Flavobacterium filum sp. nov., isolated from a wastewater treatment plant in Korea

Seung Hyun Ryu,¹ Minjeong Park,¹ Yeji Jeon,¹ Jung Ro Lee,¹ Woojun Park² and Che Ok Jeon¹

Correspondence
Che Ok Jeon
cojeon@gnu.ac.kr

¹Division of Applied Life Science, EB-NCRC, PMBBRC, Gyeongsang National University, Jinju 660-701, Republic of Korea

²Division of Environmental Science and Ecological Engineering, Korea University, Seoul 136-701, Republic of Korea

A Gram-negative bacterium, designated strain EMB34^T, was isolated from a wastewater treatment plant in Korea. Growth was observed between 10 and 40 °C (optimum, 25–35 °C) and between pH 6.0 and 9.5 (optimum, pH 7.5–8.0). The cells were non-motile rods, linked with extracellular fibrils. The predominant fatty acids of strain EMB34^T were iso-C_{15:0}, C_{15:0}, iso-C_{15:1} G, iso-C_{16:0} 3-OH and iso-C_{15:0} 3-OH and the strain contained phosphatidylethanolamine and phosphatidylinositol as the polar lipids. The G + C content of the genomic DNA was 34.2 mol% and the major quinone was menaquinone-6. Comparative 16S rRNA gene sequence analyses showed that strain EMB34^T formed a distinct phyletic line within the genus *Flavobacterium*. The levels of 16S rRNA gene sequence similarity with other *Flavobacterium* species were less than 94.5 %. On the basis of phenotypic, chemotaxonomic and molecular data, it is clear that strain EMB34^T represents a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium filum* sp. nov. is proposed. The type strain is EMB34^T (=KCTC 12610^T=DSM 17961^T).

Since the genus *Flavobacterium*, which belongs to the phylum *Bacteroidetes*, was established (Bergey *et al.*, 1923), its description has been emended repeatedly and many novel *Flavobacterium* species have been described from diverse habitats such as micromats, fresh water and seawater, Antarctic lakes, soil, gut of an earthworm and sediments (Bernardet *et al.*, 1996; McCammon & Bowman, 2000; Van Trappen *et al.*, 2004; Kämpfer *et al.*, 2006; Cousin *et al.*, 2007). Physiological characteristics of *Flavobacterium* strains are also very diverse: they can be psychrophilic, psychrotolerant or mesophilic, they can be halotolerant, halophilic or sensitive to salts and they produce a variety of enzymes (Humphry *et al.*, 2001; Tamaki *et al.*, 2003; Aslam *et al.*, 2005; Zhang *et al.*, 2006), suggesting that they may have important roles in environmental habitats. Activated sludge processes have been used to remove organic compounds as well as nutrients from wastewater and insights regarding their bacterial communities are a prerequisite for understanding the

mechanisms involved. Therefore, efforts have been made in our laboratory to isolate and characterize members of the bacterial community in activated sludge (Park *et al.*, 2006, 2007). Here we describe the taxonomic characterization of a novel species belonging to the genus *Flavobacterium*.

Strain EMB34^T was isolated from a wastewater treatment plant in Korea that performed enhanced biological phosphorus removal. The sludge sample was serially diluted with 1 % (w/v) saline solution, spread on R2A agar (Difco) and incubated at 20 °C for 5 days. The strain was grown routinely aerobically on R2A agar at 30 °C for 3 days.

Sequencing of the 16S rRNA gene of strain EMB34^T was carried out as described previously by Lane (1991). The resulting 16S rRNA gene sequence (1436 nucleotides) was compared with available 16S rRNA gene sequences from GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation, and gene sequences were aligned with those of closely related species by using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using three different algorithms, neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP), available in the PHYLIP software, version 3.6 (Felsenstein, 2002). Sequence similarity values between the novel strain and related organisms were computed using the program FASTA3 in EBI (<http://>

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EMB34^T is DQ372981.

