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Flavobacterium amnigenum sp. nov. Isolated from a River

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A yellowish, flexirubin-pigment-producing strain I3-3^T was isolated from river water in Iksan, the Republic of Korea. The strain was gram-negative, aerobic, non-motile, showed catalase and oxidase activities, and could grow at a temperature range of 10–35°C, pH 5.0–10 and 0–2.0% (w/v) of NaCl. The major fatty acids were iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). The isolate contained phosphatidylethanolamine, one aminolipid, and two unidentified lipids as the major polar lipids. Menaquinone-6 (MK6) was the major respiratory quinone. The G+C content of the genomic DNA of strain I3-3^T was 35.6%. Comparison of the 16S rRNA gene sequence with the sequences of the closely related type strains showed highest sequence similarity of 96.95% and 96.93% to *Flavobacterium nitrogenifigens* NXU-44^T and *Flavobacterium compostarboris* 15C3^T, respectively. Based on phenotypic and phylogenetic distinctiveness, strain I3-3^T is considered as a member of novel species within the genus *Flavobacterium*, for which *Flavobacterium amnigenum* sp. nov. is proposed. The type strain is I3-3^T (=KCTC 52884^T =NBRC 112871^T).

Keywords: *Flavobacterium amnigenum*, freshwater, polyphasic characterization

Introduction

The genus *Flavobacterium* belonging to the family *Flavobacteriaceae* was proposed to accommodate gram-negative, rod-shaped, aerobic, yellow-pigment-producing, non-spore-forming, motile by gliding, and chemo-organotrophic bacteria [1]. They have menaquinone-6 (MK-6) as the major respiratory quinone. DNA G+C content of the genus is in the range of 30–52 mol% [2]. Production of yellow pigments, catalase activity, having no requirement for sodium ions or seawater, inability to produce indole and growth under anaerobic conditions or at above 37°C are the key features of the genus [3]. Currently, the genus *Flavobacterium* contains around 203 species with validly published names (http://www.bacterio.net/flavobacterium.html) and have been isolated from various habitats including fresh and marine water, soil, diseased fish, and microbial mats [4, 5]. During an attempt to study the distribution and diversity of antibiotic resistant bacteria in water bodies, strain I3-3^T was obtained from a river sample. Present work used a polyphasic approach to indicate the taxonomic position of strain I3-3^T and to propose it as a novel species within the genus *Flavobacterium*.

Materials and Methods

Strain Isolation and Culture Condition

A water sample from a river in Iksan, the Republic of Korea, was serially diluted 10 fold in saline (NaCl, 0.85% (w/v)) and 100 µl of each dilution was spread on to Mueller Hinton (MH) agar plates containing gentamycin (8 µg/ml). After incubation at 30°C for 48 h, a yellow-pigmented colony was selected and designated as strain I3-3^T. The isolate was purified by repeated streaking on MH agar plates, maintained under refrigeration at 4°C, and preserved as glycerol (20%, v/v) stock at −80°C. For comparative taxonomic studies, the type strains of *Flavobacterium nitrogenifigens* NXU-44^T (=KACC 14224^T) and *Flavobacterium compostarboris* 15C3^T (=BCCM/LMG 28694^T) were purchased from Belgian Co-ordinated Collections of Microorganisms (BCCM/

LMG) and Korean Agricultural Culture Collection (KACC), respectively.

16S rRNA Gene Sequencing and Phylogenetic Analysis

For 16S rRNA gene sequencing, total genomic DNA was isolated with a bacterial genomic DNA isolation kit (Exgene Cell SV mini, GeneAll). PCR amplification and sequencing of the 16S rRNA gene were performed using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTAC CTTGTTACGACTT-3′) as described previously by Padakandla *et al*. [6]. The determined 16S rRNA gene sequences were assembled using SeqMan software (Lasergene, DNASTAR) and the contig sequence (1,482 bp) of strain I3-3^T was compared with available 16S rRNA gene sequences of culturable species in the EzBioCloud database (http://www.ezbiocloud.net/) [7]. Phylogenetic analysis with 16S rRNA gene sequences of strain I3-3^T and the most closely related type strains belonging to the family *Flavobacteriaceae* was performed using maximum likelihood (ML), neighbour-joining (NJ) and minimum evolution (ME) methods in MEGA6 software [8]. Evolutionary distances were calculated using Kimura's 2-parameter correction in a complete deletion procedure for ML tree and pairwise deletion procedure for NJ and ME trees [9]. Percentage support values were obtained using a bootstrap procedure with 1,000 replications [10]. The obtained 16S rRNA gene sequence of strain I3-3^T was deposited under the GenBank/EMBL/DDBJ accession number MH013305.

