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Herbidospora osyris sp. nov., isolated from surfacesterilized tissue of Osyris wightiana Wall. ex Wight

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An endophytic actinomycete, strain YIM 65070<sup>T</sup>, was isolated from surface-sterilized tissue of Osyris wightiana Wall. ex Wight collected from Yunnan province, south-west China, and characterized by using a polyphasic approach. Strain YIM 65070<sup>T</sup> had morphological and chemotaxonomic markers that were consistent with its classification in the genus Herbidospora. Phylogenetic analysis based on almost complete 16S rRNA gene sequences indicated that strain YIM 65070<sup>T</sup> was phylogenetically very closely related to *Herbidospora cretacea* IFO 15474<sup>T</sup>. DNA-DNA hybridization experiments confirmed the separate genomic status of strains YIM 65070<sup>T</sup> and *H. cretacea* DSM 44071<sup>T</sup>. Moreover, strain YIM 65070<sup>T</sup> could be distinguished from H. cretacea DSM 44071<sup>T</sup> by differences in several phenotypic characteristics such as tolerance to NaCl, degradation activity, utilization of sole carbon and nitrogen sources and the cellular fatty acid contents. On the basis of phenotypic and phylogenetic evidence, strain YIM 65070<sup>T</sup> was identified as a novel species of the genus Herbidospora, for which the name Herbidospora osyris sp. nov. is proposed, with YIM 65070<sup>T</sup> (=CCTCC AA 208019<sup>T</sup>=DSM 45214<sup>T</sup>) as the type strain.

The genus Herbidospora of the family Streptosporangiaceae was first described by Kudo et al. (1993). At the time of writing, the genus comprises only one recognized species, Herbidospora cretacea (Kudo et al., 1993). The members of this genus produce branching substrate mycelia, but no distinct aerial hyphae. Short chains of non-motile spores (10-30 spores per chain) are borne on the tips of sporophores, which are derived from the vegetative mycelia in clusters. Cell walls contain meso-diaminopimelic acid and N-acetylated muramic acid, but lack a significant amount of glycine. Whole-cell hydrolysates contain a trace amount of madurose. The phospholipid pattern is the type PIV pattern of Lechevalier et al. (1977). The cellular fatty acid composition is characterized by the presence of isohexadecanoic, *n*-hexadecanoic, *n*-heptadecenoic, 10methyl heptadecanoic and 2-hydroxy acids. The predominant menaquinone for the genus is MK-10(H<sub>4</sub>) and MK-10(H<sub>6</sub>), MK-10(H<sub>2</sub>), MK-10(H<sub>0</sub>) and MK-9(H<sub>4</sub>) are also present as minor components. The G+C contents of the genomic DNA are 69-71 mol%.

sequence of strain YIM 65070<sup>T</sup> is FJ214356.

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Strain YIM 65070<sup>T</sup> was isolated as part of a discovery and identification programme for endophytic actinomycetes from plants in Yunnan province, south-west China. Strain YIM 65070<sup>T</sup> was isolated from surface-sterilized tissue of Osyris wightiana Wall. ex Wight. The surface sterilization and isolation procedures were performed according to previously described methods (Li et al., 2009). The purified strain was routinely cultured on YIM 38 medium [4 g malt extract, 4 g yeast extract, 4 g glucose, vitamin mixture (0.25 mg biotin and 0.5 mg each of p-aminobenzoic acid, calcium pantothenate, inositol, niacin, pyridoxine-HCl, riboflavin and thiamine-HCl), 20 g agar; pH 7.2] at 28 °C and stored as a glycerol suspension (20 %, v/v) at -70 °C.

Genomic DNA extraction, amplification and 16S rRNA gene sequencing were performed as described previously by Li et al. (2007). An almost complete 16S rRNA gene sequence of strain YIM 65070<sup>T</sup>, comprising 1425 bp, was obtained and aligned with corresponding sequences of H. cretacea and other members of the family Streptosporangiaceae (retrieved from the GenBank/EMBL/DDBJ database) using CLUSTAL\_X (Thompson et al., 1997). The resulting alignment was corrected manually and a phylogenetic tree was constructed using the neighbour-joining (Saitou & Nei, 1987) tree-making algorithm from MEGA

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene

version 4.0 (Tamura *et al.*, 2007). The topologies of the resultant trees were evaluated by using bootstrap analysis (Felsenstein, 1985) based on 1000 resampled datasets.

