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TAXONOMIC DESCRIPTION

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Flavobacterium keumense sp. nov., isolated from freshwater

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Abstract

A yellow-pigmented, oxidase-positive, catalase-negative, Gram-stain-negative, rod-shaped, aerobic and non-motile bacterial strain designated K3R-10^T was isolated from a freshwater source. The strain grew over a temperature range from 4 to 35 °C (optimum, 30 °C), pH range pH 6–8 (optimum, pH 7) and in the presence of 0–0.5 % NaCl (optimum, 0 %). Phylogenetic analysis based on 16S rRNA gene sequence revealed that strain K3R-10^T belonged to the genus *Flavobacterium* and shared close similarities with *Flavobacterium succinicans* LMG 10402^T (97.0 %), *Flavobacterium chungangense* LMG 26729^T (96.4 %), *Flavobacterium branchiophilum* IFO 15030^T (96.4 %) and *Flavobacterium piscis*412R-09^T (96.3 %), but formed a distinct phylogenetic line of its own in the phylogenetic trees. The polar lipids consisted of phosphatidylethanolamine, an unidentified aminolipid and three unidentified phospholipids. The DNA G+C content was 35.4 mol%, MK-6 was the major isoprenoid quinone, and homospermidine was the predominant polyamine. The predominant cellular fatty acids were iso-$C_{15:0}$ 3-OH, iso-$C_{15:0}$, a summed feature comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ and iso-$C_{15:1}$ G. The absence of aminophospholipid, acid production from carbohydrates, DNA G+C content and colony morphology differentiated strain K3R-10^T from related species of the genus *Flavobacterium*. Thus, on the basis of phenotypic, chemotaxonomic and phylogenetic features, strain K3R-10^T evidently represents a novel species in the genus *Flavobacterium*, for which the name *Flavobacterium keumense* sp. nov. is proposed. The type strain is K3R-10^T (=JCM 31220^T=KCTC 52563^T).

The genus *Flavobacterium*, the type genus of the family *Flavobacteriaceae* belonging to the phylum *Bacteroidetes*, was first proposed by Bergey *et al.* [1] and currently contains over 150 species with validly published names (www.bacterio.net/index.html). Members of the genus are rod-shaped, yellow-pigmented, Gram-stain-negative, aerobic and motile by gliding or non-motile, and contain menaquinone with six isoprene units (MK-6) as the major or only respiratory quinone [2]. Homospermidine is the major polyamine, and the DNA G+C contents are in the range of 30–52 mol% [2]. In addition, a summed feature comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, iso-$C_{15:0}$ 3-OH, iso-$C_{17:0}$ 3-OH, $C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{17:1}\omega9c$, iso-$C_{16:0}$ 3-OH, anteiso-$C_{15:0}$ and $C_{16:0}$ are also present in most strains. Members of this genus are present in a wide range of habitats including soil, freshwater, fish tissues, sediments and wastewater, and some of them are aetiological agents of fish diseases [2, 3]. Several species are associated with digestion of polysaccharides and proteins [4], bioremediation in soils [5] and marine sediments [6], plant protection and growth-promoting ability [7], and plant glycan metabolism [8].

A recent study on the bacterial diversity of major freshwater bodies in Korea using a pyrosequencing approach identified the genus *Flavobacterium* as one of the main bacterial groups in freshwater environments [9]. In this study, a bacterial isolate belonging to the genus *Flavobacterium* was isolated from Keum River. Water samples were collected, serially diluted and incubated on R2A (BD) agar plates at 30 °C for 7 days. A single colony designated strain K3R-10^T was selected, purified and routinely subcultured on R2A agar at 30 °C. Stock solutions were maintained in glycerol suspension (20 %, v/v) at −70 °C. The strain was then characterized following the standards proposed by Bernardet *et al.* [10].

