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Dinghuibacter silviterrae gen. nov., sp. nov., isolated from forest soil

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A novel Gram-stain negative, non-motile, rod-shaped, aerobic bacterial strain, designated DHOA34^T, was isolated from forest soil of Dinghushan Biosphere Reserve, Guangdong Province, China. Comparative 16S rRNA gene sequence analysis showed that it exhibited highest similarity with *Flavisolibacter ginsengiterrae* Gsoil 492^T and *Flavitalea populi* HY-50R^T, at 90.89 and 90.83 %, respectively. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, DHOA34^T formed an independent lineage within the family *Chitinophagaceae* but was distinct from all recognized species and genera of the family. The major cellular fatty acids of DHOA34^T included iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$). The DNA G+C content was 51.6 mol% and the predominant quinone was menaquinone 7 (MK-7). Flexirubin pigments were produced. The phenotypic, chemotaxonomic and phylogenetic data demonstrate consistently that strain DHOA34^T represents a novel species of a new genus in the family *Chitinophagaceae*, for which the name *Dinghuibacter silviterrae* gen. nov., sp. nov. is proposed. The type strain of *Dinghuibacter silviterrae* is DHOA34^T (=CGMCC 1.15023^T=KCTC 42632^T).

The family *Chitinophagaceae*, belonging to the class *Sphingobacteriia* of the phylum *Bacteroidetes*, was proposed by Kämpfer *et al.* (2011) with *Chitinophaga* (Kämpfer *et al.*, 2006) as its type genus. Members of the family *Chitinophagaceae* possess the following characteristics: cells are often thin rods, non-motile, aerobic or facultatively anaerobic with limited fermentative capabilities; the major menaquinones are of MK-7 type; and the whole-cell fatty acid profile contains iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH as major components. At the time of writing, the family *Chitinophagaceae* encompasses 24 genera, including *Cnuella* (Zhao *et al.*, 2014), *Vibrionimonas* (Albert *et al.*, 2014), *Arachidicoccus* (Madhaiyan *et al.*, 2015), *Flaviaesturariibacter* (Kang *et al.*, 2015) and *Hydrobacter* (Eder *et al.*, 2015) proposed recently. During the course of a study on the diversity of bacterial communities associated with soil of the forest of Dinghushan Biosphere Reserve, Guangdong Province, China (112° 31′ E 23° 10′ N), a bacterial strain designated DHOA34^T was isolated. Based on its distinct physiological and phylogenetic properties, we propose that DHOA34^T represents a novel species of a new genus in the family *Chitinophagaceae*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DHOA34^T is KM389530.

Three supplementary figures are available with the online Supplementary Material.

For isolation of DHOA34^T, the soil sample was thoroughly suspended with 100 mM PBS (pH 7.0) and the suspension was spread on full-strength R2A agar (Difco) plates after serial dilution. Single colonies were purified by transferring them onto new R2A agar plates. A bacterial strain that formed yellow colonies, designated DHOA34^T, was isolated. As it grew poorly on R2A plates, growth of DHOA34^T on various dilution levels of nutrient agar (NA), Luria–Bertani agar (LB), trypticase soy agar (TSA), R2A agar and MacConkey agar was evaluated and the results showed that best growth was achieved on 1 : 6-diluted R2A agar at 28 °C for 5 days. The isolate was then routinely propagated aerobically under the above conditions for all taxonomic experiments carried out in this study except when otherwise stated.

