Actinomadura rupiterrae sp. nov., isolated from cliff soil

Soon Dong Lee

Correspondence

Soon Dong Lee
sdlee@jejunu.ac.kr

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

A novel actinomycete strain, designated CS5-AC15^T, was isolated from a soil sample collected from a cliff on Mara Island, Jeju, Republic of Korea, and subjected to a polyphasic taxonomic analysis. The isolate produced well-developed, yellow substrate mycelium and white aerial mycelium that differentiated into straight or flexuous chains of smooth-surfaced spores. 16S rRNA gene sequence analyses showed that the organism belonged to the family *Thermomonosporaceae* and formed a tight cluster with the type strain of *Actinomadura oligospora* (97.4 % sequence similarity). Chemotaxonomic characteristics were consistent with its assignment to the genus *Actinomadura* in that the isolate had *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell wall, madurose as the characteristic sugar, *N*-acetyl type of murein in the peptidoglycan, MK-9(H₆) and MK-9(H₈) as major menaquinones and a polar lipid profile containing diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and unknown phospholipids. Mycolic acids were not detected. The predominant fatty acids were C_{16:0}, C_{18:1ω9c} and iso-C_{16:0}. The DNA G + C content was 70.9 mol%. DNA relatedness of strain CS5-AC15^T and *A. oligospora* JCM 10648^T was 37.9 ± 0.7 %. On the basis of the phenotypic, phylogenetic and DNA–DNA hybridization data, strain CS5-AC15^T is assigned to a novel species of the genus *Actinomadura*, for which the name *Actinomadura rupiterrae* sp. nov. is proposed (type strain CS5-AC15^T = KCTC 19559^T = DSM 45251^T).

The genus *Actinomadura* (Lechevalier & Lechevalier, 1970b) belongs to the family *Thermomonosporaceae* Stackebrandt *et al.* 1997 emend. Zhang *et al.* 2001, together with the genera *Actinoallomurus*, *Actinocorallia*, *Spirillospora* and *Thermomonospora*, and at the time of writing consisted of 46 recognized species, including the recently described species *Actinomadura miaoliensis* (Tseng *et al.*, 2009), *A. keratinilytica* (Puhl *et al.*, 2009), *A. flavalba* (Qin *et al.*, 2009), *A. sputi* (Yassin *et al.*, 2010), *A. scrupuli* (Lee & Lee, 2010), *A. apis*, *A. rifamycinii* (Promnuan *et al.*, 2011) and *A. meridiana* (Lee, 2012). Members of the genus contain *meso*-diaminopimelic acid as the diagnostic diamino acid and madurose as the characteristic sugar in the cell wall, with a murein structure of the acetyl type, and possess predominant menaquinones MK-9(H₄), MK-9(H₆) and MK-9(H₈), phospholipid type PI (diphosphatidylglycerol and phosphatidylinositol present as major phospholipids), fatty acid type 3a [branched saturated and unsaturated fatty acids and tuberculostearic acid (TBSA)] and DNA G + C contents of 65–73 mol% (Kroppenstedt & Goodfellow, 1991; Meyer, 1989). Morphologically, the genus is characterized by the production of well-developed, non-fragmenting vegetative hyphae and aerial mycelium,

on the tips of which spore chains of various lengths are arranged in straight, hooked or spiral form. The smooth, spiny or warty-surfaced spores are non-motile, oval or rod-shaped. This genus can be readily differentiated from the other genera of the family *Thermomonosporaceae* on the basis of a combination of morphological and chemotaxonomic characteristics (Kroppenstedt & Goodfellow, 2006). In this paper, the classification and identification of an *Actinomadura* strain that was isolated from soil is described by a polyphasic taxonomic approach.

Strain CS5-AC15^T was isolated from a soil sample taken from a cliff on Mara Island, Jeju, Republic of Korea. Bacterial isolation was performed by using the procedure described by Lee & Jeong (2006) and starch-casein agar (Küster & Williams, 1964) as the isolation medium. The pure culture was maintained on International *Streptomyces* Project (ISP) medium 2 (Shirling & Gottlieb, 1966) and as mycelial fragments or spores in 20 % (v/v) glycerol at –80 °C. For phenotypic analysis and DNA–DNA hybridization, *A. oligospora* JCM 10648^T was grown on ISP medium 2 at 30 °C.

