NOTE

***Amycolatopsis eurytherma* sp. nov., a thermophilic actinomycete isolated from soil**

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The taxonomic positions of two thermophilic actinomycetes isolated from soil were established in a polyphasic taxonomic study. The organisms were shown to have phenotypic properties typical of members of the genus *Amycolatopsis* and formed a distinct phyletic line in the *Amycolatopsis methanolica* 16S rDNA subclade. They also had many phenotypic properties in common and formed a genomic species that was closely related to, albeit distinct from, the type strain of *A. methanolica*. A range of phenotypic properties distinguished the isolates from representatives of all validly described species of *Amycolatopsis*. Genotypic and phenotypic data show that the two strains should be classified in the genus *Amycolatopsis* as a novel species, *Amycolatopsis eurytherma* sp. nov.; the type strain is strain NT202^T (= DSM 44348^T = NCIMB 13795^T).

Keywords: *Amycolatopsis eurytherma* sp. nov., polyphasic taxonomy, 16S rRNA sequencing, DNA–DNA relatedness

The genus *Amycolatopsis* belongs to the family *Pseudonocardaceae* (Embley *et al.*, 1988a; Warwick *et al.*, 1994), which also encompasses the genera *Actinobispora*, *Actinopolyspora*, *Kibdelosporangium*, *Prauserella*, *Pseudonocardia*, *Saccharomonospora*, *Saccharopolyspora* and *Thermocrisum*. Members of these genera share several genotypic and phenotypic features that distinguish them from all other actinomycete taxa, including the family *Actinosynnemataceae* (Labeda & Kroppenstedt, 2000) and the genus *Crossiella* (Labeda, 2001), which also belong to the suborder *Pseudonocardineae* (Stackebrandt *et al.*, 1997). Strains assigned to the family *Pseudonocardaceae* typically contain *meso*-diaminopimelic acid (*meso*-A₂pm), arabinose and galactose in the wall peptidoglycan (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), *N*-acetylated muramic acid residues and fatty acids rich in iso- and anteiso-branched components, but lack mycolic acids (Takahashi, 2001). A combination of chemical and morphological markers are available to distinguish *Amycolatopsis* from all of the other genera classified in the family *Pseudonocardaceae* (Kim & Goodfellow, 1999).

Considerable interest is being shown in the genus *Amycolatopsis*, as it contains organisms that are the source of many bioactive compounds, two of which, rifamycin and vancomycin, are currently commercially important. The genus was proposed by Lechevalier *et al.* (1986) to accommodate four species that had previously been misclassified in the genus *Nocardia*. The 13 validly described species that currently constitute the genus *Amycolatopsis* form a clade within the evolutionary radiation occupied by the suborder *Pseudonocardineae*. Members of three of these species, namely *Amycolatopsis fastidiosa*, *Amycolatopsis methanolica* and *Amycolatopsis thermoflava*, grow well between 50 and 60 °C (Henssen *et al.*, 1987; De Boer *et al.*, 1990; Chun *et al.*, 1999) and can thereby be considered as thermophilic actinomycetes (Cross, 1968; Brock, 1986). A fourth species, *Amycolatopsis sacchari*, contains organisms that are moderately thermophilic (Goodfellow *et al.*, 2001). In contrast, organisms assigned to the remaining species, i.e. *Amycolatopsis alba*, *Amycolatopsis albidoflavus*, *Amycolatopsis azurea*, *Amycolatopsis coloradensis*, *Amycolatopsis japonica*, *Amycolatopsis mediterranei*, *Amycolatopsis orientalis* (the type species), *Amycolatopsis rubida* and *Amycolatopsis sulphurea*, are mesophilic (Huang *et al.*, 2001; Lee & Hah, 2001). Members of all of these species can be separated from one another by using a battery of biochemical, morphological and physiological properties (Goodfellow

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Abbreviation: *meso*-A₂pm, *meso*-diaminopimelic acid.

