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Flavobacterium granuli sp. nov., isolated from granules used in a wastewater treatment plant

Zubair Aslam, Wan-Taek Im, Myung Kyum Kim and Sung-Taik Lee

Department of Biological Sciences, Korea Advanced Institute of Science and Technology,
373-1, Guseong-dong, Yuseong-gu, Daejeon, 305-701, Republic of Korea

Correspondence
Sung-Taik Lee
e_stlee@kaist.ac.kr

A Gram-negative, rod-shaped, non-spore-forming bacterium (designated strain Kw05^T) was isolated from granules used in the wastewater treatment plant of a beer-brewing factory in Kwang-Ju, Republic of Korea. On the basis of 16S rRNA gene sequence similarity, strain Kw05^T was shown to belong to the family *Flavobacteriaceae*, and was most closely related to *Flavobacterium limicola* (96·6 %), *Flavobacterium hibernum* (96·3 %), *Flavobacterium hydatis* (96·1 %) and *Flavobacterium xinjiangense* (96·1 %). The G+C content of the genomic DNA of strain Kw05^T was 36·2 mol%, within the range of 32–37 mol% for the genus *Flavobacterium*. Chemotaxonomic data (major menaquinone MK-6; major fatty acids iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$ 3-OH and iso-$C_{17:1}\omega 9c$) supported the classification of strain Kw05^T within the genus *Flavobacterium*. Kw05^T therefore represents a novel species, for which the name *Flavobacterium granuli* sp. nov. is proposed. The type strain is Kw05^T (=KCTC 12201^T=IAM 15099^T).

The genus *Flavobacterium*, which belongs to the *Cytophaga–Flavobacterium–Bacteroides* group, was proposed by Frankland in 1889 (Bergey *et al.*, 1923). The formal description of the genus has been emended several times since then. At present, the genus comprises 28 species. Bernardet *et al.* (1996) proposed an amendment to the description, which states that the genus *Flavobacterium* represents predominantly gliding, pigmented bacteria that have menaquinone-6 as the primary respiratory quinone. Species of the genus *Flavobacterium* have DNA G+C contents of 32–37 mol%. Through emendation of classification, several species previously placed in the genus *Flavobacterium* have been reclassified and placed in new or different genera, including the genera *Microbacterium* (Takeuchi & Hatano, 1998), *Salegentibacter* (McCammon & Bowman, 2000) and *Planococcus* (Nakagawa *et al.*, 1996). Several species previously placed in other genera, including *Cytophaga* and *Flexibacter*, have been reclassified and placed in the genus *Flavobacterium* (Bernardet *et al.*, 1996). *Flavobacterium* species have been isolated from diverse habitats such as fresh and salt water, soil, sediment, sea ice, diseased fish and microbial mats.

Strain Kw05^T was isolated from granules used in the wastewater treatment plant of a beer-brewing factory in Kwang-Ju, Republic of Korea. Anaerobic granules are bacterial aggregates that result from the flocculation of sludge in an upflow anaerobic sludge blanket (UASB) reactor (de Zeeuw & Lettinga, 1980). They are composed of micro-organisms, inorganic nuclei and extracellular polymers (Fukuzaki *et al.*, 1991; Shen *et al.*, 1993). Great attention has been paid to the internal structure and catalytic activities of these granules (MacLeod *et al.*, 1990; Schmidt & Ahring, 1996). In our laboratory, the relationship between the structure and resistance to toxic chemicals of anaerobic granules from a brewery wastewater treatment UASB reactor was studied (Bae & Lee, 1999; Bae *et al.*, 2000). In a series of studies, we attempted to isolate micro-organisms from the anaerobic granules in order to investigate the community structure based on a culture system. Interestingly, the granules contained aerobic bacteria even though they had been kept under anaerobic conditions for 2 years. Strain Kw05^T was one of the dominant bacterial isolates that was grown under aerobic conditions.

A polyphasic approach, including phylogenetic analysis based on 16S rRNA gene sequences, chemotaxonomic and phenotypic properties, was conducted to determine the precise taxonomic position of strain Kw05^T. The results obtained indicated that it can be assigned as a member of the genus *Flavobacterium*, but it is clearly distinguished from recognized *Flavobacterium* species. Here, we propose Kw05^T as the type strain of a novel species, *Flavobacterium granuli* sp. nov.

Abbreviation: UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Kw05^T is AB180738.

Fatty acid profiles of strain Kw05^T and related type strains of *Flavobacterium* species are available as supplementary material in IJSEM Online.

