ORIGINAL ARTICLE

Actinoplanes rishiriensis sp. nov., a novel motile actinomycete isolated by rehydration and centrifugation method

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The motile actinomycete strain RI50-RCA114^T was isolated using rehydration and centrifugation method from a soil sample obtained from Rishiri Island in Japan. The taxonomic status of this organism was established using a polyphasic approach. Cells of strain RI50-RCA114^T were Gram positive, aerobic, motile and formed irregular sporangia. The strain grew in the presence of 0–2% (w/v) NaCl, between pH 5 and 8, and over a temperature range of 20–37°C, with optimal growth at 30°C. Whole-cell hydrolysates of the strain contained *meso*-diaminopimelic acid, galactose, glucose and mannose, in addition to one unidentified *O*-methyl-hexose. The predominant menaquinone was MK-9(H₄), and the major polar lipids comprised phosphatidylethanolamine, diphosphatidylglycerol and phosphatidyl-*N*-methylethylethanolamine. The major cellular fatty acids were iso-C_{16:0}, iso-C_{15:0} and anteiso-C_{17:0}. Comparative 16S ribosomal RNA gene sequence analysis revealed that strain RI50-RCA114^T had the closest sequence similarity with *Actinoplanes globisporus* JCM 3186^T (97.6%). However, DNA–DNA hybridization assays as well as physiological and biochemical analyses differentiated strain RI50-RCA114^T from its closest phylogenetic relative. On the basis of these data, we propose that strain RI50-RCA114^T (=NBRC 108556^T=BCC 49184^T) be classified as the type strain of a novel *Actinoplanes* species and named *Actinoplanes rishiriensis* sp. nov.

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INTRODUCTION

The genus *Actinoplanes*^{1–3} that currently comprises 30 species is a member of the family *Micromonosporaceae*.^{4,5} All species in this genus are motile with respect to spores and the formation of spherical, subspherical, cylindrical or highly irregular sporangia.^{3,6} Several researchers have described selective isolation techniques for motile actinomycetes including those used in the genus *Actinoplanes*.^{6,7} For example, rehydration and centrifugation method is a highly selective method of isolation based on differential centrifugation and chemotactic properties.⁸ Recently, novel actinomycetes have been screened by using rehydration and centrifugation method, including *A. tovensis*, *A. tereljensis* and *Luteipulveratus mongoliensis*.^{9,10} The genus *Actinoplanes* produces several types of antibiotics including peptides/depsipeptides, polyene-type macrolides and aromatic compounds.⁶ Representative antibiotics originated from *Actinoplanes* species are teichomycins, purpurumycin, mycoplanecins and lipiarmycin.^{11–14} Glucose/xylose isomerase was isolated from species of *Actinoplanes missouriensis*.^{15,16} Thus, the discovery of additional species of this genus will contribute both to an understanding of their ecological roles and to the provision of bioresources for industrial applications.

While screening for motile actinomycetes from a cool temperate area, we discovered strain RI50-RCA114^T in a soil sample collected from Rishiri Island, Hokkaido, Japan. The aim of the present study was to determine the taxonomic position of strain RI50-RCA114^T using a polyphasic taxonomic approach, which included chemotaxonomic, morphological, physiological, molecular and genomic characterizations.

RESULTS AND DISCUSSION

The almost complete 16S ribosomal RNA (rRNA) gene sequence (1477 nucleotide) of strain RI50-RCA114^T was compared with sequences of known bacterial species on the EzTaxon server (<http://www.eztaxon.org/>).¹⁷ Results showed that this strain has the highest sequence similarity of 97.6% to *Actinoplanes globisporus* JCM 3186^T, followed by *Actinoplanes brasiliensis* DSM 43805^T (97.3%) and *Actinoplanes deccanensis* NBRC 13994^T (96.6%). The similarity of the 16S rRNA gene sequence to members of the genus *Actinoplanes* ranged from 95.0%–97.6%. The phylogenetic tree constructed with 16S rRNA gene sequence data by neighbor-joining method showed that strain RI50-RCA114^T formed a monophyletic clade with *A. globisporus*, and

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this was supported by the maximum parsimony and maximum-likelihood method (Figure 1). However, *A. brasiliensis* did not form a cluster with strain RI50-RCA114^T and *A. globisporus* in any of the three tree-making algorithms.

