Flavobacterium croceum sp. nov., isolated from activated sludge

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A Gram-negative, non-motile, rod-shaped bacterium, designated strain EMB47^T, was isolated from activated sludge performing enhanced biological phosphorus removal in a sequencing batch reactor. Growth was observed between 10 and 40 °C (optimum, 25–35 °C) and between pH 5.0 and 8.5 (optimum, pH 7.5–8.0). The predominant fatty acids of strain EMB47^T were iso-C_{16:0} 3-OH, iso-C_{15:1} G, C_{15:0}, iso-C_{15:0}, iso-C_{14:0} and iso-C_{16:0} and it contained phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylcholine as polar lipids. The G + C content of the genomic DNA was 40.8 mol% and the major quinone was MK-6. Comparative 16S rRNA gene sequence analyses showed that strain EMB47^T formed a distinct phyletic line within the genus *Flavobacterium*. The levels of 16S rRNA gene sequence similarity with respect to *Flavobacterium* species were below 94.7 %. On the basis of the phenotypic, chemotaxonomic and molecular data, strain EMB47^T represents a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium croceum* sp. nov. is proposed. The type strain is EMB47^T (= KCTC 12611^T = DSM 17960^T).

Since the genus *Flavobacterium*, belonging to the phylum *Bacteroidetes* (formerly the *Cytophaga-Flavobacterium-Bacteroides* group), was emended (Bernardet *et al.*, 1996), several novel *Flavobacterium* species have been described: these have been isolated from diverse habitats such as micromats, fresh water, seawater, Antarctic lakes, soil, the gut of an earthworm and from sediments (McCammon & Bowman, 2000; Van Trappen *et al.*, 2002, 2004; Zhu *et al.*, 2003; Horn *et al.*, 2005). The physiological characteristics of members of the genus *Flavobacterium* are also 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). These findings suggest that the genus *Flavobacterium* may have important environmental roles. Activated-sludge processes with cyclic changes of anaerobic and aerobic conditions have been used to remove phosphate from wastewater and are increasingly used to reduce the eutrophication process in lakes (Mino

et al., 1987; Jeon & Park, 2000). An insight into the bacterial community responsible for phosphorus removal is a prerequisite for understanding and controlling the enhanced biological phosphorus removal mechanism and its processes. Therefore, efforts have been made in our laboratory to isolate and characterize members of the bacterial community found in activated sludge that perform enhanced biological phosphorus removal. Here we describe the taxonomic characterization of a novel species belonging to the genus *Flavobacterium*.

Strain EMB47^T was isolated from activated sludge that was performing enhanced biological phosphorus removal in a laboratory-scale sequencing batch reactor. Sodium acetate was supplied as a sole carbon source, and the operation of the sequencing batch reactor was as described elsewhere (Jeon *et al.*, 2003). The sludge sample was diluted serially with 1 % (w/v) saline solution and spread on R2A agar (Difco) maintained at 20 °C for 5 days. Subcultivation was performed on R2A agar maintained at 30 °C for 3 days.

Sequencing of the 16S rRNA gene was carried out as described previously (Lane, 1991). The resulting 16S rRNA gene sequence (1439 nt) of strain EMB47^T was compared with 16S rRNA gene sequences available from GenBank,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EMB47^T is DQ372982.

A transmission electron micrograph showing the general morphology of strain EMB47^T is available as a supplementary figure in IJSEM Online.

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 by using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using three different methods, namely the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in PHYLIP, version 3.6 (Felsenstein, 2002). Values for sequence similarity between the novel strain and other 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 was performed according to the algorithm of the Kimura two-parameter model (Kimura, 1980) of the neighbour-joining method in the PHYLIP software package. A phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain EMB47^T formed a phyletic lineage with *Flavobacterium columnare* IAM 14301^T and *Flavobacterium saliperosum* AS 1.3801^T within the genus *Flavobacterium*, supported by a high bootstrap value (99.0%) (Fig. 1). The overall topology of the trees constructed using the maximum-likelihood and maximum-parsimony algorithms (data not shown). Comparative 16S rRNA gene sequence analyses showed that the novel isolate was most closely related to *Flavobacterium aquatile* ATCC 11947^T, *F. saliperosum* AS 1.3801^T and *F. columnare* IAM 14301^T, with similarities of 94.2, 93.9 and 92.6%, respectively.

