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Mucilaginibacter limnophilus sp. nov., isolated from a lake[§]

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A polyphasic taxonomy approach was used to characterize strain YBJ-36^T, isolated from a freshwater lake in Taiwan. Phylogenetic analyses, based on 16S rRNA gene sequences and coding sequences of an up-to-date bacterial core gene set (92 protein clusters), indicated that strain YBJ-36^T formed a phylogenetic lineage in the genus *Mucilaginibacter*. 16S rRNA gene sequence similarity indicated that strain YBJ-36^T is closely related to species within the genus *Mucilaginibacter* (93.8–97.8% sequence similarity) and is most similar to *Mucilaginibacter fluminis* TTM-2^T (97.8%), followed by *Mucilaginibacter roseus* TTM-1^T (97.2%). Microbiological analyses demonstrated that strain YBJ-36^T is Gram-negative, aerobic, non-motile, rod-shaped, surrounded by a thick capsule, and forms pink-colored colonies. Strain YBJ-36^T grew between 20–40°C (optimal range, 35–37°C), pH 5.5–7.0 (optimal pH of 6) and 0–2% NaCl (optimal concentration, 0.5%). The predominant fatty acids of strain YBJ-36^T are iso-$C_{15:0}$ and summed feature 3 ($C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*), the major polar lipid is phosphatidylethanolamine, the major polyamine is homospermidine, and the major isoprenoid quinone is MK-7. The draft genome is approximately 4.63 Mb in size with a G+C content of 42.8 mol%. Strain YBJ-36^T exhibited less than 35% DNA-DNA relatedness with *Mucilaginibacter fluminis* TTM-2^T and *Mucilaginibacter roseus* TTM-1^T. Based on phenotypic and genotypic properties and phylogenetic inference, strain YBJ-36^T should be classified in a novel species of the genus *Mucilaginibacter*, for which the name *Mucilaginibacter limnophilus* sp. nov. is proposed. The type strain is YBJ-36^T (= BCRC 81056^T = KCTC 52811^T = LMG 30058^T).

Keywords: *Mucilaginibacter limnophilus* sp. nov., *Bacteroidetes*, *Sphingobacteriia*, *Sphingobacteriales*, *Sphingobacteriaceae*, polyphasic taxonomy

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Introduction

The genus *Mucilaginibacter* (type species, *Mucilaginibacter paludis*) was first proposed by Pankratov *et al.* (2007) and was subsequently amended by Urai *et al.* (2008), Baik *et al.* (2010), and Chen *et al.* (2014). *Mucilaginibacter* belongs to the family *Sphingobacteriaceae*, order *Sphingobacteriales*, class *Sphingobacteriia*, and phylum *Bacteroidetes* (Ludwig *et al.*, 2011). To date, the genus *Mucilaginibacter* contains more than 50 validly named species published on the List of Prokaryotic Names with Standing in Nomenclature (Parte, 2018). The genus *Mucilaginibacter* inhabits several different ecological niches and has been found in peat bog, wetland, marine sand, soil, plants, fresh water, tidal flat sediment, volcanic forest, and a wastewater treatment facility. Members of the genus *Mucilaginibacter* are Gram-negative rod-shaped bacteria, non-motile, non-spore-forming, strictly aerobic or facultative anaerobic, chemoorganotrophic, with a DNA G+C content ranging from 39.1 to 49.8 mol% (Pankratov *et al.*, 2007; Baik *et al.*, 2010; Kim *et al.*, 2012; Chen *et al.*, 2014).

While investigating bacterial biodiversity in the Beigang Old River Park Lake in the vicinity of Yunlin County, Taiwan, a large number of bacterial strains were isolated by dilution plating. Among these, a bacterial strain designated YBJ-36^T that produces pink colonies was found to represent a novel species of the genus *Mucilaginibacter*, based on phenotypic and phylogenetic analyses. In the present study, a polyphasic taxonomy approach was used to clarify the taxonomic characterization of strain YBJ-36^T.

Materials and Methods

Bacterial strains and culture conditions

A freshwater sample was collected from the Beigang Old River Park Lake (GPS location: 23°35′37″ N 120°18′35″ E) in the vicinity of Yunlin County, Taiwan. The water sample was spread on R2A agar (BD Difco) plates by the standard dilution plating method. After incubation of the plates at 25°C for 3 days, strain YBJ-36^T was isolated from one of these plates and then repeatedly sub-cultured under the same conditions, to obtain a pure culture which colony was confirmed by stereo microscopic examination (S APO; Leica). The isolate was then stored at -80°C in R2A broth (BD Difco) with 20% (v/v) glycerol or by lyophilization. The strain was deposited in the the Bioresource Collection and Research Center (BCRC 81056^T), the Korean Collection for Type Cultures (KCTC 52811^T) and the Belgian Co-ordinated Collection of Microorganisms (BCCM; LMG 30058^T). *Mucilaginibacter fluminis* TTM-2^T (= BCRC 80785^T) and *Mucilaginibacter amnicola* TAPP7^T (= BCRC 80976^T) were obtained from the Bioresource Collection and Research Center (BCRC).

