Flavobacterium defluvii sp. nov., isolated from activated sludge

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A Gram-negative bacterium, designated strain EMB117^T, was isolated from a municipal wastewater treatment plant and characterized by polyphasic taxonomy. The cells were non-spore-forming rods that showed gliding motility. Optimal growth occurred at 25–30 °C and pH 7.0–8.0. Strain EMB117^T contained phosphatidylethanolamine as the predominant polar lipid, and the major fatty acids were iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{15:0} 3-OH and summed feature 3 (C_{16:1} ω7c and/or iso-C_{15:0} 2-OH). The G + C content of the genomic DNA was 33.5 mol% and the major isoprenoid quinone was MK-6. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain EMB117^T belonged to the genus *Flavobacterium* and was most closely related to *Flavobacterium johnsoniae* DSM 425^T (97.8 % sequence similarity). The DNA–DNA relatedness between strain EMB117^T and *F. johnsoniae* ATCC 17061^T was about 18 %. On the basis of the phenotypic, chemotaxonomic and molecular data, strain EMB117^T represents a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium defluvii* sp. nov. is proposed. The type strain is EMB117^T (=KCTC 12612^T = DSM 17963^T).

Since the description of the genus *Flavobacterium* was emended by Bernardet *et al.* (1996), many additional *Flavobacterium* species have been described from diverse environmental habitats, such as microbial mats from Antarctic lakes, freshwater, seawater, sea ice, soil, the gut of an earthworm, sediments and wastewater treatment plants (McCammon & Bowman, 2000; Van Trappen *et al.*, 2002; Zhu *et al.*, 2003; Horn *et al.*, 2005; Kim *et al.*, 2006; Yi & Chun, 2006). Their physiological characteristics 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). Hence, the genus *Flavobacterium* may have diverse environmental functions. Recently, a novel Gram-negative bacterium, designated strain EMB117^T, was isolated from activated sludge from a domestic wastewater treatment plant. On the basis of phenotypic and phylogenetic investigations,

EMB117^T represents a novel species in the genus *Flavobacterium*.

Strain EMB117^T was isolated from activated sludge from a domestic wastewater treatment plant in Pohang, Korea. The sludge sample was diluted serially with 1 % (w/v) saline solution and spread on R2A (Difco) agar at 20 °C for 2 days. Subculturing was done using R2A agar at 30 °C for 1 day.

Sequencing of the 16S rRNA gene was carried out as described previously (Lane, 1991). The resulting 16S rRNA gene sequence (1436 nt) of strain EMB117^T was compared with 16S available rRNA gene sequences (from GenBank), using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) to determine an approximate phylogenetic affiliation; gene sequences were aligned with those of closely related species using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in PHYLIP software, version 3.6 (Felsenstein, 2002). The values for sequence similarity between the novel strain and related members of the genus *Flavobacterium* were computed using SIMILARITY MATRIX, version 1.1 (Ribosomal Database Project II; <http://35.8.164.52/html/>; Cole *et al.*, 2003). A bootstrap analysis

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EMB117^T is DQ372986.

A transmission electron micrograph of cells of strain EMB117^T is available as a supplementary figure in IJSEM Online.

was performed according to the Kimura two-parameter model (Kimura, 1980) of the neighbour-joining method in the PHYLIP package. The phylogenetic analysis based on 16S rRNA gene sequences indicated that strain EMB117^T formed a phyletic lineage with *Flavobacterium johnsoniae* DSM 425^T with a relatively low bootstrap value (62.0 %) (Fig. 1). The overall tree topology of the neighbour-joining tree was supported by the maximum-likelihood and maximum-parsimony trees (data not shown). Comparative 16S rRNA gene sequence analyses showed that the isolate was most closely related to *F. johnsoniae* DSM 425^T, with a 16S rRNA gene sequence similarity of 97.8 %; the sequence similarities with respect to other *Flavobacterium* species were less than 97.0 %.

DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness between strain EMB117^T and *F. johnsoniae* ATCC 17061^T. Extracted genomic DNAs were fragmented with *Hae*III for slot hybridization (Lim *et al.*, 2005). Digested DNAs were diluted serially and loaded into slots, with three replications, and each DNA was used individually as a labelled DNA probe for cross-hybridization. Random primed DNA labelling with digoxigenin-dUTP and hybridization (hybridization at 40 °C; washing at 60 °C) were performed using the DIG High Prime DNA labelling kit (Roche Applied Science) according to the manufacturer's instructions and standard procedures (Sambrook & Russell, 2001; Lim *et al.*, 2005). Signals from the series of dilutions were quantified using GelDoc scanning software (Bio-Rad). The signals produced by self-hybridization were inferred as representing 100 %, and relatedness values (percentages) were calculated from triplicate samples. The DNA–DNA relatedness between

strain EMB117^T and *F. johnsoniae* ATCC 17061^T was about 18 %, clearly below the 70 % threshold generally accepted for species delineation (Stackebrandt *et al.*, 2002).

The DNA G+C content of strain EMB117^T, determined using HPLC apparatus fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; Grom) according to the method of Tamaoka & Komagata (1984), was found to be 33.5 mol%.

Analysis of the 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 cellular membrane contained iso-C_{15:0} (19.8 %), iso-C_{17:0} 3-OH (13.1 %), iso-C_{15:0} 3-OH (11.2 %), iso-C_{15:1} G (7.6 %), C_{15:0} (6.4 %) and summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH, 10.1 %) as the major fatty acids. The polar lipid composition was dominated by phosphatidylethanolamine. The major respiratory lipoquinone of strain EMB117^T was MK-6. The fatty acid composition, the major lipoquinone and polar lipid and the DNA G+C content of strain EMB117^T 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).

Gram staining was performed using a bioMérieux Gram-stain kit according to the manufacturer's instructions. Cell morphology, flagellation 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.*, 2005). The physiological characteristics of strain EMB117^T were examined by growing the isolate in R2A broth at various temperatures and pH values. R2A media with different pH values were prepared as described previously (Gomori, 1955). Salt tolerance was tested using R2A broth supplemented with 0–3 % (w/v) NaCl for 5 days at 30 °C. Duplicate antibiotic tests were performed using the diffusion method on R2A agar at 30 °C with filter-paper discs (8 mm diameter) 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). The diameters of inhibition zones were measured after 2 days. Oxidase activity was tested by determining the 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 extracellular glycans was investigated using the KOH and the Congo red tests, respectively, according to the minimal standards for the description of novel taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). The hydrolysis of casein, gelatin, Tweens 80 and 20, aesculin, urea, tyrosine,

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain EMB117^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates, when greater than 50 %. *Capnocytophaga ochracea* ATCC 33596^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

starch and carboxymethylcellulose was investigated on R2A agar after 7 days incubation, according to methods described previously (Lanyi, 1987; Gerhardt *et al.*, 1994). Nitrate reduction was determined 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 (bioMérieux) at 30 °C. Growth under anaerobic conditions was determined in an anaerobic chamber (H₂/CO₂/N₂, 5:10:85) at 30 °C on R2A agar.

Anaerobic growth was not observed after 7 days at 30 °C on R2A agar, but the strain showed weak growth after 16 days. As strain EMB117^T did not reduce nitrate to nitrite, it was unable to grow by anaerobic respiration using nitrate or nitrite as an electron acceptor, as reported for *Flavobacterium denitrificans* (Horn *et al.*, 2005) and some *F. johnsoniae* strains (Stanier, 1947). Consequently, strain EMB117^T probably grew anaerobically by fermenting carbohydrates, as reported for *Flavobacterium hydati* (Strohl & Tait, 1978) and *Flavobacterium succinicans* (Anderson & Ordal, 1961).

The phenotypic features of strain EMB117^T are presented in the species description. Strain EMB117^T showed the characteristics typical of members of the genus *Flavobacterium* (Bernardet *et al.*, 2002) but could be differentiated from related *Flavobacterium* species by means of a number of traits, which are listed in Table 1. The physiological, biochemical and phylogenetic data support the description of strain EMB117^T as the type strain of a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium defluvii* sp. nov. is proposed.

Description of *Flavobacterium defluvii* sp. nov.

Flavobacterium defluvii (de.flu'vi.i. L. gen. n. *defluvii* of sewage).

