Actinomycetospora rishiriensis sp. nov., isolated from a lichen

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An actinomycete, strain RI109-Li102^T, was isolated from a lichen sample obtained from Rishiri Island in Japan. Cells of strain RI109-Li102^T were Gram-positive, aerobic and non-motile and formed bud-like spore chains. The isolate grew with 0–3% (w/v) NaCl, at pH 5–9 and at 10–30 °C (optimum 30 °C). The whole-cell hydrolysate contained *meso*-diaminopimelic acid, arabinose and galactose. The predominant menaquinone was MK-8(H₄) and the diagnostic phospholipids were phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and diphosphatidylglycerol. The major cellular fatty acids were iso-C_{16:0} and iso-C_{16:1} H. Comparative 16S rRNA gene sequence analysis revealed that strain RI109-Li102^T was most closely related to *Actinomycetospora corticicola* 014-5^T (99.0% rRNA gene sequence similarity) and *Actinomycetospora chiangmaiensis* YIM 0006^T (98.4%). However, DNA–DNA hybridization assays, as well as physiological and biochemical analyses, showed that strain RI109-Li102^T could be differentiated from its closest phylogenetic relatives. It is proposed that strain RI109-Li102^T (=NBRC 106356^T =KCTC 19782^T) be classified as the type strain of a novel species, with the name *Actinomycetospora rishiriensis* sp. nov.

The genus *Actinomycetospora* was first established by Jiang *et al.* (2008) and its description was later emended by Tamura *et al.* (2011). At the time of writing, eight species have been confirmed as members of the genus *Actinomycetospora*. With the exception of the tree-bark-derived species *Actinomycetospora corticicola*, all species of this genus were originally isolated from soil samples. Over the past few decades, several researchers have screened soil- and plant-related samples for actinomycetes to serve as potential sources of pharmaceutical compounds (Kitouni *et al.*, 2005; Loqman *et al.*, 2009; Qin *et al.*, 2009). In addition to tree bark, rhizosphere and leaves, lichen has also been found to be a novel source of a diverse group of actinomycetes, including the family *Pseudonocardaceae* (González *et al.*, 2005). Recently, *Nocardioideis exalbidus* (Li *et al.*, 2007) and *Leifsonia lichenia* (An *et al.*, 2009) were isolated from lichen collected in Japan. The discovery of additional species of the genus *Actinomycetospora* will contribute to a better understanding of their ecological

roles and the possible provision of bioactive compounds for industrial applications.

During the screening for lichen-associated actinomycetes, using a serial dilution-plating method, a strain was found to form *Pseudonocardia*-like colonies on humic acid-vitamin agar (Hayakawa & Nonomura, 1987; containing 1^{−1}: 20 mg nalidixic acid and 50 mg cycloheximide) after incubation for 2 weeks at 30 °C. The isolate, designated RI109-Li102^T, was isolated from a homogenized lichen sample collected from Rishiri Island, Hokkaido, Japan.

Colony morphology of strain RI109-Li102^T was recorded after 14 days at 28 °C on nutrient agar and other morphological features were analysed by light and scanning electron microscopy. Spore motility was evaluated by light microscopy using a hanging-drop method (Gerhardt, 1981). Gram staining was performed by Hucker's method (Gerhardt, 1981). To determine the conditions for growth on nutrient agar, strain RI109-Li102^T was incubated for 7 and 14 days at 5, 10, 20, 25, 30, 37 and 45 °C and at pH 4–12 (at intervals of one pH unit). Growth at 5 and 10 °C was also assayed after 6 weeks. Growth with 0–7% (w/v) NaCl (in increments of 1%) was evaluated after 14 and 21 days. API ZYM and API Coryne kits (bioMérieux) were used to

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RI109-Li102^T is AB581530.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

investigate several physiological and biochemical characteristics, according to the manufacturer's instructions. Assimilation of carbon sources at a final concentration of 1% (w/v) was tested using ISP 9 as the basal medium (Shirling & Gottlieb, 1966).

