Acidipila dinghuensis sp. nov., an acidobacterium isolated from forest soil

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An aerobic, chemoheterotrophic, non-motile, capsule-forming bacterium designated DHOF10^T was isolated from a soil sample collected from the forest of Dinghushan Biosphere Reserve, Guangdong Province, PR China. Strain DHOF10^T was able to grow at pH 3.5–8.0 (optimum pH 4.0–4.5) and at 10–37 °C (optimum 28–37 °C). NaCl tolerance was up to 1.0 % (w/v). Major fatty acids consisted of iso- $C_{15:0}$, $C_{18:1}\omega_9c$ and $C_{16:1}\omega_7c$. The quinone was MK-8 and the DNA G + C content was 56.3 mol%. The polar lipids consisted of phosphatidylethanolamine, an unidentified aminolipid, an unidentified phospholipid, two unidentified aminophospholipids and two unidentified polar lipids. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolate was a member of genus *Acidipila* of the phylum *Acidobacteria*, with the highest 16S rRNA gene sequence similarity of 97.3 % to *Acidipila rosea* AP8^T. On the basis of phylogenetic, phenotypic, physiological and chemotaxonomic distinctiveness, strain DHOF10^T represents a novel species of the genus *Acidipila*, for which the name *Acidipila dinghuensis* sp. nov. is proposed. The type strain is DHOF10^T (=CGMCC 1.13007^T=KCTC 42631^T).

Acidobacteria is a phylum of great diversity rivalling the phylum Proteobacteria according to culture-independent studies (Janssen, 2006). The phylum Acidobacteria is currently composed of 26 distinct subdivisions (Barns et al., 2007) based on the analysis of all available 16S rRNA gene sequences, and members of subdivisions 1, 2, 3, 4 and 6 are detected most abundantly in soil environments (Janssen, 2006; Jones et al., 2009). Acidobacteria can be detected in a large variety of habitat types, especially high-latitude terrestrial ecosystems (Campbell et al., 2010; Männistö et al., 2007; Pankratov et al., 2008). However, the phylum contains a very limited number of cultured representatives mainly due to difficulties in cultivation. Even if some species could be cultured, these members may grow too slowly to develop visible colonies within a week (Eichorst et al., 2007; Koch et al., 2008; Pankratov et al., 2008, 2012). As a result, little is known about the ecological roles of acidobacteria in their natural habitats.

The genus *Acidipila* was established by Okamura *et al.* (2011) as a member of subdivision 1 with *Acidipila rosea*

A supplementary figure is available with the online Supplementary Material.

as the type species. Characteristics of cell morphology, pigmentation, motility, carbon nutrition and cellular fatty acid profiles clearly distinguished this genus from the closest genus, *Acidobacterium*.

Dinghushan biosphere reserve (DHSBR) lies in the middle part of Guangdong Province, PR China (23° 10' N 112° 31' E). The mean annual temperature and rainfall are 21 °C and 1927 mm, respectively (Huang & Fan, 1982; Mo *et al.*, 2003). The soil in the study sites is acidic, lateritic, red earth with a pH of 4.0–4.5. Liu *et al.* (2012) studied the bacterial communities in the forest soils of DHSBR by means of 16S rRNA gene clone library analyses, and found that *Acidobacteria* dominate in these acidic soils. A strain designated DHOF10^T was isolated from a soil sample recently collected from DHSBR. Here, we report the characteristics of this strain and propose that it represents a novel species of the genus *Acidipila*.

The soil sample was collected from the upper (0-25 cm) layer of forest soil in DHSBR in September 2013. For isolation, a medium modified from MM1 (Pankratov & Dedysh, 2010), designated MM1F medium (0.04 g MgSO₄. 7H₂O, 0.02 g CaCl₂. 2H₂O, 0.05 g yeast extract and 0.5 g fructose per litre distilled water; pH 5.0), was used. Each plate was inoculated with 100 µl soil suspension (serially diluted in PBS) and incubated at 28 °C for 2 weeks. Cultivation resulted in the isolation of a strain

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The GenBank/EMNL/DDBJ accession number for the 16S rRNA gene sequence of strain DHOF10^T is KM083127.

that was designated DHOF10^T. The isolate was stored in 25 % glycerol at -80 °C and in liquid nitrogen.

