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Hymenobacter knuensis sp. nov., Isolated From River Water

Leonid N. Ten[1] · Jae-Jin Lee[1] · Yeon-Hee Lee[1] · Su-Jin Park[1] · Seung-Yeol Lee[1] · Sangkyu Park[1] · Dae Sung Lee[2] · In-Kyu Kang[3] · Myung Kyum Kim[4] · Hee-Young Jung[1,5]

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Abstract A Gram-stain-negative, non-motile, non-spore-forming, rod-shaped, aerobic bacterial strain, designated 16F7C-2^T, was isolated from the Han River, South Korea, and was characterized taxonomically using a polyphasic approach. Comparative 16S rRNA gene sequence analysis showed that strain 16F7C-2^T belonged to the family *Cytophagaceae* in the phylum Bacteroidetes and was most closely related to *Hymenobacter algoricola* VUG-A23a^T (98.3%) and *Hymenobacter fastidiosus* VUG-A124^T (97.7%). The G+C content of the genomic DNA of strain 16F7C-2^T was 63.4 mol%. The detection of menaquinone MK-7 as the predominant respiratory quinone; a fatty acid profile with summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$; 19.5%), summed feature 4 ($C_{17:1}$ iso I/$C_{17:1}$ anteiso B; 15.9%), $C_{15:0}$ iso (12.6%), $C_{16:1}\omega5c$ (10.5%), and $C_{15:0}$ anteiso (10.4%) as the major components; and a polar lipid profile with phosphatidylethanolamine as the major component also supported the affiliation of strain 16F7C-2^T to the genus *Hymenobacter*. The DNA–DNA relatedness between strain 16F7C-2^T and *H. algoricola* JCM 17214^T and *H. fastidiosus* JCM17224^T were 45.2 ± 5.8 and 40.3 ± 2.9%, respectively, clearly showing that the isolate constitutes a new genospecies. Strain 16F7C-2^T could be clearly differentiated from its closest neighbors on the basis of its phenotypic, genotypic, and chemotaxonomic features. Therefore, strain 16F7C-2^T represents a novel species of the genus *Hymenobacter*, for which the name *Hymenobacter knuensis* sp. nov. is proposed. The type strain is 16F7C-2^T (=KCTC 52538^T = JCM 31814^T).

Introduction

The genus *Hymenobacter* was first proposed by Hirsch et al. [10], and its description was later emended by Buczolits et al. [2]. Members of the genus *Hymenobacter* are aerobic, non-motile, Gram-negative, and red-to-pinkish rods that are characterized chemotaxonomically as having MK-7 as the predominant respiratory quinone; the fatty acid profile with major amounts of iso-$C_{15:0}$, anteiso-$C_{15:0}$, summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$) and summed feature 4 (iso-$C_{17:1}$ I/anteiso-$C_{17:1}$ B); and the polar lipid profile containing phosphatidylethanolamine as the major component and several unknown aminolipids, aminophospholipids, glycolipids, phospholipids, and polar lipids. At the time of writing, the genus *Hymenobacter* comprised 37 species with *H. roseosalivarius* as the type species (http://www.bacterio.net/hymenobacter.html). In addition, several other strains were recently proposed as novel *Hymenobacter* species and are awaiting notification [12, 14, 18, 21, 28]. Type strains of *Hymenobacter* species have been recovered from a wide range of natural sources, including aqueous environments such as lakes [11], estuaries [13], coastal seawaters

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✉ Hee-Young Jung
heeyoung@knu.ac.kr

[1] School of Applied Biosciences, Kyungpook National University, Daegu 41566, Republic of Korea

[2] Department of Environmental Engineering, Kyungpook National University, Daegu 41566, Republic of Korea

[3] Department of Horticultural Science, Kyungpook National University, Daegu 41566, Republic of Korea

[4] Department of Bio and Environmental Technology, Seoul Women's University, Seoul 01797, Republic of Korea

[5] Institute of Plant Medicine, Kyungpook National University, Daegu 41566, Republic of Korea

[12], glaciers [17, 21], snow in Antarctica [18], and wetlands [14]. In the present report, we describe a strain, designated 16F7C-2^T, which was isolated from a water sample collected from the Han River in Seoul, Republic of Korea. To determine the taxonomic position of strain 16F7C-2^T phylogenetic analysis based on 16S rRNA gene sequences, DNA–DNA hybridization and analysis of the phenotypic, genotypic, and chemotaxonomical characteristics of the isolate were performed. On the basis of the results obtained in this study, we propose strain 16F7C-2^T to be placed in the genus *Hymenobacter* as the type strain of a novel species.

