A new genus of the order Actinomycetales, *Virgosporangium* gen. nov., with descriptions of *Virgosporangium ochraceum* sp. nov. and *Virgosporangium aurantiacum* sp. nov.

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Four motile spored strains were isolated from soil samples collected in Japan. The cultures formed long, narrow sporangia on short sporangiophores directly on the substrate mycelium. The sporangia develop singly or in clusters above the surface of the substrate. Each sporangium contains a single row of six or more motile spores. Glutamic acid, glucosamine, glycine, alanine and 3-OH-diaminopimelic acid are present in the cell wall; the whole-cell sugars are 3-O-methylmannose, rhamnose, mannose, arabinose, galactose, xylose and glucose; and the predominant menaquinones are 10(H₄), 10(H₆) and 10(H₈). The diagnostic phospholipid is phosphatidylethanolamine. The acyl type of the muramic acid is glycolyl. The G+C content is 71 mol%. Mycolic acids are absent. The chemotaxonomic data indicate that these strains belong to the family Micromonosporaceae. Analysis of 16S rDNA sequences suggested that these organisms fall into a distinct clade within the family Micromonosporaceae for which a new genus, *Virgosporangium* gen. nov., is proposed containing the species *Virgosporangium ochraceum* sp. nov. (strains YU655-43^T, YU793-41 and YU794-41) and *Virgosporangium aurantiacum* sp. nov. (strain YU438-5^T).

Keywords: *Virgosporangium* gen. nov., family Micromonosporaceae, 16S rDNA, *Virgosporangium ochraceum* sp. nov., *Virgosporangium aurantiacum* sp. nov.

INTRODUCTION

The family *Micromonosporaceae* Krasil'nikov 1938 was originally created on a morphological basis. Goodfellow *et al.* (1990) emended the family and added the genera *Actinoplanes*, *Dactylosporangium* and *Pilimelia* on the basis of numerical and chemical analyses. The genera *Catenuloplanes* (Yokota *et al.*, 1993) and *Couchioplanes* (Tamura *et al.*, 1994) were subsequently added on the basis of phylogenetic analysis. Koch *et al.* (1996) emended the description of the family to reflect its phenotypic and chemotaxonomic heterogeneity. Later, Stackebrandt *et al.* (1997) emended the family on the basis of 16S rDNA/rRNA sequence phylogenetic clustering and

the presence of taxon-specific 16S rDNA/rRNA signature nucleotides.

During taxonomic studies of rare actinomycetes, four strains have been isolated, YU438-5^T, YU655-43^T, YU793-41 and YU794-41, from soil samples from Yamanashi and Okinawa prefectures, Japan. These strains produce sporangia that typically contain a single row of six or more motile spores, and have 3-OH-diaminopimelic acid and glycine in the cell wall. On the basis of their 16S rDNA sequences, the isolates phylogenetically fall within the family *Micromonosporaceae*, but form a lineage distinct from described organisms. Therefore, we propose that the isolates should be included in a new genus, *Virgosporangium*, the name being derived from the virgate sporangia of the organism.

METHODS

Micro-organisms and culture conditions. Strains YU438-5^T (= IFO 16421^T) and YU655-43^T (= IFO 16418^T) were

The GenBank accession numbers for the 16S rDNA sequences determined in this work are: AB006167 (*Virgosporangium ochraceum* YU655-43^T), AB006162 (*Virgosporangium ochraceum* YU793-41), AB006161 (*Virgosporangium ochraceum* YU794-41) and AB006169 (*Virgosporangium aurantiacum* YU438-5^T).