Genomic DNA was extracted using a commercial DNA extraction kit (Solgent). The 16S rRNA gene was amplified using the bacterial universal primers 27F and 1429R [11], and the purified PCR products were sequenced using the service of Macrogen (Korea). The closest phylogenetic neighbours on the basis of the resultant 16S rRNA gene sequence were identified using EzBioCloud (www.ezbiocloud.net/) [12]. Multiple sequence alignment was carried out using MEGA 6.0 [13]. Evolutionary distances were computed with the Jukes and Cantor model, and phylogenetic trees were reconstructed with the neighbour-joining [14], maximum-likelihood [15] and maximum-parsimony [16]

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Two supplementary figures and one supplementary table are available with the online Supplementary Material.

methods using MEGA 6.0. Bootstrap values based on 1000 replications were used to evaluate the confidence levels of the trees. Gaps were treated using pairwise deletion (neighbour-joining) or complete deletion (maximum-likelihood and maximum-parsimony).

Strain K3R-10^T formed a stable clade with species of the genus *Flavobacterium* and a distinct phyletic line in the phylogenetic tree (Fig. 1). Strain K3R-10^T shared the highest 16S rRNA gene sequence similarities with *Flavobacterium succinicans* LMG 10402^T (97.0 %), *Flavobacterium chungangense* LMG 26729^T (96.4 %), *Flavobacterium branchiophilum* IFO 15030^T (96.4 %) and *Flavobacterium piscis* 412R-09^T (96.3 %). Such levels of 16S rRNA gene sequence similarity with neighbouring species were far below the suggested threshold of 98.5–98.7 % for species distinction [9, 17].

Based on the 16S rRNA gene sequence similarity, *F. succinicans* KACC 11420^T and *F. chungangense* KACC 13353^T were obtained from the Korean Agricultural Culture Collection (KACC) and used as reference strains for further comparative studies.

For morphological, physiological and biochemical characterization, strain K3R-10^T and reference strains were grown on R2A agar for 2 days at 30 °C unless otherwise stated. Gram staining was performed using the standard method. Growth at different temperatures (4, 10, 15, 20, 25, 30, 32, 35, 37 and 40 °C) was tested on R2A agar plates, and pH range (4–10, at intervals of 1 pH unit) and NaCl concentrations (0–5 %, w/v) for growth were tested using R2A broth. Growth on R2A, Anacker Ordal, nutrient, MacConkey and tryptic soy agars (Difco) was also investigated. Oxidase and catalase activities were tested by bubble production using 1 % (w/v) tetramethyl-*p*-phenylenediamine and colour change using 3 % (v/v) hydrogen peroxide, respectively. Biochemical tests were performed using the API 20NE and API 50CH kits (bioMérieux) according to the manufacturer's instructions. Tests for degradation and hydrolysis of casein

