Actinomadura scrupuli sp. nov., isolated from rock

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A novel actinomycete, designated strain R-Ac121^T, was isolated from a small stone collected from an agricultural field in Jeju, Republic of Korea. The organism formed abundant vegetative mycelium that was branched and twisted. The reverse colour of colonies was brownish-yellow. Non-motile, wrinkled arthrospores were produced directly on the substrate mycelium. Aerial mycelium and sporangia were not observed. A neighbour-joining tree based on 16S rRNA gene sequences indicated that the isolate formed a distinct clade within the radiation of the family *Thermomonosporaceae*. The highest 16S rRNA gene sequence identity was found with the type strain of *Spirillospora rubra* (97.3 % sequence similarity) followed by those of *Actinoallomurus purpureus* (97.0 %), *Actinomadura alba* (96.5 %), *Actinomadura fibrosa* (96.5 %) and *Actinomadura echinospora* (96.4 %). The cell wall contained meso-diaminopimelic acid. The whole-cell sugars were rhamnose, glucose, ribose, xylose and arabinose. The polar lipids included diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The major menaquinone was MK-9(H₈) and the predominant fatty acids were iso-C_{16:0}, C_{16:0}, C_{17:1ω8c} and 10-methyl C_{17:0}. The DNA G+C content was 71.8 mol%. The combination of morphological, chemotaxonomic and phylogenetic data clearly supports the separation of the organism from recognized species of the genus *Actinomadura* and related genera. On the basis of the data presented here, strain R-Ac121^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura scrupuli* sp. nov. is proposed. The type strain is strain R-Ac121^T (=KCTC 19488^T =DSM 45225^T).

The genus *Actinomadura* was established by Lechevalier & Lechevalier (1970) and its description has been emended by Zhang *et al.* (1998, 2001) and Miyadoh & Miyara (2001). Phylogenetically, the genus belongs to the family *Thermomonosporaceae*, together with the genera *Actinocorallia*, *Actinoallomurus*, *Spirillospora* and *Thermomonospora* (Zhang *et al.*, 1998; Trujillo & Goodfellow, 2003; Tamura *et al.*, 2009). At the time of writing, the genus encompasses 40 recognized species and two subspecies (<http://www.bacterio.cict.fr/a/actinomadura.html>), which were mostly isolated from soil. In this study, we describe the polyphasic taxonomic characterization of an *Actinomadura*-like strain isolated from rock.

Strain R-Ac121^T was isolated from a small stone collected from an agricultural field in Jeju, Republic of Korea. An unwashed stone (1 g) was ground into powder using a pestle and suspended in 10 ml sterile distilled water. Aliquots of serial dilutions were spread onto starch-casein agar (1 % soluble starch, 0.03 % casein, 0.2 % KNO₃, 0.2 % NaCl, 0.2 % KH₂PO₄, 0.002 % CaCO₃, 0.005 % MgSO₄·7H₂O, 0.001 % FeSO₄·7H₂O and 1.8 % agar in distilled water, pH 7.2) and the plates were incubated at 30 °C for 2 weeks. A single colony was selected and further streaked on ISP

(International *Streptomyces* Project) medium 2 (Shirling & Gottlieb, 1966). The pure culture was maintained as 20 % (v/v) glycerol suspensions at –20 and –80 °C.

For cultural characterization, strain R-Ac121^T was grown for 21 days at 30 °C on ISP media 2, 3, 4, 5, 6 and 7 (Shirling & Gottlieb, 1966), nutrient agar (NA; Difco), trypticase soy agar (TSA; Difco), oatmeal-nitrate agar (Prauser & Bergholz, 1974), humic acid-vitamin (HV) agar (Nonomura & Ohara, 1969) and water agar (15.0 g agar, 1000 ml tap water). Cell morphology was observed by scanning electron microscopy (model JSM 6700F; JEOL), using cells grown on water agar for 3 weeks. The specimen was fixed for 1 h with 1 % OsO₄ and dehydrated with graded series of ethanol and then ethanol/isoamyl acetate solutions. After critical-point drying with liquid carbon dioxide, the specimen was coated with platinum before observation. Temperature and pH ranges for growth and NaCl tolerance were determined on ISP medium 2. Utilization of carbohydrates as sole carbon sources was tested on ISP medium 9 (Shirling & Gottlieb, 1966), with each filter-sterilized carbon source being used at a final concentration of 1 % (w/v) for carbohydrates and alcohols and 0.1 % (w/v) for organic acids. Gram staining, oxidase and catalase activities and degradation abilities were determined using the methods described by Lee *et al.* (2008).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R-Ac121^T is FM210339.

