Edaphobacter dinghuensis sp. nov., an acidobacterium isolated from lower subtropical forest soil

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An aerobic bacterium, designated DHF9^T, was isolated from a soil sample collected from the lower subtropical forest of Dinghushan Biosphere Reserve, Guangdong Province, PR China. Cells were Gram-stain-negative, non-motile, short rods that multiplied by binary division. Strain DHF9^T was an obligately acidophilic, mesophilic bacterium capable of growth at pH 3.5–5.5 (optimum pH 4.0) and at 10–33 °C (optimum 28–33 °C). Growth was inhibited at NaCl concentrations above 2.0 % (w/v). The major fatty acids were iso-$C_{15:0}$, $C_{16:0}$ and $C_{16:1}\omega7c$. The polar lipids consist of phosphatidylethanolamine, two unidentified aminolipids, two unidentified phospholipids, two unidentified polar lipids and an unidentified glycolipid. The DNA G+C content was 57.7 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain belongs to the genus *Edaphobacter* in subdivision 1 of the phylum *Acidobacteria*, with the highest 16S rRNA gene sequence similarity of 97.0 % to *Edaphobacter modestus* Jbg-1^T. Based on phylogenetic, chemotaxonomic and physiological analyses, it is proposed that strain DHF9^T represents a novel species of the genus *Edaphobacter*, named *Edaphobacter dinghuensis* sp. nov. The type strain is DHF9^T (=DSM 29920^T=CGMCC 1.12997^T).

The *Acidobacteria* is a large and diverse bacterial phylum that has been detected in a wide variety of environments based on 16S rRNA gene-based surveys. The phylum is currently divided into 26 phylogenetically defined subdivisions (Barns *et al.*, 2007), of which the subdivisions 1, 2, 3, 4 and 6 are detected most abundantly in soil environments (Janssen, 2006; Jones *et al.*, 2009). The biodiversity of the phylum is potentially as great as that of the phylum *Proteobacteria* (Janssen, 2006). However, so far there are only 31 species belonging to 16 genera in six of the subdivisions in this phylum with validly published names, with most of them belonging to subdivision 1.

Koch *et al.* (2008) isolated two bacterial strains from alpine and forest soils, and proposed that they represented two species of a novel genus, *Edaphobacter*, as a member of subdivision 1. Cells of the genus *Edaphobacter* are beige, short, ovoid rods that do not form a capsule or spore, and stain Gram-negative. Both species of this genus tested positive for catalase, whereas only *Edaphobacter modestus* contained cytochrome *c* oxidase. Typical growth substrates are glucose, lactose, glutamate and glutamine.

Dinghushan Biosphere Reserve (DHSBR) lies in the middle part of Guangdong Province, PR China (23° 10′ N 112° 31′ E). The soil at the study sites is acidic, lateritic red earth with a pH of 4.0–4.5. The mean annual temperature and rainfall are 21 °C and 1927 mm, respectively (Huang & Fan, 1982; Mo *et al.*, 2003). Bacterial communities in the forest soils of DHSBR were investigated using 16S rRNA gene clone library analysis and the results showed that *Acidobacteria* dominate in these acidic soils (Liu *et al.*, 2012). Recently, a bacterial strain designated DHF9^T was isolated from the same habitat. Here, we report the taxonomic characteristics of strain DHF9^T and propose the isolate represents a novel species of the genus *Edaphobacter*.

Soil samples were collected from the upper (0–25 cm) layer of forest soil in DHSBR in May 2013. For isolation, a medium modified from MM1 (Pankratov & Dedysh, 2010), designated MM1F medium (0.04 g $MgSO_4 . 7H_2O$, 0.02 g $CaCl_2 . 2H_2O$, 0.05 g yeast extract and 0.5 g fructose in 1 l distilled water; pH 5.0), was used. The soil samples were serially diluted with PBS buffer (pH 5.0) and plated on MM1F

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DHF9^T is KM083126.