Materials and Methods

Isolation of Bacterial Strain and Culture Condition

Strain 16F7C-2^T was isolated from the Han River in Seoul, Republic of Korea (37°31′40″N, 126°56′06″E). The collected water samples were filtered through membrane filters (pore size, 0.2 μm; Millipore); filtrate was discarded; the filters were vortexed in 10 mL of distilled water; and the obtained sample was spread on R2A agar plates (Difco). The plates were incubated aerobically at 25 °C for 1 week. Single colonies on these plates were purified by transferring them onto fresh plates and incubating them again under the same conditions. After isolation, strain 16F7C-2^T was cultivated by being transferred onto R2A agar every month. Stock cultures were preserved as a suspension in glycerol (20%, w/v) at −70 °C. This isolate was deposited in the Korean Collection for Type Cultures (KCTC) and the Japan Collection of Microorganisms (JCM). Two reference type species of the genus *Hymenobacter* were obtained from JCM.

Phenotypic and Biochemical Characteristics

Gram reaction was determined using Gram staining [26] and nonstaining [1] methods. The cell morphology and motility of strain 16F7C-2^T were observed under a Olympus light microscope (×1000 magnification) and a transmission electron microscope (HT7700, Hitachi, Japan), with cells grown for 3 days at 25 °C on R2A agar. Catalase and oxidase tests were performed according to the procedures outlined by Cappuccino and Sherman [3]. The effect of pH on growth was evaluated in R2A broth medium using three different buffers: sodium acetate buffer (for pH 4.0–6.0), potassium phosphate buffer (for pH 7.0–8.0), and Tris buffer (for pH 9.0–10.0). Growth at 4, 10, 15, 20, 25, 30, 37, and 42 °C was assessed on R2A agar after 7 days of incubation. Salt tolerance was tested in R2A broth supplemented with 0.5, 1, 2, 3, 4, 5, and 10% (w/v) NaCl after 7 days of incubation. Enzyme activities, assimilation of carbon sources, acid production from substrates, and other physiological characteristics were determined by inoculating API ZYM, API 20 NE, API ID 32 GN, and API 50CH strips according to the manufacturer's instructions (bioMérieux).

16S rRNA Gene Sequencing and Phylogenetic Analysis

16S rRNA gene sequence analysis of strain 16F7C-2^T was performed as described by Weisburg et al. [31] using the universal bacterial primers 27F (5′-AGAGTTTGATCC TGCTCAG-3′), 518F (5′-CCAGCAGCCGCGGTAATA CG-3′), 800R (5′-TACCAGGGTATCTAATCC-3′), and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). Full sequences of the 16S rRNA gene were assembled using SeqMan software (DNASTAR, Madison, WI, USA). Phylogenetic neighbors were identified, and pairwise 16S rRNA gene sequence similarities were calculated using both the EzTaxon-e server [15] and BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rRNA gene sequences of 41 related taxa were obtained from the GenBank. The recovered sequences were aligned with the sequence of strain16F7C-2^T using the program Clustal X [29]. Gaps and 5′ and 3′ ends of the alignment were edited manually using the BioEdit program [8]. Evolutionary distance matrices for the neighbor-joining algorithm were calculated using Kimura's two-parameter model [16]. Tree topologies were inferred by the neighbor-joining [24], maximum-likelihood [5], and maximum-parsimony [7] methods using the program MEGA7 [20]. A bootstrap analysis with 1000 replicate datasets was performed to assess the support of clusters [6].

Chemotaxonomic Analyses

Cellular fatty acids were analyzed using cultures grown on R2A agar for 7 days at 18 °C. The cellular fatty acid saponification, extraction, and methylation were performed according to Sherlock Microbial Identification System (MIDI) protocol. Fatty acid methyl esters were then analyzed by gas chromatography using the Microbial Identification software package (TSBA, version 6.0) [25]. Polar lipids were extracted using the procedure described by Minnikin et al. [23] and identified by two-dimensional thin layer chromatography (TLC), followed by spraying with appropriate detection reagents [19]. Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum, and re-extracted in n-hexane/water (1:1, v/v). The extract was purified using Sep-Pak Silica Vac Cartridges (Waters) and then analyzed by high-performance liquid chromatography (HPLC) as described previously [9].