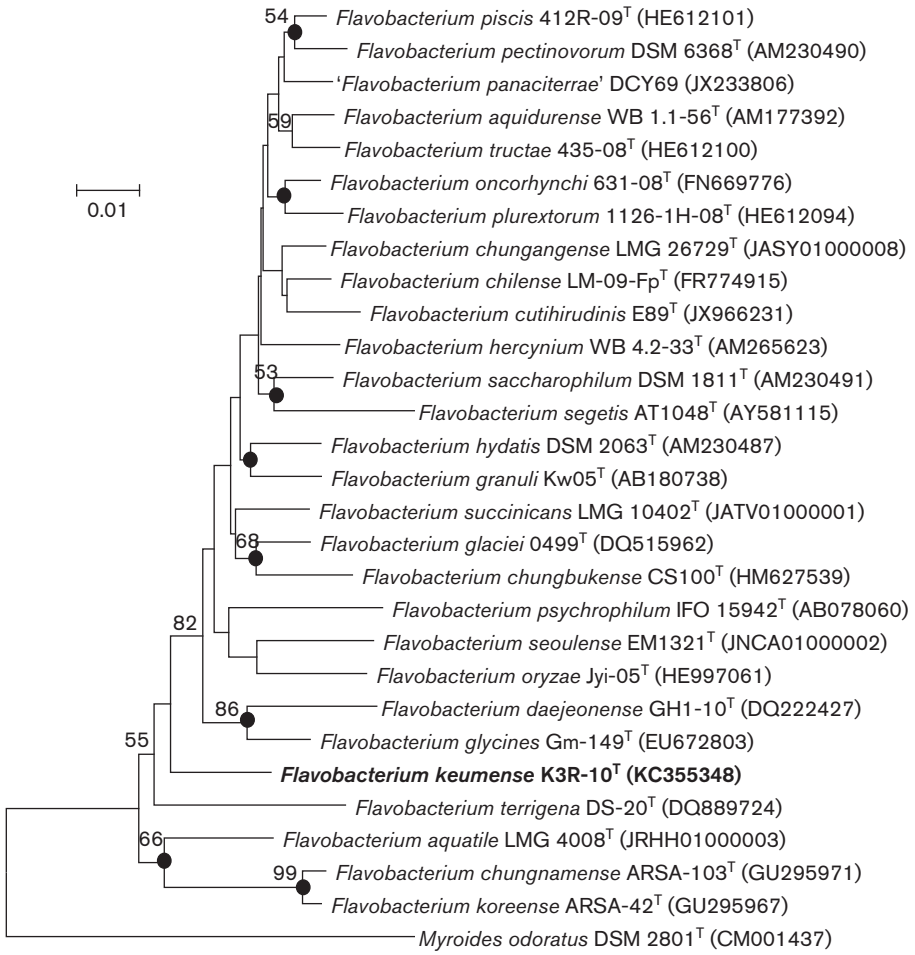

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain K3R-10^T with related taxa. Numbers at nodes indicate bootstrap percentages (>50 %) based on 1000 resampled datasets. Filled circles at nodes indicate branches that were also recovered in the tress generated with the maximum-likelihood and maximum-parsimony methods. Bar, 0.01 substitutions per nucleotide position. *Myroides odoratus* DSM 2801^T (GenBank accession CM001437) was used as the outgroup.

(skimmed milk, 5 %, w/v), starch (0.3 %, w/v), Tween 80 (1 %, v/v) and tributyrin (glyceryl tributyrate, 1 %, v/v) were carried out on R2A agar. Hydrolysis of carboxymethyl cellulose (1 %, w/v), DNA and tyrosine was tested on carboxymethyl cellulose-trypan blue agar [18], DNase test agar (BBL) with methyl green and tyrosine agar [19], respectively. Other enzyme activities were tested using the API ZYM kit according to the manufacturer's instructions (bioMérieux). Flexirubin-type pigments were detected using the bathochromic shift test with 20 % KOH solution, and Congo red adsorption was tested as described by Bernardet *et al.* [10]. Anaerobic growth was tested on R2A agar in an anaerobic chamber (Genbag) at 30 °C for 2 weeks. Gliding motility was tested by stab inoculation on 0.3 % R2A agar [20] and also by using the hanging-drop technique [10].

Strain K3R-10^T was Gram-stain-negative, aerobic, non-motile and rod-shaped (Fig. S1, available in the online Supplementary Material). No growth was observed under anaerobic conditions. Colonies were yellow-pigmented, circular, smooth and convex with entire edges. The colony size ranged from 1 to 2 mm in diameter. Strain K3R-10^T was able to grow at temperatures of 4–35 °C (optimum, 30 °C), pH 6–8 (optimum, pH 7) and in the presence of 0–0.5 % NaCl (optimum, 0 %). Other biochemical and physiological properties of strain K3R-10^T are listed in the species description and Tables 1 and S1.

For cellular fatty acid analysis, strain K3R-10^T and the reference strains were grown on R2A agar plates for 2 days at 30 °C. Cellular fatty acids were extracted according to the procedures for the MIDI Sherlock Microbial Identification System and analysed by gas chromatography using the Sherlock system version 6.1 and RTSBA6 database (MIDI). Polar lipids were extracted from freeze-dried cells according to the method of Minnikin *et al.* [21] and analysed by two-dimensional TLC. The first direction was developed in a chloroform/methanol/water mixture (65 : 25 : 3.8, by vol.) while the second direction was developed in a chloroform/methanol/acetic acid/water mixture (40 : 7.5 : 6 : 1.8, by vol.). The polar lipids with specific functional groups were detected using molybdophosphoric acid for total lipids, ninhydrin for aminolipids and molybdenum blue for phospholipids. Respiratory quinones were extracted from freeze-dried cells according to the method of Collins *et al.* [22] using chloroform/methanol, purified with Sep-Pak Vac silica cartridges (Waters) and analysed by HPLC as described by Komagata and Suzuki [23] using *F. chungangense* KACC 13353^T as a reference. The DNA G+C content was determined using the T_m method of Gonzalez and Saiz-Jimenez [24]. Polyamines were also extracted from freeze-dried cells following the procedure of Busse and Auling [25]. The polyamines extracted were applied to TLC plates and run on a solvent of ethyl acetate/cyclohexane (2 : 3, v/v) mixture. Spots were visualized using UV light at 360 nm and confirmed with ninhydrin.