For isolation of aerobic bacteria, brownish black granules (around 2 mm in diameter) from a brewery wastewater treatment UASB reactor were homogenized using an Ace homogenizer (Nihonseiki Kaisha Ltd). The suspension was spread on R2A agar plates (Difco) after being serially diluted with 50 mM phosphate buffer (pH 7·0). The plates were incubated at 30 °C for 2 weeks. Single colonies on the plates were purified by transferring them onto new plates and incubating again under the same conditions. The purified colonies were tentatively identified based on partial sequences of the 16S rRNA gene. Strain Kw05^T was one of the dominant isolates that appeared on the plates following aerobic incubation.

Gram reaction was performed using the non-staining method as described by Buck (1982). Cell morphology was observed under a Nikon light microscope at ×1000 magnification, with cells grown for 3 days at 30 °C on R2A agar. Catalase and oxidase tests were performed by the procedures outlined by Cappuccino & Sherman (2002). Substrate utilization as the sole carbon source and physiological characteristics were determined with API 32GN, API 20E and API 20NE galleries according to the manufacturer's instructions (bioMérieux). Congo red absorption, presence of gliding motility (Bernardet *et al.*, 2002), production of flexirubin-type pigments (Reichenbach, 1989), degradation of DNA [using DNA agar (Difco) supplemented with 0·01 % toluidine blue (Merck)], degradation of casein, chitin, starch and L-tyrosine (Atlas, 1993), production of a brown diffusible pigment on L-tyrosine agar and precipitation on egg-yolk agar (Atlas, 1993) were also investigated; reactions were read after 5 days. Hydrolysis of carboxymethyl cellulose was tested as described by Ten *et al.* (2004). Growth at different temperatures and pH was assessed after 5 days of incubation. Salt tolerance was tested on R2A medium supplemented with 1–10 % (w/v) NaCl after 7 days of incubation. Duplicate antibiotic-sensitivity tests were performed using filter-paper discs containing the following:

Table 1. Selected characteristics of *Flavobacterium granuli* sp. nov. Kw05^T and its nearest phylogenetic neighbours within the genus *Flavobacterium*

Taxa: 1, *Flavobacterium granuli* Kw05^T; 2, *F. hydatis*; 3, *F. saccharophilum*; 4, *F. pectinovorum* (data for taxa 2–4 are from Bernardet *et al.*, 1996); 5, *F. frigidarium* (Humphry *et al.*, 2001); 6, *F. hibernum* (McCammon *et al.*, 1998); 7, *F. limicola* (Tamaki *et al.*, 2003); 8, *F. omnivorum*; 9, *F. xinjiangense* (Zhu *et al.*, 2003); 10, *F. degerlachei*; 11, *F. frigoris*; 12, *F. micromati* (Van Trappen *et al.*, 2004). +, Positive; (+), positive, weak or delayed response; −, negative; V, variable among strains; ND, not defined. All species shown are negative for precipitate formation on egg-yolk agar, indole production and arginine dihydrolase activity and are positive for production of catalase and degradation of aesculin.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Growth on:												
TSA	+	+	+	+	+	+	+	+	−	+	+	(+)
Nutrient agar	+	+	+	+	+	+	+	+	(+)	+	−	+
Growth at 25 °C on agar	+	+	+	+	−	+	+	−	−	+	(+)	(+)
Flexirubin-type pigment	−	+	+	+	−	+	−	−	−	−	−	−
Congo red absorption	−	−	−	−	+	−	+	ND	ND	−	−	−
Glucose utilization	+	+	+	+	+	+	+	+	+	+	+	−
Acid produced aerobically from carbohydrate	−	+	+	+	−	+	−	−	−	−	−	−
Oxidase production	+	V	−	+	+	−	+	+	+	+	+	+
Urease	+	ND	ND	ND	−	−	+	−	−	−	−	−
Degradation of:												
Tyrosine	−	+	+	+	−	+	+	−	−	−	+	−
Gelatin	−	+	+	+	+	+	+	−	+	−	−	−
Casein	−	+	+	+	+	+	+	+	+	−	+	−
Carboxymethyl cellulose	−	ND	+	+	−	−	−	+	−	−	−	−
Agar	−	ND	+	−	−	−	+	ND	ND	−	−	−
Pectin	ND	+	+	+	−	−	−	+	−	−	−	−
Chitin	−	(+)	−	+	−	−	−	+	+	−	−	−
Alginate	ND	−	ND	+	−	−	−	ND	ND	−	−	−
Starch	−	+	+	+	−	+	+	+	−	+	+	−
DNA	−	+	−	+	−	+	−	−	−	−	−	−
β-Galactosidase activity (ONPG)	+	+	+	+	−	+	−	+	−	−	−	−
H_2S production	−	−	+	ND	−	−	−	−	+	−	−	−
Nitrate reduction	−	+	+	+	−	+	−	+	−	−	V	−
DNA G+C content (mol%)	36	34	33	35	35	36	35	35	34	34	34	33