Cell morphology and the presence of flagellum were observed under a Nikon light microscope at ×10 000 magnification and transmission electron microscopy (JEM-1400; Jeol) with cells grown for up to 10 days at 28 °C on 1 : 6-diluted R2A agar. Gliding motility was checked by observing the edges of colonies formed on 1 : 6-diluted R2A agar and using the hanging drop technique as described by Bernardet *et al.* (2002). Catalase activity was determined by assessing bubble production in 3 % (v/v) H_2O_2, and oxidase activity was determined using oxidase test strips [1 % (w/v) tetramethyl-*p*-phenylenediamine]. Single carbon source assimilation by strain DHOA34^T

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Table 1. Differential phenotypic characteristics between strain DHOA34^T and the type strains of related genera of the family *Chitinophagaceae*

Strains: 1, DHOA34^T; 2, *Flavisolibacter ginsengiterrae* Gsoil 492^T; 3, *Cnuella takakiae* RG1-1^T; 4, *Chitinophaga terrae* KP01^T (1–4, data from this study); 5, *Flavitalea populi* HY-50R^T (Wang *et al.*, 2011); 6, *Sediminibacterium goheungense* HME7863^T (Kang *et al.*, 2014). All strains are negative for chitinase, glucose fermentation, indole and H_2S production, reduction of nitrates to nitrites/nitrogen, arginine dihydrolase, and assimilation of capric acid, malic acid, L-arabinose, gluconate and citric acid. In API ZYM test strips, all strains are positive for α-glucosidase, *N*-acetyl-β-glucosaminidase, alkaline phosphatase, esterase (C8), β-glucosidase, leucine arylamidase and valine arylamidase but negative for urease, arginine dihydrolase, β-glucuronidase and lipase (C14). +, Positive; −, negative; w, weak reaction; ND, not determined.

Characteristic	1	2	3	4	5	6
Gliding motility	−	−	+	−	−	−
Colony colour	Yellow	Yellow	Orange	Yellow	Yellow	Yellow
Maximum NaCl (%, w/v)	1.0	3.0	2.0	2.5	2.0	1.0
Oxidase	−	+	−	−	−	−
Catalase	−	−	+	+	+	+
Growth at 37 °C	+	−	−	+	+	+
pH range	4.0–8.0	6.0–8.5	6.0–10.0	6.0–9.5	5.0–8.0	6.0–8.5
Hydrolysis of:						
Gelatin	−	−	−	−	−	+
Casein	−	−	+	−	−	−
Assimilation of:						
Adipic acid	+	−	−	−	−	−
D-Ribose	−	−	−	+	−	−
Glucose	+	w	+	−	+	−
D-Mannose	+	−	+	+	−	−
L-Rhamnose	+	+	+	−	−	−
Inositol	+	−	−	−	−	−
Mannitol	+	−	−	−	−	+
N-Acetylglucosamine	+	−	+	+	+	−
Salicin	+	−	+	+	+	−
Maltose	−	+	+	+	+	−
α-Melibiose	w	w	+	−	+	−
Sucrose	+	+	w	+	+	+
Glycogen	−	+	+	−	−	−
L-Fucose	−	w	w	+	−	+
2-Ketogluconate	−	+	−	+	−	−
5-Ketogluconate	−	+	−	−	−	+
Histidine	+	+	+	−	−	−
L-Proline	−	+	+	−	−	+
L-Alanine	+	−	+	−	−	−
3-Hydroxybenzoate	−	−	−	−	−	+
L-Serine	−	−	+	−	−	−
Enzyme activities:						
β-Galactosidase	+	+	−	+	+	−
α-Galactosidase	+	−	+	w	+	+
Acid phosphatase	+	+	+	+	ND	+
α-Chymotrypsin	−	+	−	−	−	+
Cystine arylamidase	w	+	+	−	+	+
Esterase (C4)	w	w	+	−	+	+
α-Fucosidase	w	−	−	+	−	w
α-Mannosidase	w	−	−	−	+	−
Trypsin	−	−	+	w	−	−
Naphthol-AS-BI-phosphohydrolase	w	+	−	−	+	−
DNA G+C content (mol%)	51.6	43.0	49.1	46.3	46.8	42.7

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Table 2. Cellular fatty acid composition (%) of strain DHOA34^T and the type strains of related genera of the family *Chitinophagaceae*

Strains: 1, DHOA34^T; 2, *Flavisolibacter ginsengiterrae* Gsoil 492^T; 3, *Cnuella takakiae* RG1-1^T; 4, *Chitinophaga terrae* KP01^T (1–4, data from this study); 5, *Flavitalea populi* HY-50R^T (Wang *et al.*, 2011); 6, *Sediminibacterium goheungense* HME7863^T (Kang *et al.*, 2014). –, Not detected; TR, trace (<1 %).