A transmission electron micrograph showing cells of strain EMB34^T linked by extracellular fibrils is available as a supplementary figure with the online version of this paper.

www.ebi.ac.uk/fasta33/nucleotide.html). Bootstrap analysis was performed according to the Kimura two-parameter model (Kimura, 1980) of the NJ method in the PHYLIP package. Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain EMB34^T formed a distinct phyletic lineage within the genus *Flavobacterium* (Fig. 1). The overall topologies of the ML and MP trees were essentially the same as that of the NJ tree (data not shown). Comparative 16S rRNA gene sequence analyses showed that strain EMB34^T was most closely related to *Flavobacterium flevense* ATCC 27944^T, *Flavobacterium aquatile* ATCC 11947^T and *Flavobacterium omnivorum* JCM 11313^T, with similarities of 94.4, 94.1 and 93.9%, respectively. Sequence similarities with other *Flavobacterium* species were less than 93.5%.

Gram staining was performed using a bioMérieux Gram Stain kit according to the instructions of the manufacturer. Cell morphology, flagella and gliding motility were studied using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) as described previously (Jeon *et al.*, 2005) and by Bernardet *et al.* (2002). The physiological characteristics of strain EMB34^T were examined by growing the isolate on R2A agar at various temperatures (5–50 °C at 5 °C intervals) and in R2A broth adjusted to various pH values (5.0–10.0 at 0.5 pH unit intervals) (Gomori, 1955). Salt tolerance was tested on R2A agar supplemented with 0–3% (w/v, 0.5% intervals) NaCl for 5 days at 30 °C. Antibiotic susceptibility tests were performed in duplicate using filter-paper discs (diameter, 8 mm) containing the following antibiotics: ampicillin (10 µg), polymyxin B (100 U), streptomycin (50 µg), penicillin G (10 IU), chloramphenicol (100 µg), gentamicin

(30 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg), carbenicillin (100 µg) and novobiocin (50 µg). Oxidase activity was tested by determining oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by determining the production of oxygen bubbles in a 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, according to the minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Hydrolysis of casein, gelatin, Tween 80, Tween 20, aesculin, urea, tyrosine, starch and carboxymethylcellulose was investigated on R2A agar after 7 days incubation at 30 °C, according to previously described methods (Lanyi, 1987; Gerhardt *et al.*, 1994). Nitrate reduction was performed according to the method of Lanyi (1987) and acid production from carbohydrates was tested as described by Leifson (1963). Additional enzymic activities and biochemical features were determined using API ZYM and API 20E kits as recommended by the manufacturer (bioMérieux), except that the kits were incubated for 3 days at 30 °C.

Transmission electron micrographs showed that the cells were surrounded and linked with what appeared to be extracellular fibrils (see Supplementary Fig. S1 in IJSEM Online). Phenotypic characteristics of strain EMB34^T are presented in Table 1 and in the description of the novel species. Some of the characteristics are in accordance with those of members of the genus *Flavobacterium*, whereas others allow the differentiation of strain EMB34^T from closely related *Flavobacterium* species (Table 1).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain EMB34^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates, when greater than 50%. *Capnocytophaga ochracea* ATCC 33596 was used as an outgroup. Bar, 0.01 changes per nucleotide position.

Table 1. Characteristics of strain EMB34^T and related *Flavobacterium* species

Taxa: 1, strain EMB34^T; 2, *F. aquatile* ATCC 11947^T (data from Bernardet *et al.*, 1996); 3, *F. flevense* ATCC 27944^T (Bernardet *et al.*, 1996; Van Trappen *et al.*, 2004); 4, *F. omnivorum* JCM 11313^T (Zhu *et al.*, 2003); 5, *F. saliperosum* CGMCC 1.3801^T (Wang *et al.*, 2006); 6, *F. croceum* EMB47^T (Park *et al.*, 2006); 7, *F. soli* DS-6^T (Yoon *et al.*, 2006); 8, *F. johnsoniae* (Bernardet *et al.*, 1996); 9, *F. psychrophilum* (Bernardet *et al.*, 1996). +, Positive; –, negative; v, variable among strains; (v), variable among references according to Bernardet *et al.* (1996); (+), weakly positive; NA, not available.