The phylogenetic positions of the organisms revealed that strain YIM  $65070^{T}$  formed a separate clade with *H. cretacea* (Fig. 1). A high sequence similarity value of 99.9 % was found between strains YIM  $65070^{T}$  and *H. cretacea* IFO  $15474^{T}$ . In order to investigate the genomic relatedness between these two strains, DNA–DNA hybridization was performed according to the method described by He *et al.* (2005). The experiment was performed with three replications. The level of DNA–DNA relatedness (62.9 % mean value, SD 3.4 %) supported the findings that these two strains belong to different genomic species.

Cultural characteristics were determined after 3 weeks incubation at 28 °C, according to the methods of the International *Streptomyces* Project (ISP; Shirling & Gottlieb, 1966). Czapek's agar and nutrient agar were prepared as described by Dong & Cai (2001). Colour determination was performed by using colour chips from the ISCC–NBS colour charts (standard samples, no. 2106) (Kelly, 1964). After incubation on YIM 38 medium at 28 °C for 27 days, morphological properties were examined using a light microscope (BH-2; Olympus) and a scanning electron microscope (Philips XL30; ESEM-TMP).

Growth at various temperatures, pH values and NaCl concentrations was examined according to Xu *et al.* (2005) using YIM 38 medium as the basal medium. Carbon source utilization was determined according to the methods of Shirling & Gottlieb (1966) and Gordon *et al.* (1974). Oxidase activity was determined from the oxidation of tetramethyl-*p*-phenylenediamine. Catalase activity was determined with  $3 \% H_2O_2$  according to standard methods. Other physiological and biochemical features were tested using standard procedures (Goodfellow, 1971; Williams *et al.*, 1983).

Freeze-dried cells for chemotaxonomic analysis were obtained from cultures grown in tryptic soy broth (TSB) for 5 days at 28 °C. The analysis of the cellular fatty acid composition was accomplished by following the instructions of the Microbial Identification System (MIDI) (Sasser, 1990). The amino acids and sugars of the whole-cell hydrolysates were determined using TLC as described by Staneck & Roberts (1974). Phospholipids were identified according to published procedures (Minnikin *et al.*, 1979; Collins & Jones, 1980). Menaquinones were extracted (Collins *et al.*, 1977) and separated by HPLC (Tamaoka *et al.*, 1983). The G+C content of the genomic DNA was determined by the HPLC method according to Mesbah *et al.* (1989).

Strain YIM 65070<sup>T</sup> produced branched and unfragmented substrate mycelia; no distinct aerial hyphae were found with light or scanning electron microscopy. Straight, short chains of non-motile spores (10-25 spores per chain) were borne on the tips of sporophores, which were derived from the vegetative mycelia in clusters. Spores were oval and the spore surface was smooth (Fig. 2). The cultural characteristics of isolate YIM 65070<sup>T</sup> are shown in Table 1. The vegetative mycelia were yellow-white to yellowish brown, no soluble pigments were produced on any of the media tested. Sporulation was poor and when sporulation occurred, the surface of the colony was white. These morphological properties were consistent with those of the genus Herbidospora. Physiological and biochemical test results are given in detail in Table 2 and in the species description.

Strain YIM 65070<sup>T</sup> contained *meso*-diaminopimelic acid as the diagnostic diamino acid of the cell-wall peptidoglycan. The whole-cell hydrolysates contained ribose, glucose, galactose, madurose and trace amounts of mannose. The quinone profile was composed of the compounds MK- $10(H_4)$ , MK- $10(H_2)$ , MK- $10(H_0)$ , MK- $9(H_4)$ , MK- $10(H_6)$ , MK- $9(H_2)$  and MK- $9(H_0)$  in the ratio 53:29:5:5:4:2:2.