Genomic DNA was extracted using a commercial bacterial genomic DNA-extraction kit (Omega Bio-Tek). The 16S rRNA gene was amplified using the universal primers 27F and 1492R (Lane, 1991). The amplified gene fragment was cloned into pMD18-T vector (TaKaRa) using the TA cloning method. Sequences were determined by the Sanger sequencing method employing a 3730XL DNA analyser (Applied Biosystems) and the AmpliTag FS Big Dye terminator cycle sequencing kit (Applied Biosystems). The full sequence of the 16S rRNA gene was compiled using EditSeq software (DNASTAR) and compared with those available in the GenBank database using the BLAST program (Altschul et al., 1997). Related sequences were retrieved from the GenBank database and aligned using the program CLUSTAL W of MEGA 5.05 software (Tamura et al., 2011). Phylogenetic reconstructions were also performed in MEGA 5.05 using the neighbour-joining and maximum-likelihood methods with maximum composite likelihood and Tamura-Nei model (Tamura et al., 2011). Bootstrap analyses were based on 1000 replications. Evolutionary distances were calculated using MEGALIGN software (DNASTAR).

16S rRNA gene sequence analysis showed that strain DHOF10^T has the closest relationship to Acidipila rosea AP8^T with 97.3 % sequence similarity. Among the species with validly published names, Terriglobus tenax DRP 35^T (95.4 % 16S rRNA gene sequence similarity), Acidobacterium capsulatum ATCC 51196^T (95.3 %) and Acidicapsa *ligni* WH120^T (95.2 %) were the closest relatives of the isolate. DNA-DNA hybridization was omitted since the 16S rRNA gene sequence similarity was lower than the threshold value (98.2 %) proposed by Meier-Kolthoff et al. (2013). In treeing analyses, strain DHOF10^T always belonged to a phylogenetic lineage defined by Acidobacterium capsulatum ATCC 51196^T and Acidipila rosea AP8^T, with Acidipila rosea AP8^T as its nearest phylogenetic neighbour irrespective of the algorithm used. The maximumlikelihood tree is not shown since the topology of it was similar to that of the neighbour-joining tree (Fig. 1). The above analyses based on 16S rRNA gene sequences showed that strain DHOF10^T should represent a novel species of the genus Acidipila.

Strain DHOF10^T was maintained on GYS agar (pH 5.0) (Hiraishi *et al.*, 1998) at 28 °C during the course of this study. Since *Acidipila rosea* AP8^T was the closest relative of the novel isolate, it was used as the reference organism in this study. For all comparative tests between these two strains, both strains were grown on the same medium under the same conditions.

Strain DHOF10^T formed circular (1.9–2.6 mm in diameter), smooth, slightly convex, mucous colonies after growing on GYS agar at 28 °C for 2 weeks. Colonies were slightly translucent and white to light beige. Cells of strain DHOF10^T were Gram-negative, non-motile cocci or coccobacilli (0.6–1.1 μ m wide and 1.0–1.4 μ m long), multiplied by binary fission and occurred mainly singly with a small amount in pairs (Fig. 2a).

For transmission electron microscopic observations, cells grown on GYS agar for 2 weeks were negative stained using 2 % uranyl acetate in water. For ultrathin sections, cells were pre-fixed with 1.5 % (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 6.5) for 4 h at 4 °C and posted-fixed in 1 % (w/v) OsO4 in the same buffer for 4 h at 20 °C. After dehydration in an ethanol series, samples were embedded in Spurr epoxy resin and ultrathin sections were performed with a diamond knife and mounted on uncoated copper grids. All electron micrographs were taken with a IEOL transmission electron microscope (IEM 100CXII or JEM 1400). Electron microscopic observations showed that strain DHOF10^T possessed a typical Gramnegative cell wall that was covered with a thick capsule. No flagellum or pilus was seen, but numerous glycogenlike inclusions were regularly observed (Fig. 2b, c).

Motility and anaerobic growth were tested using the classical protocol where the bacteria were inoculated by piercing into a semisolid GYS medium (0.5 % agar, pH 4.5) in a test tube. Bacterial growth was observed on the top third and along the inoculate line only, indicating that strain $DHOF10^{T}$ was aerobic and non-motile.

