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Sporichthya brevicatena sp. nov.

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A strain was isolated from soil that formed short chains of aerial mycelium dividing into motile spores on an agar medium, but not substrate mycelium. The organism had menaquinone 9(H_8), LL-diaminopimelic acid in the cell wall amino acids and a DNA G+C content of 71 mol%. On the basis of the morphological and chemotaxonomic characteristics and phylogenetic analysis, this strain was classified into the genus *Sporichthya*. DNA–DNA hybridization revealed that the strain differed from the known species, *Sporichthya polymorpha*. Therefore, a new species, *Sporichthya brevicatena* sp. nov., is proposed. The type strain is *S. brevicatena* YU720-21^T (= IFO 16195^T).

Keywords: suborder *Frankineae*, *Sporichthya brevicatena* sp. nov.

INTRODUCTION

The genus *Sporichthya* established by Lechevalier *et al.* (1968) has LL-diaminopimelic acid in its peptidoglycan and forms short spore chains. Although the appearance of *Sporichthya* clearly distinguishes it from the genus *Streptomyces*, it is classified among related organisms of the genus *Streptomyces* in *Bergey's Manual of Systematic Bacteriology* (Locci, 1989) because its cell wall is of type I. Rainey *et al.* (1993) revealed that *Sporichthya polymorpha* represents a novel line of descent within the order *Actinomycetales* by phylogenetic analysis based on 16S rDNA sequences. Stackebrandt *et al.* (1997) placed the genus *Sporichthya* in the family *Sporichthyaceae* of the suborder *Frankineae*. The families belonged to the suborder *Frankineae*, which contains the genera *Frankia* (Lechevalier & Lechevalier, 1989a), *Sporichthya* (Lechevalier *et al.*, 1968), *Geodermatophilus* (Luedemann, 1968), *Blastococcus* (Ahrens & Moll, 1970), *Acidothermus* (Mohagheghi *et al.*, 1986) and *Microsphaera* (Yoshimi *et al.*, 1996). Recently, *Cryptosporangium* (Tamura *et al.*, 1998) was proposed as a new genus belonging to the family *Sporichthyaceae*.

During studies on rare actinomycetes, we isolated a strain from soil, YU720-21^T, which produced short spore chains. The isolate contained LL-diaminopimelic acid in the peptidoglycan. Its morphological and chemotaxonomic characteristics, phylogenetic analysis and DNA–DNA hybridization data indicated that the isolate belonged to the genus *Sporichthya* but was taxonomically distinct from *S. polymorpha*.

In this paper, we describe the characterization and classification of the isolate and propose a new species of the genus *Sporichthya* for strain YU720-21^T, *Sporichthya brevicatena* sp. nov.

METHODS

Micro-organisms and culture conditions. Strain YU720-21^T was isolated from soil samples collected in Yamanashi prefecture, Japan, on humic acid/vitamin (HV) agar (Hayakawa & Nonomura, 1987) by a capillary method (Hayakawa *et al.*, 1992) improved to include vanillin as a chemoattractant. *S. polymorpha* IFO 12702^T was used for comparison with the isolate. To obtain freeze-dried cells for chemotaxonomic analyses, the culture was grown on yeast extract/starch agar (containing 2 g yeast extract, 10 g soluble starch and 18 g agar in 1000 ml distilled water, pH 7·0) at 28 °C for 5–7 d.

Morphological and physiological characteristics. The culture grown on HV agar and yeast extract/starch agar for 3–14 d at 28 °C was observed with a light microscope and 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 cells through freeze-drying and sputter-coating the preparation 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 the media tested were recorded after 5 d incubation at 28 °C according to the International

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Abbreviations: ISP, International *Streptomyces* Project; NJ method, neighbour-joining method.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AB0006164.

Streptomyces Project (ISP) (Shirling & Gottlieb, 1966). Colours are described in common terminology.

ISP medium 9 was used as basal medium for utilization of sugars. Inorganic salts/starch agar (ISP medium 4) was used for determination of starch hydrolysis. Growth temperature was determined on yeast extract/starch agar. Calcium malate medium (calcium malate 1%, NH_4Cl 0·05%, K_2HPO_4 0·05%, agar 1·8%, pH 7·0) was used for determining solubility of calcium malate. ISP medium 8 was used for nitrate determination. Gelatin medium (gelatin 20%, pH 7·0) and glucose/peptone/gelatin medium (glucose 2%, peptone 0·5%, gelatin 20%, pH 7·0) were used for determination of gelatin liquefaction. Skim milk (10%; Difco) was used for determination of milk peptonization.