A supplementary figure is available with the online Supplementary Material.

medium. Each plate was inoculated with 100 µl suspension and incubated at 28 °C for 2 weeks. Cultivation resulted in the isolation of a strain that was designated DHF9^T. The strain was maintained on GYS agar (GYS medium solidified with 1.5 % agar; pH 5.0) (Hiraishi *et al.*, 1998) for several months initially. To improve the growth rate of strain DHF9^T, the following media (all pH 5.0) were tested: MM1F medium, HD medium (1 : 10-diluted) (Koch *et al.*, 2008), GYS medium and AI medium [0.1 g glucose, 0.1 g pectin, 0.1 g soluble starch, 0.1 g xylan, 0.1 g KH_2PO_4, 0.2 g $(NH_4)_2SO_4$, 0.05 g $MgSO_4 . 7H_2O$, 0.02 g $CaCl_2 . 2H_2O$, 0.2 g NaCl and 0.1 g yeast extract in 1 l distilled water]. Compared to AI medium and MM1F medium, 1 : 10-diluted HD medium and GYS medium were better media for strain DHF9^T. To be consistent with the two species of the genus *Edaphobacter* with validly published names, strain DHF9^T was maintained on 1 : 10-diluted HD medium. The isolate was stored both in 25 % glycerol in a freezer at −80 °C and in liquid nitrogen. Genomic DNA was extracted using a commercial bacterial genomic DNA extraction kit (Omega Bio-Tek). The nearly complete 16S rRNA gene was amplified with primers 27F and 1492R (Lane, 1991), and cloned into the pMD18-T vector (TaKaRa) using the TA cloning method. Sequences were determined by Sanger sequencing employing the 3730XL DNA analyser (Applied Biosystems) and the AmpliTaq 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 seqeuences available in the GenBank database using the BLAST program (Altschul *et al.*, 1997). The 16S rRNA gene sequences of related taxa were also retrieved from the GenBank database. Multiple alignment of sequence data and phylogenetic analyses were carried out using the program CLUSTAL W with MEGA5 software package (Tamura *et al.*, 2011). Trees were reconstructed using neighbour-joining and maximum-likelihood methods with maximum-composite-likelihood and Tamura–Nei model, respectively, for nucleotide substitutions (Tamura *et al.*, 2011). The significance levels of interior branch points were determined by bootstrap analysis (1000 data resamplings). Evolutionary distances were calculated using MEGALIGN software (DNASTAR).

16S rRNA gene sequence analysis showed that strain DHF9^T has the closest relationship to *E. modestus* Jbg-1^T (97.0 % 16S rRNA gene sequence similarity) and *Edaphobacter aggregans* Wbg-1^T (96.5 %). The strain also displayed 94.8–96.2 % 16S rRNA gene sequence similarity to members of the genus *Terriglobus*, 94.0–96.2 % to members of the genus *Granulicella*, and 95.1 % to *Bryocella elongata* SN10^T. DNA–DNA hybridization was omitted since the 16S rRNA gene sequence similarity value between strain DHF9^T and its closest described species *E. modestus* Jbg-1^T (97.0 %) is lower than the threshold (98.2 %) proposed by Meier-Kolthoff *et al.* (2013). A neighbour-joining phylogenetic tree (Fig. 1) showed that the isolate was placed within a phylogenetic lineage defined by members of the genus *Edaphobacter*, with *E. modestus* Jbg-1^T as the closest relative. The topology of the maximum-likelihood tree (data not shown) was similar to that of the neighbour-joining tree. The evolutionary distances between strain DHF9^T and *Edaphobacter aggregans* Wbg-1^T and *Edaphobacter modestus* Jbg-1^T are 0.034 and 0.038, respectively, which are higher than that between the two recognised species of the genus *Edaphobacter* (0.021), indicating that strain DHF9^T should represent a novel species of the genus *Edaphobacter*.

The morphology of strain DHF9^T was studied using cultures grown on both liquid and solid media. After cultivation for 2 weeks on 1 : 10-diluted HD agar, colonies were circular, smooth, slightly convex, beige, translucent, mucous and 1.0–3.0 mm in diameter. Cells were non-spore-forming, non-capsule-forming, non-motile, short rods (0.5–0.7 µm wide and 0.8–1.4 µm long) that stained Gram-negative, reproduced by binary fission and occurred singly or in pairs (Fig. 2a).