The major differences between strain K3R-10^T and closely related species included the major fatty acids, DNA G+C content, enzyme activities and carbon source utilization. These differences are listed in Tables 1, 2 and S1. MK-6 and homospermidine were the only isoprenoid quinone and polyamine, respectively, which is typical for members of the genus *Flavobacterium*. The major polar lipids were phosphatidylethanolamine, which is also characteristic for the genus, and an unidentified aminolipid and three unidentified phsopholipids were also present (Fig. S2). The polar lipids of strain K3R-10^T were almost identical to those of *F. chungangense* KACC 13353^T. However, strain K3R-10^T lacked aminophospholipid, which is present in *F. succinicans* [26].

The major fatty acids were iso-$C_{15:0}$ 3-OH, iso-$C_{15:0}$, a summed feature consisting of $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ and iso-$C_{15:1}$ G (Table 2). The fatty acid profiles of strain

Table 1. Differential phenotypic properties of strain K3R-10^T and closely related species of the genus *Flavobacterium*

Strains: 1, K3R-10^T; 2, *F. succinicans* KACC 11420^T; 3, *F. chungangense* KACC 13353^T. +, Positive; −, negative; NA, no data available. In API 20NE tests, all strains were positive for aesculin and *p*-nitrophenyl β-D-glucopyranoside (PNPG) hydrolysis and negative for indole production, glucose fermentation, arginine hydrolysis and urea hydrolysis. In API ZYM tests, all strains were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, and were negative for lipase (C14), trypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. All strains were positive for the hydrolysis of aesculin and starch and negative for the hydrolysis of Tween 80 and carboxymethyl cellulose.

Characteristic	1	2	3
Isolation source*	Freshwater	Freshwater fish	Freshwater lake
Growth range (optimum)*			
Temperature (°C)	4–35 (30)	NA (25–30)	5–35 (30)
pH	6.0–8.0 (7.0)	NA (7.0–7.5)	5.0–8.0 (6)
NaCl (%, w/v)	0–0.5 (0)	NA	0–4.0 (0)
Nitrate/nitrite reduction	−/−	−/−	+/−
Hydrolysis of:			
Gelatin	+	−	+
Casein	−	−	+
DNA	−	−	+
Assimilation of (API 20NE):			
Glucose	−	−	+
Arabinose	−	−	+
Mannose	−	−	+
Maltose	−	−	+
Enzyme activity (API ZYM)			
Valine arylamidase	+	−	+
Cystine arylamidase	+	−	−
α-Chymotrypsin	+	−	−
β-Galactosidase	−	−	+
α-Glucosidase	+	−	+
β-Glucosidase	+	−	+
DNA G+C content (mol%)*	35.4	36.1	34.5

*Data for reference species were taken from Feng *et al.* [26] and Kim *et al.* [28]. All other data were obtained from this study.

K3R-10^T and the reference strains were distinguishable in that the proportion of iso-$C_{15:0}$ 3-OH, the most abundant fatty acid in K3R-10^T, was 20.97 % compared with 7.02 and 8.65 % in *F. succinicans* KACC 11420^T and *F. chungangense* KACC 13353^T, respectively. Detailed differences in the fatty acid profiles of K3R-10^T and the reference strains are shown in Table 2.

The DNA G+C content of strain K3R-10^T was 35.4 (±0.6) mol%, which was different from those of the reference strains albeit within the range (30–52 mol%) described for members of the genus *Flavobacterium* [2, 27]. The major polar lipids, fatty acids, isoprenoid quinone, polyamine and DNA G+C content were all in accordance with those of members of the genus *Flavobacterium*.

On the basis of phenotypic, phylogenetic and chemotaxonomic evidence, strain K3R-10^T should be classified as a representative of a novel species under the genus *Flavobacterium*, for which the name *Flavobacterium keumense* sp. nov. is proposed.

