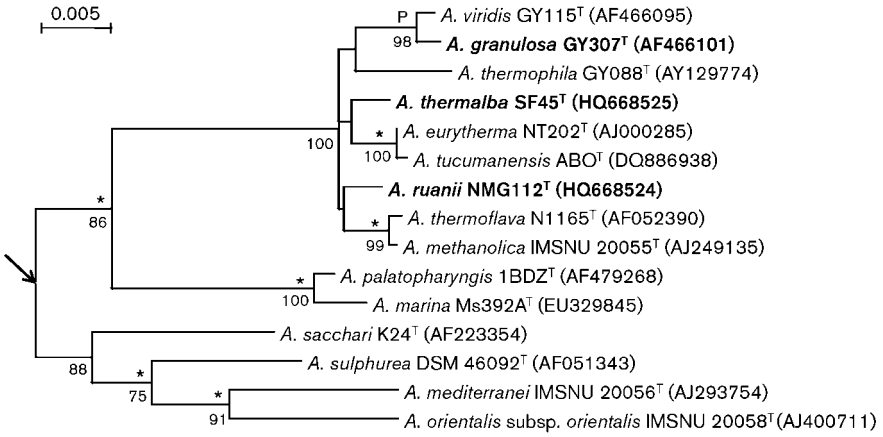


**Fig. 1.** Neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing relationships between isolates GY307$^T$, NMG112$^T$ and SF45$^T$ and the type strains of species classified in the *Amycolatopsis methanolica* subclade. Asterisks indicate branches of the tree that were also found with the maximum-likelihood and maximum-parsimony tree-making algorithms; P indicates branches that were recovered with the maximum-parsimony method. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets; only values >50 % are given. The arrow indicates the inferred root position by using *Prauserella rugosa* DSM 43194$^T$ (AF051342) and *Saccharomonospora viridis* NCIB 9602$^T$ (Z38007) as outgroups. Bar, 0.005 substitutions per nucleotide position.

SF45$^T$ and *A. eurytherma* NT202$^T$, even though these pairs of strains showed lower 16S rRNA gene sequence similarities than between the type strains of species classified in the *A. methanolica* subclade (Chun *et al.*, 1999; Albarracín *et al.*, 2010). Levels of DNA–DNA relatedness between the three pairs of strains were determined in triplicate by using the nitrocellulose membrane-filter hybridization procedure described by Seldin & Dubnau (1985). The DNA probes were labelled by using the non-radioactive digoxigenin High Prime System (Roche), hybridized DNA was visualized by using digoxigenin luminescent detection kits (Roche) and DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). The resultant mean ($\pm$SD) levels of DNA–DNA relatedness were 50.0$\pm$5.0 % between strain GY307$^T$ and *A. viridis* GY115$^T$, 42.2$\pm$2.7 % between strain NMG112$^T$ and *A. methanolica* IMSNU 20055$^T$, and 39.5$\pm$3.4 % between strain SF45$^T$ and *A. eurytherma* NT202$^T$; these values are well below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne *et al.*, 1987).

Repetitive DNA fingerprinting was performed in duplicate on the three novel isolates and the type strains of species classified in the *A. methanolica* subclade by using the BOX A1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′), following the protocol described by Versalovic *et al.* (1994). The resultant fingerprints were analysed by using Bionumerics version 2 software (Applied Maths). Each fingerprint was normalized by using a standard molecular marker and the bands were defined prior to cluster analysis. A similarity matrix was calculated with the pairwise Pearson product–moment correlation coefficient (Pearson, 1926) followed by construction of a dendrogram via the unweighted-pair-group method with arithmetic averages algorithm (UPGMA; Sokal & Michener, 1958). The three novel strains formed single-membered clusters at the 92 % cut-off point in each of the duplicate sets (Fig. 2).

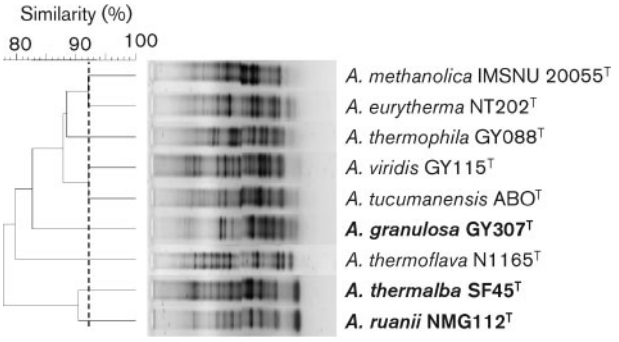


**Fig. 2.** Dendrogram showing clustering of BOX A1R fingerprints of isolates GY307$^T$, NMG112$^T$ and SF45$^T$ and the type strains of species classified in the *A. methanolica* 16S rRNA gene subclade (Pearson correlation; 1–100 %). The dashed line indicates the 92 % cut-off value.