Motility and anaerobic growth were tested using the classical protocol where the bacteria were inoculated by piercing into a semi-solid GYS medium (0.5 % agar, pH 4.0) in a test tube. Bacterial growth was observed on the top third and along the inoculate line only, indicating that strain DHF9^T was aerobic and non-motile.

For transmission electron microscopy, cells were grown on GYS agar for 2 weeks. Whole-cell negative staining was performed using 2 % uranyl acetate in water. For preparation of 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) osmium tetroxide 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 cut with a diamond knife and mounted on uncoated copper grids. All electron micrographs were taken with a JEOL transmission electron microscope (JEM 100CXII or JEM 1400). Electron microscopy revealed that strain DHF9^T has a typical cell-wall structure of Gram-negative bacteria. However, unlike *E. modestus* Jbg-1^T and *E. aggregans* Wbg-1^T, no extracellular tubular structures or small fibres were revealed in strain DHF9^T. Numerous glycogen-like inclusions were also regularly observed (Figs 2b, c).

Growth ranges and optima of temperature were determined on solid medium HD (1 : 10-diluted, 2 % agar) with temperatures ranging from 4 to 42 °C. Growth ranges and optima of pH were investigated in liquid medium HD (1 : 10-diluted) at pH 3.0–9.0 using phosphoric acid-citrate or Tris/HCl as a buffer system. Salt tolerance was tested in liquid medium HD (1 : 10-diluted) supplemented with 0–5 % (w/v) NaCl at intervals of 0.5 %. Growth was monitored by measuring optical density at 600 nm after incubation for 2 weeks. Growth was detected between 10 and 33 °C, with optimal growth occurring between 28 and 33 °C. The pH range for growth was pH 3.5–5.5, (optimum pH 4.0), indicating that the isolate is acidophilic. Growth of strain DHF9^T was inhibited at NaCl concentrations above 2.0 % (w/v).