Growth of strain DHOF10^T under a variety of conditions, including pH 3.0–9.0 and NaCl concentrations of 0–5.0 % (w/v) was examined using liquid GYS medium. Phosphoric acid-citrate and Tris/HCl were used as buffer systems to adjust the pH of the medium. Growth was assessed by measuring the OD₆₀₀ of the liquid culture after incubation for 2 weeks. Growth at 4, 10, 16, 23, 28, 33, 37 and 42 °C was determined on GYS agar. Strain DHOF10^T grew at pH 3.5–8.0, with optimum growth at pH 4.0–4.5. Growth of the isolate was detected at temperatures between 10 and 37 °C, but not at 4 or 42 °C; optimal growth occurred between 28 and 37 °C. Growth was inhibited with NaCl concentrations above 1.0 % (w/v).

The range of carbon sources utilized by strain DHOF10^T was examined in three replicates using API 50 CH and API 20 NE kits (bioMérieux). Some items unavailable in API kits were tested by cultivating the bacteria in 96-well plates with mineral base RM2 medium (Hiraishi & Kitamura, 1984) supplemented with the carbon source to be tested (10 mM) and 0.03 % yeast extract (Okamura et al., 2011) for up to 14 days at 28 °C. Yeast extract was required for good growth on single carbon sources (Männistö et al., 2011). The control incubations were run in parallel under the same conditions but without the examined substrate. Growth was assessed by measuring the OD_{600} . The carbon sources tested and their effects on growth are given in the species description and Table 1. The results on the ability of Acidipila rosea AP8^T to utilize L-serine, tartrate, L-histidine, inositol, gluconate and ethanol in this study differed from those reported by (Okamura et al., 2011). Like Acidipilla rosea AP8^T, sugars were good carbon sources for the



Fig. 1. 16S rRNA gene sequence-based neighbour-joining tree (1353 nt positions used for analysis) showing the phylogenetic relationships of strain DHOF10^T and related representatives of subdivision 1 of the phylum *Acidobacteria*. Bootstrap values (1000 data resamplings) >50 % are shown at nodes. *Aridibacter famidurans* A22_HD_4H^T (GenBank accession no. KF245634), *Aridibacter kavangonensis* Ac_23_E3^T (KF245633), *Blastocatella fastidiosa* A2-16^T (JQ309130), '*Candidatus* Chloracidobacterium thermophilum' (CP002514) and *Pyrinomonas methylaliphatogenes* K22^T (AM749787) of subdivision 4 were used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

growth of strain DHOF10^T. From the six amino acids and nine sugar-alcohols tested, few were utilized by strain DHOF10^T. Growth substrates specific for strain DHOF10^T compared to *Acidipilla rosea* AP8^T were the sugars starch, glycogen, gentiobiose, L-xylose and L-fucose, the organic acids gluconate and succinate, the sugar-alcohols inositol, D-mannitol and xylitol, and the glycoside salicin. Ethanol, D-arabinose and L-glutamate promoted growth of *Acidipila rosea* AP8^T but not of strain DHOF10^T.

Enzyme activity profiles were tested using API 20 NE and API ZYM kits (bioMérieux). Catalase activity was determined by assessing bubble production in 3 % (v/v)

 H_2O_2 , and oxidase activity was determined using 1 % (w/v) tetramethyl-p-phenylenediamine (Gerhardt *et al.*, 1981). The enzyme activities of strain DHOF10^T, compiled in Table 1 and the species description, were similar to those of *Acidipila rosea* AP8^T except β -glucuronidase activity, which was negative in strain DHOF10^T but weakly positive in *Acidipila rosea* AP8^T.