The GenBank accession number for the 16S rDNA sequence of strain NT202^T (= DSM 44348^T = NCIMB 13795^T) is AJ000285.

et al., 2001; Huang *et al.*, 2001; Lee & Hah, 2001). It has also been proposed that '*Nocardia lactamdurans*' NRRL 3802 be classified in the genus *Amycolatopsis* as '*Amycolatopsis lactamdurans*' (Barreiro *et al.*, 2000).

The present study was designed to establish the taxonomic position of two *Amycolatopsis*-like soil isolates, strains NT202^T and NT303, which formed a distinct cluster in a numerical taxonomic survey of thermophilic actinomycetes (Sahin, 1995). Genotypic and phenotypic data showed that these strains should be recognized as a novel species of *Amycolatopsis*, for which the name *Amycolatopsis eurytherma* sp. nov. is proposed.

Strains NT202^T and NT303 were isolated from scrubland and arid soil samples collected in Madurai, India, and Van, Turkey, respectively. The organisms were isolated on starch/casein agar (Küster & Williams, 1964) that had been supplemented with cycloheximide (50 µg ml⁻¹) and rifampicin (0.5 µg ml⁻¹) and incubated at 55 °C for 7 days. The two isolates, together with *A. methanolica* NCIMB 11946^T and *Streptomyces thermovulgaris* DSM 40444^T, were maintained on modified Bennett's agar (Jones, 1949) at room temperature and as suspensions of mycelial fragments in glycerol (20%, v/v) at -20 °C. Biomass for the chemosystematic and molecular systematic analyses was obtained by growing the isolates in 250 ml shake flasks (ca. 150 r.p.m.) for 3 days at 45 °C in 50 ml trypticase soy broth (Difco). Cultures were checked for purity, killed by shaking with formalin (1%, v/v) and the biomass was harvested by centrifugation. Cells needed for chemosystematic studies were washed in distilled water and freeze-dried; those required for molecular systematic investigations were washed in NaCl/EDTA buffer (0.1 M EDTA, pH 8.0, 0.1 M NaCl) and stored at -20 °C until needed. The same procedures were used to prepare biomass from the *A. methanolica* and *S. thermovulgaris* strains that were included in the DNA-DNA relatedness studies.

Morphological properties of isolates NT202^T and NT303 were observed after growth on Czapek-Dox (Weyland, 1969) and oatmeal agars (ISP medium 3; Shirling & Gottlieb, 1966) for 5 days at 45 °C using a JOEL JSM-51 scanning electron microscope and a Nikon Optiphot binocular light microscope fitted with long working distance objectives.

Phenotypic tests were carried out using media and methods described previously (De Boer *et al.*, 1990; Goodfellow *et al.*, 1997, 2001) with isolates grown for 7 days at 45 °C.

Standard TLC or HPLC procedures were used to determine the isomeric form of A₂pm, the presence of mycolic acids and whole-organism fatty acid, isoprenoid quinone and polar lipid profiles of strain NT202^T (Kim *et al.*, 1999; Minnikin *et al.*, 1980). Whole-organism sugars were analysed by GC as their alditol acetates using the method of Englyst & Cummings (1984). The G+C ratio of chromosomal DNA was

determined by the HPLC method (Tamaoka & Komagata, 1984), as described by Kim *et al.* (1998).

Extraction of chromosomal DNA and PCR amplification of 16S rDNA were achieved using procedures described previously (Kim *et al.*, 1999). Similarly, 16S rRNA genes of the isolates were amplified by PCR and sequenced directly using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The resultant 16S rDNA sequences were aligned manually using the PHYLIP program (J. Chun, personal communication) against corresponding sequences of representatives of the family *Pseudonocardiaceae* retrieved from the DDBJ, EMBL and GenBank databases. Unrooted evolutionary trees were inferred using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices were generated for the least-squares and neighbour-joining algorithms by using the method of Jukes & Cantor (1969). All phylogenetic analyses were carried out using the PHYLIP suite of programs (Felsenstein, 1993). Topologies of the resultant trees were evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based on 1000 replicates using 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 using *Pseudonocardia thermophila* ATCC 19285^T (accession no. X53195) as the outgroup organism.

