Longispora fulva sp. nov., isolated from a forest soil, and emended description of the genus *Longispora*

Hatsumi Shiratori-Takano, Koji Yamada, Teruhiko Beppu and Kenji Ueda

Correspondence

Hatsumi Shiratori-Takano
siratori@brs.nihon-u.ac.jp

Life Science Research Center, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa 252-0880, Japan

A novel actinomycete, strain KZ0017^T, was isolated from a forest soil collected in Ohnuma, Fukushima, Japan. Strain KZ0017^T formed spore chains borne on top of short sporophores arising from vegetative hyphae. Spores were non-motile and cylindrical with smooth surfaces. Strain KZ0017^T contained meso-diaminopimelic (A₂pm) acid, 3-OH A₂pm, D-glutamic acid, glycine and L-alanine in the cell-wall peptidoglycan, and xylose, mannose, galactose, rhamnose and ribose in cell-wall hydrolysates. The acyl type of the cell-wall polysaccharides was glycolyl. The predominant menaquinones were MK-10(H₄) and MK-10(H₆); MK-10(H₈) was a minor component. The polar lipids contained diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol and several unknown lipids and glycolipids. The major fatty acids were iso-C_{16:0}, 10-methyl-C_{17:0} and iso-C_{17:1ω9c}. The DNA G + C content was 70.7 mol%. The 16S rRNA gene sequence of the isolate formed a monophyletic cluster with the single member of the genus *Longispora* in the family *Micromonosporaceae*. On the basis of morphological, chemotaxonomic and phylogenetic properties, strain KZ0017^T represents a novel species of the genus *Longispora*, for which the name *Longispora fulva* sp. nov. is proposed; the type strain is KZ0017^T (=NBRC 105670^T=DSM 45356^T).

The genus *Longispora* of the family *Micromonosporaceae* was proposed recently by Matsumoto *et al.* (2003) based on the identification of *Longispora albida* K97-0003^T, the producer of an anti-HIV substance (Chiba *et al.*, 2001, 2004). This genus is characterized by the presence of meso-diaminopimelic acid (A₂pm), glycine and alanine in the cell wall, and arabinose, galactose and xylose in whole-cell hydrolysates. *Longispora albida* can be distinguished from members of other genera in the family *Micromonosporaceae* by its respiratory quinones [major, MK-10(H₄) and MK-10(H₆); minor, MK-10(H₈)] and phospholipids (type II). The organism shows better growth on gellan gum media than on agar media and forms characteristic spore chains consisting of cylindrical smooth spores on the top of short sporophores branching from vegetative hyphae. Phylogeny based on 16S rRNA gene sequences clearly showed that *Longispora* is a novel genus within the family *Micromonosporaceae*. However, it currently contains only a single species, *L. albida*.

In our recent study on the diversity of high-CO₂-dependent bacteria (Ueda *et al.*, 2008), a filamentous bacterium

(KZ0017^T) was isolated from soil collected at a group of old zelkova trees (*Zelkova serrata*). Strain KZ0017^T produced a yellow ochre pigment and an antibiotic that inhibited growth of *Bacillus subtilis* when it was cultured under a high (5 %) CO₂ atmosphere. This report deals with the taxonomic characteristics of this isolate. Overall, the data indicate that this isolate represents a novel species of the genus *Longispora*.

The soil from which strain KZ0017^T was isolated was collected in a forest in Ohnuma, Fukushima Prefecture, Japan. For the isolation, 1.0 g soil sample was suspended in 20 ml distilled water and, after serial dilution, plated on peptone-meat extract (PM) gellan gum medium [per litre distilled water: 0.1 g peptone (Wako), 0.1 g meat extract (Kyokuto), 0.05 g NaCl, 1.5 g MgSO₄ · 7H₂O, 15.0 g gellan gum (Wako), pH 7.5; chemicals were purchased from Kokusan unless indicated otherwise]. Plates were incubated at 28 °C for 2 weeks under a normal atmosphere or under a 5 % CO₂ atmosphere using a CO₂ incubator (model 5400; Napco). Despite the fact that strain KZ0017^T produced an antibiotic only under high CO₂ conditions, it grew as well under normal air as under the high CO₂ air. Hence, strain KZ0017^T was grown aerobically under a normal atmosphere for 4–35 days at 28 °C on yeast extract-malt extract agar [International *Streptomyces* Project (ISP) 2 medium; Shirling & Gottlieb, 1966], unless noted otherwise, to characterize its physiological properties. For storage, cells

Abbreviations: A₂pm, diaminopimelic acid; ISP, International *Streptomyces* Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Longispora fulva* KZ0017^T is AB489859.

