Amycolatopsis thailandensis sp. nov., a poly(L-lactic acid)-degrading actinomycete, isolated from soil

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A novel actinomycete that was capable of degrading poly(L-lactic acid), strain CMU-PLA07^T, was isolated from soil in northern Thailand. Strain CMU-PLA07^T had biochemical, chemotaxonomic, morphological and physiological properties that were consistent with its classification in the genus *Amycolatopsis*. 16S rRNA gene sequence analysis showed that the isolate formed a phyletic line within the genus *Amycolatopsis*. Strain CMU-PLA07^T was most similar to *Amycolatopsis coloradensis* IMSNU 22096^T (99.5% 16S rRNA gene sequence similarity) and *Amycolatopsis alba* DSM 44262^T (99.4%). However, strain CMU-PLA07^T was distinguishable from the type strains of species of the genus *Amycolatopsis* on the basis of DNA–DNA relatedness and phenotypic data. Therefore, strain CMU-PLA07^T is considered to represent a novel species of the genus *Amycolatopsis*, for which the name *Amycolatopsis thailandensis* sp. nov. is proposed. The type strain is CMU-PLA07^T (=JCM 16380^T=BCC 38279^T).

Biodegradable plastics are increasingly considered to be the most promising alternative to disposable plastic. Poly (L-lactic acid) (PLA) is a biodegradable plastic that can be produced by fermentation of renewable resources. It is expected that PLA will replace plastics from petrochemicals (Tokiwa & Calabia, 2006; Li *et al.*, 2008). PLA-degrading actinomycetes were first reported by Pranamuda *et al.* (1997) and identified as members of the genus *Amycolatopsis*, which is well known for its high PLA-degrading activity. The taxonomy of the genus *Amycolatopsis* is based on chemotaxonomic characteristics and phylogenetic study of 16S rRNA gene sequences (Lechevalier *et al.*, 1986; Mertz & Yao, 1993; Labeda, 1995; Lee *et al.*, 2000; Lee, 2009). At the time of writing, the genus *Amycolatopsis* consisted of 38 recognized species and many novel species have been reported from various environmental sources (Bian *et al.*, 2009; Lee, 2009). This study investigated the taxonomic position of the novel PLA-degrading strain CMU-PLA07^T, a member of the genus *Amycolatopsis*.

Strain CMU-PLA07^T was isolated from natural park soil in northern Thailand. A soil sample (1 g) was suspended in 10 ml basal medium [containing 0.4% (NH₄)₂SO₄, 0.2% K₂HPO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O and 0.1% yeast extract] with a 25 mg sample of 0.1% (w/v) PLA film, as described by Tomita *et al.* (1999), and incubated at 30 °C with rotary shaking at 150 r.p.m. for 14 days. The enriched culture broth was spread onto 0.1% (w/v) emulsified PLA agar and incubated at 30 °C for 14 days.

The cultural and morphological characteristics were observed on ISP media as described by Shirling & Gottlieb (1966). The colours of aerial mycelium, substrate mycelium and soluble pigment were observed after incubation for 14 days at 30 °C using a NBS/IBCC colour chart (Mundie, 1995). The morphological features of the mycelium and spores were observed by light microscopy (BH-2; Olympus) and scanning electron microscopy (model JSM-5910; JEOL).

Bacterial biomass for chemotaxonomic studies was prepared by culturing the isolate in ISP 2 medium with rotary shaking at 150 r.p.m. at 27 °C for 4 days. Cells were harvested and then freeze-dried. The cell-wall diamino-pimelic acid (DAP) isomers were examined by two-dimensional TLC (Tokyo Kasei) as described by Harper & Davis (1979) and by HPLC as described by Yokota *et al.* (1993). The cell-wall sugar composition was analysed by

Abbreviations: DAP, diaminopimelic acid; PLA, poly(L-lactic acid).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CMU-PLA07^T is FJ581021.

A supplementary figure and a supplementary table are available with the online version of this paper.

GLC. Sugars were converted into their alditol acetates by the method of Sawardeker *et al.* (1965). Chloroform solutions of the alditol acetate derivatives were injected into a Shimadzu GC-17A gas chromatograph equipped with an Rtx 2330 column (0.32 mm × 30 m; temperature 175–240 °C). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and purified by TLC using hexane/diethyl ether (85:15, v/v) as the solvent and the menaquinone fraction was analysed by HPLC (Collins & Jones, 1981). The cell-wall amino acid composition was determined by HPLC according to the methods of Schleifer & Kandler (1972) and Harper & Davis (1979). Cellular fatty acids were extracted according to the protocol of the MIDI system (Microbial ID). Peaks were automatically integrated and identified using the Microbial Identification software (Sasser, 1990). Polar lipids were analysed by two-dimensional TLC (Minnikin *et al.*, 1979). The DNA G+C content was determined by HPLC as described by Mesbah *et al.* (1989).