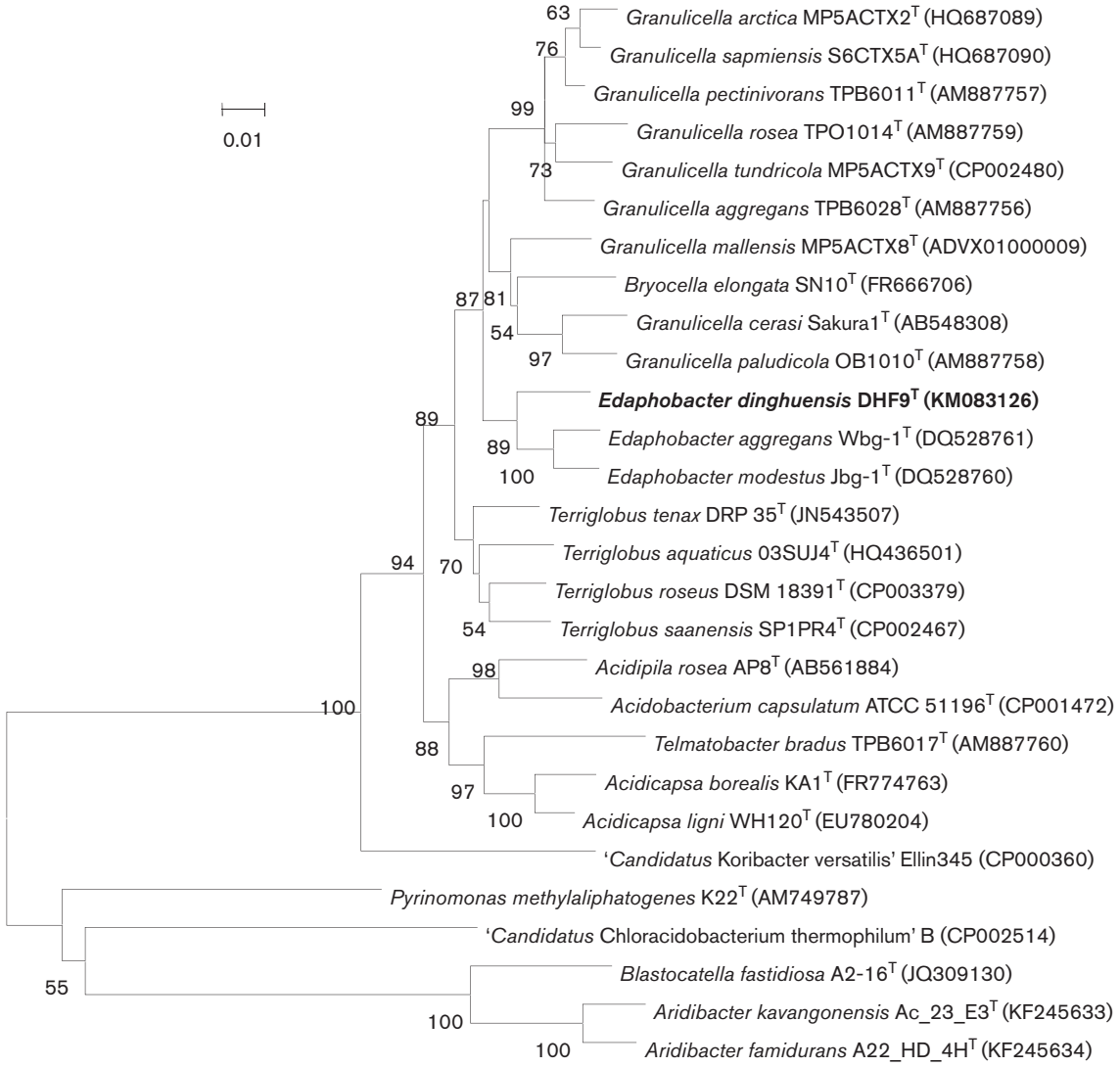

Fig. 1. 16S rRNA gene sequence-based neighbour-joining tree (1353 nt positions used for analysis) showing phylogenetic relationships of strain DHF9^T and related representatives of subdivision 1 of the phylum *Acidobacteria*. Bootstrap values (1000 data resamplings) >50 % are shown at nodes. *Aridibacter 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' B (CP002514) and *Pyrinomonas methylaliphatogenes* K22^T (AM749787) of subdivision 4 were used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

The range of carbon sources that strain DHF9^T and the reference strain *E. modestus* Jbg-1^T utilized were tested using API 50CH and API 20NE kits (bioMérieux) and in 96-well plates with mineral base RM2 (Hiraishi & Kitamura, 1984) supplemented with 10 mM carbon source and 0.03 % yeast extract (Okamura *et al.*, 2011). Yeast extract was required for good growth on single carbon sources (Männistö *et al.*, 2011). Control incubations were run in parallel under the same conditions but without the substrate examined. Growth was monitored by measuring optical density at 600 nm, and the final reading was taken after 2 weeks of incubation at 28 °C. The carbon sources tested and their effects on growth are given in the species description and are shown in Table 1. Like *E. modestus* Jbg-1^T and *E. aggregans* Wbg-1^T, strain DHF9^T had poor capacity to utilize amino acids, and sugars were the preferred growth substrates. For the sugars tested, lactose, D-glucose, Casamino acids (0.1 %, w/v) and yeast extract (0.1 %, w/v) were utilized by all three strains. None of the strains utilized dulcitol, D-fucose, melezitose, ribitol, L-sorbose, malate, citrate, L-alanine, benzoate, caprylate, L-histidine, methanol, L-serine, ethanol, acetate or lactate, while cellobiose, D-arabinose, D-galactose, D-mannose, gluconic acid, maltose, *N*-acetylglucosamine, pyruvate, raffinose and succinate were utilized by strain DHF9^T only.

Enzyme activity profiles, urease hydrolysis, indole production and the Hugh–Leifson test were analysed with