Morphological, Physiological, and Biochemical Characterization

Growth of isolate I3-3^T and two reference strains, NXU-44^T and 15C3^T, at 30°C for 48 h were tested on the following bacteriological media such as nutrient agar (NA), brain heart infusion agar, R2A agar, tryptic soy agar (TSA), Luria-Bertani agar (LB) and MacConkey agar. Since all three strains showed optimum growth on TSA, further cultural and morphological comparative characterization was conducted on TSA/tryptone soy broth (TSB) following the recommended standards proposed by Bernardet *et al*. [11] and Tindall *et al*. [12]. Gram reaction was performed using Gram stain kit (Difco) according to the manufacturer's instructions and also by non-staining KOH method [13]. Following the protocol mentioned earlier by Sirra *et al*. [14], cell shape and size was assessed using a Supra 40VP field emission scanning electron microscope (Zeiss). Motility was observed with 1,000 × magnification using a phase-contrast light microscope (Primo Star; Zeiss). Spore formation was investigated by staining the cells of 72 h old cultures with malachite green according to Schaeffer and Fulton [15]. Oxidase activity was determined by 1% aqueous solution of *N,N,N,N*-tetramethyl-*p*-phenylenediamine dihydrochloride and catalase activity was examined using 3% (v/v) H_2O_2 solution [16]. According to Bernardet *et al.* [11], the presence of flexirubin-type pigments was tested using 20% (w/v) KOH solution and the presence of extracellular galactosamine glycan was investigated by Congo red absorption method in which colonies on agar plates were directly flooded with 0.01% aqueous Congo red solution. Capnophilic growth was observed on TSA incubating in a CO_2 incubator (5% CO_2) for 72 h. The temperature range for growth was examined at 5–50°C with an interval of 5°C on TSB. Tolerance to NaCl was investigated in TSB supplemented with 0.5–8.0% (w/v) NaCl (0.5% interval) and pH tolerance was tested on the same medium by adjusting pH to 4–12 (in increments of 1.0 pH unit) using phosphate-citrate (4.0–6.5), Tris-HCl (7.0–9.0), $NaHCO_3$-NaOH (9.5–11.0), and Na_2HPO_4-NaOH (11.5–12.0) buffer systems.

Hydrolysis of skim milk (1%, w/v), carboxymethyl-cellulose (CMC) (0.5%, w/v), casein (1%, w/v), starch (1%, w/v), tween-20 (1%, v/v), and tween-80 (1%, v/v) was observed on 1.5% agar and clear zone formation around colonies was recorded after 48 h incubation [17]. Susceptibility of strain I3-3^T and the two reference strains against various antibiotics was conducted by the Kirby-Bauer disc diffusion method [18] after spreading cell suspensions on MH agar plates. The discs contained the following antibiotics (μg/disc): gentamicin (120), amoxicillin (30), cephalexin (30), tetracycline (30), erythromycin (15), tylosin (30), kanamycin (30), ciprofloxacin (5), clindamycin (2), vancomycin (30), sulfamethoxazole (50), trimethoprim (5), ofloxacin (5), lincomycin (15), norfloxacin (10), tobramycin (30), imipenem (10), penicillin (10), oxytetracycline (30), ampicillin (10), and amikacin (30). The antibiotic susceptibility to the cell growth was determined after 24 h incubation. Following the manufacturer's instructions, acid production from carbohydrates, enzyme activities, and other biochemical tests were performed using API 50CH, API 20E, API 20NE, and API ZYM kits (bioMerieux).