**Fig. 1.** Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain YIM 65070<sup>T</sup> and representative species of the family *Streptosporangiaceae*. Bootstrap values (expressed as percentages of 1000 replications) >50 % are given at the nodes. Bar, 1 nt substitution per 200 nt.



Fig. 2. Scanning electron micrograph of cells of strain YIM  $65070^{T}$  after growth on YIM 38 medium at 28 °C for 27 days. Bar, 5  $\mu$ m.

Phosphatidylethanolamine, phosphatidylinositol and two unknown phosphoglycolipids were the major phospholipids, with moderate amounts of glucosamine-containing phospholipid, diphosphatidylglycerol and two phosphatidylinositol mannosides. The fatty acid contents of strains YIM  $65070^{T}$  and *H. cretacea* DSM  $44071^{T}$  are shown in Table 3. The G+C content of the genomic DNA of strain YIM  $65070^{T}$  was 70.4 mol%.

In the phylogenetic analysis, strain YIM 65070<sup>T</sup> was found to be closely related to Streptosporangium claviforme DSM 44127<sup>T</sup>, Acrocarpospora corrugata DSM 43316<sup>T</sup>, Acrocarpospora macrocephala IFO 16266<sup>T</sup>, Acrocarpospora pleiomorpha R-31<sup>T</sup>, Planotetraspora silvatica TT 00-51<sup>T</sup>, Planotetraspora mira IFO  $15435^{T}$  and Planotetraspora thailandica BCC 21825<sup>T</sup>. The 16S rRNA gene sequence similarities between these species were 98.9%, 97.9%, 97.0%, 96.9%, 97.8%, 97.6% and 97.5%, respectively. According to the results obtained by Petrolini et al. (1992), the morphological features of S. claviforme DSM 44127<sup>T</sup> were similar to those of strains YIM 65070<sup>T</sup> and *H. cretacea* DSM 44071<sup>T</sup>. When the results of phylogenetic analysis were analysed, it was found that the taxonomic position of S. claviforme DSM 44127<sup>T</sup> remained ambiguous and it is suggested that further characteristics should be determined. Isolate YIM 65070<sup>T</sup> and H. cretacea DSM 44071<sup>T</sup> could be distinguished from the genera Acrocarpospora and

Table 1.	Cultural	characteristics	of strain	YIM	65070	

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Planotetraspora by means of their morphological properties and predominant menaquinones. Both strains YIM 65070<sup>T</sup> and *H. cretacea* DSM 44071<sup>T</sup> produce non-fragmented branched substrate mycelia, but true aerial hyphae are not formed. Short chains of non-motile spores (10-30 spores per chain) are borne on the tips of sporophores, which are derived from the vegetative mycelia in clusters. Members of genus Acrocarpospora produce spherical and club-shaped structures on the tips of the aerial mycelium, these structures contain coiled spore chains and the spores are non-motile (Tamura et al., 2000). Members of genus Planotetraspora produce long, cylindrical sporangia at the ends of short sporangiophores in aerial hyphae, with each sporangium containing four spores in a single row and the spores may exhibit motility (Tamura & Sakane, 2004). In addition to the morphological differences, strains YIM  $65070^{T}$  and *H. cretacea* DSM  $44071^{T}$  have MK-10(H<sub>4</sub>) as the predominant menaquinone, however,  $MK-9(H_4)$  is the major component among members of the genera Acrocarpospora and Planotetraspora. Both morphological and chemotaxonomic properties indicated that strains YIM 65070<sup>T</sup> and *H. cretacea* DSM 44071<sup>T</sup> were distinct from their phylogenetic neighbours.

