Description of four new species of the genus *Kineosporia*: *Kineosporia succinea* sp. nov., *Kineosporia rhizophila* sp. nov., *Kineosporia mikuniensis* sp. nov. and *Kineosporia rhamnosa* sp. nov., isolated from plant samples, and amended description of the genus *Kineosporia*

Takuji Kudo, Keisuke Matsushima,† Takashi Itoh, Junko Sasaki and Ken-ichiro Suzuki

Author for correspondence: Takuji Kudo. Tel: +81 48 462 1111. Fax: +81 48 462 4617.
e-mail: kudo@ulmus.riken.go.jp

Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-0198, Japan

Eleven motile spore-bearing actinomycetes were isolated from various plant samples and were studied to determine their taxonomic positions. The isolates showed colony appearance and morphology similar to those of the 'spore-dome actinomycetes' previously described by L. G. Willoughby in 1969. Although the isolates showed variety in composition of isomers of 2,6-diaminopimelic acid in the cell walls and in whole-cell sugar patterns, all of the isolates were classified in the genus *Kineosporia* Pagani and Parenti 1978 emend. Itoh *et al.* 1989 on the basis of their morphological and other chemotaxonomic characteristics, i.e. menaquinone, phospholipid and cellular fatty acid compositions. This assignment to the genus was also supported by a phylogenetic analysis using the 16S rRNA gene sequences. By DNA–DNA hybridization experiment, five genospecies were recognized among the isolates, and one of them showed high levels with the type strain of the type species, *Kineosporia aurantiaca*, which is the sole member of the genus. These genospecies can be distinguished from each other by their cultural, physiological and biochemical characteristics, and the other four genospecies should be placed into four new species for which the names *Kineosporia succinea* (type strain I-273^T = JCM 9957^T), *Kineosporia rhizophila* (type strain I-449^T = JCM 9960^T), *Kineosporia mikuniensis* (type strain I-463^T = JCM 9961^T) and *Kineosporia rhamnosa* (type strain I-132^T = JCM 9954^T) are proposed.

Keywords: *Kineosporia succinea*, *Kineosporia rhizophila*, *Kineosporia mikuniensis*, *Kineosporia rhamnosa*, actinomycetes

INTRODUCTION

The genus *Kineosporia* was first described by Pagani & Parenti (1978) for a single strain of an aerobic actinomycete that lacked aerial mycelia and formed

round-to-pyriiform sporangia, each of which contained a single zoospore. The ultrastructural study of this strain by Barnard & Parenti (1983) supported the light microscopy observation in the original description and showed that the motile spore had a polar tuft of flagella. The genus was also characterized in the original description by the presence of LL-2,6-diaminopimelic acid (LL-A₂pm) and glycine as cell wall components [cell wall type I of Lechevalier & Lechevalier (1970)] and arabinose, xylose and galactose as whole-cell sugars.

Subsequently, Itoh *et al.* (1989) amended this genus from viewpoints of morphology and chemotaxonomy.

† **Present address:** Discovery Research Laboratories II, Shionogi & Co., Ltd, Sagisu, Fukushima-ku, Osaka 553-0002, Japan.

Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S sequences are AB003931 (*K. aurantiaca* JCM 3230^T), AB003932 (*K. succinea* I-273^T), AB003933 (*K. rhizophila* strain I-449^T), AB003934 (*K. rhamnosa* I-113) and AB003935 (*K. rhamnosa* I-132^T).

According to the amended description, the type strain of the type species formed spore clusters on the sporophores and catenated spores around the central projection of the colony as well as single spores covered with a thin sheath that Pagani & Parenti (1978) called 'sporangia'. Cell walls prepared from the shake-flask culture contained not only LL- A_2 pm plus glycine but also a significant amount of meso- A_2 pm, and the spore fraction collected from the culture on an agar plate contained predominantly meso- A_2 pm. The whole-cell sugar components were galactose, glucose, mannose and ribose, but neither arabinose nor xylose, which were reported to be present in the original description (Pagani & Parenti, 1978), were detected. The major menaquinone and diagnostic phospholipid were MK-9(H_4) and phosphatidylcholine [phospholipid pattern PIII of Lechevalier *et al.* (1977)], respectively. The cellular fatty acids were composed of normal, mono-unsaturated, 10-methyl and 2-hydroxy acids, but neither iso- nor anteiso-branched acids were detected. At present, the genus *Kineosporia* is still constituted by a single species *Kineosporia aurantiaca* proposed in the original description of this genus.

Furthermore, Itoh *et al.* (1989) suggested that the morphological and chemical characteristics of the genus *Kineosporia* are similar to those of so-called 'spore-dome actinomycetes' originally described by Willoughby (1969). Cross and co-workers (Makkar & Cross, 1982; Cross & Makkar, 1986; Eke *et al.*, 1989) also isolated actinomycete strains of this group and found their isolates to contain MK-9(H_4) as a major menaquinone and to be heterogeneous in composition of A_2 pm isomers. They also showed heterogeneity of their isolates by a numerical phenetic analysis based on morphological and biochemical properties. However, they did not discuss relationship between the 'spore-dome actinomycetes' and the genus *Kineosporia*, and did not propose any new taxa for their isolates.