Fatty acid	1	2	3	4	5	6
iso-$C_{13:0}$	–	1.1	8.3	–	6.1	4.1
iso-$C_{13:0}$ 3-OH	–	1.8	6.0	–	4.8	–
iso-$C_{14:0}$	3.0	–	–	–	TR	3.6
$C_{14:0}$	1.3	TR	TR	TR	–	TR
iso-$C_{15:0}$ 3-OH	4.8	1.4	TR	5. 4	–	3.0
iso-$C_{15:0}$	21.2	30.1	17.3	29.7	19.6	20.3
anteiso-$C_{15:0}$	9.3	10.2	2.2	–	4.6	2.7
iso-$C_{15:1}$ G	TR	5.1	–	–	11.7	25.3
anteiso-$C_{15:1}$ A	TR	2.6	–	–	2.9	1.6
$C_{15:1}\omega6c$	–	TR	1.5	–	–	–
$C_{15:0}$	–	–	–	–	1.2	–
$C_{15:0}$ 2-OH	1.4	TR	TR	TR	–	1.6
$C_{15:0}$ 3-OH	–	–	–	–	–	3.2
$C_{16:1}\omega5c$	2.3	–	–	8.8	2.5	–
$C_{16:0}$	3.7	5.8	5.9	1.3	2.0	2.7
iso-$C_{16:0}$	3.5	TR	–	TR	TR	1.6
$C_{16:0}$ 2-OH	2.1	TR	TR	2.5	–	–
$C_{16:0}$ 3-OH	1.9	TR	TR	3.4	TR	2.4
iso-$C_{16:0}$ 3-OH	1.3	TR	TR	TR	TR	6.6
iso-$C_{16:1}$ H	–	TR	1.7	–	–	–
$C_{17:0}$	–	–	–	8.1	–	–
iso-$C_{17:0}$	–	3.1	2.4	–	4.1	–
anteiso-$C_{17:1}$ A	–	TR	1.6	–	–	–
$C_{17:1}\omega6c$	4.7	–	1.6	–	1.2	–
$C_{17:0}$ 2-OH	TR	TR	TR	TR	TR	TR
$C_{17:0}$ 3-OH	TR	TR	TR	–	TR	2.1
iso-$C_{17:0}$ 3-OH	24.9	15.2	15.8	16.2	19.3	9.6
$C_{18:1}$ 2-OH	–	–	–	1.5	–	–
$C_{18:3}\omega6c$ (6,9,12)	–	–	–	2.0	–	–
Summed features*						
1	–	2.7	3.5	–	5.6	–
3	10.5	9.7	14.9	10.2	6.9	3.7
4	–	1.1	1.1	TR	1.8	–
5	–	–	TR	–	1.4	–
6	–	–	–	4.0	–	–
9	–	TR	6.0	–	–	–

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 1 comprises iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH; summed feature 3 comprises $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; summed feature 4 comprises iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B; summed feature 5 comprises $C_{18:0}$ ante and/or $C_{18:2}\omega6,9c$; summed feature 6 comprises $C_{19:1}\omega11c$ and/or anteiso-$C_{19:1}\omega9c$; summed feature 9 comprises iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$.

was tested using the API 50CH kit (bioMérieux). Additional physiological and biochemical properties were determined using API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Hydrolysis of casein and starch (Brown, 1985) and chitin (Singh *et al.*, 1999) was assessed as described in the original publications. Growth at various temperatures (4, 10, 16, 23, 28, 33, 37 and 42 °C) and pH 3.0–11.0 (intervals of 0.5 pH units) was investigated after 5 days of incubation in 1 : 6-diluted R2A broth. The pH of the R2A broths was achieved by using the buffer system dibasic sodium phosphate/citric acid (for pH 3.0–7.0) and Tris/HCl (for pH 7.5–11.0). Salt tolerance was tested in 1 : 6-diluted R2A broth supplemented with 0–5 % (w/v) NaCl (at 0.5 % intervals) after 5 days of incubation at 28 °C. The test for flexirubin-like pigments was conducted by a fast shift of colony

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colour from yellow to reddish brown after the addition of 20 % KOH and the retention of the original colour upon addition of HCl (Fautz & Reichenbach, 1980; Reichenbach *et al.*, 1980).

