Chitinophaga dinghuensis sp. nov., isolated from soil

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A Gram-reaction-negative, aerobic, non-motile bacterial strain, DHOC24^T, was isolated from the forest soil of Dinghushan Biosphere Reserve, Guangdong Province, PR China. Strain DHOC24^T underwent a shape change during the course of culture from long filamentous cells (10–30 × 0.4–0.5 μm) at 2 days to coccobacilli (0.5–1.0 × 0.7–1.0 μm) at 15 days after inoculation. It grew optimally at 28–33 °C and pH 6.5–7.5.The major quinone of strainDHOC24^T was MK-7, the main fatty acids were iso-$C_{15:0}$, $C_{16:1}\omega5c$ and iso-$C_{17:0}$ 3-OH and the DNA G+C content was 43.1 mol%. On the basis of 16S rRNA gene sequence analysis, the strain was found to be affiliated with members of the genus *Chitinophaga*, but was clearly separated from established species of the genus. Strain DHOC24^T was most closely related to *Chitinophaga jiangningensis* JN53^T (98.3 % 16S rRNA gene sequence similarity) and *Chitinophaga terrae* KP01^T (97.9 %). DNA–DNA hybridization study showed relatively low relatedness values (32.1 %) of strain DHOC24^T with *C. jiangningensis* JN53^T. The phenotypic, chemotaxonomic and phylogenetic data showed that strain DHOC24^T represents a novel species of the genus *Chitinophaga*, for which the name *Chitinophaga dinghuensis* sp. nov. is proposed. The type strain is DHOC24^T (=CGMCC 1.12995^T=DSM 29821^T).

The genus *Chitinophaga*, which is the type genus of the recently proposed family *Chitinophagaceae* (Kämpfer *et al.*, 2011), was described by Sangkhobol & Skerman (1981) with *Chitinophaga pinensis* as its type species. Members of the genus *Chitinophaga* are Gram-negative-staining, filamentous or rod-shaped, aerobic (Chung *et al.*, 2012), and contain predominantly iso-$C_{15:0}$, $C_{16:1}\omega5c$ and moderate amounts of $C_{16:0}$, iso-$C_{15:0}$ 3-OH and iso-$C_{17:0}$ 3-OH. Strains contain MK-7 as the major menaquinone and the total DNA G+C mol % content is in the range of 40.7–53.4 % (Kämpfer *et al.*, 2011). At the time of writing, the genus *Chitinophaga* comprises 22 species with validly published names. Among them, seven were proposed in the past three years, *C. cymbidii* (Li *et al.*, 2013), *C. taiwanensis* (Li *et al.*, 2013), *C. polysaccharea* (Han *et al.*, 2014), *C. jiangningensis* (Wang *et al.*, 2014), *C. costaii* (Proença *et al.*, 2014), *C. longshanensis* (Gao *et al.*, 2015) and *C. qingshengii* (Cheng *et al.*, 2015). In this study, a bacterial strain (DHOC24^T) that was phylogenetically closely related to the genus *Chitinophaga* was taxonomically characterized.

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Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DHOC24^T is KM389531.

Three supplementary figures are available with the online Supplementary Material.

Strain DHOC24^T was isolated from the forest soil at Dinghushan Biosphere Reserve, Guangdong Province, PR China (23° 10′ N 112° 31′ E). 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. The plates were incubated at 28 °C for 5 days and single colonies were purified by transfer onto new plates. Strain DHOC24^T and the reference strains *Chitinophaga jiangningensis* JN53^T and *Chitinophaga terrae* KP01^T were routinely cultured on full-strength R2A agar at 28 °C for all taxonomic experiments.