Genomic Analysis

High molecular mass DNA from strain 16F7C-2^T and the two most closely related strains, *H. algoricola* JCM 17214^T and *H. fastidiosus* JCM17224^T, were prepared according to the standard CTAB/NaCl protocol [32]. DNA–DNA hybridization was performed fluorometrically using the method of Ezaki et al. [4], with photobiotin-labeled DNA probes and microdilution wells. The highest and lowest values from five replications for each sample were excluded, and the means of the remaining three values were recorded as the average DNA–DNA hybridization value. The standard deviation was also calculated based on these three values. Genomic DNA G + C content was determined by a reverse-phase HPLC analysis of individual nucleosides, resulting from DNA hydrolysis and dephosphorylation using nuclease P1 and alkaline phosphatase [22]. Single-stranded DNA from salmon testes (Sigma D7656; DNA G + C content, 41.2 mol%) was used as a standard.

Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequence of strain 16F7C-2^T determined in this study was deposited in NCBI GenBank/EMBL/DDBJ under the accession number LC177334. The accession numbers of the reference strains that closely related to strain 16F7C-2^T are indicated in Fig. 1.

Results and Discussion

Morphological and Phenotypic Characteristics

Cells of strain 16F7C-2^T were Gram-stain-negative, non-spore-forming, and pink pigment-producing rods without flagella (Supplementary Fig. S1). Growth was observed at 15–30 °C and pH 4–8, with an optimal temperature of 25 °C and pH of 7. Growth occurred on R2A and NA agar, but not on TSA and LB agar. The isolate tolerated 1% (w/v) NaCl, but not 2%. Other phenotypic features of strain 16F7C-2^T are summarized in the species description. Phenotypic and chemotaxonomic characteristics that differentiate the novel strain from its closest neighbors in the genus

