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Flavobacterium koreense sp. nov., *Flavobacterium chungnamense* sp. nov., and *Flavobacterium cheonanense* sp. nov., Isolated from a Freshwater Reservoir

Siwon Lee[1], Hang-Yeon Weon[2], Soo-Jin Kim[2], and Tae-Young Ahn[1]*

[1]*Department of Microbiology, Dankook University, Cheonan 330-714, Republic of Korea*
[2]*Applied Microbiology Division, National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon 441-707, Republic of Korea*

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Taxonomic studies were performed on three strains isolated from Cheonho reservoir in Cheonan, Korea. The isolates were Gram-negative, aerobic, rod-shaped, non-motile, catalase-positive, and oxidase-positive. Colonies on solid media were cream-yellow, smooth, shiny, and circular. Phylogenetic analysis of the 16S rRNA gene sequences revealed that these strains belong to the genus *Flavobacterium*. The strains shared 98.6-99.4% sequence similarity with each other and showed less than 97% similarity with members of the genus *Flavobacterium* with validly published names. The DNA-DNA hybridization results confirmed the separate genomic status of strains ARSA-42^T, ARSA-103^T, and ARSA-108^T. The isolates contained menaquinone-6 as the predominant menaquinone and iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, iso-$C_{15:1}$ G, and iso-$C_{16:0}$ 3-OH as the major fatty acids. The genomic DNA G+C content of the isolates were 31.4-33.2 mol%. According to the phenotypic and genotypic data, these organisms are classified as representative of three novel species in the genus *Flavobacterium*, and the name *Flavobacterium koreense* sp. nov. (strain ARSA-42^T =KCTC 23182^T =JCM 17066^T =KACC 14969^T), *Flavobacterium chungnamense* sp. nov. (strain ARSA-103^T =KCTC 23183^T =JCM 17068^T =KACC 14971^T), and *Flavobacterium cheonanense* sp. nov. (strain ARSA-108^T =KCTC 23184^T =JCM 17069^T =KACC 14972^T) are proposed.

Keywords: freshwater, *Bacteroidetes*, *Flavobacterium*

The genus *Flavobacterium* (family *Flavobacteriaceae*, order *Flavobacteriales*, class *Flavobacteria*, phylum *Bacteroidetes*) was created by Bergey *et al*. (1923), with *Flavobacterium aquatile* as the type species. Several species previously placed in the genus *Flavobacterium* have been reclassified and placed in new or different genera, including the genera *Empedobacter* (Vandamme *et al*., 1994), *Microbacterium* (Takeuchi and Hatano, 1998), *Salegentibacter* (McCammon and Bowman, 2000), and *Sphingobacterium* (Yabuuchi *et al*., 1983; Holmes, 1992). The genus is Gram-negative, aerobic, rod-shaped, and yellow-pigmented bacteria that usually have gliding motility and have a DNA G+C content of 30-41 mol% (Bernardet and Bowman, 2006; Park *et al*., 2006). Species of the genus have been isolated worldwide from habitats such as freshwater sediments, glacier ice, soil, Antarctic habitats, gut of an earthworm, and bacterial aggregates of a wastewater treatment plant (Cousin *et al*., 2007). This suggests that they may have important roles in the environment. Recently, several novel species belonging to the genus have been isolated from various freshwater environments (Wang *et al*., 2006; Kim *et al*., 2009; Qu *et al*., 2009). In this paper, we reported the taxonomic characterization of *Flavobacterium*-like bacterial strains, ARSA-42^T, ARSA-103^T, and ARSA-108^T, which were isolated from a freshwater reservoir of Cheonan, Korea.

* For correspondence. E-mail: ahnty@dankook.ac.kr; Tel: +82-41-550-3451; Fax:+82-41-550-3450

Materials and Methods

Bacterial strains

Strains ARSA-42^T, ARSA-103^T, and ARSA-108^T were isolated from Cheonho reservoir (36° 49′ N 127° 10′ E) in the Republic of Korea during July 2009, by using the standard dilution plating technique. The strains were isolated on R2A medium (Difco, USA) after incubation for 48 h at 25°C and produced cream-yellow, semi-transparent colonies. Subculturing was performed on R2A medium incubated at 25°C for 4 days, and the isolates were maintained as glycerol suspensions (20%, w/v) at -70°C. Reference strain, *Flavobacterium aquatile* JCM 20475^T, was obtained from the Japan Collection of Microorganisms (JCM, Japan). Three strains were deposited in KCTC (Korean Collection for Type Cultures), JCM, and KACC (Korean Agricultural Culture Collection).

