Flavobacterium Difficile sp. nov., Isolated from a Freshwater Waterfall

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Abstract

A bacterial strain designated KDG-16^T is isolated from a freshwater waterfall in Taiwan and characterized to determine its taxonomic affiliation. Cells of strain KDG-16^T are Gram-stain-negative, strictly aerobic, motile by gliding, rod-shaped and form light yellow colonies. Optimal growth occurs at 20-25 °C, pH 6-7, and with 0% NaCl. Phylogenetic analyses based on 16S rRNA gene sequences and an up-to-date bacterial core gene set reveal that strain KDG-16^T is affiliated with species in the genus *Flavobacterium*. Analysis of 16S rRNA gene sequences shows that strain KDG-16^T shares the highest similarity with *Flavobacterium terrigena* DSM 17934^T (97.7%). The average nucleotide identity, average amino acid identity and digital DNA-DNA hybridization values between strain KDG-16^T and the closely related *Flavobacterium* species are below the cut-off values of 95-96, 90 and 70%, respectively, used for species demarcation. Strain KDG-16^T contains iso-C_{15:0}, iso-C_{15:1} G and iso-C_{17:0} 3-OH as the predominant fatty acids. The polar lipid profile consists of phosphatidylethanolamine, one uncharacterized aminophospholipid, one uncharacterized phospholipid, two uncharacterized aminolipids and two uncharacterized lipids. The major polyamine is homospermidine. The major isoprenoid quinone is MK-6. Genomic DNA G+C content of strain KDG-16^T is 31.6%. Based on the polyphasic taxonomic data obtained, strain KDG-16^T is considered to represent a novel species in the genus *Flavobacterium*, for which the name *Flavobacterium difficile* sp. nov. is proposed. The type strain is KDG-16^T (=BCRC 81194^T =LMG 31332^T).

Introduction

The genus *Flavobacterium* (type species, *Flavobacterium aquatile*), a member of the family *Flavobacteriaceae* in the order *Flavobacteriales* of class *Flavobacteriia* within the phylum *Bacteroidetes* (2011), was first established by Bergey et al. (1923) and emended by Bernardet et al. (1996), Dong et al. (2013), Kang et al. (2013) and Kuo et al. (2013). The genus *Flavobacterium* comprises 242 species with validly published names so far stated on the List of Prokaryotic Names with Standing in Nomenclature (<https://lpsn.dsmz.de/genus/flavobacterium>). Species of the genus *Flavobacterium* have been isolated from habitats worldwide such as animal, plant, algae, freshwater, seawater, sediment, soil, compost, lagoon, glacier, decaying wood, water reservoir, activated sludge, sequencing batch reactor and wastewater treatment plant sources (Bernardet et al. 1996, 2006, 2011; Dong et al. 2013; Fujii et al. 2014; Kang et al. 2013; Kuo et al. 2013; Liu et al. 2010; Yoon et al. 2007). The present study was carried out to clarify the taxonomic position of a putative novel species belonging to the genus *Flavobacterium*, designated KDG-16^T, by a polyphasic taxonomic approach.

Materials And Methods

Bacterial isolation and culture conditions

During a survey on cultivable bacterial resources from freshwater environment, a water sample (20 °C, pH 7, 0% NaCl) was collected from the Dajin Waterfall (GPS location: 22° 51' 40" N 120° 38' 43" E) in the Sandimen Township of Pingdong County, Taiwan on 5 April 2015 (Supplementary Fig. S1). The water sample was plated on R2A agar medium (BD Difco) through serial dilution technique. After incubation at 25 °C for 3 days, strain KDG-16^T was isolated as a single light yellow colony and subjected to detailed taxonomy analyses. Strain KDG-16^T was sub-cultured on R2A agar and stored at -80 °C in R2A broth with 20% (v/v) glycerol or stored following lyophilization. Strain KDG-16^T has been deposited to Bioresource Collection and Research Center, Taiwan (BCRC 81194^T) and BCCM/LMG Bacteria Collection, Belgium (LMG 31332^T). *Flavobacterium terrigena* DSM 17934^T, *Flavobacterium urocaniciphilum* JCM 19142^T and *Flavobacterium aquatile* DSM 1132^T were obtained from corresponding culture collections. The three type strains were used as reference strains and evaluated together with strain KDG-16^T under identical experimental conditions.

