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Flavobacterium aquaticum sp. nov., a Member of the *Bacteroidetes* Isolated from a Freshwater Reservoir[§]

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A novel bacterial strain, designated ARSA-111^T, was isolated from a freshwater reservoir in Cheonan, Korea. Phylogenetic analysis based on 16S rRNA gene sequences suggested that the isolate belonged to the genus *Flavobacterium* of phylum *Bacteroidetes*. The 16S rRNA gene sequence of strain ARSA-111^T showed a high degree of sequence similarity to those of *Flavobacteium cheonanense* KACC 14972^T (97.3%), *F. aquatile* JCM 20475^T (97.1%), and other type strains of the genus *Flavobacterium* (< 97.0%). The phylogenetic tree and network analysis (i.e. median-joining) based on 16S rRNA gene sequences showed that strain ARSA-111^T is most closely related to *F. aquatile* JCM 20475^T. DNA-DNA hybridization experiment revealed < 70% of genomic relatedness among strain ARSA-111^T, *F. aquatile* JCM 20475^T and *F. cheonanense* KACC 14972^T. The isolate had iso-$C_{15:1}$, iso-$C_{15:0}$, and iso-$C_{15:0}$ 3-OH as predominant cellular fatty acids and MK-6 as a predominant menaquinone. The genomic DNA G+C content of the isolate was 35.6 mol%. On the basis of these data, strain ARSA-111^T is considered to be a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium aquaticum* sp. nov. is proposed. The type strain is strain ARSA-111^T (=KACC 14973^T =KCTC 23185^T = JCM 17070^T).

Keywords: *Flavobacterium*, *Bacteroidetes*, median-joining, network

Introduction

The genus *Flavobacterium* (in family *Flavobacteriaceae* of phylum *Bacteroidetes*) was introduced by Bergey *et al.* (1923), with *F. aquatile* as the type species. Its description was emended by Bernardet *et al.* (1996). The genus comprises Gram-negative, aerobic rods that are usually motile by means of gliding, contains menaquinone-6 (MK-6) as a sole or major respiratory quinone, produce yellow carotenoid and/or flexirubin pigments, and has a DNA G+C content in the range of 30–41 mol% (Bernardet and Bowman, 2011). Species of the genus have been isolated from diverse habitats that include freshwater sediments, glacier ice, soil, Antarctic habitats, earthworm gut, wastewater treatment bacterial aggregates, and freshwater shrimp worldwide (Cousin *et al.*, 2007; Sheu *et al.*, 2011). The physiological characteristics of members of the genus are also diverse; they can be psychrophilic, psychrotolerant or mesophilic and halotolerant, halophilic or sensitive to salts. They produce a variety of enzymes (Park *et al.*, 2006), suggesting that they may have important roles in the environment (Lee *et al.*, 2011). Recently, several novel species isolated from various freshwater environments have been added to the genus, (Wang *et al.*, 2006; Cousin *et al.*, 2007; Kim *et al.*, 2009; Qu *et al.*, 2009; Lee *et al.*, 2011, 2012). In this paper, we report the taxonomic characterization of *Flavobacterium*-like bacterial strain, ARSA-111^T, which was isolated from a freshwater reservoir in Cheonan, Korea.

Materials and Methods

Bacterial strain

Strain ARSA-111^T was isolated from a freshwater lake (Cheonho reservoir) located at Dankook University in Cheonan, Korea (36° 49′ N 127° 10′ E) and inoculated on R2A agar (Difco, USA) pH 7.2±0.2 at 25°C for 4 days. After primary isolation and purification, the strain was cultivated at 25°C on the same medium. The isolate was routinely cultured on R2A agar at 25°C and preserved as a glycerol suspension (20% of glycerol and 80% of ddH_2O, v/v) at -80°C. We deposited this organism to the Korean Agricultural Culture Collection (KACC 14967^T), Korean Collection for Type Cultures (KCTC 23180^T), and Japan Collection of Microorganisms (JCM 17064^T). Reference strain, *F. aquatile* JCM 20475^T was obtained from the JCM, while *F. cheonanense* ARSA-108^T, *F. koreense* ARSA-42^T, *F. chungnamense* ARSA-103^T, and *F. dankookense* ARSA-19^T were previously isolated in our laboratory (Lee *et al.*, 2012). These strains were used as reference strains for most phenotypic tests.