Phylogenetic analysis and DNA-DNA hybridization

Genomic DNA extraction and PCR amplification of 16S rRNA gene were performed as described by Lee *et al*. (2009). Almost complete 16S rRNA gene sequences of strains ARSA-42^T (1409 nt), ARSA-103^T (1401 nt), and ARSA-108^T (1397 nt) were determined. Nucleotide similarity values were calculated using the EzTaxon server ver. 2.1 [htty://www.EzTaxon.org/; Chun *et al*. (2007)]. We aligned the most closely related sequences with the sequences of reference strains, using the software BioEdit ver. 7.0.0 (Hall, 1999). The phylogenetic tree was constructed using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4.1 (Tamura *et al*., 2007). Distance calculation (distance options according to the Kimura 2-parameter) and clustering with the neighbour-joining was performed,

and bootstrap values were estimated based on 1,000 replications. The taxonomic relationships among strain ARSA-42^T, ARSA-103^T, and ARSA-108^T were further examined using DNA-DNA hybridization. DNA-DNA hybridization was carried out as 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 Molecular Biochemicals). Reassociation was conducted at 65°C. The hybridized DNA was visualized using the DIG luminescent detection kit (Roche, Germany). DNA-DNA relatedness was quantified by using a densitometer (Bio-Rad, USA).

Morphological and biochemical characteristics

Gram staining was performed using a Sigma-Aldrich Gram-stain kit, according to the manufacturer's instructions. Cell morphology was ascertained by light microscopy using a CHT microscope (Olympus, Japan) at ×1,000 magnification, with cells grown for 4 days at 25°C on R2A medium. Motility test was carried out on R2A medium containing 0.5% of agar. Catalase and oxidase were determined by using the methods described by Smibert and Krieg (1994). Growth was tested on nutrient agar (NA, Difco), Trypticase soy agar (TSA, Difco), Anacker and Ordal's agar (Anacker and Ordal, 1955), and MacConkey agar (Difco). The temperature range (4, 10, 15, 20, 25, 30, 37, and 45°C) and pH range (pH 5.0-11.0 at intervals of 0.5 pH unit) were determined by using R2A medium. Tolerance to salinity was tested in R2A agar medium supplemented with 0-2.0% NaCl (w/v) at 0.5% intervals after 4 days of incubation at 25°C. The optimum temperature and pH were tested by R2A broth for 14 days. Anaerobic growth was evaluated on R2A agar in a GasPak anaerobic system (BBL, USA). 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 (Bernardet *et al*., 2002) under a CHT microscope (Olympus). Production of flexir-