Mucilaginibacter roseus TTM-1^T (= KCTC 42273^T), *Mucilaginibacter defluvii* A5^T (= KCTC 23922^T), *Mucilaginibacter lutimaris* BR-3^T (= KCTC 23461^T), and *Mucilaginibacter litoreus* BR-18^T (= KCTC 23697^T) were obtained from the Korean Collection for Type Cultures (KCTC). All six type strains were grown under the same conditions and used as reference strains for comparative taxonomic analyses.

Morphological, physiological, and biochemical characterization

Cell morphology of strain YBJ-36^T was observed by phase-contrast microscopy (DM 2000; Leica). Motility was tested using the hanging drop method (Beveridge *et al.*, 2007), and the Spot Test Flagella Stain (BD Difco) was used for flagellum staining. Gliding motility was studied using phase-contrast microscopy as described by Bernardet *et al.* (2002). The Gram Stain Set S kit (BD Difco) and the Ryu non-staining KOH method (Powers, 1995) were used to perform the Gram reaction. The presence of flexirubin and carotenoid types of pigments was investigated as described by Reichenbach (1992) and Schmidt *et al.* (1994). Poly-β-hydroxybutyrate granule accumulation was examined as described by Schlegel *et al.* (1970) and Spiekermann *et al.* (1999). The presence of a capsule was assessed using the Hiss staining method as described by Beveridge *et al.* (2007). Colony morphology was observed on R2A agar by using a stereoscopic microscope (SMZ 800; Nikon).

The physiological characteristics of strain YBJ-36^T and the six closest relatives were examined by growing bacteria at various pH values, temperatures and NaCl concentrations. The pH range for bacterial growth was estimated by measuring the optical densities (wavelength 600 nm) of R2A broth cultures. The pH of the medium was adjusted prior to sterilization to pH 4.0–9.0 (at intervals of 0.5 pH unit) using the following biological buffers (Breznak and Costilow, 2007): 100 mM citrate/Na_2HPO_4 (pH 4.0–5.5), 100 mM phosphate (pH 6.0–7.5), and 100 mM Tris (pH 8.0–9.0). The temperature range for growth was determined on R2A agar at 4, 10, 15, 20, 25, 30, 35, 37, 40, 45, and 50°C. To investigate the tolerance to NaCl, R2A broth was prepared according to the formula of the BD Difco medium with NaCl concentration adjusted to 0, 0.5% and 1.0–6.0%, w/v (at intervals of 1.0%). Growth under anaerobic conditions was determined after incubating bacterial strain in anaerobic jars by using AnaeroGen anaerobic system envelopes (Oxoid) at 30°C for 15 days. Growth was tested on nutrient agar, trypticase soy agar, R2A agar and LB agar (all from Difco) under aerobic condition at 30°C.

Catalase activity was determined by bubble production in 3% (v/v) hydrogen peroxide and oxidase activity was assessed colorimetrically using tetramethyl *p*-phenylenediamine. DNA hydrolysis was investigated on DNase test agar (BD Difco). Hydrolysis of casein (2% skimmed milk, w/v), starch (2.5% soluble starch, w/v), lecithin (10%, w/v), corn oil (3%, w/v) and Tweens 20, 40, 60, and 80 (1%, w/v) was determined using the methods of Tindall *et al.* (2007). Chitin hydrolysis was assessed on chitinase-detection agar and visualized by the formation of clear zones around the colonies as described by Wen *et al.* (2002). Hydrolysis of carboxymethyl cellulose (CM-cellulose) was tested as described by Bowman (2000) using R2A agar as the basal medium. Additional biochemical tests were performed using API ZYM, API 20NE, and API 50CH kits (all from bioMérieux) and carbon source utilization was evaluated using the GN2 MicroPlate (Biolog). All commercial phenotypic tests were performed according to the manufacturers' instructions.

Sensitivity to antibiotics was tested by the disc diffusion method after spreading cell suspensions (0.5 McFarland standard) on R2A agar plates. The discs (Oxoid) contained the following antibiotics: ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), penicillin G (10 U), rifampicin (5 μg), streptomycin (10 μg), sulfamethoxazole (23.75 μg)/trimethoprim (1.25 μg), and tetracycline (30 μg). The effect of antibiotics on cell growth was assessed after 3 days at 30°C. A strain was considered as susceptible when the diameter of the inhibition zone was > 12 mm, moderately susceptible at 10–12 mm and resistant at < 10 mm as described by Nokhal and Schlegel (1983).

