Actinopolymorpha cephalotaxi sp. nov., a novel actinomycete isolated from rhizosphere soil of the plant *Cephalotaxus fortunei*

Li-Jie Yuan,^{1,2†} Yu-Qin Zhang,^{1†} Li-Yan Yu,¹ Cheng-Hang Sun,¹ Yu-Zhen Wei,¹ Hong-Yu Liu,¹ Wen-Jun Li³ and Yue-Qin Zhang¹

Correspondence

Wen-Jun Li

wjli@ynu.edu.cn

Yue-Qin Zhang

zyq_0525@yahoo.com.cn

¹Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, PR China

²Department of Biology, North China Coal Medical College, Tangshan, Hebei 063000, PR China

³The Key Laboratory for Microbial Resources of the Ministry of Education, PR China and Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan 650091, PR China

An actinomycete, strain I06-2230^T, was isolated from rhizosphere soil of the plant *Cephalotaxus fortunei*, collected from Yunnan province, south China. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate belongs to the genus *Actinopolymorpha*. Cells grew on agar surfaces, with no penetration even after prolonged cultivation. Aerial hyphae were absent. Cells were irregularly shaped and remained attached as chains or aggregates. Chemotaxonomic data, which showed LL-diaminopimelic acid in the cell wall, glucose as the whole-cell sugar, type PI phospholipids and MK-9(H₄) as the predominant menaquinone, supported the affiliation of strain I06-2230^T to the genus *Actinopolymorpha*. The major fatty acids were iso-C_{15:0}, iso-C_{16:0} and iso-C_{16:1} H. The genomic DNA G+C content was 69.3 mol%. DNA–DNA hybridization data, in combination with chemotaxonomic, physiological and biochemical data, demonstrated that strain I06-2230^T should be classified as representing a novel species of the genus *Actinopolymorpha*. The name *Actinopolymorpha cephalotaxi* sp. nov. is proposed, with strain I06-2230^T (=DSM 45117^T=CCM 7466^T=KCTC 19293^T) as the type strain.

The genus *Actinopolymorpha* was first proposed by Wang *et al.* (2001) to accommodate a highly pleomorphic and considerably salt-tolerant actinomycete with LL-diaminopimelic acid in the cell wall, type PI phospholipids and MK-9(H₆) as the predominant menaquinone. At the time of writing, the genus comprised two species with validly published names, *Actinopolymorpha singaporensis* (Wang *et al.*, 2001) and *Actinopolymorpha rutila* (Wang *et al.*, 2008).

During our screening research on the plant rhizosphere microbial flora of China, strain I06-2230^T was recovered and its taxonomic position was investigated using a polyphasic approach. Strain I06-2230^T was isolated from rhizosphere soil of *Cephalotaxus fortunei*, collected from Yunnan province, south China, using the dilution plating method and incubation at 28 °C for 21 days. The medium

used for selective isolation contained (l⁻¹): 5 g starch, 5 g glycerol, 1 g proline, 1 g (NH₄)₂SO₄, 1 g NaCl, 2 g CaCl₂, 1 g K₂HPO₄, 1 g MgSO₄·7H₂O, 50 mg K₂Cr₂O₇, 25 mg aztreonam and 15.0 g agar and had a final pH 7.2. The strain was maintained on ISP 2 agar slants (Shirling & Gottlieb, 1966) at 4 °C and stored as 20% (w/v) glycerol suspensions at –20 °C. Biomass for chemical and molecular systematic studies was obtained by cultivation in shake flasks (160 r.p.m.) using tryptic soy broth (TSB; Difco) at 28 °C for 6 days.

For the observation of colony and cultural characteristics, ISP 2, 3, 4 and 5 agar (Shirling & Gottlieb, 1966), Czapek's solution agar (Waksman, 1961), nutrient agar (Difco) and tomato paste–oatmeal agar (Waksman, 1961) were inoculated with strain I06-2230^T. Cultures were incubated at 28 °C and observations were recorded after 7, 14, 21 and 28 days. For cell morphology, the coverslip technique, as described by Kawato & Shinobu (1959), was used on ISP 2 agar. Some gold-coated dehydrated specimens of 14-day cultures were observed by using scanning electron microscopy (Quanta; FEI). Strain I06-2230^T grew well on agar surfaces, but without penetration even after prolonged

†These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain I06-2230^T is EU438909.

Scanning electron micrographs and a table of cellular fatty acid contents are available as supplementary material with the online version of this paper.

cultivation. Aerial hyphae were absent. The colonies were paste-like and initially smooth and later wrinkled. Irregular cells were observed (Supplementary Fig. S1, available in IJSEM Online). Cells appeared to divide through apical and lateral budding and remained attached after division, often forming short chains and small aggregates. The colour of the colonies on ISP 3, 4 and 5 agar, Czapek's solution agar, nutrient agar and tomato paste–oatmeal agar was buff or yellow and on ISP 2 agar was brilliant orange. Buff diffusible pigment was produced on ISP 3 agar, nutrient agar and tomato paste–oatmeal agar.