Growth and pigmentation of strain CS5-AC15^T were investigated on various media: yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salts-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7)

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CS5-AC15^T is FM210337.

A supplementary table is available with the online version of this paper.

(Shirling & Gottlieb, 1966). Results for cultural and morphological characterization were recorded after 2 weeks of incubation at 30 °C. Spore chain morphology and spore ornamentation were observed by using scanning electron microscopy (JSM-6500; JEOL). The specimen for electron microscopy was fixed, dehydrated, critical-point-dried and coated with gold as described by Lee & Jeong (2006) before observation.

Strain CS5-AC15^T grew well on ISP media 2, 3 and 4 and moderately on ISP 5, but poor or no growth was observed on ISP media 6 and 7. Substrate mycelium was well developed, branched and light to strong yellow in colour. White aerial mycelium was produced abundantly on ISP media 3 and 4 and differentiated into straight or flexuous chains of smooth-ornamented spores. Ten or more spores were present per chain (Fig. 1). No soluble pigments were produced on any of the tested media.

Unless otherwise indicated, physiological properties were determined using ISP 2 as the basal medium. Growth was examined at 4, 10, 20, 30, 37, 42 and 45 °C and at pH 4–12 (at intervals of 1 pH unit). The results were recorded after 14 days of incubation at or below 30 °C and after 5 days of incubation at or above 37 °C. Tolerance of NaCl for growth was tested on ISP 2 supplemented with 1–9 % (w/v) NaCl (at intervals of 1.0 %). Tolerance of pH and NaCl for growth was observed on plates incubated at 30 °C for 14 days. Decomposition of hypoxanthine, DL-tyrosine and xanthine was tested as described by Gordon *et al.* (1974). Oxidase activity was determined according to the method of MacFaddin (1980). Catalase and urease activities, H₂S production, nitrate reduction and hydrolysis of casein and gelatin were tested as described by Lee & Jeong (2006). Cellulose hydrolysis was determined on ISP medium 2 supplemented with 0.5 % (w/v) CM-cellulose (Sigma). Aesculin hydrolysis was tested on agar medium containing

0.3 % (w/v) yeast extract, 0.05 % (w/v) ferric ammonium citrate, 1 % (w/v) aesculin and 0.75 % (w/v) agar, and blackening of the medium was recorded as positive after incubation for 14 days at 30 °C. Hydrolysis of DNA and starch was determined using DNase test agar (Difco) and starch agar (Difco), respectively. Utilization of substrates as sole carbon and energy sources was determined using ISP 9 supplemented with carbohydrates and alcohols at final concentrations of 1 % (w/v) and organic acids at final concentrations of 0.1 % (w/v). The physiological properties of strain CS5-AC15^T are given in the species description and in Table 1.

Chromosomal DNA extraction, amplification of the 16S rRNA gene by PCR and its sequencing were performed as described previously (Lee, 2006). The CLUSTAL_X program (Thompson *et al.*, 1997) was used for multiple alignments of related sequences. Phylogenetic analyses were performed using several programs contained in the PHYLIP package (Felsenstein, 1993).

A partial 16S rRNA gene sequence (1437 nt) was determined for strain CS5-AC15^T. A phylogenetic tree was reconstructed from evolutionary distances (Jukes & Cantor, 1969) by using the neighbour-joining method (Saitou & Nei, 1987), based on a total of 1284 nt present in all strains between positions 95 and 1460 (*Escherichia coli* numbering; Brosius *et al.*, 1978). A neighbour-joining tree (Fig. 2) indicated that strain CS5-AC15^T belonged to the family *Thermomonosporaceae*

Table 1. Characteristics that differentiate strain CS5-AC15^T from the type strain of *A. oligospora*

Strains: 1, strain CS5-AC15^T; 2, *A. oligospora* JCM 10648^T. All data were obtained in this study, except for the cultural characteristics of *A. oligospora*, which were taken from Mertz & Yao (1986).