Fig. 1. Light micrograph (a) and scanning electron micrograph (b) of isolate YU655-43^T, and scanning electron micrograph of isolate YU438-5^T (c) grown on yeast extract-starch agar for 2 weeks at 28 °C. Bars: (a) 10 µm, (b) 1 µm, (c) 5 µm.

isolated from a soil sample from a vegetable field in Yamanashi prefecture, strain YU793-41 (= IFO 16419) was isolated from a soil sample from a mulberry field in Okinawa prefecture, and YU794-41 (= IFO 16420) was isolated from a soil sample from a potato field in Okinawa prefecture, Japan. All were isolated on humic acid-vitamin (HV) agar (Hayakawa & Nonomura, 1987) by the capillary method (Palleroni, 1980; Hayakawa *et al.*, 1992) using vanillin as an attractant. Freeze-dried cells for chemotaxonomic analyses were grown in yeast extract-glucose broth (10 g yeast extract, 10 g D-glucose per litre distilled water, pH 7.0) on a rotary

shaker at 28 °C. *Actinoplanes philippinensis* IFO 13878^T, *Dactylosporangium thailandense* IFO 12593^T, *Micromonospora chalcone* IFO 13503^T and *Catellatospora citrea* subsp. *citrea* IFO 14495^T were analysed to compare whole-cell sugar patterns.

Cultural, morphological and physiological characteristics.

Cultures grown on HV agar and yeast extract-starch agar (containing 2 g yeast extract and 10 g soluble starch per litre distilled water, pH 7.0) for 3–14 d at 28 °C were observed with a light microscope and with a scanning electron microscope (model JSM-5400; JEOL). Samples for scanning electron microscopy were prepared by cutting a block from an agar plate containing the strain, fixing the block in osmium tetroxide vapour at room temperature for 4 h, dehydrating the cells by freeze-drying and then sputter-coating them with palladium under vacuum.

Motility was observed with a light microscope using cells grown on HV agar for 7–10 d at 28 °C, then incubated at 28 °C for 30–60 min in sterile water. Cultural characteristics on media according to the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966) and on nutrient agar (Difco) were recorded after 14 d incubation at 28 °C. Colours are described in common terminology, and colours were also determined more exactly by comparison with the colour chips given by Rayner (1970).

Physiological characteristics were determined according to Seino *et al.* (1985). ISP medium 9 was used as a basal medium to examine utilization of sugars. Inorganic salts-starch agar (ISP medium 4) was used to determine starch hydrolysis. Growth temperature was determined on yeast extract-starch agar. Calcium malate medium (10 g calcium malate, 0.5 g NH₄Cl, 0.5 g K₂HPO₄, 18 g agar per litre distilled water, pH 7.0) was used to determine solubility of calcium malate. ISP medium 8 was used to determine nitrate reduction. Gelatin medium (200 g gelatin per litre distilled water, pH 7.0) and glucose-peptone-gelatin medium (20 g glucose, 5 g peptone and 200 g gelatin per litre distilled water, pH 7.0) were used to determine gelatin liquefaction. Skim milk (10%, w/v; Difco) was used for determination of coagulation and peptonization of milk.

Analyses of chemotaxonomic characteristics. Analyses of whole-cell sugar patterns, cell wall amino acids, menaquinones, cellular fatty acids, isomers of diaminopimelic acid, acyl type of the peptidoglycan, mycolic acid and DNA base composition were performed as described previously (Tamura *et al.*, 1994).

Preparation of DNA and determination of G+C content.

Genomic DNA was prepared according to the method of Ezaki *et al.* (1990). The G+C content of the DNA was determined by the method of Mesbah *et al.* (1989) after treatment with P1 nuclease and alkaline phosphatase, and by HPLC using a Shimadzu LC-6AD apparatus equipped with a column of Cosmosil 5C18-AR (4.6 × 150 mm; Nacalai Tesque).

PCR amplification and sequencing of 16S rDNA.

16S rDNA was amplified by PCR and the resulting products directly sequenced using previously described protocols (Tamura *et al.*, 1998). The products were analysed with a Pharmacia ALF DNA Sequencer II according to the manufacturer's protocol.

Phylogenetic analysis. 16S rDNA sequences of the strains and reference organisms were aligned with that of *Streptomyces ambofaciens* (Pernodet *et al.*, 1989). The CLUSTAL W version 1.7 software package (Thompson *et al.*, 1994) was used to generate the evolutionary distances (the K_{nuc} value of

Table 1. Cultural characteristics of isolates YU438-5^T and YU655-43^T

YU793-41 and YU794-41 showed cultural characteristics similar to those of YU655-43^T.