Phenotypic characterization was carried out using established methods. Growth with 0–9% (w/v) NaCl, at 5, 10, 17, 25, 30, 37 and 45 °C and at pH 4.0–11.0 was tested on ISP 2. Carbohydrate utilization was investigated on ISP 9 at 30 °C for 14 days. Acid production from carbohydrates and degradation of casein, aesculin, starch, L-tyrosine and urea were examined using the method described by Gordon *et al.* (1974). Catalase activity was determined by assessing bubble production in 3% (v/v) H₂O₂. Enzyme activity was determined using the API ZYM system (bioMérieux) according to the manufacturer's instructions.

Genomic DNA was prepared according to a modified cetyltrimethylammonium bromide method (Murray &

Thompson, 1980). The 16S rRNA gene of strain CMU-PLA07^T was amplified by PCR using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3'). The PCR products were purified using a Nucleospin Extract II PCR purification kit (Macherey-Nagel) and sequenced by Macrogen. The 16S rRNA gene sequence was compared with the sequences obtained from public databases and aligned using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic and molecular evolution analysis was conducted using MEGA version 4.0 (Tamura *et al.*, 2007). Tree topologies were evaluated by bootstrap analysis with 1000 resamplings. DNA–DNA relatedness studies were carried out in duplicate with five replications between strain CMU-PLA07^T and its closest phylogenetic neighbours using photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989) and a multi-well plate reader (CytoFluoR; PerSeptive Biosystems).

The morphological characteristics of strain CMU-PLA07^T were typical for members of the genus *Amycolatopsis*. The isolate grew well on all ISP media tested and formed powdery circular colonies that were covered with white to pale yellow aerial mycelium on different ISP media. On ISP 2, 5 and 7 media, aerial mycelium was abundant, which fragmented into long strands and had smooth-surfaced conidia (see Supplementary Fig. S1, available in IJSEM Online). Substrate mycelium was yellowish brown and well developed on ISP 3, 4, 5 and 7 media. No diffusible pigments were produced on any of the media tested.

The cell-wall hydrolysates contained *meso*-diaminopimelic acid (*meso*-DAP) and arabinose and galactose as major sugars (cell-wall type IV; Lechevalier & Lechevalier, 1970).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain CMU-PLA07^T and related members of the genus *Amycolatopsis*. Bootstrap values (>50) based on 1000 resamplings are shown at branch nodes. Asterisks indicate that the corresponding nodes were also found in trees generated with the minimum-evolution and maximum-parsimony algorithms. *Pseudonocardia autotrophica* IMSNU 20050^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

The cell-wall amino acids were alanine, glutamate, *meso*-DAP and glycine. The menaquinone was MK-9(H₄). The polar lipid pattern was composed of phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylmethylethylethanolamine. All of the chemotaxonomic properties of strain CMU-PLA07^T were consistent with those of the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Henssen *et al.*, 1987; Kim & Goodfellow, 1999; Tan *et al.*, 2006).

The fatty acid analysis was performed with strain CMU-PLA07^T and its closest phylogenetic neighbours. The fatty acid profiles contained a mixture of saturated and branched-chain acids. The predominant fatty acids of strain CMU-PLA07^T were iso-C_{15:0} (21.47%), C_{16:0} (20.72%) and iso-C_{16:0} (14.41%). Strain CMU-PLA07^T differed from *Amycolatopsis coloradensis* JCM 9869^T and *Amycolatopsis alba* JCM 10030^T by containing minor amounts of C_{16:0} 9-methyl but not anteiso-C_{16:0} or anteiso-C_{17:0} 2-OH (see Supplementary Table S1).

The neighbour-joining tree based on 16S rRNA gene sequences revealed that strain CMU-PLA07^T belonged to the genus *Amycolatopsis* and formed a clade with *A. coloradensis* IMSNU 22096^T and *A. alba* DSM 44262^T, which was supported by a bootstrap value of 99% (Fig. 1). The minimum-evolution and maximum-parsimony algorithms resulted in trees with similar topologies. Strain CMU-PLA07^T was most closely related to *A. coloradensis* IMSNU 22096^T (99.5% 16S rRNA gene sequence similarity) and *A. alba* DSM 44262^T (99.4%). Strain CMU-PLA07^T displayed low DNA–DNA relatedness with *A. coloradensis* JCM 9869^T and *A. alba* JCM 10030^T (49 ± 1.4 and 45 ± 2.8%, respectively). Both values were significantly less than the 70% cut-off value recommended for the delineation of bacterial species by Wayne *et al.* (1987). Some members of the genus *Amycolatopsis* share high 16S rRNA gene sequence similarity but less than 70% DNA–DNA relatedness. For example, the type strains of *Amycolatopsis lexingtonensis* and *Amycolatopsis pretoriensis* share 99.9% 16S rRNA gene sequence similarity and 54.1% DNA–DNA relatedness (Labeda *et al.*, 2003). Similarly, the type strains of *Amycolatopsis methanolica* and *Amycolatopsis thermoflava* share 99.8% 16S rRNA gene sequence similarity and only 21% DNA–DNA relatedness (Chun *et al.*, 1999). The DNA G+C content of strain CMU-PLA07^T was 67 mol%.