Chemotaxonomic characterization

The fatty acid profile was analyzed on cells grown on R2A at 30°C for 3 days. The physiological age of the different bacterial cultures at the time of harvesting was standardized by the choice of sector from a quadrant streak on R2A agar plates, according to the MIDI protocol. Fatty acid methyl esters were prepared and separated according to the standard protocol of MIDI (Sherlock Microbial Identification System, version 6.0), analyzed by GC (Hewlett-Packard 5890 Series II) and identified by using the RTSBA6.00 database of the microbial identification system (Sasser, 1990). The polar lipids were extracted from cells grown on R2A at 30°C for 3 days, and analyzed by two-dimensional TLC according to Embley and Wait (1994). Ethanolic molybdophosphoric acid (10%) was used for the detection of the total polar lipids, ninhydrin for amino lipids, the Zinzadze reagent for phospholipids, Dragendorff reagent for choline-containing lipids and the α-naphthol reagent for glycolipids. The sphingolipids were analyzed as described by Yoon *et al.* (2012), with DL-dihydrosphingosine used as a standard. Polyamines were extracted and analysis was carried out as described by Busse and Auling (1988) and Busse *et al.* (1997). Cells were cultivated in R2-PYE broth (per L: 0.75 g peptone from casein, 0.75 g yeast extract, 0.3 g K_2HPO_4, 0.024 g $MgSO_4$, pH 7.2) at 30°C for 3 days, and homogenized in 0.2 M perchloric acid ($HClO_4$) and centrifuged. Polyamines in the resultant supernatant were treated with dansyl chloride solution (7.5 μg/ml in acetone), and analyzed by HPLC on a D-7000 high-speed liquid chromatograph (Hitachi) and UV-VIS detector L-7420 (Hitachi). Isoprenoid quinones were extracted from cells grown on R2A at 30°C for 3 days, and purified according to the method of Collins (1994) and analyzed by HPLC with a Spherisorb ODS column using methanol/1-chlorobutane (100:10, v/v) as mobile phase (1.5 ml/min).

Phylogenetic analyses based on 16S rRNA gene

Genomic DNA was isolated using a bacterial genomic DNA purification kit (DP02-150, GeneMark). Primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGG

AGGTGATCCAGCC-3′) were used for amplification of bacterial 16S rRNA genes by PCR (Weisburg *et al.*, 1991; Anzai *et al.*, 1997). The 16S rRNA gene was sequenced and analyzed as described previously by Chen *et al.* (2001). The sequence fragments were assembled by manual adjustments to obtain a nearly full-length 16S rRNA gene sequence. The novel sequence was compared with those available from the EzBioCloud (Yoon *et al.*, 2017). The 16S rRNA gene sequences of novel strain and closely related type strains were aligned using the fast secondary-structure aware Infernal aligner available in the Ribosomal Database Project (RDP) (Nawrocki and Eddy, 2007). Phylogenetic analysis was performed by using the software package BioEdit (Hall, 1999) and MEGA 7 (Kumar *et al.*, 2016). Phylogenetic trees were inferred by using three different methods, the neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge and Farris, 1969) methods. Evolutionary distance matrices for the neighbor-joining algorithm were calculated using Kimura's two-parameter model (Kimura, 1983). Bootstrap analysis with 1000 resamplings was conducted to obtain confidence levels for the branch nodes (Felsenstein, 1993).

Genomic analysis and genome annotation

The whole genome sequence was prepared by the Genomics BioSci & Tech. Co., Ltd. (Taiwan) using the Illumina NextSeq sequencer platform and using MultiQC v1.2 for evaluating read quality (Ewels *et al.*, 2016). The whole genome was assembled using SPAdes (version 3.10.1) (Bankevich *et al.*, 2012), and gene prediction and annotation by Prokka pipe-