Purified DNA was prepared from the test strains using the procedure described by Mordarski *et al.* (1976). The quality of the DNA preparations was checked by taking spectrophotometric readings and by agarose gel electrophoresis of the samples (Sambrook *et al.*, 1989). Duplicate experiments were carried out to determine DNA relatedness values between strains (Table 1) using the nitrocellulose membrane filter technique (Mordarski *et al.*, 1976; Stackebrandt *et al.*, 1981). High-molecular-mass DNA was dissolved using the alkaline extraction method and immobilized single-stranded DNA was prepared by allowing denatured DNA solutions to filter by gravity or low vacuum through nitrocellulose membrane filters (0.2 µm pore size; Sartorius). Filter discs (5 mm) loaded with about 12 µg DNA were punched from the nitrocellulose membranes. DNA preparations (Table 1) were labelled with deoxy[1',2',5'-³H]cytidine 5'-triphosphate using a nick translation kit. The filter disc preparations were pre-incubated in 5 × Denhardt's solution (Sambrook *et al.*, 1989) at 60 °C for 3–5 h. Overnight hybridizations were carried out in 200 µl 3 × SSC solution (Sambrook *et al.*, 1989) containing 35% (w/v) deionized formamide and approximately 50 000 c.p.m. labelled DNA at 60 °C. The concentration of SSC and the hybridization temperature were designed to achieve optimal hybridization conditions, that is, 25 °C below the melting temperature assuming that the mean DNA

Table 1. Mean levels of DNA relatedness between strains NT202^T and NT303 and *A. methanolica* NCIMB 11946^T

The pairwise DNA relatedness values shown (%) are means from two sets of hybridizations conducted using the nitrocellulose filter method. In each hybridization, one of a pair of DNA preparations was labelled. ND, Not determined.

Test strain	Source of labelled DNA		
	NCIMB 11946 ^T	NT202 ^T	NT303
<i>A. methanolica</i> NCIMB 11946 ^T	100	ND	ND
Strain NT202 ^T	60	100	ND
Strain NT303	58	93	100
<i>Streptomyces thermovulgaris</i> DSM 40444 ^T *	5	2	0

* Phylogenetically distant control strain.

G+C content of *Amycolatopsis* strains is 70 mol%. After hybridization, the nitrocellulose filters were washed in 3 × SSC, thoroughly dried and baked at 80 °C for 50 min. The amount of bound probe DNA was estimated by scintillation counting and relatedness values were expressed as the percentage of the probe bound (mean duplicate hybridizations) relative to the homologous reaction.

An almost complete 16S rDNA sequence was generated for strain NT202^T (1477 nt). Phylogenetic comparison of this sequence with corresponding sequences of representatives of the suborder *Pseudonocardineae* showed that the strain belongs to the family *Pseudonocardiaceae* (data not shown). The high 16S rRNA gene sequence similarities found between the tested strain and representatives of the genus *Amycolatopsis* (93.9–98.8%) support inclusion of strain NT202^T in this genus.

It is apparent from Fig. 1 that *Amycolatopsis* strains form two multimembered groups, the *A. methanolica* and *A. orientalis* subclades, each of which is supported by high bootstrap values. The remaining thermophilic strain, *A. fastidiosa* IMSNU 20054^T, forms a distinct phyletic line at the foot of the *Amycolatopsis* 16S rDNA tree. Strain NT202^T belongs to the *A. methanolica* subclade and shares a 16S rDNA nucleotide similarity value of 98.8% with the type strains of *A. methanolica* and *A. thermoflava*, a value that corresponds to 17 differences at 1456 and 1476 nt sites, respectively. This clearly indicates that strain NT202^T forms a new centre of taxonomic variation in the genus *Amycolatopsis*, as the type strains of *A. methanolica* and *A. thermoflava* have a 16S rDNA similarity value of 99.8%, which is equivalent to three differences at 1456 nt sites, but show a DNA relatedness value of only 21% (Chun *et al.*, 1999), a value well below the 70% cut-off point for strains belonging to the same genomic species (Wayne *et al.*, 1987). The *A. methanolica* subclade was recognized in all three phylogenetic analyses and was supported by a bootstrap value of 100% using the neighbour-joining method.