The whole-cell hydrolysate of strain RI50-RCA114^T contained meso-diaminopimelic acid, galactose, glucose, mannose and one unidentified *O*-methyl-hexose.¹⁸ The major menaquinone was MK-9 (H₄). The predominant polar lipids were phosphatidylethanolamine, diphosphatidylglycerol (phospholipid type PII)¹⁹ and phosphatidyl-*N*-methylethylethanolamine (Supplementary Figure S1). Table 1 summarizes the cellular fatty-acid profiles of strain RI50-RCA114^T and its two closest phylogenetic relatives. The major fatty acids (>10% of the total) detected in strain RI50-RCA114^T were iso-C_{15:0} (12.4%), iso-C_{16:0} (35.6%) and anteiso-C_{17:0} (11.9%), a profile quite similar to *A. globisporus*.⁹ In contrast, *A. brasiliensis* was differentiated from RI50-RCA114^T and *A. globisporus* based on the presence of anteiso-C_{15:0} in the fatty-acid profile.⁹ The DNA G+C content of strain RI50-RCA114^T was 71.2 mol%. On the basis of phylogenetic and chemotaxonomic findings, strain RI50-RCA114^T was identified as a member of the genus *Actinoplanes*. Strain RI50-RCA114^T belonging to the genus *Actinoplanes* was further characterized on the basis of comparisons with its phylogenetic relative, *A. globisporus*.

Strain RI50-RCA114^T formed light brown to pale reddish orange substrate mycelia without aerial mycelia (Figure 2). The diameter of irregular sporangia that were directly formed from substrate mycelia ranged from 3 to 5 µm. Strain RI50-RCA114^T grew in the pH range of 5–8 and in the presence of 0–2% NaCl (*w/v*), with optimal growth conditions being pH 7.0 and 0% NaCl (*w/v*). The temperature range for growth was 20–37 °C, with the optimum of 30 °C. Strain

RI50-RCA114^T was differentiated from *A. globisporus* by the catalase reaction, gelatin liquefaction, degradation of calcium malate and Tween 20, utilization of lactose and enzyme activity of naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-mannosidase, valine arylamidase and the range of pH of growth (Table 2).

The DNA–DNA hybridization values between strain RI50-RCA114^T and *A. globisporus* NBRC 13912^T were <48%, which is <70% cut-off point recommended for the assignment of bacterial strains to the same genomic species.²⁰

On the basis of the phenotypic and genotypic characteristics, strain RI50-RCA114^T represents a novel species within the genus *Actinoplanes*, for which the name *Actinoplanes rishiriensis* sp. nov. is proposed.

Description of *Actinoplanes rishiriensis* sp. nov.

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

Cells are aerobic and Gram positive. Aerial mycelia are absent and vegetative mycelia have branched hyphae. Irregular sporangia are formed directly from substrate mycelia. Sporangiospores are motile. The substrate mycelium is light brown to pale reddish–orange, and soluble pigment is not produced. Whole-cell hydrolysates contain meso-diaminopimelic acids, galactose, glucose, mannose and one unidentified *O*-methyl-hexose. The major fatty acids (>10% of total) are iso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. The polar lipid profile consists of phosphatidylethanolamine, phosphatidyl-*N*-methylethylethanolamine and diphosphatidylglycerol. MK-9 (H₄) is the major menaquinone. The organism grows at 20–37 °C, but not at 15 and 45 °C, in the presence of 0–2% NaCl (*w/v*), and at an initial pH

Figure 1 Phylogenetic tree derived from 16S rRNA gene sequences shows relationships between strain RI50-RCA114^T and members of genus *Actinoplanes*. Root position of neighbour-joining tree was determined using *Dactylosporangium aurantiacum* DSM 43157^T (X93191) as outgroup. The tree was constructed using neighbor-joining and K_{nuc} values.³⁹ Only bootstrap values >50% are shown (1000 resamplings) at branching points. Solid circles indicate that corresponding nodes were also recovered in maximum parsimony and maximum-likelihood algorithms.⁴⁰ Bar, 0.005 K_{nuc} .

