Virgisporangium aliadipatigenens sp. nov., Isolated from Soil in Iriomote Island and Emended Description of the Genus *Virgisporangium*

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During a study of the distribution of actinomycetes in a subtropical zone in Japan, two strains forming short sporangiophores on the substrate mycelium were isolated from soil. The 16S rRNA gene sequences of the isolates indicated that these organisms formed a monophyletic cluster with members of the genus *Virgisporangium* in the family *Micromonosporaceae*. These strains formed narrow sporangia on short sporangiophores directly above the substrate mycelium. The sporangia contained motile spores. The strains contained 3-OH-diaminopimelic acid in the cell wall and glucose, ribose, mannose, galactose, xylose and 3-O-methylmannose as whole-cell sugars. The predominant menaquinones were MK-10(H₄) and MK-10(H₆). The diagnostic phospholipid was phosphatidylethanolamine. *Cis* 9 C_{17:1} and C_{17:0} were detected as the major cellular fatty acids. The G+C content of the DNA was 71.7 mol%. DNA–DNA relatedness analysis showed that the two isolates represented the same genomic species. The results of morphological, chemotaxonomic and 16S rRNA gene sequence analyses, as well as DNA–DNA hybridization studies, confirmed that these isolates certainly belonged to a new species of the genus *Virgisporangium*. We propose a novel taxon of the genus *Virgisporangium* as *Virgisporangium aliadipatigenens* sp. nov., with the type strain IR20-55^T (= NBRC 105644^T).

INTRODUCTION

The genus *Virgisporangium* was firstly described by Tamura *et al.* (2001) for actinomycete strains that are characterized by motile spores, and contain 3-OH-diaminopimelic acid (3-OH-A₂pm) in the cell wall and 3-O-methylmannose as a whole-cell sugar. The original spelling *Virgosporangium* was corrected by the List Editor of the *International Journal of Systematic and Evolutionary Microbiology* (2001). This genus currently contains only two species, namely, *Virgisporangium ochraceum* and *Virgisporangium aurantiacum*, both of which were isolated in Japan.

During studies of the inventories of actinomycetes from a subtropical area of Japan, strains IR20-55^T and IR08-25 were isolated from soil in Iriomote island, Okinawa, Japan, using the rehydration and centrifugation method (Hayakawa *et al.*, 2000), a highly selective isolation method for motile actinomycetes. Comparative 16S rRNA gene sequence analysis revealed that these resided in the family *Micromonosporaceae* and were closely related to the genus *Virgisporangium*. Their phenotypic and phylo-

genetic characteristics, coupled with data on genomic DNA–DNA relatedness, suggested that these strains should be classified as a novel species. Accordingly, we propose the name *Virgisporangium aliadipatigenens* sp. nov. for strains IR20-55^T and IR08-25.

MATERIALS AND METHODS

Strains IR20-55^T and IR08-25 were isolated from soil collected in Iriomote Island, Japan using the rehydration and centrifugation method (Hayakawa *et al.*, 2000) with humic acid-vitamin (HV) agar (Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l⁻¹) and nalidixic acid (20 mg l⁻¹). After aerobic incubation at 28°C for 3 weeks, colonies were transferred and purified on yeast extract-starch (YS) agar (2 g yeast extract, 10 g soluble starch and 15 g agar l⁻¹; pH 7.3).

The morphological features of the strains grown on YS medium or HV agar were observed by light and scanning electron microscope (model JSM-6060; JEOL). For electron microscopy, agar blocks containing microbial growth were fixed with 1% osmium tetroxide, dehydrated through

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a graded series of ethanol and isoamyl acetate and critical-point-dried. The gold-coated specimens were then observed using a scanning electron microscope. The motility of the spores was observed under a light microscope using cells grown on HV agar at 28°C for 21 days. Spore suspensions were obtained from the agar surface and mixed with phosphate buffer (K-salts; 0.01 M, pH 7.0) containing 10% soil extract (Henrich, 1947) and incubated at 30°C for 1 hour.

The physiological characteristics and the growth range for temperatures and pH values were tested on YS broth. The cultural and biochemical characteristics were examined using methods described previously (Gordon *et al.*, 1974; Shirling & Gottlieb, 1966; Williams *et al.*, 1983). The colours of the colonies were determined according to a Mycological Color Chart (Rayner, 1970). The utilization of carbohydrates as sole carbon sources was tested using ISP medium 9 (Difco) containing 0.1% yeast extract and B-vitamins (Hayakawa & Nonomura, 1987) as a basal medium. API ZYM and API Coryne tests (bioMérieux) were used according to the manufacturer's protocol to determine the physiological and biochemical characteristics of the strains.