Standard procedures were used to determine the isomers of diaminopimelic acid (Staneck & Roberts, 1974), the acyl type of murein (Uchida *et al.*, 1999), sugars (Schaal, 1985), menaquinones and polar lipids (Minnikin *et al.*, 1984), by using the type strains of *A. methanolica*, *A. tucumanensis* and *A. viridis* as controls. All of the isolates contained *meso*-diaminopimelic acid, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV after Lechevalier & Lechevalier, 1970), together with *N*-acetylated muramic acid, and major amounts of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethylethanolamine, phosphatidylglycerol and phosphatidylinositol (Fig. S1 available in IJSEM Online; phospholipid pattern II *sensu* Lechevalier *et al.*, 1977). The predominant isoprenologue of strains GY307$^T$ and NMG112$^T$ was tetra-hydrogenated menaquinone with nine isoprene units [MK-9($H_4$)] and that of isolate SF45$^T$ was MK-9($H_2$). These results are in line with those of previous studies, as representatives of *Amycolatopsis* species contain di-, tetra-, hexa- or octa-hydrogenated menaquinones with nine isoprene units as predominant components (Lechevalier *et al.*, 1986; Yassin *et al.*, 1991; Huang *et al.*, 2004; Wink *et al.*, 2003; Albarracín *et al.*, 2010). In contrast, *Amycolatopsis halophila* and *Amycolatopsis nigrescens* strains contain MK-8($H_4$) and MK-11($H_4$) as respective predominant isoprenologues (Groth *et al.*, 2007; Tang *et al.*, 2010). Furthermore, the type strain of *Amycolatopsis decaplanina* is reported to have a mixture of tetra-hydrogenated menaquinones with eight and nine isoprene units (Wink *et al.*, 2004). It is apparent that the predominant menaquinones vary considerably in the genus (Tan & Goodfellow, 2012), although some of this variation may be attributed to the stage of the growth cycle from which biomass is taken (Yassin *et al.*, 1991). Consequently, all of these chemical properties support the assignment of the isolates to the genus *Amycolatopsis* (Tan & Goodfellow, 2012).

Fatty acids extracted from the isolates and from the type strains of species classified in the *A. methanolica* subclade were methylated then analysed by using the standard Sherlock Microbial Identification (MIDI) system, version 5 (MIDI, 1999; Sasser, 1990). All of the strains contained iso-$C_{16:0}$ as the predominant fatty acid (29.5–48.7 % of the total fatty acids) and major proportions of $C_{16:0}$ (6.5–13.2 %) and anteiso-$C_{17:0}$ (6.5–18.3), results that are in line with those from previous studies (De Boer *et al.*, 1990; Chun *et al.*, 1999; Albarracín *et al.*, 2010). It can be seen from the fatty acid profiles shown in Table S1 that some of the minor components are discontinuously distributed and that, apart from the type strain of *A. viridis*, all of the organisms contain major proportions of iso-$C_{16:0}$ 2-OH (7.3–16.4 %).

The novel isolates and the type strains of species classified in the *A. methanolica* subclade were examined for phenotypic properties known to be of value in *Amycolatopsis* systematics (De Boer *et al.*, 1990; Kim *et al.*, 2002; Tan & Goodfellow, 2012). Additional biochemical features were obtained by using API ZYM test strips (bioMérieux),

following the manufacturer's protocol. The isolates were readily distinguished from one another and from the type strains of their nearest neighbours by using a combination of phenotypic properties (Table 1). Isolate GY307$^T$, unlike the type strain of *A. viridis*, produced acid from dextrin, *meso*-erythritol, methyl α-D-glucoside, raffinose, D-ribose, sucrose and D-xylose, and produced trypsin, but did not grow at 10 °C. Similarly, isolate NMG112$^T$ could be distinguished from the type strains of *A. methanolica* and *A. thermoflava* by its ability to produce acid from dextrin, *meso*-erythritol, D-galactose, *myo*-inositol, maltose, D-mannitol and methyl α-D-glucoside and by its inability to produce naphthol-AS-BI-phosphohydrolase. In turn, isolate SF45$^T$, unlike the type strain of *A. eurytherma*, formed acid from maltose, methyl α-D-glucoside, raffinose and D-ribose, produced β-glucosidase, did not degrade hypoxanthine