Table 1 Cellular fatty acid profiles of strain RI50-RCA114^T and its closest phylogenetic relatives

Fatty acid	1	2	3
<i>Saturated straight-chain</i>			
C _{15:0}	—	1.0	3.0
C _{16:0}	—	3.6	1.7
C _{17:0}	1.5	3.0	5.1
C _{18:0}	—	3.8	1.4
<i>Saturated branched</i>			
iso-C _{14:0}	3.3	—	1.5
iso-C _{15:0}	12.4	17.6	10.1
anteiso-C _{15:0}	4.3	3.4	24.5
iso-C _{16:0}	35.6	20.1	17.1
anteiso-C _{16:0}	—	1.4	—
C _{16:0} 9-methyl	3.7	—	—
iso-C _{16:0} 2-OH	3.1	7.9	—
iso-C _{17:0}	5.5	9.7	2.1
anteiso-C _{17:0}	11.9	10.3	18.8
iso-C _{17:0} 2-OH	—	1.7	2.2
anteiso-C _{17:0} 2-OH	—	4.7	—
C _{17:0} 10-methyl	1.2	—	—
iso-C _{18:0}	1.3	—	—
<i>Unsaturated</i>			
C _{16:1} ω9c	1.3	—	—
Anteiso-C _{17:1}	1.4	—	—
C _{17:1} ω9c	6.9	1.3	7.0
C _{18:1} ω9c	3.5	3.9	1.9

Abbreviations: Strain 1, *A. rishiriensis* RI50-RCA114^T; Strain 2, *A. globisporus* NBRC 13912^T (data from Ara et al.¹¹); Strain 3, *A. brasiliensis* NBRC 13938^T (data from Ara et al.¹¹). —, undetectable or detected in trace amounts. Bold entries indicate >10% of the total fatty acids.

of 5–8, with an optimum of pH 7. Nitrate is reduced and the catalase reaction is negative. Tyrosine, casein, starch and Tween 20 are hydrolyzed, but not gelatin and calcium malate. Urease is weakly positive. L-arabinose, D-mannose, xylose, lactose, rhamnose and sucrose are utilized as sole carbon sources, but not sodium acetate, sorbitol, glycerol or D-ribose. The API ZYM, tests for alkaline phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, β-glucosidase and N-acetyl-β-glucosaminidase are positive. Tests for esterase (C4), esterase lipase (C8), β-galactosidase and α-glucosidase are weakly positive. Activities of lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, α-mannosidase and α-fucosidase are negative. The DNA G+C content of the type strain is 71.2 mol%.

The type strain is RI50-RCA114^T (=NBRC 108556^T=BCC 49184^T), isolated from a soil sample collected on Rishiri Island, Hokkaido, Japan.

MATERIALS AND METHODS

Isolation and maintenance of organism

A soil sample collected from Rishiri Island, Hokkaido, Japan was air-dried for 7 days at room temperature and passed through a 2-mm mesh sieve. Actinomycetes were then isolated as follows. The sample was processed using rehydration and centrifugation method as described by Hayakawa et al.,⁸ spread on humic acid-vitamin agar²¹ containing nalidixic acid (20 mg l⁻¹) and cycloheximide (50 mg l⁻¹), and then incubated at 30°C for 2 weeks. Thereafter, strain RI50-RCA114^T was isolated and transferred to oatmeal-yeast extract-glucose-glycerol (OYGG) agar²² and maintained in 20% (v/v) glycerol at –80°C.

Figure 2 Scanning electron micrographs of strain RI50-RCA114^T cultured on HA for 2 weeks at 30°C. Bar indicates 10 (a) or 1 (b) μm.