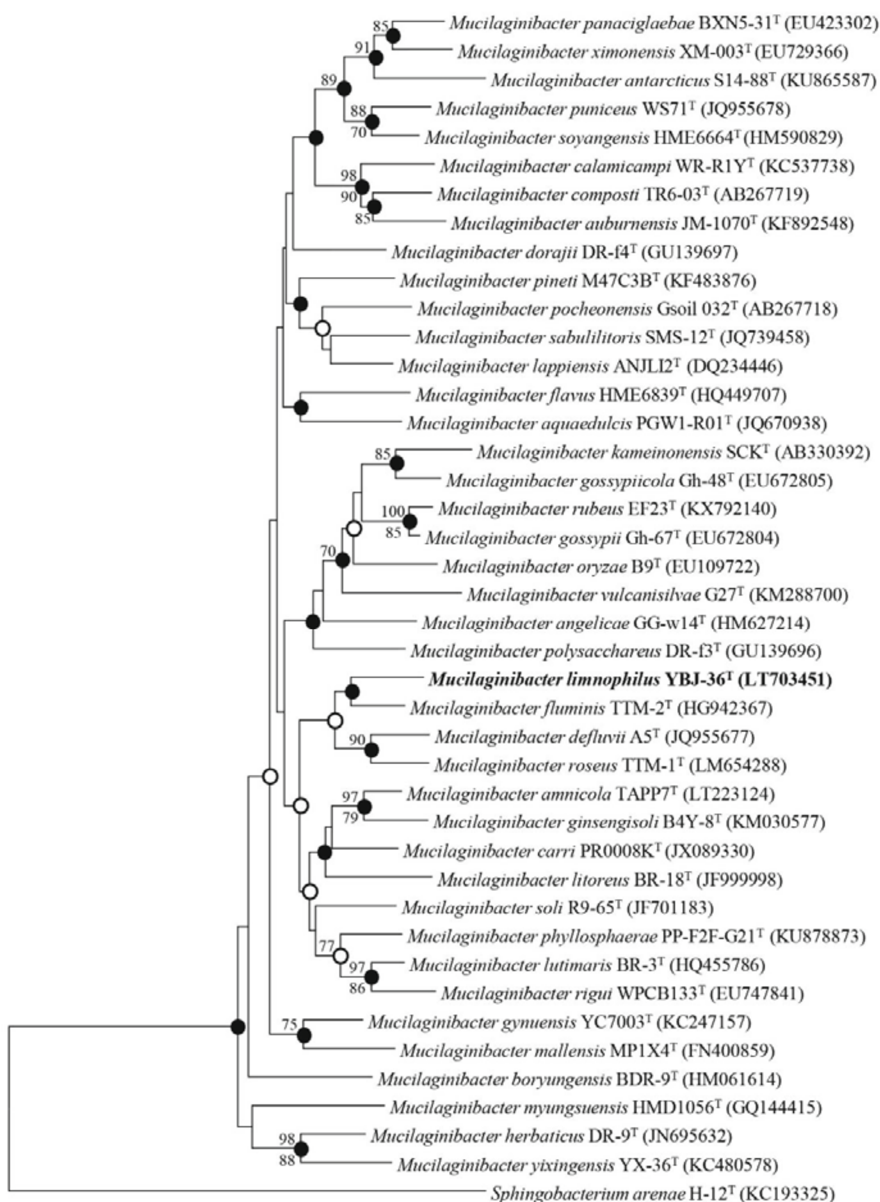

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Mucilaginibacter limnophilus* YBJ-36^T and closely related strains. Numbers at nodes are bootstrap percentages > 70% based on the neighbor-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms. Filled circles indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony tree-making algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Sphingobacterium arenae* H-12^T was used as an out-group. Bar, 0.01 substitutions per nucleotide position.

line (Seemann, 2014). The estimated genome-sequence-based digital DNA-DNA hybridization (dDDH) values were calculated as described by Meier-Kolthoff *et al.* (2013). Average nucleotide identity (ANI) calculations were performed by OrthoANI analysis (Lee *et al.*, 2016). An up-to-date bacterial core gene set (UBCG, concatenated alignment of 92 core genes) and pipeline was utilized for phylogenetic tree construction as described by Na *et al.* (2018). 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 as described by Huerta-Cepas *et al.* (2016, 2017). For gene prediction, these sequences were also submitted to Rapid Annotation of microbial genomes using Subsystem Technology (RAST) (Aziz *et al.*, 2008; Overbeek *et al.*, 2014).

DNA-DNA hybridization

DNA-DNA hybridization experiments were carried out at 55°C in triplicate with photobiotin-labelled probes by the method of Ezaki *et al.* (1989). The signal produced by self-hybridization was taken as 100%, and percentage homology values were calculated from duplicate samples.

Nucleotide and whole genome sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the whole genome of *Mucilaginibacter limnophilus* strain YBJ-36^T are LT703451 and SACK-00000000.

Results and Discussion

Phylogenetic and genomic analyses

Comparative analysis of the 16S rRNA gene sequence of strain YBJ-36^T (1,401 nucleotides) was performed. Phylogenetic analyses revealed that strain YBJ-36^T belonged to the genus *Mucilaginibacter* within the family *Sphingobacteriaceae*. In the neighbour-joining tree, strain YBJ-36^T formed a monophyletic cluster along with *M. fluminis* TTM-2^T, *M. roseus* TTM-1^T, and *M. defluvii* A5^T (Fig. 1). The overall topologies of the maximum-parsimony and maximum-likelihood trees were similar (Supplementary data Fig. S1). Sequence similarity calculations (conducted on > 1,400 bp) indicated that strain YBJ-36^T is closely related to species of the genus *Mucilaginibacter* (93.8–97.8% sequence similarity) and has high sequence similarity to *M. fluminis* TTM-2^T (97.8%), *M. roseus* TTM-1^T (97.2%), *M. gynuensis* YC7003^T (96.8%), *M. herbaticus* DR-9^T (96.7%), and *M. defluvii* A5^T (96.7%). Sequence similarities < 96.6% were observed with all other type strains of *Mucilaginibacter* species listed in Fig. 1.

To further investigate the taxonomic rank of strain YBJ-36^T, whole genome sequencing and assembly was performed. The genome sequence of strain YBJ-36^T is 4.63 Mb (sequencing metrics included an average coverage of 454X, and 40 contigs with an N_{50} value of 417,795 bp). Gene prediction and annotation identified 4,025 protein encoding genes, seven rRNA genes, and 38 tRNA genes. Comparison of the PCR-amplified 16S rRNA gene sequence with the corresponding sequence from the genomic sequencing effort revealed a single base difference between the two sequences, located in the reverse primer (primer 1541R) area. The DNA G+C content of strain YBJ-36^T, calculated directly from the genome sequence, is 42.8 mol%, which is within the range of 39.1–49.8 mol% reported for the genus *Mucilaginibacter* (Kim *et al.*, 2012; Chen *et al.*, 2014).