**Table 1.** Characteristics differentiating isolates GY307$^T$, NMG112$^T$ and SF45$^T$ from one another and from their closest phylogenetic relatives in the genus *Amycolatopsis*

Strains: 1, GY307$^T$; 2, NMG112$^T$; 3, SF45$^T$; 4, *A. eurytherma* DSM 44348$^T$; 5, *A. methanolica* KCTC 9411$^T$; 6, *A. thermoflava* KCTC 9833$^T$; 7, *A. thermophila* NRRL B-24836$^T$; 8, *A. tucumanensis* DSM 45259$^T$; 9, *A. viridis* NRRL B-24837$^T$. All data are from this study.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Properties on oatmeal agar | | | | | | | | | |
| Formation/colour of aerial hyphae | White | White | White | White | White | White | − | − | White |
| Colour of substrate mycelium | Yellow–green | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow–brown | Yellow | Yellow–green |
| Production of soluble pigment | − | − | − | − | − | + | − | − | − |
| Acid production from: | | | | | | | | | |
| (+)-L-Arabinose | + | + | + | + | + | + | − | + | + |
| Dextrin | + | + | + | + | − | − | − | − | − |
| *meso*-Erythritol | + | + | + | + | − | + | − | + | − |
| (+)-D-Galactose | + | + | + | + | + | + | − | + | + |
| *myo*-Inositol | − | + | + | + | − | − | − | − | − |
| (+)-D-Lactose | + | + | + | + | + | + | − | + | + |
| Maltose | − | + | + | − | − | − | − | − | − |
| (−)-D-Mannitol | − | + | + | + | − | + | − | + | − |
| Melibiose | − | − | − | − | − | + | − | − | − |
| Methyl α-D-glucoside | + | + | + | − | − | + | − | + | − |
| Raffinose | + | + | + | − | + | + | − | + | − |
| (+)-D-Ribose | + | + | + | − | + | + | − | − | − |
| (−)-D-Sorbitol | − | + | + | + | + | + | − | + | − |
| (−)-D-Sucrose | + | + | − | − | + | − | − | + | − |
| Trehalose | + | − | + | + | + | + | − | − | + |
| (+)-D-Xylose | + | + | + | + | + | + | − | + | − |
| Degradation of: | | | | | | | | | |
| Allantoin | − | − | + | + | − | + | − | − | − |
| Hypoxanthine | + | + | − | + | + | + | − | − | + |
| Uric acid | + | + | − | − | + | + | − | + | + |
| Xylan | + | + | − | − | − | − | + | + | + |
| API ZYM tests | | | | | | | | | |
| *N*-Acetyl-β-glucosaminidase | − | + | − | + | + | + | + | − | − |
| Acid phosphatase | + | + | − | + | + | + | + | + | + |
| Alkaline phosphatase | + | + | + | + | + | + | − | − | + |
| Cystine arylamidase | + | − | − | + | + | + | − | − | + |
| Esterase lipase (C8) | + | + | + | + | + | − | + | + | + |
| β-Galactosidase | + | − | − | − | − | − | − | − | + |
| β-Glucosidase | − | − | + | − | + | − | − | + | − |
| β-Glucuronidase | + | − | − | − | − | − | − | − | + |
| Leucine arylamidase | + | + | + | + | + | + | − | + | + |
| Lipase (C14) | + | − | − | + | + | − | − | − | + |
| Naphthol-AS-BI-phosphohydrolase | + | − | + | + | + | + | + | − | + |
| Trypsin | + | − | − | + | + | + | − | − | − |
| Valine arylamidase | + | − | − | + | + | + | − | − | + |
| Growth at 10 °C | − | − | − | − | + | − | + | − | + |

and did not produce *N*-acetyl-β-glucosaminidase, acid phosphatase, cystine arylamidase, lipase (C4), trypsin or valine arylamidase.

The genotypic and phenotypic data presented here show that the three isolates can be distinguished from one another and from the type strains of species classified in the *A. methanolica* 16S rRNA gene subclade. We therefore suggest that strains GY307$^T$, NMG112$^T$ and SF45$^T$ represent three novel species of the genus *Amycolatopsis*, for which the names *Amycolatopsis granulosa* sp. nov., *Amycolatopsis ruanii* sp. nov. and *Amycolatopsis thermalba* sp. nov., respectively, are proposed.

