Flavobacterium anatoliense sp. nov., isolated from fresh water, and emended description of Flavobacterium ceti

Murat Kacagan, Kadiyie Inan, Ali Osman Belduz and Sabriye Canakci

Karadeniz Technical University, Faculty of Sciences, Department of Biology, 61080 Trabzon, Turkey

A Gram-staining-negative, catalase- and oxidase-positive, strictly aerobic, rod-shaped bacterial strain isolated from fresh water in Trabzon, Turkey and designated MK3T was characterized by phenotypic and molecular methods in order to determine its phylogenetic position. On the basis of 16S rRNA gene sequence similarity, strain MK3T was shown to belong to the genus Flavobacterium, being most closely related to Flavobacterium ceti CECT 7184T (93.6 %). Sequence similarity with other species of the genus Flavobacterium with validly published names was less than 91.6 %. Phenotypic and chemotaxonomic data supported the affiliation of strain MK3T to the genus Flavobacterium. The only menaquinone was MK-6; the major fatty acids were iso-C15:0 (45.2%), summed feature 9 (C16:0 10-methyl and/or iso-C17:1ω8c; 20.4%) and summed feature 3 (C16:1ω7c and/or C16:1ω6c; 13.3%) and the major polar lipids were phosphatidylethanolamine, one unidentified aminophospholipid and two unidentified phospholipids. The G+C content of the genomic DNA was 38.6 mol%. The results of physiological and biochemical tests allowed strain MK3T to be distinguished phenotypically from Flavobacterium ceti CECT 7184T. Strain MK3T, therefore, represents a novel species of the genus Flavobacterium, for which the name Flavobacterium anatoliense sp. nov. is proposed. The type strain is MK3T (=LMG 26441T=NCCB 100384T). An emended description of Flavobacterium ceti is also proposed.

The genus Flavobacterium, belonging to the phylum Bacteroidetes (formerly the Cytophaga–Flavobacterium–Bacteroides group), was proposed by Bergey et al. (1923) and its description was considerably emended by Bernardet et al. (1996). Flavobacterium species have been described from diverse environmental habitats, such as microbial mats from lakes, fresh water, seawater, sea ice, soil, the gut of an earthworm, the lung and liver of a beaked whale, sediments and wastewater treatment plants (McCaman & Bowman, 2000; Van Trappen et al., 2002; Zhu et al., 2003; Horn et al., 2005; Kim et al., 2006; Yi & Chun, 2006; Yoon et al., 2006; Vela et al., 2007; Ryu et al., 2007). Physiological characteristics of Flavobacterium strains are also very diverse: they can be psychrophilic, psychrotolerant or mesophilic, and halotolerant, halophilic or sensitive to salts. They produce a variety of enzymes (Humphry et al., 2001; Tamaki et al., 2003; Aslam et al., 2005; Zhang et al., 2006). These findings suggest that these bacteria may have important roles in the uptake and degradation of organic matter in aquatic environments (Kirchman, 2002). Indeed, many species of the genus Flavobacterium are able to hydrolyse organic polymers such as complex polysaccharides (Bernardet et al., 1996).

Strain MK3T was isolated from a sample of fresh water collected from the Yomra river 204 m above sea level and 7 km inland from the coast in Trabzon, Turkey. Temperature and pH of the sample were 20 °C and pH 8.0, respectively. The water sample was filtered within 2 h of sampling through Millipore membrane filters with a pore size of 0.45 μm. The filters were deposited on Degryse 162 agar and incubated at 28 °C for 3 days (Kristjansson & Alfredsson, 1983). Degryse 162 agar contained (l

**Abbreviations:** FAME, fatty acid methyl ester; PS, phosphatidylserine. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of Flavobacterium anatoliense MK3T is JF825522.
Degryse 162 broth supplemented with 20% glycerol (v/v). Growth was tested on Degryse 162 agar, nutrient agar (NA; Merck), Luria–Bertani agar (LB; Himedia), MacConkey agar (Oxoid) and trypticase soy agar (TSA; Oxoid).