For fatty acid analysis of DHOA34^T, 40 mg of bacterial cells was scraped from the culture plates and the fatty acid methyl esters were obtained by saponification, methylation and extraction using the methods of Miller (1982) and Kuykendall *et al.* (1988). The fatty acid methyl ester mixtures were then separated using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID).

Polar lipids of DHOA34^T were extracted, examined by two-dimensional TLC and identified by using previously described procedures (Collins & Jones, 1980; Minnikin *et al.*, 1979). Two-dimensional migration was performed on each plate using chloroform/methanol/water (65 : 25 : 4, by vol.) as the first solvent and chloroform/acetic acid/methanol/water (80 : 18 : 12 : 5, by vol.) as the second solvent (Collins & Jones, 1980; Minnikin *et al.*, 1979). Total polar lipids were revealed by spraying with ethanolic molybdatophosphoric acid, and other plates were sprayed with ninhydrin for aminolipids. Menaquinones were isolated according to the methods of Minnikin *et al.* (1984) and identified by HPLC as described by Kroppenstedt (1982).

Genomic DNA was prepared according to the method of Wilson (1987). DNA for determination of the G+C content was isolated as described by Nielsen *et al.* (1995) and determined by HPLC as described by Mesbah *et al.* (1989).

The 16S rRNA gene of DHOA34^T was PCR-amplified using universal primers 27F and 1492R (Lane, 1991). The resultant sequence was used as a query to search the EzTaxon-e database (Chun *et al.*, 2007; Kim *et al.*, 2012) and the sequences of related taxa were retrieved. Phylogenetic analysis of 16S rRNA gene sequences was performed using MEGA version 5.0 software (Tamura *et al.*, 2011), and clustering was determined using the neighbour-joining, maximum-parsimony and maximum-likelihood methods. The robustness of the topology in each of the resultant trees was evaluated by bootstrap analyses based on 1000 replications.

Cells of DHOA34^T were Gram-stain-negative, aerobic, non-motile, rod-shaped, 0.3–0.7 μm wide and 0.5–1.5 μm long (Fig. S1, available in the online Supplementary Material). Colonies after 5 days of incubation on 1 : 6-diluted R2A agar at 28 °C were smooth, convex, regular, opaque and fresh yellow-coloured. Growth occurred at 16–37 °C (optimum 28–30 °C), at pH 4.0–8.0 (optimum pH 6.0–7.0) and in the presence of 0–1 % (w/v) NaCl (optimal growth without NaCl). DHOA34^T was able to grow on R2A agar and TSA, but not NA, LB agar or MacConkey agar, with optimal growth achieved on 1 : 6-diluted R2A agar. Oxidase and catalase tests were negative. Flexirubin pigments were produced. The physiological characteristics of strain DHOA34^T are summarized in the species description, and differential characteristics with the type strains of genera of the family *Chitinophagaceae* are presented in Table 1.

The major fatty acids of DHOA34^T were iso-$C_{17:0}$ 3-OH (24.9 %), iso-$C_{15:0}$ (21.2 %), summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, 10.5 %) and anteiso-$C_{15:0}$ (9.3 %), which are characteristic of members of the family *Chitinophagaceae* (Table 2).