### Description of *Amycolatopsis granulosa* sp. nov.

*Amycolatopsis granulosa* (gra.nu.lo′sa. L. n. *granulum* a small grain; L. fem. suff. *-osa* suffix meaning full of; N.L. fem. adj. *granulosa* granular).

Aerobic, Gram-positive, non-acid–alcohol-fast, non-motile, catalase-positive actinomycete. Forms abundant, white aerial hyphae and a yellow–green substrate mycelium that fragments into cylindrical elements (0.4–0.5 × 1.0–1.2 μm) with a granular ornamentation on modified Bennett's agar supplemented with mannitol and soybean flour; diffusible pigments are not formed on this medium. Grows at 20–50 °C and pH 5.0–7.0 but not in the presence of NaCl (5 %, w/v). Acid is produced from D-fructose, D-mannose and melezitose, but not from D-arabitol, dulcitol, glycogen or xylitol. Chitin, gelatin and starch are degraded, but not cellulose, guanine, tyrosine or xanthine. Does not produce chymotrypsin, α-galactosidase, α-glucosidase, α-mannosidase or α-fucosidase (API ZYM tests). Additional phenotypic test results are given in the main text and in Table 1. Chemotaxonomic properties are typical of the genus.

The type strain, GY307$^T$ (=NCIMB 14709$^T$=NRRL B-24844$^T$), was isolated from an arid soil sample collected from Marla, Australia. The species description is based on a single strain and hence serves as the description of the type strain.

### Description of *Amycolatopsis ruanii* sp. nov.

*Amycolatopsis ruanii* (ru.a′ni.i. N.L. gen. masc. n. *ruanii* of Ruan, named after Jisheng Ruan in recognition of his many contributions to the systematics of actinomycetes).

Aerobic, Gram-positive, non-acid–alcohol-fast, non-motile, catalase-positive actinomycete. Forms abundant white aerial hyphae and an extensively branched yellow substrate mycelium that fragments into granular ornamented cylindrical elements (0.3–0.5 × 1.2–1.7 μm) on modified Bennett's agar supplemented with mannitol and soybean flour; diffusible pigments are not formed on this medium. Grows at 20–50 °C, at pH 4.0–10.0 and in the presence of NaCl (7.0 %, w/v). Acid is produced from D-fructose, glycogen, D-mannose, melezitose and xylitol, but not from D-arabitol or dulcitol. Degrades chitin, gelatin and starch, but not cellulose, guanine, tyrosine or xanthine. Does not produce chymotrypsin, α-galactosidase, α-glucosidase, α-mannosidase or α-fucosidase (API ZYM tests). Additional phenotypic test results are given in the main text and in Table 1. Chemotaxonomic properties are typical of the genus.

The type strain, NMG112$^T$ (=NCIMB 14711$^T$=NRRL B-24848$^T$), was isolated from an arid soil sample. The species description is based on a single strain and hence serves as the description of the type strain.

### Description of *Amycolatopsis thermalba* sp. nov.

*Amycolatopsis thermalba* (therm.al′ba. Gr. n. *thermê* heat; L. fem. adj. *alba* white; N.L. fem. adj. *thermalba* thermophilic and white).

Aerobic, Gram-positive, non-acid–alcohol-fast, non-motile, catalase-positive actinomycete. Forms an extensively branched substrate mycelium that fragments into smooth-walled, cylindrical elements (0.3–0.4 × 1.5–1.8 μm). Abundant, white sterile aerial hyphae, a yellow substrate mycelium and a medium olive brown diffusible pigment are produced on modified Bennett's agar supplemented with mannitol and soybean flour. Grows at 20–50 °C, at pH 4.0–10.0 and in the presence of NaCl (7.0 %, w/v). Acid is produced from D-fructose, glycogen, D-mannose, melezitose and xylitol, but not from D-arabitol or dulcitol. Gelatin and starch are degraded, but not chitin, cellulose, guanine, tyrosine or xanthine. Does not produce chymotrypsin, α-fucosidase, α-galactosidase, α-glucosidase or α-mannosidase (API ZYM tests). Additional phenotypic test results are given in the main text and in Table 1. Chemotaxonomic properties are typical of the genus.

The type strain, SF45$^T$ (=NCIMB 14705$^T$=NRRL B-24845$^T$), was isolated from an arid soil sample collected at Marla, Australia. The species description is based on a single strain and hence serves as the description of the type strain.

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