Morphological, physiological, and biochemical characterizations

Cell morphology of strain KDG-16^T was observed by phase-contrast microscopy (DM 2000; Leica) and transmission electron microscopy (Model H-7500; Hitachi) using cells grown on R2A agar at 25 °C for 3 days. The gliding motility was studied using phase-contrast microscopy as described by Bernardet et al. (2002). Gram-staining was performed with Stain Set S kit (BD Difco). Colony morphology was investigated on R2A agar using a stereoscopic microscope (SMZ 800; Nikon). The presence of flexirubin and carotenoid types of pigments was examined as described by Reichenbach (1992) and Schmidt et al. (1994). The physiological characteristics of strain KDG-16^T and the three reference strains were examined by growing bacteria at various temperatures, pH values and NaCl concentrations. The temperature range for bacterial growth was determined on R2A agar at 4, 10, 15, 20, 25, 30, 35, 37, 40, 45 and 50 °C. The pH range for bacterial growth was estimated at pH 4–9 (at intervals of 0.5 pH unit) using R2A broth at 25 °C. The pH of the medium was adjusted prior to sterilization to pH 4–9 using the biological buffers such as citrate/Na₂HPO₄, phosphate and Tris (Breznak and Costilow 2007). To measure the tolerance to NaCl, R2A broth was prepared and contained NaCl concentration, 0, 0.5% and 1–5%, w/v (at intervals of 1%). Growth under anaerobic conditions was determined after streaking strain KDG-16^T on R2A agar and on R2A agar supplemented with nitrate (0.1% KNO₃) and then incubated in anaerobic jars with AnaeroGen anaerobic system envelopes (Oxoid) at 25 °C for 21 days. Bacterial growth was studied on R2A agar, nutrient agar, Luria-Bertani agar and trypticase soy agar (all from Difco) under aerobic condition at 25 °C for 21 days.

Activities of catalase, oxidase, DNase, urease and lipase (corn oil), and hydrolysis of starch, casein, lecithin and Tweens 20, 40, 60 and 80 were analyzed according to the methods described by Tindall et al. (2007). Chitin hydrolysis was evaluated on chitinase-detection agar as described by Wen et al. (2002) and carboxymethyl cellulose (CM-cellulose) hydrolysis was assessed using R2A agar as the basal medium as

the method described by Bowman (2000). Utilization of carbon sources was investigated in a basal medium as described by Chang et al. (2004). Substrates were added at a concentration of 0.1% (w/v or v/v). Incubation was prolonged for 21 days at 25 °C under aerobic condition and bacterial growth was examined every two days. Additional biochemical tests were carried out using API ZYM, API 20NE and API 50CH kits (all from bioMérieux) according to the manufacturers' instructions. Sensitivity of strain KDG-16^T to antibiotics was tested by the disc diffusion method on R2A agar at 25 °C for 3 days using the approach described by Nokhal and Schlegel (1983). The discs (Oxoid) contained the following antibiotics: rifampicin (5 µg), ampicillin (10 µg), gentamicin (10 µg), streptomycin (10 µg), kanamycin (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), tetracycline (30 µg), penicillin G (10 U) and sulfamethoxazole (23.75 µg) plus trimethoprim (1.25 µg).

Chemotaxonomy

The fatty acid profiles of strain KDG-16^T and the three reference strains were analyzed from cells that grown on R2A agar at 25 °C for 3 days. Fatty acid methyl esters were prepared and separated using a standard MIDI protocol (Sherlock Microbial Identification System, version 6.0), analyzed by GC (Hewlett-Packard 5890 Series II) and identified by using the RTSBA6.00 database (Sasser 1990). Polar lipids were extracted and analyzed by two-dimensional TLC as described by Embley and Wait (1994). Ethanolic molybdophosphoric acid was used for the detection of the total polar lipids, ninhydrin for amino lipids, the α -naphthol reagent for glycolipids and the Zinzadze reagent for phospholipids. Polyamines were extracted and identified according to the methods described by Busse and Auling (1988) and Busse et al. (1997). Cells of strain KDG-16^T were cultivated in R2-PYE medium as described by Kämpfer et al. (2007) at 25 °C for 3 days, and homogenized in 0.2 M perchloric acid (HClO₄) and centrifuged. Polyamines in the resultant supernatant were treated with dansyl chloride solution (7.5 µg ml⁻¹ in acetone), and analyzed by HPLC on a D-7000 high-speed liquid chromatograph and UV-VIS detector L-7420 (Hitachi). Isoprenoid quinones were extracted and purified according to the method of Collins (1994) and analyzed by HPLC with a Spherisorb ODS column.

