Herbidospora yilanensis sp. nov. and *Herbidospora daliensis* sp. nov., from sediment

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Two actinomycete strains, 0351M-12^T and 0385M-1^T, were isolated from sediment samples collected from Yilan County and Dali City in Taiwan, respectively. The two isolates displayed characteristics which were similar to those of the genus *Herbidospora*. They both produced branched and unfragmented substrate mycelia, and aerial hyphae were not produced on Bennett's, glucose-asparagine, Hickey-Tresner, sucrose-nitrate, yeast extract-malt extract, or inorganic salts-starch agars. Short spore-chains were borne on the tips of sporophores arising from the substrate mycelia on oatmeal and oatmeal-nitrate agars. The spore chains contained 10–20 non-motile, smooth-surfaced, oval spores. Galactose, glucose, mannose and madurose were the whole-cell sugars in both strains and *meso*-diaminopimelic acid was present in their peptidoglycans. The phospholipid pattern was PIV. The predominant menaquinone was MK-10(H₄) in both strains. Mycolic acids were not detected. Major cellular fatty acids were iso-C_{16:0} and 10-methyl C_{17:0}. The DNA G + C contents of the two new isolates were 70.6 mol% (0351M-12^T) and 70.7 mol% (0385M-1^T), respectively. The relatedness between the isolates and *Herbidospora cretacea* NBRC 15474^T was 40.7–48.5%. The value of DNA–DNA hybridization between strain 0351M-12^T and strain 0385M-1^T was 53.6–54.9%. These two isolates could also be distinguished from each other and from *H. cretacea* NBRC 15474^T by differences in several phenotypic characteristics. We therefore propose the names *Herbidospora yilanensis* sp. nov. and *Herbidospora daliensis* sp. nov. for these novel bacteria, with strains 0351M-12^T (=FIRDI 003^T =BCRC 16875^T =LMG 24337^T) and 0385M-1^T (=FIRDI 004^T =BCRC 16876^T =LMG 24336^T) as the type strains, respectively.

The genus *Herbidospora* was described by Kudo *et al.* (1993) and contains only one species, *Herbidospora cretacea*, at the time of writing. *H. cretacea* produces branching substrate mycelia, but no distinct aerial hyphae. Short chains of spores (10–30 spores per chain) are borne on the tips of sporophores arising directly from the agar surface. The isomer of diaminopimelic acid (A₂pm) is the *meso* isomer. The whole-cell sugars are glucose, mannose, ribose and a trace amount of madurose. The acyl type of muramic acid in the cell wall is the acetyl type. The phospholipid pattern is the type PIV pattern of Lechevalier *et al.* (1977). The major fatty acids are straight, iso-

branched, 10-methyl, and 2-hydroxyl acids. Mycolic acids are absent. The predominant menaquinone is MK-10(H₄); small amounts of MK-10(H₆), MK-10(H₂), MK-10(H₀) and MK-9(H₄) are also present. The G + C content of the genomic DNA is 69–71 mol%.

During our search for novel actinomycetes, we isolated some novel strains from sediments in Taiwan. In this study, we describe two isolates, 0351M-12^T and 0385M-1^T, belonging to the genus *Herbidospora*. On the basis of polyphasic taxonomic characterization that combined phenotypic properties, phylogenetic and genetic data, we propose that the two strains should be classified as novel species of genus *Herbidospora* as *Herbidospora yilanensis* sp. nov. and *Herbidospora daliensis* sp. nov.

Strains 0351M-12^T and 0385M-1^T were isolated from sediment samples from a dry lake in Yilan county and a river in Dali city in Taiwan, respectively. The wet clay sediment samples were dried at room temperature for 7 days. Approximately 2 g of each sediment sample was suspended in 18 ml of sterile distilled water, subjected to shaking for 1 h and allowed to settle. The suspensions (100 µl) were plated on humic acid-vitamin (HV) agar

Abbreviations: A₂pm, diaminopimelic acid; GlcNU, a phospholipid of unknown structure containing glucosamine; PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-monomethylethanolamine.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 0351M-12^T and 0385M-1^T are DQ246623 and AY749433, respectively.