A supplementary table and figures are available with the online version of this paper.

were maintained by using a Microbank (Pro-Lab Diagnostics) or as glycerol (10 %, v/v)/trehalose (0.5 %, w/v) suspensions at -80°C . *L. albida* K97-0003^T (=NBRC 100759^T) was used as a reference strain.

Cell morphology was observed under a VE-8800 scanning electron microscope (Keyence) using cells grown on ISP 2 medium and inorganic salts-starch agar (ISP 4 medium) for 1–5 weeks at 28°C . For scanning electron microscopy, cells were fixed with 4.0 % (v/v) osmium tetroxide vapour. ISP media were prepared as described by Shirling & Gottlieb (1966). Cultural and physiological characteristics were determined as described previously (Gordon *et al.* 1974; Hamada, 2001; Shirling & Gottlieb, 1966). Growth of the isolate was assessed at various temperatures (5, 10, 12, 15, 20, 25, 28, 30, 33, 35, 37 and 40°C) and salinities [0, 0.4, 0.8, 1.0, 1.2, 1.4, 1.5, 2.0, 4.0, 7.0, 10 and 13 % (w/v) NaCl] after cultivation for 5–10 days on ISP 2 medium (NaCl plates were incubated at 28°C). The pH range for growth was assessed in ISP 2 broth at 28°C at 0.5 pH unit intervals between pH 5.0 and 12.0 using 100 mM citrate phosphate (pH 5.0–5.5), sodium phosphate (pH 6.0–8.5), sodium carbonate (pH 9.0–10.0) and sodium sesquicarbonate/NaOH (pH 10.5–12.0) buffers. Colours of the cultures were determined according to the *Munsell Book of Color* (Munsell Color Co. Inc., 1976) and *A Mycological Colour Chart* (Rayner, 1970). The Gram reaction was performed following the method described by Bartholomew & Mittwer (1952). Acid-fastness was determined by using carbol-fuchsin solution for cell staining, acid alcohol treatment and counter-staining with methylene blue (Ziehl–Neelsen staining method).

Strain KZ0017^T exhibited good growth on ISP 2 medium, ISP 4 medium and glucose-peptone agar; moderate growth on oatmeal agar (ISP 3 medium), glycerol-asparagine agar (ISP 5 medium), glucose-asparagine agar, nutrient agar, water-proline agar (1 % proline, tap water) and sucrose-nitrate agar; poor growth on tyrosine agar (ISP 7 medium), glucose-nitrate agar, 1/10 V8 agar and 1/10 V8-calcium chloride agar (Matsumoto *et al.*, 2003); and almost no growth on HV agar (Hayakawa & Nonomura, 1989) or peptone-yeast extract-iron agar (ISP 6 medium). The colour of the colonies was light greyish olive (Munsell 5Y 6/8) on ISP 2, light greenish yellow (Munsell 7.5Y 9/4) on ISP 4, yellowish white (Munsell 5Y 9/3) on glucose-asparagine agar, olive-ochre (Munsell 5Y 6/6) on glucose-peptone agar, pale yellow (Munsell 2.5Y 9/2) on ISP 3, ISP 5, nutrient agar, water-proline agar and sucrose-nitrate agar, and white on ISP 7, glucose-nitrate agar, 1/10 V8 agar and 1/10 V8-calcium chloride agar. Colonies on ISP 2 medium were solid and wrinkled with a wavy surface.

