***Amycolatopsis thermoflava* sp. nov., a novel soil actinomycete from Hainan Island, China**

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A soil isolate, which had been assigned to the genus *Nocardia*, was shown to have properties consistent with its classification in the genus *Amycolatopsis*. An almost complete nucleotide sequence of the 16S rDNA of the strain was determined following cloning and sequencing of the amplified gene. The sequence was aligned with those available for members of the family *Pseudonocardiaceae* and related taxa and phylogenetic trees were inferred using three tree-making algorithms. The organism consistently formed a distinct monophyletic clade with the type strain of *Amycolatopsis methanolica*, but DNA–DNA relatedness data showed that the two strains belonged to distinct genomic species. The organism was also distinguished from the type strains of all validly described species of *Amycolatopsis* using a battery of phenotypic properties. The genotypic and phenotypic data show that the strain merits recognition as a new species of the genus *Amycolatopsis*. The name proposed for the new species is *Amycolatopsis thermoflava* sp. nov. The type strain is IFO 14333^T.

Keywords: *Amycolatopsis thermoflava* sp. nov., polyphasic taxonomy, 16S rDNA sequencing

INTRODUCTION

The genus *Amycolatopsis* was proposed by Lechevalier *et al.* (1986) for mycolateless actinomycetes previously classified in the genus *Nocardia*. Members of the genus are aerobic, Gram-positive, non-acid-fast, non-motile organisms which form a branched substrate mycelium that fragments into squarish elements. When formed, aerial hyphae may be sterile or differentiate into long chains of smooth-walled, squarish to ellipsoidal spore-like structures. The organism contains *meso*-diaminopimelic acid (A₂pm), arabinose and galactose (wall

chemotype IV *sensu* Lechevalier & Lechevalier, 1970), is rich in fatty acids of the iso- and anteiso-branched series, contains di-, tetra- and hexahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue and phosphatidylethanolamine and phosphatidylglycerol as major polar lipids with diphosphatidylglycerol, phosphatidylinositol and phosphatidylinositol mannosides variably present. The G+C content of the DNA is in the range 66–73 mol%. The genus encompasses ten validly described species and forms a phyletic line within the evolutionary radiation occupied by the family *Pseudonocardiaceae* (Embley *et al.*, 1988; Warwick *et al.*, 1994; Kim & Goodfellow, 1999).

The present investigation was designed to establish the taxonomic position of a soil isolate, designated N1165^T, which had been classified as a new species of

The GenBank accession numbers for 16S rDNA sequences of *Amycolatopsis alba* DSM 44262^T, *Amycolatopsis coloradensis* NRRL 3218^T, *Amycolatopsis sulphurea* DSM 46092^T and *Amycolatopsis thermoflava* N1165^T are AF051340, AF051341, AF051343 and AF052390, respectively.

Fig. 1. Unrooted neighbour-joining tree based on nearly complete 16S rDNA sequences showing relationships between strain N1165^T and representatives of the family Pseudonocardiaceae and related taxa. The asterisks indicate the branches that were also recovered using the Fitch-Margoliash and maximum-parsimony methods. The numbers at the nodes indicate the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets. Scale bar, 0.01 nucleotide substitutions per nucleotide position.

Nocardia, namely '*Nocardia thermoflava*', on the basis of a few biochemical, morphological and physiological properties (Lu & Yan, 1983). This name was neither included in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980) nor recognized in the current edition of *Bergey's Manual of Systematic Bacteriology* (Williams *et al.*, 1989). Genotypic and phenotypic data show that '*Nocardia thermoflava*' strain N1165^T should be recognized as a new species of *Amycolatopsis* for which the name *Amycolatopsis thermoflava* sp. nov. is proposed.

METHODS

Test strains and culture conditions. '*Nocardia thermoflava*' strain N1165^T, which was received from the Institute of Fermentation in Kyoto (IFO 14333^T), was isolated from a heat-treated soil sample collected in Hainan Island, China by Lu & Yan (1983). Strain N1165^T, *Amycolatopsis alba* DSM 44262^T, *Amycolatopsis azurea* NRRL 11412^T,

Amycolatopsis coloradensis NRRL 3218^T, *Amycolatopsis fastidiosa* NRRL B-16697^T (=ATCC 31181^T), *Amycolatopsis japonica* DSM 44213^T, *Amycolatopsis mediterranei* ATCC 13685^T, *Amycolatopsis methanolica* IFO 15065^T (=NCIB 11946^T), *Amycolatopsis orientalis* NRRL 2450^T (=IFO 12806^T) and *Amycolatopsis sulphurea* DSM 46092^T were maintained on glucose-yeast extract agar (GYEA; Gordon & Mihm, 1962) at 4 °C and as suspensions of mycelial fragments and spores in glycerol (20%, v/v) at -20 °C. Biomass for the chemotaxonomic and molecular systematic studies was prepared by growing strains in shake flasks of modified Sauton's broth (Mordarska *et al.*, 1972) for 10 d at 30 °C. Cultures were checked for purity, harvested by centrifugation, washed with distilled water and freeze-dried.

