Flavobacterium fluvii sp. nov., isolated from stream sediment

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An aerobic, yellow-pigmented, Gram-staining-negative bacterium, designated strain H7^T, was isolated from sediment of Gazwa stream in Jinju city, South Korea. Cells of strain H7^T were non-motile, straight rods that produced flexirubin pigments and showed catalase- and oxidase activities. Strain H7^T contained iso-C_{15:0}, anteiso-C_{15:0} and summed feature 3 (comprising C_{16:1ω7c} and/or iso-C_{15:0} 2-OH) as the main fatty acids and menaquinone-6 (MK-6) as the major isoprenoid quinone. The DNA G+C content of strain H7^T was 37.2 mol%. Comparative 16S rRNA gene sequence analysis showed that strain H7^T formed a distinct phyletic line within the genus *Flavobacterium*. Based on 16S rRNA gene sequence similarities, the novel strain was most closely related to *Flavobacterium limicola* ST-82^T and *Flavobacterium resistens* BD-b365^T with 97.2% and 97.0% gene sequence similarities, respectively. The DNA–DNA relatedness of the novel strain with these species was only 18% and 14%, respectively. On the basis of phenotypic data and molecular properties, strain H7^T represents a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium fluvii* sp. nov. is proposed. The type strain is H7^T (=KACC 12818^T=DSM 19978^T).

The genus *Flavobacterium*, which belongs to the family *Flavobacteriaceae*, phylum *Bacteroidetes*, was proposed by Bergey *et al.* (1923) and the description was considerably emended by Bernardet *et al.* (1996). The genus *Flavobacterium* comprises Gram-staining-negative, strictly aerobic, predominantly gliding, pigmented rods with menaquinone-6 (MK-6) as the major respiratory quinone and with DNA G+C contents of between 30 and 41 mol% (Bernardet & Bowman, 2006; Park *et al.*, 2006). Species of the genus *Flavobacterium* with diverse physiological characteristics have been isolated from varied habitats such as microbial mats, freshwater and seawater, Antarctic lakes, green house soil, beaked whales, earthworm gut and contaminated sediments (McCammon & Bowman, 2000; Humphry *et al.*, 2001; Tamaki *et al.*, 2003; Van Trappen *et al.*, 2005; Cousin *et al.*, 2007; Park *et al.*, 2007; Ryu *et al.*, 2007, 2008; Vela *et al.*, 2007; Weon *et al.*, 2007; Jit *et al.*, 2008). This suggests that they may have important roles in the environment. The taxonomic characterization of a

novel species belonging to the genus *Flavobacterium* is reported in this paper.

Soil sediment was collected from the Gazwa stream in Jinju city (35° 11' N 128° 05' E) in Gyeongnam province, South Korea. The sediment was diluted serially using 0.9% (w/v) saline and diluted solutions were spread on R2A agar (Difco) plates and incubated at 25 °C for 5 days. Strain H7^T was isolated and routinely grown on R2A agar aerobically at 30 °C for 3 days, except where indicated otherwise. The strain was stored at –80 °C in R2A broth supplemented with 10% (v/v) glycerol for preservation.

Amplification and sequencing of the 16S rRNA gene of strain H7^T was carried out as described by Kim *et al.* (2008). Briefly, a single colony grown on R2A agar was resuspended in 100 µl 5% (w/v) Chelex-100 solution (Bio-Rad) and boiled for 10 min to prepare crude genomic DNA lysates. PCR amplification of 16S rRNA genes from the crude lysates was performed using the universal primer F1 (5'-AGAGTTTGATCMTGGCTCAG-3') and R13 (5'-TACGGYTACCTTGTTACGACTT-3') as described previously (Lu *et al.*, 2006). The resulting 16S rRNA gene sequence (1479 nucleotides) was checked manually for quality and gaps and compared with sequences from

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H7^T is EU109724.

GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation. It was then aligned with sequences of closely related members of the family *Flavobacteriaceae* by using CLUSTAL W software (Thompson *et al.*, 1994). The sequence similarity values between the new isolate and related bacteria were evaluated using the EzTaxon Server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic trees were constructed by using the neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) algorithms available in the PHYLIP software, version 3.6 (Felsenstein, 2002). The resulting tree topologies were evaluated using bootstrap analysis based on 1000 resampled datasets with the PHYLIP package.

DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness between strain H7^T and the related strains *Flavobacterium limicola* ST-82^T and *Flavobacterium resistens* BD-b365^T using the fluorometric microplate method (Ezaki *et al.*, 1989). Fluorometric data recorded after reaction of the substrates for 30 min were used for the calculation of DNA–DNA hybridization values. The signals produced by homologous reactions were inferred as 100 % and percentages of DNA–DNA relatedness were calculated from the mean values of five replications.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain H7^T formed a distinct phylogenetic line within the genus *Flavobacterium* (Fig. 1), although the bootstrap value was low (16.5 %). The overall topology of the ML and MP trees was essentially the same as that of the NJ tree (data not shown). Comparative 16S rRNA gene sequence analysis showed that the new isolate was most closely related to *F. limicola* ST-82^T and *F. resistens* BD-b365^T with similarities of 97.2 % and 97.0 %, respectively. The 16S rRNA gene sequence similarities of the new isolate with other species of the genus *Flavobacterium* were less than 97.0 %. The DNA–DNA relatedness values for strain H7^T with *F. limicola* ST-82^T and *F. resistens* BD-b365^T were 18 % and 14 %, respectively, which are clearly below the 70 % threshold generally accepted for species delineation (Rosselló-Mora & Amann, 2001).

Growth was tested on R2A, Luria–Bertani (LB), tryptic soy (TSA, Difco) and nutrient (NA, Difco) agars at 30 °C. Temperatures and pH values for growth of strain H7^T were examined by growing the isolate on R2A agar at 5–35 °C at 5 °C intervals and in R2A broth adjusted to pH 5.0–10.0 at 0.5 pH unit intervals for 5 days. The pH was adjusted prior to sterilization by the addition of HCl or NaOH and measured again after sterilization. Gram staining was performed using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Cell morphology, the presence of flagella and gliding motility were studied using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) as described previously (Bernardet *et al.*, 2002; Jeon *et al.*, 2004). Salt tolerance was tested in R2A broth prepared with 0–3 % (w/v) NaCl at

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain H7^T and related taxa. Bootstrap values >50 % are shown as percentages of 1000 replicates. *Capnocytophaga granulosa* LMG 16022^T (U41347) was used as an outgroup (not shown). Bar, 0.01 changes per nucleotide position.

0.5 % intervals for 5 days at 30 °C. Oxidase activity was tested by oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. The production of flexirubin type pigments and of extracellular glycans was investigated using the KOH and Congo red tests, respectively, following the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Nitrate reduction was assessed according to the method of Lányi (1987) and acid production from carbohydrates was tested as described by Leifson (1963). Antibiotic susceptibility tests were performed in duplicate on R2A agar at 30 °C for 3 days using filter-paper discs (diameter, 8 mm) containing the following antibiotics (µg per disc unless otherwise indicated): ampicillin (10), polymyxin B (100 U), streptomycin (50), penicillin G (10 U), gentamicin (30), chloramphenicol (100), tetracycline (30), kanamycin (30), lincomycin (15), oleandomycin (15),

carbenicillin (100) and novobiocin (50). Hydrolysis of carboxymethylcellulose, aesculin, casein, Tween 80, Tween 20, tyrosine, gelatin and starch was investigated on R2A agar after a 7-day incubation and was conducted according to previously described methods (Lányi, 1987; Smibert & Krieg, 1994). Additional enzymic activities and biochemical features were determined using API ZYM, API 20NE and API 20E kits (bioMérieux) and the utilization or oxidation of carbon sources was determined using the GN MicroPlate system (Biolog) according to the manufacturers' instructions. Anaerobic growth was assessed on R2A agar under anaerobic (with 4–10 % CO₂) conditions using the GasPak Plus system (BBL) at 30 °C for 15 days. The hydrolysis and utilization tests were performed in parallel on strain H7^T and on the type strains of its closest phylogenetic neighbours *F. limicola* ST-82^T, *F. resistens* BD-b365^T, *Flavobacterium psychrolimnae* LMG 22018^T and *Flavobacterium xinjiangense* JCM 11314^T.

Strain H7^T grew at 30 °C on R2A, LB, TSA and nutrient (NA, Difco) agars. The new isolate grew optimally in R2A broth without the addition of NaCl and growth was severely inhibited in the presence of >1 % (w/v) NaCl. Anaerobic growth was not observed after 15 days at 30 °C on R2A agar. The phenotypic features of strain H7^T are presented in Table 1 and in the species description. Some of them were in accordance with the characteristics of members of the genus *Flavobacterium*, whereas others enabled the differentiation of strain H7^T from closely related species of the genus *Flavobacterium* (Table 1).