Tables showing cultural characteristics of strains 0351M-12^T and 0385M-1^T, fatty acid methyl ester profiles and levels of DNA relatedness among isolates 0351M-12^T, 0385M-1^T and *H. cretacea* NBRC 15474^T are available with the online version of this paper.

(Hayakawa & Nonomura, 1987) supplemented with cycloheximide (50 mg l⁻¹) and nalidixic acid (20 mg l⁻¹) and incubated at 28 °C for 4 weeks. The two strains were purified and maintained on oatmeal agar slants and as suspensions of spores or mycelial fragments in glycerol (20 %, v/v) stored at -20 °C.

Morphological characteristics of the two strains were observed by scanning electron microscopy (S-4700, Hitachi) following incubation on oatmeal agar for 14 days at 28 °C and fixation in 4 % osmium tetroxide solution. The samples were then dehydrated through a series of ethanol-acetone solutions and critical point dried. Cultural characteristics were tested by using 21-day-old cultures grown at 28 °C on various media (Supplementary Table S1 available in IJSEM Online). The *ISCC-NBS Colour-Name Charts* (Kelly, 1964) were used for determining colour designations of the substrate mycelium. Media and procedures used for determination of physiological and biochemical features and carbon source utilization were those described by Shirling & Gottlieb (1966), Goodfellow (1971) and Gordon *et al.* (1974).

The isomer of A₂pm and the sugars in whole-cell hydrolysates were determined by the methods of Hasegawa *et al.* (1983). The presence of mycolic acids was examined by TLC following Minnikin *et al.* (1975), and phospholipids were extracted and identified following the method of Minnikin *et al.* (1984). Menaquinones were extracted and purified by the method of Collins *et al.* (1977), then analysed by HPLC (model 600, Waters) using a Nova-Pak C18 column. The cellular fatty acid compositions of strains 0351M-12^T and 0385M-1^T were determined by using the Sherlock Microbial Identification System (MIDI). Extracts of the methylated fatty acids were prepared according to the protocol provided by the manufacturer and analysed with a 5890 gas chromatograph equipped with a flame-ionization detector and an automatic injector with a G1512A controller (Hewlett Packard). Identification of the peaks was made by comparing the results with the built-in TSBA 40 database (MIDI).

Total DNA was extracted from 14-day-old cells by using the Qiagen genomic DNA kit. The G+C content of the DNA was determined by the HPLC method of Tamaoka & Komagata (1984). The 16S rRNA gene was PCR-amplified by using the method of Nakajima *et al.* (1999) and was sequenced directly on an ABI model 3730 automatic DNA sequencer by using a PRISM ready reaction dye primer cycle sequencing kit (Applied Biosystems). Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of the sequences by CLUSTAL_X (Thompson *et al.*, 1997). Genetic distances were calculated using the Kimura two-parameter model (Kimura, 1980, 1983) and clustering was performed by using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of

the neighbour-joining data by performing 1000 resamplings (Felsenstein, 1985).

Strains 0351M-12^T and 0385M-1^T produced branched and unfragmented substrate mycelia; no distinct aerial hyphae were found. Short spore chains were borne on the tips of sporophores arising from the substrate mycelia. The spore chains were composed of 10–20 non-motile, smooth-surfaced, oval spores. Scanning electron micrographs of strains 0351M-12^T and 0385M-1^T are shown in Fig. 1 and Fig. 2. The cultural characteristics of strains 0351M-12^T and 0385M-1^T are shown in Supplementary Table S1. Growth was good on most of the media tested and the substrate mycelia on most media were yellow. Sporulation occurred on oatmeal-nitrate, yeast-extract-starch (only strain 0351M-12^T), oatmeal, inorganic salts-starch (only strain 0385M-1^T) and glycerol-asparagine (only strain 0351M-12^T) agars. No soluble pigments were produced on any of the media tested. The results of the physiological and biochemical tests are indicated in Table 1 and in the species descriptions.