Cell morphology was observed under a Nikon light microscope at × 1000 magnification, and the presence of flagellum was investigated by transmission electron microscopy (JEM-1400; JEOL) with cells grown for up to 15 days at 28 °C on R2A agar. A Gram reaction was performed using the non-staining method, as described by Buck (1982). Gliding motility was checked by observing the edges of colonies formed on R2A agar and using the hanging-drop technique as described by Bernardet *et al.* (2002). Carbon source utilization and enzyme activities of strain DHOC24^T and the reference strains were analysed with API 50CH, API 20NE and API ZYM galleries (bioMérieux). Hydrolysis of casein and starch (Brown, 1985), cellulose (Hendricks *et al.*, 1995), chitin (Singh *et al.*, 1999), and Tweens 20 and 80 (Atlas, 1993) were determined as described in the original publications. Catalase activity was determined by assessing bubble production in 3 % (v/v) H_2O_2, and oxidase activity was determined using

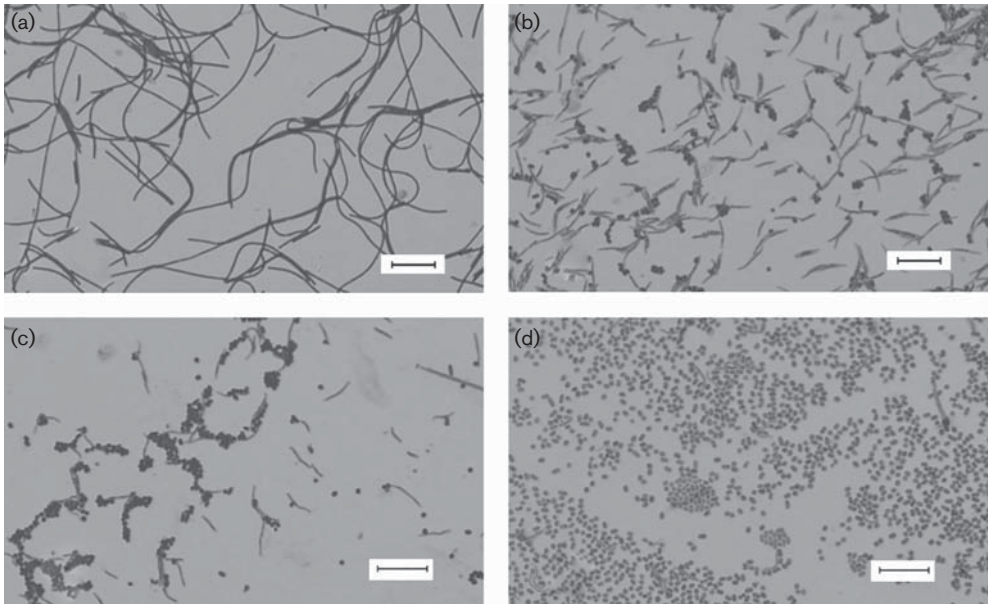

Fig. 1. Optical micrographs of cells of strain DHOC24^T grown on R2A plates at 28 °C for 1 (a), 4 (b), 8 (c) and 15 (d) days. Bars, 10 μm (a), 5.0 μm (b, c, d).

oxidase test strips [1 % (w/v) tetramethyl-*p*-phenylenediamine]. Growth on nutrient agar (NA), Luria–Bertani (LB) agar, TSA and R2A agar was evaluated at 28 °C and pH 7.0. To determine the optimal temperature and pH for growth of strain DHOC24^T, cultures were incubated in R2A broth at 4, 10, 16, 23, 28, 33, 37 and 42 °C at pH 7.0, and at pH 5.0–11.0 (at 0.5 unit intervals) at 28 °C. The pH values were achieved by adjusting the media with dibasic sodium phosphate/citric acid buffer (for pH 5.0–7.0) or Tris/HCl buffer (for pH 7.5–11.0). Salt tolerance was tested in R2A broth supplemented with 0–5 % (w/v) NaCl (at 0.5 % intervals) and incubated for 5 days at 28 °C. The presence of flexirubin-type pigments were demonstrated by a fast shift of colony 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).