Average nucleotide identity (ANI) values between the genome of strain YBJ-36^T and type strains of other *Mucilaginibacter* species with publicly available genome sequences were calculated. ANI values were found to be between 70.0–71.4% (Supplementary data Table S1), which are lower than the previously proposed 95–96% cut-off threshold for species delimitation (Richter and Rosselló-Móra, 2009). In ad-

Table 1. Differential characteristics of *Mucilaginibacter limnophilus* YBJ-36^T and phylogenetically closely related *Mucilaginibacter* species

Strains: 1, Strain YBJ-36^T; 2, *M. fluminis* TTM-2^T; 3, *M. roseus* TTM-1^T; 4, *M. defluvii* A5^T; 5, *M. lutimaris* BR-3^T; 6, *M. litoreus* BR-18^T; 7, *M. amnicola* TAPP7^T; 8, *M. paludis* TPT56^T. Data were obtained in this study except the G+C content of *M. fluminis* TTM-2^T (Sheu *et al.*, 2016), *M. roseus* TTM-1^T (Chen *et al.*, 2016), *M. defluvii* A5^T (Hwang *et al.*, 2014), *M. lutimaris* BR-3^T (Kim *et al.*, 2012), *M. litoreus* BR-18^T (Yoon *et al.*, 2012) and *M. amnicola* TAPP7^T (Chen *et al.*, 2018), and data for *M. paludis* TPT56^T from Pankratov *et al.* (2007) and Aydogan *et al.* (2017). +, Positive reaction; -, negative reaction; w, weakly positive reaction. All strains are positive for oxidase, β-galactosidase (PNPG), alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and *N*-acetyl-β-glucosaminidase activities, hydrolysis of esculin. In the API 50CH test system, all strains can produce acids from D-mannose, esculin, trehalose and gentiobiose. All strains are negative for: Gram staining; indole production; D-glucose acidification; arginine dihydrolase, C14 lipase, trypsin and α-chymotrypsin activities; assimilation of caprate, adipate and phenyl-acetate; hydrolysis of casein, gelatin and chitin.

Characteristic	1	2	3	4	5	6	7	8
Colony pigmentation	pink	light pink	pink	pale pink	light pink	light pink	pink	light pink to reddish
Temperature range for growth (°C) (optimum)	20–40 (35–37)	15–37 (30)	10–37 (30–37)	10–37 (25)	4–37 (25)	10–40 (25–30)	10–30 (15–20)	2–33 (20)
pH range for growth (optimum)	5.5–7 (6)	4–8 (6)	6–8 (6–7)	6–8 (7)	6–7 (6)	6–7 (6)	4–8 (6)	4.2–8.2 (6–6.5)
NaCl range for growth (%, w/v) (optimum)	0–2 (0.5)	0–1 (0.5)	0–2 (0.5)	0–2 (0–0.5)	0–1 (0)	0–2 (0)	0–1 (0.5)	0–1
Anaerobic growth	-	-	-	-	+	+	-	+
Nitrate reduction	-	-	-	-	-	-	-	+
Hydrolysis of :								
Starch	+	+	+	+	w	-	-	+
CM-cellulose	+	+	+	+	-	-	-	-
Urea	-	-	-	-	-	-	-	+

Table 1. Continued

Characteristic	1	2	3	4	5	6	7	8
Assimilation of (API 20NE):								
Glucose	+	+	+	+	+	+	+	-
Arabinose	-	+	+	+	-	-	-	-
Mannose	+	+	+	+	+	+	+	-
Mannitol	-	-	-	-	-	-	-	w
N-Acetyl-glucosamine	+	+	+	+	+	+	+	-
Maltose	+	+	+	+	+	+	+	-
Gluconate	-	-	-	-	-	+	-	-
Malate	-	-	-	-	-	+	-	-
Citrate	-	-	-	-	-	+	-	-
Enzymatic activities (API ZYM):								
C4 esterase	+	-	-	-	w	w	+	+
C8 esterase lipase	+	-	-	-	+	w	+	+
Valine arylamidase	+	+	+	+	+	+	+	-
Cystine arylamidase	+	-	+	+	+	+	+	-
α-Galactosidase	+	+	+	+	+	+	+	-
β-Galactosidase	+	-	-	-	+	+	+	+
β-Glucuronidase	-	-	-	-	-	-	+	-
β-Glucosidase	+	-	-	+	+	+	+	-
α-Mannosidase	+	-	+	+	+	+	+	-
α-Fucosidase	+	-	-	-	-	-	+	-
Acid production (API 50CH):								
Erythritol	-	+	-	-	-	-	-	-
D-Arabinose	+	+	+	+	-	w	+	-
L-Arabinose	-	+	+	+	-	-	-	-
D-Ribose	-	+	+	-	-	-	-	-
D-Xylose	+	+	+	+	-	w	+	-
Methyl β-xyloside	w	+	+	-	-	-	+	-
D-Galactose	+	+	+	+	+	+	+	-
D-Glucose	+	+	+	+	+	+	+	-
D-Fructose	+	+	+	+	-	+	+	-
L-Rhamnose	w	+	+	+	-	w	-	-
Methyl α-mannoside	+	+	+	+	+	+	+	-
Methyl α-glucoside	+	+	+	+	+	+	+	-
N-Acetylglucosamine	+	+	+	+	+	+	+	-
Amygdaline	+	+	+	+	+	+	+	-
Arbutin	-	+	+	+	+	+	+	-
Salicin	+	+	+	+	+	+	+	-
Cellobiose	+	+	+	+	+	+	+	-
Maltose	-	+	+	+	+	+	+	+
Lactose	-	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	-
Sucrose	+	+	+	+	+	+	+	-
Inulin	+	+	+	+	-	-	+	-
Melezitose	w	+	+	+	-	w	+	-
Raffinose	+	+	+	+	+	+	+	-
Starch	+	+	+	+	-	w	+	-
Glycogen	+	+	+	+	-	-	-	-
D-Turanose	+	+	+	+	+	+	+	-
D-Lyxose	+	-	-	-	-	-	+	-
L-Fucose	w	+	+	+	-	-	-	-
D-Arabitol	-	+	-	-	-	-	-	-
5-Ketogluconate	+	+	+	w	-	-	-	-
DNA G+C content (mol%)	42.8	46.6	45.1	41.2	49.8	42.4	45.6	46.1