Isoprenoid quinones were analysed using HPLC (LC-20A; Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250 × 4.6 mm; Kromasil, Akzo Nobel) as described previously (Komagata & Suzuki, 1987). Cellular fatty acid analysis using cells grown on NA plates at 20 °C for 3–5 days was performed in parallel for strain H7^T and the type strains of its closest phylogenetic neighbours *F. limicola* ST-82^T, *F. resistens* BD-b365^T, *F. xinjiangense* JCM 11314^T and *F. psychrolimnae* LMG 22018^T. However, *F. xinjiangense* JCM 11314^T was cultivated at 12 °C for 5 days for the cellular fatty acid analysis because this strain does not grow at 20 °C (Zhu *et al.*, 2003). Analysis of fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID, Inc.). The DNA G+C content of strain H7^T was determined using HPLC with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM) according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone of strain H7^T was menaquinone-6 (MK-6). The major cellular fatty acids were iso-C_{15:0} (18.2 %), anteiso-C_{15:0} (15.6 %), summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 9.1 %), iso-C_{17:0} 3-OH (6.2 %) and iso-C_{15:0} 3-OH (6.1 %). The complete fatty acid contents of strain H7^T and its closest phylogenetic neighbours are given in Table 2. The DNA G+C content of strain H7^T was 37.2 mol%. The

Table 1. Differential characteristics of strain H7^T and closely related species of the genus *Flavobacterium*

Strains: 1, H7^T (this study); 2, *F. limicola* ST-82^T (Tamaki *et al.*, 2003); 3, *F. resistens* BD-b365^T (Ryu *et al.*, 2008); 4, *F. psychrolimnae* LMG 22018^T (Van Trappen *et al.*, 2005); 5, *F. xinjiangense* JCM 11314^T (Zhu *et al.*, 2003). All strains are positive for the following characteristics: catalase, oxidase, hydrolysis of aesculin, utilization (Biolog) of α-cyclodextrin, dextrin, glycogen, cellobiose, α-D-glucose, maltose, turanose, glycyl-L-aspartic acid, L-ornithine, L-proline, L-threonine, uridine, glucose 1-phosphate and glucose 6-phosphate. All strains are negative for the following characteristics: Gram staining, nitrate reduction, hydrolysis of Tween 40, Tween 80, citrate and CM-cellulose, utilization (Biolog) of Tween 80, D-arabitol, D-erythritol, D-galactonic acid lactone, *cis*-aconitic acid, D-glucosaminic acid, *p*-hydroxyphenylacetic acid, L-pyrogutamic acid, D-serine, DL-carnitine, 2-aminoethanol, 2,3-butanediol and glycerol. +, Positive; –, negative; (+), weakly positive.

Characteristic	1	2	3	4	5
Gliding motility	–	–	+	–	–
Flexirubin type pigments	+	–	+	–	–
Growth on:					
Nutrient agar*	+	+	+	+	+
Trypticase soy agar	+	+	+	+	–
Growth at 20 °C	+	+	+	+	–
β-Galactosidase activity (API 20E)*	+	–	+	–	–
H ₂ S production (API 20E)*	–	–	–	–	+
Hydrolysis of:*					
Casein	–	+	+	+	+
Gelatin	–	+	–	–	+
Starch	–	+	+	+	–
Tyrosine	–	+	–	–	–
Utilization (Biolog) of:*					
Acetic acid	(+)	+	+	+	(+)
N-Acetyl D-galactosamine	+	–	–	–	(+)
L-Arabinose	+	–	(+)	–	–
D-Arabitol	–	–	–	–	(+)
Gentiobiose	+	(+)	+	–	+
D-Mannitol	–	–	–	–	(+)
Melibiose	(+)	+	+	+	(+)
Methyl β-D-glucoside	–	–	+	–	+
L-Rhamnose	+	–	–	–	–
Sucrose	+	(+)	–	(+)	+
DNA G+C content (mol%)	37.2	35.0	35.4	34.5	34.4

*All results from this study.

major lipoquinone, major fatty acids and DNA G+C content were in line with those of members of the genus *Flavobacterium* (Tamaki *et al.*, 2003; Zhu *et al.*, 2003; Bernardet & Bowman, 2006; Vela *et al.*, 2007). Therefore, the physiological, biochemical and phylogenetic properties of strain H7^T support its description as a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium fluvii* sp. nov. is proposed.

