Amycolatopsis echigonensis sp. nov. and *Amycolatopsis niigatensis* sp. nov., novel actinomycetes isolated from a filtration substrate

Linxian Ding, Taketo Hirose and Akira Yokota

Correspondence

Linxian Ding

linxian@iam.u-tokyo.ac.jp

Laboratory of Bioresources, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

The taxonomic position of two actinomycete strains, LC2^T and LC11^T, isolated from a filtration substrate made from Japanese volcanic soil, was determined using a polyphasic approach. The strains grew at temperatures from 5 to 45 °C, on media of pH between 6 and 11 and in the presence of 7 % NaCl. The major menaquinone was MK-9(H₄). The major fatty acid was iso-C_{16:0}. A phylogenetic tree based on 16S rRNA gene sequences showed that the two strains formed a distinct evolutionary lineage within the genus *Amycolatopsis*. On the basis of their morphological, physiological and genotypic characteristics, the isolates are proposed to represent two novel species of the genus *Amycolatopsis*, for which the names *Amycolatopsis echigonensis* sp. nov. (type strain LC2^T = IAM 15387^T = CCTCC AB206019^T), and *Amycolatopsis niigatensis* sp. nov. (type strain LC11^T = IAM 15388^T = CCTCC AB206020^T) are proposed.

The genus *Amycolatopsis* was established by Lechevalier *et al.* (1986) and was assigned to the family *Pseudonocardaceae* (Embley *et al.*, 1988; Warwick *et al.*, 1994). The principal characteristics of this genus are the presence of a type IV cell wall (*meso*-diaminopimelic acid and cell-wall sugars consisting of arabinose and galactose; Lechevalier & Lechevalier, 1970; Lechevalier *et al.*, 1986) and MK-9(H₄) menaquinones and the absence of mycolic acids. During the first 13 years of its existence, only 10 novel species were described for this genus, but, since 2000, many novel species have been described from soil, fresh water, human and animal clinical sources, natural caves, prairie soil and other natural areas. At the time of writing, the genus contains 34 species (as well as four subspecies).

In this investigation, we describe two actinomycetes, LC2^T and LC11^T, which were isolated from a filtration substrate composed of volcanic soil. They are moderately thermophilic actinomycetes that can grow at 45 °C. Genotypic and phenotypic analyses indicated that these isolates should be classified within two novel species of the genus *Amycolatopsis*.

We used a filtration substrate as the substrate for the isolation of bacterial strains on NY medium (1.6 g nutrient broth, 0.5 g yeast extract, 1000 ml deionized water and 1.5 % agar, pH 7.0) with cycloheximide (0.05 g l⁻¹) and kabicidin (0.1 g l⁻¹). All of the isolates were maintained on NY medium or oatmeal agar and incubated at 27 °C.

Determination of the optimum temperature for growth and pH tolerance was carried out by incubating in both nutrient broth and nutrient agar. NaCl tolerance was determined in nutrient broth amended with 3, 5 and 7 % NaCl and incubated at 27 °C. Air-dried smears prepared at 48 h intervals were Gram-stained to determine the Gram reaction, and investigation of the cell morphology was done after incubating for 2 weeks. Biochemical tests were performed with API 50CH, API ZYM and API CORYNE strips (bioMérieux) according to the manufacturer's instructions.

Genomic DNA preparation and PCR amplification of the 16S rRNA gene were performed as described previously by Ding & Yokota (2002, 2004). Nucleotide sequences were obtained automatically using an Applied Biosystems DNA sequencer (model 377) and software provided by the manufacturer. Alignment was performed using CLUSTAL_X version 1.83 (Thompson *et al.*, 1997). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) and the maximum-likelihood method (Felsenstein, 1981) and parsimony analysis based on 1348 nucleotides present in all strains. The evolutionary distance matrix was calculated using Kimura's two-parameter method (Kimura, 1980). The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1000 sampling replications according to the method of Felsenstein (1985). The PHYLIP package (version 3.5c; Felsenstein, 1993) was used for all phylogenetic analyses.

Genomic DNA was prepared using a method described previously (Sambrook *et al.*, 1989). The G + C content of

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains LC2^T and LC11^T are AB248535 and AB248537.

the DNA was determined by HPLC (Mesbah *et al.*, 1989). DNA–DNA relatedness experiments were performed by a modification of the microplate method of Ezaki *et al.* (1989), using photobiotin-labelled DNA and microdilution wells, as described by Willems *et al.* (2001). The DNA–DNA hybridization temperature was 55 °C.