During a search of actinomycetes from various plant samples, we isolated 11 strains showing morphological characteristics similar to a 'spore-dome' structure from leaf litter and sphagnum around hydrosphere and roots. In this paper we report taxonomic characterization and classification of these isolates, and propose the following four new species of the genus *Kineosporia*: *Kineosporia succinea*, *Kineosporia rhizophila*, *Kineosporia mikuniensis* and *Kineosporia rhamnosa*.

METHODS

Bacterial strains and cultivation. Eleven strains isolated from plant samples in Japan (Table 1) were investigated. The leaf, root or sphagnum samples were dried at 28 °C for at least 1 week and were ground with a blender after adding sterile water. The suspension was incorporated into an agar medium containing 0.2 g yeast extract (Difco), 50 mg cycloheximide, 50 mg nystatin and 15 g agar in 1000 ml distilled water (pH not adjusted), and this preparation was poured into a Petri dish. After incubation at 28 °C for more than 2 weeks, colonies that appeared were picked up with a sterile needle under a stereomicroscope and transferred to an agar plate of yeast extract-starch agar containing 2 g yeast

extract, 10 g soluble starch and 15 g agar in 1000 ml distilled water (pH 7.3). *K. aurantiaca* JCM 3230^T was also used for the comparative study.

These organisms were routinely cultivated on yeast extract-starch agar at 28 °C for 10 to 14 d and maintained at 8 °C. Biomass for chemotaxonomic analyses and DNA preparation were obtained from cultures grown in yeast extract-glucose broth containing 10 g yeast extract and 10 g D-glucose in 1000 ml distilled water (pH 7.2) on a rotary shaker at 30 °C. Spore fraction used for analysis of A_2 pm isomers was obtained as described previously (Itoh *et al.*, 1989).

Morphology. Cultures were grown on yeast extract-starch agar for 14 d at 28 °C and observed with a light microscope and a Hitachi S-2400 scanning electron microscope. The specimens for scanning electron microscopy were prepared as described previously (Itoh *et al.*, 1989).

Cultural, physiological and biochemical characteristics. Cultural characteristics were tested by using 21-d cultures grown at 28 °C on four selected agar media (Table 2). Colour designations and colour names of colonies were described by comparison with colour chips from the *Color Harmony Manual* (Jacobson *et al.*, 1958). Decomposition of urea was examined on Christensen agar (Difco) supplemented with 2% (w/v) urea, and decomposition of the other compounds was examined on the basal medium of Gordon *et al.* (1974). Utilization of carbohydrates as sole carbon sources was tested by using neutralized yeast nitrogen base without amino acids (Difco) as a basal medium according to the method of Stevenson (1967). Utilization of organic acids was examined on the defined medium of Gordon *et al.* (1974). Tolerance to NaCl was determined by using yeast extract-starch agar supplemented with various concentrations (0–10%, w/v) of NaCl at 28 °C.

Chemotaxonomy. A_2 pm and its isomers (LL and/or meso) in the cell wall peptidoglycan were detected by using TLC as described by Stanek & Roberts (1974). To determine the composition of the A_2 pm isomers, a neutral amino acid fraction was separated from a whole-cell hydrolysate and analysed by using HPLC as follows. A whole-cell hydrolysate was loaded onto a column (5 × 50 mm) packed with Dowex 50W × 8 (200–400 mesh, H⁺ form) and washed with 4 ml H₂O. An amino acid fraction was eluted with 3 M NH₄OH. The amino acid fraction was then loaded onto the second column packed with Dowex 1 × 8 (200–400 mesh, CH₃COO⁻ form) and a neutral and basic amino acid fraction was eluted with H₂O. This fraction was loaded onto the third column packed with Dowex 50W × 8 (200–400 mesh, NH₄⁺ form) and a neutral amino acid fraction was eluted with H₂O. The neutral amino acid fraction was reacted with a chiral reagent [(+)-1-(9-fluorenyl)ethyl chloroformate] and applied to HPLC as described by Einarsson & Josefsson (1987). The acyl group of muramic acid in peptidoglycan was determined by the method of Uchida & Aida (1984). Whole-cell sugars were analysed by the TLC method of Stanek & Roberts (1974) and by the HPLC method of Mikami & Ishida (1983). 3-O-Methylrhamnose was identified by comparison of *R_f* values on cellulose and silica gel TLC using various solvent systems (Asano *et al.*, 1989b) and of retention times on HPLC with a whole-cell hydrolysate of *Catellatospora ferruginea* JCM 7544^T (Asano *et al.*, 1989b, 1990). Phospholipids were extracted and identified by the method of Minnikin *et al.* (1984). Methyl esters of cellular fatty acids were prepared by the direct transmethylation method with methanolic hydrochloride (Suzuki & Komagata, 1983), and were analysed by GLC as described previously (Kudo *et al.*, 1993). Isoprenoid quinones were

Table 1. List of the isolates used in this study and their sources

Strain no.	Source
I-092 (= JCM 9952)	Leaves of cat-tail (<i>Typha latifolia</i>), pond in Saitama, Japan
I-113 (= JCM 9953)	Leaves of cat-tail (<i>Typha latifolia</i>), pond in Saitama, Japan
I-132 ^T (= JCM 9954 ^T)	Leaves of cat-tail (<i>Typha latifolia</i>), pond in Saitama, Japan
I-225 (= JCM 9955)	Fallen leaves, Saitama, Japan
I-229 (= JCM 9956)	Fallen leaves, Saitama, Japan
I-273 ^T (= JCM 9957 ^T)	Fallen leaves, Saitama, Japan
I-279 (= JCM 9958)	Fallen leaves, Saitama, Japan
I-281 (= JCM 9959)	Fallen leaves, Saitama, Japan
I-449 ^T (= JCM 9960 ^T)	Root of galingale (<i>Cyperus microiria</i>), Saitama, Japan
I-463 ^T (= JCM 9961 ^T)	Sphagnum, Mt Mikuni in Gumma, Japan
I-468 (= JCM 9962)	Sphagnum, Mt Mikuni in Gumma, Japan