Analyses of chemotaxonomic characteristics. The analyses of whole-cell sugars, cellular fatty acids, isoprenoid quinones and DNA base composition were performed as described previously (Tamura *et al.*, 1994). The isomers of diaminopimelic acid were determined by TLC (Hasegawa *et al.*, 1983).

Preparation of DNA. Total DNA was prepared according to the method of Saito & Miura (1963) with modifications. Cells were harvested from 5 ml yeast extract/starch agar. After washing three times with 2 ml 50 mM EDTA (pH 7·5), cells were suspended in 500 µl 5 mM EDTA (pH 8·0). The cell suspension was transferred to a 1·5 ml Eppendorf microtube and 50 µl lytic solution [5 mg achromopeptidase ml^{-1} and 7·5 mg lysozyme ml^{-1} (both from Wako Pure Chemical Industries) in 10 mM Tris/HCl, pH 8·0] was added for spheroplasting and the mixture was incubated for 2 h at 37 °C. Spheroplasts were lysed gently by adding 50 µl 20% SDS and incubating for 10 min at 65 °C. Phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by phenol extraction was carried out and crude DNA was obtained by 2-propanol precipitation. The precipitate was rinsed with 70% ethanol, dried and dissolved in 0·2 ml 1 × TE (1 mM EDTA, 10 mM Tris/HCl, pH 8·0). After addition of 150 µl ice-cold ethanol, the precipitate was harvested by centrifugation (12000 r.p.m., 5 min) and resuspended completely in 0·3 ml 10 × TE. After RNase A and RNase T_1 (both from Sigma) and proteinase K (Wako Pure Chemical Industries) treatments, phenol/chloroform/isoamyl alcohol (25:24:1) extraction followed by phenol extraction was carried out and DNA was obtained by 2-propanol precipitation. The precipitate was rinsed with 70% ethanol, dried and dissolved in 50 µl distilled water. The concentration of DNA solution was measured by using a photometer (Beckman DU-65). DNA samples having an A_{260}/A_{280} ratio of 1·8–1·9 were used in the DNA similarity experiment.

PCR amplification and sequencing of 16S rDNA. 16S rDNA was amplified by PCR (Saiki *et al.*, 1988) using TaKaRa *Taq* (Takara Shuzo) and a pair of primers, 5′-GAGTTTGATCCTGGCTCAG-3′ [identical to positions 9–27 in the *Escherichia coli* numbering system (Brosius *et al.*, 1978)] and 5′-AAGGAGGTGATCCAGCC-3′ (complementary to positions 1541–1525). The conditions for thermal cycling were denaturation of the target DNA at 96 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and primer extension at 72 °C for 2 min. At the end of cycling, the reaction mixture was held at 72 °C for 5 min, then cooled to 4 °C.

The 1·5 kb amplified 16S rDNA was subjected to cycle sequencing with a ThermoSequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) according to the manufacturer's protocol, with the following primers: 5′-GAGTTTGATCCTGGCTCAG-3′ (complementary to positions 9–27); 5′-CTCCTACGGGAGGCAGCAG-3′ (complementary to positions 339–357); 5′-GTATTACCGCGGCTGCTG-3′ (complementary to positions 536–519); 5′-GGATTAGATACCCTGGTAGTC-3′ (complementary to positions 785–805); 5′-CTACCAGGGTATCTAAT-3′ (complementary to positions 803–787); 5′-GCAACGAGCGCAACCC-3′ (complementary to positions 1099–1114); 5′-AGGGTTGCGCTCGTTG-3′ (complementary to positions 1115–1100); 5′-GCGGTGTGTACAAGGCCC-3′ (complementary to positions 1382–1365); and 5′-AAGGAGGTGATCCAGCC-3′ (complementary to positions 1541–1525). The conditions for thermal cycling were denaturation of the target DNA at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 0·5 min, primer annealing at 55 °C for 0·5 min, and primer extension at 72 °C for 1 min. The products were analysed with a Pharmacia *ALF* DNA Sequencer II according to the manufacturer's protocol.

Phylogenetic analysis. 16S rDNA sequences of the strain and reference organisms were aligned against that of *Streptomyces ambofaciens* (Pernodet *et al.*, 1989). The CLUSTAL W v.1.6 software (Thompson *et al.*, 1994) package was used to generate the evolutionary distances [the K_{nuc} value of Kimura (1980)] and the similarity values; the phylogenetic tree was constructed by the neighbour-joining (NJ) method (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.