Gram staining was performed using a bioMérieux Gram stain kit according to the manufacturer's instructions. Cell

morphology, 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.*, 2005). The physiological characteristics of strain EMB47^T were examined by growing the isolate on R2A medium at different temperatures and pH values. R2A media with different pH values were prepared as described previously (Gomori, 1955). Salt tolerance was tested on R2A media supplemented with 0–3% (w/v) NaCl for 5 days at 30 °C. Duplicate antibiotic-susceptibility tests were performed using filter-paper discs (diameter, 8 mm) containing 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) or novobiocin (50 µg). Oxidase activity was tested by means of the oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck). Catalase activity was evaluated by monitoring the production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution. The production of flexirubin-type pigments and extracellular glycans was investigated using the KOH and Congo red tests, respectively, following the minimal standards for the *Flavobacteriaceae* (Bernardet *et al.*, 2002). The hydrolysis of casein, gelatin, hypoxanthine, Tweens 80 and 20, aesculin, urea, tyrosine, starch, carboxymethylcellulose and xanthine was investigated after 7 days incubation on R2A agar, according to previously described methods (Lanyi, 1987; Gerhardt *et al.*, 1994). Nitrate reduction was determined according to the method of Lanyi (1987), and acid production from carbohydrates was tested for as described by Leifson (1963). Additional

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain EMB47^T and related taxa. Bootstrap values are expressed as percentages of 1000 replicates; only values greater than 50% are shown. *Capnocytophaga ochracea* ATCC 33596^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

enzymic activities and biochemical features were determined using API ZYM and API 20E kits at 30 °C as recommended by the manufacturer (bioMérieux).

Growth of strain EMB47^T was observed at temperatures between 10 and 45 °C, the optimum being at 25–35 °C. The strain grew at pH values in the range 5.5–8.5, the optimum being pH 7.5–8.0. The cells were Gram-negative, straight or slightly curved rods 0.3–0.5 µm in width and 1.0–3.2 µm in length (see Supplementary Fig. S1 available in IJSEM Online). The isolate grew optimally on R2A medium without the addition of NaCl, and growth was severely inhibited on R2A medium containing more than 1 % (w/v) NaCl. Other phenotypic features of strain EMB47^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 EMB47^T from closely related *Flavobacterium* species (Table 1).

Analysis of the fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Analyses of the polar lipids and isoprenoid quinones were carried out using the methods described by Komagata & Suzuki (1987). The

DNA G + C content of strain EMB47^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 EMB47^T was MK-6. The cellular membrane of the strain contained iso-C_{16:0} 3-OH (16.5 %), iso-C_{15:1} G (11.5 %), C_{15:0} (10.8 %), iso-C_{15:0} (9.3 %), iso-C_{14:0} (8.7 %) and iso-C_{16:0} (8.5 %) as the major fatty acids, a profile resembling those determined for *Flavobacterium* species (Bernardet *et al.*, 2002; Wang *et al.*, 2006; Yoon *et al.*, 2006). The predominant polar lipid of strain EMB47^T was phosphatidylethanolamine, and small amounts of phosphatidylglycerol and phosphatidylcholine were also present. The G + C content of the genomic DNA of strain EMB47^T was 40.8 mol%. The fatty acid composition, major lipoquinone, major polar lipid 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). Therefore, the physiological, biochemical and phylogenetic data for strain EMB47^T support its description as a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium croceum* sp. nov. is proposed.

Description of *Flavobacterium croceum* sp. nov.

Flavobacterium croceum (cro'ce.um. L. neut. adj. *croceum* yellow).

Cells form yellow, slightly raised, circular colonies with entire margins on R2A agar. Growth occurs optimally at 25–35 °C and pH 7.5–8.0. Cells are Gram-negative, non-motile rods 0.3–0.5 µm wide and 1.0–3.2 µm long at 30 °C on R2A agar. Nitrate is not reduced to nitrite. Anaerobic growth is not observed at 7 days at 30 °C on R2A agar, but weak growth occurs after 16 days. Catalase-negative and oxidase-positive. NaCl concentrations above 1 % severely inhibit growth. Casein and gelatin are hydrolysed. Hypoxanthine, Tweens 80 and 20, aesculin, urea, tyrosine, starch, carboxymethylcellulose and xanthine are not hydrolysed. Congo red is not absorbed by colonies and flexirubin-type pigments are not produced. Negative for indole, H₂S and acetoin production and for citrate utilization (API 20E kit). Produces acid from raffinose, D-glucose, *myo*-inositol, lactose, L-arabinose and melibiose, but not from D-fructose, D-galactose, D-mannose, D-mannitol, arbutin or salicin. Produces alkaline phosphatase and leucine arylamidase, but not esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase or urease. Weak enzymic activities are observed for valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and tryptophan deaminase. Strain EMB47^T is resistant to polymyxin B, gentamicin, kanamycin, oleandomycin and neomycin and is sensitive to ampicillin, streptomycin, penicillin G, chloramphenicol, tetracycline, lincomycin,

Table 1. Characteristics of strain EMB47^T and related *Flavobacterium* species

Taxa: 1, strain EMB47^T; 2, *F. aquatile* (Bernardet *et al.*, 1996); 3, *F. columnare* (Tamaki *et al.*, 2003); 4, *F. saliperosum* AS 1.3801^T (Wang *et al.*, 2006); 5, *Flavobacterium psychrophilum* (Bernardet *et al.*, 1996; Tamaki *et al.*, 2003). +, Positive; –, negative; v, variable among strains; V, variable among references; (+), weakly positive; NA, not available. All strains could degrade casein but not carboxymethylcellulose.