Cells are Gram-negative, non-spore-forming rods, 0.4–0.5 µm wide and 2.3–6.5 µm long. Some cells show a knar (see Supplementary Fig. S1 available in IJSEM Online). Cells are devoid of flagella, but gliding motility is observed. Colonies on R2A agar are pale yellow, glistening, translucent, slightly sticky, irregular, slightly raised and have curled margins. Grows at 10–40 °C (optimum, 25–30 °C) and at pH 5.0–10.0 (optimum, pH 7.0–8.0). Grows optimally in plain R2A broth (no added NaCl) and in R2A broth to which 1 % (w/v) NaCl has been added. Growth in R2A broth is severely inhibited by the addition of more than 2 % (w/v) NaCl. Grows at 30 °C on R2A agar, Luria–Bertani agar, tryptic soy agar (Difco) and nutrient agar (Difco). Grows well under aerobic conditions. Growth is weak and delayed under anaerobic conditions. Catalase-positive and oxidase-negative. Nitrate is not reduced to nitrite. Casein, Tween 20, urea, aesculin, gelatin and carboxymethylcellulose are hydrolysed. Tween 80, tyrosine and starch are not hydrolysed. Congo red is not adsorbed by colonies, and

Table 1. Differential characteristics of strain EMB117^T and related *Flavobacterium* species

Taxa: 1, strain EMB117^T; 2, *F. johnsoniae* (data from Bernardet *et al.*, 1996); 3, *F. denitrificans* (Horn *et al.*, 2005); 4, *F. soli* (Yoon *et al.*, 2006); 5, *F. daejeonense* (Kim *et al.*, 2006); 6, *F. aquatile* (Bernardet *et al.*, 1996). Symbols: +, positive; –, negative; d, variable among strains; v, variable among references according to Bernardet *et al.* (1996); (+), weakly positive; NA, no data available.

Characteristic	1	2	3	4	5	6
Facultative anaerobe	(+)	d	+	–	–	–
Growth on nutrient agar	+	+	+	+	+	–
Gliding motility	+	+	NA	–	+	+
Flexirubin-type pigments	+	+	+	–	–	–
Acid from carbohydrate	+	+	–	–	+	+
Congo red adsorption	–	v	NA	–	–	–
Degradation of:						
Gelatin	+	+	NA	+	–	v
Casein	+	+	NA	–	–	+
Starch	–	+	NA	–	+	v
Carboxymethylcellulose	+	+	NA	–	–	–
Aesculin	+	+	NA	–	+	v
Tyrosine	+	+	NA	–	–	v
Brown pigment on tyrosine agar	–	d	NA	NA	NA	–
β-Galactosidase activity	(+)	+	NA	–	+	v
Nitrate reduction	–	d	+	–	+	v
DNA G + C content (mol%)	33.5	35.2	35	36.9	35	33

flexirubin-type pigments are produced. Negative for indole, H₂S and acetoin production and for citrate utilization (API 20E). Produces acid from raffinose, D-glucose, *myo*-inositol, lactose, L-arabinose, melibiose, D-fructose, D-galactose, D-mannose, D-mannitol and arbutin, but not from salicin. Produces alkaline phosphatase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase in API ZYM kits, but does not produce esterase (C4), lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-mannosidase or α-fucosidase. Weak enzymic activities are observed for esterase lipase (C8), trypsin, acid phosphatase and β-galactosidase. Resistant to polymyxin B, ampicillin, gentamicin, kanamycin, lincomycin, oleandomycin, neomycin, carbenicillin and novobiocin. Sensitive to streptomycin, penicillin G, chloramphenicol and tetracycline. Phosphatidylethanolamine is the predominant polar lipid. The major isoprenoid quinone is MK-6. The cellular fatty acids are iso-C_{15:0} (19.8 %), iso-C_{17:0} 3-OH (13.1 %), iso-C_{15:0} 3-OH (11.2 %), iso-C_{15:1} G (7.6 %), C_{15:0} (6.4 %), iso-C_{16:0} 3-OH (5.6 %), C_{16:0} 3-OH (4.5 %), anteiso-C_{15:0} (4.4 %), C_{17:1}ω6c (2.4 %), iso-C_{16:0} (2.3 %), iso-C_{17:0}ω9c (2.3 %), C_{15:0} 3-OH (1.9 %), C_{16:0} (1.9 %), C_{17:0} 3-OH (1.0 %), summed feature 2 (iso-C_{16:1} I and/or C_{14:0} 3-OH, 1.2 %), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 10.1 %) and traces (<1 %) of C_{15:1}ω6c, iso-C_{14:0} and C_{17:1}ω8c. The DNA G + C content is 33.5 mol% (HPLC).

The type strain, EMB117^T (=KCTC 12612^T=DSM 17963^T), was isolated from sludge that performed enhanced biological phosphorus removal in a laboratory-scale sequencing batch reactor.

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