Determination 16S rRNA gene sequence and phylogenetic analysis

Genomic DNA was extracted using a bacterial genomic DNA purification kit (DP02-150, GeneMark) for 16S rRNA gene analysis. The 16S rRNA gene was amplified using the universal primer set (27F and 1541R) and then sequenced using four primers (27F, 520F, 800R and 1541R) (Weisburg et al. 1991; Anzai et al. 1997). The sequence obtained was compared with those available from EzBioCloud (Yoon et al. 2017). Multiple sequence alignment was carried out using the clustal W (Larkin et al. 2007) and BioEdit software (Hall 1999). Phylogenetic analyses were performed with the neighbour-joining (NJ) (Saitou and Nei 1987), maximum-likelihood (ML) (Felsenstein 1981) and maximum-parsimony (MP) (Kluge and Farris 1969) methods in the program MEGA 7 (Kumar et al. 2016). Bootstrap analysis was calculated based on 1000 resamplings.

Genome sequencing, analysis and comparison

Genome sequencing was performed by the Genomics BioSci & Tech. Co., Ltd. (Taipei, Taiwan, ROC) using the Illumina NextSeq sequencer platform and using MultiQC v1.2 for evaluating read quality (Ewels et al. 2016). The sequence assembly was performed using SPAdes (version 3.10.1) (Bankevich et al. 2012). Gene prediction and annotation was carried out using the Prokka pipeline (Seemann 2014). The protein encoding genes were classified into functional categories based on eggNOG (evolutionary genealogy of genes: Nonsupervised Orthologous Groups)-Mapper using precomputed cluster and phylogenies from the eggNOG database according to Huerta-Cepas et al. (2016, 2017). Digital DNA-DNA hybridization (dDDH) analysis was performed on the DSMZ Genome-to-Genome Distance Calculator platform as described by Meier-Kolthoff et al. (2013). Average nucleotide identity (ANI) values were calculated by OrthoANI analysis (Lee et al. 2016). Average amino acid identity (AAI) calculations were performed (<http://enve-omics.ce.gatech.edu/>). A genome-based phylogenetic tree was reconstructed using an up-to-date bacterial core gene set (UBCG, concatenated alignment of 92 core genes) as described by Na et al. (2018). For genome comparison, the genome sequences of strain KDG-16^T and three genome sequences from the phylogenetically closely related *Flavobacterium* species were annotated by the NCBI Prokaryotic Genome Annotation Pipeline and Rapid Annotation of microbial genomes using Subsystem Technology (RAST) server as described by Overbeek et al. (2014). Comparative gene contents were analyzed using an enhanced software platform EDGAR 2.0 according to the method described by Blom et al. (2016).

Results And Discussion

16S rRNA gene sequencing and phylogenetic analysis

The sequenced length of the 16S rRNA gene for strain KDG-16^T was 1435 bp (GenBank accession number MH54470). The sequence similarity calculations revealed that strain KDG-16^T was most closely related to the species of the genus *Flavobacterium*, and had the highest sequence similarity with *F. terrigena* DSM 17934^T (97.7%), followed by *F. glaciei* 0499^T (95.2%) and *F. urocaniciphilum* JCM 19142^T (95.1%). Sequence similarities < 95.1% were observed with the type strains of all other species listed in Fig. 1. And, strain KDG-16^T shared 94.2% similarity with the type strain of the type species of the genus, *F. aquatile* DSM 1132^T. Phylogenetic analysis based on 16S rRNA gene sequence indicated that strain KDG-16^T formed a separate phylogenetic branch cluster with *F. terrigena* DSM 17934^T and *F. urocaniciphilum* JCM 19142^T within the genus *Flavobacterium* in the neighbour-joining tree (Fig. 1). The overall topologies of the maximum-likelihood and maximum-parsimony trees were similar.

Whole genome analysis, average nucleotide identity and average amino acid identity calculations, digital DNA-DNA hybridization scores and UBCG phylogenetic tree construction

The genome of strain KDG-16^T was 2.96 Mb (GenBank accession number NZ_JAAJBT000000000) with G + C content of 31.6%. It was composed of 33 contigs with an average coverage of 321x and a N50 size of 307762 bp. A single copy of the 16S rRNA gene was found in the annotated genome, which showed 100% similarity to the amplified 16S rRNA gene sequence. A total of 2586 protein encoding genes, 3 rRNA

genes and 46 tRNA genes predicted. According eggNOG database, the 2586 protein encoding genes in strain KDG-16^T genome were classified into 20 functional categories (Supplementary Table S1). Most of coding sequences are classified as general function prediction only (R, 8.9%), followed by those identified as having roles in cell wall/membrane/envelope biogenesis (M, 7.7%), functional unknown (S, 6.2%), translation, ribosomal structure and biogenesis (J, 5.5%) and amino acid transport and metabolism (E, 5.3%).