The results of the physiological and biochemical characterization are given in the species description and the phenotypic characteristics that differentiated strain CMU-PLA07^T from its closest phylogenetic neighbours, *A. coloradensis* JCM 9869^T and *A. alba* JCM 10030^T, are given in Table 1. Strain CMU-PLA07^T differed from the reference strains in its ability to grow at 37 °C and to produce acid from maltose and D-ribose.

On the basis of phenotypic, chemotaxonomic and genotypic characteristics, strain CMU-PLA07^T represents

Table 1. Phenotypic and biochemical characteristics of strain CMU-PLA07^T and its closest phylogenetic neighbours

Strains: 1, CMU-PLA07^T; 2, *A. coloradensis* JCM 9869^T; 3, *A. alba* JCM 10030^T. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3
Production of diffusible pigments	–	+	–
Nitrate reduction	+	+	–
Growth at/in:			
37 °C	+	–	–
7% NaCl	+	–	+
Utilization of:			
Sucrose	–	w	+
Raffinose	+	w	+
Cellulose	–	–	+
Degradation of:			
Urea	+	–	+
Acid production from:			
D-Galactose	+	+	w
Lactose	+	–	+
Maltose	+	–	–
Melibiose	+	–	w
Raffinose	+	–	+
D-Ribose	+	–	–
L-Sorbose	–	w	–
Trehalose	+	+	w
Enzyme activity			
Cystine arylamidase	w	+	+
β-Galactosidase	–	–	+
α-Galactosidase	w	–	–
Lipase (C14)	–	+	–
Urease	+	–	+

a novel species of the genus *Amycolatopsis*, for which the name *Amycolatopsis thailandensis* sp. nov. is proposed.

Description of *Amycolatopsis thailandensis* sp. nov.

Amycolatopsis thailandensis (thai.land.en'sis. N.L. fem. adj. *thailandensis* pertaining to Thailand, the source of the soil from which the type strain was isolated).

Gram-positive, aerobic, non-motile and non-acid-fast actinomycete, which forms white colonies on ISP 2. Aerial mycelium is white to pale yellow with long fragmented strands of smooth-surfaced conidia. Substrate mycelium is yellowish brown. No diffusible pigment is produced. On ISP 2, grows at pH 5.0–11.0 (optimum pH 6.0–10.0), at 10–37 °C (optimum 25–37 °C), but not at 45 °C, and with 7% (w/v) NaCl. Cell-wall hydrolysates contain *meso*-DAP, arabinose and galactose. The menaquinone is MK-9(H₄). The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylmethylethylethanolamine. The major fatty acids (>10%) are iso-C_{15:0}, C_{16:0} and iso-C_{16:0}. Arabinose, fructose, glucose, inositol, mannitol, raffinose and xylose are utilized as sole carbon sources,

but not cellulose, rhamnose or sucrose. Nitrate is reduced to nitrite and gelatin is hydrolysed. Acid is produced from cellobiose, D-fructose, D-galactose, D-glucose, inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, raffinose, D-ribose, trehalose and D-xylose, but not from L-arabinose, L-rhamnose, D-sorbitol or L-sorbose. Casein, aesculin, starch, L-tyrosine and urea are degraded, but not adenine. Positive for acid phosphatase, N-acetyl- β -glucosamidase, alkaline phosphatase, catalase, esterase, esterase lipase (C8), α -glucosidase, β -glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, pyrazinamidase, pyrrolidonyl arylamidase, urease and valine arylamidase, weakly positive for α -chymotrypsin, cystine arylamidase, α -galactosidase and trypsin, but negative for α -fucosidase, β -galactosidase, β -glucuronidase, lipase (C14) and α -mannosidase.

The type strain, CMU-PLA07^T (=JCM 16380^T=BCC 38279^T), was isolated from soil in northern Thailand and is able to degrade poly(L-lactic acid). The DNA G+C content of the type strain is 67 mol%.

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