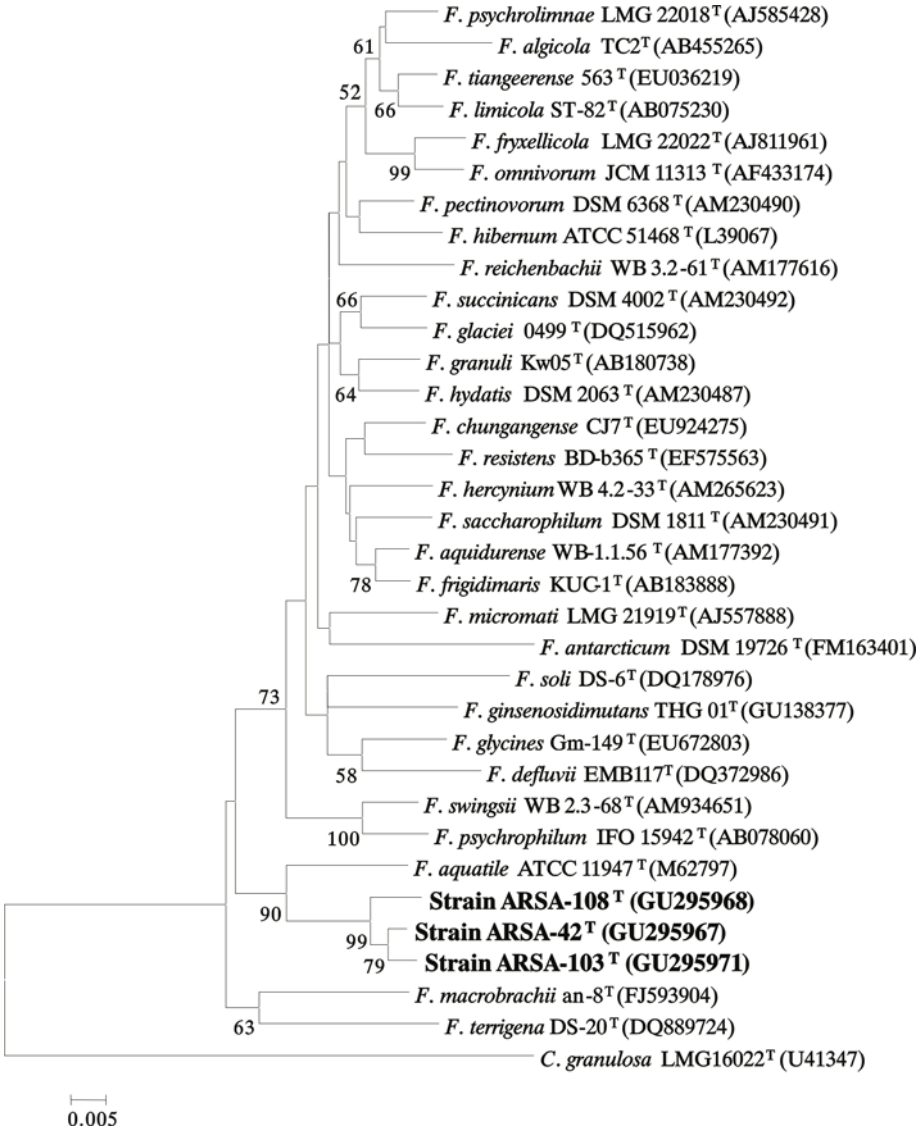

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strains ARSA-42^T, ARSA-103^T, and ARSA-108^T and their closest relatives. *Capnocytophaga granulosa* LMG16022^T (U41347) was used as the out-group. Numbers at nodes indicate levels of bootstrap support based on 1,000 resampled datasets. Bar, 0.005 nucleotide substitutions per nucleotide position.

Table 1. Levels of DNA-DNA hybridization among novel strain ARSA-42^T, ARSA-103^T, and ARSA-108^T

Strain	DNA-DNA relatedness (%) of labeled DNA from		
	1	2	3
Strain ARSA-42^T	-	59.85	69.75
Strain ARSA-103^T	64.85	-	41.50
Strain ARSA-108^T	62.65	40.05	-

ubin-type pigments and congo red adsorption were investigated using 20% KOH test and 0.01% aqueous solution, according to the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al*., 2002). Hydrolysis of casein, starch, CM-cellulose, chitin, DNA, pectin, Tween 80, tyrosine, xanthine, and hypoxanthine were determined using the methods of Smibert and Krieg (1994). Additional biochemical tests were performed using API 20NE and Vitek kits (all from bioMérieux, France). Enzyme activity tests were conducted by using the API ZYM galleries, according to the manufacturer's instructions (bioMérieux).

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 enzymically degraded into nucleosides. As a reference strain for G+C content analysis, *Escherichia 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).

Determination of isoprenoid quinone and fatty acids

Isoprenoid quinone was extracted from lyophilized cells and analyzed by HPLC as described by Groth *et al*. (1996). Cultures for determination of the fatty acid composition were grown on R2A agar at 25°C for 4 days. The fatty acid composition was analyzed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification (MIDI, 1999) software package.

Results and Discussion

Phylogenetic analysis and DNA-DNA hybridization

Strains ARSA-42^T, ARSA-103^T, and ARSA-108^T showed 16S rRNA gene sequence similarity of 92.6-96.3% with the type strains of recognized species of the genus *Flavobacterium* and showed the highest similarity to *Flavobacterium aquatile* JCM

Table 2. Differential characteristics of the novel strains and closely related type strains

Strains: 1, ARSA-42^T; 2, ARSA-103^T; 3, ARSA-108^T; 4, *F. aquatile* JCM 20745^T; 5, *F. macrobrachii* an-8^T (data from Sheu *et al*., in press). +, positive; -, negative; w, weak positive; ND, no data. All species shown here are positive for starch, catalase and oxidase (N,N,N,N-tetramethyl-p-phenylenediamine) and negative for CM-cellulose and chitin.