Carbohydrate utilization and enzyme activities were determined using the API 50 CH and API ZYM systems (bioMérieux), respectively, according to the manufacturer's instructions, after growth at 4, 10, 20, 28–37 (with intervals of 1 °C), 40 and 45 °C on ISP 2 agar for 15–30 days. The ability of the strain to grow at different NaCl concentrations (0, 1, 3, 5–20 %, with intervals of 0.5 %, w/v) was determined according to Wang *et al.* (2001). pH tolerance (pH 5.0–11.0, with intervals of 0.5 pH units) was examined with TSB medium. Other physiological and biochemical tests were performed according to the established methods of Williams *et al.* (1983) and Kämpfer *et al.* (1991). Good growth occurred at 20–28 °C and no growth

occurred at 4 °C or above 37 °C. Growth was observed at initial pH values between pH 6.0 and 8.0 and on TSB medium containing 0–5 % NaCl. Physiological features that differentiate the novel isolate from *A. rutila* YIM 45725^T and *A. singaporensis* KCTC 19907^T are given in Table 1 and other phenotypic characteristics are presented in the species description.

The whole-cell sugar composition and diagnostic isomers of diaminopimelic acid were analysed by TLC as described by Lechevalier & Lechevalier (1980). Menaquinones were extracted by using the method of Collins (1985) and analysed by HPLC (Groth *et al.*, 1997). The phospholipids were extracted, examined by two-dimensional TLC and identified as described by Minnikin *et al.* (1984). The fatty acids were prepared and analysed according to the instructions of the standard Sherlock Microbial Identification system (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Strain I06-2230^T contained LL-diaminopimelic acid as the diamino acid and glucose as the whole-cell sugar. The phospholipids comprised phosphatidylinositol, phosphatidylglycerol and phosphatidyl-inositol mannosides. The predominant menaquinone was MK-9(H₄). Small amounts of MK-9(H₆), MK-9(H₈) and MK-10(H₄) were also present. The major fatty acids were

Table 1. Differential characteristics of strain I06-2230^T and its closest phylogenetic neighbours

Strains: 1, I06-2230^T (*Actinopolymorpha cephalotaxi* sp. nov.); 2, *A. rutila* YIM 45725^T; 3, *A. singaporensis* KCTC 19907^T. Data were taken from this study. All strains were positive for utilization of D-fructose, D-galactose, α-D-glucose, D-mannitol, L-rhamnose, sucrose, D-xylose, *myo*-inositol and sorbitol and hydrolysis of gelatin. All strains were negative for starch degradation and were sensitive to 0.1 % phenol. +, Positive; –, negative.

Characteristic	1	2	3
Penetrative growth of vegetative hyphae (ISP 3 agar)	–	–	+
Aerial hyphae (ISP 2 agar)	–	+	–
Soluble pigment	+	–	–
Growth conditions			
Temperature (°C)	20–28	15–37	25–37
NaCl maximum (%)	5	5	15
Utilization of:			
D-Arabinose	+	+	–
Glucosamine	+	+	–
Raffinose	+	+	–
Nitrate reduction	+	–	+
Milk coagulation and peptonization	+	–	+
Acid production from:			
Glucose	–	+	–
Lactose (5 %)	–	+	+
D-Ribose	–	+	+
Enzyme activities			
Esterase	+	–	–
Esterase lipase	+	–	+
Urease	+	–	–
Whole-cell sugars	Glucose	Ribose, galactose, glucose	Rhamnose, ribose, glucose
Phospholipids*	PI, PG, PIM	PI, PG, PIM, DPG	PI, PG, PIM, DPG
Predominant menaquinone	MK-9(H ₄)	MK-9(H ₄)	MK-9(H ₆)

*PI, Phosphatidylinositol; PG, phosphatidylglycerol; PIM, phosphatidylinositol mannosides; DPG, diphosphatidylglycerol.

iso-C_{16:1} H (32.9%), iso-C_{16:0} (21.4%) and iso-C_{15:0} (12.0%). The detailed profile is given in Supplementary Table S1 (in IJSEM Online).

