Amycolatopsis minnesotensis sp. nov., isolated from a prairie soil

Soon Dong Lee,¹ Linda L. Kinkel² and Deborah A. Samac²

Correspondence

Soon Dong Lee
sdlee@cheju.ac.kr

¹Department of Science Education, Cheju National University, Jeju 690-756, Republic of Korea

²Department of Plant Pathology, University of Minnesota, St Paul, MN 55108, USA

Two actinomycete strains, 32U-2^T and 32U-4, were isolated from a prairie soil in Minnesota and subjected to characterization by means of polyphasic taxonomy. The 16S rRNA gene sequences were determined following PCR amplification and cloning. A phylogenetic analysis, based on comparative analysis of 16S rRNA gene sequences, indicated that the organisms consistently formed a well-separated, distinct sub-branch within the radiation of the genus *Amycolatopsis* of the family *Pseudonocardiaceae*. The levels of 16S rRNA sequence similarity between the isolates and the type strains of recognized *Amycolatopsis* species ranged from 94.1 to 97.9 %. The highest levels of sequence similarity were found between the isolates and *Amycolatopsis coloradensis* (97.6–97.9 %), *Amycolatopsis alba* and *Amycolatopsis orientalis* (97.3–97.6 %) and *Amycolatopsis lurida* (97.2–97.5 %). Chemotaxonomic characteristics supported the phylogenetic relationships between the organisms and members of the genus *Amycolatopsis*. However, a broad range of phenotypic and genetic data revealed that the isolates should be classified as novel species of the genus *Amycolatopsis*, for which the name *Amycolatopsis minnesotensis* sp. nov. is proposed. The type strain is 32U-2^T (=KCCM 42246^T = NRRL B-24435^T).

During an investigation of the genetic diversity of streptomycete populations in prairie soils with different nitrogen-input histories at the Cedar Creek Natural History Area, an NSF-LTER site in central Minnesota, two *Amycolatopsis* strains were isolated, using oatmeal agar [the LTER (Long Term Ecological Research) program was established by the National Science Foundation (NSF) in 1980 to support research on long-term ecological phenomena in the United States; <http://www.lternet.edu/>]. The taxonomic status of these strains was studied by using polyphasic approaches encompassing morphological, physiological and chemotaxonomic characterization in addition to phylogenetic analysis based on 16S rRNA gene sequence comparisons.

The genus *Amycolatopsis*, which was proposed by Lechevalier *et al.* (1986) for aerobic, amycolate, nocardioform actinomycetes, is well defined as a result of chemotaxonomic characterization (Lechevalier *et al.*, 1986; Henssen *et al.*, 1987; Mertz & Yao, 1993; Yassin *et al.*, 1993) and phylogenetic analyses based on the comparison of 16S rRNA gene sequences (Embley *et al.*, 1988; Warwick *et al.*, 1994) and currently contains 26 species with validly published names. Members of the genus *Amycolatopsis*, which belongs to the family *Pseudonocardiaceae* (Embley *et al.*, 1988; Warwick *et al.*, 1994), are represented chemotaxonomically by the

following features: wall chemotype IV (*meso*-diaminopimelic acid, arabinose and galactose in cell-wall hydrolysates); a tetrahydrogenated menaquinone with nine isoprene units (MK-9(H₄)) as the major menaquinone; a phospholipid pattern of type PII (phosphatidylethanolamine as a diagnostic phospholipid); fatty acid profiles that include complex mixtures of saturated and branched-chain acids; and the absence of mycolic acids.

It is evident from our study that the two isolates could be readily differentiated from all *Amycolatopsis* species with validly published names on the basis of a battery of phenotypic and genetic data and merit recognition as a novel species.

Strains 32U-2^T and 32U-4 were isolated from a prairie soil by using the dilution plating method with oatmeal agar and were maintained as 20 % (v/v) glycerol suspensions at –70 and –20 °C. Morphological and cultural characteristics were determined by cultivating the isolates at 30 °C for 7–14 days on several media, such as yeast extract/malt extract agar (ISP 2 medium), oatmeal agar (ISP 3 medium) and ISP 4 medium (Shirling & Gottlieb, 1966). Specimens with growth were critical-point-dried, coated with gold and then observed under a scanning electron microscope (model JSM 5410LV; JEOL). The organisms produced well-developed, branched vegetative mycelium bearing white-coloured aerial mycelium. The reverse sides of colonies were light yellow in colour. Both types of hyphae fragmented into rod-shaped cells.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 32U-2^T and 32U-4 are DQ076482 and DQ076483, respectively.