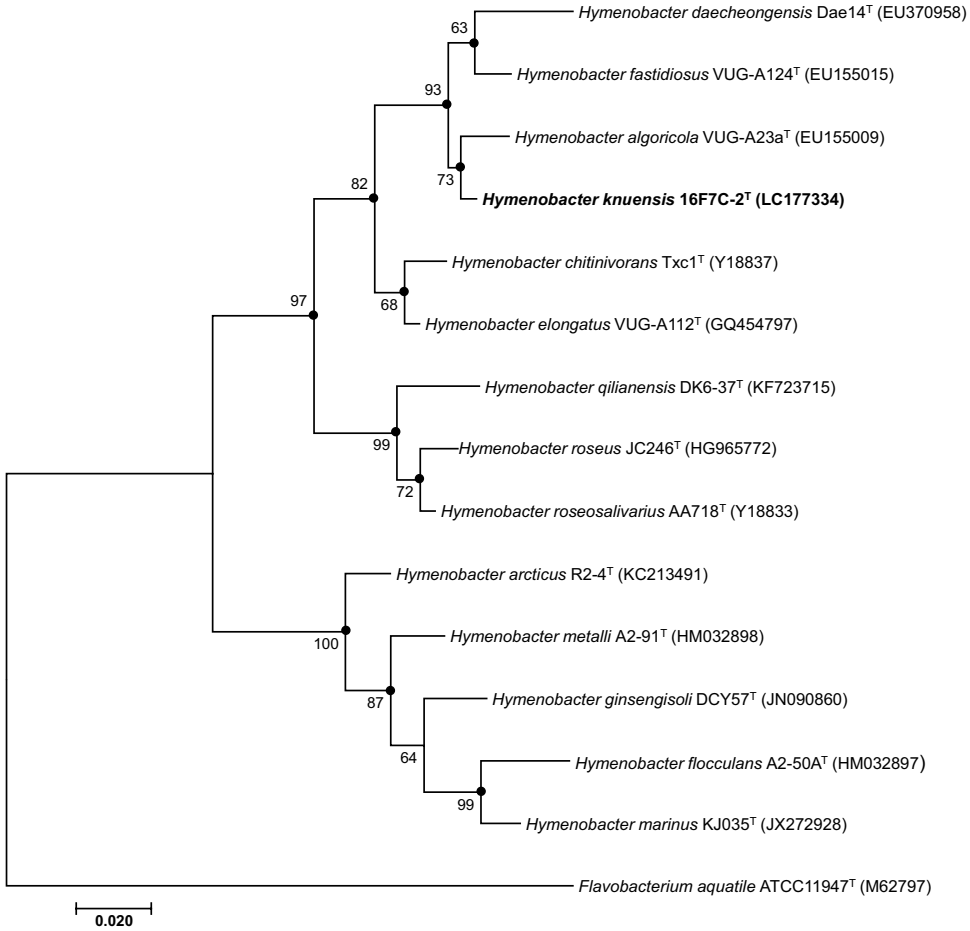

Fig. 1 Maximum-likelihood tree, based on 16S rRNA gene sequences, showing relationships between strain 16F7C-2^T and members of the genus *Hymenobacter*. Bootstrap values (based on 1000 replications) greater than 60% are shown at branching points. *Filled circles* indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Accession numbers are shown in parentheses. *Flavobacterium aquatile* ATCC 11947^T (M62797) was used as an outgroup. *Bar* 0.02 substitutions per nucleotide position

Hymenobacter are listed in Table 1. In particular, strain 16F7C-2^T could be differentiated readily from its phylogenetically closely related type strains of *H. algoricola* and *H. fastidiosus*, based on its inability to grow at 10 °C, pH 9, or 1.0% NaCl, and its ability to grow on NA agar, to hydrolyze aesculin, to produce catalase and *β*-galactosidase, and to utilize D-sorbitol.

Phylogenetic Analysis

The 16S rRNA gene sequence of strain 16F7C-2^T was a continuous stretch of 1458 bp. On the basis of 16S rRNA gene sequence similarity values, the closest relatives of strain 16F7C-2^T were *Hymenobacter algoricola* VUG-A23a^T (98.3%) and *Hymenobacter fastidiosus* VUG-A124^T (97.7%) [17]. Levels of sequence similarity to other genera (*Adhaeribacte*r and *Nibribacter*) were less than 90%. The phylogenetic position of the new isolate, determined using various tree-making algorithms (maximum-likelihood, neighbor-joining, and maximum parsimony), revealed that strain 16F7C-2^T appeared within the genus *Hymenobacter* and clustered with *H. algoricola* VUG-A23a^T with moderate bootstrap values of 73, 78, and 70%, respectively (Fig. 1, Supplementary Fig.

Table 1 Differential phenotypic characteristics of strain 16F7C-2^T and its phylogenetically closest relatives in the genus *Hymenobacter*

Characteristic	1	2	3
Cell size (width x length; μm)	0.8–1.0×2.6–4.1	0.5–0.8×1.0–2.0[a]	0.5–1.0×1.0×3.0[a]
Colony color on R2A	pink	red-pink	red-pink
Growth on/at			
NA agar	+	−	−
4 or 10 °C	−	+	+
25 or 30 °C	+	−	−
pH 5	+	−	−
pH 9–10	−	+	+
0.5 or 1.0% NaCl	+	−	−
Aesculin hydrolysis	+	−	−
Gelatin hydrolysis	+	+	−
Enzyme activities			
Catalase	+	−	−
α-Chymotrypsin	−	−	w
β-Galactosidase	+	−	−
α-Glucosidase	w	−	−
β-Glucosidase	w	−	−
Trypsin	−	+	w
Assimilation of			
L-Fucose	w	−[a]	−[a]
D-Glucose	−	+	−
Glycogen	w	−[a]	−[a]
D-Maltose	−	−[a]	w[a]
D-Sucrose	w	−[a]	−[a]
DL-3-Hydroxybutyrate	w	−[a]	−[a]
D-Sorbitol	+	−[a]	−[a]

All data were obtained in this study, unless otherwise noted

(+) positive, (−) negative, *w* weakly positive reaction

All strains were positive for oxidase, acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase (C8), leucine arylamidase, lipase (C14), naphtol-AS-BI-phosphohydrolase, and valine arylamidase

All strains were negative for Gram reaction, nitrate reduction, indole production, glucose fermentation, *N*–acetyl-*β*-glucosaminidase, arginine dihydrolase, *α*-fucosidase, *α*-galactosidase, *β*-glucuronidase, α-mannosidase, urease and assimilation of acetate, *N*-acetyl-D-glucosamine, adipate, L-alanine, L-arabinose, caprate, citrate, gluconate, L-histidine, *m*-inositol, DL-lactate, L-malate, D-mannitol, D-mannose, phenyl acetate, L-proline, propionate, L-rhamnose, and L-serine