According to the results of simultaneous experiments, many phenotypic differences between strains YIM 65070<sup>T</sup> and *H. cretacea* DSM 44071<sup>T</sup> were observed, including differences in tolerance to NaCl, hydrolysis of Tween 80, utilization of sole carbon and nitrogen sources and the fatty acid contents (Tables 2 and 3). Strain YIM 65070<sup>T</sup> tolerated NaCl concentrations of 3 % (w/v) and did not hydrolyse Tween 80. It utilized amygdalin, arbutin, glycerol, inositol, melibiose, raffinose, salicin, sodium DLmalate, D-tagatose, trehalose and turanose as sole carbon sources, but none of these sources were used by H. cretacea DSM 44071<sup>T</sup>. Strain YIM 65070<sup>T</sup> utilized adenine, Lcysteine and xanthine, none of which were used by H. cretacea DSM 44071<sup>T</sup>. In contrast, *H. cretacea* DSM 44071<sup>T</sup> utilized L-arginine, L-asparagine, DL-methionine and Lvaline. The cellular fatty acid profile of strain YIM 65070<sup>T</sup> did not contain any 2-hydroxy acids, but minor to trace amounts of C<sub>16:0</sub> iso 2-OH, C<sub>17:0</sub> 2-OH, C<sub>15:0</sub> 2-OH, C<sub>16:0</sub> 2-OH and C<sub>17:0</sub> iso 2-OH were detected in H.

Media	Growth	Reverse side colour	Sporulation	Aerial mass colour
Czapek's agar	Good	Yellow-white	Poor	White
Glycerol-asparagine agar (ISP 5)	Poor	Pale orange–yellow	None	-
Inorganic salts-starch agar (ISP 4)	Moderate	Yellowish brown	Poor	White
Nutrient agar	Poor	Grey–yellow	None	-
Oatmeal agar (ISP 3)	Poor	Yellow-white	None	-
Potato dextrose agar (PDA)	Poor	Grey–yellow	None	-
Yeast extract-malt extract agar (ISP 2)	Good	Grey–yellow	Moderate	White
YIM 38 agar	Good	Grey-yellow	Moderate	White

**Table 2.** Physiological characteristics that differentiate strainYIM $65070^{T}$  from its closest phylogenetic neighbourH. cretacea DSM  $44071^{T}$ 

Strains: 1, YIM 65070<sup>T</sup>; 2, DSM 44071<sup>T</sup>. +, Positive; -, negative; w, weakly positive.

Characteristic	1	2
Tween 80 hydrolysis	-	+
Growth in 3 % NaCl	+	_
Utilization as sole carbon sources:		
Amygdalin	+	-
Arbutin	+	_
Glycerol	+	-
Inositol	+	-
Melibiose	+	-
Raffinose	+	-
Salicin	+	-
Sodium DL-malate	+	-
D-Tagatose	+	-
Trehalose	+	-
Turanose	+	-
Utilization as sole nitrogen sources:		
Adenine	+	-
L-Arginine	_	+
l-Asparagine	—	+
L-Cysteine	+	-
DL-Methionine	_	W
l-Valine	_	+
Xanthine	+	-

*cretacea* DSM 44071<sup>T</sup>. Although the major components of fatty acids of these two strains were qualitatively similar, the contents were significantly different.

On the basis of the results obtained in this study, the level of DNA–DNA relatedness (<70%) and many phenotypic characteristics supported the conclusion that strains YIM 65070<sup>T</sup> and *H. cretacea* DSM 44071<sup>T</sup> belong to separate species. Therefore, it is suggested that strain YIM 65070<sup>T</sup> represents a novel species of the genus *Herbidospora*, for which the name *Herbidospora osyris* sp. nov. is proposed.

## Description of Herbidospora osyris sp. nov.

Herbidospora osyris (o.sy'ris. N.L. gen. n. of Osyris, the plant genus from which this species was isolated).

Forms yellow–white to yellowish brown substrate mycelia on the media tested. When sporulation occurs, the surface of the colony is white. No soluble pigments are produced on nutrient, Czapek's, ISP 2, ISP 3, ISP 4, ISP 5, PDA or YIM 38 agars. Short chains of spores (10–25 spores per chain) are borne at the tips of sporophores, which are derived from the vegetative mycelia in clusters. Growth occurs at 10–37 °C and pH 6.0–8.0. NaCl is tolerated at up to 3 % (w/v). Catalase is produced. Negative for the Voges– Proskauer and methyl red tests, for the oxidase reaction, for production of H<sub>2</sub>S, for nitrate reduction and for milk **Table 3.** Fatty acid profiles (%) of strains YIM  $65070^{T}$  and *H. cretacea* DSM  $44071^{T}$ 

Strains: 1, YIM 65070<sup>T</sup>; 2, DSM 44071<sup>T</sup>.