Table 2. Cultural characteristics of *Kineosporia* strains

Growth of all strains was good on yeast extract-starch agar and oatmeal agar; growth of all strains was poor on inorganic salts-starch agar (ISP medium no. 4). Colonies of all strains were colourless on inorganic salts-starch agar (ISP no. 4). There was no diffusible pigment on yeast-malt agar or inorganic salts-starch agar (ISP no. 4). Colour names and codes were taken from the *Color Harmony Manual* (Jacobson *et al.*, 1958). ISP, International *Streptomyces* Project (Shirling & Gottlieb, 1966).

Medium	<i>K. aurantiaca</i>			<i>K. succinea</i>				<i>K. rhizophila</i>		<i>K. mikuniensis</i>	<i>K. rhamnosa</i>	
	JCM 3230 ^T	I-225	I-279	I-092	I-229	I-273 ^T	I-281	I-449 ^T	I-468	I-463 ^T	I-113	I-132 ^T
Yeast extract-starch agar												
Colour of colony	Tan (3 ie)	Light brown (3 lg)	Light tan (3 gc)	Light amber (3 ic)	Luggage tan (4 ne)	Amber (3 pe)	Light copper brown (5 pg)	Dusty orange (4 lc)	Dusty orange (4 lc)	Light tan (3 gc)	Light amber (3 ic)	Amber (3 lc)
Diffusible pigment	None	None	None	None	Yellow	Yellow	Yellow	Faint red	Faint red	None	None	None
Yeast-malt agar (ISP medium no. 2)												
Colour of colony	Light amber (3 ic)	Light amber (3 ic)	Light amber (3 ic)	Amber (3 lc)	Topaz (3 ne)	Topaz (3 ne)	Topaz (3 ne)	Amber (3 nc)	Amber (3 nc)	Amber (3 pc)	Amber (3 lc)	Amber (3 lc)
Oatmeal agar (ISP medium no. 3)												
Colour of colony	Light tan (3 gc)	Tan (3 ie)	Light amber (3 ic)	Light tan (3 gc)	Camel or tan (3 ie)	Light amber (3 lc)	Amber (3 pe)	Dusty orange (4 lc)	Dusty orange (4 lc)	Light tan (3 gc)	Russet orange (4 pc)	Russet orange (4 nc)
Diffusible pigment	None	None	None	Faint yellow	Faint yellow	Faint yellow	Faint yellow	None	None	Faint yellow	None	None

extracted as described by Collins *et al.* (1984), and analysed by HPLC and a mass spectrometer (Tamaoka *et al.*, 1983; Kudo *et al.*, 1993). Preparation and detection of methyl esters of mycolic acids were carried out as described by Tomiyasu (1982).

Preparation of DNA, DNA base composition and DNA-DNA hybridization. DNA was isolated from biomasses by the method of Saito & Miura (1963) with minor modification as follows: achromopeptidase crude (Wako), *N*-acetylmuramidase SG (Seikagaku Kogyo) and lysozyme were used for lysing cells. In case cells failed to be lysed by these enzymes, the cells were freeze-dried and mechanically ground as described by Raeder & Broda (1985).

The G+C content of DNA was determined by the HPLC method of Tamaoka & Komagata (1984). An equimolar mixture of nucleotides for analysis of DNA base composition (Yamasa Shoyu) was used as the quantitative standard. DNA-DNA relatedness was measured fluorometrically by using the microplate hybridization method described by Ezaki *et al.* (1989).

16S rDNA sequencing and phylogenetic analysis. The 16S rRNA genes of four isolates (strains I-113, I-132^T, I-273^T and

I-449^T) and *K. aurantiaca* JCM 3230^T were sequenced as described previously (Suzuki *et al.*, 1996). These sequences were manually aligned with the selected sequences (Fig. 1) obtained from the Ribosomal Database Project (Maidak *et al.*, 1994). Evolutionary distances were represented by the K_{nuc} values of Kimura (1980), and a phylogenetic tree was constructed by the neighbour-joining method of Saitou & Nei (1987) in the PHYLIP package (Felsenstein, 1995). The topology of the resultant unrooted tree was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

RESULTS

Morphological observation

All of our isolates formed central projections covered with numerous spores, termed 'spore domes' by Willoughby (1969). Single spores borne at the tips of substrate hyphae and spore clusters on a sporophore were also observed at the edges of colonies in some strains as illustrated in the amended description of the genus *Kineosporia* (Itoh *et al.*, 1989). The colonies of

Table 3. Physiological and biochemical characteristics of *Kineosporia* strains

All strains decomposed casein and urea; none decomposed adenine, tyrosine and xanthine. All strains utilized D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, L-rhamnose, starch and D-xylose; none utilized adonitol, i-erythritol, L-sorbose and xylitol. All of the strains utilized fumarate, malate and succinate; none utilized benzoate, mucate, oxalate and tartrate. All strains produced acid from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, L-rhamnose, sucrose and D-xylose; none was produced acid from adonitol and i-erythritol.