Medium	YU438-5 ^T		YU655-43 ^T	
	Growth	Colour*	Growth	Colour*
Yeast extract-malt extract agar (ISP medium 2)	Moderate	Strong vivid reddish orange (5)	Good	Strong orange (7)
Oat meal agar (ISP medium 3)	Good	Strong orange (7)	Moderate	Strong orange (7)
Inorganic salts-starch agar (ISP medium 4)	Moderate	Strong orange (7)	Poor	Colourless
Glycerol-asparagine agar (ISP medium 5)	Moderate	Strong orange (7)	Poor	Colourless
Peptone-yeast extract iron agar (ISP medium 6)	Moderate	Strong orange (7)	Moderate	Moderate orange-yellow (44)
Tyrosine agar (ISP medium 7)	Moderate	Moderate reddish brown (60) to moderate brown (63); moderate brown (87) soluble pigment	Moderate	Moderate brown (87); greyish reddish orange (59) to moderate brown (87) soluble pigment
Nutrient agar	Moderate	Strong orange (7)	Moderate	Moderate orange-yellow (44)

* Numbers in parentheses correspond to the colour chips given by Rayner (1970).

Table 2. Cellular fatty acid compositions (%) of isolates YU438-5^T, YU655-43^T, YU793-41 and YU794-41

Fatty acid	YU438-5 ^T	YU655-43 ^T	YU793-41	YU794-41
Iso-branched				
C _{15:0}	5.9	10.3	10.5	9.6
C _{16:0}	27.9	12.6	6.8	18.2
C _{17:0}	6.1	5.4	7.8	6
Anteiso-branched				
C _{15:0}	1.9	2.9	1.8	1.2
C _{17:0}	28.8	21.3	16.4	11.9
Saturated				
C _{14:0}			0.6	
C _{15:0}		2.5	1.5	2.4
C _{16:0}	1.4	2.4	4.6	2.2
C _{17:0}	5.6	8.8	8.4	11.1
C _{18:0}	4.3	1.8	7.7	2.2
C _{19:0}	1.6	1.3	2.4	1.6
Unsaturated				
C _{17:1}	3.4	8.9	2.2	7.8
C _{18:1}	9.1	13.4	19.2	14.4
C _{19:1}	3.3	8.5	8.3	11.6
Unidentified	0.8		2.0	

Kimura, 1980) and the similarity values and for the neighbour-joining (NJ) analysis (Saitou & Nei, 1987) from K_{nuc} values. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. NJplot (Perrière & Gouy, 1996) was used for constructing the phylogenetic tree.

The GenBank accession numbers for the 16S rDNA sequences determined in this work are: AB006169 (strain YU438-5^T), AB006167 (strain YU655-43^T), AB006162 (strain YU793-41) and AB006161 (strain YU794-41).

DNA-DNA hybridization. To determine DNA relatedness, the microplate-hybridization method developed by Ezaki *et*

Fig. 2. For legend see facing page.

al. (1988, 1989) was applied with minor modifications (Tamura *et al.*, 1999).

RESULTS

Morphological observations

Micrographs of 14-d-old cultures of isolates grown on HV agar are shown Fig. 1. The isolates formed rod-shaped sporangia on the substrate mycelium, which developed singly or in clusters above the surface of the substrate. Under the scanning electron microscope (Fig. 1b), each sporangium was seen to typically contain a single row of six spores or more. Globose bodies were not observed. Spores were oval to short rods (0.6–0.9 by 0.8–1.5 µm). After incubation at 28 °C for 1 h in distilled water, many spores exhibited active motility.

Cultural characteristics

Strain YU438-5^T developed scarlet to orange colonies on ISP media 2, 3, 4, 5 and 6, and on nutrient agar, and the other strains developed orange to rust colonies on ISP media 2, 3 and 6, and on nutrient agar (Table 1). All isolates produced a brownish soluble pigment on ISP medium 7. Aerial mycelium rarely developed on colonies growing on either HV agar or water agar.

