ORIGINAL ARTICLE

Actinomycetospora iriomotensis sp. nov., a novel actinomycete isolated from a lichen sample

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An actinomycete strain, IR73-Li102^T, was isolated from a lichen sample obtained from Iriomote Island, Japan, and subsequently characterized using a polyphasic approach. Comparative 16S rRNA gene sequence analysis revealed that strain IR73-Li102^T had the highest sequence similarities with *Actinomycetospora chiangmaiensis* YIM 0006^T (98.3%), *A. corticola* 014-5^T (98.1%) and *A. rishiriensis* RI109-Li102^T (98.0%). However, DNA–DNA hybridization assays, as well as physiological and biochemical analyzes, showed that strain IR73-Li102^T could be clearly differentiated from its closest phylogenetic relatives. The strain contained meso-diaminopimelic acid, and arabinose and galactose were present in whole-cell hydrolysates. The predominant menaquinone was MK-8(H₄), and the diagnostic phospholipids were phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and diphosphatidylglycerol. The major cellular fatty acid was iso-C_{16:0} (58%). The chemotaxonomic properties of strain IR73-Li102^T were consistent with those shared by members of the genus *Actinomycetospora*. On the basis of the phenotypic features and DNA–DNA hybridization data, strain IR73-Li102^T (= NBRC 106365^T = KCTC 19783^T) represents a novel species of the genus *Actinomycetospora*, for which the name *Actinomycetospora iriomotensis* sp. nov. is proposed. *The Journal of Antibiotics* (2011) 64, 289–292; doi:10.1038/ja.2011.15; published online 9 March 2011

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INTRODUCTION

Lichens are frequently found in several environments, such as tree and stone surfaces and are classified into four groups based on their growth forms: foliaceous (leaf-like), fruticose (stalked), squamulose (scale-like), and crustose (crust-like).¹ Biologically, lichens are symbiotic microorganisms that comprises a fungi and green algae and/or cyanobacteria.^{1,2} However, several researchers have identified diverse species of actinomycetes from lichens that have the potential for producing secondary metabolites, as determined by biosynthetic gene sequences.^{3,4} Recently, Motohashi *et al.*⁵ reported the isolation of a novel angucycline and butenolide from lichen-derived *Streptomyces* spp. Thus, evaluation of the diversity of culturable actinomycetes from lichens is an important task for understanding the ecological roles of actinomycetes and may allow the identification of novel bioactive compounds for industrial applications.

Previously, several rare actinomycetes were isolated from lichen samples collected in Japan, and were proposed as new taxa, named *Nocardioidea exalbidus*, *Leifsonia lichenia*, and *Actinomycetospora rishiriensis*.^{6–8} The genus *Actinomycetospora* was originally proposed by Jiang *et al.*⁹ as a member of the family *Pseudonocardiaceae*, and was later amended by Tamura *et al.*¹⁰ At the time of writing, nine *Actinomycetospora* species have been characterized by polyphasic taxonomic approaches. The type strain of the genus

Actinomycetospora, *A. chiangmaiensis*, was originally isolated from a soil sample in northern Thailand, whereas the other species were from soil, tree bark, and lichen samples collected in Japan.^{8–10}

During a screening for lichen-associated actinomycetes, we isolated strain IR73-Li102^T from a crustose lichen sample collected from Iriomote Island, Okinawa, Japan. The aim of the present study was to determine the taxonomic position of strain IR73-Li102^T using a polyphasic taxonomic approach, which included chemotaxonomic, morphological, physiological, molecular and genomic characterizations.

MATERIALS AND METHODS

Isolation and maintenance of organism

A crustose lichen sample was collected from Iriomote Island, Okinawa, Japan, and was then air dried for 7 days at room temperature. The lichen sample was homogenized with mortar and pestle in sterilized distilled water, and the suspension was spread on humic acid-vitamin (HV) agar containing nalidixic acid (20 mg l⁻¹) and cycloheximide (50 mg l⁻¹), which was then incubated at 30 °C for 2 weeks.¹¹ Following the incubation period, the strain IR73-Li102^T was isolated and transferred to a nutrient agar (NA) plate. The strain was maintained in 20% (v/v) glycerol at –80 °C until use.