Chemotaxonomic Characterization

Polar lipids were extracted from 150 mg freeze-dried cells following the integrated protocol described by Minnikin *et al*. [19] and separated by two dimensional chromatography on silica gel TLC plates (Kieselgel 60 F254, Merck). Separation was performed using chloroform/methanol/water (65:25:4, v/v/v) and chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v) as mobile phases in the first and second dimensions, respectively [20]. Plates were sprayed with 5% ethanolic molybdophosphoric acid to detect the total polar lipids, and the reagents ninhydrin, molybdenum blue or α-naphthol to detect amino or phosphate groups, respectively [21, 22]. For comparison of cellular fatty acid composition, strains I3-3^T, NXU-44^T, and 15C3^T were grown on TSA for 48 h. According to the instructions for the Microbial Identification System (Microbial ID; MIDI, version 6.0), fatty acid methyl esters were prepared, separated, and identified [23]. As described by Tamaoka *et al*. [24], quinones were extracted from wet culture pellets by shaking with chloroform/methanol (2:1, v/v) for 3–4 h. After the suspension was filtered with Whatman No.2 filter paper, the filtrate was concentrated and suspended in 100 μl chloroform/methanol (8.5:1.5, v/v). It was centrifuged with 12,000 ×*g* for 5 min and the supernatant was analyzed by HPLC (YOUNG LIN YL9100 (YL9111 Binary pump); solvent system, methanol/isopropyl ether (4:1, v/v); flow rate 1.0 ml min^{-1};

Spherisorb 5 μm ODS2 column (4.6 × 150 mm, Waters); YOUNG LIN YL9120 UV/Vis detector; detection wavelength 254 nm). Data analysis was carried out using YOUNG LIN Autochro-3000 software. Extraction and purification of genomic DNA was carried out following the protocol mentioned by Marmur *et al*. [25] and the mol% G+C of the DNA was determined by HPLC method [26].

Results and Discussion

Cells of strain I3-3^T grown on TSA were rod shaped, gram-negative and formed visible dark yellowish colonies (diameter about 2 mm) within 48 h at 30°C. The colonies were convex shaped, translucent, glistening with entire edges, and produced flexirubin type of pigment which was indicated by an immediate color shift from yellow to orange red after addition of 20% (w/v) KOH solution. Strain I3-3^T was positive for oxidase and catalase activities, but negative for Congo red absorption. Under microscopic examination, the strain was non-motile and non-spore-forming rods with 1.0–4.0 μm in length and 0.3–0.4 μm in width (Figs. S1 and S2). Optimal growth was observed on TSA and the strains could also grow on used other culture media except for MacConkey agar. Strain I3-3^T grew over a temperature range of 15–30°C with an optimum at 30°C but showed significant weak growth at temperatures 10°C and 35°C. The strain showed optimum growth at 0.5% (w/v) of NaCl. Although additional salt was not required for the growth of strain I3-3^T, it could tolerate up to 2% NaCl (w/v). Growth occurred over pH range of 5.0–10 with optimum pH of 7.0. No growth was observed in the presence of 5% CO_2. It was also observed that strain I3-3^T exhibited resistance to gentamicin, amoxicillin, erythromycin, kanamycin, vancomycin, trimethoprim, tobramycin, imipenem, amikacin, ampicillin, and penicillin. However, it was sensitive to cephalexin, tetracycline, tylosin, ciprofloxacin, clindamycin, sulfamethoxazole, ofloxacin, lincomycin, norfloxacin, and oxytetracycline. Comparison of the physiological and biochemical characteristics of strain I3-3^T with those of the closely related species was shown in Table 1.

Phylogenetic analysis revealed that the closest phylogenetic neighbors of strain I3-3^T were *Flavobacterium nitrogenifigens* NXU-44^T and *Flavobacterium compostarboris* 15C3^T with sequence similarities of 96.95% and 96.93%. Strain I3-3^T closely clustered with the members of genus *Flavobacterium* and formed a separate clade along with *Flavobacterium compostarboris* 15C3^T in the phylogenetic tree (Fig. 1). DNA G+C content of strain I3-3^T was 35.6 mol%.