ANI values were calculated between the genome of strain KDG-16^T and the type strains of other close related *Flavobacterium* species with whole genome sequence publicly available. The results showed that the ANI values were 70.7–84.2% (Supplementary Table S2), which were obviously lower than the prokaryotic species delineation threshold of 95–96% (Richter and Rosselló-Móra 2009). The dDDH values between strain KDG-16^T and the close related *Flavobacterium* species were 15.4–23.0% (Supplementary Table S2), which are evidently below the threshold of 70% for species delimitation (Goris et al. 2007). AAI calculations obtained from the comparison with *F. terrigena* DSM 17934^T, *F. urocaniciphilum* JCM 19142^T and *F. aquatile* DSM 1132^T were 89.2, 84.8 and 69.7%, respectively (Supplementary Fig. S2). The calculated AAI values were above the threshold of 60% for genus boundary and below the threshold of 90% for species demarcation (Rodriguez-R and Konstantinidis 2014). These data supported that strain KDG-16^T is a novel species in the genus *Flavobacterium*.

To infer a genome-based phylogenetic tree, UBCG was utilized for phylogenetic tree construction. The phylogenetic tree based on the coding sequences of 92 protein clusters showed that strain KDG-16^T formed a distinct phylogenetic lineage cluster with *F. terrigena* DSM 17934^T and *F. urocaniciphilum* JCM 19142^T in the genus *Flavobacterium* (Fig. 2), which indicated that strain KDG-16^T should be assigned to a novel species of the genus *Flavobacterium*.

Genome comparative analysis

The genome sequences of strain KDG-16^T and three genome sequences from the genus *Flavobacterium* were used for genome comparative analysis, including the type species *F. aquatile* DSM 1132^T isolated from deep well and two type strains, *F. terrigena* DSM 17934^T isolated from soil and *F. urocaniciphilum* JCM 19142^T isolated from wastewater treatment plant. The genome characteristics of strain KDG-16^T and these three strains is shown in Supplementary Table S3. Results revealed that some genes had all four strains in common and some genes differed among them (Table 1). Strain KDG-16^T had genes putatively encoding proteins associated with gliding motility such as gliding motility proteins, GldB, GldC, GldD, GldF, GldG, GldH, GldI, GldJ, GldK, GldL, GldM, GldN, which confirmed the observed gliding on phase-contrast microscopy (described below). Strain KDG-16^T had genes putatively encoding proteins related to carotenoid biosynthesis including polyprenyl synthetase family protein, sterol desaturase family protein, lycopene cyclase, consistent with result of absorption maximum at 455 nm of carotenoid pigments as measured by spectrophotometer (described below). Furthermore, four strains had genes putatively encoding proteins associated with gliding motility and carotenoid biosynthesis.

Table 1

Comparison of the presence and absence of selected genes among *Flavobacterium difficile* KDG-16^T and three type strains of the genus *Flavobacterium*.

Genes putatively encoding	1	2	3	4
Motility				
Proteins associated with gliding	+	+	+	+
Pigment				
Carotenoid biosynthesis	+	+	+	+
Cofactor and vitamin				
Flavodoxin	-	-	-	+
Coenzyme A biosynthesis	+	+	+	-
Cell wall and capsule				
Capsular polysaccharide biosynthesis and assembly	+	-	-	+
Virulence, disease and defense				
Tolerance to colicin E2	-	+	+	+
Arsenic resistance	+	+	-	+
Copper tolerance	+	-	-	-
Multidrug resistance efflux pumps	-	-	-	+
Multidrug resistance, tripartite systems found in Gram negative bacteria	-	+	+	-
Phage, prophage, transposable element and plasmid				
Phage tail fiber proteins	+	-	+	-
Phage lysis modules	-	-	-	+
Phage DNA synthesis	-	-	-	+
Pathogenicity island	-	+	-	-
Regulation and cell signaling				
Toxin-antitoxin replicon stabilization system	+	-	-	+
Phd-Doc, YdcE-YdcD toxin-antitoxin system	+	+	-	-
Stress response				

Strains: 1, strain KDG-16^T; 2, *F. terrigena* DSM 17934^T; 3, *F. urocaniciphilum* JCM 19142^T; 4, *F. aquatile* DSM 1132^T. +, Present; -, absent.