Characteristic	<i>K. aurantiaca</i>			<i>K. succinea</i>				<i>K. rhizophila</i>		<i>K. mikuniensis</i>	<i>K. rhamnosa</i>	
	JCM 3230 ^T	I-225	I-279	I-092	I-229	I-273 ^T	I-281	I-449 ^T	I-468	I-463 ^T	I-113	I-132 ^T
Decomposition of:												
DNA	+	+	+	+	+	+	+	+	+	NG	+	+
Aesculin	+	+	+	—	—	—	—	—	—	—	—	—
Hypoxanthine	—	—	—	+	+	+	+	—	—	—	—	—
Testosterone	+	+	+	+	+	+	+	+	+	±	—	±
Utilization of:												
L-Arabinose	+	+	+	+	+	+	+	+	+	+	±	±
D-Arabitol	+	+	+	+	+	+	+	+	+	+	—	—
Dulcitol	—	—	—	+	+	+	+	—	—	±	—	±
myo-Inositol	+	+	+	—	+	+	+	—	—	+	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+	—	—
D-Melezitose	+	+	+	+	+	+	+	+	+	+	—	—
Melibiose	+	+	+	+	+	+	+	+	+	+	—	—
Methyl α-D-glucoside	—	—	—	—	—	—	—	—	—	+	—	—
Raffinose	—	—	—	—	—	—	—	+	+	—	—	±
D-Ribose	+	+	+	+	+	+	+	+	+	+	—	—
Salicin	+	+	+	+	+	+	+	+	+	+	—	—
D-Sorbitol	—	—	—	±	+	+	+	—	—	—	+	+
Sucrose	+	+	+	+	+	+	+	+	+	±	±	±
D-Trehalose	+	+	+	+	+	+	+	+	+	+	—	—
Utilization of:												
Citrate	+	+	+	—	—	—	—	—	—	—	—	—
Acid production from:												
myo-Inositol	—	—	—	—	—	—	—	—	—	+	+	+
D-Melezitose	+	+	+	—	—	—	—	+	+	—	—	—
D-Sorbitol	—	—	—	—	—	—	—	—	—	—	+	+
NaCl tolerance (% w/v)	< 3	< 3	< 3	< 5	< 5	< 5	< 5	< 5	< 5	< 1	< 1	< 2

NG, No growth.

all our isolates released motile spores after being transferred to water.

Cultural, physiological and biochemical characteristics

Cultural characteristics of our isolates and *K. aurantiaca* JCM 3230^T are shown in Table 2. They lacked aerial mycelium on any of the agar media and their colonies were usually orange to light brown with a glossy appearance. Strains I-092, I-229, I-273^T, I-281 and I-463^T produced yellowish diffusible pigments in oatmeal agar, and strains I-449^T and I-468 produced small amounts of reddish diffusible pigments in yeast extract-starch agar. The results of physiological and biochemical tests are shown in Table 3.

Chemotaxonomic characteristics

The TLC analysis of A₂pm obtained from submerged cultures in shake flasks indicated that the strains used in this study were heterogeneous in composition of the isomers as follows: strains I-225, I-229, I-273^T and I-281 as well as *K. aurantiaca* JCM 3230^T contained both LL- and meso-A₂pm; strains I-092 and I-279 contained LL-A₂pm; and strains I-113, I-132^T, I-449^T, I-463^T and I-468 contained meso-A₂pm. The quantitative analysis by HPLC revealed that the strains mainly containing LL and meso isomers also contained meso- and LL-A₂pm as minor components, respectively (Table 4). Spore fractions of all seven strains tested contained meso-A₂pm as a major component (Table 4). This could be observed even in strain I-092 that contained

Table 4. Distribution of isomers of diaminopimelic acid (A₂pm) and composition of whole-cell sugars in *Kineosporia* strains

Gal, Galactose; Glc, glucose; Man, mannose; Ara, arabinose; Xyl, xylose; Rib, ribose; Rha, rhamnose; 3-*O*-M, 3-*O*-methylrhamnose.

Strain	Composition of isomers of A ₂ pm (%) in:				Composition of whole-cell sugar							
	Shake-flask culture		Spore fraction		Gal	Glc	Man	Ara	Xyl	Rib	Rha	3- <i>O</i> -M
	LL	meso	LL	meso								
<i>K. aurantiaca</i>												
JCM 3230 ^T	49	51	32	68	+	+	+	—	—	+	—	—
I-225	45	55	ND	ND	+	+	+	—	—	+	—	—
I-279	86	14	ND	ND	+	+	+	—	—	+	—	—
<i>K. succinea</i>												
I-092	86	14	30	70	+	+	+	—	—	+	—	—
I-229	51	49	ND	ND	+	+	+	—	—	+	—	—
I-273 ^T	42	58	21	79	+	+	+	—	—	+	—	—
I-281	37	63	ND	+	+	+	—	—	+	—	—	—
<i>K. rhizophila</i>												
I-449 ^T	19	81	19	81	+	+	+	—	—	+	—	—
I-468	21	79	ND	ND	+	+	+	—	—	+	—	—
<i>K. mikuniensis</i>												
I-463 ^T	17	83	15	85	+	+	+	—	—	+	—	—
<i>K. rhamnosa</i>												
I-113	17	83	29	71	+	+	+	—	—	+	+	+
I-132 ^T	24	76	25	75	+	+	+	—	—	+	+	+

ND, Not determined.