Phylogenetic analysis and DNA-DNA hybridization

For phylogenetic analysis, the genomic DNA of strain ARSA-19^T was extracted through the InstaGene^{TM} Matrix (Bio-Rad, USA) following the manufacturer's instructions. 16S rRNA gene was amplified by a GeneAmp PCR system 9700 (Applied Biosystems, USA) with *Taq* DNA polymerase (Bioneer, Korea) and the 8-27F and 1492-1510R universal primers (Lane, 1991). The PCR product was purified using

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the PCRquick-spin™ PCR product purification kit (iNtRON, Korea) and direct sequence determination of the purified 16S rRNA gene was performed using an ABI3730XL automated sequencer (Applied Biosystems) with sequencing primers (27F, 518F, 800R, and 1492R; Lane, 1991) at Macrogen (Korea). The identification of phylogenetic neighbors was achieved using the EzTaxon server (http://www.eztaxon.org/; Chun *et al*., 2007) and PHYDIT program version 3.1 (Chun and Bae, 2000). Sequences of related species were downloaded, together with those of *Escherichia coli* KCTC 2441^T (GenBank accession no. EU014689) and *Capnocytophaga granulosa* LMG 16022^T (U41347) used as out groups. The alignments were manually corrected using the BioEdit program (Hall, 1999). The phylogenetic tree was constructed using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.05. Distance calculation (distance options according to the Kimura 2-parameter) and clustering were done with the Neighbor-joining and maximum likelihood methods, and bootstrap values were estimated based on 1,000 replications. In addition, to examine the phylogenetic relationship of the strain ARSA-111^T and closely related strains, we constructed a median-joining network (Bandelt *et al*., 1999) using NETWORK version 4.6.0.0 available at http://www.fluxus-engineering.com/sharenet.htm. *Capnocytophaga granulosa* LMG 16022^T (GenBank accession no. U41347) was used as an out-group. The network was generated using partial 16S rRNA gene sequences (906 bp). The taxonomic relationship between strain ARSA-111^T and *F. aquatile* was further examined using DNA-DNA hybridization which was carried out following the protocol described by Seldin and Dubnau (1985). Probe labeling was conducted by using the non-radioactive DIG High Prime DNA labeling and detection starter kit II (Roche, Germany). Reassociation was conducted at 65°C. The hybridized DNA was visualized using the DIG luminescent detection kit (Roche). DNA-DNA relatedness was quantified by using a densitometer (Bio-Rad).

Phenotypic and biochemical characteristics

Cell morphology was observed by phase-contrast microscopy using an AXIO (Carl Zeiss, Germany). Gram staining was performed using a Sigma-Aldrich Gram-stain kit, according to manufacturer's instructions. Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and oxidase reagent (bioMérieux, France), respectively. Motility was tested by the hanging-drop method (Skerman, 1967) using light microscopy and a CHT microscope (Olympus, Japan) at ×1000 magnification. Growth was investigated at temperatures ranging from 5 to 45°C at intervals of 5°C and at the range of pH 5.0–11.0 at intervals of 0.5 pH units. The influence of salinity was tested using modified R2A media supplemented with 0, 0.5, 1.0, 1.5, or 2.0 (%, w/v) NaCl. To adjust the pH of the R2A medium, phosphate buffer was added and 1 N HCl or 1 N NaOH was used to achieve the desired pH. The optimal temperature and pH were tested with R2A broth for 14 days. Anaerobic growth was examined on solid R2A medium incubated in a jar with the GasPak anaerobic system (BBL, USA). Growth was tested on nutrient agar (NA, Difco), trypticase soy agar (TSA, Difco), MacConkey agar (Difco), and Anacker & Ordal's agar (Anacker and Ordal, 1955). Gliding motility was tested by observing the spread of colony edges on R2A plates (Perry, 1973) and by microscopic observation of hanging drops of a R2A broth culture under a CHT microscope (Olympus). Production of flexirubin-type pigments and Congo red adsorption were investigated using 20% KOH test and 0.01% aqueous solution, respectively, according to the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al*., 2002). Hydrolysis of starch, casein, Tween 80, CM-cellulose, chitin, xanthine, and hypoxanthine were performed by using the methods of Smibert and Krieg (1994). Substrate utilization and enzyme activities were tested by using the API 20NE, Vitek, and API ZYM galleries (bioMérieux) according to the instructions of the manufacturer.