Genes putatively encoding	1	2	3	4
NADPH:quinone oxidoreductase 2	+	-	-	-
Cluster containing glutathione synthetase	+	-	-	-
Sigma B stress response egulation	+	+	-	+
Dimethylarginine metabolism	-	-	-	+
Photosynthesis				
Proteorhodopsin	+	-	-	+
Membrane transport				
NhaA, NhaD and sodium-depedent phosphate transporter	+	+	-	+
Iron acquisition and metabolism				
Hemin transport system	+	-	+	+
Secondary metabolism				
Lanthionine synthetase	+	-	+	-
Nitrogen metabolism				
Nitrosative stress	-	-	-	+
Denitrification	-	-	-	+
Sulfur metabolism				
Galactosylceramide and sulfatide metabolism	+	-	-	+
Metabolism of aromatic compounds				
Salicylate ester degradation	+	+	-	-
Central meta-cleavage pathway of aromatic compound degradation	-	-	-	+
Lipid metabolism				
Glycerolipid and glycerophospholipid metabolism	+	-	-	+
Triacylglycerol metabolism	-	-	-	+
DNA Metabolism				
DNA repair: RecA and Rex	-	-	-	+
DNA repair: 2-phosphoglycolate salvage	-	+	+	+

Strains: 1, strain KDG-16^T; 2, *F. terrigena* DSM 17934^T; 3, *F. urocaniciphilum* JCM 19142^T; 4, *F. aquatile* DSM 1132^T. +, Present; -, absent.

Genes putatively encoding	1	2	3	4
Type I restriction-modification	-	-	+	+
Restriction-modification system	-	-	+	+
Metabolism of amino acid and derivatives				
Cyanophycin metabolism	+	+	-	+
Arginine deiminase pathway	-	+	-	-
Arginine biosynthesis extended	-	+	-	-
Arginine and ornithine degradation	+	+	-	+
Methionine and cysteine biosynthesis	+	+	+	-
Proline synthesis	-	+	+	+
Proline and 4-hydroxyproline uptake and utilization	+	+	-	-
Carbohydrate metabolism				
Central carbohydrate metabolism: glycolate and glyoxylate interconversion	-	+	+	+
Di- and oligosaccharide: trehalose biosynthesis and trehalose uptake and utilization	+	-	-	+
Di- and oligosaccharide: lactose and galactose uptake and utilization	+	-	-	+
Fermentation: lactate	-	+	-	-
Polysaccharide: glycogen metabolism	-	-	-	+
Polysaccharide: cellulosome	+	-	-	+
Strains: 1, strain KDG-16 ^T ; 2, <i>F. terrigena</i> DSM 17934 ^T ; 3, <i>F. urocaniciphilum</i> JCM 19142 ^T ; 4, <i>F. aquatile</i> DSM 1132 ^T . +, Present; -, absent.				

The most obvious difference is that only strain KDG-16^T had genes related to copper tolerance e.g. apolipoprotein *N*-acyltransferase and DUF21 domain-containing protein involved in virulence and defense, related to NADPH:quinone oxidoreductase 2 e.g. NmrA family NAD(P)-binding protein, and related to cluster containing glutathione synthetase such as 16S rRNA (uracil¹⁴⁹⁸-N³)-methyltransferase and holliday junction resolvase RuvX involved in stress response. But the other three strains, *F. terrigena* DSM 17934^T, *F. urocaniciphilum* JCM 19142^T and *F. aquatile* DSM 1132^T, did not have these related genes. Other features are that only these three strains possessed genes putatively encoding proteins regarding to tolerance to colicin E2 involved in virulence and defense, regarding to 2-phosphoglycolate salvage involved in DNA repair, regarding to proline synthesis involved in amino acid metabolism and regarding to glycolate and glyoxylate interconversion involved in central carbohydrate metabolism, but the novel strain KDG-16^T did not possess these related genes.

In addition, only strain KDG-16^T and *F. aquatile* DSM 1132^T had genes related to capsular polysaccharides biosynthesis and assembly involved in cell wall and capsule biosynthesis, related to toxin-antitoxin replicon stabilization system involved in regulation and cell signaling, related to photosynthesis, related to galactosylceramide and sulfatide metabolism, related to glycerolipid and glycerophospholipid metabolism, related to trehalose biosynthesis and trehalose uptake and utilization, related to lactose and galactose uptake and utilization involved in disaccharide and oligosaccharide metabolism, and related to cellulosome involved in polysaccharide metabolism (Supplementary Table S4). But *F. terrigena* DSM 17934^T and *F. urocaniciphilum* JCM 19142^T did not possess these related genes. Meanwhile, all four strains showed highly diverse distribution pattern in iron acquisition and metabolism, nitrogen, sulfur, aromatic compound, lipid, DNA, amino acid and derivative and carbohydrate metabolisms.

When the percentage of genes of strain KDG-16^T shared with the type species of the genus was estimated, strain KDG-16^T showed 1823 genes (69.4%) shared with *F. aquatile* DSM 1132^T (Supplementary Fig. S3A). If strain KDG-16^T, *F. terrigena* DSM 17934^T, *F. urocaniciphilum* JCM 19142^T and *F. aquatile* DSM 1132^T were analyzed together, there are 1613 genes of strain KDG-16^T in common (about 61.4% of the total number of genes). There are 427 genes presented as strain KDG-16^T specific genes (about 16.3% of the total number of genes) (Supplementary Fig. S3B). Summary, the genomic information could provide the basic knowledge related to physiological and biochemical characteristics of these stains such as metabolic ability of various nutrients, resistant ability of different toxic compounds and adaptability to environmental changes. These capabilities confer the competitive ecological advantage for bacteria to adapt to miscellaneous environments in the complicated microbial ecosystem.