The isomeric form of the diaminopimelic acid was determined by TLC from whole-cell hydrolysates using the method of Stanek & Roberts (1974). The whole-cell sugar composition was determined by TLC as described by Stanek & Roberts (1974), Hasegawa *et al.* (1983) and Lechevalier & Lechevalier (1980). Analysis of menaquinones was carried out according to the methods of Minnikin *et al.* (1984) and Kroppenstedt (1985). Mycolic acids were examined by the acid methanolysis method of Minnikin *et al.* (1980) and Yano *et al.* (1972). The strains were incubated on TSBA medium for 48 h at 30 °C. Fatty acid methyl esters were obtained from the cells by saponification, methylation and extraction. Analysis by GC was controlled by MIS software (Microbial ID Inc.). The peaks were automatically integrated and identified by the Microbial Identification software package (Sasser, 1990).

The almost-complete 16S rRNA gene sequences of strains LC2^T (1473 nt) and LC11^T (1472 nt) were determined in this study. Database sequences for other members of the genus *Amycolatopsis* were obtained from DDBJ/EMBL/GenBank for use in comparative analyses, and the phylogenetic tree was constructed. 16S rRNA gene sequence comparisons showed clearly that isolates LC2^T and LC11^T are members of the genus *Amycolatopsis*. Fig. 1 shows that these two isolates, *Amycolatopsis rubida*, *Amycolatopsis albidoflavus* and *Amycolatopsis benzoatilytica* formed a single cluster, and the sequence similarity between strains LC2^T and the type strains of *A. rubida*, *A. albidoflavus*, *A. benzoatilytica* and LC11^T was 97.6, 98.1, 97.9 and 96.4 %, respectively, while the sequence similarity of strains LC11^T and the type strains of *A. rubida*, *A. albidoflavus* and *A. benzoatilytica* was respectively 97.8, 98.2 and 96.1 %. These results suggest that strains LC2^T and LC11^T belong to two genetically distinct *Amycolatopsis* species, which are not closely related to *A. rubida*, *A. albidoflavus* and *A. benzoatilytica*. Furthermore, chromosomal DNA–DNA hybridization studies were performed to establish whether isolates LC2^T and LC11^T represent distinct species. Strain LC2^T

Fig. 1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the phylogenetic position of strains LC2^T and LC11^T within the radiation of *Amycolatopsis* species. Asterisks indicate branches of the tree that were also found using the maximum-likelihood (Felsenstein, 1981) treeing algorithm. Numbers at nodes indicate percentage levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values over 50 % are given. Bar, 0.01 nucleotide substitution per nucleotide position.

Table 1. Differential phenotypic characteristics of the novel species of the genus *Amycolatopsis*

Data for reference strains were taken from Lee & Hah (2001) (*A. albidoflavus*), Huang *et al.* (2001) (*A. rubida*) and Majumdar *et al.* (2006) (*A. benzoatilytica*). +, Positive; (+), weakly positive; –, negative; ND, no data.

Characteristic	Strain LC2 ^T	Strain LC11 ^T	<i>A. albidoflavus</i> JCM 11300 ^T	<i>A. rubida</i> JCM 10871 ^T	<i>A. benzoatilytica</i> DSM 43387 ^T
Colony colour*	W/LY	W, Y/PB	W/Y	W/Y	CC
Temperature range (°C)	5–45	5–45	10–37	10–40	10–37
pH range for growth	6–11	6–11	ND	ND	5–10
Growth in the presence of NaCl (% w/v)	3–7	3–7	7	7	5
Acid produced from:					
<i>myo</i> -Inositol	+	–	+	+	+
D-Lactose	+	–	+	–	+
D-Mannose	–	–	+	ND	+
D-Mannitol	–	+	(+)	+	–
D-Sorbitol	–	(+)	–	–	–
Nitrate reductase	+	–	+	ND	ND

*CC, Cream-coloured; LY, light yellow; PB, purple–brown; W, white; Y, yellow.

displayed low levels of DNA–DNA reassociation with *A. albidoflavus* JCM 11300^T (44.7–48.3 %), *A. rubida* JCM 10871^T (39.0–44.1 %) and strain LC11^T (58.0–62.1 %); also, strain LC11^T displayed low levels of DNA–DNA reassociation with *A. albidoflavus* JCM 11300^T (32.8–34.4 %) and *A.*

rubida JCM 10871^T (28.3–35.3 %). These results are below the cut-off point recommended for the circumscription of bacterial genomic species by Wayne *et al.* (1987), and confirm the separation of isolates LC2^T and LC11^T from *A. albidoflavus*, *A. rubida* and *A. benzoatilytica*.

Table 2. Comparison of chemotaxonomic and genetic characteristics among the novel isolates and related *Amycolatopsis* species

Data for reference strains were taken from Lee & Hah (2001) (*A. albidoflavus*), Huang *et al.* (2001) (*A. rubida*) and Majumdar *et al.* (2006) (*A. benzoatilytica*).