Extraction of genomic DNA was performed with a commercial genomic DNA extraction kit (GeneStar). The 16S

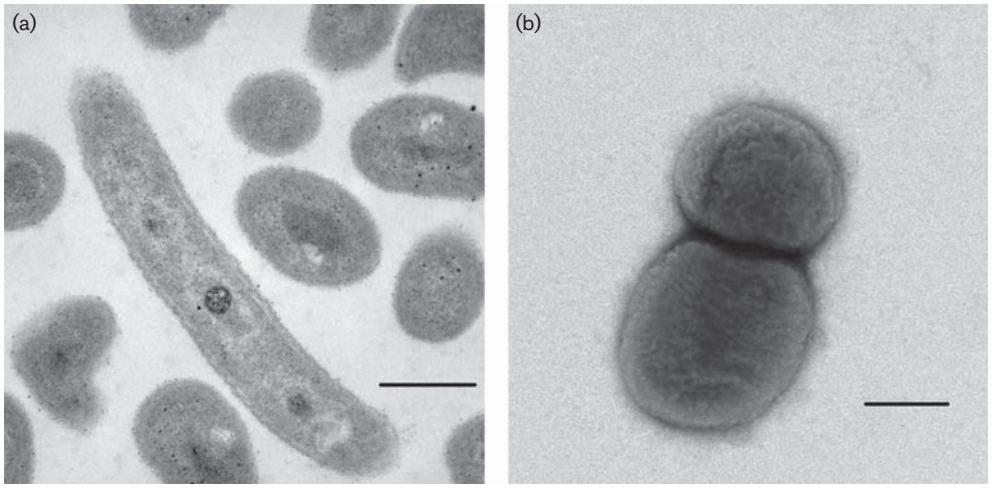

Fig. 2. Electron micrographs of strain DHOC24^T showing cross- and longitudinal-section of cells grown R2A plates at 28 °C for 1 day (a) and negatively stained cells grown on R2A plates at 28 °C for 15 days (b). Bars, 0.5 μm.

Table 1. Differential characteristics between strain DHOC24^T and the type strains of related species of the genus *Chitinophaga*

Strains: 1, DHOC24^T (data from this study); 2, *C. jiangningensis* JN53^T (this study); 3, *C. terrae* KP01^T (this study); 4, *C. eiseniae* YC6729^T (Yasir *et al.*, 2011); 5, *C. niastensis* JS16-4^T (Weon *et al.*, 2009); 6, *C. polysaccharea* MRP-15^T (Han *et al.*, 2014). All strains were negative for gliding motility. According to the API 20 NE test strips, all strains are positive for aesculin hydrolysis, but negative for starch hydrolysis, indole production and glucose fermentation. According to the API ZYM test strips, all strains are positive for acid phosphatase, alkaline phosphatase, leucine arylamidase and α-glucosidase, but negative for β-glucuronidase and lipase (C14). According to the API 50 CH test strips, all strains assimilate *N*-Acetyl-D-glucosamine, D-mannose, maltose and sucrose. +, Positive; W, weakly positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5	6
Cell length (μm)	0.5–30	2.3–2.6	0.6–0.8	1.2–1.6	1.0–7.0	0.6–0.9
Maximum NaCl for growth (%, w/v)	2.5	6	2.5	2.5	2	2
Oxidase	−	+	+	+	+	−
Catalase	+	+	+	+	+	−
Growth at 37 °C	+	+	+	+	−	+
pH range for growth	5.5–8.5	5.0–10.0	6.0–9.5	6.0–10.0	6.0–9.0	4.0–10.0
Chitinase	+	−	−	ND	−	ND
Urease	+	+	+	−	W	−
Hydrolysis of:						
Casein	+	+	−	+	−	ND
L-Arginine	+	−	W	ND	−	+
Carboxymethyl-cellulose	+	+	−	+	−	ND
Gelatin	−	+	−	+	+	−
Assimilation of:						
L-Arabinose	−	+	+	+	−	+
D-Arabinose	W	−	+	W	ND	ND
α-Melibiose	+	−	−	−	+	+
L-Rhamnose	−	+	−	+	−	+
Salicin	+	+	−	ND	+	+
D-Fucose	−	+	−	+	ND	ND
Glycogen	+	+	−	ND	−	−
Histidine	−	+	−	ND	W	−
L-Proline	−	+	−	ND	−	−
D-Ribose	W	−	+	ND	−	+
Adipate	+	+	−	ND	−	−
L-Alanine	+	W	−	ND	−	−
2-Ketogluconate	+	+	+	ND	−	−
5-Ketogluconate	+	+	−	ND	−	−
DL-malic acid	+	+	−	ND	−	ND
L-Serine	−	−	−	+	−	−
D-Sorbitol	+	−	−	ND	ND	−
Raffinose	−	+	+	−	ND	ND
D-Adonitol	+	−	−	−	ND	ND
Aspartic acid	−	+	+	+	ND	ND
Enzyme activities						
β-Galactosidase	−	−	+	+	+	+
α-Galactosidase	−	−	W	+	−	+
N-Acetyl-β-glucosaminidase	W	+	+	+	+	+
Cystine arylamidase	W	W	−	+	W	+
Esterase (C4)	+	+	−	+	−	+
Esterase (C8)	−	+	−	+	−	+
β-Glucosidase	W	+	+	+	+	+
Valine arylamidase	−	+	+	+	+	+
α-Fucosidase	−	−	+	−	+	+
Trypsin	−	−	W	+	−	+
Naphthol-AS-BI-phosphohydrolase	W	+	−	+	+	+
DNA G+C content (mol%)	43.1	49.7	46.3	48.9	43	47.9