The 16S rRNA gene of strain MK3$^T$ was selectively amplified from purified genomic DNA by using the oligonucleotide primer pair UNI16S-L and UNI16S-R (Brosius et al., 1978). PCRs were performed as follows: 2 min at 95°C, 36 cycles of 94°C for 1 min, 50°C for 45 s, 72°C for 2 min and finally 10 min at 72°C. The PCR product was cloned into the pGEM-T Easy vector system and the 16S rRNA gene sequence was determined with a model 373A DNA sequencer (Applied Biosystems) by using an ABI PRISM cycle sequencing kit at Macrogen (South Korea). The results of 16S rRNA gene sequencing were analysed using the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). The 16S rRNA gene sequences of related taxa were obtained from GenBank and edited by using the BioEdit program (Hall, 1999) and multiple alignments were performed with the CLUSTAL_X program (Thompson et al., 1997). Evolutionary distances were calculated by using Kimura’s two-parameter model (Kimura, 1980), and phylogenetic analyses were performed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. Bootstrap analysis based on 1000 replicates was also conducted in order to obtain confidence levels for the branches (Felsenstein, 1985). The phylogenetic trees were constructed using the program MEG4 (Tamura et al., 2007).

The almost-complete 16S rRNA gene sequence of strain MK3$^T$ (1404 nt) was obtained and used for the phylogenetic analyses. Strain MK3$^T$ exhibited the highest level of 16S rRNA gene sequence similarity with Flavobacterium ceti CECT 7184$^T$ (93.6%). Sequence similarity was distinctly lower with the next closest relatives (i.e. 91.6% with Flavobacterium weaverense JCM 12384$^T$, Flavobacterium beibuense DSM 21788$^T$ and Flavobacterium cheniense CGMCC 1.6844$^T$). Sequence similarity with the type strain of the type species, Flavobacterium aquatile ATCC 11947$^T$, was 89.9%. The neighbour-joining phylogenetic tree (Fig. 1) revealed that strain MK3$^T$ is a member of the genus Flavobacterium, forming a cluster with Flavobacterium ceti CECT 7184$^T$ (96% bootstrap support). The topology of the maximum-parsimony tree was essentially the same (data not shown).

Cells of strain MK3$^T$ and F. ceti CECT 7184$^T$ were Gram-stained using the method of Dussault (1955) and the KOH method (Powers, 1995). Cell morphology and gliding motility were examined by using phase-contrast microscopy (Nikon Eclipse E600) on an exponentially growing liquid culture. Gliding mobility was investigated using the hanging drop technique (Bernardet et al., 2002). The morphology, size and pigmentation of colonies were observed under optimal growth conditions on Degryse 162 agar after 1 day of incubation at 28°C. Growth was tested in Degryse 162 broth at 4–45°C (at 5°C intervals) and at pH 4.0–11.0 (at 0.5 pH unit intervals). The pH of the medium was adjusted using the following biological buffers: 30 mM C$_2$H$_5$O$_2$Na (pH 4.0–5.5); 30 mM MES (pH 5.5–6.0); 30 mM K$_2$HPO$_4$/KH$_2$PO$_4$ (pH 6.5–7.5); 30 mM Tricine (pH 8.0–9.0) and 30 mM CAPS (pH 9.5–11.0). The pH of each buffer was adjusted with HCl or NaOH, the pH values were determined at room temperature, and the pH was readjusted after sterilization. Growth was scored as the OD$$_{600}$ after 3 days of incubation. Anaerobic growth was tested using a GasPak pouch (Becton Dickinson) for 7 days. Salt tolerance was tested in Degryse 162 broth supplemented with 0–10% NaCl (w/v, at 1% intervals) for 7 days at 28°C. The sensitivity of strain MK3$^T$ to antibiotics was examined on plates of Degryse 162 agar incubated at 28°C. The following standard (6 mm) antibiotic discs (Oxoid) were used: ampicillin (10 μg), gentamicin (10 μg), cefuroxime (30 μg), cefazolin (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), imipenem (10 μg), ciprofloxacin (5 μg), amikacin (30 μg), meropenem (10 μg), ceftriaxone (30 μg), piperacillin/tazobactam (100/10 μg), cefepime (30 μg), levofloxacin (5 μg), cepofurazone (75 μg) and kanamycin (30 μg). The diameters of inhibition zones were measured after 2 days.