Fig. 1. Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rDNA sequences (at least 1372 nt) showing relationships between strain NT202^T 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) treeing algorithms. The arrow indicates the estimated root position based on *Pseudonocardia thermophila* ATCC 19285^T as the outgroup organism. Numbers at the nodes indicate the level of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. F and P indicate branches that were also recovered using the least-squares and maximum-parsimony methods, respectively.

Strain NT303 also belongs to this subclade, as its 16S rDNA nucleotide sequence was shown to be identical to that of strain NT202^T in a comparison of over 800 nt [positions 44–905, according to the *Escherichia coli* numbering system (Brosius *et al.*, 1978), which included several highly variable regions (Neefs *et al.*, 1993)]. It is evident from the present study that strains NT202^T and NT303 belong to a single genomic species that is closely related to, albeit distinct from, the type strain of *A. methanolica* (Table 1).

Table 2. Characteristics of species of the genus *Amycolatopsis*

Taxa are identified as: 1, strains NT202^T and NT303; 2, *A. alba* DSM 44262^T; 3, *A. albidoflavus* IMSNU 22139^T; 4, *A. azurea* NRRL 11412^T; 5, *A. coloradensis* NRRL 3218^T; 6, *A. fastidiosa* NRRL B-16697^T; 7, *A. japonica* DSM 44213^T; 8, *A. mediterranei* ATCC 13685^T; 9, *A. methanolica* IFO 15065^T; 10, *A. orientalis* NRRL 2450^T; 11, *A. rubida* JCM 10871^T; 12, *A. sacchari* DSM 44468^T; 13, *A. sulphurea* DSM 46092^T; 14, *A. thermoflava* IFO 14333^T. Data for the type strains were taken from Goodfellow *et al.* (2001), Huang *et al.* (2001) and Lee & Hah (2001). +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Colour of aerial mycelium*	WH	WH	WH	B	WO	P/WH	WH	WH	WH	WH	WH	WH	WH/YG	WH
Production of soluble pigment	–	–	–	+	+	+	–	–	–	–	+	–	–	+
Acid production from:														
Adonitol	+	+	+	+	–	–	+	–	+	+	+	+	–	+
L-(+)-Arabinose	+	+	+	+	–	w	+	+	–	+	+	+	–	+
D-(+)-Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Dextrin	w	+	–	+	+	w	+	+	–	+	–	+	+	–
meso-Erythritol	+	+	+	+	–	–	+	–	+	+	+	+	–	+
D-(+)-Fructose	+	+	+	+	+	w	+	+	+	+	+	+	+	+
D-(+)-Galactose	+	+	+	+	+	w	+	+	+	+	+	+	+	+
meso-Inositol	+	+	+	+	+	–	+	+	–	+	+	–	–	–
D-(+)-Lactose	w	+	+	+	–	–	+	+	–	+	–	+	–	+
D-(+)-Maltose	–	+	+	+	+	w	+	+	–	+	–	+	+	–
D(–)-Mannitol	+	+	w	+	+	–	+	+	+	+	+	+	+	+
D-(+)-Melibiose	–	+	–	+	–	–	+	+	–	–	–	–	–	+
Methyl α -D-glucoside	–	+	–	+	+	w	+	–	–	+	–	+	–	+
D-(+)-Raffinose	–	+	–	+	–	w	+	+	–	+	–	–	–	+
α -L-(+)-Rhamnose	+	–	–	–	–	–	–	+	+	+	+	+	–	–
Salicin	–	+	–	+	+	–	+	+	w	+	+	+	–	+
D(–)-Sorbitol	+	–	–	–	–	–	–	w	+	–	–	–	–	+
D(–)-Sucrose	–	+	+	+	+	w	+	+	+	+	+	+	+	–
D-(+)-Trehalose	+	w	+	+	+	w	+	+	+	+	+	+	+	+
D-(+)-Xylose	+	+	+	+	+	–	+	+	+	+	+	+	–	+
Decomposition of:														
Aesculin	–	+	+	+	+	–	+	+	w	+	+	+	–	+
Allantoin	+	+	+	–	–	–	+	–	+	–	–	+	–	+
Casein	+	+	+	+	+	+	+	+	–	+	+	+	+	+
Gelatin	+	+	+	+	+	–	+	+	+	+	w	+	+	–
Hypoxanthine	w	+	+	+	+	–	+	+	+	+	+	–	–	+
Xanthine	–	+	+	–	–	–	+	–	–	+	+	+	–	+
Growth at:														
10 °C	–	–	+	+	+	+	+	+	–	+	+	–	–	–
45 °C	+	–	–	–	–	+	–	–	+	–	–	+	–	+
Growth in the presence of NaCl (5%, w/v)	+	–	+	+	+	–	w	w	+	w	+	+	–	+
Production of:														
Amylase	–	+	–	–	+	–	+	–	–	+	–	–	–	–
Nitrate reductase	+	–	+	+	+	+	–	–	+	+	+	+	+	–
Urease	+	+	w	+	–	+	–	+	–	+	+	+	–	+