Strains 0351M-12^T and 0385M-1^T contained glucose, galactose, mannose, ribose and a trace amount of madurose in whole-cell hydrolysates, and the cell-wall peptidoglycan contained *meso*-A₂pm. The predominant menaquinone was MK-10(H₄) and minor components were MK-10(H₆) and MK-9(H₄). Mycolic acids were not detected. The phospholipids were phosphatidylethanolamine (PE), phosphatidyl-*N*-monomethylethanolamine (PME) and a phospholipid of unknown structure containing glucosamine (GlcNU) (phospholipid type PIV of Lechevalier *et al.*, 1977). The fatty acid methyl ester profiles of strains 0351M-12^T and 0385M-1^T are shown in Supplementary Table S2. The G+C contents of the genomic DNA were 70.6 and 70.7 mol%, respectively.

The almost-complete 16S rRNA gene sequences of strains 0351M-12^T (1517 nt) and 0385M-1^T (1515 nt) were determined. Preliminary comparison of the sequences against

Fig. 1. Scanning electron micrograph of strain 0351M-12^T after growth on oatmeal agar at 28 °C for 14 days. Bar, 10 μm

Fig. 2. Scanning electron micrograph of strain 0385M-1^T after growth on oatmeal agar at 28 °C for 14 days. Bar, 10 µm

the GenBank database indicated that the two isolates belonged to the family *Streptosporangiaceae* (Fig. 3) and were most closely related to *H. cretacea* NBRC 15474^T. The binary similarities of the two isolates to *H. cretacea* were 98 %. The results of DNA–DNA hybridization among isolates 0351M-12^T, 0385M-1^T and *H. cretacea* NBRC 15474^T are shown in Supplementary Table S3. The relatedness between the isolates and *H. cretacea* NBRC 15474^T was 40.7–48.5 %. The value of DNA–DNA

hybridization between strain 0351M-12^T and strain 0385M-1^T was 53.6–54.9 %.

The DNA–DNA relatedness values of the isolates to each other and to the type strain of *H. cretacea* were lower than 70 %, which is the recommended threshold value for the delineation of genomic species (Wayne *et al.*, 1987). The physiological characteristics also showed (Table 1) that the isolates were distinguishable from *H. cretacea* NBRC 15474^T. Therefore, we propose that the isolates represent novel species of the genus *Herbidospora*, for which we propose the names *Herbidospora yilanensis* sp. nov. and *Herbidospora daliensis* sp. nov.

Description of *Herbidospora yilanensis* sp. nov.

Herbidospora yilanensis (yi.lan.en'sis. N.L. fem. adj. *yilanensis* pertaining to Yilan county, Taiwan, where the micro-organism was isolated).

Cells are Gram-positive, aerobic, non-acid-fast and mesophilic. Produces branched and unfragmented substrate mycelia; no aerial hyphae are found. Short spore-chains are borne on the tips of sporophores arising from the substrate mycelium. Spore chains are composed of 10–20 non-motile, smooth-surfaced, oval spores. The substrate mycelium is yellowish white to yellow. No soluble pigments are produced. Good growth occurs on most media, but sporulation only occurs on oatmeal-nitrate, yeast-extract-starch, oatmeal and glycerol-asparagine agars. Growth occurs between 20 and 40 °C; NaCl tolerance is 1 % on nutrient agar. Decomposes starch but not adenine, aesculin, hypoxanthine, tyrosine or xanthine. Fructose, fumaric acid, galactose, glucose, glycerol, DL-lactic acid, *myo*-inositol, maltose, mannitol, mannose, melibiose, melezitose, raffinose, rhamnose, salicin, sucrose, succinic acid and trehalose are utilized. Adonitol, L-arabinose, benzoic acid, cellobiose, cellulose, dulcitol, erythritol, L-malic acid, mucic acid, propionic acid, D-ribose, D-sorbitol, L-tartaric acid and xylose are not utilized. *p*-Aminobenzoic acid, *myo*-inositol and thiamine are required for growth. Biotin, nicotinic acid, pantothenic acid and pyridoxine are not required for growth. Melanin is not produced. Lysozyme sensitive. Glucose, galactose, mannose, ribose and a trace amount of madurose occur in whole-cell hydrolysates. The cell-wall peptidoglycan contains *meso*-A₂pm. The predominant menaquinone is MK-10(H₄) and the minor components are MK-10(H₆) and MK-9(H₄). The phospholipids are PE, PME and GlcNU. The cellular fatty acid profile contains 10-methyl C_{17:0} (41.94 %), iso-C_{16:0} (18.44 %), C_{17:1ω8c} (6.34 %), C_{17:0} (4.47 %), C_{15:0} 2-OH (4.39 %), C_{16:0} (3.88 %) and C_{17:1ω6c} (3.83 %). The DNA G+C content of the type strain is 70.6 %.