streptomycin (5, 10 and 15 μg ml^{-1}) (Mast Diagnostics), tetracycline (5, 10 and 15 μg^{-1}), kanamycin (1·0, 1·5 and 2·0 mg ml^{-1}) and ampicillin (20, 25 and 30 μg ml^{-1}) (Sigma). Discs were placed on R2A plates containing cultured Kw05^T and were then incubated at 30 °C for 7 days. Growth was tested against various dissolved antibiotics in duplicate at three different concentrations. A discrete Kw05^T colony (grown at 30 °C on R2A medium) was then spread onto each plate. (Humphry *et al.*, 2001). Physiological and biochemical characteristics of strain Kw05^T and related type strains of *Flavobacterium* species are summarized in Table 1.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Im *et al.* (2004). 16S rRNA gene sequences of related taxa were obtained from GenBank. Multiple alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BIOEDIT program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed using a neighbour-joining method (Saitou & Nei, 1987) in the MEGA 2 program (Kumar *et al.*, 2001) with bootstrap values based on 1000 replications (Felsenstein, 1985) (Fig. 1).

The G+C content of the chromosomal DNA was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Quinones were extracted from cells grown on R2A broth (Difco) and analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. Cellular fatty acids were analysed in organisms grown on trypticase soy agar (TSA; Difco) for 2 days. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids analysed by GC (Hewlett Packard 6890) were identified using the Microbial Identification software package (Sasser, 1990).

Cells of strain Kw05^T are aerobic, Gram-negative and rod-shaped. Movement via flagella was not observed. Colonies grown on R2A agar plates (Difco) for 2 days were smooth, circular, non-glossy, yellow in colour and 2–4 mm in diameter. On R2A agar, strain Kw05^T was able to grow at 15–30 °C but not at 4 or 45 °C. Physiological characteristics of strain Kw05^T are summarized in the species description below and comparison of selective characteristics with related type strains of *Flavobacterium* species is given in Table 1.

The 16S rRNA gene sequence of strain Kw05^T was a continuous stretch of 1442 bp. Sequence similarity calculations based on a neighbour-joining analysis indicated that the closest relatives of strain Kw05^T were *Flavobacterium limicola* (96·6 %), *Flavobacterium hibernum* (96·3 %), *Flavobacterium hydatis* (96·1 %) and *Flavobacterium xinjiangense* (96·1 %). Lower sequence similarities (< 97·0 %) were found with all recognized species of the genus *Flavobacterium*. It has been suggested that in bacterial strains with less than 97 % 16S rRNA gene sequence similarity, levels of DNA–DNA hybridization are less than 70 % (Stackebrandt & Goebel, 1994), which defines a genomic species (Wayne *et al.*, 1987). Thus, based on the 16S rRNA gene sequence analysis, strain Kw05^T represents a novel species.

The G+C content of the genomic DNA of strain Kw05^T was 36·2 mol% and its major quinone was MK-6. The fatty acid profile of strain Kw05^T comprised iso-C$_{15:0}$ (28·2 %),

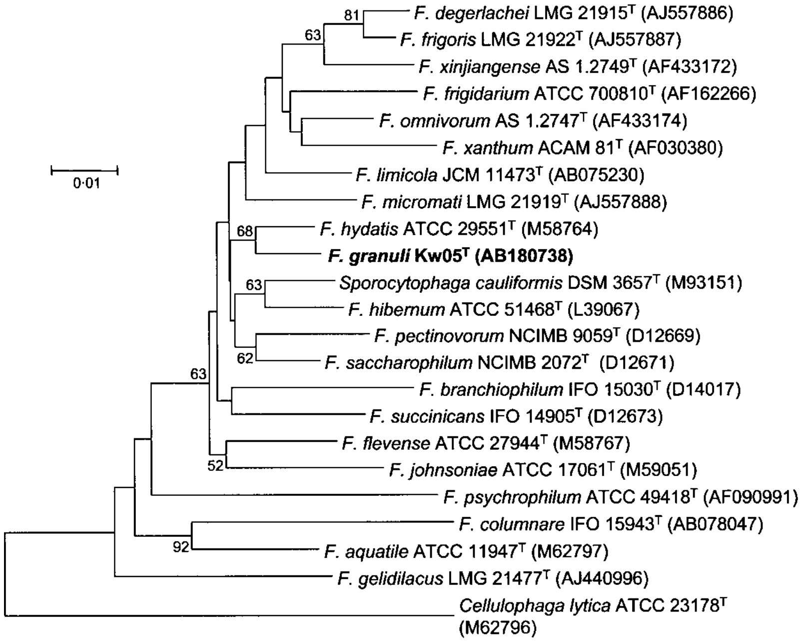

Fig. 1. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of *Flavobacterium granuli* sp. nov. strain Kw05^T with related species. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at the branch points. Bar, 0·01 substitutions per nucleotide position.