The polar lipids of strain I3-3^T were identified as

Table 1. Comparison of the physiological characteristics of strain I3-3^T with those of closely related species of the genus *Flavobacterium*.

Characteristic	1	2	3
Cell shape and size (length × diameter) (μm)	Rods 1.0-4.0 × 0.3-0.4	Rods 1.0 × 2.0	Rods 1.1 -7.2 × 0.3-0.7
Colony color	Yellow	Yellow	Orange yellow
Growth temperature range and optimum (°C)	10-35 (30)	15-30 (25)	10-30 (25)
pH range (optimum)	5.0-10 (7.0)	5.0-10 (7.0)	6.0-8.0 (6.0)
NaCl tolerance % (w/v)	0.0-2.0%	0.0-1.0%	0.0-2.0%
Oxidase	+	+	-
Hydrolysis of			
Tween-20	-	+	+
Carboxymethyl-cellulose (CMC)	-	-	+
Assimilation of:			
D-Xylose	-	w	w
D-Fructose	+	-	-
Salicin	-	w	w
D-Lactose	-	-	+
D-Saccharose	+	-	-
D-Trehalose	-	w	w
Glycogen	w	w	-
Biochemical characters			
ONPG	-	+	+
Urease	-	+	-
Voges Proskauer test	-	+	-
Nitrate reduction	+	-	-
β-glucosidase	-	-	+
DNA G+C content (mol %)	35.6%	ND	33.6%

Strains: 1, *Flavobacterium amnigenum* I3-3^T; 2, *Flavobacterium nitrogenifigens* NXU-44^T; 3, *Flavobacterium compostarboris* 15C3^T. Data obtained from the present study. +, substrate utilized /present; -, substrate not utilized/absent; w, weak growth/activity; ND, not determined. All strains are gram-negative, non-motile, and positive for utilization of citrate, hydrolysis of skim milk, starch, tween-80, esculin, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, assimilation of D-glucose, D-galactose, D-mannose, *N*-acetyl-glucosamine, amygdalin, D-cellobiose, D-maltose, amidon but negative for arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S, indole production, tryptophan deaminase, gelatinase, glucose fermentation, arginine dehydrolase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, assimilation of glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, arbutin, D-melibiose, inuline, D-melezitose, D-raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-keto-gluconate, potassium 5-keto-gluconate, capric acid, adipic acid, malic acid, and phenylacetic acid.