The type strain is 0351M-12^T (=FIRDI 003^T =BCRC 16875^T =LMG 24337^T), which was isolated from sediment from Yilan, a county in the north of Taiwan.

Table 1. Phenotypic properties useful for distinguishing strains 0351M-12^T and 0385M-1^T from *H. cretacea* NBRC 15474^T

Strains: 1, 0351M-12^T; 2, 0385M-1^T; 3, *H. cretacea* NBRC 15474^T. +, Positive; –, negative.

Characteristic	1	2	3
Decomposition of:			
Hypoxanthine	–	–	+
Aesculin	–	–	+
Carbon source utilization			
L-Arabinose	–	+	–
Glycerol	+	–	+
<i>myo</i> -Inositol	+	–	–
Mannose	+	–	–
Melibiose	+	–	+
Salicin	+	–	+
Trehalose	+	–	–
Requirement for:			
Riboflavin	+	–	+
Thiamine	+	–	+
Organic acid utilization			
Fumaric acid	+	–	–
DL-Lactic acid	+	–	–
Mucic acid	–	–	+
Succinic acid	+	–	–
NaCl tolerance (%)	1	5	1

Fig. 3. Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the phylogenetic position of strains 0351M-12^T and 0385M-1^T among species of the family Streptosporangiaceae. Numbers at nodes indicate percentage of 1000 bootstrap resamplings and only values over 50 % are given. Asterisks indicate branches of the tree that were also found using the maximum-parsimony (Kluge & Farris, 1969) algorithm. Bar, 0.01 substitutions per nucleotide position.

Description of *Herbidospora daliensis* sp. nov.

Herbidospora daliensis (da.li.en'sis. N.L. fem. adj. *daliensis* pertaining to Dali city, Taiwan, where the micro-organism was isolated).

Cells are Gram-positive, aerobic, non-acid-fast and mesophilic. Produces branched and unfragmented substrate mycelia; no aerial hyphae are found. Short spore-chains are borne on the tips of sporophores arising from the substrate mycelium. The spore chains are composed of 10–20 non-motile, smooth-surfaced, oval spores. Growth occurs between 20 and 40 °C; NaCl tolerance is 5 % on nutrient agar. Decomposes starch but not adenine, hypoxanthine, tyrosine or xanthine. L-Arabinose, fructose, galactose, glucose, maltose, mannitol, melezitose, raffinose, rhamnose and sucrose are utilized. Adonitol, benzoic acid, cellobiose, cellulose, dulcitol, erythritol, fumaric acid, glycerol, *myo*-inositol, DL-lactic acid, L-malic acid, mannose, melibiose, mucic acid, propionic acid, D-ribose, salicin, D-sorbitol, succinic acid, L-tartaric acid, trehalose and xylose are not utilized. Requires *p*-aminobenzoic acid and *myo*-inositol for growth. Biotin, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine are not required for growth. Melanin is not produced. Lysozyme sensitive. Glucose, galactose, mannose, ribose and a trace amount of madurose occur in whole-cell hydrolysates. The cell-wall peptidoglycan contains *meso*-A₂pm. The predominant menaquinone is MK-10(H₄) and the minor components

are MK-10(H₆) and MK-9(H₄). The phospholipids are PE, PME and GlcNU. The cellular fatty acid profile contains 10-methyl C_{17:0} (36.28 %), C_{17:1}ω8c (16.09 %), isoC_{16:0} (10.83 %), C_{17:0} (9.21 %), C_{15:0} 2-OH (6.66 %), C_{17:1}ω6c (3.63 %) and C_{16:0} (3.03 %). The DNA G+C content of the type strain is 70.7 %.

The type strain is 0385M-1^T (=FIRDI 004^T =BCRC 16876^T =LMG 24336^T), which was isolated from sediment from central Taiwan.

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