iso-$C_{17:0}$ 3-OH (11·2 %), iso-$C_{15:0}$ 3-OH (10·3 %), iso-$C_{17:1}\omega 9c$ (7·5 %), $C_{15:1}\omega 6c$ (5·6 %), $C_{15:0}$ (5·0 %), iso-$C_{15:1}$G (4·5 %), unknown 13·566 (4·3 %), $C_{17:1}\omega 6c$ (4·1 %), anteiso-$C_{15:0}$ (3·1 %), iso-$C_{13:0}$ (2·9 %), summed feature 4 ($C_{16:1}\omega 7c$/iso-$C_{15:0}$-2OH, 2·76 %), summed feature 5 (anteiso-$C_{17:1}$B/I, 2·2 %), iso-$C_{16:0}$ 3-OH (1·6 %), $C_{16:0}$ (1·42 %), iso-$C_{14:0}$ (1·0 %), $C_{17:1}\omega 8c$ (1·0 %) and $C_{18:0}$ (1·0 %). No significant differences in the fatty acid profiles for the other *Flavobacterium* species were found, except that some strains have $C_{16:1}\omega 7c$ as a major component. The fatty acid profiles of strain Kw05^T and related type strains of *Flavobacterium* species are available as supplementary material in IJSEM Online.

The results of our polyphasic analysis support the recognition of a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium granuli* sp. nov. is proposed.

Description of *Flavobacterium granuli* sp. nov.

Flavobacterium granuli (gra.nu′li. L. gen. n. *granuli* of a small grain, pertaining to a granule, from which the type strain was isolated).

Cells are aerobic, Gram-negative, rod-shaped, non-motile and non-gliding (i.e. non-flagellated), 0·3–0·5 μm wide by 2·0–5·0 μm long. Colonies grown on R2A are circular, convex and yellow-coloured: flexirubin-type pigments are not detected. Congo red is not absorbed. Temperature range for growth is 15–37 °C; no growth occurs at 45 °C. Optimum temperature for growth is 25–30 °C. Growth occurs in the absence of NaCl and in the presence of 1·0 % (w/v) NaCl, but not more than 2 % (w/v) NaCl. Catalase- and oxidase-positive and strictly heteroorganotrophic. Cannot grow anaerobically. H_2S is not produced. Nitrate is not reduced to nitrite. No precipitation is produced on egg-yolk agar. Urease, *β*-glucosidase and *β*-galactosidase are positive. Acetoin is produced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, gelatinase and citrate utilization are negative. Does not produce any acid or gas from glucose. Casein, xylan, chitin, DNA, tyrosine, carboxymethyl cellulose, gelatin and starch are not degraded. The following are utilized as sole carbon sources: glucose, mannose, *N*-acetylglucosamine, maltose, propionate and L-proline. The following are not utilized as sole carbon sources: L-arabinose, mannitol, gluconate, caprate, adipate, malate, citrate, phenylacetate, salicin, D-melibiose, L-fucose, D-sorbitol, valerate, histidine, 2-ketogluconate, 3-hydroxybutyrate, 4-hydroxybutyrate, rhamnose, D-ribose, inositol, D-sucrose, itaconate, suberate, malonate, acetate, lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxybenzoate and L-serine. Resistant to 20 μg ampicillin ml^{-1}, 15 μg tetracycline ml^{-1} and 15 μg streptomycin ml^{-1}, and sensitive to 0·5 mg kanamycin ml^{-1}. Major cellular fatty acids are iso-$C_{15:0}$ (28·2 %), iso-$C_{17:0}$ 3-OH (11·2 %), iso-$_{15:0}$ 3-OH (10·3 %) and iso-$C_{7:1}\omega 9c$ (7·5 %). The G+C content of the genomic DNA of the type strain is 36·2 mol% (as determined by HPLC). The type strain, Kw05^T (=KCTC 12201^T=IAM 15099^T), was isolated from granules used in the wastewater treatment plant of a beer-brewing factory in Kwang-Ju, Republic of Korea.

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