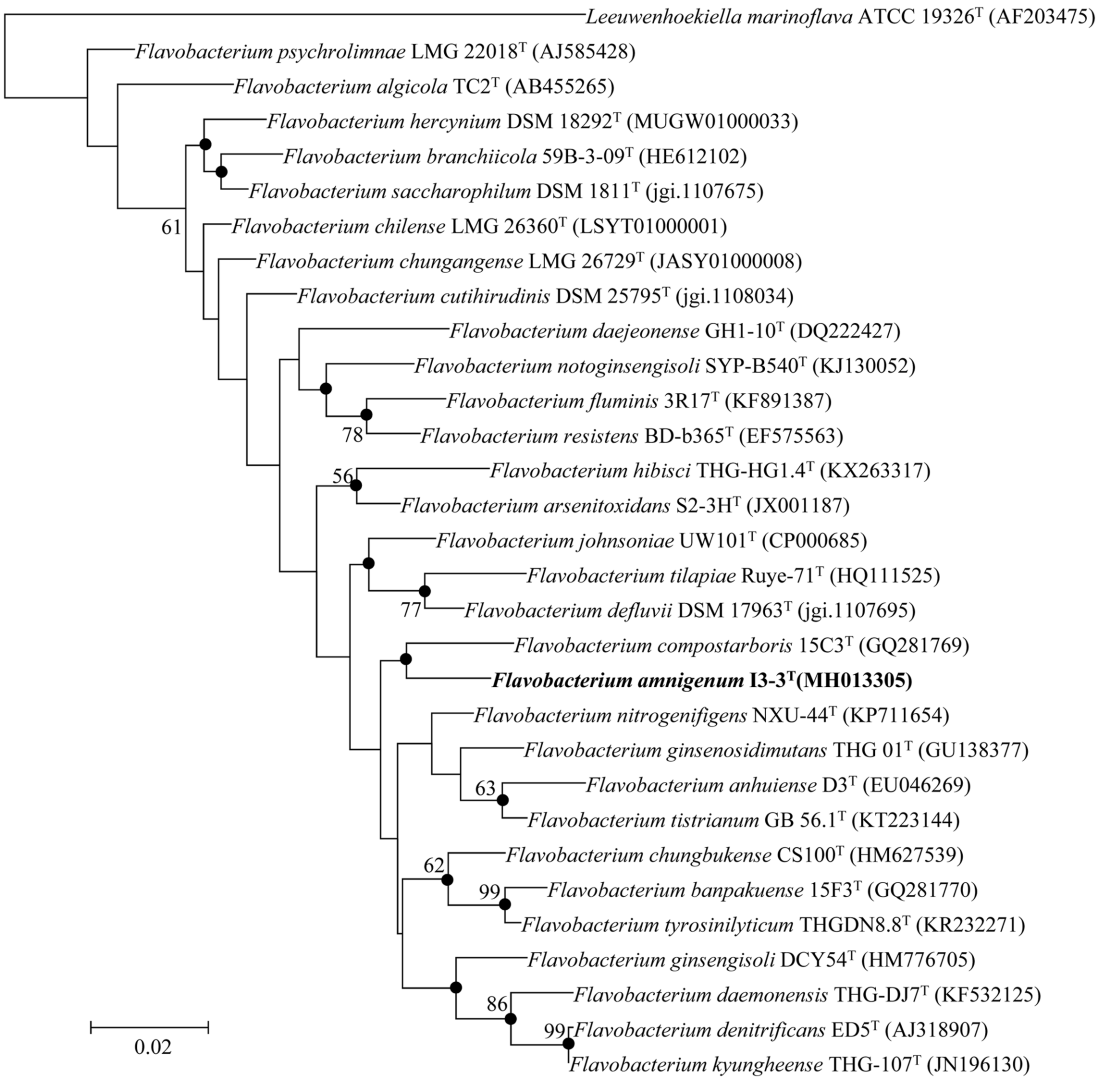

Fig. 1. Maximum likelihood tree depicting the phylogenetic position of strain I3-3^T among the members of the genus *Flavobacterium* based on 16S rRNA gene sequences from EZBioCloud database (accession numbers in parentheses).
Multiple alignment, distance calculations (according to Kimura 2-parameter model), and clustering were performed using MEGA-6 software package. Bootstrap values (≥50) based on 1,000 replications were shown at the nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees reconstructed with neighbor-joining and minimum evolution algorithms. *Leeuwenhoekiella marinoflava* ATCC 19326^T (AF203475) was used as an outgroup. Bar, 0.02 nucleotide substitutions per nucleotide position.

phosphatidylethanolamine (PE), two aminolipids (AL1 and 2), and four unidentified lipids (UL1, 2, 3, and 4) (Fig. S3). Strain I3-3^T contained iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) as the major cellular fatty acids (>10%) (Table 2). Menaquinone-6 (MK6) was the sole isoprenoid quinone in the cells of strain I3-3^T which is in accordance with the description of the *Flavobacterium* genus.

Along with the phylogenetic distinctiveness, strain I3-3^T could also be differentiated from the closely related type strains, *Flavobacterium nitrogenifigens* NXU-44^T and *Flavobacterium compostarboris* 15C3^T by phenotypic characteristics such as colony morphology, acid production, enzyme activities, and fatty acid composition (Tables 1 and 2). This justifies the placement of strain I3-3^T as a novel species within the genus *Flavobacterium* for which the name *Flavobacterium amnigenum* sp. nov. is proposed.

Description of *Flavobacterium amnigenum* sp. nov.