Characteristic	1	2	3	4	5	6	7	8	9
Growth on:									
Nutrient agar	–	–	+	+	+	–	+	+	–
Trypticase soy agar	+	(+)	+	+	(+)	(+)	+	+	–
Growth at 25 °C	+	+	+	–	+	+	+	+	–
Gliding motility	–	+	+	–	–	–	–	+	(+)
Flexirubin-type pigments	–	–	–	–	+	–	–	+	+
Acid from glucose	+	+	+	–	–	+	+	+	–
Congo red adsorption	–	–	–	NA	–	–	–	(v)	–
Degradation of:									
Gelatin	–	(v)	–	–	+	+	+	+	+
Casein	+	+	–	+	+	+	–	+	+
Starch	–	(v)	(v)	+	–	–	–	+	–
Carboxymethylcellulose	–	–	–	+	–	–	–	+	–
Aesculin	–	(v)	+	+	–	–	+	+	–
Tyrosine	–	(v)	–	–	–	–	–	+	v
Brown pigment on tyrosine agar	–	–	–	NA	+	–	NA	v	–
H ₂ S production	–	–	–	–	–	–	NA	–	–
β-Galactosidase activity	–	(v)	+	+	NA	–	–	+	–
Nitrate reduction	+	(v)	(v)	+	–	–	–	v	–
DNA G + C content (mol%)	34.2	33	35	35.2	41	40.8	36.9	34	33

Analysis of fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Analyses of polar lipids and isoprenoid quinones were carried out using the methods described by Komagata & Suzuki (1987). The DNA G + C content of strain EMB34^T was determined using a HPLC fitted 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 EMB34^T was menaquinone-6 (MK-6). The cellular membrane contained iso-C_{15:0} (19.7%), C_{15:0} (17.2%), iso-C_{15:1} G (16.8%), iso-C_{16:0} 3-OH (9.7%) and iso-C_{15:0} 3-OH (9.2%) as the major fatty acids. The polar lipid composition was dominated by a large amount of phosphatidylethanolamine, but a small amount of phosphatidylinositol was also present. The G + C content of the genomic DNA of strain EMB34^T was 34.2 mol%. The fatty acid composition, major lipoquinone and polar lipids and G + C content are in accordance with those of members of the genus *Flavobacterium* (Bernardet *et al.*, 2002; Van Trappen *et al.*, 2004, 2005; Aslam *et al.*, 2005; Wang *et al.*, 2006; Yoon *et al.*, 2006). Based on biochemical and phylogenetic data, it is concluded that strain EMB34^T represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium filum* sp. nov. is proposed.

Description of *Flavobacterium filum* sp. nov.

Flavobacterium filum [fi'lum. L. neut. n. *filum* (nominative in apposition), a thread, a *Flavobacterium* species called the thread].

Cells are Gram-negative straight rods, 0.4–0.7 µm in width and 1.3–1.8 µm in length, linked by extracellular fibrils and devoid of flagellar and gliding motility. Colonies on R2A agar are pale-yellow, glistening, translucent, sticky, slightly raised and circular with entire margins. Growth occurs at 10–40 °C (optimum, 25–35 °C) and at pH 6.0–9.5 (optimum, 7.5–8.0). Nitrate is reduced to nitrite and nitrogen is produced. Oxidase-negative and catalase-positive. Grows at 30 °C on R2A agar, Luria–Bertani agar and trypticase soy agar (Difco), but not on nutrient agar (Difco) at 30 °C. Grows optimally on R2A agar without addition of NaCl; growth is severely inhibited on R2A agar containing more than 2.0% (w/v) NaCl. No anaerobic growth after 7 days on R2A agar, but weak growth occurs after 16 days, suggesting that growth may occur by anaerobic respiration using nitrate or nitrite as an electron acceptor. Casein is hydrolysed. Gelatin, Tween 80, Tween 20, aesculin, urea, tyrosine, starch and carboxymethylcellulose are not hydrolysed. Congo red is not absorbed by colonies and flexirubin-type pigments are not produced. Indole, H₂S and acetoin are not produced and citrate is not