The total polar lipids of strain DHOA34^T were phosphatidylethanolamine, three unknown glycophospholipids and three unidentified aminophospholipids. The absence of an unknown glycolipid, an unidentified aminophospholipid and unidentified polar lipids differentiated DHOA34^T

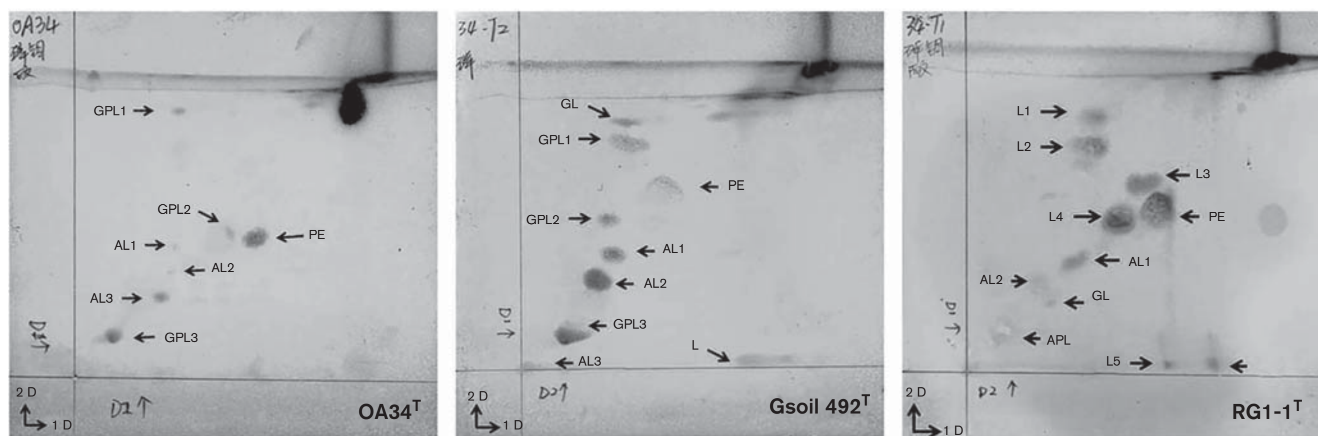

Fig. 1. Two-dimensional thin-layer chromatograms showing the polar lipid profiles of strain DHOA34^T, *Flavisolibacter ginsengiterrae* Gsoil 492^T and *Cnuella takakiae* RG1-1^T. Polar lipid chromatograms were sprayed with phosphomolybdic acid reagent following separation by two-dimensional TLC. Ascending solvent system: (1) chloroform/methanol/water (65 : 25 : 4, by vol.); (2) chloroform/acetic acid/methanol/water (80 : 18 : 12 : 5, by vol.). PE, phosphatidylethanolamine; AL, unknown aminolipid; APL, unidentified aminophospholipid; GL, unknown glycolipid; GPL, unknown glycophospholipid; L, unknown lipid.