Physiological and biochemical characteristics

All isolates utilized glucose, saccharose, D-fructose, D-mannitol, L-arabinose, glycerol, galactose and mannose. Only strain YU655-43^T utilized inositol and D-sorbitol; only strain YU438-5^T utilized lactose; strains YU438-5^T and YU655-43^T utilized melibiose; only strain YU793-41 did not utilize maltose. All isolates were positive for hydrolysis of starch, coagulation and peptonization of milk, and formation of nitrite from nitrate, and were negative for decomposition of calcium malate and gelatin liquefaction. Strain YU438-5^T grew at 15–30 °C but not at 37 °C, while strains YU655-43^T, YU793-41 and YU794-41 grew at 15–37 °C.

Chemotaxonomic characteristics

The predominant menaquinones of strains YU655-43^T and YU793-41 were MK-10(H₄) and MK-10(H₆), with minor amounts of MK-10(H₈). Strains YU438-5^T and YU794-41 had major amounts of MK-10(H₈) with MK-10(H₄) and MK-10(H₆). Isolates contained glucose, mannose, galactose, rhamnose, xylose, arabinose and 3-O-methylmannose as whole-cell sugars. The amino acids in the cell wall were 3-OH-diaminopimelic acid, alanine, glycine and glutamic acid (molar ratio, approx. 1:1:1:1); this corresponds to murein type A1_γ according to Schleifer & Kandler (1972).

Mycolic acids were not detected. The glycan moiety of the murein contained glycolyl residues. Phosphatidylethanolamine was detected as a diagnostic phospholipid, but phosphatidylinositol, phosphatidylglycerol, phosphatidylcholine and an unidentified phospholipid containing glucosamine were not always detected.

The cellular fatty acids included iso-branched, anteiso-branched, saturated and unsaturated fatty acids, corresponding to fatty acid pattern 2d according to Kroppenstedt (1985). Isolate YU438-5^T contained diagnostic amounts of iso-C_{16:0} and anteiso-C_{17:0} (> 25%), while isolates YU655-43^T, YU793-41 and YU794-41 contained relatively higher proportions of C_{15:0} and C_{18:1} with iso-C_{16:0} and anteiso-C_{17:0} (Table 2). The G+C content of the DNA of the isolates ranged from 70.7 to 71.4 mol %.

DNA–DNA hybridization

Strain YU438-5^T exhibited levels of DNA–DNA relatedness of 12–20% with the other strains. DNA relatedness among strains YU655-43^T, YU793-41 and YU794-41 was 40–60%.

Phylogenetic position

Phylogenetic analysis of 16S rDNA sequences revealed that the isolates fell within the cluster of the family *Micromonosporaceae* and represented a line of descent distinct from previously described actinomycetes of this family (Fig. 2). The 16S rDNA signature nucleotides at positions 502–543 (A–U) and 1116–1184 (U–G) of isolates were different from those of the family *Micromonosporaceae* [positions 502–543 (G–C) and 1116–1184 (C–G)]. The closest neighbours of the isolates are members of the genus *Dactylosporangium* (similarity values from 95.8 to 97.1%). Strain YU438-5^T had 98.7–98.9% similarity to other isolates. The similarity values among strains YU655-43^T, YU793-41 and YU794-41 were 99.7–99.9%.

DISCUSSION

The family *Micromonosporaceae* currently consists of the genera *Micromonospora*, *Actinoplanes*, *Dactylosporangium*, *Pilimelia*, *Catellatospora*, *Catenuloplanes*, *Couchioplanes*, *Spirilliplanes* (Tamura *et al.*, 1997) and *Verrucosipora* (Rheims *et al.*, 1998), each having distinctive morphological features and/or chemotaxonomic characteristics. *Micromonospora* species form single spores on short or long sporophores; *Actinoplanes*, *Dactylosporangium* and *Pilimelia* species form sporangia; *Catellatospora*, *Catenuloplanes*, *Couchioplanes* and *Spirilliplanes* species form spore chains; *Verrucosipora* species do not form aerial mycelium or sporangia; *Actinoplanes*, *Dactylo-*

Fig. 2. Phylogenetic tree derived from 16S rDNA sequences of isolates and members of the family *Micromonosporaceae*. *Streptomyces ambofaciens* ATCC 23877^T (M27245) was used as the root organism. Scale bar = 0.01 K_{nuc}. The numbers on the branches are confidence limits (expressed as percentages) estimated from a bootstrap analysis with 1000 replicates (only percentages above 50% are indicated).