Cell biomass for DNA extraction as well as cellular fatty acid, intact polar lipids and isoprenoid quinone analyses was obtained by scrapping colonies grown on GYS agar (pH 4.5) at 28 °C for 7–14 days. Cellular fatty acids of strain DHOF10^T and *Acidipila rosea* AP8^T were saponified,



Fig. 2. Micrographs of the cells of strain DHOF10^T. (a) Optical micrograph of the cells; (b) electron micrograph of a negatively stained cell; (c) electron micrograph of an ultrathin section of a cell. Ca, capsule; CM, cytoplasmic membrane; OM, outer membrane; In, glycogen-like inclusions. Bars, 1 μ m (a, b) and 0.5 μ m (c).

methylated, extracted and analysed using the standard method of the Microbial Identification System (MIDI Sherlock version 6.1; library TSBA6) according to the manufacturer's instructions (Sasser, 1990). Menaquinones were isolated according to Collins (1985) and separated by HPLC (Tamaoka *et al.*, 1983). Genomic DNA was extracted and purified by a standard phenol/chloroform extraction followed by ethanol precipitation (Sambrook *et al.*, 1989). Polar lipids were extracted and analysed by two-dimensional TLC according to Minnikin *et al.* (1984). The G+C content of the DNA was determined by reverse-phase HPLC of nucleosides according to Mesbah *et al.* (1989).

Strain DHOF10^T contained iso- $C_{15:0}$ (48.6 %), $C_{18:1}\omega_9c$ (17.0 %) and $C_{16:1}\omega_7c$ (11.5 %) as major cellular fatty acids (Table 2). High amounts of iso- $C_{15:0}$ are typical for all previously described subdivision 1 acidobacteria. Significant amounts of $C_{18:1}\omega_9c$ have so far been detected only in *Acidobacterium capsulatum*. However, in our study, $C_{18:1}\omega_9c$ was the second most abundant component in strain DHOF10^T but was absent from the profile of *Acidipila rosea* AP8^T, consistent with the result reported by

Okamura *et al.* (2011). The polar lipids profile of strain DHOF10^T (Fig. S1, available in the online Supplementary Material) was found to consist of phosphatidylethanolamine, an unidentified aminolipid, an unidentified phospholipid, two unidentified aminophospholipids and two unidentified polar lipids, while that of *Acidipila rosea* AP8^T lacked an unidentified aminolipid (AL) and an unidentified polar lipid (L4) but possessed two unidentified polar lipids (L2 and L3). Similar to other described subdivision 1 acidobacteria, strain DHOF10^T contained menaquinone-8 (MK-8) as the predominant isoprenoid quinone. The G+C content of the DNA of strain DHOF10^T was 56.3 mol%.

The characteristics that differentiate strain DHOF10^T from other closely related species are summarized in Table 1. Strain DHOF10^T could be clearly distinguished from *Acidipila rosea* AP8^T by colony colour, fatty acid profile and range of pH and temperature for growth. Furthermore, strain DHOF10^T differed from *Acidipila rosea* AP8^T by the absence of β -glucuronidase activity and the ability to utilize inositol, salicin, starch, glycogen, gentiobiose, gluconate, succinate, L-xylose, D-mannitol, xylitol and L-fucose, and the inability to utilize ethanol, D-arabinose and L-glutamate. 16S rRNA gene sequence similarity and cell size also distinguished strain DHOF10^T from *Acidipila rosea* AP8^T and other closely related species (Table 1).

These morphological, physiological and chemotaxonomic data, together with 16S rRNA gene sequence analyses, provide sufficient evidence to support the conclusion that strain DHOF10^T represents a novel species of the genus *Acidipia*, for which the name *Acidipila dinghuensis* sp. nov. is proposed.

Description of Acidipila dinghuensis sp. nov.

Acidipila dinghuensis (ding.hu.en'sis. N.L. fem. adj. dinghuensis pertaining to Mount Dinghu, PR China, where the soil samples containing this strain were collected).