Freeze-dried cells for chemotaxonomic studies were obtained from cultures grown in YS broth on a rotary shaker at 200 rpm at 28°C for 5 days. The A₂pm isomers and sugars of whole-cell hydrolysates were analysed using procedures described by Hasegawa *et al.* (1983) and Schaal (1985), respectively. Polar lipids were extracted and identified by two-dimensional TLC using methods described by Minnikin *et al.* (1984). Isoprenoid quinones were extracted and purified by the method of Minnikin *et al.* (1984) and determined by LC-MS. The cellular fatty acid composition was determined using the Microbial Identification System (MIDI Inc.). Identification and quantification of the peaks was performed using GC-MS. The N-acetyl group of muramic acid in peptidoglycan was determined according to the method of Uchida *et al.* (1999).

Genomic DNA was obtained using the method of Saito & Miura (1963). The G+C content of the DNA was determined by HPLC as described by Tamura *et al.* (1994). DNA-DNA hybridization was performed fluorometrically using photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989).

The 16S rRNA gene was amplified by PCR and sequenced following the procedures described by Tamura & Hatano (2001) using an ABI PRISM 3730 Genetic Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The DDBJ accession numbers for the 16S rRNA gene sequences of strains IR20-55^T and IR08-25 are AB548616 and AB548617, respectively. The 16S rRNA gene sequences obtained in the present study were aligned with the sequences of the species with validly published names from the family *Micromonosporaceae* that were available from the GenBank/EMBL/DDBJ by using the MEGA (Molecular Evolu-

tionary Genetics Analysis) version 3.1 (Kumar *et al.*, 2004) and CLUSTALX (Thompson *et al.*, 1997) programs. A phylogenetic tree was constructed using the neighbour-joining tree algorithm (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum parsimony (Fitch, 1971) methods. The resultant neighbour-joining tree topology was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

RESULTS AND DISCUSSION

Strains IR20-55^T and IR08-25 formed short sporangio-phores and narrow sporangia on these sporangiophores directly above the surface of the substrate mycelium (Fig. 1). Several spores were present per sporangium. Strains IR20-55^T and IR08-25 produced oval to short rods (0.6–0.9 by 0.8–1.4 µm) spores. The spores exhibited motility when suspended in sterile distilled water or potassium phosphate buffer (0.01 M, pH 7.0). The sporangia were well-developed on HV agar. The strains produced a pale sienna soluble pigment on ISP medium 7 (Table 1). Growth occurred at 20–37°C but not at 15°C. Optimal growth was observed at 28–37°C. The pH range for growth was 7.0–9.0. The strains did not grow at a NaCl concentration higher than 2%. Both strains utilized glucose, sucrose, galactose and mannose. Only strain IR20-55^T utilized D-fructose, maltose and xylose, and only strain IR08-25 utilized L-arabinose. The isolates were positive for starch hydrolysis. Other physiological and biochemical characteristics are shown in Table 2 and in the species description.

Cell wall analysis by HPLC and TLC indicated that the strains contained 3-OH-A₂pm. The whole-cell sugars were glucose, mannose, galactose, xylose and 3-O-methylmannose. The predominant menaquinones of strain IR20-55^T

Fig. 1. Scanning electron micrographs of isolate IR20-55^T grown on HV agar for 3 weeks at 28°C. Bar, 10 µm.

Table 1. Cultural characteristics of isolates IR20-55^T and IR08-25.

	IR20-55 ^T	IR08-25
ISP2		
Growth	Weak	Weak
Reverse colour	Orange (7)	Luteus (12)
Soluble pigment	Absent	Absent
ISP3		
Growth	Good	Good
Reverse colour	Luteus (12)	Luteus (12)
Soluble pigment	Absent	Absent
ISP4		
Growth	Good	Good
Reverse colour	Orange (7)	Luteus (12)
Soluble pigment	Absent	Absent
ISP5		
Growth	Good	Good
Reverse colour	Orange (7)	Pale luteus (11)
Soluble pigment	Absent	Absent
ISP6		
Growth	Weak	Weak
Reverse colour	Luteus (12)	Luteus (12)
Soluble pigment	Absent	Absent
ISP7		
Growth	Good	Good
Reverse colour	Umber (9)	Umber (9)
Soluble pigment	Weak sienna (8)	Weak sienna (8)
YS		
Growth	Good	Good
Reverse colour	Luteus (12)	Luteus (12) to sienna (8)
Soluble pigment	Absent	Absent

The colour codes in parentheses correspond to the colour code in *A Mycological Colour Chart* (Rayner, 1970).