Table 3. Differential characteristics of the genus *Virgosporangium* and related genera

Genus	Spore motility*	Sporangium formation*	Fatty acid type†	Cell wall type‡	Major menaquinones	Phospholipid type§
<i>Virgosporangium</i>	+	+	2d	II	MK-10(H ₄ , H ₆ , H ₈)	PII
<i>Dactylosporangium</i>	+	+	3b	II	MK-9(H ₄ , H ₆ , H ₈)	PII
<i>Actinoplanes</i>	+	+	2d	II	MK-9(H ₄), 10(H ₄)	PII
<i>Spirilliplanes</i>	+	—	2d	II	MK-10(H ₄)	PII
<i>Pilimelia</i>	+	+	2d	II	MK-9(H ₂ , H ₄)	PII
<i>Micromonospora</i>	—	—	3b	II	MK-10(H ₄ , H ₆), 9(H ₄ , H ₆)	PII
<i>Catellatospora</i>	—	—	ND	II	MK-10(H ₈ , H ₆), 9(H ₄ , H ₆)	PII
<i>Catenuloplanes</i>	+	—	2c	VI	MK-9(H ₈), 10(H ₈)	PII
<i>Couchioplanes</i>	+	—	2c	VI	MK-9(H ₄)	PIII

*+, Present; —, absent.

† According to the classification of Kroppenstedt (1985). ND, Not determined.

‡ According to the classification of Lechevalier & Lechevalier (1970).

§ According to the classification of Lechevalier *et al.* (1977).

sporangium, *Pilimelia*, *Catenuloplanes*, *Couchioplanes* and *Spirilliplanes* spores show motility; *Catenuloplanes* and *Couchioplanes* species have lysine instead of meso-diaminopimelic acid as the diamino acid of the peptidoglycan.

Like most members of the family *Micromonosporaceae*, the isolates produced orange colonies on most media. They formed rod-shaped sporangia on short sporangiophores, which resembled the extended sporangia of the genus *Dactylosporangium*. Unlike members of the genus *Dactylosporangium*, however, which usually develop three to four spores per sporangium, the isolates developed rod-shaped sporangia with six or more spores. The isolates contained 3-OH-diaminopimelic acid as the diamino acid, and meso-diaminopimelic acid was not detected. Some members of the genera *Micromonospora* and *Dactylosporangium* are known to have 3-OH-diaminopimelic acid with meso-diaminopimelic acid in the cell wall. The isolates contained arabinose and xylose as diagnostic whole-cell sugars, which corresponds to cell wall type II of Lechevalier & Lechevalier (1970). The isolates also contained 3-O-methylmannose as a whole-cell sugar. *Actinoplanes philippinensis*, *Dactylosporangium thailandense* and *Micromonospora chalcone* were also found to have this sugar and *Spirilliplanes yamanashiensis* is known to have it (Tamura *et al.*, 1997). Thus, most chemotaxonomic features are characteristic of the family *Micromonosporaceae*. The isolates were distinguished from the genus *Dactylosporangium*, their closest relatives based on 16S rDNA sequence data, by the absence of 10-methylated fatty acids and the presence of MK-10(H₄, H₆, H₈). This combination of chemotaxonomic characteristics therefore distinguished the isolates from other members of the family *Micromonosporaceae* (Table 3). Phylogenetic analysis indicated that isolates belong to the family *Micromonosporaceae* and formed a compact cluster near the genus *Dactylosporangium* (Fig. 2). The robustness of

this cluster was supported by a high bootstrap value (100%).

On the basis of morphological, physiological, chemotaxonomic (Table 3) and phylogenetic criteria, strains YU438-5^T, YU655-43^T, YU793-41 and YU794-41 are readily distinguishable from the known actinomycetes mentioned above and warrant a new taxon. Strain YU438-5^T is considered to represent a distinct species based on DNA–DNA hybridization, cellular fatty acid proportion and lack of growth at 37 °C. We propose that the isolates be classified in a new genus, *Virgosporangium* gen. nov., with the type species *Virgosporangium ochraceum* sp. nov. for strains YU655-43^T (= IFO 16418^T = JCM 11001^T), YU793-41 (= IFO 16419) and YU794-41 (= IFO 16420), and *Virgosporangium aurantiacum* sp. nov. for strain YU438-5^T (= IFO 16421^T = JCM 11002^T).