dition, the dDDH values between strain YBJ-36^T and the related *Mucilaginibacter* strains were 20.8–23.6%, which is below the 70% threshold for species delineation (Goris *et al.*, 2007). These data suggest that strain YBJ-36^T is a separate species within the genus *Mucilaginibacter*. Genome-based phylogeny was performed with a set of core genes rather than a single gene. Specifically, an up-to-date bacterial core gene set (UBCG, concatenated alignment of 92 core genes) and pipeline was utilized for phylogenetic tree construction. The constructed phylogenetic tree (based on the coding sequences of 92 protein clusters) showed that strain YBJ-36^T formed a distinct phylogenetic lineage within the genus *Mucilaginibacter* (Supplementary data Fig. S2), which also indicates that strain YBJ-36^T should be considered to be a novel species of the genus *Mucilaginibacter*.

DNA-DNA hybridization

Since complete genome information is not available for the reference species and since it was proposed that DNA-DNA relatedness below 70% can be used to define a novel species (Wayne *et al.*, 1987), DNA-DNA hybridization experiments between strain YBJ-36^T and *M. fluminis* TTM-2^T and *M. roseus* TTM-1^T were performed. DNA-DNA relatedness between YBJ-36^T and *M. fluminis* TTM-2^T and *M. roseus* TTM-1^T was found to be 34.3 ± 1.1% and 28.2 ± 1.2%, respectively. Therefore, strain YBJ-36^T can be considered to be a separate species within the genus *Mucilaginibacter*.

Morphological, physiological, and biochemical characteristics

Cells of strain YBJ-36^T are rod-shaped and are surrounded by a thick capsule (Supplementary data Fig. S3). Cells grew well on R2A agar and nutrient agar, but not on trypticase soy agar or LB agar. Strain YBJ-36^T was resistant to ampicillin, penicillin G, gentamicin, kanamycin, and chloramphenicol, and sensitive to nalidixic acid, novobiocin, rifampicin, streptomycin, tetracycline, and sulfamethoxazole/trimethoprim. Detailed phenotypic and biochemical analysis results of strain YBJ-36^T are provided in the species description, and selected characteristics of strain YBJ-36^T and the selected type strains are compared in Table 1. As shown in Table 1, phenotypic examination revealed several common traits shared between the novel strain and seven related strains (*M. fluminis* TTM-2^T, *M. roseus* TTM-1^T, *M. defluvii* A5^T, *M. lutimaris* BR-3^T, *M. litoreus* BR-18^T, *M. amnicola* TAPP7^T, and *M. paludis* TPT56^T). However, strain YBJ-36^T could be clearly differentiated from these seven related strains by a higher optimal growth temperature (35–37°C), an inability to grow at lower temperatures (< 20°C), and an inability to produce acid from maltose and lactose.

Table 2. Cellular fatty acid composition of *Mucilaginibacter limnophilus* YBJ-36^T and phylogenetically closely related *Mucilaginibacter* species
Strains: 1, Strain YBJ-36^T; 2, *M. fluminis* TTM-2^T; 3, *M. roseus* TTM-1^T; 4, *M. defluvii* A5^T; 5, *M. lutimaris* BR-3^T; 6, *M. litoreus* BR-18^T; 7, *M. amnicola* TAPP7^T; 8, *M. paludis* TPT56^T. All data were obtained from this study except data for *M. paludis* TPT56^T from Pankratov *et al.* (2007). All strains were grown on R2A agar at 30°C for 3 days. Only fatty acids with more than 1% of the total acids in at least one of the strains are shown. Major fatty acids (> 10%) are indicated in bold. TR, Trace (< 1%); -, not detected.