Morphology. The morphological properties of strain N1165^T were examined by light and scanning electron microscopy as described previously (Williams *et al.*, 1983).

Phenotypic characterization. The cultural and staining properties of the test strains were determined by using standard procedures after growth on GYEA plates at 37 °C for 7 d. After incubation, colonies were examined both by eye and microscopically. Unless otherwise stated, the remaining tests were read after 21 d at 30 °C. The degradation tests were carried out using GYEA as the basal medium and the results were read after 28 d following the procedures of Gordon *et al.* (1974). The growth and enzyme tests were performed using the media and methods employed by Goodfellow *et al.* (1997). Acid production from sugars was determined according to Gordon *et al.* (1974).

Chemotaxonomy. Analyses for diaminopimelic acid and major sugars were carried out using the procedures described by Stanek & Roberts (1974) and Lechevalier & Lechevalier (1970), respectively. Menaquinones were extracted from dried biomass (about 50 mg) using the small-scale method of Minnikin *et al.* (1984) and purified preparations were examined by electron-impact mass spectrometry, as described previously (Chun *et al.*, 1997). Fatty acid methyl esters were prepared and analysed using the MIDI system (Sasser, 1980). The presence of mycolic acids was checked by the acid methanolysis method according to Minnikin *et al.* (1980).

Determination of DNA base composition. DNA was prepared according to Chun & Goodfellow (1995). The G+C contents of the resultant preparations were determined using the thermal denaturation (T_m) method (Mandel & Marmur, 1968).

16S rDNA sequencing. Chromosomal DNA was isolated from strain N1165^T and from the type strains of *A. alba*, *A. coloradensis* and *A. sulphurea*, according to Chun & Goodfellow (1995). PCR, cloning and sequencing of the resultant 16S rDNA preparations was carried out using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an Applied Biosystems 373 DNA sequencer, as described elsewhere (Chun & Goodfellow, 1995). The resultant 16S rDNA sequences were aligned manually against corresponding sequences of representative members of the family Pseudonocardiaceae and related taxa retrieved from the GenBank database.

Unrooted evolutionary trees were inferred by using three treeing algorithms, namely the Fitch-Margoliash (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. The distance model of Jukes & Cantor (1969) was used to generate an evolutionary distance matrix. The topologies of

Table 1. Characters separating species of the genus *Amycolatopsis*

+, Positive or present; w, weak positive; —, negative or absent. Strains: 1, *A. thermoflava* N1165^T; 2, *A. alba* DSM 44262^T; 3, *A. azurea* NRRL 11412^T; 4, *A. coloradensis* NRRL 3218^T; 5, *A. fastidiosa* NRRL B-16697^T; 6, *A. japonica* DSM 44213^T; 7, *A. mediterranei* ATCC 13685^T; 8, *A. methanolica* IFO 15065^T; 9, *A. orientalis* NRRL 2450^T; 10, *A. sulphurea* DSM 46092^T.

Character	1	2	3	4	5	6	7	8	9	10
Presence of aerial mycelium	+	+	+	+	+	+	+	+	+	+
Colour of aerial mycelium:										
Blue	—	—	+	—	—	—	—	—	—	—
Purple	—	—	—	—	+	—	—	—	—	—
White	+	+	—	—	+	+	+	+	+	+
White to olive buff	—	—	—	+	—	—	—	—	—	—
Yellowish green	—	—	—	—	—	—	—	—	—	+
Production of soluble pigment	+	—	+	+	+	—	—	—	—	—
Acid production from:										
Adonitol	+	+	+	—	—	+	—	+	+	—
L(+)Arabinose	+	+	+	—	w	+	+	—	+	—
D(+)Cellobiose	+	+	+	+	+	+	+	+	+	—
Dextrin	—	+	+	+	w	+	+	—	+	+
meso-Erythritol	+	+	+	—	—	+	—	+	+	—
D(+)Fructose	+	+	+	+	w	+	+	+	+	+
D(+)Galactose	+	+	+	+	w	+	+	+	+	+
meso-Inositol	—	+	+	+	—	+	+	—	+	—
D(+)Lactose	+	+	+	—	—	+	+	—	+	—
D(+)Maltose	—	+	+	+	w	+	+	—	+	+
D(–)Mannitol	+	+	+	+	—	+	+	+	+	+
D(+)Melezitose	—	—	—	—	—	—	—	—	—	—
D(+)Melibiose	+	+	+	—	—	+	+	—	—	—
Methyl α -D-glucoside	+	+	+	+	w	+	—	—	+	—
D(+)Raffinose	+	+	+	—	w	+	+	—	+	—
L(+)Rhamnose	—	—	—	—	—	—	+	+	+	—
Salicin	+	+	+	+	—	+	+	w	+	—
D(–)Sorbitol	+	—	—	—	—	—	w	+	—	—
Sucrose	—	+	+	+	w	+	+	+	+	+
D(+)Trehalose	+	w	+	+	w	+	+	+	+	+
D(+)Xylose	+	+	+	+	—	+	+	+	+	—
Decomposition of:										
Allantoin	+	+	—	—	—	+	—	+	—	—
Casein	+	+	+	+	+	+	+	—	+	—
Aesculin	+	+	+	+	—	+	+	w	+	—
Gelatin	—	+	+	+	—	+	+	+	+	+
Hypoxanthine	+	+	+	+	—	+	+	+	+	—
Xanthine	+	+	+	—	—	+	—	+	—	—
Growth at:										
10 °C	—	—	+	+	+	+	+	—	+	—
45 °C	+	—	—	—	+	—	—	+	+	—
Growth in presence of 5 % NaCl (w/v)	+	—	+	+	—	w	w	+	w	—