LL-A₂pm as a major component in a biomass from the submerged culture. As whole-cell sugars (Table 4), galactose, glucose, mannose and ribose were detected in all of our isolates and in *K. aurantiaca* JCM 3230^T. In addition to these sugars, two strains I-113 and I-132^T contained a significant amount of rhamnose and 3-*O*-methylrhamnose.

Table 5 shows compositions of cellular fatty acids and menaquinones of the test strains. The cellular fatty acid profiles of all of our isolates and *K. aurantiaca* JCM 3230^T were mainly composed of n-16:0 and n-18:1. A significant amount of 10-methyloctadecanoic acid was also detected in all of the strains except for strains I-449^T and I-468. The seven strains except I-113, I-132^T, I-449^T, I-463^T and I-468 also had a small amount of 2-hydroxy hexadecanoic acid. Neither isomer anteiso-branched acids were observed in any strains. These fatty acid patterns correspond to fatty acid types 1a (with 10-methyl acid) and 1b (without 10-methyl acid) of Kroppenstedt (1992). All of the strains contained MK-9(H₄) as a predominant isoprenologue, and the retention time in the HPLC analysis suggested that the positions of the hydrogenation were the second and third isoprene units from the 2-methyl-1,4-naphthoquinone moiety [MK-9(II,III-H₄)]. Phosphatidylcholine was detected in all the strains as a diagnostic

phospholipid [phospholipid type PIII of Lechevalier *et al.* (1977)]. Mycolic acids and *N*-glycolated muramic acid were not detected. The DNA G+C composition of our isolates and *K. aurantiaca* JCM 3230^T ranged from 68 to 71 mol% as shown in Table 6.

16S rDNA sequence analysis

The sequences of 1432–1476 nucleotides of the 16S rDNAs were determined for *K. aurantiaca* JCM 3230^T and strains I-113, I-132^T, I-273^T and I-449^T. The sequences of the four isolates showed high similarities with that of *K. aurantiaca* JCM 3230^T and the levels of sequence similarities among these strains ranged from 96.3 to 99.8%. The data set used for construction of the phylogenetic tree contained 1055 nucleotide positions of each sequence as a result of elimination of gaps and ambiguous nucleotides from the sequences between positions 107 and 1423 [*Escherichia coli* position number (Brosius *et al.*, 1978)]. The phylogenetic tree constructed by the neighbour-joining method is shown in Fig. 1. *K. aurantiaca* and our isolates formed a coherent cluster supported by a high bootstrap value (100%) in the order *Actinomycetales* and this cluster was loosely but stably related to the genus *Kineococcus*. The cluster composed of *Kineo-*

TR, Trace amounts ($< 0.5\%$).

Table 6. DNA base composition and relatedness among *Kineosporia* strains

Strain	G + C content (mol%)	Percentage DNA complementarity with labelled DNA from:											
		JCM 3230 ^T	I-225	I-279	I-092	I-229	I-273 ^T	I-281	I-449 ^T	I-468	I-463 ^T	I-113	I-132 ^T
<i>K. aurantiaca</i>													
JCM 3230 ^T	69.5	100	93	93	37	33	34	35	40	41	38	23	19
I-225	69.1	94	100	98	32	30	31	32	37	35	34	21	17
I-279	69.6	94	93	100	28	31	30	30	33	36	33	21	19
<i>K. succinea</i>													
I-092	70.8	26	33	31	100	67	62	62	42	37	37	21	18
I-229	70.5	34	37	34	77	100	101	104	39	40	38	23	22
I-273 ^T	70.7	35	36	37	79	105	100	109	42	43	37	24	24
I-281	70.3	20	29	27	62	92	89	100	38	31	33	19	17
<i>K. rhizophila</i>													
I-449 ^T	70.0	23	32	30	35	33	34	34	100	99	36	19	20
I-468	70.2	30	30	28	36	31	31	33	90	100	38	20	18
<i>K. mikuniensis</i>													
I-463 ^T	69.4	36	35	34	39	33	32	36	40	42	100	21	17
<i>K. rhammosa</i>													
I-113	68.7	12	20	16	22	20	19	18	22	19	20	100	92
I-132 ^T	68.3	12	17	14	20	19	19	18	21	19	19	87	100

sporia aurantiaca, our isolates and *Kineococcus aurantiacus* was not affiliated in any suborder proposed by Stackebrandt *et al.* (1997).

DNA-DNA hybridization

The DNA-DNA hybridization experiments revealed that five genospecies were recognized among the 11 isolates (Table 6). One of them composed of strains I-225 and I-279 showed high levels of DNA relatedness (93–94%) with *K. aurantiaca* JCM 3230^T, while members of the other four genospecies were 41% or less related to *K. aurantiaca* JCM 3230^T. Levels of DNA relatedness among strains within the same genospecies were more than 62%, and levels among the five genospecies ranged from 12 to 42%.

