Actinocatenispora sera sp. nov., isolated by long-term culturing

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Two novel actinomycete strains, KV-744^T and KV-856, were isolated by long-term cultivation. Aerial long-chain spores were produced directly from vegetative mycelia and possessed no motility. Vegetative mycelia developed very well and exhibited fragmentation. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid, glycine, alanine and glutamic acid, and whole-cell hydrolysates contained arabinose, galactose and xylose. The acyl type of the peptidoglycan was glycolyl. The predominant menaquinone was MK-9(H₄) and mycolic acids were not detected. The diagnostic phospholipid was phosphatidylethanolamine. The predominant cellular fatty acids were 14-methylhexadecanoic (ai-C_{17:0}), 14-methylpentadecanoic (i-C_{16:0}), 15-methylhexadecanoic (i-C_{17:0}) and 13-methyltetradecanoic (i-C_{15:0}) acids. The G + C content of the DNA was 72–73 mol%. The phenotypic and chemical properties indicated that the two isolates belong to the family *Micromonosporaceae* and the 16S rRNA gene sequence analysis suggested that the closest relationship was with *Actinocatenispora thailandica*. The DNA–DNA hybridization values between strain KV-744^T or KV-856 and *A. thailandica* TT2-10^T were 42–53 %. Based on the data above, strains KV-744^T and KV-856 should be classified as representing a novel species of the genus *Actinocatenispora*, for which the name *Actinocatenispora sera* sp. nov. is proposed. The type strain is KV-744^T (=NRRL B-24477^T=NBRC 101916^T).

Members of the order *Actinomycetales* offer the possibility of discovering new bioactive compounds. Many new bioactive metabolites have been found from actinomycete strains that were isolated from soil. It is our view that an efficient way of finding new bioactive metabolites is by the discovery of novel micro-organisms, and we have focused on three techniques. The first is isolation of bacteria using agar medium supplemented with superoxide dismutase and catalase. Using this method, we have succeeded in increasing the number of bacterial colonies on isolation plates (Takahashi *et al.*, 2003). The second is isolation of bacterial strains from soil aggregates. The environment for bacteria is known to differ inside and outside soil aggregates (Vargas & Hattori, 1991). We have isolated bacterial strains separately from the inside and outside of soil aggregates and have obtained many isolates (Matsumoto *et al.*, 2006). The third technique is isolation

of slow-growing bacteria by long-term cultivation. Janssen *et al.* (2002) reported that incubation of isolation plates for at least 10 weeks resulted in an increased colony number. We conducted long-term incubation of isolation plates in order to isolate slow-growing bacteria. As a result, strains KV-744^T and KV-856 were isolated using the latter two techniques.

The genus *Actinocatenispora*, members of which produce aerial hyphae bearing spore chains, was proposed by Thawai *et al.* (2006) as a member of the family *Micromonosporaceae*. At present, the only recognized species of the genus *Actinocatenispora* is *Actinocatenispora thailandica*.

Strain KV-744^T was isolated from inside soil aggregates collected in Niigata Prefecture, Japan, following the method of Matsumoto *et al.* (2006) after 2 months of cultivation. Strain KV-856 was isolated after 3 months of cultivation by using the general dilution method from soil collected in Tokyo, Japan.

Morphological characteristics were observed using scanning electron microscopy (model JSM-5600; JEOL) following incubation on glycerol-asparagine agar (ISP 5)

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KV-744^T and KV-856 are AB263096 and AB297661, respectively.

Scanning electron micrographs showing aerial and vegetative mycelia of strain KV-744^T are available as a supplementary figure with the online version of this paper.

Fig. 1. Phylogenetic tree, derived from 16S rRNA gene sequences, constructed using the neighbour-joining method and K_{nuc} values. Only bootstrap values above 50% (percentages of 1000 replications) are shown. Solid circles indicate that the corresponding nodes were also recovered in the maximum-likelihood tree. The tree was unrooted and *Patulibacter minatonensis* was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