the resulting trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings. All of the phylogenetic analyses were carried out using the PHYLIP package (Felsenstein, 1993).

DNA–DNA relatedness studies. Levels of genomic relatedness between strain N1165^T and *A. methanolica* IFO 15065^T were determined by using a DNA–DNA slot blot hybridization method (Kafatos *et al.*, 1979), as described by Chun *et al.* (1998).

RESULTS AND DISCUSSION

Almost complete 16S rDNA sequences were obtained for strain N1165^T (1495 nt) and for the type strains of *A. alba*, *A. coloradensis* and *A. sulphurea*. Phylogenetic comparison of the sequence of strain N1165^T with corresponding nucleotide sequences of representatives of the family *Pseudonocardiaceae* show that the organism belongs to the genus *Amycolatopsis* (Fig. 1). It

is also clear from the phylogenetic trees that the strain forms a monophyletic clade with *A. methanolica*. This relationship is supported by the 100% bootstrap value recorded using the neighbour-joining method. The 16S rDNA sequence similarity between strain N1165^T and the type strain of *A. methanolica* is 98.8%; this value corresponds to 16 differences out of 1328 nt positions compared. Similarity values with the type strains of the remaining validly described *Amycolatopsis* species range from 93.8 to 94.4%.

The assignment of the test strain to the genus *Amycolatopsis* is also supported by the biochemical, chemotaxonomic, morphological and physiological data. The organism is aerobic, non-motile, Gram-positive, non-acid-alcohol-fast and forms a substrate mycelium which fragments into squarish elements. Strain N1165^T contains meso-A₂pm as the wall diamino acid, arabinose and galactose as major sugars, predominant amounts of tetrahydrogenated menaquinone with nine isoprene units and DNA with a G+C content of 75 mol%. The organism also contains major proportions of 14-methylpentadecanoic acid (29% of total cellular fatty acid composition), α -hydroxy-14-methylpentadecanoic acid (16%) and 14-methylhexadecanoic acid (14%) with smaller proportions of 13-methyltetradecanoic acid (4%), 15-methylhexadecanoic acid (5%), hexadecanoic acid (8%), hexadecenoic acid (6%), 14-methylpentadecenoic acid (5%), 2-hydroxy-16-methylheptadecanoic acid (5%) and octadecenoic acid (4%), but it lacks mycolic acids. These properties are consistent with the classification of the strain in the genus *Amycolatopsis* (Holt *et al.*, 1994).

Strain N1165^T can be distinguished from representatives of all validly described species of *Amycolatopsis*, including *A. methanolica*, using a combination of phenotypic properties (Table 1). It is also clear from DNA–DNA relatedness data that the test strain and *A. methanolica* IFO 15065^T belong to different genomic species. The two strains showed a DNA relatedness value of 21% under optimal conditions when DNA from the *A. methanolica* strain was used as the probe. When DNA from strain N1165^T was labelled, the relatedness value fell to 5%. It is evident from these genotypic and phenotypic data that strain N1165^T merits species status in the genus *Amycolatopsis*. It is proposed that the organism be classified in the genus *Amycolatopsis* as *Amycolatopsis thermoflava* sp. nov.

Description of *Amycolatopsis thermoflava* sp. nov.

Amycolatopsis thermoflava (ther.mo fla.va. Gr. n. therme heat; L. adj. flavus yellow; M.L. adj. thermo-*flava* thermophilic, yellow).

The description is based on data taken from this and an earlier study (Lu & Yan, 1983). Aerobic, Gram-positive, non-acid-alcohol-fast, non-motile actinomycete which forms an extensively branched substrate

mycelium which fragments into squarish elements (0.6–0.7 × 6.5–14.0 µm). The aerial mycelium is sterile, sparse and white, and the substrate mycelium is yellow. A yellowish diffusible pigment is produced. Produces nitrate reductase, phosphatase and urease, but not amylase. The organism, which grows between 28 and 55 °C, but not at 10 or 60 °C, is resistant to lysozyme and penicillin. Other phenotypic properties are given in Table 1. The G+C content of the DNA is 75 mol% (*T_m* method). Isolated from a soil sample collected in Hainan Island, China. The type strain is N1165^T (= IFO 14333^T). The species description is based on a single strain and hence serves as the type strain description.

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