Table 2. Cellular fatty acid contents (%) of strain H7^T and closely related species of the genus *Flavobacterium*

Strains: 1, H7^T; 2, *F. limicola* ST-82^T; 3, *F. resistens* BD-b365^T; 4, *F. psychrolimnae* LMG 22018^T; 5, *F. xinjiangense* JCM 11314^T. All data are from this study. Data are expressed as percentages of total fatty acids. Fatty acids amounting to <1.0% in all strains are not shown. tr, Trace amount (<1%); –, not detected; ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5
Straight chain:					
C _{14:0}	1.1	tr	tr	tr	tr
C _{15:0}	3.4	4.0	10.0	4.1	7.7
C _{16:0}	1.7	tr	1.7	1.2	tr
Branched:					
iso-C _{13:0}	2.6	tr	tr	tr	tr
iso-C _{14:0}	–	3.5	tr	3.1	2.5
iso-C _{15:0}	18.2	16.0	33.2	13.2	10.0
iso-C _{15:1} G	4.9	7.0	2.2	6.0	6.2
iso-C _{16:0}	tr	3.7	1.7	4.2	2.5
iso-C _{16:1} H	1.0	5.4	tr	5.4	2.8
iso-C _{17:1} ω9c	1.3	6.7	4.9	5.1	2.0
anteiso-C _{13:0}	3.1	–	–	–	–
anteiso-C _{15:0}	15.6	4.0	2.7	4.3	4.6
anteiso-C _{15:1} A	2.7	tr	–	tr	tr
anteiso-C _{17:1} ω9c	1.9	–	–	–	–
Unsaturated:					
C _{15:1} ω6c	2.3	6.8	5.6	6.2	10.4
C _{17:1} ω6c	2.1	6.1	1.8	6.4	9.8
C _{18:1} ω5c	–	1.0	tr	1.2	tr
Hydroxy:					
C _{15:0} 3-OH	–	–	–	–	1.5
C _{16:0} 3-OH	1.7	tr	1.6	tr	1.7
C _{17:0} 2-OH	2.5	tr	tr	–	tr
iso-C _{15:0} 3-OH	6.1	5.1	8.4	5.5	3.8
iso-C _{16:0} 3-OH	3.5	6.2	1.5	6.4	4.5
iso-C _{17:0} 3-OH	6.2	4.4	6.9	4.4	3.4
Summed features*					
3	9.1	9.7	5.7	11.9	14.9
4	–	1.6	2.6	1.7	–
Unknown					
ECL 11.543	1.8	1.4	tr	1.5	1.5
ECL 13.565	3.2	2.1	1.1	2.4	2.2

*Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1} ω7c and/or iso-C_{15:0} 2-OH; summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1}.

Description of *Flavobacterium fluvii* sp. nov.

Flavobacterium fluvii (flu'vi.i. L. gen. n. *fluvii* of a river, of a stream).

Cells are Gram-staining-negative rods, 0.7–0.9 µm in diameter and 2.0–3.0 µm in length. Colonies on R2A agar are yellow, convex and circular with entire margins. Growth occurs at 15–35 °C (optimum, 25–30 °C), at

pH 6.0–8.0 (optimum, pH 6.5–7.5). More than 1% (w/v) NaCl inhibits growth severely; no growth occurs in the presence of 2% NaCl. Oxidase- and catalase-positive. Nitrate is not reduced to nitrite. Gliding motility is not observed. Aesculin is hydrolysed. Tween 20, Tween 80, tyrosine, carboxymethylcellulose, gelatin, casein and starch are not hydrolysed. Flexirubin type pigments are produced. Acid is produced from raffinose, *myo*-inositol, lactose, L-arabinose, arbutin, D-fructose, salicin, D-mannose and D-galactose, but not from melibiose. In the API 20NE kit, D-glucose, L-arabinose, D-mannose and maltose are assimilated, but D-mannitol, malic acid, potassium gluconate, *N*-acetylglucosamine, capric acid, adipic acid, trisodium citrate and phenylacetic acid are not assimilated. In the API ZYM kit, alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase and α-glucosidase activities are present and weak enzymic activity is observed for naphthol-AS-BI-phosphohydrolase. No activities are found for lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, β-galactosidase, α-fucosidase or α-mannosidase. The type strain is resistant to polymyxin B (100 U), gentamicin (30 µg), kanamycin (30 µg), oleandomycin (15 µg), ampicillin (10 µg), chloramphenicol (100 µg), streptomycin (50 µg), lincomycin (15 µg), tetracycline (30 µg), carbenicillin (100 µg) and novobiocin (50 µg), but sensitive to penicillin G (10 U). The major isoprenoid quinone is MK-6. The major (>5% of the total fatty acids) cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, summed feature 3 (C_{16:1} ω7c and/or iso-C_{15:0} 2-OH), iso-C_{17:0} 3-OH and iso-C_{15:0} 3-OH.

The type strain, H7^T (=KACC 12818^T=DSM 19978^T), was isolated from sediment of the Gazwa stream, Jinju city, South Korea. The DNA G+C content of the type strain is 37.2 mol% (HPLC).

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