Cells of strain KZ0017^T were Gram-positive, non-acid-fast, aerobic and non-motile. Strain KZ0017^T formed spore chains on ISP 2 and ISP 4 media, glycerol-asparagine agar and sucrose-nitrate agar, but not on the other media described above. Short sporophores branched from the substrate mycelium. After 3 weeks cultivation on ISP 2

medium, straight chains of spores were formed on the top of sporophores (see Supplementary Fig. S1a, available in IJSEM Online). Spores were cylindrical ($0.25\text{--}0.35 \times 0.5\text{--}0.75\text{ }\mu\text{m}$) with a smooth surface. During the first 2–3 weeks of cultivation at 28°C , the morphology of strain KZ0017^T on ISP 4 medium was the same as that observed on ISP 2 medium in terms of the structure of mycelia and spore chains. However, after 4 weeks growth, a bundle-like structure of aerial hyphae was occasionally observed on ISP 4 (Supplementary Fig. S1b), but not on ISP 2 medium. Motile spores and sporangia were not observed.

Growth of strain KZ0017^T was observed at $10\text{--}33^{\circ}\text{C}$ (optimum, $28\text{--}30^{\circ}\text{C}$), at pH 6.0–8.5 (optimum, pH 6.5–7.5) and in 0–1.2 % (w/v) NaCl. Strain KZ0017^T utilized (+)-D-glucose, inositol and xylose and grew weakly on sucrose, but did not utilize (+)-L-arabinose, fructose, (+)-D-mannitol, melibiose, (+)-raffinose or rhamnose. Melanoid pigment and H_2S were not produced. Nitrate was not reduced. Hydrolysis of casein and starch was positive, but that of gelatin, cellulose, xylan and chitin was negative. Differences in physiological and biochemical characteristics between strain KZ0017^T and *L. albida* K97-0003^T are summarized in Table 1.

Biochemical characterizations and determination of the DNA G+C content of strain KZ0017^T were carried out using cells cultured in ISP 2 broth on a rocking shaker at 28°C for 4–5 days. DNA was extracted by using phenol/chloroform extraction followed by polyethylene glycol precipitation, based on the method described by Hopwood *et al.* (1985) with some modifications as follows: crude achromopeptidase (Wako), *N*-acetylmuramidase SG (Seikagaku Kogyo) and lysozyme were used for lysing cells. The DNA G+C content of strain KZ0017^T, determined by HPLC (Tamaoka & Komagata, 1984), was 70.7 mol% [mean of three measurements ($\text{SD}=0.1$)]. Polar lipid extraction and identification were performed according to Komagata & Suzuki (1987). Sugar- and choline-containing lipids were detected by using *p*-anisaldehyde reagent and Dragendorff reagent, respectively. The spots were identified by using a phospholipid kit (Funakoshi) as a standard. Menaquinones were determined by using the procedures of Nishijima *et al.* (1997). Fatty acid methyl esters were extracted and analysed according to the standard protocol of the Sherlock Microbial Identification System (version 4.5; MIDI) with the TSBA40 library. The cell wall was purified following the method of Kawamoto *et al.* (1981) and amino acids in the peptidoglycan were determined by TLC (Harper & Davis, 1979). Whole-cell sugar patterns were analysed according to the method of Becker *et al.* (1965). Isomers of A_2pm and the acyl type of the peptidoglycan were determined by the methods of Hasegawa *et al.* (1983) and Uchida & Aida (1977), respectively. The presence of mycolic acids was examined as described by Tomiyasu (1982).

The major cellular fatty acids of strain KZ0017^T were iso- $\text{C}_{16:0}$, 10-methyl- $\text{C}_{17:0}$ and iso- $\text{C}_{17:1}\omega 9\text{c}$ (see Supplementary Table S1, available in IJSEM Online), which accounted for

Table 1. Diagnostic characteristics that differentiate strain KZ0017^T from *Longispora albida* K97-0003^T

Taxa: 1, strain KZ0017^T (data from this study); 2, *Longispora albida* K97-0003^T [data from Matsumoto *et al.* (2003), except where marked]. +, Positive; –, negative.