utilized (API 20E kit). Acid is produced from raffinose, D-glucose, *myo*-inositol, lactose and melibiose, but not from D-fructose, D-galactose, D-mannose, D-mannitol, L-arabinose, arbutin or salicin. In API ZYM kits, leucine arylamidase is produced, but esterase (C4), lipase (C14), cystine arylamidase, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, acid phosphatase, tryptophan deaminase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and urease are not produced. Weak enzymic activities are observed for alkaline phosphatase, esterase lipase (C8), valine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase. Resistant to polymyxin B, oleandomycin and neomycin, but sensitive to ampicillin, streptomycin, penicillin G, chloramphenicol, tetracycline, lincomycin, carbenicillin, novobiocin, gentamicin and kanamycin. Contains a large amount of phosphatidylethanolamine and a small amount of phosphatidylinositol as the polar lipids. The major isoprenoid quinone is MK-6. The cellular fatty acids are iso-C_{15:0} (19.7%), C_{15:0} (17.2%), iso-C_{15:1} G (16.8%), iso-C_{16:0} 3-OH (9.7%), iso-C_{15:0} 3-OH (9.2%), iso-C_{16:0} (5.8%), anteiso-C_{15:0} (3.7%), iso-C_{14:0} (3.5%), iso-C_{17:0} 3-OH (3.5%), C_{15:0} 3-OH (1.9%), C_{15:1} ω 6c (1.8%), iso-C_{16:1} H (1.5%), iso-C_{14:0} 3-OH (1.5%), C_{16:0} (1.5%), C_{16:0} 3-OH (1.2%), C_{14:0} (1.0%) and iso-C_{13:0} (0.7%). The G+C content of the DNA of the type strain is 34.2 mol% (HPLC).

The type strain is EMB34^T (=KCTC 12610^T=DSM 17961^T), which was isolated in Korea from a wastewater treatment plant that performed enhanced biological phosphorus removal.

Acknowledgements

This study was supported by grants from MOST/KOSEF to the Environmental Biotechnology National Core Research Center (grant no. R15-2003-012-02002-0) and to the 21C Frontier Microbial Genomics and Application Center Program (grant no. MG05-0104-4-0), Ministry of Science and Technology, Korea. S.H.R. and M.P. were supported by scholarships from the BK21 program of the Ministry of Education and Human Resources Development in Korea.

References

- Aslam, Z., Im, W. T., Kim, M. K. & Lee, S. T. (2005). *Flavobacterium granuli* sp. nov., isolated from granules used in a wastewater treatment plant. *Int J Syst Evol Microbiol* **55**, 747–751.
- Bergey, D. H., Harrison, F. C., Breed, R. S., Hammer, B. W. & Huntoon, F. M. (1923). *Bergey's Manual of Determinative Bacteriology*, 1st edn. Baltimore, MD: Williams & Wilkins.
- Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996). Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatidis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.
- Bernardet, J. F., Nakagawa, Y. & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family

Flavobacteriaceae and emended description of the family. *Int J Syst Evol Microbiol* **52**, 1049–1070.

Cousin, S., Pauker, O. & Stackebrandt, E. (2007). *Flavobacterium aquidurens* sp. nov. and *Flavobacterium hercynium* sp. nov., from a hard-water creek. *Int J Syst Evol Microbiol* **57**, 243–249.

Felsenstein, J. (2002). PHYLIP (phylogeny inference package), version 3.6a. Department of Genome Sciences, University of Washington, Seattle, USA.

Gerhardt, P., Murray, R. G. M., Wood, W. A. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt. Washington DC: American Society for Microbiology.

Gomori, G. (1955). Preparation of buffers for use in enzyme studies. *Methods Enzymol* **1**, 138–146.