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 (Baek et al., 2006). 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 was determined by observing clear zones surrounding colonies on skim milk agar (Atlas, 1993). Hydrolysis of starch, chitin, Tween 20, Tween 80, aesculin, tyrosine, carboxymethylcellulose and DNA was investigated according to previously described methods (Bernardet et al., 2002; Smibert & Krieg, 1994; Bowman et al., 1996; Bowman, 2000). In addition, the API 20E and Viktek Gram Negative Identification Card (GNI) microtest systems (bioMérieux) were used at 28°C for 18–24 h according to the manufacturer’s instructions. Sporulation was tested on solid maintenance medium (Smibert & Krieg, 1994).

Strain MK3$^T$ grew optimally on Degryse 162 agar at 28°C, yielding dark yellow, convex and circular colonies. Cells were strictly aerobic, Gram-staining-negative, non-motile rods that were able to grow at 6–40°C and at pH 5.5–10.0. Other biochemical and physiological properties of strain MK3$^T$ are presented in Table 1 and in the species description.

Fatty acid methyl esters (FAMEs) of strain MK3$^T$ and F. ceti CECT 7184$^T$ were obtained from 40 mg wet cells collected from Degryse 162 agar plates after incubation at 28°C for 24 h in the late-exponential growth phase by saponification, methylation and extraction using minor modifications of described methods (Kuykendall et al., 1988; Miller, 1982). The FAME mixtures were analysed...
using the instant FAME method of the Sherlock Microbial Identification System (MIDI) version 6.0B using an AgilentTec model 6890N gas chromatogram and identified with the MIDI RTSBA60 database (Sasser, 1990). Peaks were automatically integrated and percentages were calculated using the MIS Standard Software. Flavobacterium anatoliense sp. nov. was identified among the species listed below:

- Flavobacterium gillisiae ACAM 601\textsuperscript{T} (U85989)
- Flavobacterium degerlachei LMG 21915\textsuperscript{T} (AJ557886)
- Flavobacterium frigoris LMG 21922\textsuperscript{T} (AJ557887)
- Flavobacterium xinjiangense JCM 11314\textsuperscript{T} (AF433173)
- Flavobacterium limicola DSM 15094\textsuperscript{T} (AB075230)
- Flavobacterium xanthum ACAM 81\textsuperscript{T} (AF030380)
- Flavobacterium omnivorum JCM 11313\textsuperscript{T} (AF433174)
- Flavobacterium frigidarium ATCC 700810\textsuperscript{T} (AF162266)
- Flavobacterium psychrophilum ATCC 49418\textsuperscript{T} (AM988681)
- Flavobacterium micromai LMG 21919\textsuperscript{T} (AJ557888)
- Flavobacterium pectinovorum NCMB 9059\textsuperscript{T} (AM230590)
- Flavobacterium hibernal ATCC 51468\textsuperscript{T} (L39067)
- Flavobacterium hibernal ATCC 29651\textsuperscript{T} (M230487)
- Flavobacterium aquidenum WB 1.1-56\textsuperscript{T} (AM177392)
- Flavobacterium frigidimaris KUC-1\textsuperscript{T} (AB183888)
- Flavobacterium hervincum WB 4.2-33\textsuperscript{T} (AM265623)
- Flavobacterium saccharophilum NCMB 2072\textsuperscript{T} (AM230491)
- Flavobacterium succicincans IFO 14905\textsuperscript{T} (AM230492)
- Flavobacterium soli DS-6\textsuperscript{T} (DQ178976)
- Flavobacterium daejeonense DSM 17708\textsuperscript{T} (DQ222427)
- Flavobacterium johnsoniae ATCC 17061\textsuperscript{T} (CP000685)
- Flavobacterium flexens ATCC 27944\textsuperscript{T} (AM230486)
- Flavobacterium antarcticum AT1026\textsuperscript{T} (AY581113)
- Flavobacterium tegetincula ACAM 602\textsuperscript{T} (U85887)
- Flavobacterium weaverense JCM 12384\textsuperscript{T} (AY581114)
- Flavobacterium segetis AT1048\textsuperscript{T} (AY581115)
- Flavobacterium gelidilacus LMG 21477\textsuperscript{T} (AJ440996)
- Flavobacterium aquatile ATCC 11947\textsuperscript{T} (M62797)
- Flavobacterium cheni NBRC 103934\textsuperscript{T} (EF407880)
- Flavobacterium croceum DSM 17960\textsuperscript{T} (DQ372982)
- Flavobacterium indicum DSM 17447\textsuperscript{T} (AY904351)
- Flavobacterium columnare IAM 14301\textsuperscript{T} (AB078047)
- Flavobacterium saliperorum JCM 13331\textsuperscript{T} (DQ021903)
- Flavobacterium anatoliense MK3\textsuperscript{T} (JF825522)
- Flavobacterium ceti CECT 7184\textsuperscript{T} (AM292800)
- Flavobacterium beibensis LMG 25233\textsuperscript{T} (GQ245972)
- Flavobacterium rivuli DSM 21788\textsuperscript{T} (AM934661)
- Leeuwenhoekiella marinoflava ATCC 19326\textsuperscript{T} (AB880577)