Phenotypic and biochemical characteristics

Cells of strain KDG-16^T were Gram-stain-negative, strictly aerobic, catalase-positive, oxidase-negative, rod-shaped and motile by gliding (Supplementary Fig. S4). Colonies of strain KDG-16^T were light yellow. The optimal growth temperature, pH and NaCl concentration were 20–25 °C, 6–7 and 0%, respectively. Strain KDG-16^T was sensitive to chloramphenicol, kanamycin, nalidixic acid, novobiocin, rifampicin, streptomycin, tetracycline, gentamicin, ampicillin and penicillin G, and resistant to sulfamethoxazole plus trimethoprim. Detailed results from the phenotypic and biochemical analyses of strain KDG-16^T are provided in the species description, Table 2 and Supplementary Table S5.

Table 2

Differential characteristics of *Flavobacterium difficle* KDG-16^T and phylogenetically closely related *Flavobacterium* species.

Characteristic	1	2	3	4
Isolation source	waterfall	soil	wastewater treatment plant	deep well
Colony pigmentation	light yellow	dark yellow	bright yellow	pale yellow
Temperature range for growth (°C) (optimum)	15–30 (20–25)	10–30 (25)	10–37 (30)	10–37 (20–30)
pH range for growth (optimum)	5.5-9 (6–7)	6–8 (6.5-7)	6.5–8.5 (7)	6.5-8 (7)
Flexirubin-type pigments	-	+	-	-
Growth on:				
Nutrient agar	-	+	+	-
Hydrolysis of:				
Casein	+	+	-	+
Starch	+	-	-	+
DNA	+	+	-	-
Gelatin	+	+	+	-
Urea	-	+	-	-
Enzymatic activities:				
Oxidase	-	+	+	+
C4 esterase	+	-	-	+
C8 esterase lipase	+	+	-	+
C14 lipase	+	-	-	-
Cystine arylamidase	+	+	-	+

Strains: 1, strain KDG-16^T; 2, *F. terrigena* DSM 17934^T; 3, *F. urocaniciphilum* JCM 19142^T; 4, *F. aquatile* DSM 1132^T. All data from this study. +, positive reaction; -, negative reaction. All strains are aerobic, positive for activities of catalase, alkaline phosphatase, leucine arylamidase and valine arylamidase, positive for utilization of mannose and Tween 40 as carbon source. All strains are negative for Gram staining; nitrate reduction; indole production; glucose acidification; activities of arginine dihydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase; utilization of gluconate, citrate, L-glutamic acid, L-leucine, L-serine and L-proline as carbon source; and hydrolysis of esculin, chitin and CM-cellulose.

Characteristic	1	2	3	4
Trypsin	+	+	-	-
α -Chymotrypsin	+	-	+	-
Acid phosphatase	+	-	-	+
Naphthol-AS-BI-phosphohydrolase	+	-	+	+
α -Glucosidase	+	-	-	+
Carbon source utilization:				
D-Glucose	+	+	+	-
D-Fructose	-	+	+	+
Maltose	-	+	+	-
L-Arabinose	+	-	+	-
Dextrin	-	+	+	+
Sucrose	-	-	-	+
D-Cellobiose	-	+	+	+
D-Trehalose,	-	-	-	+
L-Rhamnose	-	+	+	-
N-Acetyl-glucosamine	-	+	+	+
D-Mannitol	-	-	-	+
D-Sorbitol	-	+	+	-
Acetate	+	+	-	-
L-Histidine	-	+	+	-
Tween 80	-	+	+	+

Strains: 1, strain KDG-16^T; 2, *F. terrigena* DSM 17934^T; 3, *F. urocaniciphilum* JCM 19142^T; 4, *F. aquatile* DSM 1132^T. All data from this study. +, positive reaction; -, negative reaction. All strains are aerobic, positive for activities of catalase, alkaline phosphatase, leucine arylamidase and valine arylamidase, positive for utilization of mannose and Tween 40 as carbon source. All strains are negative for Gram staining; nitrate reduction; indole production; glucose acidification; activities of arginine dihydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase; utilization of gluconate, citrate, L-glutamic acid, L-leucine, L-serine and L-proline as carbon source; and hydrolysis of esculin, chitin and CM-cellulose.