Determination of DNA G+C content

To estimate the G+C content of chromosomal DNA, the genomic DNAs of strains were extracted and purified as described by Moore and Dowhan (1995) and enzymatically degraded into nucleosides. As a reference strain for G+C content analysis, *E. coli* ATCC 35607 was obtained from KACC. The DNA G+C content was determined as described by Mesbah *et al*. (1989), using reversed-phase high-performance liquid chromatography (HPLC, Supelco).

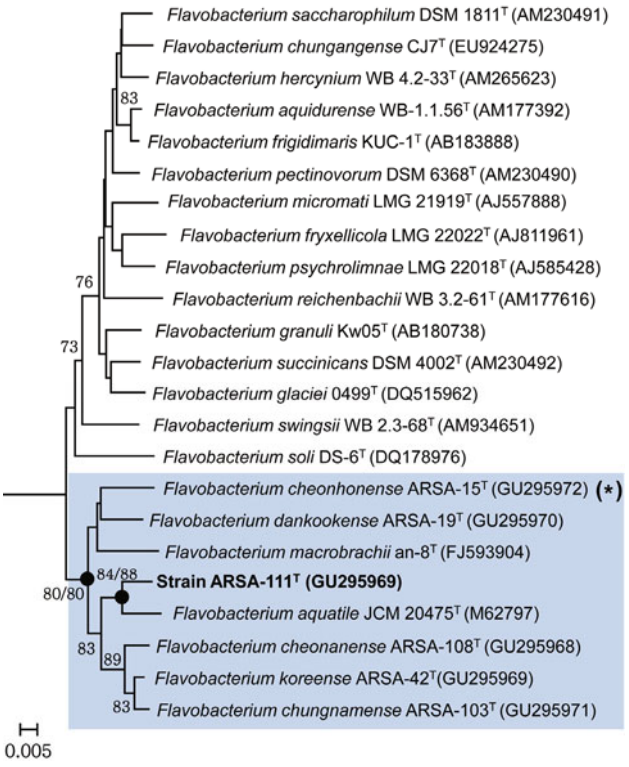

Fig. 1. Neighbor-joining phylogenetic tree showing the phylogenetic position of novel species based on 16S rRNA gene sequences. Evolutionary distances were computed using the Kimura 2-parameter model. Closed circle represents that the corresponding branches were also recovered in the neighbor-joining and maximum likelihood trees. *E. coli* KCTC 2441^T (EU014689) was used as an out-group. Bootstrap values (>70%) based on 1,000 replications are shown. The network of Fig. 1 (*) clade was reconstructed. Bar, 0.005 substitutions per nucleotide position.

Chemotaxonomy

All tests of chemotaxonomic characterization were obtained from cells grown in R2A agar at 25°C for 4 days. Isoprenoid quinones and polar lipids were obtained using the methods of Minnikin *et al*. (1984). Fatty acid profiles of strain ARSA-111^T and five reference strains were determined on the identical conditions. The fatty acid composition was analyzed by gas chromatography (model 7890; Hewlett Packard, USA) using the Sherlock Microbial Identification (MIDI) system version 6.1.