Aerial mycelium of the isolates was not observed on the media used in this study.

were MK-10(H₄), MK-10(H₆) and MK-10(H₈). The predominant menaquinone of IR08-55 was MK-10(H₄), with small amounts of MK-10(H₆) and MK-10(H₈). Mycolic acids were absent. Phosphatidylethanolamine was detected as a diagnostic phospholipid, but other major polar phospholipids were not detected. The acyl type of the cell wall polysaccharides was glycolyl. The G+C contents of the DNAs of the two strains were 71.7 mol%. The major fatty acids of the isolates were *cis* 9 C_{17:1} (34.6–39.8%) and C_{17:0} (21.8–25.3%), as identified by GC-MS and the retention times of GC analysis. The predominant cellular fatty acids were different from the two known species (Table 3).

Almost complete gene sequences for the 16S rRNA genes of strains IR20-55^T and IR08-25 were determined. Phylogenetic analysis revealed that the isolates were affiliated within the cluster of the genus *Virgisporangium* (Fig. 2). The similarity values of the 16S rRNA gene sequences

Table 2. Differential phenotypic properties of the isolates IR20-55^T, IR08-25 and the type strains of phylogenetically related *Virgisporangium* species.

Characteristic	1	2	3	4
Growth on sole carbon sources:				
D-Fructose	+	–	+	+
D-Mannitol	–	–	+	+
L-Arabinose	–	+	+	+
Glycerol	+	–	+	+
Inositol	–	–	+	–
D-Sorbitol	–	–	+	–
Lactose	–	–	–	+
Melibiose	–	–	+	+
Maltose	+	–	+	+
Xylose	+	–	+	+
Growth in				
NaCl 2%	–	–	+	+
Assimilation of calcium malate	+	–	–	–
Nitrate reduction	–	++	+	+
Growth at				
15°C	–	–	+	+
37°C	++	++	+	–
Peptonization of Milk	–	–	+	+
API ZYM:				
Alkaline phosphatase	+	w	+	+
Esterase (C-4)	–	w	w	w
Trypsin	+	–	+	+
Chymotrypsin	–	+	+	+
Acid phosphatase	+	–	–	w
α-Galactosidase	+	–	–	w
N-Acetyl-β-glucosaminidase	–	–	+	+

Strains indicated as: 1, IR20-55^T; 2, IR08-25; 3, *V. ochraceum* NBRC 10618^T; 4, *V. aurantiacum* NBRC 16421^T. +, positive; –, negative; w, weakly positive.

Data were taken from present study and Tamura *et al.* (2001).

between the isolates and the type strains of validated *Virgisporangium* species were 97.0–97.5%. The sequence similarity between the isolates IR20-55^T and IR08-25 was 99.8%. Signature nucleotides of the genus *Virgisporangium* at positions 502–543 (A–U) and 1116–1184 (U–G) (Tamura *et al.*, 2001) were present in the 16S rRNA gene sequences of strains IR20-55^T and IR08-25. The DNA–DNA relatedness values between strains IR20-55^T and IR08-25 were 81 and 106%, indicating that the two strains belong to one species. The values ranged from 6% to 21% for DNA–DNA relatedness between strain IR20-55^T and *Virgisporangium ochraceum* NBRC 16418^T and *Virgisporangium aurantiacum* NBRC 16421^T.

On the basis of the results of phylogenetic, chemotaxonomic, physiological and biochemical analysis and DNA–DNA hybridization tests, we propose a novel *Virgisporangium* species, namely, *Virgisporangium aliadipatigenens* sp. nov. for strains IR20-55^T and IR08-25.

Table 3. Cellular fatty acid composition (%) of isolates IR20-55^T, IR08-25, *Virgisporangium ochraceum* NBRC 16418^T and *Virgisporangium aurantiacum* NBRC 16421^T.