Genomic DNA extraction and PCR amplification of the 16S rRNA gene sequence were performed according to the procedure described by Xu *et al.* (2003). Purified PCR products were sequenced with an automatic sequencer (ABI PRISM 3730 XL; Applied Biosystems). The almost-complete 16S rRNA gene sequence of strain I06-2230^T (1441 bp) was aligned with *Actinopolymorpha* nucleotide sequences retrieved from GenBank using CLUSTAL X version 1.8 (Thompson *et al.*, 1997). Subsequently, a phylogenetic tree was constructed using MEGA version 2.1 (Kumar *et al.*, 2001). Distances were calculated using distance options according to the Kimura two-parameter model (Kimura, 1980, 1983) and clustering was performed with the neighbour-joining method of Saitou & Nei (1987). Bootstrap resampling analysis, according to the method of Felsenstein (1985), with 1000 resamplings was used to evaluate the phylogenetic tree topology. The 16S rRNA gene sequence comparisons clearly showed that strain I06-2230^T was a member of the family *Nocardioideae*. Strain I06-2230^T was phylogenetically closely related to *A. rutila* YIM 45725^T and *A. singaporensis* KCTC 19907^T, with 16S rRNA gene sequence similarity values of 99.5 and 98.9%, respectively, which indicated the affiliation of strain I06-2230^T to the genus *Actinopolymorpha*. Strain I06-2230^T formed a distinct branch with *A. rutila* YIM 45725^T in the family *Nocardioideae* in the neighbour-joining phylogenetic tree (Fig. 1).

DNA–DNA hybridizations between strain I06-2230^T and *A. rutila* YIM 45725^T and *A. singaporensis* KCTC 19907^T

were performed to confirm whether the isolate represented a novel species. The thermal renaturation method (De Ley *et al.*, 1970) was used, with a UV-1700 spectrophotometer (Shimadzu) equipped with a DCW-2008 thermo bath. The DNA–DNA relatedness values between strain I06-2230^T and *A. rutila* YIM 45725^T and *A. singaporensis* KCTC 19907^T were 33.81% and 9.13%, respectively, which were much lower than 70%, the threshold value considered for the delineation of genomic species (Wayne *et al.*, 1987). Therefore, the strain could not be classified in any of the recognized species of the genus *Actinopolymorpha*. The genomic DNA G+C content of strain I06-2230^T was determined using the thermal denaturation (*T_m*) method (Marmur & Doty, 1962) with *Escherichia coli* AS1.365 as a control. The DNA G+C content was 69.3 mol%.

On the basis of the phenotypic and genotypic data presented above, we propose that strain I06-2230^T represents a novel species of the genus *Actinopolymorpha*, for which the name *Actinopolymorpha cephalotaxi* sp. nov. is proposed.

Description of *Actinopolymorpha cephalotaxi* sp. nov.

Actinopolymorpha cephalotaxi (ce.pha.lo.ta'xi. N.L. gen. n. *cephalotaxi* of *Cephalotaxus*, isolated from *Cephalotaxus fortunei* from which the rhizosphere soil sample was collected).

Gram-positive. Strictly aerobic. Tolerates up to 5% NaCl on TSB medium and ISP 2 medium. Growth occurs at pH 6.0–8.0 and 20–28 °C. Cells show irregular shapes.

Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequences, showing the position of strain I06-2230^T among its phylogenetic neighbours. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Bar, 1% sequence divergence.

Cells appear to divide through apical and lateral budding and remain attached after division, often forming short chains and small aggregates. Aerial mycelia are absent on the tested media. Vegetative mycelia develop on agar surfaces. Colonies are paste-like, with buff or yellow colour on most tested media and brilliant orange on ISP 2 medium. Buff diffusible pigment is produced on ISP 3 agar, nutrient agar and tomato paste–oatmeal agar. Utilizes D-arabinose, D-fructose, D-galactose, D-mannitol, raffinose, D-xylose, glucosamine, L-rhamnose, *myo*-inositol, sorbitol, sucrose and α -D-glucose as sole carbon sources, but not acetate, citrate, gluconate, mucic acid or tartrate. Positive for gelatin hydrolysis, milk coagulation and peptonization and nitrate reduction, but negative for starch degradation, H₂S production and cellulose hydrolysis. Sensitive to 0.1 % phenol. Positive for alkaline phosphatase, esterase, esterase lipase, urease, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α - and β -galactosidases, α - and β -glucosidases, *N*-acetyl- β -glucosaminidase and α -mannosidase. The cell wall contains LL-diaminopimelic acid as the diamino acid in the peptidoglycan, without a diagnostic sugar. MK-9(H₄) is the predominant menaquinone; MK-9(H₆), MK-9(H₈) and MK-10(H₄) also can be detected. Phosphatidylinositol, phosphatidylglycerol and phosphatidylinositol mannosides (type PI phospholipids) are present. Major cellular fatty acids (>10 %) are iso-C_{16:1} H, iso-C_{16:0} and iso-C_{15:0}. The genomic DNA G + C content of the type strain is 69.3 mol%.

The type strain, I06-2230^T (=DSM 45117^T=CCM 7466^T=KCTC 19293^T), was isolated from rhizosphere soil of the plant *Cephalotaxus fortunei*, collected from Yunnan province, south China.

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