Biomass for chemotaxonomic studies was obtained by growing strain RI109-Li102^T in shake flasks containing glucose-yeast extract broth (Gordon & Mihm, 1962) for 5 days at 30 °C. After harvesting cells by centrifugation, the resultant pellet was washed twice with distilled water. The diaminopimelic acid isomer and sugars in the whole-cell hydrolysate were analysed according to the methods established by Hasegawa *et al.* (1983) and Schaal (1985), respectively. Cellular fatty acids were processed and analysed as methyl esters, following the protocol of the Sherlock Microbial Identification System (Sasser, 1990). Standard procedures were also used for the extraction and analysis of isoprenoid quinones and polar lipids (Minnikin *et al.*, 1984) and the results were compared with the appropriate controls. Chromosomal DNA from strain RI109-Li102^T was isolated and purified by the method of Saito & Miura (1963), with a minor modification (Hatano *et al.*, 2003). The DNA G+C content of strain RI109-Li102^T was determined by HPLC, as described by Tamaoka & Komagata (1984). DNA–DNA hybridization was performed as described by Kusunoki *et al.* (1991) using biotinylated DNA, with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the mean of the remaining three values was quoted as DNA–DNA relatedness.

PCR amplification of the 16S rRNA gene from strain RI109-Li102^T was performed as described by Tamura & Hatano (2001) and the resultant PCR product was sequenced directly using an ABI Prism BigDye Terminator Cycle Sequencing kit and an ABI 3730 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequence was aligned with reference sequences of the genus *Actinomyces* and representative species of the genus

Pseudonocardia available from public databases using CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed with MEGA version 4 (Tamura *et al.*, 2007) and CLUSTAL X using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Takahashi & Nei, 2000). The PhyML package (Guindon *et al.*, 2005) was used to reconstruct a maximum-likelihood tree. Topologies of reconstructed trees were evaluated by bootstrap analysis (Felsenstein, 1985) with either 1000 (neighbour-joining and maximum-parsimony) or 500 (maximum likelihood) resamplings.

The whole-cell hydrolysate of strain RI109-Li102^T contained *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV, *sensu* Lechevalier & Lechevalier, 1970). The major menaquinone was MK-8(H₄) and the predominant polar lipids were phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and diphosphatidylglycerol (phospholipid type PIII, *sensu* Lechevalier *et al.*, 1977), and phosphatidylglycerol, phosphatidylmonomethylethanolamine and unknown phospholipids were detected in trace amounts (Supplementary Fig. S1, available in IJSEM Online). The major fatty acids (>10% of the total) were iso-C_{16:0} (49.2%) and iso-C_{16:1} H (12.5%) (Supplementary Table S1). The DNA G+C content was 73.1 mol%. Strain RI109-Li102^T displayed morphological properties typical of the genus *Actinomyces* (Tamura *et al.*, 2011) and formed bud-like spore chains similar to those of *Actinomyces* *chiangmaiensis* directly from vegetative mycelium (Supplementary Fig. S2). The spores were oval- and rod-shaped (width 0.4–0.6 µm; length 0.9–1.3 µm) with a smooth surface.

The nearly complete 16S rRNA gene sequence (1468 nt) of strain RI109-Li102^T was compared with sequences from members of the genus *Actinomyces* and related taxa. The 16S rRNA gene sequence similarities between strain RI109-Li102^T and members of the genus *Actinomyces* ranged from 97.3 to 99.0%. The isolate was most closely related to *A. corticicola* 014-5^T (99.0% 16S rRNA gene

Fig. 1. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences showing the relationship of strain RI109-Li102^T with the genus *Actinomyces* and related taxa. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood algorithms. Bar, 0.005 K_{nuc} .

Table 1. DNA–DNA relatedness between strain RI109-Li102^T and its closest phylogenetic neighbours in the genus *Actinomycetospira*

Strain	DNA–DNA relatedness (%) with labelled DNA from:		
	1	2	3
1. <i>A. rishiriensis</i> RI109-Li102 ^T	100	33.7 ± 1.3	18.3 ± 0.6
2. <i>A. corticicola</i> NBRC 103689 ^T	42.1 ± 1.7	100	52.1 ± 1.3
3. <i>A. chiangmaiensis</i> NBRC 104400 ^T	41.2 ± 13.4	49.3 ± 0.9	100

sequence similarity) and *A. chiangmaiensis* YIM 0006^T (98.4%). The phylogenetic tree reconstructed with the neighbour-joining method showed that strain RI109-Li102^T formed a separate branch within the cluster containing *A. corticicola* 014-5^T and *A. chiangmaiensis* YIM 0006^T (Fig. 1). Moreover, the same topology was also recovered with the maximum-likelihood and maximum-parsimony methods, strongly supported by high bootstrap values.