Fatty acid	IR20-55 ^T	IR08-25	<i>V. ochraceum</i> NBRC 16418 ^T	<i>V. aurantiacum</i> NBRC 16421 ^T
iso-C _{14:0}	—	—	0.7	1.3
C _{14:0}	—	—	—	0.4
iso-C _{15:0}	1.0	2.3	3.4	6.2
anteiso-C _{15:0}	0.6	—	0.9	2.6
C _{15:0}	4.2	1.6	0.8	0.5
iso-C _{16:1} G	—	—	—	1.9
iso-C _{16:1} H	—	—	0.7	—
iso-C _{16:0}	7.3	5.2	40.1	41.54
<i>cis</i> 9-C _{16:1}	1.1	2.1	—	—
anteiso-C _{15:0} 2-OH	—	—	0.9	—
C _{16:0}	3.1	6.6	1.5	1.8
C _{16:0} 9-methyl?	—	1.0	—	0.7
anteiso-C _{17:1} C	0.6	—	—	1.3
iso-C _{17:0}	1.0	3.7	3.3	2.8
anteiso-C _{17:0}	4.9	3.0	13.2	18.4
<i>cis</i> 9-C _{17:1}	39.8	34.6	8.1	2.8
C _{17:0}	25.3	21.8	4.7	2.7
10-methyl C _{17:0}	1.0	0.9	0.9	—
iso-C _{18:0}	0.5	0.4	1.2	0.8
<i>cis</i> 9-C _{18:1}	4.6	10.3	13.1	8.2
iso-C _{17:0} 2-OH	—	—	—	1.2
Summes Feature 7	0.9	1.8	—	—
C _{18:0}	1.6	3.0	1.3	1.5
Sum in feature 8	—	—	5.0	1.3
Sum in feature 9	—	—	—	2.4

Data were taken from the present study. Values are percentage of total fatty acids. —, not detected.

Fatty acid identities were determined from the Microbial Identification System software (MIDI Inc.) peak-naming table.

Emended description of *Virgisporangium Tamura et al. 2001*

The following features are added to those given in the current description of *Virgisporangium* by Tamura *et al.* (2001). The major fatty acids are iso-C_{16:0} and anteiso-C_{17:0}, or *cis* 9-C_{17:1} and C_{17:0}. The G+C content of the genomic DNA is 71–72 mol%.

Description of *Virgisporangium aliadipatigenens* sp. nov.

Virgisporangium aliadipatigenens (a.li.a.di.pa.ti.ge'nens. L. adj. and pronoun *alius*, other, another, different; N.L. n. *acidum adipatum*, fatty acid; L. part. adj. *genens*, producing; N.L. part. adj. *aliadipatigenens*, producing different fatty acids)

Gram-positive, non-acid-fast and aerobic bacterium with branching hyphae. The sporangia were well-developed on HV agar. Non-fragmenting orange to luteus coloured substrate mycelia are present. Light brown soluble pigment is produced on tyrosine agar (ISP medium 7). Slender sporangia are formed on short sporangiophores on the substrate mycelium. Several spores are present per sporangium, and the spores are oval to short rods (0.6–0.9 by 0.8–

1.5 µm); they are motile when suspended in sterile distilled water or potassium phosphate buffer (0.01 M, pH 7.0). Growth occurs between pH 7 and 9, and 20°C and 37°C. Does not grow in 2% NaCl. Nitrate reduction is a variable property. Starch hydrolysis is positive. Gelatin liquefaction, milk peptonization and urea hydrolysis are negative. Glucose, sucrose, galactose and mannose are utilized, but D-mannitol, inositol, D-sorbitol, lactose and melibiose are not. Leucine arylamidase, β-galactosidase and α-glucosidase were detected by the API ZYM enzyme assay; lipase (C-14), valine arylamidase, cysteine arylamidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are negative. The major cellular fatty acids are *cis* 9 C_{17:1} and C_{17:0}. The G+C content of the DNA is 71.7 mol%. The type strain is IR20-55^T (= NBRC 105644^T), which was isolated from the soil in a sugarcane field in Iriomote Island, Japan.

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Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strains IR20-55^T and IR08-25 to the family Micromonosporaceae. The tree was constructed using the neighbour-joining method and K_{nuc} values (Saitou & Nei, 1987). *Actinocatenispora thailandica* TT2-10^T (AB107233) was used as an out-group. The numbers at branch nodes represent bootstrap percentages (1000 resamplings; only values over 50% are given). Dots indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 0.01 K_{nuc} .

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