Characteristic	1	2	3	4	5
Growth on:					
Nutrient agar	+	+	+	-	w
Tryptic soy agar	-	-	-	+	-
Gliding motility	-	-	-	+	-
Flexirubin-type pigments	+	+	+	-	-
Range of:					
Temperature (°C)	4-37	10-30	4-37	10-30	15-30
pH	6.0-9.5	6.5-9.5	6.5-9.5	6.5-9.5	7.0-8.0
Growth in NaCl (%, w/v)					
1.0	-	-	+	+	+
1.5	-	-	-	+	-
Hydrolysis of:					
Casein	-	-	-	+	+
Tween 80	-	-	-	-	-
DNA	-	-	-	-	+
Esculin	+	-	-	+	-
Gelatine	+	-	-	-	+
Assimilation of:					
Courmaric acid	+	-	-	-	ND
Capric acid	w	-	-	w	-
Enzymatic activities					
Lipase	+	+	-	-	-
Esterase (C4)	-	-	-	+	-
Esterase lipase (C8)	-	+	-	+	+
Crystine arylamidase	+	-	-	+	-
Acid phospatase	+	-	-	-	-
Naphtol-AS-BI-phosphohydrolase	+	+	-	-	+
α-Glucosidase	-	-	-	+	-
DNA G+C content (mol%)	31.5	33.2	31.4	34.7	39.8

20745^T (95.6, 95.7, and 96.3%, respectively). The three strains occupied independent evolutionary lineages within the genus *Flavobacterium*, forming a clade with *F. aquatile* JCM 20745^T (Fig. 1). The analysis of pairwise similarities based on the PHYDIT version 3.2 program indicated that the isolates shared 98.6-99.4% similarity with each other. The results of DNA-DNA hybridization showed less than 70% relatedness among strains ARSA-42^T, ARSA-103^T, and ARSA-108^T (Table 1).

Morphological and biochemical characteristics

The phenotypic characteristics of strains are given in Table 2 and in the species description. Strains ARSA-42^T, ARSA-103^T, and ARSA-108^T could be clearly distinguished from their closest phylogenetic neighbour, *F. aquatile* JCM 20475^T, and *F. macrobrachii* an-8^T.

Determination of DNA G+C content

The DNA G+C content of strains ARSA-42^T, ARSA-103^T, and ARSA-108^T were 31.5, 33.2, and 31.4 mol%, respectively, which were slightly different from that of *F. aquatile* JCM 20475^T (34.7 mol%) and *F. macrobrachii* an-8^T (39.8 mol%). However, the values still lie in the range expected for members of the same genus.

Determination of isoprenoid quinone and fatty acids

Cells of isolates contained menaquinone 6 (MK-6) as the major respiratory quinone. The major cellular fatty acids of strain ARSA-42^T were iso-$C_{16:0}$ 3-OH (13.8%), iso-$C_{15:1}$ G (12.7%), and iso-$C_{15:0}$ (12.0%). Strain ARSA-103^T consisted of iso-$C_{15:0}$ (21.0%), iso-$C_{15:0}$ 3-OH (14.5%), and iso-$C_{16:0}$ 3-OH (14.3%) as the major cellular fatty acids. Strain ARSA-108^T contained iso-$C_{16:0}$ 3-OH (23.6%), iso-$C_{15:0}$ (12.9%), and iso-$C_{15:1}$ G (10.5%) as the predominant fatty acids. Detailed fatty acid profiles are shown in Table 3.

Taxonomic conclusions

According to the phenotypic and genotypic data, we conclude that strains ARSA-42^T, ARSA-103^T, and ARSA-108^T are representative of three novel species of the genus *Flavobacterium*, for which the names *F. koreensis* sp. nov., *F. chungnamense* sp. nov., and *F. cheonanense* sp. nov., respectively, are proposed.

Description of *Flavobacterium koreense* sp. nov.

Flavobacterium koreense (Ko.re.en'se. N.L. neut. adj. Koreense, of or belonging to Korea)

Cells are Gram-negative, rod-shaped, and non-motile. After 2 days incubation at 25°C on R2A, colonies are circular and

Table 3. Cellular fatty acid compositions of novel strains and closely related strains
Strains: 1, ARSA-42^T; 2, ARSA-103^T; 3, ARSA-108^T; 4, *F. aquatile* JCM 20745^T; 5, *F. macrobrachii* an-8^T (data from Sheu *et al.*, in press). -, trace (fatty acid that accounts for <1% of the total).