Measurement of DNA similarity. The microplate-hybridization method developed byEzaki *et al.* (1988, 1989) was applied with minor modifications to determine DNA similarity. A heat-denatured sample DNA (1 µg) was immobilized in a well of a microplate (Immuno plate II; Nunc) at 28 °C for 3 h. The microplate was dried at 60 °C for 3 h. Probe DNA was prepared by labelling with photobiotin acetate (Bresatec) according to the supplier's instructions. Before hybridization with probe DNA, 0·2 ml prehybridization solution [50% deionized formamide, 2 × SSC (1 × SSC is 0·15 M NaCl, 0·015 M trisodium citrate, pH 7·0), 5 × Denhardt's solution (1 × Denhardt's solution is 0·02% bovine serum albumin, 0·02% polyvinylpyrrolidone and 0·02% Ficoll 400) and heat-denatured and sonicated salmon testes DNA (0·1 mg ml^{-1})] was added to the well and the plate was incubated for 30 min at 37 °C. The prehybridization solution was discarded and 0·1 ml hybridization solution (2·5% dextran sulfate and 2 µg heat-denatured probe DNA ml^{-1}) was added to the well. Hybridization was performed at 55 °C for 3 h and then wells were washed four times with 0·3 ml 1 × SSC. After washing, 0·1 ml PBS (8 mM Na_2HPO_4, 1·5 mM KH_2PO_4, 137 mM NaCl, 2·7 mM KCl, pH 7·2) containing 0·5% bovine albumin and 0·1% streptoavidin-β-galactosidase conjugate (Boehringer Mannheim) was added to the well and the plate was kept at 37 °C for 10 min at room temperature. After incubation at 37 °C for 10 min, wells were washed four times with 0·3 ml 1 × SSC. For the β-galactosidase assay, 0·2 ml PBS containing 1 mM $MgCl_2$ and 0·1 mg 4-methylumbelliferyl-β-D-galactopyranoside ml^{-1} (Wako Pure Chemical Industries) was added and plates were incubated for an appropriate time at 37 °C and fluorescence intensity was measured in wells with a microplate reader MTP-32 (Corona Electric) every 15 min. DNA similarity was calculated by using the following equation:

$$\text{DNA similarity } (\%) = 100\,(X - N)/(P - N),$$

where X is fluorescence intensity of unknown DNA, P is fluorescence intensity of DNA used in making the probe (positive reference) and N is fluorescence intensity of calf thymus DNA (negative reference).

RESULTS AND DISCUSSION

Morphological observations

Strain YU720-21^T was observed in 4–5 d cultures on HV agar and yeast extract starch agar (Fig. 1). Substrate mycelium was not observed. The aerial hyphae were maintained upright on the surface of the medium. Most spore chains were short, but some were long (15–20 μm). Ordinary cells were rod-shaped or oval (approximate i.d. 0·5–1·5 μm) and many spores showed motility after incubation at 28 °C for 30 min in distilled water.

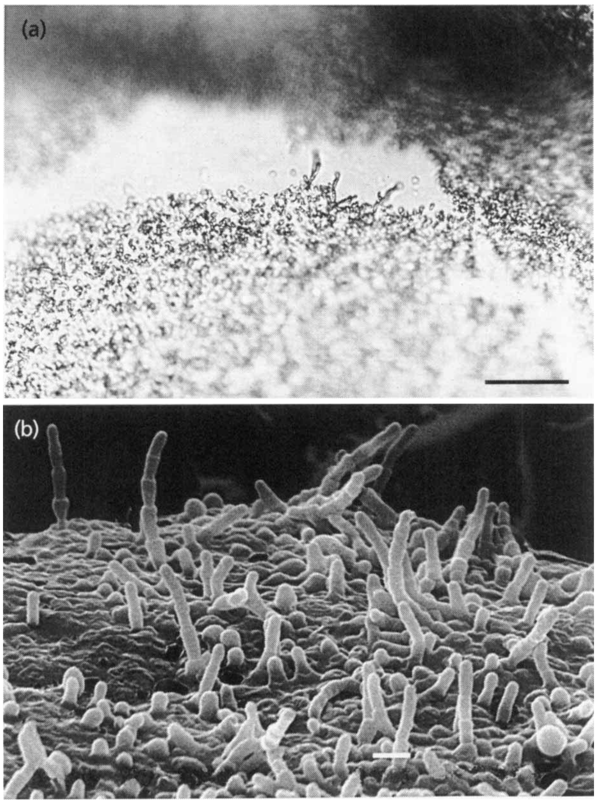

Fig. 1. (a) Light micrograph of isolate YU720-21^T grown on HV agar for 5 d at 28 °C (bar, 20 μm) and (b) scanning electron micrograph of isolate YU720-21^T grown on yeast extract/starch agar for 4 d at 28 °C (bar, 1 μm).