rRNA gene was PCR-amplified using universal primers 27F and 1492R (Lane, 1991). The 16S rRNA gene sequence of strain DHOC24^T was first analysed using EzTaxon-e (http://www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012) and NCBI (http://www.ncbi.nlm.nih.gov) to determine the most closely related type strains. Phylogenetic analyses of 16S rRNA gene sequences were performed using MEGA version 5.0 software (Tamura *et al.*, 2011). Phylogenetic trees were reconstructed with neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-likelihood (ML) and maximum-parsimony (MP) methods. The robustness of the topology in the phylogenetic trees was evaluated by bootstrap analyses based on 1000 replications (Felsenstein, 1985). The DNA G+C content was determined by means of HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989). DNA–DNA hybridizations with *C. jiangningensis* JN53^T was determined using a UV/VIS spectrophotometer (UV1201; Rayleigh) as described by De Ley *et al.* (1970).

Cellular fatty acids of strain DHOC24^T and the reference strains were analysed by using biomass collected from batch cultures grown on R2A agar at 28 °C for 48 h. Fatty acid methyl esters were obtained from 40 mg cells by saponification, methylation and extraction using minor modifications of the method of Miller (1982) and Kuykendall *et al.* (1988). The fatty acid methyl esters mixtures were separated using Sherlock Microbial Identification System (MIS; MIDI).

Cultures for polar lipid analysis of strain DHOC24^T were grown on R2A plates and incubated at 28 °C for 5 days. Polar lipids were extracted and loaded onto thin-layer silica gel 60 plates (Merck). 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. Quinones were isolated according to the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt, 1982).

Cells of strain DHOC24^T were Gram-reaction-negative, and underwent a shape change during the course of culture development. When plated onto a fresh solid medium for 2 days, the isolates grew into long (10–30 × 0.4–0.5 μm) filamentous cells (Figs. 1a and 2a), which then divided gradually into shorter filamentous cells (Figs. 1b and c), and finally coccobacilli (0.5–1.0 × 0.7–1.0 μm) 15 days after inoculation (Figs. 1d and 2b). This characteristic differs significantly from the closely related *C. jiangningensis* JN53^T and *C. terrae* KP01^T. Colonies grown on R2A agar plates for 5 days were smooth, circular, sticky and yellowish. The strain grew well on R2A, TSB, LB and NA media supplemented with 1.5 % agar. Strain DHOC24^T was catalase-positive, but exhibited a negative reaction for oxidase, which is positive for *C. jiangningensis* JN53^T and *C. terrae* KP01^T (Table 1). Flexirubin-type pigments were not found.

The cellular fatty acid profile of strain DHOC24^T was generally similar to those of the type strains of closely related species of the genus *Chitinophaga* (*C. jiangningensis* JN53^T, *C. terrae* KP01^T, *C. polysaccharea* MRP-15^T, *C. niastensis* JS16-4^T and *C. eiseniae* YC6729^T), with iso-$C_{15:0}$ (37.68 %), $C_{16:1}\omega5c$ (28.0 %) and iso-$C_{17:0}$ 3-OH (14.4 %) as the major cellular

Table 2. Cellular fatty acid contents (%) of strain DHOC24^T and the type strains of related species of the genus *Chitinophaga*

Strains: 1, DHOC24^T (data from this study); 2, *C. jiangningensis* JN53^T (this study); 3, *C. terrae* KP01^T (this study); 4, *C. eiseniae* YC6729^T; (Yasir *et al.*, 2011); 5, *C. niastensis* JS16-4^T (Weon *et al.*, 2009); 6, *C. polysaccharea* MRP-15^T (Han *et al.*, 2014). −, Not detected.

