**Flavobacterium croceum** sp. nov., isolated from activated sludge

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A Gram-negative, non-motile, rod-shaped bacterium, designated strain EMB47ᵀ, 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 EMB47ᵀ were iso-C₁₆:0 3-OH, iso-C₁₅:0 3-OH, C₁₅:0 3-OH, iso-C₁₅:0 3-OH, iso-C₁₄:0 and iso-C₁₆:0 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 EMB47ᵀ formed a distinct phylogenetic 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 EMB47ᵀ represents a novel species within the genus **Flavobacterium**, for which the name *Flavobacterium croceum* sp. nov. is proposed. The type strain is EMB47ᵀ (= KCTC 12611ᵀ = DSM 17960ᵀ).

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 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 EMB47ᵀ 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 30 °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 EMB47ᵀ was compared with 16S rRNA gene sequences available from GenBank.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EMB47ᵀ is DQ372982.

A transmission electron micrograph showing the general morphology of strain EMB47ᵀ 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 neighbour-joining tree was supported by those 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), carbencillin (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

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**Capnocytophaga ochracea** ATCC 33596\(^T\) (L14635)

**Flavobacterium psychrophilum** ATCC 49418\(^T\) (AF109099)

**Flavobacterium denitrificans** ED\(^T\) (AJ318907)

**Flavobacterium soli** DS-6\(^T\) (DQ178976)

**Flavobacterium flavescens** ATCC 27944\(^T\) (MS8767)

**Flavobacterium gelidilacus** LMG 21477\(^T\) (AJ440996)

**Flavobacterium jeotgerincola** ACAM 602\(^T\) (U85887)

**Flavobacterium antarcticum** AT1026\(^T\) (AY581113)

**Flavobacterium branchophilum** IFO 15030\(^T\) (D14017)

**Flavobacterium hydatis** ATCC 29551\(^T\) (MS8764)

**Flavobacterium hibernum** ATCC 51468\(^T\) (L39067)

**Flavobacterium pectinovorum** DSM 6368\(^T\) (AM230490)

**Flavobacterium microaerum** LMG 21919\(^T\) (AJ557888)

**Flavobacterium xanthum** ACAM 81\(^T\) (AF030380)

**Flavobacterium omnivorum** JCM 11131\(^T\) (AF433174)

**Flavobacterium gililliseconds** ACAM 601\(^T\) (L85889)

**Flavobacterium sinjiense** JCM 11314\(^T\) (AF433173)

**Flavobacterium aquatile** ATCC 11947\(^T\) (M62797)

**Flavobacterium columnare** IAM 14301\(^T\) (AB010951)

**Flavobacterium saliperosum** AS 1.3801\(^T\) (DQ021903)

**Strain EMB47\(^T\)** (DQ372982)

**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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{T} was MK-6. The cellular membrane of the strain contained iso-C\textsubscript{16:0} 3-OH (16:5%); iso-C\textsubscript{15:1} G (11:5%); C\textsubscript{15:0} (10:8%); iso-C\textsubscript{15:0} (9:3%); iso-C\textsubscript{14:0} (8:7%) and iso-C\textsubscript{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\textsuperscript{T} was phosphatidylethanolamine, and small amounts of phosphatidyglycerol and phosphatidylcholine were also present. The G+C content of the genomic DNA of strain EMB47\textsuperscript{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\textsuperscript{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, H\textsubscript{2}S 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 (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 trypthphan deaminase. Strain EMB47\textsuperscript{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\textsuperscript{T} and related Flavobacterium species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Growth on:</td>
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<tr>
<td>Nutrient agar</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Trypticase soy agar</td>
<td>(+)</td>
<td>(+)</td>
<td>−</td>
<td>(+)</td>
<td>−</td>
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<tr>
<td>Growth at 25 °C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Gliding motility</td>
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<td>+</td>
<td>+</td>
<td>−</td>
<td>(+)</td>
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<td>Flexirubin-type pigments</td>
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<td>−</td>
<td>+</td>
<td>+</td>
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<td>Acid from glucose</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>Congo red adsorption</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>Degradation of:</td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>+</td>
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<td>Starch</td>
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<td>−</td>
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<tr>
<td>Aesculin</td>
<td>−</td>
<td>V</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Tyrosine</td>
<td>−</td>
<td>V</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Brown pigment on tyrosine agar</td>
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<td>−</td>
<td>v</td>
<td>+</td>
<td>−</td>
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<tr>
<td>H\textsubscript{2}S production</td>
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<td>−</td>
<td>−</td>
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<td>−</td>
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<tr>
<td>β-Galactosidase activity</td>
<td>−</td>
<td>V</td>
<td>−</td>
<td>NA</td>
<td>−</td>
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<tr>
<td>Nitrate reduction</td>
<td>−</td>
<td>V</td>
<td>V</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>40–8</td>
<td>33</td>
<td>32</td>
<td>41</td>
<td>33</td>
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</table>
The 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**