Fatty acid	1	2	3	4	5	6	7	8
Straight chain:								
$C_{12:0}$	TR	1.5	1.4	1.3	1.3	1.4	2.0	-
$C_{14:0}$	TR	1.1	TR	1.0	1.2	1.3	1.5	TR
$C_{15:0}$	-	-	-	-	-	-	-	3.2
$C_{16:0}$	8.2	4.2	2.5	4.9	6.1	7.0	8.8	3.1
$C_{18:0}$	1.8	TR	TR	3.2	3.7	5.3	3.2	TR
Branched:								
anteiso-$C_{14:0}$	TR	1.8	1.6	2.3	2.0	2.5	3.8	-
anteiso-$C_{15:0}$	-	-	-	-	TR	-	-	**21.1**
anteiso-$C_{17:0}$	-	-	-	-	-	-	-	4.5
iso-$C_{15:0}$	**36.4**	**37.5**	**33.3**	**25.3**	**24.5**	**24.4**	**18.5**	**15.7**
iso-$C_{16:0}$	-	-	-	-	TR	-	-	1.5
iso-$C_{17:0}$	2.5	1.7	-	1.2	-	1.0	1.4	TR
Unsaturated:								
$C_{15:1}$ $\omega 6c$	TR	TR	-	-	TR	-	2.2	TR
$C_{16:1}$ $\omega 5c$	2.0	3.5	5.5	5.6	4.3	4.4	5.7	5.8
$C_{18:1}$ $\omega 9c$	1.8	TR	TR	2.9	3.2	3.6	2.6	TR
Hydroxy:								
$C_{16:0}$ 3-OH	TR	TR	TR	TR	1.2	1.5	2.1	TR
iso-$C_{15:0}$ 3-OH	2.0	1.6	1.9	2.4	2.1	1.4	TR	1.8
iso-$C_{17:0}$ 3-OH	8.4	8.7	6.9	8.2	8.0	7.8	6.5	8.9
Summed features*								
3	**31.6**	**33.6**	**39.1**	**35.0**	**38.8**	**35.0**	**38.7**	**24.7**
9	2.3	3.8	6.7	5.7	2.6	2.4	2.0	TR

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 cannot be separated by GLC using the MIDI system. Summed feature 3 comprised $C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$ and summed feature 9 comprised $C_{16:0}$ 10-methyl and/or iso-$C_{17:1}$ $\omega 9c$.

Chemotaxonomic characteristics

The predominant cellular fatty acids (i.e., > 10% of total fatty acids) of strain YBJ-36^T are iso-$C_{15:0}$ (36.4%) and summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$; 31.6%). The fatty acid profile of strain YBJ-36^T is similar to those of the seven related *Mucilaginibacter* species described above, although there are differences in the relative proportions of some components (Table 2). The major fatty acids of this group are iso-$C_{15:0}$ and summed feature 3 and the major hydroxy fatty acid is iso-$C_{17:0}$ 3-OH, which are the main fatty acids found in *Mucilaginibacter* species (Pankratov *et al*., 2007; Baik *et al*., 2010). However, *M. paludis* TPT56^T contains a larger amount of anteiso-$C_{15:0}$, which is different than the other strains.

Strain YBJ-36^T exhibited a complex polar lipid profile consisting of phosphatidylethanolamine (PE), two uncharacterized aminophospholipids (APL1 and APL2), one uncharacterized sphingolipid (SL1), two uncharacterized phospholipids (PL1 and PL2), and four uncharacterized lipids (L1-L4) (Supplementary data Fig. S4). The predominant polar lipid detected in strain YBJ-36^T was PE, which is consistent with previous descriptions of *Mucilaginibacter* species (Jiang *et al*., 2012; Kim *et al*., 2012; Yoon *et al*., 2012; Chen *et al*., 2014; Hwang *et al*., 2014). Strain YBJ-36^T also contains sphingolipid, which is the characteristic polar lipid of *Sphingobacteriaceae* the family members (Steyn *et al*., 1998). Polyamines detected in strain YBJ-36^T were homospermidine (HSPD, 98.5%) and putrescine (PUT, 1.5%) (Supplementary data Fig. S5). HSPD was the main polyamine detected in strain YBJ-36^T, which is consistent with the known polyamine profile of the family *Sphingobacteriaceae* (Steyn *et al*., 1998). The predominant respiratory quinone detected in strain YBJ-36^T was menaquinone (MK-7), which is typical of members of the genus *Mucilaginibacter* (Pankratov *et al*., 2007; Baik *et al*., 2010).

Insight from genome sequence of the new taxon

Based on eggNOG classification, the protein encoding genes in strain YBJ-36^T genome are classified into 22 functional categories (Supplementary data Table S2). Most of coding sequences are classified as functional unknown (S, 22.9% of all assigned eggNOG), followed by those identified as having roles in carbohydrate transport and metabolism (G, 9.2%), cell wall/membrane/envelope biogenesis (M, 8.7%), transcription (K, 6.6%), amino acid transport and metabolism (E, 6.4%), and signal transduction mechanisms (T, 5.9%).

According to Rapid Annotation of microbial genomes using Subsystem Technology (RAST), strain YBJ-36^T genome contains 295 genes involved in carbohydrate metabolism including central carbohydrate metabolism, aminosugars, di- and oligosaccharides, one-carbon metabolism, organic acids, polysaccharides, monosaccharides and fermentation. There are 50 genes in strain YBJ-36^T genome related with resistance to antibiotics such as multidrug resistance-tripartite systems found in Gram-negative bacteria, resistance to fluoroquinolones, beta-lactamase, multidrug resistance efflux pumps and related with resistance to toxic compounds such as copper homeostasis, cobalt-zinc-cadmium resistance, zinc resistance, arsenic resistance and resistance to chromium compounds. Thirty-two genes in strain YBJ-36^T genome are assigned as putative functions in synthesis of capsular and extracellular polysaccharides including dTDP-rhamnose synthesis, capsular heptose biosynthesis, polysaccharide deacetylases and rhamnose containing glycans. In addition, 33 genes in strain YBJ-36^T genome are annotated with putative functions related to phages, prophages, transposable elements and plasmids. Except for one gene has a relationship with phages and prophages, the rest genes are associated with the conjugative transposon, *Bacteroidales*.