Results and Discussion

Phylogenetic analysis and DNA-DNA hybridization

The 16S rRNA gene sequence of strain ARSA-111^T comparised 1408 nucleotides. Preliminary sequence comparisons with 16S rRNA gene sequences retrieved from the EzTaxon and GenBank database indicated that strain ARSA-111^T belonged to the family *Flavobacteriaceae* of the *Bacteroidetes*. The 16S rRNA gene sequence of strain ARSA-111^T as similarly ranged from 91.3–97.4% as compared to members of the genus *Flavobacterium*, with the highest similarity to *F. cheonanense* KACC 14972^T (97.4%), followed by *F. aquatile*

Table 1. Phenotypic differences between *Flavobacterium aquaticum* sp. nov. and closest phylogenetic neighbors
Strains: 1, *F. aquaticum* ARSA-111^T; 2, *F. aquatile* JCM 20475^T (data from Lee *et al*., 2011); 3, *F. cheonanense* KACC 14972^T (data from Lee *et al*., 2011); 4, *F. koreense* KACC 14969^T (data from Lee *et al*., 2011); 5, *F. chungnamense* KACC 14971^T (data from Lee *et al*., 2011); 6, *F. dankookense* KACC 14968^T (data from Lee *et al*., 2012). +, Positive; -, negative; w, weak positive; Y, yellow; PY, pale yellow; CY, cream yellow; PE, phosphatidylethanolamine; L, unidentified lipid; AL, unknown aminolipid. All strains are positive for hydrolysis of starch, catalase, and oxidase. All strains are negative for Tween 80, CM-cellulose, chitin, tyrosine, xanthine and hypoxanthine.

Characteristic	1	2	3	4	5	6
Colony pigmentation	Y	PY	CY	CY	CY	Y
Growth on:						
Nutrient agar	+	-	+	+	+	+
Tryptic soy agar	-	+	-	-	-	-
Gliding motility	-	+	-	-	-	-
Flexirubin-type pigments	+	-	+	+	+	+
Growth at:						
4°C	-	-	+	+	-	+
37°C	+	-	+	+	-	+
pH 10	-	+	+	+	+	+
1% NaCl (w/v)	+	+	-	-	-	+
API 20NE & Vitek						
Esculine ferric citrate	-	+	-	+	w	-
Gelatine	-	-	-	w	-	-
4-Nitrophenyl-βD-galactopyranoside	-	w	-	w	w	-
Capric acid	-	w	-	w	-	w
Ala-Phe-Pro-arylamidase	-	+	+	+	+	+
Lipase	-	-	-	+	+	+
Glu-Gly-Arg-arylamidase	-	-	-	+	-	-
API ZYM						
Esterase (C4)	-	+	-	-	-	+
Esterase lipase (C8)	-	+	-	-	+	+
Crystine arylamidase	-	+	+	-	-	-
Acid phospatase	+	-	-	+	-	+
Naphtol-AS-BI-phosphohydrolase	+	-	-	+	+	+
α-Glucosidase	-	+	-	-	-	-
Polar lipid						
PE	+	+	+	+	+	+
L1	-	+	+	+	+	+
L2	-	+	+	+	+	+
AL1	+	+	+	+	+	+
AL2	+	-	+	-	-	+
AL3	-	+	+	+	+	+
AL4	-	-	+	-	-	+
AL5	-	+	-	-	-	-
AL6	-	+	-	-	-	-
AL7	-	+	-	-	-	-
DNA G+C content (mol%)	35.6	34.7	31.4	31.5	33.2	33.3