Characteristic	1	2
Temperature range for growth (°C)	10–33	12–37
Growth at pH 9.0	–	+
Growth in 1.5 % (w/v) NaCl	–	+
Growth/colour on:*		
ISP 2	Good/light greyish olive	Good/yellowish white
ISP 4	Good/light greenish yellow	–
ISP 6	–	Moderate/pale yellow
Glucose-asparagine agar	Moderate/yellowish white	Moderate/white
Glucose-peptone agar	Good/olive–ochre	Poor/pale yellow
Sucrose-nitrate agar	Moderate/pale yellow	–
Utilization of carbon sources:		
Inositol	+	–
Xylose	+	–
DNA G + C content (mol%)	70.7	70

*Data for both strains from this study.

68.8 % of the total fatty acids of cells of strain KZ0017^T grown in ISP 2. A similar fatty acid profile was determined for *L. albida* K97-0003^T in this study (Supplementary Table S1, available in IJSEM Online). These profiles corresponded to fatty acid type 3b according to Kroppenstedt (1985). Strain KZ0017^T could be differentiated from *L. albida* K97-0003^T by the absence of the saturated fatty acids C_{15:0}, C_{17:0} and C_{19:0} and the unsaturated fatty acid iso-C_{18:1} H (Supplementary Table S1). Strain KZ0017^T contained diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine, phosphatidylinositol, three unknown lipids (L1, L2 and L3) and unknown glycolipids (GLs). Phosphatidylcholine and phospholipids containing glucosamine were not detected (Supplementary Fig. S2a, available in IJSEM Online). The polar lipid pattern of strain KZ0017^T was similar to that of *L. albida* K97-0003^T (Supplementary Fig. S2b, available in IJSEM Online), but the predominant polar lipids of strain KZ0017^T differed from those of *L. albida* K97-0003^T in terms of the higher proportion of diphosphatidylglycerol and hydroxyphosphatidylethanolamine.

The cell wall of strain KZ0017^T contained meso-A₂pm, 3-OH A₂pm, D-glutamic acid, glycine and L-alanine. The peptidoglycan type according to Schleifer & Kandler (1972) was A1γ. Whole-cell sugars of KZ0017^T contained xylose, mannose, galactose, rhamnose and ribose, but not arabinose. The acyl type of the cell-wall polysaccharides was glycolyl. Mycolic acids were absent. The major menaquinones were MK-10(H₄) and MK-10(H₆); MK-10(H₈) was detected as a minor component. Most features with regard to the cell-wall components and quinone profile supported the affiliation of strain KZ0017^T to the genus *Longispora* (Matsumoto *et al.*, 2003). However, the whole-cell sugar pattern of strain KZ0017^T differed from

that of *L. albida* K97-0003^T in terms of the absence of arabinose and the presence of rhamnose, and the presence of mannose and ribose as minor components.

16S rRNA genes were amplified by PCR with a bacterial domain-specific primer set, 27F/1492R (Wang *et al.*, 2007). PCR was performed on a T1 Thermocycler (Biometra) with Ex Taq polymerase (Takara-shuzo). The PCR protocol included: an initial denaturation period of 4 min at 94 °C; 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; 72 °C for 3 min; and incubation at 4 °C until further processing. The nearly complete 16S rRNA gene sequence of strain KZ0017^T (1417 bp) was determined by a direct method using a BigDye terminator v3.1 cycle sequencing kit on an ABI 3130 Genetic Analyzer (Applied Biosystems). Analysis of sequence data was performed by using the MEGA version 3.1 software package (Kumar *et al.*, 2004), after multiple alignment of the sequences by CLUSTAL W (Thompson *et al.*, 1994) and SeaView (Galtier *et al.*, 1996). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. Bootstrap analysis (1000 replications) was carried out to evaluate the topology of the resulting tree (Felsenstein, 1985). An evolutionary distance matrix for the neighbour-joining method was generated according to the Kimura two-parameter model (Kimura, 1983).