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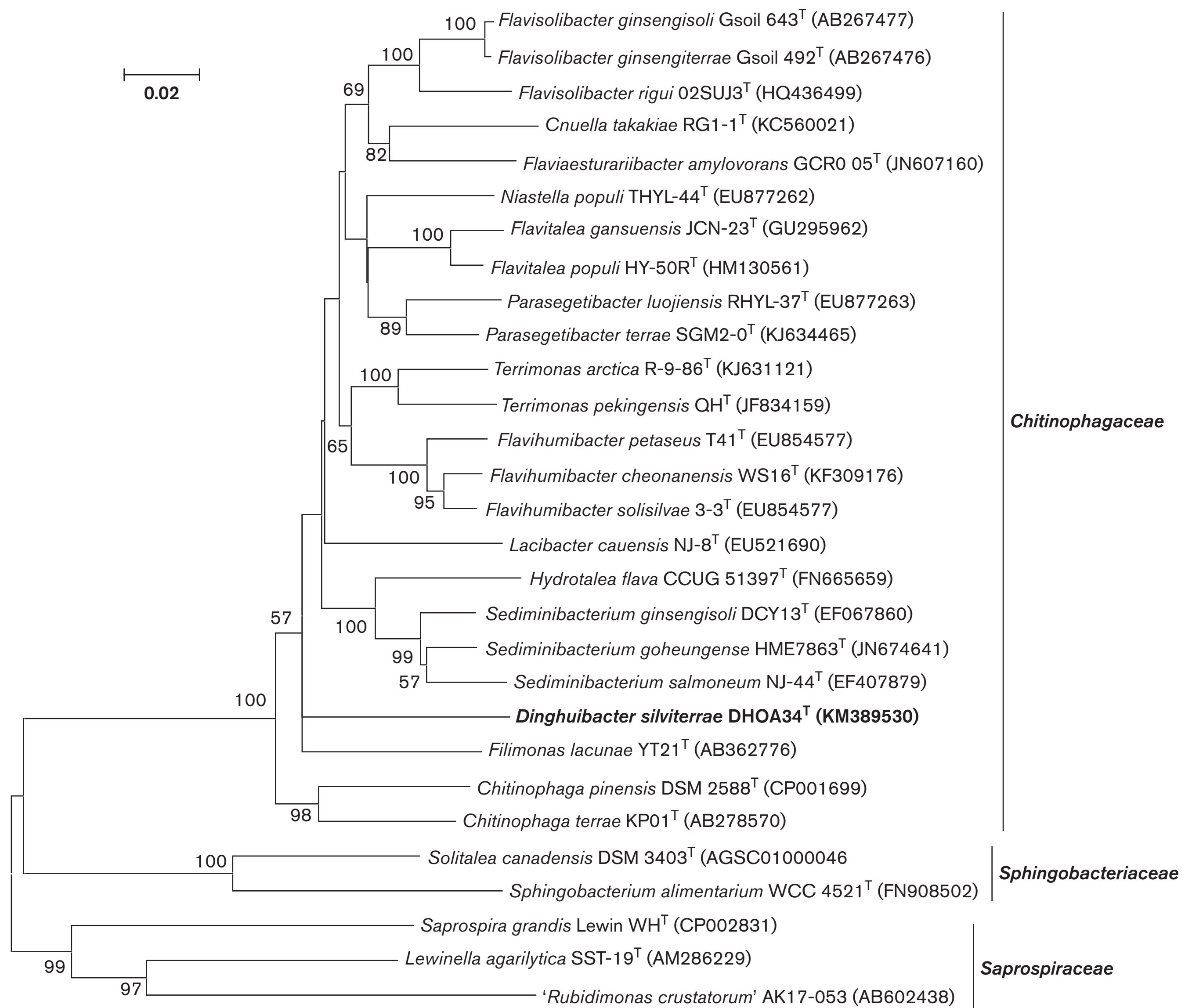

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain DHOA34^T and closely related species of the order *Sphingobacteriales*. Numbers at branching points are bootstrap values >50 %. Bar, 0.02 substitutions per nucleotide position.

from its closest relatives *Flavisolibacter ginsengisoli* Gsoil 643^T and *Cnuella takakiae* RG1-1^T (Fig. 1). MK-7 was detected as the major respiratory quinone of strain DHOA34^T, which is characteristic of species of the family *Chitinophagaceae*.

The almost-complete 16S rRNA gene sequence of strain DHOA34^T, comprising a continuous stretch of 1487 nt, was determined and compared with sequences of representatives of the order *Sphingobacteriales*. Comparative 16S rRNA gene sequence analysis showed that strain DHOA34^T exhibited highest similarity with *Flavisolibacter ginsengiterrae* Gsoil 492^T and *Flavitalea populi* HY-50R^T, but the values were only 90.89 and 90.83 %, respectively. In the neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain DHOA34^T and those of representative taxa of the order *Sphingobacteriales*, strain DHOA34^T formed an independent cluster with members of the family *Chitinophagaceae* but distinct from any described species and genera of the family (Fig. 2), indicating that it represents a novel species of a new genus of the family *Chitinophagaceae*. A similar phylogenetic relationship was revealed in the trees generated with the maximum-parsimony (Fig. S2) and maximum-likelihood (Fig. S3) algorithms. The genomic DNA G+C content of strain DHOA34^T was 51.6 mol%.