Fatty acid	1	2	3	4	5	6
$C_{14:0}$	0.61	0.9	0.8	4.4	1.8	1.73
$C_{14:0}$ 2-OH	−	0.27	−	−	−	−
iso-$C_{15:0}$ 3-OH	5.19	4.10	5.38	3.5	4.7	2.03
$C_{15:0}$ 2-OH	−	0.53	0.38	−	−	−
iso-$C_{15:0}$	37.68	27.15	29.71	30.9	36.6	37.68
anteiso-$C_{15:0}$	0.18	−	−	−	1.2	2.24
$C_{16:1}\omega11c$	4.42	0.75	0.41	−	−	−
$C_{16:1}\omega5c$	28.0	21.44	8.84	21.3	32.5	34.18
$C_{16:0}$	1.69	3.07	1.28	6.1	6.5	5.48
iso-$C_{16:0}$	0.23	0.85	0.86	−	−	1.14
$C_{16:0}$ 2-OH	−	2.24	2.52	−	−	−
$C_{16:0}$ 3-OH	0.81	1.88	3.38	1.4	1.4	−
iso-$C_{16:0}$ 3-OH	0.69	0.62	0.71	−	−	−
$C_{17:0}$	−	5.39	8.14	−	−	−
iso-$C_{17:0}$	0.67	0.73	−	−	−	−
$C_{17:0}$ 2-OH	0.3	0.34	0.32	−	−	−
iso-$C_{17:0}$ 3-OH	14.4	14.79	16.22	9.4	9.2	1.43
$C_{17:0}$ 3-OH	−	0.87	−	−	−	−
$C_{18:1}\omega9c$	−	0.60	0.71	−	−	−
$C_{18:0}$	−	0.24	0.19	−	−	−
$C_{18:0}$ 2-OH	−	0.33	−	−	−	−
$C_{18:1}$ 2-OH	−	−	1.53	−	−	−
$C_{18:3}\omega6c$ (6,9,12)	−	−	1.97	−	−	−
Summed features*						
2	0.65	0.48	0.53	4.7	−	−
3	2.38	10.33	10.17	−	4.4	2.57
4	1.86	−	0.72	−	−	−
6	−	−	4.00	−	−	−
8	−	0.91	1.24	−	−	−

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 comprises12 : 0 aldehyde and/or unknown 10.9525; summed feature 3 comprises iso-$C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 4 comprises iso-$C_{17:1}$ iso I and/or anteiso B; summed feature 6 comprises $C_{19:1}\omega11c$ and/or anteiso-$C_{19:1}\omega9c$; summed feature 8 comprises $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$.

fatty acids. However, strain DHOC24^T differed from *C. jiangningensis* JN53^T and *C. terrae* KP01^T by having larger amounts of $C_{16:0}\omega 11c$ (4.42 %), and lacking $C_{16:0}$ 2-OH and $C_{17:0}$ (Table 2). The major polar lipids of strain DHOC24^T were phosphatidylethanolamine and some unidentified aminophospholipids (AL1–7) and lipids (L1–2) were also detected (Fig. S1, available in the online supplementary material). Although strain DHOC24^T shared phosphatidylethanolamine as the major polar lipid with *C. jiangningensis* and *C. eiseniae*, unidentified aminophospholipids AL1–4, AL7 and polar lipid L2 were only detected in strain DHOC24^T. The major respiratory quinone was MK-7, as is the case for the known species of the genus *Chitinophaga* (Kämpfer *et al.*, 2006).