Table 2. Fatty acid content (percentage) of strain K3R-10^T and related species of the genus *Flavobacterium*

Strains: 1, K3R-10^T; 2, *F. succinicans* KACC 11420^T; 3, *F. chungangense* KACC 13353^T. All data are from this study. TR, Trace amount (<1.0 %); ND, not detected.

Fatty acid	1	2	3
Straight-chain (saturated)			
$C_{14:0}$	1.9	TR	1.8
$C_{16:0}$	3.4	9.0	5.3
Branched-chain			
iso-$C_{13:0}$	5.4	TR	TR
iso-$C_{14:0}$	1.4	TR	TR
iso-$C_{15:0}$	18.9	25.7	29.0
iso-$C_{15:0}$ 3-OH	21.0	7.0	8.7
iso-$C_{16:0}$	TR	4.8	TR
iso-$C_{16:0}$ 3-OH	TR	2.0	TR
iso-$C_{17:0}$	TR	2.4	TR
iso-$C_{17:0}$ 3-OH	2.1	7.9	6.1
anteiso-$C_{15:0}$	6.4	7.8	3.36
anteiso-$C_{17:0}$	TR	1.7	ND
Unsaturated			
iso-$C_{15:1}$ G	8.7	2.1	5.0
$C_{15:1}\omega 6c$	2.7	TR	8.9
$C_{17:1}\omega 6c$	1.1	TR	4.3
$C_{17:1}\omega 8c$	TR	TR	1.0
anteiso-$C_{17:1}\omega 9c$	ND	2.7	ND
Hydroxylated			
$C_{15:0}$ 2-OH	TR	1.1	TR
$C_{15:0}$ 3-OH	3.1	ND	2.2
$C_{16:0}$ 3-OH	2.6	3.6	3.7
$C_{17:0}$ 2-OH	TR	2.2	TR
Summed features*			
2	1.8	TR	1.2
3	8.9	7.7	12.1
9	2.6	5.7	1.6

*Summed features comprise groups of two or more fatty acids that cannot be separated by the MIDI detection system. Summed features contain: 2, $C_{12:0}$ aldehyde and/or unknown equivalent chain-length (ECL) 10.9525; 3, $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$; 9, $C_{16:0}$ 10-methyl and/or iso-$C_{17:1}\omega 9c$.

DESCRIPTION OF *FLAVOBACTERIUM KEUMENSE* SP. NOV.

Flavobacterium keumense (keum.en′se. N.L. neut. adj. *keumense* belonging to Keum River, where the type strain was isolated).

Cells are rod-shaped, Gram-stain-negative, aerobic, non-motile and yellow-pigmented. Cell size ranges from approximately 0.5–1 µm in width to 1.5–2.0 µm in length. After incubation for 3 days at 30 °C on R2A, colonies are yellow, circular, opaque and smooth with entire margins. Growth occurs on nutrient, Anacker Ordal and R2A agars, but not on TSA or MacConkey agars. Oxidase-positive but catalase-negative. Growth occurs at temperatures of between 4 and 35 °C (optimum, 30 °C) on R2A agar. The pH range for growth is pH 6–8 (optimum, 7), and no growth occurs with greater than 0.5 % (w/v) NaCl in R2A broth. Flexirubin-type pigments are absent, and Congo red adsorption is negative. Hydrolysis of starch, tyrosine, aesculin, gelatin and *p*-nitrophenyl β-D-galactopyranoside (PNPG) is positive while that of casein, carboxymethyl cellulose, chitin, DNA and agar is negative. Alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase activities are positive based on API ZYM. Carbohydrate assimilation on API 20NE is negative for all substrates tested, but acid is produced from glucose, fructose, mannose, manopyranoside, glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, gentiobiose and turanose on API 50CH. MK-6 is the only respiratory quinone, and homospermidine is the only polyamine. The major polar lipid is phosphatidylethanolamine, and an unidentified aminolipid and three unidentified phospholipids are also present. The predominant fatty acids are iso-$C_{15:0}$ 3-OH, iso-$C_{15:0}$, a summed feature comprising $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$ and iso-$C_{15:1}$ G.

The type strain, K3R-10^T (=JCM 31220^T=KCTC 52563^T), was isolated from a river. The DNA G+C content of the type strain is 35.4 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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