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Based on the distinct phenotypic, chemotaxonomic and genetic characteristics of strain DHOA34[T], we propose that it represents a novel species of a new genus in the family *Chitinophagaceae*, phylum *Bacteroidetes*, for which the name *Dinghuibacter silvaterrae* gen. nov., sp. nov. is proposed.

Description of *Dinghuibacter* gen. nov.

Dinghuibacter (Ding.hu.i.bac′ter. N.L. masc. n. *bacter* a rod; N.L. masc. n. *Dinghuibacter* a rod from Dinghushan Biosphere Reserve, Guangdong Province, China, where the organism was first isolated).

Cells are aerobic, Gram-stain-negative, non-motile rods. Colonies are smooth, convex, regular and fresh yellow-coloured. Flexirubin pigments are produced. Major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$) and anteiso-$C_{15:0}$. The major polar lipids are phosphatidylethanolamine, glycophospholipids and aminophospholipids. The predominant quinone is MK-7. Phylogenetically, the genus *Dinghuibacter* is a member of the family *Chitinophagaceae*, phylum *Bacteroidetes*. The type species is *Dinghuibacter silviterrae*.

Description of *Dinghuibacter silviterrae* sp. nov.

Dinghuibacter silviterrae (sil.vi.ter′rae. L. n. *silva* wood, forest; L. n. *terra* soil; N.L. gen. n. *silviterrae* of soil from a forest).

Displays the following characteristics in addition to those given for the genus. Cells are 0.3–0.7 μm wide and 0.5–1.5 μm long. Colonies on 1:6-diluted R2A agar are smooth, convex, regular and fresh yellow-coloured after 5 days of incubation at 28 °C. Growth occurs at 16–37 °C (optimum 28–30 °C) and at pH 4.0–8.0 (optimum pH 6.0–7.0). NaCl inhibits growth at concentrations above 1% (w/v). Grows on R2A agar (with optimal growth on 1:6-diluted R2A agar) and TSA, but not NA, LB agar or MacConkey agar. Enzyme activities, substrate assimilation and other physiological characteristics are indicated in Table 1. Catalase and oxidase are negative. Indole and H_2S are not produced. Hydrolyses aesculin but not chitin, urea, casein, L-arginine or CM-cellulose. Produces β-galactosidase, α-galactosidase, *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, β-glucosidase, esterase (C8), leucine arylamidase, valine arylamidase and α-glucosidase, but not arginine dihydrolase, α-chymotrypsin, β-glucuronidase, lipase (C14) or trypsin. In the API ZYM system, weakly positive for cystine arylamidase, esterase (C4), α-fucosidase, α-mannosidase and naphthol-AS-BI-phosphohydrolase. Positive reactions are observed for assimilation of adipic acid, D-galactose, glucose, D-fructose, D-mannose, L-rhamnose, inositol, mannitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, salicin, lactose, sucrose, melezitose, raffinose, starch and gentiobiose. Weak assimilation is observed for α-melibiose, histidine, L-alanine and trehalose. Assimilation of capric acid, lactate, DL-malic acid, phenylacetic acid, citric acid, L-proline, 3-hydroxybenzoate, L-serine, glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-adonitol, methyl β-D-arabinopyranoside, L-sorbose, dulcitol, D-sorbitol, amygdalin, arbutin, aesculin, (+)-cellobiose, maltose, glycogen, xylitol, D-lyxose, D-tagatose, DL-fucose, DL-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate is negative. Nitrate cannot be reduced to nitrite or nitrogen. The total polar lipids are phosphatidylethanolamine, three unknown glycophospholipids and three unidentified aminophospholipids.

The type strain is DHOA34[T] (=CGMCC 1.15023[T]=KCTC 42632[T]), which was isolated from forest soil of Dinghushan Biosphere Reserve, Guangdong Province, China. The DNA G+C content of the type strain is 51.6 mol%.

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