Phenotypic characterization

Strain IR73-Li102^T was grown on both HV and oatmeal–YGG agar for 14 days at 28 °C, and morphological features were then analyzed by light and scanning

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electron microscopy (JEOL, JSM-6500F).¹² Spore motility was evaluated by light microscopy using a hanging drop method with spores suspended in sterilized distilled water. Gram staining was performed by the Hucker method.¹³ To determine the optimal temperature and pH for growth, strain IR73-Li102^T was incubated for 7 and 14 days on NA at temperatures of 5, 10, 20, 25, 30, 37 and 45 °C, and at pHs ranging from 4–12 (in 1 pH unit intervals). Growth at 5 and 10 °C was assayed after a 6-week incubation period. Growth at different NaCl concentrations (0–7%, w/v, in 1% increments) was evaluated after 14- and 21-day incubations on NA. API ZYM and API Coryne Biochemical Test Kits (bioMérieux, Tokyo, Japan) were used to evaluate 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 medium 9 as the basal medium.¹⁴ Antimicrobial activities against selected Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi were assayed using an overlay method.¹⁵ Briefly, spot-inoculated, 10-day-old colonies of strain IR73-Li102^T on NA plates were overlaid with 5 ml of sloppy NA/YEPD agar inoculated with the test organisms. The sizes of the zones of inhibition around the colonies were recorded after 24 h at 30 °C.

Chemotaxonomy

Biomass for chemotaxonomic studies was obtained by growing strain IR73-Li102^T in shake flasks containing glucose–yeast extract broth for 5 days at 30 °C.¹⁶ After harvesting cells by centrifugation, the resultant pellet was washed twice with distilled water. Diaminopimelic acid isomers and sugars in whole-cell hydrolysates were then analyzed on the basis of the methods established by Hasegawa *et al.*¹⁷ and Schaal,¹⁸ respectively. Cellular fatty acids were processed and analyzed as methyl esters, following the protocol of the MIDI Sherlock Microbial Identification System.¹⁹ Standard procedures were also used for the extraction and analysis of isoprenoid quinones and polar lipids, and the results were compared with the appropriate controls.²⁰ The DNA G+C content of strain IR73-Li102^T was determined by HPLC, as described by Tamaoka and Komagata.²¹

Molecular analysis

Chromosomal DNA from strain IR73-Li102^T was isolated and purified by the method of Saito and Miura,²² with a minor modification.²³ PCR amplification of the 16S rRNA gene from strain IR73-Li102^T was performed as described by Tamura *et al.*,²⁴ and the resultant PCR product was purified using a MonoFas DNA Purification Kit (GL Sciences, Tokyo, Japan). The purified PCR product was directly sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a Genetic Analyzer automated DNA sequencer (model 3730; Applied Biosystems). The obtained

16S rRNA gene sequence was compared with published 16S rRNA gene sequences of bacterial type strains using the EzTaxon server (<http://www.eztaxon.org/>).²⁵ For phylogenetic analyses, 16S rRNA gene sequences were collected from the EMBL/GenBank/DBJ database and aligned using the CLUSTAL_X program.²⁶ Phylogenetic trees were constructed with the CLUSTAL_X program and Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 using the neighbor-joining and maximum parsimony methods.^{26–29} The PHYML software package was used to construct a maximum-likelihood tree.³⁰ The topologies of the constructed trees were evaluated by bootstrap analysis with either 1000 (neighbor-joining and maximum-parsimony methods) or 500 resamplings (maximum-likelihood method).³¹

DNA–DNA hybridization

DNA–DNA hybridizations were performed as described by Kusunoki *et al.*,³² 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 the DNA–DNA relatedness value.

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain IR73-Li102^T determined in this study has been deposited in the DDBJ database under the accession number AB581531.

RESULTS AND DISCUSSION

The nearly complete 16S rRNA gene sequence (1428 nucleotide) of strain IR73-Li102^T was compared with sequences of recognized bacterial species on the EzTaxon server. The highest similarity value (98.3%) was observed with *A. chiangmaiensis* YIM 0006^T followed by *A. corticola* 014-5^T (98.1%) and *A. rishiriensis* RI109-Li102^T (98.0%). The 16S rRNA gene sequence similarities to members of the genus *Actinomycetospora* ranged from 97.0 to 98.4%.^{8–10} The phylogenetic tree constructed with 16S rRNA gene sequence data of members of the genus *Actinomycetospora* and related genera using the neighbor-joining method showed that strain IR73-Li102^T formed a monophyletic clade with *A. chiangmaiensis*, *A. corticola* and *A. rishiriensis* (Figure 1). Moreover, this clade was strongly supported by the maximum-likelihood and maximum parsimony trees with high bootstrap values.