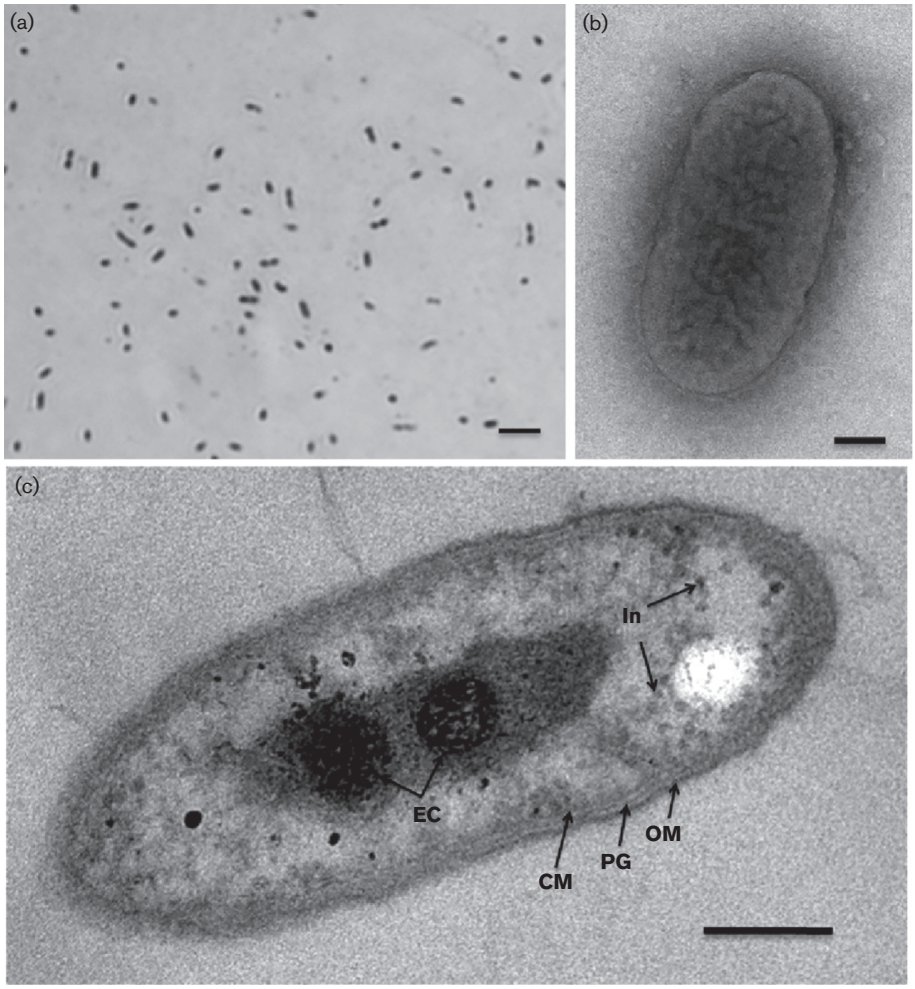

Fig. 2. Micrographs of the cells of strain DHF9^T. (a), Optical micrograph of the cells; (b), electron micrograph of a negatively stained cell; (c), electron micrograph of an ultrathin section of a cell. CM, cytoplasmic membrane; OM, outer membrane; PG, peptidoglycan layer; EC, electron-dense centre; In, glycogen-like inclusions. Bars, 2 μm (a) and 0.2 μm (b and c).

API 20NE 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). Strain DHF9^T showed the following enzyme activities: *N*-acetyl-*β*-glucosaminidase, alkaline phosphatase, esterase lipase (C8), *β*-glucosidase, esterase (C4), leucyl arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-fucosidase and α-mannosidase. The absence of enzyme activities of oxidase, valyl arylamidase and α-chymotrypsin clearly distinguished strain DHF9^T from *E. modestus* Jbg-1^T.

Cell biomass of strain DHF9^T for cellular fatty acid analyses was harvested from batch cultures grown on 1 : 10 diluted HD medium (pH 4.5) at 28 °C for 14 days. *E. modestus* Jbg-1^T maintained on 1 : 10 diluted HD medium (pH 4.5) was used as a reference organism. Cellular fatty acids were saponified, methylated, extracted and analysed by using the standard method of the Sherlock Microbial Identification System (MIDI, version 6.1; library TSBA6) according to the manufacturer's instructions (Sasser, 1990). Strain DHF9^T contained iso-$C_{15:0}$ (36.7 %), $C_{16:0}$ (10.2 %) and $C_{16:1}\omega7c$ (31.6 %) as the major cellular fatty acids (Table 2). High amounts of iso-$C_{15:0}$ and $C_{16:1}\omega7c$ are typical for the genus *Edaphobacter* and the closely related genera *Terriglobus*, *Bryocella* and *Granulicella*.