Strain R-Ac121^T showed good growth on ISP media 2, 3, 4, 5 and 7, NA, oatmeal-nitrate agar, HV agar and water agar and poor growth on ISP medium 6 and TSA. Vegetative mycelium was abundant, branched and twisted. The reverse colour of the colonies was brownish-yellow. Wrinkled arthrospores were borne directly on the substrate mycelium (Fig. 1). Aerial mycelium and soluble pigments were not produced on any of the tested media. Sporangia were not observed. Other physiological and biochemical properties are given in the species description and Table 1.

Genomic DNA was extracted and purified as described by Hopwood *et al.* (1985). Amplification of the 16S rRNA gene by PCR and sequencing of the PCR product were performed as described by Lee & Lee (2008). The 16S rRNA gene sequence was aligned with corresponding sequences obtained from GenBank by using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic analyses were performed by using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Fitch, 1971) and maximum-parsimony (Felsenstein, 1981) methods. A phylogenetic tree was constructed by using the NEIGHBOR program in the PHYLIP package (Felsenstein, 1993) from evolutionary distances calculated with the coefficient of Jukes & Cantor (1969). The robustness of the tree topology was assessed by using bootstrap analysis with 1000 replicated datasets (Felsenstein, 1985).

An almost-complete 16S rRNA gene sequence (1402 nt) of strain R-Ac121^T determined in this study was compared with the corresponding sequences of members of the genus *Actinomadura* and related taxa. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences indicated that strain R-Ac121^T formed a distinct clade within the radiation of the family *Thermomonosporaceae* (Fig. 2). The highest 16S rRNA gene sequence similarity of

Fig. 1. Scanning electron micrograph of cells of strain R-Ac121^T grown on water agar for 3 weeks at 30 °C. Bar, 1 µm.

strain R-Ac121^T to type strains of recognized species of the *Thermomonosporaceae* was found with *Spirillospora rubra* (97.3 % sequence similarity), *Actinoallomurus purpureus* (97.0 %), *Actinomadura alba* (96.5 %), *Actinomadura fibrosa* (96.5 %) and *Actinomadura echinospora* (96.4 %).

Cell biomass for chemotaxonomic analyses was obtained from cultures grown in ISP 2 broth for 7 days at 30 °C. The isomer of diaminopimelic acid in the cell-wall peptidoglycan was determined by the method of Stanek & Roberts (1974). Whole-cell sugars were analysed as described by Saddler *et al.* (1991). Respiratory quinones were extracted according to Collins (1985) and identified by HPLC (Kroppenstedt, 1985). Analysis of polar lipids was performed by TLC as described previously (Minnikin *et al.*, 1975). Cellular fatty acid methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (version 6; MIDI).

Strain R-Ac121^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid and rhamnose, glucose, ribose, xylose and arabinose as whole-cell sugars. Madurose (3-*O*-methyl D-galactose), a characteristic sugar found in most species of the family *Thermomonosporaceae* (Lechevalier & Gerber, 1970), was not detected in our analysis by GC. Like strain R-Ac121^T, a few strains of the genus *Thermomonospora* and thermophilic *Actinomadura* species either lack madurose or synthesize it in trace amounts (Kroppenstedt, 1987). The polar lipids included diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The menaquinone composition was MK-9(H₆), MK-9(H₈) and MK-9(H₄) in the ratio of 80:14:6. The cellular fatty acid profile of strain R-Ac121^T consisted of saturated, unsaturated, branched and 10-methyl fatty acids. The cellular fatty acid composition was iso-C_{16:0} (23.8 %), C_{16:0} (11.8 %), C_{17:1ω8c} (10.7 %), 10-methyl C_{17:0} (10.7 %), C_{18:1ω9c} (8.7 %), 10-methyl C_{18:0} (6.6 %), C_{17:0} (4.4 %), C_{18:0} (4.3 %), C_{15:0} (4.2 %), C_{16:1ω7c} and/or iso-C_{15:0} 2-OH (3.6 %), C_{19:1ω11c} and/or unknown 18.756 (1.2 %), iso-C_{14:0} (1.1 %) and iso-C_{18:0} (1.1 %). The G + C content of the DNA, as determined by HPLC (Mesbah *et al.*, 1989), was 71.8 mol%.