DISCUSSION

According to the amended description of the genus *Kineosporia* (Itoh *et al.*, 1989), this genus has the following morphological characteristics: colonies lack aerial mycelia and form central (dome) projections having a conical crateriform or cerebriform shape; numerous zoospores are released from the surfaces of colonies immersed in water; the central projections seem to show hyphae radiating toward the surfaces; catenated spores are formed around the central projections; single spores are attached to the ends of vegetative hyphae at the edges of colonies; and some colonies have small dome projections that bear a number of spore clusters with sporophores, which are bunched together in the agar. These characteristics of sporulation correspond to those of spore domes and spore heads described by Willoughby (1969) and are consistent with those of our isolates.

The chemotaxonomic characteristics of our isolates, with the exception of the ratio of isomers of A₂pm in the cell wall and whole-cell sugar patterns, are almost homogeneous and coincide with those of the genus *Kineosporia*. The menaquinone composition of our isolates [MK-9(H₄)] also agreed with the result of Cross & Makkar (1986) on their spore dome isolates. Strains I-449^T and I-468 do not have 10-methyloctadecanoic acid in their fatty acid composition, while most strains including *K. aurantiaca* JCM 3230^T have a significant amount of this acid. However, such a difference is not substantial for classification because 10-methylation of fatty acids secondarily occurs in the corresponding monounsaturated fatty acids, e.g. 10-methyloctadecanoic acid is derived from octadecenoic acid) (Suzuki & Komagata, 1983).

Diamino acids in cell walls have historically been one of the most important criteria for classification of actinomycetes and related bacteria at the genus level. However, the strains used in this study showed variety in the ratio of A₂pm isomers and can be classified into LL-plus-*meso* type (strains I-225, I-229, I-273^T and I-281 and JCM 3230^T), LL type (strains I-092 and I-279) and *meso* type (strains I-113, I-132^T, I-449^T, I-463^T and I-468). Cross & Makkar (1986) and Eke *et al.* (1989) also reported heterogeneity of their spore-dome isolates that contained LL- or *meso*-A₂pm or both of the two isomers. Eke *et al.* (1989) compared the isomers derived from shake-flask cultures and fractionated spores and substrate mycelia to explain this phenomenon. They indicated that the spore fraction had cell walls that contained *meso*-A₂pm and the substrate mycelium fraction contained the LL isomer. From this result they concluded that the proportions of the two isomers detected in shake-flask cultures varies with

incubation time and the strain used. In our results of the A_2pm analyses, there were three patterns in composition of the isomers. Two patterns of them, i.e. the LL-plus-*meso* type and the LL type, can be explained by the interpretation of Eke *et al.* (1989), and this is supported by our result that a large amount of the *meso* isomer was detected in spore fraction of strain I-092 predominantly containing LL- A_2pm in its shake-flask culture. Therefore, the amount of *meso*- A_2pm in shake-flask cultures of the LL-plus-*meso* type and the LL type depends on the degree of sporulation and is influenced by culture condition or ability to sporulate, and there was no substantial difference between the two types. This is also supported by the result of our DNA-DNA hybridization experiment (Table 6). For example, strain I-279 of the LL type showed high similarity with *K. aurantiaca* JCM 3230^T and strain I-225 of the LL-plus-*meso* type. On the other hand, it is difficult to apply the interpretation mentioned above to strains of the *meso* type because proportion of spores to mycelia in the liquid medium cannot be so high as indicated by the amount of *meso*- A_2pm . In fact, microscopic observation indicated that mycelia were much more dominant in the liquid cultures of the strains of the *meso* type. Therefore, it is proper to consider that a large amount of the *meso* isomer exists in the substrate mycelia of the strains of the *meso* type. The result of our DNA-DNA hybridization experiment (Table 6) shows that the five strains of the *meso* type are clearly separated from strains of the LL-plus-*meso* and LL types.

K. aurantiaca JCM 3230^T and our isolates contained galactose, glucose, mannose and ribose as whole-cell sugars in common, which are not considered to be diagnostic for actinomycete taxonomy. On the other hand, strains I-113 and I-132^T, which had *meso*- A_2pm as a major component of the cell walls, contained rhamnose and 3-*O*-methylrhamnose in the whole cells. Rhamnose is characteristic of the genera *Saccharothrix* (Labeda *et al.*, 1984; Grund & Kroppenstedt, 1989) and *Kutzneria* (Stackebrandt *et al.*, 1994), and 3-*O*-methylrhamnose was detected in cell walls of some species of the genus *Catellatospora* (Asano *et al.*, 1989a, b). The genus *Catellatospora* (Asano & Kawamoto, 1986; Asano *et al.*, 1989a) can be divided into two groups, i.e. members of one group contain menaquinones with nine isoprene units (*Catellatospora citrea* and *Catellatospora tsunoense*) and members of the other group contain menaquinones with 10 isoprene units (*Catellatospora ferruginea* and *Catellatospora matsumotoense*). 3-*O*-Methylrhamnose was detected in members of only the two species of the latter group. The recent phylogenetic study (Koch *et al.*, 1996) suggested the possibility that these two groups are separated at the genus level, and this means that 3-*O*-methylrhamnose may be used as a criterion at the genus level.