The characteristics of strain DHOC24^T are summarized in the species description, and differential characteristics of the strain are compared with those of the closely related species of the genus *Chitinophaga* in Tables 1 and 2. Strain DHOC24^T could be distinguished from *C. jiangningensis* JN53^T and *C. terrae* KP01^T by its distinct change in cell morphology during the course of culture development (from filament to coccobacilli), which was not observed on either of its two closest relatives.

A total of 1490 bp of the 16S rRNA gene sequence of strain DHOC24^T was determined. Comparative 16S rRNA gene sequence analysis showed that strain DHOC24^T was most closely related to members of the genus *Chitinophaga*, with similarities of 98.29 % and 97.9 % to *C. jiangningensis* JN53^T and *C. terrae* KP01^T, respectively. In the NJ tree (Fig. 3) based on 16S rRNA gene sequences, strain DHOC24^T and *C. jiangningensis* JN53^T formed an independent cluster with a bootstrap value of 52 %, and the two species formed an independent cluster with *C. terrae* KP01^T with a bootstrap value of 89 % (Fig. 3). The topology of ML and MP trees (Figs S2 and S3) were similar to the NJ tree. The DNA G+C content was 43.1 mol%. DNA–DNA

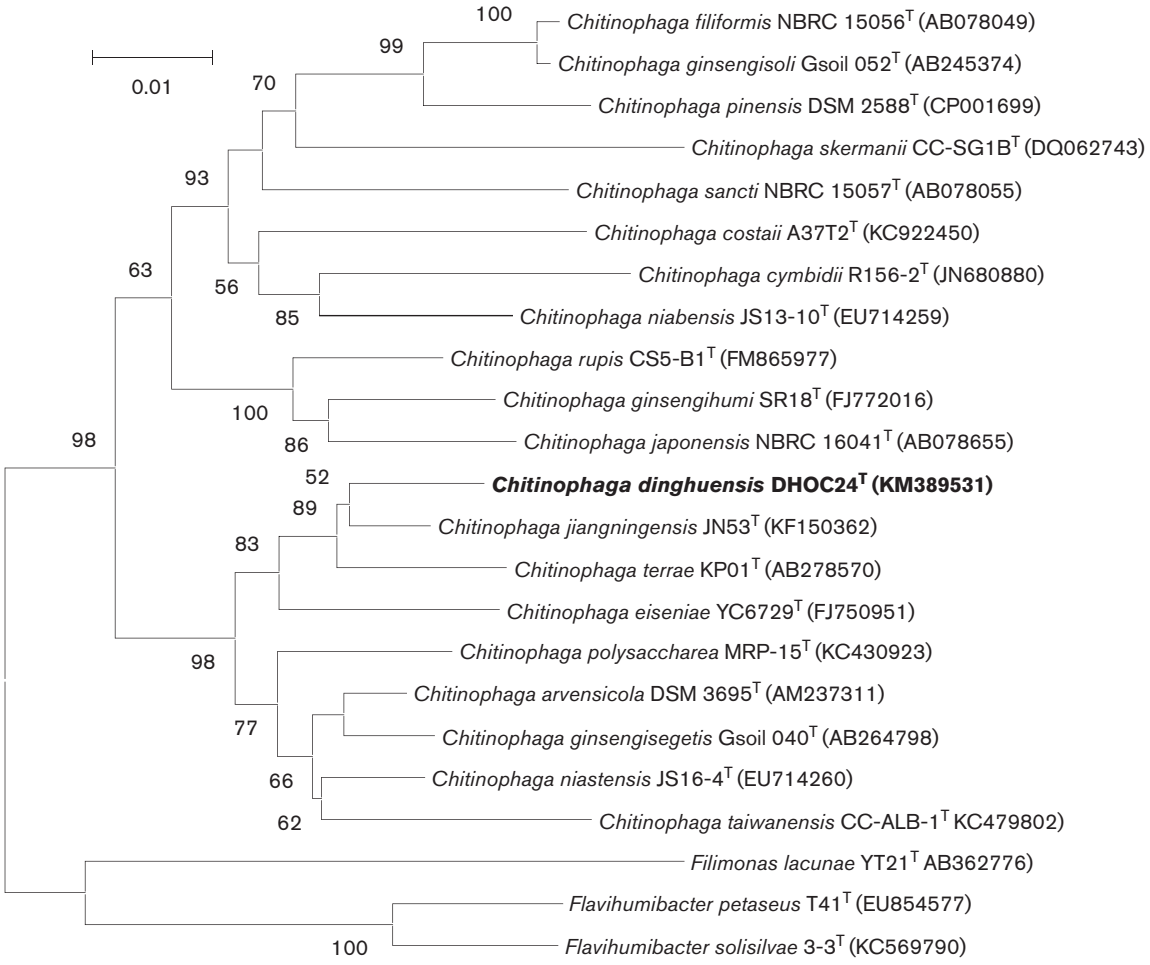

Fig. 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain DHOC24^T, species of the genus *Chitinophaga* and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at branch points. *Filimonas lacunae* YT21^T, *Flavihumibacter petaseus* T41^T and *Flavihumibacter solisilvae* 3-3^T were used as outgroups. Bar, 0.01 substitutions per nucleotide position.

hybridization study showed relatively low relatedness values of strain DHOC24^T with *C. jiangningensis* JN53^T (32.1 %). These results indicated that strain DHOC24^T represents a novel species of the genus *Chitinophaga*.

On the basis of the data and observations described above, strain DHOC24^T should be assigned to the genus *Chitinophaga* as the type strain of a novel species, for which the name *Chitinophaga dinghuensis* sp. nov. is proposed.

Description of *Chitinophaga dinghuensis* sp. nov.

Chitinophaga dinghuensis (ding.hu.en′sis N.L. fem. adj. *dinghuensis* referring to Dinghushan Biosphere Reserve, Guangdong Province, PR China, where the organism was first isolated).