Characteristic	Strain LC2 ^T	Strain LC11 ^T	<i>A. albidoflavus</i> JCM 11300 ^T	<i>A. rubida</i> JCM 10871 ^T	<i>A. benzoatilytica</i> DSM 43387 ^T
Fatty acid (%)					
iso-C _{14:0}	6.5	7.1	9.8	1.2	11.3
C _{14:0}	0.4	0.4	1.1	0.2	0.5
iso-C _{15:0}	7.0	9.3	8.7	22.7	6.8
anteiso-C _{15:0}	16.7	7.7		5.9	3.0
C _{15:0}	5.2	4.5	5.8	4.0	14.6
C _{15:1} ω6 <i>c</i>	3.6		2.3	1.6	12.8
iso-C _{16:0}	31.9	40.0	26.1	11.2	12.8
C _{16:0}	2.5	2.1	10.2	5.6	5.2
iso-C _{16:1} H	1.3	2.6			0.2
iso-C _{17:0}	1.1	1.6	1.6	8.1	0.4
anteiso-C _{17:0}	6.9	6.9		12.4	0.8
C _{17:0}	3.4	2.6	7.8	9.9	11.3
C _{17:1} ω6 <i>c</i>	5.6	6.6	4.4	2.9	
C _{17:1} ω8 <i>c</i>	2.4	1.7		1.6	
C _{18:0}			6.2		0.4
Summed feature 3*	4.5	5.3		11.6	6.8
Major quinone	MK-9(H ₄)	MK-9(H ₄)	MK-9(H ₄)	MK-9(H ₆)	ND
DNA G + C content (mol%)	72.7	72.4	68.5	67.4	ND

*Summed feature 3 contains C_{16:1}ω7*c* and/or iso-C_{15:0} 2-OH.

The classification of isolates LC2^T and LC11^T within the genus *Amycolatopsis* is supported by phenotypic, morphological and biochemical characteristics. Differential morphological and physiological characteristics are shown in Table 1. Both isolates are Gram-positive, aerobic, catalase-positive and oxidase-negative. Results of chemotaxonomic analysis of the isoprenoid menaquinone, fatty acid compositions and DNA G + C content of isolates LC2^T and LC11^T are shown in Table 2. The major isoprenoid menaquinone was MK-9(H₄). The major cellular fatty acid was iso-C_{16:0} (31.9 and 40.0 % of the total in strains LC2^T and LC11^T), and other fatty acids (over 5 % of the total) included iso-C_{14:0}, iso-C_{15:0}, anteiso-C_{15:0}, C_{15:0}, anteiso-C_{17:0} and C_{17:1ω6c}. The isolates contained *meso*-diaminopimelic acid as the cell-wall diamino acid and arabinose and galactose as major whole-cell sugars (type IV cell wall, according to Lechevalier & Lechevalier, 1970). The strains did not contain mycolic acids. Other morphological and physiological characteristics are listed in the species descriptions.

On the basis of the phenotypic, chemotaxonomic and phylogenetic data, strains LC2^T and LC11^T merit recognition as representatives of novel species of the genus *Amycolatopsis*. We propose the names *Amycolatopsis echigonensis* sp. nov. and *Amycolatopsis niigatensis* sp. nov. for these taxa.

Description of *Amycolatopsis echigonensis* sp. nov.

Amycolatopsis echigonensis (e.chi.go.nen'sis. N.L. fem. adj. *echigonensis* referring to Echigo, the old name of Niigata Prefecture, Japan, from where the type strain was isolated).

Cells are aerobic, non-motile and Gram-positive and form a well-developed aerial and vegetative mycelium. The aerial mycelium is white and the vegetative mycelium is light yellow. The temperature range for growth is 5–45 °C and the optimum temperature for growth is 30 °C. Grows on medium containing 3–7 % NaCl. The pH range for growth is 6–11, and the optimum is pH 9. Catalase-positive and oxidase-negative. Acid is produced from D-xylose, D-galactose, L-rhamnose, inositol and D-lactose. Production of acid from erythritol, D-adonitol and D-glucose is weak. Using the API CORYNE and API ZYM systems, positive reactions are observed for nitrate reductase, pyrazinamidase, α-glucosidase, β-glucuronidase, β-galactosidase, urease, gelatin hydrolysis, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, α-galactosidase, alkaline phosphatase, N-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase and α-mannosidase. No activity is detected for β-glucosidase, lipase (C14) or α-fucosidase; pyrrolidonyl arylamidase and α-chymotrypsin activities are weak. The predominant fatty acid is iso-C_{16:0}. The major menaquinone is MK-9(H₄). The G + C content of the type strain is 72.7 mol%.

The type strain is strain LC2^T (=IAM 15387^T =CCTCC AB206019^T), which was isolated from a filtration substrate made from volcanic soil, Niigata, Japan.

Description of *Amycolatopsis niigatensis* sp. nov.

Amycolatopsis niigatensis (ni.i.ga.ten'sis. N.L. fem. adj. *niigatensis* referring to Niigata Prefecture, Japan, from where the type strain was isolated).