Strains 1, *H. knuensis* 16F7C-2^T; 2, *H. algoricola* JCM 17214^T; 3, *H. fastidiosus* JCM17224^T

[a]Data from reference [17]

S2). As mentioned above, the level of 16S rRNA gene sequence similarity between strain 16F7C-2^T and two closest *Hymenobacter* species is higher than the threshold generally employed for the delineation of novel species (i.e., 97% similarity or below) [27, 30]. Thus, to verify the taxonomic status of strain 16F7C-2^T, DNA–DNA hybridization was performed with the most closely related members in the genus *Hymenobacter*.

Chemotaxonomic Characteristics

The fatty acid profile of strain 16F7C-2^T was characterized by the presence of summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$; 19.5%), summed feature 4 ($C_{17:1}$ iso I/$C_{17:1}$ anteiso B; 15.9%), $C_{15:0}$ iso (12.6%), $C_{16:1}\omega5c$ (10.5%), and $C_{15:0}$ anteiso (10.4%) as major fatty acids. This cellular fatty acid profile was similar to that of the phylogenetically closest relatives *H. algoricola* VUG-A23a^T and *H. fastidiosus* VUG-A124^T (Table 2) and those of other members of the genus *Hymenobacter* [10, 11, 13, 18, 28]. However, some qualitative and quantitative differences in fatty acid content could be observed between strain 16F7C-2^T and its closest neighbors. In particular, strain 16F7C-2^T could be differentiated from its abovementioned phylogenetically closest relatives by its higher content of $C_{15:0}$ anteiso and $C_{17:0}$ iso 3-OH; the presence of small amount of $C_{16:0}$ N alcohol; the absence of $C_{15:1}$ iso G; and lower $C_{16:0}$ content. The major polar lipid found in strain 16F7C-2^T was phosphatidylethanolamine (PE), similar to that in other *Hymenobacter* species [2, 12–14]. In addition, the polar lipid profile of the isolate included moderate amounts of three unknown polar lipids (L_3, L_5 and L_6) and minor amounts of five unknown aminolipids (AL_1-AL_5), four unknown aminophospholipids (APL_1-APL_4), an unknown glycolipid (GL), and three unknown polar lipids (L_1, L_2, and L_4) (Supplementary Fig. S3). The predominant isoprenoid quinone in strain 16F3Y-2^T was a menaquinone with seven isoprene units (MK-7), which is the major respiratory quinone found in members of the genus *Hymenobacter* [2, 12, 14, 18, 21, 28].

Genomic Characteristic

The DNA–DNA relatedness values of strain 16F7C-2^T to its closest phylogenetic relatives, *H.algoricola* JCM 17214^T and *H. fastidiosus* JCM17224^T, were 45.2 ± 5.8% and 40.3 ± 2.9%, respectively, which was sufficiently low [27, 30] to assign strain 16F7C-2^T as a novel species of genus *Hymenobacter*. The DNA G + C content of strain 16F7C-2^T was 63.4 mol%, which lies within the range observed for recognized members of the genus *Hymenobacter* [2, 11, 13].

Table 2 Cellular fatty acid profiles of strain 16F7C-2^T and its closest phylogenetic neighbors in the genus *Hymenobacter*

Fatty acids	1	2	3
Saturated			
$C_{14:0}$	tr	1.3	tr
$C_{15:0}$ iso	12.6	8.7	22.2
$C_{15:0}$ anteiso	10.4	7.5	tr
$C_{15:0}$ 2-OH	1.0	tr	nd
$C_{15:0}$ iso 3-OH	4.0	2.9	3.2
$C_{16:0}$	1.4	10.6	2.1
$C_{16:0}$ iso	1.5	1.9	2.2
$C_{16:0}$ N alcohol	1.8	nd	nd
$C_{17:0}$ iso	2.7	tr	3.3
$C_{17:0}$ anteiso	1.4	1.0	nd
$C_{17:0}$ 2-OH	1.4	1.3	nd
$C_{17:0}$ iso 3-OH	7.1	3.9	5.1
$C_{18:0}$	tr	6.8	tr
Unsaturated			
$C_{15:1}$ iso G	nd	tr	2.3
$C_{15:1}\omega6c$	tr	tr	2.2
$C_{16:1}$ iso H	2.5	2.9	3.1
$C_{16:1}\omega5c$	10.5	12.5	12.3
$C_{17:1}\omega6c$	tr	tr	2.7
Summed feature 1[a] ($C_{15:1}$ iso H/$C_{13:0}$ 3-OH)	1.0	1.3	1.8
Summed feature 3[a] ($C_{16:1}\omega7c/C_{16:1}\omega6c$)	19.5	15.7	12.4
Summed feature 4[a] ($C_{17:1}$ iso I/$C_{17:1}$ anteiso B)	15.9	13.6	19.5
Summed feature 5[a] ($C_{18:0}$ anteiso/$C_{18:2}\omega6,9c$)	nd	nd	2.1
Summed feature 9[a] ($C_{17:1}$ iso $\omega9c/C_{16:1}$ 10-methyl)	tr	tr	1.0