Fig. 1. Scanning electron micrograph of strain CS5-AC15^T, showing straight to flexuous chains of smooth ornamented spores, after growth for 2 weeks at 30 °C on ISP 4 medium. Bar, 1 µm.

Characteristic	1	2
Growth on ISP media 3 and 4	Good	Poor
Aerial mycelium on ISP media 3 and 4	Abundant	Trace
Growth at 2 % (w/v) NaCl	–	+
Decomposition of:		
Casein	–	+
DNA	–	+
Utilization of:		
Adonitol	–	+
Cellobiose	–	+
Citrate	+	–
Dextrin	+	–
D-Galactose	–	+*
Lactose	+	–
Melezitose	+	–
D-Mannitol	+	–
D-Ribose	–	+
L-Sorbose	–	+
Sucrose	+	–

*Different response reported previously (Mertz & Yao, 1986).

Fig. 2. Neighbour-joining (Saitou & Nei, 1987) tree showing the phylogenetic position of strain CS5-AC15^T within the radiation of the family *Thermomonosporaceae*. Evolutionary distances were calculated using the model of Jukes & Cantor (1969). *Nocardiopsis dassonvillei* DSM 43111^T (GenBank accession no. X97886) was used as an outgroup (not shown). Asterisks indicate branches also found in the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees. Bootstrap values (>50%) are indicated at each node. Bar, 0.01 substitutions per nucleotide position.

and was closely related to *A. oligospora* (97.4% sequence similarity to the type strain). This branching pattern was supported by a moderate bootstrap value (69%) and was also found in trees generated by the maximum-parsimony and maximum-likelihood algorithms. Strain CS5-AC15^T showed 16S rRNA gene sequence similarity values lower

than 96.8% to other species of the genus *Actinomadura* and related taxa.

The following chemotaxonomic characteristics were analysed, with biomass obtained from cultures grown in ISP 2 broth for 7 days at 30 °C: the isomer of diaminopimelic

acid (Staneck & Roberts, 1974), whole-cell sugars (Becker *et al.*, 1965), menaquinones (Kroppenstedt, 1985), *N*-acyl type of murein in the cell wall (Uchida & Aida, 1984), polar lipids (Minnikin *et al.*, 1977) and mycolic acids (Minnikin *et al.*, 1980). For fatty acid analysis, strain CS5-AC15^T and *A. oligospora* JCM 10648^T were grown on ISP 2 for 6 days at 30 °C. Methyl esters of cellular fatty acids were prepared and analysed by gas chromatography according to the instructions of the Sherlock Microbial Identification System (version 2.11; MIDI), using the AEROBE package including the TSBA (version 3.9), CLIN (version 3.9) and MI7H10 (version 3.8) databases for identification of fatty acids.

The cell wall of strain CS5-AC15^T contained meso-diaminopimelic acid as the diagnostic diamino acid and madurose and glucose as whole-cell sugars, showing that it possessed cell-wall type III (Lechevalier & Lechevalier, 1970a) and whole-cell sugar pattern B (Lechevalier, 1968). The acyl type of murein in the peptidoglycan was acetyl. The menaquinones of strain CS5-AC15^T were MK-9(H₆) (62%), MK-9(H₈) (31%) and MK-9(H₄) (7%). *A. oligospora* JCM 10648^T contained a similar menaquinone profile, at a peak ratio of 71:27:2 in this study. The polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-inositol and unknown phospholipids, showing a phospholipid type PI pattern (Lechevalier *et al.*, 1981). Mycolic acids were not detected. The fatty acid profile of strain CS5-AC15^T contained saturated, unsaturated iso-branched and 10-methyl-branched fatty acids. The predominant fatty acids (>10% of the total) were C_{16:0} (31.1%), C_{18:1ω9c} (21.5%) and iso-C_{16:0} (11.1%). *A. oligospora* JCM 10648^T showed a similar profile, but differed from strain CS5-AC15^T in the relative proportions of 10-methyl C_{18:0} (TBSA) and iso-C_{16:0} (see Table S1, available in IJSEM Online). The G+C content of the DNA was 70.9 mol%, as determined by HPLC (Mesbah *et al.*, 1989).