JCM 20745^T (97.1%), *F. koreense* KACC 14969^T (96.6%), *F. dankookense* KACC 14968^T (96.5%), *F. chungnamense* KACC 14971^T (96.1%), *F. macrobrachii* an-8^T (95.9%), and <95% to other *Flavobacterium* species. Neighbor-joining and maximum likelihood trees placed strain ARSA-111^T in the genus *Flavobacterium*, most closely related to *F. aquatile* (Fig. 1). In addition, we applied the median-joining network method (Bandelt *et al*., 1999) using partial 16S rRNA gene sequences of seven strains belonging to a single clade (Supplementary data Fig. S1). This network shows the comprehensive sequential order in which the species diversified according to the number of substitutions. As shown in Supplementary data Fig. S1, a 35 nucleotide difference between 16S rRNA gene sequences of the strain ARSA-111^T, *F. aquatile* JCM 20745^T (97.1%), and *F. chungnamense* KACC 14971^T was evident. The result of the network study was congruent with the phylogenetic inference based on the neighbor-joining study. DNA-DNA hybridization revealed less than 70% relatedness among strain ARSA-111^T, *F. cheonanense* KACC 14972^T and *F. aquatile* JCM 20475^T (data not shown).

Phenotypic characteristics and DNA G+C content

Colonies of strain ARSA-111^T were yellow pigmented, semi-translucent, smooth and short rods (0.4–0.6 μm width × 0.9–1.4 μm length) on R2A agar for 4 days. Cells of the isolate were Gram-negative, aerobic, non-motile, and non-gliding. Strain ARSA-111^T grew well on R2A, nutrient agar, and Anacker & Ordal's agar, while no growth was observed on TSA and MacConkey agar. Phenotypic characteristics of strain ARSA-15^T and closely related species are shown in Table 1. The DNA G+C content of the isolate was 35.6 mol%, a value consistent with those of other *Flavobacterium* species [30–41 mol%; Bernardet and Bowman (2011); Table 1].

Chemotaxonomy

The major respiratory quinone of strain ARSA-111^T was MK-6. The polar lipids consisted of phosphatidylethanolamine and two unknown aminolipids (AL1 and AL2) as major components (Table 1 and Supplementary data Fig. S2). The dominant fatty acids of strain ARSA-111^T were iso-$C_{15:1}$ (19.1%), iso-$C_{15:0}$ (13.9%), and iso-$C_{15:0}$ 3-OH (10.0%). The proportion of iso-$C_{13:0}$, iso-$C_{15:1}$, anteiso-$C_{15:1}$, $C_{15:0}$ 2-OH, and $C_{17:0}$ 2-OH were larger than those recorded for other type species and smaller than $C_{15:1}$ *ω6c*, $C_{17:1}$ *ω9c*, and $C_{15:0}$ 3-OH. Especially, the proportion of $C_{15:1}$ *ω6c* was different between our isolate (3.0%) and *F. aquatile* (20.4%). The de-