Cells are Gram-negative, capsule-forming, non-motile, aerobic cocci or coccobacilli $(0.6-1.1 \times 1.0-1.4 \ \mu m)$ dividing by binary fission. Colonies on GYS agar are circular (1.9-2.6 mm in diameter), smooth, convex, slightly translucent, mucous, and white to light beige. Growth occurs at pH 3.5-8.0 (optimum pH 4.0-4.5) and at 10-37 °C (optimum 28-37 °C). NaCl inhibits growth at concentrations above 1.0 % (w/v). Carbon sources utilized include Dglucose, D-galactose, D-mannose, D-xylose, L-xylose, L-arabinose, L-rhamnose, L-fucose, D-fructose, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, turanose, gentiobiose, raffinose, melezitose, starch, glycogen, inositol, inulin, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, methyl β -D-xylopyranoside, N-acetylglucosamine, amygdalin, aesculin, arbutin, salicin, gluconate, succinate, pyruvate, tartrate, capric acid, D-ornithine, D-mannitol, glycerol, xylitol, L-serine, tryptone (0.1 %, w/v), Casamino acids (0.1 %, w/v) and yeast extract (0.1 %, w/v). Does not utilize D-ribose, D-arabinose, L-sorbose, D-sorbitol,

Characteristic	1	2	3	4	ъ
Origin	Forest soil	Acid mine drainage/acidic soil	Acidic mineral environment	Sphagnum peat	Sphagnum peat/decaying wood
Cell size (μm)	$0.6 - 1.1 \times 1.0 - 1.4$	0.5–0.8	$0.3 - 0.8 \times 1.1 - 2.3$	$0.4 - 0.6 \times 2.0 - 10.0$	$0.6 - 0.9 \times 1.0 - 3.0$
Capsule formation	+	+	+	ND	+
Motility	I	1	+	+	1
pH for growth					
Range	3.5-8.0	3.0-6.0	3.0-6.0	3.0-7.5	3.5-7.3
Optimum	4.0 - 4.5	4.5	ND	4.5 - 5.0	5.0-5.5
Temperature for growth (°C)					
Range	10–37	22–37	25-37	4–35	10–33
Optimum	28–37	30	ND	20–28	22–28
Colony colour	White or light beige	Pale pink	Pale orange or orange	Milk-white or beige	Pale pink
DNA G+C content (mol%)	56.3	59.5*	59.7-60.8	57.6	54.1
Carbon source utilization					
Inositol	+	I	ND	I	ND
Salicin	+	I	ND	+	+
Starch	+	1	+	+	+
Glycogen	+	1	ND	ND	ND
Gentiobiose	+	1	+	ND	ND
Gluconate	+	1	ND	ND	ND
Succinate	+	1	ND	I	1
L-Xylose	W	1	ND	ND	ND
D-Mannitol	Μ	I	I	I	I
Xylitol	W	1	ND	ND	ND
L-Fucose	W	1	ND	ND	ND
Ethanol	I	+	1	I	1
D-Arabinose	I	W	ND	+	+
L-Glutamate	I	W	1	ND	ND
Enzymic activities					
Catalase	I	1	+	Μ	+
eta-Glucuronidase	I	W	+	+	+
Alkaline phosphatase	+	+	1	I	+
Esterase lipase (C 8)	W	+	I	I	+
Leucine arylamidase	+	+	1	I	+

Table 1. Major characteristics that distinguish strain DHOF10^T from other closely related species

Table 2. Whole-cell fatty acid compositions of strain DHOF10^T and other closely related species

Strains: 1, DHOF10^T (data from this study); 2, *Acidipila rosea* AP8^T (this study); 3, *Acidobacterium capsulatum* ATCC 51196^T and 1372 (Okamura *et al.*, 2011); 4, *Telmatobacter bradus* TPB6017^T (Pankratov *et al.*, 2012); 5, *Acidicapsa borealis* KA₁^T (Kulichevskaya *et al.*, 2012). Strain DHOF10^T and *Acidipila rosea* AP8^T were grown on GYS agar (pH 4.5) at 28 °C for 7–14 days. Values are percentages of total fatty acids. Major components (>5 %) are highlighted in bold. –, Not detected.