Growth characteristics

Table 1 shows the growth characteristics of the isolate and *S. polymorpha* IFO 12702^T. The isolate formed greyish-white colonies on most agar media. It showed good growth on glycerol/asparagine agar (ISP medium 5) and tyrosine agar (ISP medium 7). Traces of moderate brown soluble pigments were observed when the isolate was cultured over 1 week on tyrosine agar (ISP medium 7).

Physiological characteristics

Table 2 shows the physiological properties of the isolate and *S. polymorpha* IFO 12702^T. Strain YU720-21^T utilized glucose, inositol, raffinose, saccharose, D-xylose, glycerol, lactose, D-galactose and melibiose as a sole carbon source. It was positive for nitrate reduction and negative for peptonization of milk, gelatin liquefaction, hydrolysis of starch and decomposition of calcium malate. It grew at 15–37 °C; good growth occurred at 20–30 °C.

Chemotaxonomic characteristics

Table 3 shows the menaquinone composition, whole-cell sugars and the G+C content of the DNA. The major menaquinone of the isolate was MK-9(H_8); small amounts of MK-8(H_6) and MK-9(H_6) were also present. The cell walls of strain YU720-21^T contained LL-diaminopimelic acid, indicating wall chemotype I according to Lechevalier & Lechevalier (1970). The isolate contained large amounts of glucose and small amounts of mannose and galactose in the whole-cell hydrolysates. The G+C content of DNA was 71

Table 1. Cultural characteristics of isolate YU720-21^T and *S. polymorpha* IFO 12702^T

Medium	YU720-21^T			IFO 12702^T	
	Growth	Colour of colony	Soluble pigment	Growth	Colour of colony
Yeast extract/malt extract agar (ISP medium 2)	Moderate	Greyish-white		Moderate	Greyish-white
Oatmeal agar (ISP medium 3)	Moderate	Yellowish-white		Poor	Colourless
Inorganic salts/starch agar (ISP medium 4)	Moderate	Greyish-white		Moderate	Greyish-white
Glycerol/asparagine agar (ISP medium 5)	Good	Greyish-white		Good	Greyish-white
Peptone/yeast extract iron agar (ISP medium 6)	Poor	Greyish-white		Poor	Colourless
Tyrosine agar (ISP medium 7)	Good	Greyish-white	Moderate brown	Good	Greyish-white

Table 2. Phenotypic characteristics of isolate YU720-21^T and *S. polymorpha* IFO 12702^T

Characteristic	**YU720-21^T**	**IFO 12702^T**
Utilization of:*		
D-Glucose	+	+
Inositol	+	+
Raffinose	+	+
Saccharose	+	±
D-Fructose	–	–
D-Mannitol	±	–
L-Rhamnose	–	+
D-Xylose	+	–
Glycerol	+	–
Lactose	+	+
D-Galactose	+	+
Maltose	±	±
D-Mannose	±	+
Melibiose	+	–
Solubility of calcium malate†	–	–
Starch hydrolysis†	–	–
Nitrite from nitrate†	+	+
Peptonization of milk†	–	–
Gelatin liquefaction†	–	–
Pigmentation in:†		
ISP-6	–	–
ISP-7	Weak (brown)	–
Growth temperature (°C):*		
10	–	–
15	±	–
20	+ +	+
25	+ +	+
30	+ +	+
37	±	+
45	–	–

* Growth: + +, good; +, moderate; ±, poor; –, none.

† +, positive; –, negative.

mol%. Table 4 shows the major cellular fatty acids of the isolate, iso-$C_{16:0}$, $C_{17:1}$, $C_{17:0}$ and 10-methyl $C_{18:0}$. The fatty acid type of the isolate is 3a according to Kroppenstedt (1985).