Cells are aerobic, non-motile and Gram-positive and form a well-developed aerial and vegetative mycelium. The aerial mycelium is white or light yellow; the vegetative mycelium is purple or purple-brown. The temperature range for growth is 5–45 °C and the optimum temperature for growth is 30 °C. Grows in presence of 3–7 % NaCl. The pH range for growth is 6–11 and the optimum is pH 9. Catalase-positive and oxidase-negative. Acid is produced from D-galactose and L-rhamnose; no acid is produced from D-xylose, inositol or D-lactose; acid production for erythritol, D-adonitol and D-glucose is weak. Using the API CORYNE and API ZYM systems, positive reactions are observed for pyrazinamidase, pyrrolidonyl arylamidase, α-glucosidase, β-glucuronidase, β-galactosidase, urease, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, β-glucosidase, alkaline phosphatase, N-acetyl-β-glucosaminidase, α-chymotrypsin and α-mannosidase. No activity is detected for nitrate reductase, esterase (C4), α-galactosidase, lipase (C14) or α-fucosidase; gelatin hydrolysis and acid phosphatase are weak. The predominant fatty acid is iso-C_{16:0}. The major menaquinone is MK-9(H₄). The G + C content of the DNA of the type strain is 72.4 mol%.

The type strain is strain LC11^T (=IAM 15388^T =CCTCC AB206020^T), which was isolated from a filtration substrate made from volcanic soil, Niigata, Japan.

Acknowledgements

We thank Professor Dr Hans G. Trüper and Dr Jean Euzéby for their help regarding the Latin nomenclature.

References

- Ding, L. & Yokota, A. (2002). Phylogenetic analysis of the genus *Aquaspirillum* based on 16S rRNA gene sequences. *FEMS Microbiol Lett* **212**, 165–169.
- Ding, L. & Yokota, A. (2004). Proposals of *Curvibacter gracilis* gen. nov., sp. nov. and *Herbaspirillum putei* sp. nov. for bacterial strains isolated from well water and reclassification of [*Pseudomonas*] *huttiensis*, [*Pseudomonas*] *lanceolata*, [*Aquaspirillum*] *delicatum* and [*Aquaspirillum*] *autotrophicum* as *Herbaspirillum huttiense* comb. nov., *Curvibacter lanceolatus* comb. nov., *Curvibacter delicatus* comb. nov. and *Herbaspirillum autotrophicum* comb. nov. *Int J Syst Evol Microbiol* **54**, 2223–2230.
- Embley, T. M., Smida, J. & Stackebrandt, E. (1988). The phylogeny of mycolate-less wall chemotype IV actinomycetes and description of *Pseudonocardia* fam. nov. *Syst Appl Microbiol* **11**, 44–52.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Felsenstein, J. (1993). PHYLIP (phylogeny inference package) version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Hasegawa, T., Takizawa, M. & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* **29**, 319–322.
- Huang, Y., Qi, W., Lu, Z., Liu, Z. & Goodfellow, M. (2001). *Amycolatopsis rubida* sp. nov., a new *Amycolatopsis* species from soil. *Int J Syst Evol Microbiol* **51**, 1093–1097.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kroppenstedt, R. M. (1985). Fatty acid and menaquinone analysis of actinomycetes and related organisms. In *Chemical Methods in Bacterial Systematics*, pp. 173–199. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Lechevalier, M. P. & Lechevalier, H. A. (1970). Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int J Syst Bacteriol* **20**, 435–443.
- Lechevalier, H. A. & Lechevalier, M. P. (1980). The chemotaxonomy of actinomycetes. In *Actinomycete Taxonomy* (Society for Industrial Microbiology Special Publication no. 6), pp. 277–284. Edited by A. Dietz & D. W. Thayer. Arlington, VA: Society for Industrial Microbiology.
- 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.
- Majumdar, S., Prabhakaran, S. R., Shivaji, S. & Lal, R. (2006). Reclassification of *Amycolatopsis orientalis* DSM 43387 as *Amycolatopsis benzoatilytica* sp. nov. *Int J Syst Evol Microbiol* **56**, 199–204.
- 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., 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.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*. MIDI Technical Note 101. Newark, DE: MIDI Inc.
- 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.
- Warwick, S., Bowen, T., McVeigh, H. & 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.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Willems, A., Doignon, B. F., Goris, J., Coopman, R., de Lajudie, P., De Vos, P. & Gillis, M. (2001). DNA–DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* **51**, 1315–1322.
- Yano, I., Saito, K., Fukukawa, Y. & Kusunose, M. (1972). Structural analysis of molecular species of nocardomycetic acids from *Nocardia erythropolis* by the combined system of gas chromatography and mass spectrometry. *FEBS Lett* **21**, 215–219.