Determination of cellular fatty acids, polar lipids, polyamines and isoprenoid quinones

The major cellular fatty acids (> 10% of the total fatty acids) of strain KDG-16^T were iso-C_{15:0} (22.9%), iso-C_{15:1} G (15.3%) and iso-C_{17:0} 3-OH (11.2%). The fatty acid composition of strain KDG-16^T and three type strains was shown in Table 3. Strain KDG-16^T and the three reference strains had iso-C_{15:0}, iso-C_{15:1} G and iso-C_{17:0} 3-OH as the predominant cellular fatty acids. Their fatty acid compositions were similar, with slightly differences in the proportions of some fatty acids. Strain KDG-16^T had a complex polar lipid profile consisting of phosphatidylethanolamine (PE), one uncharacterized aminophospholipid (APL), one uncharacterized phospholipid (PL), two uncharacterized aminolipids (AL1 and AL2) and two uncharacterized lipids (L1 and L2) (Supplementary Fig. S5). PE was the major polar lipid of strain KDG-16^T consistent with previous descriptions of *Flavobacterium* species (Dong et al. 2013; Kang et al. 2013). Strain KDG-16^T contained homospermidine (HSPD, 88.5%) as the major polyamine and spermidine (SPD, 11.5%) as the minor component (Supplementary Fig. S6). HSPD was the major polyamine of strain KDG-16^T in line with that of other *Flavobacterium* species which polyamine composition was analyzed (Bernardet et al. 1996, 2011). The major respiratory quinone of strain KDG-16^T was menaquinone (MK-6) (Supplementary Fig. S7). The major respiratory quinone was MK-6, which is a common feature of the members of the family *Flavobacteriaceae* (Bernardet et al. 2002). Overall chemotaxonomic characterization based on the fatty acid, polar lipid, polyamine and quinone suggested the inclusion of strain KDG-16^T as a member of the genus *Flavobacterium*.

Table 3

Cellular fatty acid composition of *Flavobacterium difficile* KDG-16^T and phylogenetically closely related *Flavobacterium* species.

Fatty acid	1	2	3	4
Straight chain:				
C _{16:0}	1.1	1.9	3.1	2.2
C _{18:0}	-	-	-	1.8
Branched:				
iso-C _{14:0}	-	-	-	2.5
iso-C _{15:0}	22.9	26.0	30.9	38.4
iso-C _{15:1} G	15.3	13.4	16.2	14.1
iso-C _{16:0}	5.0	6.3	4.0	-
iso-C _{16:1} H	2.1	2.1	-	-
anteiso-C _{15:0}	2.3	3.4	-	6.3
Unsaturated:				
C _{15:1} ω6c	9.5	6.1	7.2	2.5
C _{17:1} ω6c	1.9	1.8	2.2	-
C _{17:1} ω8c	1.1	1.3	-	-
Hydroxy:				
C _{15:0} 3-OH	2.1	-	2.7	-
C _{16:0} 3-OH	1.3	1.1	2.5	1.3

Strains: 1, strain KDG-16^T; 2, *F. terrigena* DSM 17934^T; 3, *F. urocaniciphilum* JCM 19142^T; 4, *F. aquatile* DSM 1132^T. All data are from this study. Strains were grown on R2A agar at 25 °C for 3 days. Data are presented as percentages of the total fatty acids; the major fatty acids (> 10%) are in bold type. Only fatty acids representing more than 1% of the total fatty acids of at least one of the strains are shown. -, not detected.

For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. *cis* isomer is indicated by the suffix *c*. *Summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete ECLs as well as those where the ECLs are not reported separately. Summed feature 3 was listed as C_{16:1} ω6c and/or C_{16:1} ω7c and summed feature 9 as iso-C_{17:1} ω9c and/or 10-methyl C_{16:0}.

Fatty acid	1	2	3	4
iso-C _{15:0} 3-OH	8.8	7.2	7.8	2.1
iso-C _{16:0} 3-OH	2.7	1.7	1.8	-
iso-C _{17:0} 3-OH	11.2	10.9	12.4	16.2
Summed features*				
3	4.6	4.4	3.4	5.5
9	5.2	9.4	2.9	4.1
<p>Strains: 1, strain KDG-16^T; 2, <i>F. terrigena</i> DSM 17934^T; 3, <i>F. urocaniciphilum</i> JCM 19142^T; 4, <i>F. aquatile</i> DSM 1132^T. All data are from this study. Strains were grown on R2A agar at 25 °C for 3 days. Data are presented as percentages of the total fatty acids; the major fatty acids (> 10%) are in bold type. Only fatty acids representing more than 1% of the total fatty acids of at least one of the strains are shown. -, not detected.</p>				
<p>For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. <i>cis</i> isomer is indicated by the suffix <i>c</i>. *Summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete ECLs as well as those where the ECLs are not reported separately. Summed feature 3 was listed as C_{16:1} ω6<i>c</i> and/or C_{16:1} ω7<i>c</i> and summed feature 9 as iso-C_{17:1} ω9<i>c</i> and/or 10-methyl C_{16:0}.</p>				