* B, Blue; P, purple; WH, white; WO, white to olive-buff; YG, yellowish green.

Assignment of strain NT202^T to the genus *Amycolatopsis* is also supported by chemotaxonomic data. The organism was found to contain meso-A₂pm as the wall diaminopimelic acid, arabinose and galactose as major sugars, tetrahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue and a phospholipid pattern consisting of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol

dimannoside, phosphatidylmethylethanolamine and an unidentified polar lipid, that is, it has phospholipid pattern type II *sensu* Lechevalier *et al.* (1977). Hydroxyphosphatidylethanolamine is present in members of most *Amycolatopsis* species, including *A. methanolica* and *A. thermoflava* (Yassin *et al.*, 1993; Chun *et al.*, 1999), but was not detected in strain NT202^T. The organism did contain 13-methyltetradecanoic acid (iso-15:0; 4% of the total fatty acid

content), 14-methylpentadecanoic acid (iso-16:0; 46%), hexadecanoic acid (16:0; 23%), 15-methylhexadecanoic acid (iso-17:0; 6%), 14-methylhexadecanoic acid (anteiso-17:0; 9%), heptadecanoic acid (17:0; 3%) and octadecanoic acid (18:0; 3%), though hydroxy fatty acids were not detected. The organism did not contain mycolic acids. The G+C content of the DNA was 73.9 ± 0.9 mol%. The chemical profile of strain NT202^T serves to distinguish it from all other wall chemotype IV actinomycetes, except those classified in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Embley *et al.*, 1988b; Yassin *et al.*, 1991; Kim & Goodfellow, 1999).

It is clear from the numerical phenetic survey of Sahin (1995) that strains NT202^T and NT303 have many phenotypic properties in common. These organisms are aerobic, non-motile, Gram-positive, non-acid-alcohol-fast, catalase-positive actinomycetes that form branched substrate hyphae that fragment into squarish, rod-like elements when grown on modified Bennett's (Jones, 1949) and Czapek–Dox (Weyland, 1969) agars. The substrate mycelium carries sparse, white, sterile aerial hyphae. All of these properties are consistent with the classification of the strains in the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Holt *et al.*, 1994). Additional phenotypic properties are shown in Table 2. It is also evident from these data that the tested strains can be distinguished from representatives of all of the validly described *Amycolatopsis* species by a range of biochemical, degradative and physiological properties.