DNA–DNA relatedness between strain RI109-Li102^T and *A. corticicola* NBRC 103689^T and *A. chiangmaiensis* NBRC 104400^T ranged from 18.3% to 42.1% (Table 1), which was well below the 70% cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987). Strain RI109-Li102^T could also be distinguished from its closest phylogenetic neighbours on comparison of biochemical and phenotypic characteristics (Table 2), particularly D-melezitose utilization. Strain RI109-Li102^T could also be differentiated from *A. corticicola* NBRC 103689^T by pyrrolidonyl arylamidase, urea hydrolysis and NaCl concentration range for growth and from *A. chiangmaiensis* NBRC 104400^T by alkaline phosphatase and temperature range for growth. Detailed results of these analyses are provided in Table 1 and the species description. The cultural characteristics of strain RI109-Li102^T are summarized in Supplementary Table S2.

On the basis of the phenotypic, phylogenetic and chemotaxonomic analysis, strain RI109-Li102^T represents a novel species within the genus *Actinomycetospira*, for which the name *Actinomycetospira rishiriensis* sp. nov. is proposed.

Description of *Actinomycetospira rishiriensis* sp. nov.

Actinomycetospira rishiriensis (ri.shi.ri.en'sis. N.L. fem. adj. *rishiriensis* of or pertaining to Rishiri Island, Hokkaido, Japan, where the organism was originally isolated).

Aerobic, Gram-positive, non-motile actinomycete that forms oval- and rod-shaped spores. Colonies are light to moderate orange–yellow and have a powdery surface. The substrate mycelium is long, well developed and branched. Whole-cell hydrolysate contains *meso*-diaminopimelic acid, arabinose and galactose. The major fatty acids (>10%) are iso-C_{16:0} and iso-C_{16:1} H. The polar lipid profile contains phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and diphosphatidylglycerol. MK-8(H₄) is the major menaquinone. Melanoid pigments are not formed on ISP 6 or ISP 7. Growth occurs at 10–30 °C, but not at 5, 37 or 45 °C. Grows with 0–3% NaCl (w/v) and at pH 5–9 (optimum pH 7). Aesculin, gelatin and starch are hydrolysed. Nitrate is not reduced and urease is not produced.

Table 2. Characteristics that differentiate strain RI109-Li102^T from its closest phylogenetic neighbours in the genus *Actinomycetospira*

Strains: 1, *Actinomycetospira rishiriensis* sp. nov. RI109-Li102^T; 2, *A. corticicola* NBRC 103689^T; 3, *A. chiangmaiensis* NBRC 104400^T; 4, *A. chibensis* TT04-21^T; 5, *A. chlora* TT07I-57^T; 6, *A. cinnamomea* IY07-53^T; 7, *A. lutea* TT00-04^T; 8, *A. straminea* IY07-55^T; 9, *A. succinea* TT00-49^T. Data for columns 1–3 were taken from this study and for columns 4–9 from Tamura *et al.* (2011). +, Positive; w, weakly positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9
Pyrrolidonyl arylamidase	–	+	–	+	+	–	–	+	w
Urea hydrolysis	–	+	–	–	–	–	–	–	+
Esterase (C4)	–	w	–	+	–	+	+	+	w
Valine arylamidase	+	w	+	w	+	–	–	+	+
Alkaline phosphatase	–	–	+	+	ND	+	ND	ND	ND
Utilization of D-melezitose	–	+	+	ND	ND	ND	ND	ND	ND
Conditions for growth									
pH	5–9	5–9	5–8	7–8	5–8	5–8	5–8	6–8	5–9
NaCl (%)	0–3	0–7	0–3	0–1	0–1	0–7	0–1	0–5	0–7
Temperature (°C)	10–30	10–30	20–37	20–28	15–28	20–37	20–28	20–28	15–37

Trehalose, turanose, D-fructose, D-galactose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, D-sorbitol, sucrose and xylitol are used as sole carbon sources, but erythritol, L-arabitol, D-arabinose and melezitose are not. With API ZYM and API Coryne, positive for acid phosphatase, esterase lipase (C8), α -galactosidase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, pyrrolidonyl arylamidase and hydrolysis of gelatin and aesculin, weakly positive for lipase (C14) and trypsin and negative for alkaline phosphatase, esterase (C4), β -galactosidase, α - and β -glucosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, pyrazinamidase, α -mannosidase, urease and α -fucosidase.

The type strain is RI109-Li102^T (=NBRC 106356^T =KCTC 19782^T), isolated from a lichen sample collected on Rishiri Island, Hokkaido, Japan. The DNA G+C content of the type strain is 73.1 mol%.

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