The genus *Virgosporangium* should be considered as a member of the family *Micromonosporaceae* Stackebrandt *et al.* 1997 on the basis of the comparative analysis of 16S rRNA sequences described here.

Description of *Virgosporangium* gen. nov.

Virgosporangium (Vir.go.spo.ran'gi.um. Gr. n. *virga* rod; Gr. n. *spora* a seed, spore; Gr. n. *argeion* vessel; M.L. neut. n. *Virgosporangium* an organism with rod-shaped, spore-containing vessels, i.e. sporangia).

Cells are Gram-positive, non-acid-fast, aerobic organisms with branching hyphae. Non-fragmentary substrate mycelia are present. Slender sporangia are formed on short sporangiophores on the substrate mycelium. Each sporangium typically contains a single row of six or more spores. Spores are oval or short rods (0.6–0.9 by 0.8–1.5 µm) and exhibit motility. Strictly aerobic. Good growth occurs between 20 and 30 °C. In general, the vegetative mycelia are yellow to orange. Cell walls contain glutamic acid, glycine, alanine and

3-OH-diaminopimelic acid. Wall chemotype is II according to Lechevalier & Lechevalier (1970), and the peptidoglycan type is presumed to be A1 γ according to Schleifer & Kandler (1972). Mannose, 3-O-methylmannose, rhamnose, glucose, arabinose, xylose and galactose are detected as whole-cell sugars. The major cellular fatty acid is anteiso-C_{17:0}. The major menaquinones are MK-10(H₄) and MK-10(H₆). Phosphatidylethanolamine is present as the diagnostic phospholipid (phospholipid pattern type PII). The acyl type of cell wall polysaccharide is glycolyl. Mycolic acid is not detected. The G + C content of DNA is 71 mol %. Habitat is soil. The type species is *Virgosporangium ochraceum*.

Description of *Virgosporangium ochraceum* sp. nov.

Virgosporangium ochraceum (och.ra'ce.um. M.L. neut. adj. *ochraceum* rust-coloured).

Morphological, chemotaxonomic and general characteristics are as given above for the genus. Brownish soluble pigment is produced on tyrosine agar (ISP medium 7). Gelatin liquefaction is negative. Hydrolyses starch. Does not decompose calcium malate. Coagulation and peptonization of milk are positive. Optimum temperature for growth is 15–30 °C. Grows at 37 °C. Does not grow on 7% NaCl. Glucose, D-fructose, D-xylose, L-arabinose, glycerol and mannose are utilized, but inulin is not. The major cellular fatty acids are iso-C_{15:0}, iso-C_{16:0}, C_{17:0} and C_{18:1} with anteiso-C_{17:0}. The G + C content of DNA is 71 mol %. Habitat is soil. The type strain is YU655-43^T (= IFO 16418^T = JCM 11001^T).

Description of *Virgosporangium aurantiacum* sp. nov.

Virgosporangium aurantiacum (au.ran.ti'ac.um. M.L. neut. adj. *aurantiacum* orange-coloured).

Morphological, chemotaxonomic and general characteristics are as given above for the genus. Brownish soluble pigment is produced on tyrosine agar (ISP medium 7). Gelatin liquefaction is negative. Hydrolyses starch. Does not decompose calcium malate. Coagulation and clearing of milk are positive. Optimum temperature for growth is 15–30 °C. Does not grow at 37 °C. Does not grow on 4% NaCl. Glucose, D-fructose, D-xylose, L-arabinose, glycerol, maltose and mannose are utilized, but inositol, D-sorbitol and inulin are not. The major cellular fatty acids are iso-C_{16:0} with anteiso-C_{17:0}. The G + C content of DNA is 71 mol %. Habitat is soil. The type strain is YU438-5^T (= IFO 16421^T = JCM 11002^T).

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