Characteristic	1	2	3	4	5
Growth on:					
Nutrient agar	–	–	–	+	–
Trypticase soy agar	(+)	(+)	–	(+)	–
Growth at 25 °C	+	+	+	+	–
Gliding motility	–	+	+	–	(+)
Flexirubin-type pigments	–	–	+	+	+
Acid from glucose	+	+	–	–	–
Congo red adsorption	–	–	+	–	–
Degradation of:					
Gelatin	+	V	+	+	+
Starch	–	V	–	–	–
Aesculin	–	V	–	–	–
Tyrosine	–	V	–	–	v
Brown pigment on tyrosine agar	–	–	v	+	–
H ₂ S production	–	–	+	–	–
β -Galactosidase activity	–	V	–	NA	–
Nitrate reduction	–	V	V	–	–
DNA G + C content (mol%)	40.8	33	32	41	33

carbenicillin and novobiocin. The strain contains a large amount of phosphatidylethanolamine and small amounts of phosphatidylglycerol and phosphatidylcholine as the polar lipids. The major isoprenoid quinone is MK-6. The cellular fatty acids are iso-C_{16:0} 3-OH (16.5%), iso-C_{15:1} G (11.5%), C_{15:0} (10.8%), iso-C_{15:0} (9.3%), iso-C_{14:0} (8.7%), iso-C_{16:0} (8.5%), iso-C_{15:0} 3-OH (5.9%), ante-iso-C_{15:0} (5.1%), iso-C_{16:1} G (2.9%), iso-C_{17:0} 3-OH (2.7%), iso-C_{14:0} 3-OH (2.6%), C_{15:0} 2-OH (2.0%), anteiso-C_{15:1} A (1.6%), C_{15:0} 3-OH (1.3%), C_{16:0} (1.0%), iso-C_{13:0} (1.0%), C_{16:0} 3-OH (0.6%), iso-C_{12:0} (0.5%), anteiso-C_{13:0} (0.5%), C_{17:0} 3-OH (0.5%) and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, 3.6%). DNA G+C content is 40.8 mol% (HPLC).

The type strain, EMB47^T (= KCTC 12611^T = DSM 17960^T), was isolated from sludge that performed enhanced biological phosphorus removal in a laboratory-scale sequencing batch reactor.

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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.
- 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.
- Cole, J. R., Chai, B., Marsh, T. L. & 8 other authors (2003). The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**, 442–443.
- Felsenstein, J. (2002). PHYLIP (phylogeny inference package), version 3.6a. Distributed by the author. 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.
- Horn, M. A., Ihssen, J., Matthies, C., Schramm, A., Acker, G. & Drake, H. L. (2005). *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72, N₂O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*. *Int J Syst Evol Microbiol* **55**, 1255–1265.
- 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. & Park, J. M. (2000). Enhanced biological phosphorus removal in a sequencing batch reactor supplied with glucose as a sole carbon source. *Water Res* **34**, 3470–3480.
- Jeon, C. O., Lee, D. S. & Park, J. M. (2003). Microbial communities in activated sludge performing enhanced biological phosphorus removal in a sequencing batch reactor. *Water Res* **37**, 2195–2205.
- 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**, 743–746.
- 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**, 161–208.
- 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: Wiley.
- 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 *Salagentibacter salegens* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **50**, 1055–1063.
- Mino, T., Arun, V., Suzuki, Y. T. & Matsuo, T. (1987). Effect of phosphorus accumulation on acetate metabolism in the biological phosphorus removal process. In *Biological Phosphate Removal from Wastewaters*, pp. 27–38. Edited by R. Ramadori. Oxford, UK: Pergamon Press.
- 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 reverse-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., Mergaert, J., Van Eygen, S., Dawyndt, P., Cnockaert, M. C. & Swings, J. (2002). Diversity of 746 heterotrophic bacteria isolated from microbial mats from ten Antarctic lakes. *Syst Appl Microbiol* **25**, 603–610.
- Van Trappen, S., Vandecastelaere, 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.

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.