The genotypic and phenotypic data indicate that strains NT202^T and NT303 form a novel species in the genus *Amycolatopsis*. It is therefore proposed that these organisms be classified in this genus as *Amycolatopsis eurytherma* sp. nov., with strain NT202^T as the type strain.

Description of *Amycolatopsis eurytherma* sp. nov.

Amycolatopsis eurytherma (eur.y.ther'ma. Gr. pref. *eury* many; Gr. n. *therme* heat; N.L. fem. adj. *eurytherma* grows over a wide temperature range).

The description is based on data taken from this and an earlier study (Sahin, 1995). Aerobic, non-motile, Gram-positive, non-acid-alcohol-fast, catalase-positive actinomycetes that form extensively branched, substrate hyphae that fragment into squarish, rod-like elements (approx. $0.4\text{--}0.5 \times 0.7\text{--}1.6$ µm). The substrate mycelium carries sparse, white, sterile aerial hyphae. A yellow substrate mycelium is formed on modified Bennett's and glucose/yeast extract agars, but distinct substrate mycelial pigments are not evident on Czapek–Dox, glycerol/asparagine or oatmeal agars; diffusible pigments are not formed on any of these media. Melanin pigments are not produced on peptone/yeast extract/iron or tyrosine agars. Growth occurs between 25 and 55 °C, but not at 10 or 60 °C, and at pH 6.0–9.0, but not at pH 5.0 or 10.0. Elastin, L-tyrosine and xylan are degraded, but not adenine,

aesculin, chitin or guanine. Grows on adonitol, L-(+)-arabinose, arabitol, D-(+)-cellobiose, D-(+)-fructose, D-(+)-galactose, *meso*-inositol, D-(+)-lactose, D-(+)-mannitol, D-(+)-mannose, D-(+)-melibiose, D-(+)-melezitose, α-L-rhamnose, D-(+)-ribose, D-(+)-sorbitol, starch, D-(+)-sucrose, D-(+)-trehalose, D-(+)-turanose, xylitol and D-(+)-xylose (all at 1%, w/v) as sole sources of carbon and energy for growth, but not on D-(+)-raffinose (1%, w/v). Sensitive to the following (concentrations given in µg ml⁻¹ in parentheses): amikacin (4, 8 and 16), bacitracin (32), doxycycline (16, 32 and 64), fusidic acid (8), gentamicin sulfate (64), lividomycin A (16), neomycin sulfate (32), novobiocin (16 and 32), streptomycin sulfate (64), ticarcillin (16 and 32) and tyrothricin (16 and 32). Resistant to the following (concentrations given in µg ml⁻¹, except for penicillin G, in parentheses): ampicillin (8, 16 and 32), bacitracin (16), carbenicillin (12), cefoxitin (16 and 32), cephaloridine (32, 64 and 128), cephradine (8, 16 and 32), doxycycline hydrochloride (4), ethionamide (16), fusidic acid (4), gentamicin sulfate (32), gramicidin (8), lincomycin hydrochloride (32, 64 and 128), isoniazid (16), lividomycin A (4 and 8), nalidixic acid (32), neomycin sulfate (8), novobiocin (4), oleandomycin phosphate (16, 32, 64 and 128), penicillin G (5, 10 and 15 i.u.), polymyxin B sulfate (16 and 32), rifampicin (16, 32 and 64), spiramycin (10), streptomycin sulfate (4, 8, 16, 32 and 64), tetracycline hydrochloride (4, 8, 16 and 32), vancomycin (16, 32 and 64) and viomycin sulfate (20). Additional phenotypic properties are shown in Table 2. Isolated from arid and scrubland soil. In addition to these properties, the type strain contains DNA with a G+C ratio of 73.1 ± 0.9 mol%.

The type strain is strain NT202^T (= DSM 44348^T = NCIMB 13795^T).

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