for 3 weeks at 27 °C and fixation with 4% osmium tetroxide vapour. Cultural and physiological characteristics were observed after incubation for 3 weeks at 27 °C. The ability of the test strain to grow on a range of sole carbon sources at 1% (w/v) was determined using carbon utilization media (Pridham & Gottlieb, 1948) (Nihon Pharmaceutical Co., Ltd). NaCl tolerance and pH and temperature ranges for growth were determined using 1/5-strength nutrient agar. Isomers of diaminopimelic acid in whole-cell hydrolysates were determined using TLC following standard methods (Becker *et al.*, 1965; Hasegawa *et al.*, 1983), and *N*-acyl types of muramic acid were determined using the method of Uchida & Aida (1977). Purified cell wall was obtained using the method of Kawamoto *et al.* (1981), and the amino acid composition of hydrolysed cell walls was determined using cellulose TLC with *n*-butanol/acetic acid/H₂O (4:1:2). Whole-cell sugars were analysed according to Becker *et al.* (1965), the presence of mycolic acids was examined by TLC following Tomiyasu (1982) and phospholipids were extracted and identified according to the method of Minnikin *et al.* (1977). Menaquinones were extracted and purified using the method of Collins *et al.* (1977) and were then analysed by HPLC (model 802-SC; Jasco) on a chromatograph equipped with a CAPCELL PAK C18 column (Shiseido) (Tamaoka *et al.*, 1983). Analysis of fatty acids was performed according to the procedures of the Sherlock Microbial Identification System (Microbial ID). Chromosomal DNA was isolated as described by Saito & Miura (1963), with some modifications. The DNA base composition was estimated by using the HPLC method of Tamaoka & Komagata (1984). DNA–DNA hybridization experiments were performed as described by Ezaki *et al.* (1989). DNA for analysis of 16S rRNA gene sequences was prepared by using the method of Yu *et al.* (2002). The 16S rRNA gene was amplified by PCR using previously described methods (Takahashi *et al.*, 2002) and was sequenced directly on an ABI model 3130 automatic DNA sequencer using a BigDye terminator cycle sequencing kit (Applied

Biosystems). The CLUSTAL W software package (Thompson *et al.*, 1994) was used for multiple alignments with selected sequences for calculating evolutionary distances (Kimura, 1980), and similarity values and a phylogenetic tree were constructed based on the neighbour-joining method (Saitou & Nei, 1987). Data were resampled with 1000 bootstrap replications (Felsenstein, 1985). For the construction of a phylogenetic tree using the maximum-likelihood method (Felsenstein, 1981), the PHYLIP software package was used. Sequence similarity values were determined by visual comparison and manual calculation.

Whole-cell hydrolysates of strains KV-744^T and KV-856 contained arabinose, galactose and xylose. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid, glycine, alanine and glutamic acid. The acyl type of the peptidoglycan was glycolyl. The predominant menaquinone was MK-9(H₄). Mycolic acids were not detected. The phospholipid detected was phosphatidylethanolamine, but phosphatidylcholine, phosphatidylglycerol and an unidentified phospholipid containing glucosamine were absent, which corresponds to phospholipid pattern II *sensu* Lechevalier *et al.* (1977). The above data suggest that the strains are members of the family *Micromonosporaceae*. The DNA G + C contents were 72 and 73 mol%, respectively. Almost complete 16S rRNA gene sequences were determined for strains KV-744^T and KV-856 and were compared with 16S rRNA gene database sequences of the genera of the family *Micromonosporaceae* (Fig. 1). Strains KV-744^T and KV-856 were most closely related to *A. thailandica* TT2-10^T, the type species of the genus *Actinocatenispora*, and the 16S rRNA gene sequence similarity values were 99.2 and 99.3%, respectively. Based on the phylogenetic analysis and the chemotaxonomic data, it is clear that strains KV-744^T and KV-856 belong to the genus *Actinocatenispora*.

Strains KV-744^T and KV-856 were isolated from plates after culture for 2 or 3 months. However, once they started to grow, growth was fast. Good growth in 3 days of

Table 1. Differential characteristics of strains KV-744^T and KV-856 and *A. thailandica* TT2-10^T

All are positive for utilization of D-glucose, adonitol, L-rhamnose, xylitol and D-xylose as carbon sources. All are negative for utilization of D-fructose, glycerol, maltose, D-mannitol, melibiose, D-ribose and sucrose as carbon sources. All are positive for coagulation and peptonization of milk. All are negative for production of melanoid pigment, hydrolysis of starch and liquefaction of gelatin.