Polar lipids were extracted and analysed by two-dimensional TLC according to Minnikin *et al.* (1984). The total polar lipids of strain DHF9^T (Fig. S1, available in the online Supplementary Material) were phosphatidylethanolamine, two unidentified aminolipids, two unidentified phospholipids, two unidentified polar lipids and an unidentified glycolipid. The G+C content of the DNA was determined by reverse-phase HPLC of nucleosides according to Mesbah *et al.* (1989). The DNA G+C content of strain

DHF9^T is 57.7 mol%, which is slightly higher than the values reported for recognized species of the genus *Edaphobacter* (55.8 and 56.9 mol%; Koch *et al.*, 2008).

Strain DHF9^T could be distinguished from the two recognized species of the genus *Edaphobacter* by acidophilic level (Table 1). The similarity and evolutionary distances based on 16S rRNA gene sequences among strain DHF9^T and the two members of the genus *Edaphobacter* also provide support for the conclusion that strain DHF9^T should represent a novel species of the genus *Edaphobacter*. Furthermore, the ability to utilize cellobiose, D-arabinose, D-galactose, D-mannose, maltose, myo-inositol, tartrate *N*-acetylglucosamine, pyruvate, raffinose and succinate, the inability to utilize L-glutamate, and the negative reaction of catalase, valyl arylamidase and α-chymotrypsin also differs from the two recognized species the genus *Edaphobacter* (Table 1). The negative reaction of oxidase and inability to utilize D-lyxose, D-mannitol, D-sorbitol and xylitol distinguish strain DHF9^T from *E. modestus* Jbg-1^T (Table 1).

In conclusion, based on phylogenetic, morphological, physiological and chemotaxonomic analyses, strain DHF9^T represents a novel species of the genus *Edaphobacter*, for which the name *Edaphobacter dinghuensis* sp. nov. is proposed.

Description of *Edaphobacter dinghuensis*, sp. nov.

Edaphobacter dinghuensis (ding.hu.en′sis. N.L. fem. adj. *dinghuensis* pertaining to Mount Dinghu, PR China, the source of the soil from which the type strain was isolated).

Cells are Gram-stain-negative, aerobic, short rods measuring 0.8–1.4 × 0.5–0.7 μm, and multiply by binary fission. On 1 : 10-diluted HD medium, colonies are circular, smooth, slightly convex, light beige, translucent, mucous and 1.0–3.0 mm in diameter. The temperature range for growth is 10–33 °C (optimum 28–33 °C). The pH range for growth is pH 3.5–5.5 (optimum pH 4.0). Usable carbon sources are D-arabinose, L-arabinose, D-xylose, D-galactose, D-glucose, D-mannose, D-fructose, inositol, methyl α-D-mannopyranoside, *N*-acetylglucosamine, amygdalin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, starch, gentiobiose, turanose, gluconate, glucose, tryptone (0.1 %, w/v), yeast extract (0.1 %, w/v), Casamino acids (0.1 %, w/v), succinate, oxalic acid, pyruvate, glycerol, L-rhamnose, glycogen, L-fucose, capric acid, tartrate, malate and D-ornithine. Those not utilized are glycerol, erythritol, D-ribose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, arbutin, melezitose, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate, adipic acid, malate, citrate, phenylacetic acid, acetate, methanol, ethanol, lactate, octoic acid, L-alanine, L-glutamate, L-serine, DL-aspartic acid, benzoate, L-histidine and D-glucuronic acid. Positive for alkaline phosphatase, esterase lipase (C8), β-glucosidase, *N*-acetyl-β-glucosaminidase,

Table 1. Major characteristics that distinguish strain DHF9^T from type strains of other closely related species *Edaphobacter*

Strains: 1, DHF9^T (data from this study); 2, *E. modestus* Jbg-1^T (this study except where indicated); 3, *E. aggregans* Wbg-1^T (Koch *et al.*, 2008). +, Positive; w, weakly positive; −, negative.