Fatty acid	1	2	3	4	5
iso-$C_{10:0}$	-	-	-	-	1.0
iso-$C_{14:0}$	2.8	2.4	5.3	1.1	1.4
$C_{14:0}$	-	-	-	-	2.1
iso-$C_{14:0}$ 3-OH	1.1	-	1.5	-	-
iso-$C_{15:1}$	-	-	-	-	7.6
iso-$C_{15:0}$	12.0	21.0	12.9	17.7	18.1
anteiso-$C_{15:0}$	5.2	6.6	3.5	10.2	2.1
iso-$C_{15:0}$ 2-OH	1.4	-	-	-	
iso-$C_{15:0}$ 3-OH	11.5	14.5	9.9	3.7	8.8
iso-$C_{15:1}$ G	12.7	8.0	10.5	9.9	-
anteiso-$C_{15:1}$ A	2.1	-	-	1.8	-
$C_{15:0}$ 2-OH	-	-	-	-	1.6
$C_{15:0}$ 3-OH	2.1	3.2	1.8	5.6	2.6
$C_{15:1}\omega6c$	3.8	7.5	6.0	20.4	3.7
$C_{15:1}\omega8c$	-	-	-	1.2	-
iso-$C_{16:1}$	-	-	-	-	1.6
iso-$C_{16:0}$	4.9	5.1	7.1	1.5	4.5
iso-$C_{16:0}$ 3-OH	13.8	14.3	23.6	3.1	9.1
iso-$C_{16:1}$ H	3.3	-	4.9	1.1	-
$C_{16:0}$	1.3	-	-	-	5.5
$C_{16:0}$ 3-OH	2.2	-	-	-	4.2
iso-$C_{17:0}$ 3-OH	10.4	12.3	9.0	4.8	7.9
iso-$C_{17:1}\omega9c$	2.2	2.6	1.4	1.7	1.3
$C_{17:0}$ 2-OH	1.7	-	-	1.7	-
$C_{17:0}$ 3-OH	-	-	-	1.1	1.3
$C_{17:1}\omega6c$	-	-	-	5.4	-
$C_{18:0}$	-	-	-	-	1.7
Summed feature 3[a]	3.4	2.6	2.6	3.1	9.6

[a] Summed features 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 $C_{16:1}\omega7c$.

cream-yellow color. The organism has properties of adherence. Grows on NA but not on MacConkey, DNase test agar, and TSA medium. Colonies on Anacker and Ordal's agar are round and 0.1-0.3 mm in diameter after 10 days incubation. Growth occurs at 4-37°C (optimum, 25°C), pH 6.0-9.5 (optimum, 7.0-8.0), and in the presence of 0-0.5% NaCl (optimum, 0%). Anaerobic growth does not occur. Congo red is not absorbed by the colonies. Flexirubin-type pigments are not produced (KOH test-negative). Positive for catalase and oxidase activity with N,N,N,N-tetramethyl-p-phenylenediamine. Starch is hydrolyzed, but casein, Tween 80, CM-cellulose, chitin, tyrosine, pectin, xanthine, and hypoxanthine are not. Nitrate is not reduced. In the API 20NE and Vitek system, positive for esculin, gelatin, PNPG (β-galactosidase), capric acid, Ala-Phe-Pro-arylamidase, L-proline arylamidase, lipase, tyrosine arylamidase, phosphatase, and Glu-Gly-Arg-arylamidase. Enzymic activity is detected for alkaline phosphatase, leucine arylazmidase, valine arylamidase, acid phospatase, and naphtol-AS-BI-phosphohydrolase. The major fatty acids (>7% of the total fatty acids) are iso-$C_{16:0}$ 3-OH (13.8%), iso-$C_{15:1}$ G (12.7%), iso-$C_{15:0}$ (12.0%), iso-$C_{15:0}$ 3-OH (11.5%), and iso-$C_{17:0}$ 3-OH (10.4%). MK-6 is the major respiratory quinone. The G+C content of the DNA of the type strain is 31.5 mol% (HPLC). The GenBank accession number for 16S rRNA gene sequence is GU295967. The type strain is ARSA-42^T (=KCTC 23182^T =JCM 17066^T =KACC 14969^T), isolated from a freshwater reservoir of Cheonan, Korea.