Characteristic	Strain KV-744 ^T	Strain KV-856	<i>A. thailandica</i> TT2-10 ^T
Utilization of:			
L-Arabinose	—	—	+
D-Cellobiose	—	—	+
myo-Inositol	—	—	+
D-Mannose	—	—	+
Raffinose	—	—	+
Trehalose	—	—	+

cultivation could be achieved after successive inoculations in liquid media. Both strains grew well on yeast extract-malt extract agar and peptone-yeast extract-iron agar, but aerial spores did not appear after incubation for 3 weeks at 27 °C. Aerial spores were produced directly from vegetative mycelia that grew slightly on glycerol-asparagine agar, tyrosine agar, sucrose-nitrate agar and glycerol-calcium malate agar. The spores were cylindrical, 0.5–0.6 × 0.8–1.0 µm and had a smooth surface, and spore chains had more than 20 spores (see Supplementary Fig. S1a, b, available in IJSEM Online). Vegetative mycelia developed well and fragmentation occurred (Supplementary Fig. S1c, d) after 21 days of cultivation. Flagellated spores were not observed.

The physiological differences between strains KV-744^T and KV-856 and *A. thailandica* TT2-10^T are shown in Table 1. The dominant cellular fatty acids of the two strains and *A. thailandica* TT2-10^T were 14-methylhexadecanoic (ai-C_{17:0}), 14-methylpentadecanoic (i-C_{16:0}), 15-methylhexadecanoic (i-C_{17:0}) and 13-methyltetradecanoic (i-C_{15:0}) acids (Table 2).

At present, the genus *Actinocatenispora* comprises only one species, *A. thailandica*. The DNA–DNA hybridization values were 97–100 % between strains KV-744^T and KV-856, 45–47 % between strain KV-744^T and *A. thailandica* TT2-10^T and 42–53 % between strain KV-856 and *A. thailandica* TT2-10^T. The DNA–DNA hybridization values are below the 70 % cut-off value, although the strains exhibited a high level of sequence similarity with *A. thailandica* TT2-10^T. Therefore, we propose that strains KV-744^T and KV-856 represent a novel species, *Actinocatenispora sera* sp. nov.

Description of *Actinocatenispora sera* sp. nov.

Actinocatenispora sera (se'ra. L. fem. adj. *sera* late).

Spores are cylindrical (0.5–0.6 × 0.8–1.0 µm) and have a smooth surface. Temperature range for growth and the

Table 2. Cellular fatty acid compositions (%) of strains KV-744^T and KV-856 and *A. thailandica* TT2-10^T

Data for *A. thailandica* TT2-10^T are taken from Thawai *et al.* (2006). Fatty acids that represented less than 1 % in all strains are omitted. —, Not detected.

Fatty acid	Strain KV-744 ^T	Strain KV-856	<i>A. thailandica</i> TT2-10 ^T
Saturated			
C _{14:0} 2-OH	2.1	2.6	1.6
10-Methyl C _{16:0}	1.7	—	—
C _{16:0}	0.4	1.1	0.3
Unsaturated			
C _{18:1} ω7c	—	—	1.8
C _{18:3} ω6c	—	—	1.2
Branched			
i-C _{14:0}	1.6	—	2.6
i-C _{14:0} 3-OH	9.2	11.5	3.9
i-C _{15:0}	14.2	14.5	20.2
ai-C _{15:0}	4.1	4.4	6.0
i-C _{16:0}	21.6	15.3	26.2
i-C _{16:1}	1.4	2.7	0.9
i-C _{17:0}	15.5	11.9	11.3
ai-C _{17:0}	24.6	23.7	19.3
ai-C _{17:1}	1.3	3.3	0.6

optimum range are 13–37 and 18–25 °C, respectively. Growth occurs at pH 6–9. Melanoid pigments are not produced. Negative for the liquefaction of gelatin and hydrolysis of starch and positive for the reduction of nitrate and coagulation and peptonization of milk. Adonitol, D-glucose, L-rhamnose, xylitol and D-xylose are utilized, but L-arabinose, D-cellobiose, D-fructose, glycerol, myo-inositol, maltose, D-mannose, D-mannitol, melibiose, raffinose, D-ribose, trehalose and sucrose are not. Cellulose is not decomposed. Does not grow in the presence of 5 % NaCl. Predominant cellular fatty acids are ai-C_{17:0}, i-C_{16:0}, i-C_{17:0} and i-C_{15:0}. The G + C content of the DNA of the type strain is 72 mol%.

The type strain, KV-744^T (=NRRL B-24477^T=NBRC 101916^T), was isolated from soil in Niigata Prefecture, Japan.

Acknowledgements

This study was supported in part by a Grant of the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) and a JSPS Grant-in-Aid for Science Research foundation.

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