In conclusion, the genome information of strain YBJ-36^T provides the basis for understanding the properties that this novel stain may have the potential abilities in metabolism of various carbohydrates, fermentation, synthesis of capsule, resistance to antibiotics and toxic compounds, and horizontal transfer of genetic elements between bacterial species. These abilities may confer a competitive ecological advantage to this strain in the complex microbial ecosystem.

Taxonomic conclusion

Based on the phylogenetic analysis presented in this work, strain YBJ-36^T occupies a distinct position within the genus *Mucilaginibacter*. The sufficiently low degree of DNA-DNA relatedness between strain YBJ-36^T and the closely related *Mucilaginibacter* species (< 35%) differentiated these two strains from each other. Chemotaxonomic data of fatty acids, polar lipids, polyamine, and quinone for strain YBJ-36^T and genomic DNA G+C content were consistent with the genus *Mucilaginibacter*. Distinct differences in certain phenotypic, biochemical, chemotaxonomic, and genotypic characteristics between strain YBJ-36^T and the closely related *Mucilaginibacter* species were observed. We consider strain YBJ-36^T to be a novel species of the genus *Mucilaginibacter*, for which we propose the name *Mucilaginibacter limnophilus* sp. nov.

Description of *Mucilaginibacter limnophilus* sp. nov.

Mucilaginibacter limnophilus (lim.no′phi.lus. Gr. n. *limnos* lake; L. adj. *philus* from Gr. adj. *philos* loving, friendly to; N.L. masc. adj. *limnophilus* lake-loving).

Cells are Gram-negative, aerobic, non-motile, rod-shaped and surrounded by a thick capsule. After incubation on R2A agar 48 h at 30°C, the mean cell size is 0.4–0.5 μm in width and 0.7–1.5 μm in length. Colonies on R2A agar are pink, mucoid, convex and circular with irregular, curled margins. The colony size is approximately 4.0–5.5 mm in diameter after 48 h at 30°C. Growth occurs at 20–40°C (optimal range, 35–37°C), at pH 5.5–7 (optimal pH of 6) and with 0–2% NaCl (optimal concentration, 0.5%). Negative for poly-β-hydroxybutyrate accumulation. Flexirubin-type pigments are not produced. Carotenoid pigments are present with maximum absorption at 485 nm. Positive for oxidase and catalase activities and hydrolysis of starch and CM-cellulose. Negative for urease and lipase activities and hydrolysis of casein, chitin, lecithin, DNA, Tweens 20, 40, 60, and 80. In API 20NE tests, positive for aesculin hydrolysis and β-galactosidase activity, and assimilation of glucose, mannose, *N*-acetyl-glucosamine and maltose, and negative for nitrate reduction, indole production, D-glucose acidification, argi-

nine dihydrolase and urease activities, gelatin hydrolysis, and assimilation of arabinose, mannitol, gluconate, caprate, adipate, malate, citrate and phenyl-acetate. In the API ZYM kit, alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities are present, but C14 lipase, trypsin, α-chymotrypsin, and β-glucuronidase activities are absent. Acids are produced from D-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-mannoside, methyl α-glucoside, *N*-acetylglucosamine, amygdalin, esculin, salicin, cellobiose, melibiose, sucrose, trehalose, inulin, raffinose, starch, glycogen, gentiobiose, D-turanose, D-lyxose, and 5-ketogluconate, and weakly produced from methyl β-xyloside, L-rhamnose, melezitose, and L-fucose. Negative for erythritol, L-arabinose, D-ribose, glycerol, L-xylose, D-adonitol, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, arbutin, D-maltose, D-lactose, xylitol, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate, and 2-ketogluconate. The following compounds are utilized as sole carbon sources in the GN2 microplate: α-cyclodextrin, dextrin, glycogen, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, D-galactose, gentibiose, α-D-glucose, maltose, D-mannose, melibose, β-methyl-D-glucoside, sucrose, trehalose, and D-turanose. All other substrates in the GN2 microplate are not utilized. The major fatty acids (> 10% of total fatty acids) are iso-$C_{15:0}$ and summed feature 3 ($C_{16:1}\ \omega 7c$ and/or $C_{16:1}\ \omega 6c$). The respiratory quinone is MK-7. The polar lipid profile consists of phosphatidylethanolamine, two uncharacterized aminophospholipids, one uncharacterized sphingolipid, two uncharacterized phospholipids and four uncharacterized lipids. The major polyamine is homospermidine. The DNA G+C content of the type strain is 42.8 mol%.

The type strain is YBJ-36^T (= BCRC 81056^T = KCTC 52811^T = LMG 30058^T) isolated from the freshwater of the Beigang Old River Park Lake in the vicinity of Yunlin County, Taiwan. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the whole genome of *Mucilaginibacter limnophilus* strain YBJ-36^T are LT703451 and SACK00000000, respectively.

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