A whole-cell hydrolysate of strain IR73-Li102^T contained *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV).³³ The major menaquinone was observed only a MK-8 (H₄) and minor menaquinones were not observed. The predominant polar lipids were

Figure 1 Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship of strain IR73-Li102^T to a member of the genus *Actinomycetospora* and related taxa. The tree was constructed using the neighbor-joining method and K_{nuc} values.²⁸ Only bootstrap values above 50% are shown (1000 resamplings) at the branching points. Solid circles indicate that the corresponding nodes were also recovered in maximum parsimony and maximum-likelihood algorithms.²⁹ Bar, 0.005 K_{nuc} .

Table 1 Cellular fatty acid profiles of strain IR73-Li102^T and its closest phylogenetic relatives

Fatty acid	1	2	3	4
iso-C _{14:0}	4.1	1.4	—	1.1
iso-C _{15:0}	1.6	—	—	3.2
iso-C _{16:1} H	8.9	12.5	7.7	2.6
iso-C _{16:0}	58.1	49.4	49.5	29.8
C _{16:1} cis 9	7.5	7.3	7.5	17.6
C _{16:0}	4.5	3.6	2.4	10.8
10M C _{16:0}	4.6	9.4	17	7.2
anteiso-C _{17:0}	—	—	—	5.5
C _{17:1} cis 9	2.3	5.2	4.4	4.4
20H iso-C _{16:0}	1.6	3.3	4	—
C _{17:0}	—	—	—	1.6
10M C _{17:0}	1.6	7.9	7.6	1.3

Strain: 1, *A. iriomotensis* IR73-Li102^T; 2, *A. rishiriensis* RI109-Li102^T (data from Yamamura et al.⁸); 3, *A. corticola* O14-5^T (data from Tamura et al.¹⁰); and 4, *A. chiangmaiensis* NBRC 104400^T (data from Tamura et al.⁹). —, not detected or detected in trace amounts. > 10% of the total fatty acids are shown as bold.

Table 2 Differential characteristics of strain IR73-Li102^T from its closest phylogenetic species

Characteristic	1	2	3	4
Pyrrolidonyl arylamidase	—	+	+	—
Urea hydrolysis	+	—	+	—
Esterase (C-4)	+	—	w	—
Valine arylamidase	—	+	w	+
Alkaline phosphatase	—	—	—	+
Acid phosphatase	—	+	+	+
α-Glucosidase	—	+	+	+
Utilization of;				
D-Galactose	—	+	+	+
D-Mannose	—	+	+	+
D-Melezitose	—	—	+	+
Growth pH	5–8	5–9	5–9	5–8
Tolerance to NaCl (%)	0–3	0–3	0–7	0–3
Growth temperature (°C)	20–30	10–30	10–30	20–37

Antimicrobial to:

<i>Bacillus subtilis</i>	—	w	w	—
<i>Pseudomonas syringae</i>	—	—	+	w
<i>Candida albicans</i>	—	—	+	—
<i>Alternaria brassicicola</i>	—	—	w	—
<i>Aspergillus niger</i>	w	w	+	—

Strains: 1, *A. iriomotensis* IR73-Li102^T; 2, *A. rishiriensis* RI109-Li102^T; 3, *A. corticola* NBRC 103689^T; and 4, *A. chiangmaiensis* NBRC 104400^T. +, positive; w, weakly positive; —, negative. All reported data were obtained in the present study.

Figure 2 Scanning electron micrographs of strain IR73-Li102^T cultured on nutrient agar for 2 weeks at 30 °C. Bar indicates 10 μm (a) or 1 μm (b).

phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and diphosphatidylglycerol (phospholipid type PIII).³⁴ The cellular fatty acid profiles of strain IR73-Li102^T and its three closest phylogenetic relatives are summarized in Table 1. In contrast to *A. chiangmaiensis*, *A. corticola* and *A. rishiriensis*, the only major fatty acid (> 10% of the total) detected in strain IR73-Li102^T was iso-C_{16:0} (58.1%), which represents a differentiating characteristic of these related species. The DNA G+C content of strain IR73-Li102^T was 74.0 mol%. On the basis of the phylogenetic and chemotaxonomic analyses, strain IR73-Li102^T was identified as a member of the genus *Actinomycetospira*. Interestingly, the previously reported *Actinomycetospira* species were mainly isolated from subtropical/tropical region.^{9,10} A similar tendency was also reported by González et al.³ It is considered that the abundance or diversity of *Actinomycetospira* correlated to the climate. However, ecological futures of lichen-associated actinomycetes, including *Actinomycetospira* were not clarified yet. The further characterization of the strain IR73-Li102^T belonging to the genus *Actinomycetes* was examined on the basis of the comparisons with the three phylogenetically-related species.