Conclusion, phenotypic characterization demonstrated that strain KDG-16^T shared several traits in common with the three reference strains. Furthermore, strain KDG-16^T could be obviously distinguished from these three strains by several properties listed in Table 2. Based on the data obtained from 16S rRNA gene sequence and whole genome sequence comparison, strain KDG-16^T occupies a distinct position within the genus *Flavobacterium* that is supported by the phenotypic, biochemical and chemotaxonomic features. Strain KDG-16^T represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium difficile* is propose.

Description of *Flavobacterium difficile* sp. nov.

Flavobacterium difficile (dif.fi.ci'le. L. neut. adj. *difficile* difficult, referring to difficulties in cultivating the type strain).

Cells are Gram-stain-negative, strictly aerobic, rod-shaped and motile by gliding. Cells grow well on R2A agar, but not on nutrient, trypticase soy and Luria-Bertani agars. After 3 days of incubation on R2A agar at 25 °C, the mean cell size is 0.4-0.5 μ m in width and 1.2–1.8 μ m in length. Colonies on R2A agar are light yellow, convex and circular with regular margins. Growth occurs at 15–30 °C (optimum, 20–25 °C), at pH 5.5-9 (optimum, pH 6–7) and with 0-0.5% NaCl (optimum, 0%). Positive for catalase activity and negative for oxidase activity. Positive for hydrolysis of starch, casein, DNA and Tweens 20 and 80. Negative for hydrolysis of chitin, CM-cellulose, corn oil, lecithin and Tweens 40 and 60. Carotenoid pigments are

present with maximum absorption at 455 nm. Flexirubin-type pigments are not produced. Positive for gelatin hydrolysis. Alkaline phosphatase, C4 esterase, C8 esterase lipase, C14 lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α -glucosidase activities are present. Acids are produced from esculin, starch, glycogen and 5-ketogluconate. Growth under aerobic condition is positive on: D-glucose, D-mannose, L-arabinose, acetate and Tween 40. The predominant fatty acids are iso-C_{15:0}, iso-C_{15:1} G and iso-C_{17:0} 3-OH. The polar lipid profile consists of phosphatidylethanolamine, one uncharacterized aminophospholipid, one uncharacterized phospholipid, two uncharacterized aminolipids and two uncharacterized lipids. Homospermidine is the major polyamine and spermidine is the minor polyamine. The major respiratory quinone is MK-6. The DNA G + C content of the type strain is 31.6%.

The type strain is KDG-16^T (= BCRC 81194^T = LMG 31332^T) isolated from the Dajin Waterfall in the Sandimen Township of Pingdong County, Taiwan. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the whole genome of *Flavobacterium difficile* KDG-16^T are MH544703 and NZ_JAAJBT000000000, respectively.

Abbreviations

eggNOG, evolutionary genealogy of genes: Nonsupervised Orthologous Groups; UBCG, up-to-date bacterial core gene set; ANI, average nucleotide identity; AAI, average amino acid identity; dDDH, digital DNA-DNA hybridization; PE, phosphatidylethanolamine; APL, uncharacterized aminophospholipid; PL, uncharacterized phospholipid; AL, uncharacterized aminolipid; L, uncharacterized lipid; HSPD, homospermidine; SPD, spermidine; MK-6, menaquinone-6.

Declarations

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Compliance with ethical standards

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figures

Figure 1

Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Flavobacterium difficile* KDG-16T and other *Flavobacterium* species. Numbers at nodes are bootstrap percentages (> 70%) based on the neighbour-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms. Filled circles indicate nodes that are also found with the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate nodes that are also recovered with

the maximum-parsimony algorithm. *Lutibacter flavus* IMCC1507T was used as an out-group. Bar, 0.01 substitutions per nucleotide position.

Figure 2

Phylogenetic tree inferred using UBCGs (concatenated alignment of 92 core genes) showing the position of *Flavobacterium difficile* KDG-16T and closely related taxa within the genus *Flavobacterium*. The number of single gene trees supporting a branch in a UBCG tree is calculated and designated the Gene

Support Index (GSI). The GSIs (> 60) are given at branching points. *Lutibacter flavus* DSM 27993T was used as an out-group. Bar, 0.05 substitutions per position.

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