Chromosomal DNA used for genetic characterization was extracted and purified with the Wizard genomic DNA purification kit (Promega) according to the instructions of the manufacturer. The 16S rRNA gene of the chromosomal DNA was amplified by a PCR and cloned into *Escherichia coli* JM109 (Promega) as described previously (Lee *et al.*, 2000). The resultant inserts were sequenced using an ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). These experiments were performed in the first author's laboratory in Korea. The sequences determined in this study were aligned and compared with the corresponding sequences of all *Amycolatopsis* species with validly published names by using the CLUSTAL X program (Thompson *et al.*, 1997) and then manually optimized according to the secondary structure of the *E. coli* sequence (Brosius *et al.*, 1978). A phylogenetic analysis was performed using the neighbour-joining method (Saitou & Nei, 1987). Evolutionary distances for the neighbour-joining method were computed by using the method of Jukes & Cantor (1969). The confidence level of the tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining data, using 1000 replications.

The almost-complete 16S rRNA gene sequences for the isolates 32U-2^T and 32U-4 each comprised continuous stretches of 1510 nt. A total of 1347 unambiguous aligned positions present in all strains between *E. coli* positions 72 and 1452 were used for phylogenetic analysis. *Pseudonocardia*

thermophila was used as an outgroup for tree construction. A phylogenetic tree (Fig. 1) based on 16S rRNA gene sequence comparisons showed that the isolates form a distinct lineage within the radius of the genus *Amycolatopsis*, this being supported by a bootstrap value of 100 %. The organisms showed 16S rRNA gene sequence similarity of 99.7 % to each other. The levels of 16S rRNA gene sequence similarity between the isolates and the type strains of recognized *Amycolatopsis* species ranged from 94.1 to 97.9 %. Of these species, *Amycolatopsis coloradensis* (97.6–97.9 %), *Amycolatopsis orientalis* and *Amycolatopsis alba* (97.3–97.6 %) and *Amycolatopsis lurida* (97.2–97.5 %) showed the highest levels of 16S rRNA gene sequence similarity with respect to the isolates.

For chemotaxonomic characterization, the organisms were cultivated on trypticase soy broth (Difco) for 3 days at 30 °C with shaking. After the purity had been checked, the cells were harvested by centrifugation and washed twice with distilled water. Analyses of the diaminopimelic acid isomer, the sugar compositions of whole-cell hydrolysates, the acyl type of the murein, mycolic acids, polar lipids, lipoquinones and DNA G+C content were carried out by using previously described methods (Staneck & Roberts, 1974; Uchida & Aida, 1984; Minnikin *et al.*, 1977, 1980, 1984; Mesbah *et al.*, 1989; Saddler *et al.*, 1991). Cellular fatty acid methyl esters were prepared by alkaline methanolysis (Minnikin, 1988) and analysed by GC with an Agilent mode 6850 gas chromatograph as described previously (Lee & Hah, 2001).

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strains 32U-2^T and 32U-4 within a taxon encompassing representatives of the genus *Amycolatopsis*. The tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). *Pseudonocardia thermophila* was used as an outgroup. Numbers at branching nodes are percentages of occurrence in 1000 bootstrapped trees (only values greater than 40 % are shown). Bar, 1 substitution per 100 nt.

The chemotaxonomic properties supported the phylogenetic assignment of the isolates within the radiation of the genus *Amycolatopsis*, as follows. The organisms were characterized by a type IV cell wall (*meso*-diaminopimelic acid, arabinose and galactose in whole-cell hydrolysates), MK-9(H₄) as a major menaquinone and muramic acid of the *N*-acetylated type. Mycolic acids were not detected. The DNA G + C contents of strains 32U-2^T and 32U-4 were 69.5 and 70.2 mol%, respectively. The polar lipid profiles were characterized by the presence of considerable amounts of phosphatidylmethylethanolamine, diphosphatidylglycerol and phosphatidylinositol, but the organisms showed minor differences in the relative amounts of phosphatidylethanolamine. The cellular fatty acids comprised a mixture of saturated and branched-chain acids, and were characterized by the predominance of iso-hexadecanoic acid (i-C_{16:0}). Small amounts of unsaturated and branched hydroxy fatty acids were also detected. The cellular fatty acid profiles of the isolates are shown in Table 1.

Acid production from carbohydrates was determined by using Bacto OF basal medium (Difco) that included each filter-sterilized compound at a final concentration of 1%. Decomposition of adenine, hypoxanthine, DL-tyrosine and xanthine was assessed as described previously (Gordon *et al.*, 1974). Catalase activity was determined with a 3% (v/v) hydrogen peroxide solution. The temperature for growth was tested at 10, 30, 37 and 45 °C. The production of hydrogen sulfide was detected on trypticase soy broth by using lead acetate strips. Urease activity was determined by

means of a colour change in Bacto urea broth (Difco). Hydrolysis of casein, gelatin and starch, and nitrate reduction, were examined by using previously described methods (MacFaddin, 1981). NaCl tolerance was studied on ISP 2 medium containing NaCl at final concentrations of 2, 3, 5, 7 and 10% (w/v).