To evaluate differences in compositions of the A_2pm isomers and in presence or absence of rhamnose and 3-*O*-methylrhamnose, a phylogenetic analysis of our

isolates and *K. aurantiaca* JCM 3230^T based on 16S rDNA sequences was performed by using the following strains: strains JCM 3230^T and I-273^T were used as representatives of the LL-plus-*meso* type, strains I-113, I-132^T and I-449^T were representatives of the *meso* type, and strains I-113 and I-132^T were also representatives of the rhamnose- and 3-*O*-methylrhamnose-containing group. The sequences among these strains showed high similarity ranging from 96.3 to 99.8%. Fig. 1 shows that strains of the *meso* type and the rhamnose- and 3-*O*-methylrhamnose-containing strains form a coherent cluster with *K. aurantiaca* JCM 3230^T and strain I-273^T, supported by a high bootstrap value (100%). On the basis of morphology and chemotaxonomy mainly based on the lipid profiles, and the phylogenetic analysis, we conclude that all of our isolates should be classified in the genus *Kineosporia*.

Itoh *et al.* (1989) described in their emendation of the genus *Kineosporia* that LL- and *meso*- A_2pm were localized in the mycelia and in the spores, respectively, and did not refer to the presence of strains containing rhamnose and 3-*O*-methylrhamnose as whole-cell sugars. Therefore, further emendation of the genus *Kineosporia* as proposed below is necessary for placing the strains of the *meso* type and the rhamnose- and 3-*O*-methylrhamnose-containing strains into the genus *Kineosporia*.

The 16S rDNA analysis in this study also suggested the phylogenetic position of the genus *Kineosporia*. Stackebrandt *et al.* (1997) classified almost all the genera constituting the order *Actinomycetales* under the 10 suborders and 30 families based on the phylogenetic analyses of the 16S rRNA/rDNA sequence data, but the six genera, including *Kineosporia* and *Kineococcus*, were excluded from the classification system proposed by them owing to uncertainty of the phylogenetic positions. Fig. 1 indicates that the genera *Kineosporia* and *Kineococcus* form a cluster with a high bootstrap value (98%) and do not belong to any suborder previously described. Furthermore, the evolutionary distance between the two genera seems to be distant enough to separate them at the family level. Valid descriptions of a new suborder and new families for these genera will require confirming the phylogenetic position by using some algorithms other than the neighbour-joining method and searching for signature nucleotides that distinguish the *Kineosporia*-*Kineococcus* cluster and between these two genera.

Classification at the species level was based on results of the DNA-DNA hybridization experiment and cultural, physiological and biochemical characterization. As shown in Table 6, our isolates and *Kineosporia aurantiaca* JCM 3230^T were classified into five genospecies. Since the first one contains strain JCM 3230^T, strains I-225 and I-279 should be identified as *K. aurantiaca*. The second including four strains consists of the rest of LL-plus-*meso* and LL types. The third to fifth are constituted by strains of the *meso* type, and two strains of the fifth one are the rhamnose

and 3-*O*-methylrhamnose-containing strains. These five genospecies can be clearly distinguished by their phenotypic characteristics as shown in Tables 2 and 3, and this means that each genospecies should be considered an independent species. Therefore, we propose four new species of the genus *Kineosporia*, *Kineosporia succinea* sp. nov., *Kineosporia rhizophila* sp. nov., *Kineosporia mikuniensis* sp. nov. and *Kineosporia rhamnosa* sp. nov., for the second to fifth genospecies, respectively.

Amended description of the genus *Kineosporia* Pagani and Parenti 1978

The amended description below is based on the previous amended description (Itoh *et al.*, 1989) except for compositions of A₂pm isomers and whole-cell sugars.

Aerobic, Gram-positive and non-acid-fast. Colonies on agar medium lack aerial mycelia, form central projections with radiating vegetative hyphae, and are occasionally accompanied by bunches of spore clusters in the agar. Mature colonies have a gelatinous matrix which confers a glossy appearance. Spores, which are spherical to ovoid or pyriform with a long axis of 1–2 µm, are catenated around the central projection or are located singly or in aggregates at the tips of hyphae. The spores are motile with polar tufts of flagella. The peptidoglycan contains almost equivalent LL- and meso-A₂pm, or mainly LL- or meso-A₂pm. The spores contain predominantly meso-A₂pm accompanied by glycine. The whole-cultured organism contains galactose, glucose, mannose and ribose. In addition to these sugars, rhamnose and 3-*O*-methylrhamnose may be present. Phosphatidylcholine is a diagnostic phospholipid, and MK-9(II,III-H₄) is present as a major component. The cellular fatty acid profile is composed mainly of n-hexadecanoic acid and n-octadecanoic acid, and lacks iso- and anteiso-branched fatty acids. 10-Methyl octadecanoic acid and 2-hydroxy hexadecanoic acids are also present in some strains. Mycolic acids are absent. The G + C content of the DNA ranges from 69 to 71 mol %. The type species is *K. aurantiaca*.

Description of *Kineosporia succinea* sp. nov.

Kineosporia succinea (suc.ci.nea. L. fem. adj. *succinea* amber-coloured).