On R2A agar, colonies are yellowish, circular, sticky and slightly convex. Young cultures contain long (10–30 μm × 0.4–0.5 μm) filamentous cells, which divide into shorter filamentous cells and finally coccobacilli (0.5–1.0 μm × 0.7–1.0 μm). Cells are Gram-reaction-negative, aerobic and non-motile. Growth occurs at pH 5.5–8.5 and between 10–37 °C (optimum growth at pH 6.5–7.5 and 28–33 °C). NaCl inhibits growth at concentrations above 2.5 % (w/v) (optimum growth in the presence of 0–0.5 % NaCl). Catalase-positive but oxidase-negative. Neither indole nor H_2S are produced. Hydrolyses aesculin, chitin, urea, casein, L-arginine and carboxymethyl-cellulose, but not Tweens 80 and 20. Produces acid phosphatase, alkaline phosphatase, leucine arylamidase, α-glucosidase and esterase (C4), but not β-glucuronidase, lipase (C14), β-galactosidase, α-galactosidase, α-chymotrypsin, esterase (C8), valine arylamidase, α-fucosidase, α-mannosidase or trypsin. Weakly positive for *N*-acetyl-β-glucosaminidase, cystine arylamidase, β-glucosidase and naphthol-AS-BI-phosphohydrolase. In single carbon source utilisation tests, positive reactions are observed for *N*-Acetyl-D-glucosamine, D-mannose, maltose, sucrose, gluconate, α-melibiose, salicin, L-fucose, glycogen, adipate, L-alanine, 2-ketogluconate, 5-ketogluconate, DL-malic acid, D-sorbitol, and D-adonitol, but negative reactions are observed for L-arabinose, L-rhamnose, D-fucose, histidine, L-proline, capric acid, inositol, mannitol, propionic acid, L-serine, raffinose and aspartic acid. The major fatty acids are iso-$C_{15:0}$, $C_{16:1}\omega5c$ and iso-$C_{17:0}$ 3-OH. The major polar lipids are phosphatidylethanolamine and some unidentified aminophospholipids (AL1–7) and lipids (L1–2) are also detected. MK-7 is the predominant quinone.

The type strain is DHOC24^T (=CGMCC 1.12995^T= DSM 29821^T), and was isolated from the forest soil of Dinghushan Biosphere Reserve, Guangdong Province, PR China. The DNA G+C content of the type strain is 43.1 mol%.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (project nos 31030015 and J1310025). We would like to thank Professor Wenjun Li in School of Life Sciences, Sun Yat-sen University for technical support.

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