DNA–DNA hybridization

The level of DNA relatedness between strain YU720-21^T and *S. polymorpha* IFO 12702^T was less than 30% (20–28%). The results of DNA–DNA hybridization studies showed that strain YU720-21^T is a distinct species from *S. polymorpha* IFO 12702^T.

Phylogenetic position

The 16S rDNA sequence of strain YU720-21^T from positions 28–1524 [*Escherichia coli* numbering system (Brosius *et al.*, 1978), total 1490 bases] was determined.

Table 3. Chemotaxonomic characteristics of isolate YU720-21^T

Characteristic	**YU720-21^T***
Menaquinone composition:	
MK-8(H_6)	23·4
MK-9(H_6)	13·6
MK-9(H_8)	62·9
Sugar composition of whole cells:	
Glucosamine	13·2
Ribose	ND
Mannose	6·0
Arabinose	ND
Galactose	16·2
Xylose	ND
Glucose	46·8
G+C content of DNA	71·0

ND, Not detected.

* Values given as percentages except DNA G + C content which is given in mol%.

Table 4. Cellular fatty acid compositions of isolate YU720-21^T and *S. polymorpha*

Fatty acid	**YU720-21^T**	***S. polymorpha****
Iso-branched fatty acids		
$C_{16:0}$	24·4	8·9
$C_{18:0}$	Trace	7·5
Saturated fatty acids		
$C_{14:0}$		4·5
$C_{15:0}$	2·8	6·1
$C_{16:0}$	6·6	24·4
$C_{17:0}$	17·1	
$C_{18:0}$	1·8	
Unsaturated fatty acids		
$C_{16:1}$	2·5	9·9
$C_{17:1}$	18·5	20·9
$C_{18:1}$	2·5	5·3
10-methyl fatty acids		
$C_{18:0}$	17·5	
$C_{19:0}$	3·4	
Unidentified fatty acids	3·0	

* Data from Rainey *et al.* (1993).

For the reference organisms, the positions at which secondary structures varied between strains and at which sequences were not determined were excluded from the phylogenetic analysis, in which a total of 1112 nt were compared after elimination of these sites. Fig. 2 depicts the phylogenetic tree obtained by applying the NJ method to K_{nuc} values.

Strain YU720-21^T formed a monophyletic cluster with *S. polymorpha*. The fidelity of this cluster was supported by bootstrap analysis at a confidence level

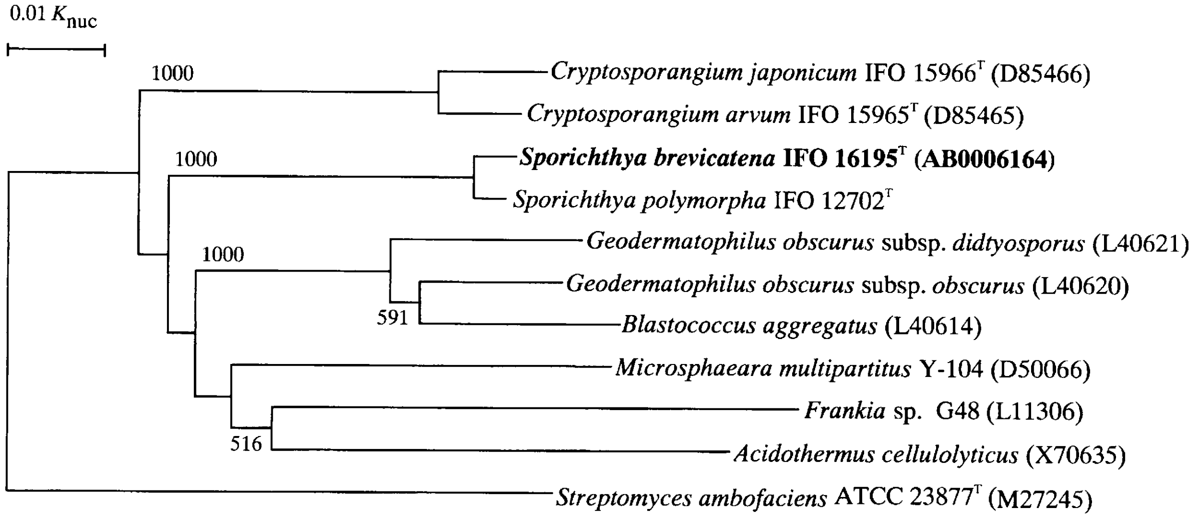

Fig. 2. Phylogenetic tree derived from 16S rDNA sequences of the suborder *Frankineae*. *Streptomyces ambofaciens* ATCC 23877^T (M27245) was used as the root organism. Bar, 0·01 K_{nuc} in nucleotide sequences. The numbers on the branches are confidence limits estimated from a bootstrap analysis with 1000 replicates (only values above 50 % are indicated).