A phylogenetic tree (Fig. 2) showed that strain CS5-AC15^T belongs to the family *Thermomonosporaceae* and is closely related to *A. oligospora*. The combination of morphological and chemotaxonomic data supports the assignment of the strain to the genus *Actinomadura*. Phenotypic features that differentiate strain CS5-AC15^T from *A. oligospora* JCM 10648^T are shown in Tables 1 and S1. Strain CS5-AC15^T showed good growth and produced abundant aerial mycelium on ISP media 3 and 4, in contrast to *A. oligospora* (Mertz & Yao, 1986). Strain CS5-AC15^T can be further differentiated from *A. oligospora* by utilization of carbohydrates and hydrolysis of casein and DNA (Table 1).

DNA–DNA hybridization experiments were performed according to the photobiotin-labelling method of Ezaki *et al.* (1989). Genomic DNA was extracted and purified by the method of Hopwood *et al.* (1985). Five replications were carried out for each DNA pairing and the highest and lowest scores for each DNA pairing were excluded. Finally, DNA relatedness values were recorded as mean ± SD values for the remaining three scores (Yoon *et al.*, 2007). DNA–DNA

relatedness between strain CS5-AC15^T and *A. oligospora* JCM 10648^T was 37.9 ± 0.7%, well below the 70% cut-off value recommended for the delineation of bacterial genospecies (Wayne *et al.*, 1987).

Based on the phenotypic, phylogenetic and DNA–DNA hybridization data presented here, strain CS5-AC15^T is considered to represent a novel species of the genus *Actinomadura*, for which the name *Actinomadura rupiterrae* sp. nov. is proposed.

Description of *Actinomadura rupiterrae* sp. nov.

Actinomadura rupiterrae (ru.pi.ter'rae. L. fem. n. *rupes* cliff; L. fem. n. *terra* soil; N.L. gen. n. *rupiterrae* of cliff soil, referring to the sample from which the type strain was isolated).

Catalase-positive. Oxidase-negative. Grows well on ISP 2, 3 and 4 media. Substrate mycelium is well-developed, branched and light to strong yellow in colour. Aerial mycelium is produced abundantly on ISP 3 and 4 and differentiates into straight to flexuous chains of ten or more spores. The spore surface is smooth. Growth occurs at 20–42 °C (optimum, 37–42 °C), at pH 5–11 (optimum, pH 6–8) and in the presence of up to 1% (w/v) NaCl. Aesculin and gelatin are hydrolysed, but casein, DNA, CM-cellulose, starch and urea are not. Hypoxanthine, DL-tyrosine and xanthine are not decomposed. H₂S production and nitrate reduction are not observed. Citrate, dextran, D-glucose, lactose, D-mannitol, melezitose and sucrose are utilized as sole carbon sources. Acetate, adonitol, D- and L-arabinose, benzoate, 2,3-butanediol, cellobiose, dulcitol, meso-erythritol, formate, D-fructose, D-galactose, glycerol, myo-inositol, inulin, DL-malate, maltose, methyl α-D-glucoside, methyl α-D-mannoside, 1,2-propanediol, raffinose, L-rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, succinate, trehalose, DL-tartrate, D-xylitol and D-xylose are not utilized. meso-Diaminopimelic acid is the diagnostic diamino acid and madurose is the characteristic sugar (type III/B cell wall). The acyl type of muramic acid is acetyl. The major menaquinones are MK-9(H₆) and MK-9(H₈). The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-inositol and unknown phospholipids. Mycolic acids are absent. The predominant fatty acids are C_{16:0}, C_{18:1ω9c} and iso-C_{16:0}.

The type strain, CS5-AC15^T (=KCTC 19559^T =DSM 45251^T), was isolated from a soil sample collected from a cliff on Mara Island, Jeju, Republic of Korea. The G+C content of the DNA of the type strain is 70.9 mol%.

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