Description of *Flavobacterium chungnamense* sp. nov.

Flavobacterium chungnamense (chun.gna.men'se. N.L. neut. adj. chungnamense, of or belonging to Chungnam)

Cells are Gram-negative, rod-shaped, and non-motile. After 2 days incubation at 25°C on R2A, colonies are circular and cream-yellow color. The organism has properties of adherence. Grows on NA but not on MacConkey, DNase test agar, and TSA medium. Colonies on Anacker and Ordal's agar are round and 0.1-0.5 mm in diameter after 10 days incubation. Growth occurs at 10-30°C (optimum, 25°C), pH 6.5-9.5 (optimum, 7.5-8.0) and in the presence of 0-0.5% NaCl (optimum, 0%). Anaerobic growth does not occur. Congo red is not absorbed by the colonies. Flexirubin-type pigments are not produced. Positive for catalase and oxidase activity with N,N,N,N-tetramethyl-p-phenylenediamine. Starch is hydrolyzed, but casein, Tween 80, CM-cellulose, chitin, tyrosine, pectin, xanthine, and hypoxanthine are not. Nitrate is not reduced. In the API 20NE and Vitek system, positive for PNPG (β-galactosidase), Ala-Phe-Pro-arylamidase, L-proline arylamidase, lipase, tyrosine arylamidase, and phosphatase. Enzymic activity is detected for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, and naphtol-AS-BI-phosphohydrolase. The major fatty acids (>7% of the total fatty acids) are iso-$C_{15:0}$ (21.0%), iso-$C_{15:0}$ 3-OH (14.5%), iso-$C_{16:0}$ 3-OH (14.3%), iso-$C_{17:0}$ 3-OH (12.3%), iso-$C_{15:1}$ G (8.0%), and $C_{15:1}\omega6c$ (7.5%). MK-6 is the major respiratory quinone. The G+C content of the DNA of the type strain is 33.2 mol%. The GenBank accession no. for 16S rRNA gene sequence is GU295971. The type strain is ARSA-103^T (=KCTC 23183^T =JCM 17068^T =KACC 14971^T), isolated from a freshwater reservoir of Cheonan, Korea.

Description of *Flavobacterium cheonanense* sp. nov.

Flavobacterium cheonanense (che.o.nan.en'se. N.L. neut. adj. cheonanense, of or belonging to Cheonan)

Cells are Gram-negative, rod-shaped, and non-motile. After 2 days incubation at 25°C on R2A, colonies are circular and cream-yellow color. The organism has properties of strong adherence to agar. Grows on NA but not on MacConkey, DNase test agar and TSA medium. Colonies on Anacker and Ordal's agar are round and 0.1-0.7 mm in diameter after 10 days incubation. Growth occurs at 4-37°C (optimum, 25°C), pH 6.5-9.5 (optimum, 7.5-8.0) and in the presence of 0-1.0% NaCl (optimum, 0%). Anaerobic growth does not occur. Congo red is not absorbed by the colonies. Flexirubin-type pigments are not produced. Positive for catalase and oxidase activity with N,N,N,N-tetramethyl-p-phenylenediamine. Starch is hydrolyzed, but casein, Tween 80, CM-cellulose, chitin, tyrosine, pectin, xanthine, and hypoxanthine are not. Nitrate is not reduced. In the API 20NE and Vitek system, positive for Ala-Phe-Pro-arylamidase, L-proline arylamidase, tyrosine arylamidase, and phosphatase. Enzymic activity is detected for alkaline phosphatase, leucine arylamidase, valine arylamidase, and Crystine arylamidase. The major fatty acids (>7% of the total fatty acids) are iso-$C_{16:0}$ 3-OH (23.6%), iso-$C_{15:0}$ (12.9%), iso-$C_{15:1}$ G (10.5%), iso-$C_{15:0}$ 3-OH (9.9%), iso-$C_{17:0}$ 3-OH (9.0%), and iso-$C_{16:0}$ (7.1%). MK-6 is the major respiratory quinone. The G+C content of the DNA of the type strain is 31.4 mol%. The GenBank accession number for 16S rRNA gene sequence is GU295968. The type strain is ARSA-108^T (=KCTC 23184^T =JCM 17069^T =KACC 14972^T), isolated from a freshwater reservoir of Cheonan, Korea.

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