The five genera *Actinoallomurus*, *Actinocorallia*, *Actinomadura*, *Spirillospora* and *Thermomonospora* of the family *Thermomonosporaceae* are similar in most of their chemotaxonomic characteristics with the exception of polar lipid profiles (Kroppenstedt, 1985; Zhang *et al.*, 2001; Tamura *et al.*, 2009). However, they can be differentiated from one another by a combination of morphological and chemotaxonomic features. The 16S rRNA gene tree (Fig. 2) reveals that strain R-Ac121^T formed a distinct sublineage within the family *Thermomonosporaceae*. Of its phylogenetic neighbours, *S. rubra* showed the highest sequence similarity (97.3 %) to strain R-Ac121^T but it, together with *Spirillospora albida*, differs morphologically from strain R-Ac121^T in that they form sporangia containing motile spores. *Actinomadura fibrosa* produces thick fibres on

Table 1. Characteristics that differentiate strain R-Ac121^T from its phylogenetic neighbours

Taxa: 1, R-Ac121^T; 2, *Actinallomurus purpureus* TTN02-30^T (data from Tamura *et al.*, 2009); 3, *Actinomadura alba* YIM 45681^T (Wang *et al.*, 2007); 4, *Actinomadura echinospora* DSM 43163^T (Miyadoh *et al.*, 1990; Holt *et al.*, 1994; Wang *et al.*, 2007); 5, *Actinomadura fibrosa* ATCC 49459^T (Mertz & Yao, 1990; Wink *et al.*, 2003); 6, *Spirillospora* (Vobis & Kothe, 1989; Wink, 2001; Zhang *et al.*, 2001). +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6
Colour of growth on ISP						
4						
Aerial mycelium	None	White	White	Yellow–pink	Pink	White
Substrate mycelium	Brownish-yellow	Pale yellow to reddish-purple	Greyish-yellow	Greyish-yellow	Brown	Beige or red to reddish-brown
Diffusible pigment	None	None	None	Tan	None	None
Production of sporangia	–	–	–	–	–	+
Production of spores	+	+	+	+	–	–
Motility of spores	–	–	–	–	–	+
Optimal temperature (°C)	30	25–30	28	35–40	37	25
Utilization of:						
Glycerol	+	+	–	w	ND	ND
myo-Inositol	–	–	–	–	+	–*
Raffinose	–	ND	–	–	+	–*
L-Rhamnose	+	–	–	–	+	–*
D-Xylose	+	ND	+	+	–	–*
D-Mannitol	–	ND	+	+	–	+
Liquefaction of gelatin	+	+	+	+	+	–*
Hydrolysis of starch	–	+	+	–	+	ND
Nitrate reduction	+	–	+	–	+	–
Polar lipids†	DPG, PG, PI	DPG, PG	DPG, PG, PI, PIM	DPG, PG, PI, PIM	DPG, PI	DPG, PI, PIM
Major menaquinone(s)	9(H ₆)	9(H ₆), 9(H ₈)	9(H ₄), 9(H ₆), 9(H ₂)	9(H ₆)	9(H ₆)	9(H ₄), 9(H ₆)
Major fatty acids (>10%)	i-C _{16:0} , C _{16:0} , C _{17:1} ω8c, 10-Me C _{17:0}	i-C _{16:0} , ai-C _{17:0}	i-C _{16:1} H, C _{17:1} ω8c, C _{16:0} , 10-Me C _{17:0}	C _{17:0} , i-C _{18:0} , 10-Me C _{17:0} , TBSA	i-C _{16:0} , TBSA	C _{16:0} , C _{18:1} , i-C _{16:0} *, TBSA
Cell-wall sugar(s)§	Ara, Glc, Rha, Rib, Xyl	Mad, Gal	Gal, Glc, Mad, Rib, Xyl	Mad	Gal, Glc, Mad, Man, Rib	Mad
DNA G + C content (mol%)	71.8	70.0	66.5	74.0	ND	71.0–73.0

*Detected only in the type strain of *Spirillospora albida* (Vobis & Kothe, 1989; Wink, 2001; Zhang *et al.*, 2001).

†DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides.

‡i, iso-branched; ai, anteiso-branched; Me, methyl; TBSA, tuberculostearic acid (10-methyl C_{18:0}).

§Ara, Arabinose; Gal, galactose; Glc, glucose; Mad, madurose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

the substrate mycelium, but strain R-Ac121^T produces wrinkled arthrospores (Fig. 1). *Actinomadura alba*, *Actinomadura echinospora* and members of the genus *Actinallomurus* are similar to strain R-Ac121^T in that they produce arthrospores, but they can be differentiated from the isolate in some chemotaxonomic characters (i.e. cell-wall sugars, menaquinones and fatty acids). Differential characteristics of strain R-Ac121^T from its phylogenetic neighbours are given in Table 1.

On the basis of the morphological, chemotaxonomic and phylogenetic data, strain R-Ac121^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura scrupuli* sp. nov. is proposed.

Description of *Actinomadura scrupuli* sp. nov.

Actinomadura scrupuli (scru'pu.li. L. gen. n. *scrupuli* of a small stone, referring to the isolation of the type strain).

Aerobic, Gram-stain-positive. Cells grow well on ISP media 2, 3, 4, 5 and 7, NA, oatmeal-nitrate agar, HV agar and water agar plates and show poor growth on ISP medium 6 and TSA. The organism forms abundant vegetative mycelium that is branched and twisted. The reverse colour of the colonies is brownish-yellow. Aerial mycelium and soluble pigments are not produced on any of the tested media. Wrinkled arthrospores (0.9–1.1 µm in diameter) are produced directly from the substrate mycelium on water agar. Sporangia are not observed. Growth occurs at

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain R-Ac121^T within the radiation of the family *Thermomonosporaceae*. The tree was constructed from an evolutionary distance matrix by using the neighbour-joining method. The sequence of *Nocardiopsis dassonvillei* DSM 43111^T (GenBank accession no. X97886; not shown) was used as an outgroup. Asterisks represent branches also found in both maximum-likelihood and maximum-parsimony trees. Numbers at nodes indicate levels of bootstrap support (>50%) based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 0.01 substitutions per site.

pH 6.1–8.1. The temperature range for growth is 20–30 °C. Good growth occurs in the absence of NaCl. Nitrate is reduced to nitrite. Aesculin and gelatin are degraded but not casein, cellulose, chitin, DNA, elastin, hypoxanthine, starch, L-tyrosine or xanthine. Acetate, adonitol, L-arabinose, cellobiose, dextran, D-fructose, D-galactose, D-glucose, glycerol, lactose, DL-malate, melibiose, L-rhamnose, D-sorbitol, L-sorbose, succinate, sucrose, trehalose and D-xylose can be utilized as carbon sources, but D-arabinose, benzoate, citrate, dulcitol, *meso*-erythritol, formate, *myo*-inositol, inulin, D-mannose, melezitose, methyl α -D-glucoside, methyl α -D-mannoside, D-mannitol, raffinose, L-ribose, salicin, L-tartrate and D-xylitol are not utilized. *meso*-Diaminopimelic acid and rhamnose, glucose, ribose, xylose and arabinose are the diagnostic diamino acid and whole-cell sugars, respectively. The polar lipids include diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The major menaquinone is

MK-9(H₆). The predominant fatty acids are iso-C_{16:0}, C_{16:0}, C_{17:1 ω 8c} and 10-methyl C_{17:0}. The G + C content of the DNA of the type strain is 71.8 mol%.

The type strain, R-Ac121^T (=KCTC 19488^T =DSM 45225^T), was isolated from a small stone in Jeju, Republic of Korea.

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