Substrate mycelia are orange to light brown (especially on yeast extract-starch agar). Produces faint yellowish diffusible pigments on oatmeal agar. Decomposes casein, DNA, hypoxanthine, testosterone and urea, but not adenine, aesculin, tyrosine and xanthine. Utilizes L-arabinose, D-arabitol, D-cellobiose, dulcitol, D-fructose, D-galactose, D-glucose, D-lactose, maltose, D-mannitol, D-melezitose, melibiose, L-rhamnose, D-ribose, salicin, starch, sucrose, D-trehalose and D-xylose as a sole carbon source, but not adonitol, i-erythritol, methyl α-D-glucoside, raffinose, L-sorbose

and xylitol. Utilizes fumaric acid, L-malic acid and succinic acid, but not benzoic acid, citric acid, mucic acid, oxalic acid and L-tartaric acid. Produces acids from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, L-rhamnose, sucrose and D-xylose, but not adonitol, i-erythritol, *myo*-inositol, D-melezitose and D-sorbitol. Tolerates up to 4% (w/v) NaCl. Mesophilic. The G + C content of the DNA is 70–71 mol %. Contains almost the same amount of LL- and meso-diaminopimelic acid or a major amount of LL-diaminopimelic acid in the cell wall. Contains neither rhamnose nor 3-*O*-methylrhamnose in the whole-cell hydrolysate. The type strain is strain I-273^T (= JCM 9957^T).

Description of *Kineosporia rhizophila* sp. nov.

Kineosporia rhizophila (rhi.zo.phi'la. Gr. n. *rhizoma* root; Gr. adj. *philus* loving; M.L. fem. adj. *rhizophila* root-loving).

Substrate mycelia are orange to light brown (especially on yeast extract-starch agar). Produces faint reddish diffusible pigments on yeast extract-starch agar. Decomposes casein, DNA, testosterone and urea, but not adenine, aesculin, hypoxanthine, tyrosine and xanthine. Utilizes L-arabinose, D-arabitol, D-cellobiose, D-fructose, D-galactose, D-glucose, D-lactose, maltose, D-mannitol, D-melezitose, melibiose, raffinose, L-rhamnose, D-ribose, salicin, starch, sucrose, D-trehalose and D-xylose as a sole carbon source, but not adonitol, dulcitol, i-erythritol, *myo*-inositol, methyl α-D-glucoside, D-sorbitol, L-sorbose and xylitol. Utilizes fumaric acid, L-malic acid and succinic acid, but not benzoic acid, citric acid, mucic acid, oxalic acid and L-tartaric acid. Produces acids from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, D-melezitose, L-rhamnose, sucrose and D-xylose, but not adonitol, i-erythritol, *myo*-inositol and D-sorbitol. Tolerates up to 4% (w/v) NaCl. Mesophilic. The G + C content of the DNA is 70 mol %. Contains a major amount of meso-diaminopimelic acid in the cell wall. Contains neither rhamnose nor 3-*O*-methylrhamnose in the whole-cell hydrolysate. The type strain is strain I-449^T (= JCM 9960^T).

Description of *Kineosporia mikuniensis* sp. nov.

Kineosporia mikuniensis (mi.ku.ni.en'sis. M.L. fem. adj. *mikuniensis* derivative of Mt Mikuni, Gumma Prefecture, Japan, the location from which the type strain was isolated).

Substrate mycelia are orange to light brown (especially on yeast extract-starch agar). Produces faint yellowish diffusible pigments in oatmeal agar. Decomposes casein and urea, but not aesculin, hypoxanthine, tyrosine and xanthine. Utilizes L-arabinose, D-arabitol, D-cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, D-lactose, maltose, D-mannitol, D-melezitose, melibiose, methyl α-D-glucoside, L-rhamnose, D-ribose, salicin, starch, D-trehalose and D-xylose as a

sole carbon source, but not adonitol, i-erythritol, raffinose, D-sorbitol, L-sorbose and xylitol. Utilizes fumaric acid, L-malic acid and succinic acid, but not benzoic acid, citric acid, mucic acid, oxalic acid and L-tartaric acid. Produces acids from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, L-rhamnose, sucrose and D-xylose, but not adonitol, i-erythritol, D-melezitose and D-sorbitol. Does not grow in the presence of 1% (w/v) NaCl. Mesophilic. The G+C content of the DNA is 69 mol%. Contains a major amount of *meso*-diaminopimelic acid in the cell wall. Contains neither rhamnose nor 3-*O*-methylrhamnose in the whole-cell hydrolysate. The type strain is strain I-463^T (= JCM 9961^T). Monotypic.

Description of *Kineosporia rhamnosa* sp. nov.

Kineosporia rhamnosa (rham.no'sa. M.L. fem. adj. *rhamnosa* pertaining to rhamnose).

Substrate mycelia are orange to light brown (especially on yeast extract-starch agar). Does not produce any diffusible pigments in yeast extract-starch and oatmeal agar media. Decomposes casein, DNA and urea, but not aesculin, hypoxanthine, tyrosine and xanthine. Utilizes D-cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, maltose, D-mannitol, L-rhamnose, D-sorbitol, starch and D-xylose as a sole carbon source, but not adonitol, D-arabitol, i-erythritol, D-lactose, D-melezitose, melibiose, methyl α -D-glucoside, D-ribose, salicin, L-sorbose, D-trehalose and xylitol. Utilizes fumaric acid, L-malic acid and succinic acid, but not benzoic acid, citric acid, mucic acid, oxalic acid and L-tartaric acid. Produces acids from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, L-rhamnose, D-sorbitol, sucrose and D-xylose, but not adonitol, i-erythritol and D-melezitose. Does not grow in the presence of 2% (w/v) NaCl. Mesophilic. The G+C content of the DNA is 68–69 mol%. Contains a major amount of *meso*-diaminopimelic acid in the cell wall. Contains rhamnose and 3-*O*-methylrhamnose in the whole-cell hydrolysate. The type strain is strain I-132^T (= JCM 9954^T).

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