Fatty acid	1	2	3	4	5
Saturated					
C _{14:0}	1.7	0.6	t-1.0	0.1	0.4
C _{15:0}	_	-	1.0	_	_
C _{16:0}	8.6	9.7	4.0-4.3	1.5	5.9
C _{17:0}	2.7	4.5	1.4-1.9	0.2	-
C _{18:0}	3.7	1.3	13.3-13.8	0.5	1.6
C _{20:0}	-	1.0	_	_	-
Unsaturated					
$C_{14:1}\omega 5c$	_	0.2	_	_	-
$C_{15:1}\omega_{6c}$	-	1.6	_	_	-
$C_{16:1}\omega7c$	11.5†	24.6†	3.5-4.0	0.4	0.8
$C_{17:1}\omega 8c$	1.5	0.3	2.3-3.9	_	-
$C_{18:1}\omega_9c$	17.0	-	12.4-15.2	_	1.9
Methyl-branched					
iso-C _{13 : 0}	_	0.2	_	_	-
iso-C _{15 : 0}	48.6	52.3	55.8-65.0	69.8	55.4
iso-C _{15 : 1} F	_	_	_	1.6	-
iso-C _{15 : 1} ω6 <i>c</i>	_	_	_	_	1.9
anteiso-C _{15:0}	-	-	_	_	0.3
iso-C _{16 : 0}	_	0.2	_	_	-
iso-C _{17 : 0}	_	2.6	2.2-3.0	5.4	3.8
iso-C _{17 : 1} ω8c	_	-	_	_	25.5
iso- $C_{17 \pm 1}\omega 8t$	_	_	_	_	0.6
iso-C _{17 : 1} ω9c	_	_	_	14.8	-
anteiso-C _{17:0}	_	_	_	1.1	2.0
Summed features*					
1	1.1	-	-	1.9§	-
8	_	0.5	-	_	-
9	2.3	0.4	_	-	-

*Summed features are groups of two or more fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 contained iso- $C_{15:1}$ H and/or $C_{13:0}$ 3-OH; summed feature 8 contained $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$; summed feature 9 contained iso- $C_{17:1}\omega 9c$ and/or $C_{16:0}$ 10-methyl.

†Determined as $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ in this study.

‡Determined as $C_{16:1}\omega 7c$ and/or $C_{15:0}$ 2-OH by Pankratov *et al.* (2012).

\$Determined as iso- $C_{15:1}$ I and/or $C_{13:0}$ 3-OH by Pankratov *et al.* (2012).

D-lyxose, D-tagatose, D-fucose, phenylacetic acid, octoic acid, oxalic acid, L-alanine, L-histidine, DL-aspartic acid, Dglucuronic acid, 2-ketogluconate, 5-ketogluconate, adipic acid, malate, citrate, acetate, lactate, benzoate, L-glutamate,

Characteristic		1	2	4	Ĵ
Cystine arylamidase	M	M	Ι	I	+
Trypsin	Μ	M	Ι	I	ND
α-Chymotrypsin	I	Ι	I	Ι	-/+
α-Galactosidase	Μ	+	W	+	+
α-Glucosidase	Μ	M	M	+	+
N -Acetyl- β -glucosaminidase	Μ	+	+	+	+
α-Mannosidase	I	I	I	Ι	+
∞-Fucosidase	Μ	+	Ι	+	+
*Data from Olcomura at al (2011)					
Data HUIII UKAIIIMI a El Mi. (2011)	_				

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Table 1. cont.

methanol, ethanol, dulcitol, erythritol, D-adonitol, D-arabitol or L-arabitol as carbon sources. Positive result in tests for the following enzyme activities: acid phosphatase, alkaline phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, α fucosidase, N-acetyl- β -glucosaminidase, leucine arylamidase, cystine arylamidase, valine arylamidase, trypsin, esterase (C4) and esterase lipase (C8); negative result in tests for α -mannosidase, β -glucuronidase, α -chymotrypsin, lipase (C14), reduction of nitrates, indole production, glucose fermentation, arginine dihydrolase, urease, protease, catalase and oxidase. The major cellular fatty acids are iso- $C_{15:0}$, $C_{18:1}\omega_9c$ and $C_{16:1}\omega_7c$. The polar lipids consist of phosphatidylethanolamine, an unidentified aminolipid, an unidentified phospholipid, two unidentified aminophospholipids and two unidentified polar lipids. The quinone is MK-8.

The type strain, $DHOF10^{T}$ (=CGMCC 1.13007^T= KCTC 42631^T), was isolated from soil in Dinghushan Biosphere Reserve, Guangdong Province, PR China. The G+C content of the DNA of the type strain is 56.3 mol%.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (project nos J1310025 and 31030015).

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