Strains 1, *H. knuensis* 16F7C-2^T; 2, *H. algoricola* VUG-A23a^T; 3, *H. fastidiosus* VUG-A124^T. All data were taken from this study after growth on R2A agar at 18 °C for 7 days. Values are percentages, and only fatty acids accounting for more than 1% in at least one of the strains are indicated

nd not detected; tr trace (<1.0%)

[a]Summed feature contained two fatty acids, which could not be separated by GLC with the MIDI system

Taxonomic Conclusion

Phenotypic and phylogenetic characteristics of strain 16F7C-2^T showed that it was a member of the genus *Hymenobacter*. However, there are several phenotypic differences between strain 16F7C-2^T and its phylogenetically closest relatives (Table 1). Thus, phenotypic results as well as DNA–DNA hybridization data indicated that strain 16F7C-2^T can be delineated from its closest neighbors *H. algoricola* VUG-A23a^T and *H. fastidiosus* VUG-A124^T. Therefore, on the basis of the data presented, strain 16F7C-2^T should be classified as a novel species of the genus *Hymenobacter*, for which the name *Hymenobacter knuensis* sp. nov. is proposed.

Description of *Hymenobacter knuensis* sp. nov

Hymenobacter knuensis (knu.en'sis. N.L. masc. adj. *knuensis* of or belonging to KNU, Kyungpook National University, where the taxonomic study was performed).

Cells are Gram-stain-negative, non-motile, aerobic rods, 0.8–1.0 μm wide, and 2.6–4.1 μm long. After 7 days of incubation at 25 °C on R2A agar, colonies are pink-colored, circular, smooth, translucent, and slimy. Catalase and oxidase activities are positive. In API 20 NE tests, it shows positive for *β*-galactosidase, aesculin, and gelatin hydrolysis, but negative for nitrate reduction, indole production, glucose fermentation, urea hydrolysis and arginine dihydrolase activity. It utilizes L-fucose (weakly, w), glycogen (w), 4-hydroxybenzoate (w), DL-3-hydroxybutyrate (w), itaconate (w), D-sorbitol, suberate, and D-sucrose (w), but none of other substrates tested in API 32 GN and API 20 NE systems (Supplementary Table S1). In API ZYM tests, it shows positive for acid phosphatase, alkaline phosphatase, cysteine arylamidase, esterase (C4) (weak, w), esterase (C8) (w), *β*-galactosidase, *α*-glucosidase (w), *β*-glucosidase (w), leucine arylamidase, lipase (C14) (w), naphthol-AS-BI-phosphohydrolase, and valine arylamidase; negative for other enzyme activities. Acid is produced from amidon, arbutin, aesculin, D-fructose, D-galactose, gentiobiose, glycogen, inulin (weakly, w), 5-ketogluconate, D-lactose, D-maltose, D-mannose (w), D-raffinose, L-rhamnose (w), D-sucrose, and D-trehalose (w), but not from other substrates tested in the API 50CH system. The major cellular fatty acids are summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$), summed feature 4 ($C_{17:1}$ iso I/$C_{17:1}$ anteiso B), $C_{15:0}$ iso, $C_{16:1}\omega5c$, and $C_{15:0}$ anteiso. The predominant menaquinone is MK-7. Phosphatidylethanolamine is the major polar lipid. The G+C content is 63.4 mol%. The type strain 16F7C-2^T (=KCTC 52537^T=JCM 31814^T) was isolated from a water sample collected from the Han River in Seoul (37°31′40″N, 126°56′06″E), South Korea.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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