The strains, whilst showing slight differences in their 16S rRNA gene sequences and some chemical properties, showed the same results for a broad range of physiological characteristics. They produced acid from D-arabinose, D-cellobiose, D-fructose, methyl α -D-mannoside, L-ribose, salicin, D-trehalose, D-xylose, D-xylose, glycerol, *myo*-inositol and D-mannitol but did not produce acid from inulin, D-melezitose, D-raffinose, L-sorbose, 2,3-butanediol, dulcitol or 1,2-propanediol. H₂S production and gelatin liquefaction were observed. Both isolates degraded casein, hypoxanthine and DL-tyrosine. Other physiological properties that differentiate the isolates from their phylogenetic neighbours are listed in Table 2.

Table 1. Cellular fatty acid compositions (%) of strains 32U-2^T and 32U-4

The isolates also contained an unknown fatty acid (3.1 and 3.5% in strains 32U-2^T and 32U-4) at a retention time between that of C_{16:0} and i-C_{17:1}. tr, Trace amount; –, not detected.

Fatty acid	32U-2 ^T	32U-4
Saturated acids		
C _{13:0}	tr	1.0
C _{14:0}	2.4	2.8
C _{15:0}	7.2	7.4
C _{16:0}	13.8	13.1
C _{17:0}	10.7	10.2
C _{18:0}	4.7	4.7
Branched acids		
i-C _{14:0}	2.5	2.1
i-C _{15:0}	12.3	14.2
i-C _{16:0}	23.1	19.1
i-C _{17:0}	4.7	5.1
ai-C _{15:0}	4.5	4.4
ai-C _{17:0}	7.2	7.2
Unsaturated acids		
C _{16:1}	1.5	2.2
C _{18:1cis}	–	1.5

Table 2. Physiological properties that distinguish strain 32U-2^T from its phylogenetic neighbours

Strains: 1, 32U-2^T (strain 32U-4 gave identical results); 2, *A. alba* IMSNU 22095^T; 3, *A. coloradensis* IMSNU 22096^T; 4, *A. lurida* IMSNU 20057^T; 5, *A. orientalis* IMSNU 20058^T. Data are from Lee & Hah (2001) and this study. +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5
Acid production from:					
L-Arabinose	+	+	–	+	+
Dextran	–	–	+	ND	+
D-Galactose	+	+	–	–	–
D-Glucose	+	–	–	–	–
D-Lactose	+	+	–	+	+
Maltose	+	+	–	+	+
D-Mannose	+	–	–	–	–
Melibiose	–	+	–	–	–
Methyl α -D-glucoside	–	+	+	–	+
D-Raffinose	–	+	–	–	–
L-Rhamnose	+	–	–	–	–
Sucrose	+	–	–	–	+
Adonitol	+	+	–	+	+
<i>meso</i> -Erythritol	+	+	–	+	+
D-Sorbitol	+	–	–	–	–
D-Xylitol	+	–	–	–	–
Nitrate reductase activity	+	–	+	–	+
Decomposition of:					
Starch	–	+	+	–	+
Urea	+	+	–	+	+
Xanthine	–	+	–	+	+
Growth at 37 °C	–	+	+	+	+
Growth on 5% NaCl	+	+	–	–	–

On the basis of the phenotypic and genotypic data, it was evident that strains 32U-2^T and 32U-4 represent a novel species of the genus *Amycolatopsis*, for which the name *Amycolatopsis minnesotensis* sp. nov. is proposed.

Description of *Amycolatopsis minnesotensis* sp. nov.

Amycolatopsis minnesotensis (min.ne.sot.en'sis. N.L. fem. adj. *minnesotensis* of Minnesota, the origin of the soil sample from which the type strain was isolated).

Forms well-developed aerial and vegetative mycelia that fragment into rod-shaped elements. The aerial mycelium is white and the vegetative mycelium is yellow. Aerobic, Gram-positive, not acid-alcohol-fast. Catalase-positive. H₂S production is observed. Nitrate is reduced to nitrite. Growth occurs between 10 and 30 °C. Growth does not occur at 37 °C. Acid is produced from D-arabinose, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, D-lactose, maltose, D-mannose, methyl α-D-mannoside, L-rhamnose, L-ribose, salicin, sucrose, D-trehalose, D-xylose, L-xylose, adonitol, *meso*-erythritol, glycerol, *myo*-inositol, D-mannitol, D-sorbitol and D-xylitol. No acid is produced from dextran, inulin, D-melezitose, melibiose, methyl α-D-glucoside, D-raffinose, L-sorbose, 2,3-butanediol, dulcitol or 1,2-propanediol. Casein, gelatin, hypoxanthine, DL-tyrosine and urea are decomposed but starch and xanthine are not. Growth occurs in the presence of 5 % NaCl but not 7 % NaCl. The phospholipid profile contains diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylmethyl-ethanolamine, phosphatidylinositol and an unknown phospholipid (a phospholipid type PII pattern). Mycolic acids are not present. The major cellular fatty acids are i-C_{16:0} (23.1 %), C_{16:0} (13.8 %), i-C_{15:0} (12.3 %) and C_{17:0} (10.7 %). The DNA G + C content is 69.5 mol%.