The 16S rRNA gene sequence of strain KZ0017^T was compared with those from GenBank/EMBL/DDBJ using the program BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequence similarity of strain KZ0017^T to the type strain of *L. albida* was 96.4 % indicating that strain KZ0017^T represents a novel species in the genus *Longispora* (Stackebrandt &

Goebel, 1994). The neighbour-joining tree based on 16S rRNA gene sequences, which was also supported by the maximum-parsimony method, showed that strain KZ0017^T fell in the cluster of the family *Micromonosporaceae* and formed a monophyletic cluster with *L. albida* K97-0003^T (Fig. 1).

Strain KZ0017^T differed from *L. albida* K97-0003^T, its closest relative, in terms of growth characteristics and colony colour on several ISP media, temperature and pH ranges for growth, substrate utilization patterns and DNA G + C content (Table 1). In addition, strain KZ0017^T was sensitive to 1.5 % (w/v) NaCl, whereas *L. albida* K97-0003^T was not. Furthermore, fatty acid and polar lipid profiling clearly differentiated strain KZ0017^T from *L. albida*

K97-0003^T (see Supplementary Table S1 and Supplementary Fig. S2, available in IJSEM Online).

On the basis of the data presented, it is proposed that strain KZ0017^T represents a novel species of the genus *Longispora*, for which the name *Longispora fulva* sp. nov. is proposed.

Description of *Longispora fulva* sp. nov.

Longispora fulva (ful'va. L. fem. adj. *fulva* tawny, yellowish brown, the colour of the colonies).

Cells are Gram-positive, non-acid-fast, aerobic and non-motile. Straight chains of spores are formed on the top of short sporophores branching from the substrate mycelium. Spores are cylindrical (0.25–0.35 × 0.5–0.75 µm) and

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain KZ0017^T and the type strains of related genera in the family *Micromonosporaceae*. Numbers at nodes are bootstrap values (expressed as percentages of 1000 resampled datasets; only values >70 % are shown). Branching points supported by the maximum-parsimony method are indicated by closed circles. Bar, 0.01 substitutions per nucleotide position.

exhibit a smooth surface. Grows at 10–33 °C and pH 6.0–8.5; optimum growth is at 28–30 °C and pH 6.5–7.5. The salinity (NaCl) range for growth is 0–1.2 % (w/v); optimum growth is in 0–0.4 % (w/v) NaCl. No growth is seen in the presence of 1.5 % (w/v) NaCl. Utilizes (+)-D-glucose, inositol, xylose and sucrose as sole carbon sources. Does not grow on (+)-L-arabinose, fructose, (+)-D-mannitol, melibiose, (+)-raffinose or rhamnose. Melanoid pigment is not produced. Negative for H₂S production and nitrate reduction. Positive for hydrolysis of casein and starch, but negative for hydrolysis of gelatin, cellulose, xylan and chitin. Mycolic acids are absent. The cell wall contains *meso*-A₂pm, 3-OH A₂pm, D-glutamic acid, glycine and L-alanine. Xylose, rhamnose and galactose are detected as the major whole-cell sugars; minor components are mannose and ribose. The acyl type of the cell-wall muramic acid is glycolyl. The major isoprenoid quinones are MK-10(H₄) and MK-10(H₆); MK-10(H₈) is a minor component. The major fatty acids are iso-C_{16:0}, 10-methyl-C_{17:0} and iso-C_{17:1ω9c}. The predominant polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, hydroxyphosphatidylethanolamine and phosphatidylinositol; several unknown lipids and glycolipids are detected as minor components.

The type strain is KZ0017^T (=NBRC 105670^T=DSM 45356^T), isolated from soil collected at a group of old zelkova trees in Ohnuma, Fukushima, Japan. The genomic G+C content of the type strain is 70.7 mol%.

Emended description of the genus *Longispora* Matsumoto *et al.* 2003

The description is as given by Matsumoto *et al.* (2003) with the following amendments. Galactose and xylose are the major sugars in whole-cell hydrolysates; arabinose, rhamnose, mannose and ribose may be detected. The diagnostic phospholipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol (phospholipid type II). In addition, hydroxyphosphatidylethanolamine and several unknown lipids and glycolipids may be present. The major cellular fatty acids are iso-C_{16:0}, 10-methyl-C_{17:0} and iso-C_{17:1ω9c} (fatty acid type 3b).

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