Characteristic	1	2	3
Origin	Subtropical forest soil	Alpine soil*	Boreal forest soil
Cell size (μm)	0.8–1.4 × 0.5–0.7	1.0–1.8 × 0.5–0.7	1.5–2.1 × 0.7–0.9
Motility	−	+	−
pH for growth			
Range	3.5–5.5	4.5–7.0	4.0–7.0
Optimum	4.0	5.5	5.5
Temperature for growth (°C)			
Range	10–33	15–30	15–37
Optimum	28–33	30	30
DNA G+C content (mol%)	57.7	55.8*	56.9
Carbon source utilization			
Glucuronic acid	−	−	+
L-Aspartate	−	−	+
D-Lyxose	−	+	−
D-Mannitol	−	+	−
D-Sorbitol	−	+	−
Xylitol	−	+	−
L-Glutamate	−	+	+
Cellobiose	+	−	−
D-Arabinose	+	−	−
D-Galactose	+	−	−
D-Mannose	+	−	−
Maltose	+	−	−
N-Acetylglucosamine	+	−	−
Pyruvate	+	−	−
Raffinose	+	−	−
Succinate	+	−	−
D-Fructose	+	+	−
D-Xylose	+	+	−
L-Arabinose	+	+	−
myo-Inositol	+	−	−
Trehalose	+	+	−
Tartrate	w	−	−
L-Rhamnose	w	+	−
Enzymic activities			
Oxidase	−	+	−
Catalase	−	+	+
Valyl arylamidase	−	+	w
α-Chymotrypsin	−	+	w
α-Glucosidase	−	−	w
N-Acetyl-β-glucosaminidase	+	−	+
α-Mannosidase	+	−	−

*Data from Koch *et al.* (2008).

Table 2. Whole-cell fatty acid compositions of strain DHF9^T and other members of the genus *Edaphobacter*

Strains: 1, DHF9^T (data from this study); 2, *E. modestus* Jbg-1^T (this study); 3, *E. aggregans* Wbg-1^T (Dedysh *et al.*, 2012). Values are percentages of total fatty acids. Major components (>5 %) are highlighted in bold. −, Not detected.

Fatty acid	1	2	3
Saturated			
$C_{14:0}$	1.6	2.3	1.9
$C_{15:0}$	−	−	0.1
$C_{16:0}$	**10.2**	**11.3**	**6.0**
$C_{17:0}$	2.2	0.5	−
$C_{18:0}$	4.3	0.5	0.2
$C_{20:0}$	2.0	0.23	0.9
Unsaturated			
$C_{14:1}\omega5c$	0.4	0.83	2.0
$C_{15:1}\omega6c$	2.0	0.92	0.3
$C_{16:1}\omega7c$	**31.6**†	**35.83**†	**38.9**
iso-$C_{15:1}$ G	0.2	−	−
$C_{18:1}\omega9c$	1.3	−	−
Methyl-branched			
iso-$C_{15:0}$	**36.7**	**38.9**	**46.1**
anteiso-$C_{15:0}$	0.8	−	−
iso-$C_{16:0}$	0.6	0.6	−
iso-$C_{17:1}\omega9c$	−	−	1.8
iso-$C_{17:0}$	1.5	3.05	1.2
anteiso-$C_{17:0}$	0.5	0.87	−
cyclo-$C_{17:0}$	2.3	−	−
Summed features*			
1	0.2	−	0.3
2	0.9	−	−
3	0.7	1.84	−

*Summed features represent 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 2 contained $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; summed feature 3 contained iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl.
†Determined as $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ in this study.

esterase (C4), leucyl arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, α-mannosidase, β-galactosidase, β-glucuronidase and α-fucosidase activities; negative for lipase (C14), valyl arylamidase, cystyl arylamidase, trypsin, α-chymotrypsin, α-glucosidase, catalase, oxidase, reduction of nitrates, indole production, fermentation of glucose, arginine, urease and protease. The major cellular fatty acids are iso-$C_{15:0}$, $C_{16:0}$ and $C_{16:1}\omega7c$. The polar lipids consist of phosphatidylethanolamine, two unidentified aminolipids, two unidentified phospholipids, two unidentified polar lipids and an unidentified glycolipid.

The type strain, DHF9^T (=DSM 29920^T=CGMCC 1.12997^T), was isolated from soil samples collected from Dinghushan Biosphere Reserve, located in Guangdong Province, PR China. The G+C content of the genomic DNA of the type strain is 57.7 mol% (HPLC).

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