Humphry, D. R., George, A., Black, G. W. & Cummings, S. P. (2001). *Flavobacterium frigidarium* sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. *Int J Syst Evol Microbiol* **51**, 1235–1243.

Jeon, C. O., Lim, J. M., Lee, J. M., Xu, L. H., Jiang, C. L. & Kim, C. J. (2005). Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* **55**, 1891–1896.

Kämpfer, P., Young, C. C., Sridhar, K. R., Arun, A. B., Lai, W. A., Shen, F. T. & Rekha, P. D. (2006). Transfer of [*Flexibacter*] *sancti*, [*Flexibacter*] *filiformis*, [*Flexibacter*] *japonensis* and [*Cytophaga*] *arvensicola* to the genus *Chitinophaga* and description of *Chitinophaga skermanii* sp. nov. *Int J Syst Evol Microbiol* **56**, 2223–2228.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester, UK: John Wiley and Sons.

Lanyi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.

Leifson, E. (1963). Determination of carbohydrate metabolism of marine bacteria. *J Bacteriol* **85**, 1183–1184.

McCammon, S. A. & Bowman, J. P. (2000). Taxonomy of Antarctic *Flavobacterium* species: description of *Flavobacterium gillisiae* sp. nov., *Flavobacterium tegetincola* sp. nov., and *Flavobacterium xanthum* sp. nov., nom. rev. and reclassification of [*Flavobacterium*] *salegens* as *Salegentibacter salegens* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **50**, 1055–1063.

Park, M., Lu, S., Ryu, S. H., Chung, B. S., Park, W., Kim, C. J. & Jeon, C. O. (2006). *Flavobacterium croceum* sp. nov., isolated from activated sludge. *Int J Syst Evol Microbiol* **56**, 2443–2447.

Park, M., Ryu, S. H., Vu, T. H., Ro, H. S., Yun, P. Y. & Jeon, C. O. (2007). *Flavobacterium defluvii* sp. nov., isolated from activated sludge. *Int J Syst Evol Microbiol* **57**, 233–237.

Tamaki, H., Hanada, S., Kamagata, Y., Nakamura, K., Nomura, N., Nakano, K. & Matsumura, M. (2003). *Flavobacterium limicola* sp. nov., a psychrophilic, organic-polymer-degrading bacterium isolated from freshwater sediments. *Int J Syst Evol Microbiol* **53**, 519–526.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Van Trappen, S., Vandecandelaere, I., Mergaert, J. & Swings, J. (2004). *Flavobacterium degerlachei* sp. nov., *Flavobacterium frigoris* sp. nov. and *Flavobacterium micromati* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. *Int J Syst Evol Microbiol* **54**, 85–92.

Van Trappen, S., Vandecandelaere, I., Mergaert, J. & Swings, J. (2005). *Flavobacterium fryxellicola* sp. nov. and *Flavobacterium psychrolimnae* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. *Int J Syst Evol Microbiol* **55**, 769–772.

Wang, Z.-W., Liu, Y.-H., Dai, X., Wang, B.-J., Jiang, C.-Y. & Liu, S.-J. (2006). *Flavobacterium saliperosum* sp. nov., isolated from freshwater lake sediment. *Int J Syst Evol Microbiol* **56**, 439–442.

Yoon, J.-H., Kang, S.-J. & Oh, T.-K. (2006). *Flavobacterium soli* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **56**, 997–1000.

Zhang, D. C., Wang, H. X., Liu, H. C., Dong, X. Z. & Zhou, P. J. (2006). *Flavobacterium glaciei* sp. nov., a novel psychrophilic bacterium isolated from the China No.1 glacier. *Int J Syst Evol Microbiol* **56**, 2921–2925.

Zhu, F., Wang, S. & Zhou, P. (2003). *Flavobacterium xinjiangense* sp. nov. and *Flavobacterium omnivorum* sp. nov., novel psychrophiles from the China No. 1 glacier. *Int J Syst Evol Microbiol* **53**, 853–857.