Phenotypic characterization

Strain RI50-RCA114^T grew on HA agar for 14 days at 28°C, and morphological features were analyzed using light and scanning electron microscopy (JEOL, Tokyo, Japan, JSM-6500F).²³ We used light microscopy to evaluate motility in spores suspended in hanging drops of phosphate buffer-soil extract solution.⁸ Spores were Gram-stained according to Hucker.²⁴ To determine the optimal temperature and pH for growth, strain RI50-RCA114^T was incubated for 7 and 14 days on oatmeal-YGG agar at temperatures of 5, 10, 20, 30, 37, 40 and 45°C, and at pH values ranging from 5 to 11 (in 1 pH unit intervals). Growth at 5 and 10°C was assayed after 6 weeks of incubation. Growth in various concentrations of NaCl (0–8%, w/v, in 1% increments) was evaluated after 14 and 21 days of incubation on Bennett's agar.²⁵ Several physiological and biochemical characteristics were evaluated using API ZYM Biochemical Test Kits (bioMérieux, Tokyo, Japan) according to the manufacturer's instructions. Assimilation of carbon sources at a final concentration of 1% (w/v) was tested using International *Streptomyces* Project (ISP) medium 9 as the basal medium.²⁶

Chemotaxonomy

Biomass was generated for chemotaxonomic studies by shaking strain RI50-RCA114^T in glucose-yeast extract broth for 5–7 days at 30°C.²⁷ Pelleted cells harvested by centrifugation were washed twice with distilled water. Diamino-pimelic acid isomers and sugars in whole-cell hydrolysates were then analyzed

Table 2 Differential characteristics of strain RI50-RCA114^T and its closest phylogenetic relative

Characteristic	1	2
NaCl tolerance (%)	0–2	0–3
Growth pH	5–8	5–11
Catalase reaction	–	+
Gelatin liquefaction	–	+
<i>Degradation of</i>		
Calcium malate	–	+
Tween 20	+	–
<i>Utilization of</i>		
Lactose	+	–
<i>Enzyme activity of</i>		
Naphthol-AS-BI-phosphohydrolase	+	–
β-Glucuronidase	+	–
α-Mannosidase	–	+

Abbreviations: Strain 1, *A. rishiriensis* RI50-RCA114^T; Strain 2, *A. globisporus* NBRC 13912^T.
+, positive; –, negative.

All data were generated in the present study.

based on the methods described by Hasegawa *et al.*²⁸ and Tamura *et al.*,¹⁸ respectively. Cellular fatty acids were processed and analyzed as methyl esters, following the protocol of the MIDI Sherlock Microbial Identification System.²⁹ Isoprenoid quinones and polar lipids were extracted and isolated using standard procedures,³⁰ and the results were compared with those of appropriate controls. The isoprenoid quinone content was determined using liquid chromatography/mass spectrometry described by Hamada *et al.*³¹ The polar lipids were identified by two dimensional thin-layered chromatography, followed by spraying with appropriate detection reagents according to the method of Yassin *et al.*³² The DNA G+C content of strain RI50-RCA114^T was determined by HPLC as described by Tamaoka and Komagata.³³

Molecular analysis

Chromosomal DNA from strain RI50-RCA114^T was isolated and purified as described by Saito and Miura³⁴ with a minor modification.³⁵ The 16S rRNA gene from strain RI50-RCA114^T was amplified by PCR as described by Tamura and Hatano³⁶ and the PCR product was purified using MonoFas DNA Purification Kits (GL Sciences, Tokyo, Japan). The purified PCR product was directly sequenced using ABI Prism BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and a Genetic Analyzer automated DNA sequencer (model 3730; Applied Biosystems). The 16S rRNA gene sequence was compared with published 16S rRNA gene sequences of bacterial type strains using the EzTaxon server.¹⁷ For phylogenetic analyses, 16S rRNA gene sequences were collected from the EMBL/GenBank/DNA Data Bank of Japan database and aligned using the CLUSTAL_X program.³⁷ Phylogenetic trees were constructed using the CLUSTAL_X program³⁷ and Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0³⁸ with neighbor-joining, maximum parsimony and maximum-likelihood methods.^{39,40} 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).⁴¹

DNA–DNA hybridization

DNA–DNA hybridization proceeded as described by Kusunoki *et al.*⁴² 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 is quoted as the DNA–DNA relatedness value.

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain RI50-RCA114^T determined in this study has been deposited in the DNA Data Bank of Japan database under the accession number AB641831.

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