Table 5. Differential characteristics of *Sporichthya* species

Characteristic	*S. brevicatena*	*S. polymorpha*
Colour of colonies on ISP-3	Yellowish-white	Colourless
Utilization of:*		
D-Mannitol	±	–
L-Rhamnose	–	+
D-Xylose	+	–
Glycerol	+	–
Melibiose	+	–
Major menaquinone	MK-9(H_8)	MK-9(H_6)†
Major cellular fatty acid	iso-$C_{16:0}$	$C_{16:0}$, $C_{17:1}$†

* + +, Good; +, moderate; ±, poor; –, none.

† Data from Rainey *et al.* (1993).

of 100 %. The level of 16S rDNA sequence similarity between strain YU720-21^T and *S. polymorpha* was 99·0 %.

The isolate had short aerial mycelium dividing into motile spores on the surface of solid media but no substrate mycelium, LL-diaminopimelic acid in the cell wall, and MK-9(H_8), 9(H_6), 8(H_6). Morphological features and chemotaxonomic characteristics of the isolate were similar to those of *S. polymorpha*. Furthermore, the phylogenetic tree based on 16S rDNA sequences indicated that the isolate forms a monophyletic cluster with *S. polymorpha* and could be considered as a member of the genus *Sporichthya*. In the genus *Sporichthya*, only one species was previously known, *S. polymorpha*, five strains of which have been isolated worldwide so far (Lechevalier & Lechevalier, 1989b). The chemotaxonomic characteristics were studied by Rainey *et al.* (1993). *S. polymorpha* has MK-9(H_6) and MK-9(H_8) (in a quantitative ratio of 1·5:1) as major isoprenoid quinone and $C_{16:0}$ and $C_{17:1}$. The isolate has MK-9(H_8), MK-8(H_6) and MK-9(H_6) (in a quantitative ratio of 4·6:1·7:1), and iso-$C_{16:0}$, $C_{17:0}$, $C_{17:1}$ and 10-methyl $C_{18:0}$. Therefore, on the basis of physiological and chemotaxonomic characteristics, as well as DNA–DNA hybridization, the isolate was assigned to a new species designated *Sporichthya brevicatena* sp. nov. This strain has been deposited in the Institute for Fermentation, Osaka as strain IFO 16195^T. Table 5 summarizes the differential characteristics of the new species and *S. polymorpha*.

Recently, Suzuki *et al.* (1997) reported that many *Sporichthya* species were isolated from samples collected worldwide using gellan gum instead of agar (Suzuki *et al.*, 1997). This suggests that *Sporichthya* may be commonly distributed. Further study may reveal more diversity in the genus *Sporichthya*.

Description of *Sporichthya brevicatena* sp. nov.

Sporichthya brevicatena (bre.vi.cat.e′na. L. adj. *brevis* short; L. fem. n. *catena* chain; M.L. fem. n. *brevicatena* short chain).

Gram-positive, non-acid-fast and strictly aerobic organism with short spore chains. Substrate mycelia are absent. Spores are oval or short rods (diameter 1·3–2·0 μm) and exhibit motility when suspended in sterile distilled water. The organism grows well on ISP 5 and ISP 7. In general, the spore chains are greyish-white. The temperature range for growth is 15–37 °C; good growth occurs at 20–30 °C. Reduces nitrate to nitrite. Gelatin liquefaction is negative. Does not hydrolyse starch. Does not decompose calcium malate.

Peptonization of milk is negative. Glucose, inositol, raffinose, saccharose, D-xylose, glycerol, lactose, D-galactose and melibiose are utilized as a sole carbon source. Cell walls contain LL-diaminopimelic acid. Glucose, galactose and mannose are the characteristic whole-cell sugars. Iso-$C_{16:0}$, $C_{17:0}$, $C_{17:1}$ and 10-methyl $C_{18:0}$ are the major cellular fatty acids. The DNA G+C content of the type strain is 71·0 mol%. Major menaquinones are MK-9(H_8), MK-8(H_6) and MK-9(H_6). Habitat is soil. Type strain is YU720-21^T (=IFO 16195^T).

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