Table 2. Fatty acid compositions of the novel strain and related type strains

Strains: 1, *F. aquaticum* ARSA-111^T; 2, *F. aquatile* JCM 20475^T (data from Lee *et al*., 2011); 3, *F. cheonanense* KACC 14972^T (data from Lee *et al*., 2011); 4, *F. koreense* KACC 14969^T (data from Lee *et al*., 2011); 5, *F. chungnamense* KACC 14971^T (data from Lee *et al*., 2011); 6, *F. dankookense* KACC 14968^T (data from Lee *et al*., 2012). Fatty acids amounting to less than 0.5% in all strains are not shown. Tr, traces (<0.5%); -, not detected.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{13:0}$	Tr	-	-	-	-	Tr
$C_{14:0}$	0.5	-	-	0.5	-	0.9
$C_{16:0}$	0.8	-	-	1.3	-	1.8
Branched						
iso-$C_{13:0}$	1.2	-	-	Tr	-	Tr
iso-$C_{14:0}$	2.0	1.1	5.3	2.8	2.4	2.5
iso-$C_{15:0}$	13.9	17.7	12.9	12.0	21.0	21.8
anteiso-$C_{15:0}$	9.2	10.2	3.5	5.2	6.6	4.9
iso-$C_{15:1}$	19.1	9.9	10.5	12.7	8.0	9.3
anteiso-$C_{15:1}$	4.6	1.8	-	2.1	-	0.7
iso-$C_{16:0}$	2.6	1.5	7.1	4.9	5.1	7.3
iso-$C_{16:1}$	2.0	1.1	4.9	3.3	-	2.9
Unsaturated						
$C_{15:1}$*ω6c*	3.0	20.4	6.0	3.8	7.5	4.0
$C_{15:1}$*ω8c*	-	1.2	-	-	-	-
$C_{17:1}$*ω6c*	1.2	5.4	-	-	-	1.0
iso-$C_{17:1}$*ω9c*	0.9	1.7	1.4	2.2	2.6	1.8
Hydroxy						
iso-$C_{14:0}$ 3-OH	0.8	-	1.5	1.1	-	0.7
$C_{15:0}$ 2-OH	2.3	-	-	1.4	-	0.9
$C_{15:0}$ 3-OH	1.2	5.6	1.8	2.1	3.2	1.7
iso-$C_{15:0}$ 3-OH	10.0	3.7	9.9	11.5	14.5	7.7
$C_{16:0}$ 3-OH	0.7	Tr	-	2.2	-	1.4
iso-$C_{16:0}$ 3-OH	6.9	3.1	23.6	13.8	14.3	10.4
$C_{17:0}$ 2-OH	2.2	1.7	-	1.7	-	1.5
$C_{17:0}$ 3-OH	Tr	1.1	-	1.0	-	Tr
iso-$C_{17:0}$ 3-OH	5.8	4.8	9.0	10.4	12.3	5.9
Summed feature 3[a]	7.4	3.1	2.6	3.4	2.6	8.2

[a] Summed feature represent groups two or three fatty acids that could not be separated by GLC with MIDI system. Summed feature 3 comprises iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$*ω7c*.

tailed fatty acids profile of strain ARSA-111^T is compared with those of closely related members in Table 2.

Taxonomical conclusions

According to the phenotypic and genotypic data, we conclude that strain ARSA-111^T is representative of novel species of the genus *Flavobacterium*, for which the name *Flavobacterium aquaticum* sp. nov., is proposed.

Description of *Flavobacterium aquaticum* sp. nov.

Flavobacterium aquaticum (a.qua'ti.cum. L. neut. adj. aquaticum, living, growing, or found in or by the water, aquatic).

Colonies are circular, yellow pigmented and semi-translucent on R2A agar. Cells are Gram-negative, rod-shaped (0.4–0.6 μm wide × 0.9–1.4 μm long), strictly aerobic, non-motile, non-gliding, catalase-positive, and oxidase-positive. Congo red is not absorbed by the colonies. Flexirubin-type pigments are produced. Grows on R2A, nutrient agar, and Anacker & Ordal's agar but not on MacConkey, DNase test agar, and TSA. Cells grows on R2A at 10–37°C (optimum, 25°C) but not at 5 or 45°C. The pH range for growth is 6.5–10.0 (optimum, 7.5–8.0). No growth is observed in the presence of 1.5% or higher NaCl concentration. The novel species shows hydrolysis of starch but does not hydrolyze Tween 80, casein, CM-cellulose, chitin, tyrosine, xanthine, or hypoxanthine. Strain ARSA-111^T is positive for L-proline arylamidase, tyrosine arylamidase, and phosphatase in the Vitek and API 20NE systems. In the API ZYM system, alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, and naphtol-AS-BI-phosphohydrolase are present. The predominant menaquinone is MK-6 and DNA G+C content of the type strain is 35.6 mol%. The polar lipids consist of phosphatidylethanolamine and two unknown aminolipids (AL1 and AL2) as the major components. The major fatty acids (>7% of the total fatty acids) are iso-$C_{15:1}$, iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, and Summed feature 3. The GenBank accession no. for 16S rRNA gene sequence is GU295969. The type strain, ARSA-111^T (=KACC 14973^T =KCTC 23185^T =JCM 17070^T), was isolated from Cheonho reservoir in Cheonan, Republic of Korea.

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