The strain IR73-Li102^T formed cream–yellow aerial mycelium and bud-like spores, similar in appearance to those of *A. chiangmaiensis* NBRC 104400^T directly from vegetative mycelium (Figure 2). The spores were oval and rod-shaped (width, 0.4–0.5 μm; length, 0.9–1.1 μm) with a smooth surface. No diffusible pigment was detected when the strain was cultured on ISP medium 6 or 7. Growth of strain IR73-Li102^T occurred in the pH range 5–8 and in the presence of 0–3% NaCl (w/v), with optimum growth observed at pH 7.0 and 0% NaCl (w/v). The temperature range for growth was 20–30 °C, with the optimum temperature being 30 °C. Strain IR73-Li102^T could be differentiated from its phylogenetic relatives by acid phosphatase, utilization of D-galactose and D-mannose, fatty acid profile and growth temperature (Table 2). Strain IR73-Li102^T only exhibited weakly

positive antimicrobial activities against *Aspergillus niger* NBRC 33023^T. In contrast, *A. corticola* NBRC 103689^T has both antibacterial and anti-fungal activities. The detailed physiological and biochemical properties of strain IR73-Li102^T are summarized in the species description below and Table 2.

Finally, the DNA–DNA hybridization values for strain IR73-Li102^T with *A. chiangmaiensis* NBRC 104400^T, *A. corticola* NBRC 103689^T and *A. rishiriensis* RI109-Li102^T were below 35%, which is well below the 70% cut-off point recommended for the assignment of bacterial strains to the same genomic species.³⁵

On the basis of the phenotypic and genotypic characteristics, strain IR73-Li102^T represents a novel species within the genus *Actinomycetospira*, for which the name *A. iriomotensis* sp. nov. is proposed.

Description of *Actinomycetospira iriomotensis* sp. nov.

Actinomycetospira iriomotensis (i'ri.o.mo.ten'sis. N.L. fem. adj. iriomotensis, of or pertaining to Iriomote Island, Okinawa, Japan, where the organism was originally isolated).

Aerobic, Gram-positive, non-motile actinomycete, which forms oval and rod-shaped spores on aerial mycelium. Colonies are powdery surface and have cream–yellow aerial mycelium. The substrate mycelium is long, well developed and branched. Contains meso-diaminopimelic acids, and arabinose and galactose are present in whole-cell hydrolysates. The major fatty acid (>10% of total) is iso-C_{16:0}. The polar lipid profile consists of phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and diphosphatidylglycerol. MK-8 (H₄) is the major menaquinone. Melanoid pigments are not formed on ISP medium 6 or 7. Growth occurs at 20–30 °C, but not at 15, 37 or 45 °C. Growth occurs in the presence of 0–3% NaCl (w/v) and at an initial pH of 5–8, with an optimum at pH 7. Aesculin, urea and starch are hydrolyzed, but not gelatin. Nitrate is not reduced. D-trehalose, D-turanose, D-fructose, D-glucose, glycerol, D-maltose, D-mannitol, D-sorbitol, D-sucrose and xylitol are used as the sole carbon source, but not erythritol, L-arabitol, D-arabinose, D-galactose, D-mannose or D-melezitose. In the API ZYM and Coryne systems (bioMérieux), tests for esterase (C4) and esterase lipase (C8) are positive. Naphthol-AS-BI-phosphohydrolase is weakly positive. Activities of lipase (C14), valine arylamidase, cystine arylamidase, pyrrolidonyl arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase and pyrazinamidase are negative. The DNA G+C content of the type strain is 74.0 mol%.

The type strain is IR73-Li102^T (=NBRC 106365^T = KCTC 19782^T), isolated from a lichen sample collected on Iriomote Island, Okinawa, Japan.

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