Flavobacterium amnigenum (am.ni'ge.num. L. n. *amnis* any

Table 2. Comparison of cellular fatty acid compositions of strain I3-3^T with those of its closest neighbours in the genus *Flavobacterium*.

Fatty acid	1	2	3
Saturated			
$C_{14:0}$	tr	1.2	1.0
$C_{16:0}$	4.5	5.8	3.9
Unsaturated			
$C_{15:1}\omega6c$	2.0	3.9	3.2
$C_{17:1}\omega6c$	1.6	2.3	2.6
Branched chain			
iso-$C_{15:0}$	28.3	23.7	29.3
iso-$C_{15:0}$ 3-OH	9.5	8.4	10.9
iso-$C_{15:1}$ G	5.1	3.0	6.0
iso-$C_{16:0}$ 3-OH	2.0	1.3	2.2
iso-$C_{17:0}$ 3-OH	14.0	8.7	14.7
anteiso-$C_{15:0}$	1.0	tr	1.0
Hydroxy			
$C_{15:0}$ 3-OH	-	2.2	1.7
$C_{16:0}$ 3-OH	5.3	9.2	4.1
Summed features*			
2	1.0	1.5	1.0
3	16.2	20.7	11.1
9	3.7	1.6	2.2

Strains: 1, *Flavobacterium amnigenum* I3-3^T; 2, *Flavobacterium nitrogenifigens* NXU-44^T; 3, *Flavobacterium compostarboris* 15C3^T. Data obtained from the present study using cells grown on TSA at 30°C for 48 h. Fatty acids representing <1.0% of the total in all strains are not shown. tr, Trace (<1.0%); -, not detected. Summed feature 2 comprised of iso-$C_{16:1}$ I and/or $C_{14:0}$ 3OH; summed feature 3 comprised of $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 9 comprised iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl.

broad and deep-flowing, rapid water; L. suff. genus -a *-um* (from L. v. *gigno*, to produce, give birth to, beget) born from; N.L. neut. adj. *amnigenum* coming from water).

Cells are rod shaped, gram-negative, aerobic, 1.0–4.0 μm long and 0.3–0.4 μm wide, and non-motile. Growth occurs on TSA, brain heart infusion agar, R2A, LB, and NA but not on MacConkey agar at 30°C after 48 h incubation. Colonies on TSA are circular, 1–2 mm in diameter, convex, smooth, translucent, and yellowish with entire edges. The yellow pigment of flexirubin type is non-diffusible and non-fluorescent. No gliding motility and non-swarming. Spores are not formed. Catalase and oxidase positive but negative for Congo red absorption. Growth occurs between 10°C and 35°C with an optimal temperature of 30°C. No growth occurs below 10°C or above 35°C. Growth occurs at NaCl concentrations between 0% and 2% with optimum at 0.5%. Growth occurs between pH 5.0 and 10.0 with an optimal pH 7.0. Starch, skim milk, and tween-80 are hydrolyzed but CMC, casein, and tween-20 are not. Able to assimilate D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, amygdalin, esculin, D-celiobiose, D-maltose, D-saccharose, amidon, and glycogen but not glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, L-xylose, D-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, arbutin, salicin, D-lactose, D-trehalose, D-melibiose, inuline, D-melezitose, D-raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-keto-gluconate, potassium 5-keto-gluconate, capric acid, adipic acid, malic acid, and phenylacetic acid. Showed positive reaction for citrate utilization, nitrate reduction, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and valine aryl amidase whereas negative reaction for H_2S, acetoin, and indole production, ONPG hydrolysis, tryptophane deaminase, gelatinase, urease, arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, lipase (C14), cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, and β-glucosidase. The predominant polar lipids are phosphatidylethanolamine, one aminolipid, and two unidentified lipids. The major fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and summed feature 3 comprising of $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$. Major respiratory quinone is MK-6. The DNA G+C content of the type strain is 35.6 mol%.

The type strain I3-3^T (=KCTC 52884^T =NBRC 112871^T) was isolated from a freshwater river in Iksan, the Republic of Korea.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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