Fatty acid	1	2
C <sub>13:0</sub>	3.19	1.52
$C_{14:0}$ iso	5.89	1.63
C <sub>14:0</sub>	0.99	0.56
$C_{15:0}$ iso	2.32	1.12
C <sub>15:0</sub> anteiso		0.24
C <sub>15:0</sub>	11.53	8.48
$C_{16:0}$ iso	21.82	11.85
C <sub>16:1</sub> cis 9		0.93
C <sub>16:0</sub>	1.70	2.79
C <sub>15:0</sub> 2-OH		0.96
C <sub>16:0</sub> 10-methyl		0.63
$C_{17:0}$ iso	1.27	1.10
C <sub>17:0</sub> anteiso		0.68
C <sub>17:1</sub> cis 9	9.34	16.76
C <sub>16:0</sub> iso 2-OH		2.74
C <sub>17:0</sub>	9.10	15.65
C <sub>16:0</sub> 2-OH		0.50
C <sub>17:0</sub> 10-methyl	31.73	24.53
C <sub>18:3</sub> cis 6,12,14		0.39
$C_{18:0}$ iso		0.79
C <sub>18:1</sub> cis 9		0.80
C <sub>17:0</sub> iso 2-OH		0.40
C <sub>18:0</sub>	1.12	1.59
C <sub>17:0</sub> 2-OH		1.88
TBSA C <sub>18:0</sub> 10-methyl		0.64
C <sub>19:0</sub>		0.38
C <sub>17:1</sub> iso I/antei B		0.46

coagulation and peptonization. Tweens 20 and 40 and urea are hydrolysed, but Tween 80, gelatin, starch and cellulose are not hydrolysed. Utilizes amygdalin, arbutin, cellobiose, aesculin, D-fructose, D-galactose, glycerol, inositol, maltose, D-mannitol, D-mannose, melibiose, raffinose, D-ribose, salicin, sodium DL-malate, D-tagatose, trehalose, turanose and D-xylose as sole carbon sources. D-Adonitol, Darabinose, dulcitol, erythritol, D-lactose, L-rhamnose, Dsorbitol, L-sorbose and xylitol are not utilized. Adenine, L-alanine, L-cysteine, L-cystine, L-histidine, L-lysine, Lphenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and xanthine can be used as sole nitrogen sources, but not L-arginine, L-asparagine, L-glutamic acid, glycine, hypoxanthine, DL-methionine or L-valine. The diagnostic amino acid is meso-diaminopimelic acid and ribose, glucose, galactose, madurose and trace amounts of mannose are present in the whole-cell hydrolysates. The predominant menaquinone is MK-10( $H_4$ ). MK-10( $H_2$ ), MK-10( $H_0$ ), MK-9(H<sub>4</sub>), MK-10(H<sub>6</sub>), MK-9(H<sub>2</sub>) and MK-9(H<sub>0</sub>) are also present as moderate to minor components. Phospholipids are phosphatidylethanolamine, phosphatidylinositol, two unknown phosphoglycolipids, glucosamine-containing phospholipid, diphosphatidylglycerol and two phosphatidylinositol mannosides. Fatty acids are  $\begin{array}{l} C_{17:0} \text{ 10-methyl, } C_{16:0} \text{ iso, } C_{15:0} \text{, } C_{17:1} \text{ cis } 9 \text{, } C_{17:0} \text{, } C_{14:0} \\ \text{iso, } C_{13:0} \text{, } C_{15:0} \text{ iso, } C_{16:0} \text{, } C_{17:0} \text{ iso, } C_{18:0} \text{ and } C_{14:0}. \end{array}$ 

The type strain, YIM  $65070^{T}$  (=CCTCC AA  $208019^{T}$ = DSM  $45214^{T}$ ), was isolated from a surface sterilized plant sample, *Osyris wightiana* Wall. ex Wight, collected from Yunnan province, south-west China. The G+C content of the genomic DNA of the type strain is 70.4 mol%.

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