The type strain is 32U-2^T (=KCCM 42246^T=NRRL B-24435^T), isolated from a prairie soil in Minnesota, USA. Strain 32U-4 is a reference strain.

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References

Brosius, J., Palmer, J. L., Kennedy, J. P. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4840.

Embley, T. M., Smida, J. & Stackebrandt, E. (1988). The phylogeny of mycolate-less wall chemotype IV actinomycetes and the description of *Pseudonocardia* fam. nov. *Syst Appl Microbiol* **11**, 44–52.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Gordon, R. E., Barnett, D. A., Handerman, J. E. & Pang, C. H.-N. (1974). *Nocardia coeliaca*, *Nocardia autotrophica*, and the nocardin strain. *Int J Syst Bacteriol* **24**, 54–63.

Henssen, A., Kothe, H. W. & Kroppenstedt, R. M. (1987). Transfer of *Pseudonocardia azurea* and “*Pseudonocardia fastidiosa*” to the genus *Amycolatopsis*, with emended species description. *Int J Syst Bacteriol* **37**, 292–295.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Lechevalier, M. P., Prauser, H., Labeda, D. P. & Ruan, J.-S. (1986). Two new genera of nocardioform actinomycetes: *Amycolata* gen. nov. and *Amycolatopsis* gen. nov. *Int J Syst Bacteriol* **36**, 29–37.

Lee, S. D. & Hah, Y. C. (2001). *Amycolatopsis albidoflavus* sp. nov. *Int J Syst Evol Microbiol* **51**, 645–650.

Lee, S. D., Kim, E. S. & Hah, Y. C. (2000). Phylogenetic analysis of the genera *Pseudonocardia* and *Actinobispora* based on 16S rDNA sequence studies. *FEMS Microbiol Lett* **182**, 125–129.

MacFaddin, J. F. (1981). *Biochemical Tests for Identification of Medical Bacteria*, 2nd edn. Baltimore: Williams & Wilkins.

Mertz, F. P. & Yao, R. C. (1993). *Amycolatopsis alba* sp. nov., isolated from soil. *Int J Syst Bacteriol* **43**, 715–720.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Minnikin, D. E. M. (1988). Isolation and purification of mycobacterial wall lipids. In *Bacterial Cell Surface Techniques*, pp. 125–135. Edited by I. C. Hancock & I. R. Poxton. Chichester: Wiley.

Minnikin, D. E., Alshamaony, L. & Goodfellow, M. (1977). Differentiation of *Mycobacterium*, *Nocardia*, and related taxa by thin layer chromatographic analysis of whole-cell methanolysates. *J Gen Microbiol* **88**, 200–204.

Minnikin, D. E., Hutchinson, I. G., Caldicott, A. B. & Goodfellow, M. (1980). Thin layer chromatography of methanolysates of mycolic acid-containing bacteria. *J Chromatogr* **188**, 221–233.

Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.

Saddler, G. S., Tavecchia, P., Lociuero, S., Zanol, M., Colombo, E. & Selva, E. (1991). Analysis of madurose and other actinomycete whole cell sugars by gas chromatography. *J Microbiol Methods* **14**, 185–191.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Shirling, E. B. & Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* **16**, 313–340.

Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Uchida, K. & Aida, K. (1984). An improved method for the glycolate test for simple identification of the acyl type of bacterial cell walls. *J Gen Appl Microbiol* **30**, 131–134.

Warwick, S., Bowen, T., McVeigh, H. P. & Embley, T. M. (1994). A phylogenetic analysis of the family *Pseudonocardiaceae* and the

genera *Actinokineospora* and *Saccharothrix* with 16S rRNA sequences and a proposal to combine the genera *Amycolata* and *Pseudonocardia* in an emended genus *Pseudonocardia*. *Int J Syst Bacteriol* **44**, 293–299.

Yassin, A. F., Haggenei, B., Budzikiewicz, H. & Schaal, K. P. (1993). Fatty acid and polar lipid composition of the genus *Amycolatopsis*: application of fast bombardment-mass spectrometry to the structure analysis of underivatized phospholipids. *Int J Syst Bacteriol* **43**, 414–420.