**Fig. 1.** Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain MK3\textsuperscript{T} and the type strains of representative species of the genus Flavobacterium. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at the branch points. Leeuwenhoekiella marinoflava ATCC 19326\textsuperscript{T} was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.
**Table 1. Differential characteristics of strain MK3**\(^T\) and *Flavobacterium ceti* CECT 7184**\(^T\)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell length (µm)</td>
<td>0.8–1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Colony pigmentation</td>
<td>Yellow</td>
<td>Orange</td>
</tr>
<tr>
<td>Growth on nutrient agar</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>Growth at 15 °C</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth with 5 % NaCl</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>28</td>
<td>35</td>
</tr>
<tr>
<td>Enzyme activity (VITEK 2 GNI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine (API 20E)</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>38.6</td>
<td>36.7</td>
</tr>
</tbody>
</table>

The major fatty acids of strain MK3**\(^T\)** were iso-C\(_{15:0}\) (45.2 %), summed feature 9 (C\(_{16:0}\) 10-methyl and/or iso-C\(_{17:1\alpha\text{oil}}\); 20.4 %) and summed feature 3 (C\(_{16:1\text{oil}}\) and/or C\(_{16:1\alpha\text{oil}}\); 13.3 %). The fatty acid composition of *F. ceti* CECT 7184**\(^T\)** was similar, with only minor differences in the respective proportions of the components. The complete fatty acid profiles are given in Table 2. The major fatty acids are in accordance with those of members of the genus *Flavobacterium* (Bernardet et al., 1996, 2002; Bernardet & Bowman, 2011; Vela et al., 2007).

**Table 2. Fatty acid compositions (%) of *Flavobacterium anatoliense* sp. nov. MK3**\(^T\) and *Flavobacterium ceti* CECT 7184**\(^T\)**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso-C(_{14:0})</td>
<td>1.4</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C(_{15:0})</td>
<td>45.2</td>
<td>39.7</td>
</tr>
<tr>
<td>iso-C(_{15:0}) 3-OH</td>
<td>2.3</td>
<td>5.6</td>
</tr>
<tr>
<td>C(_{15:0}) 2-OH</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>anteiso-C(_{15:0})</td>
<td>1.5</td>
<td>3.9</td>
</tr>
<tr>
<td>C(_{16:0})</td>
<td>2.5</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C(_{16:0})</td>
<td>3.0</td>
<td>1.5</td>
</tr>
<tr>
<td>iso-C(_{17:0}) 3-OH</td>
<td>4.6</td>
<td>18.6</td>
</tr>
<tr>
<td>Summed feature 9</td>
<td>20.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Summed feature 3</td>
<td>13.3</td>
<td>19.6</td>
</tr>
</tbody>
</table>

Isoprenoid quinones of strain MK3**\(^T\)** and polar lipids of strain MK3**\(^T\)** and *F. ceti* CECT 7184**\(^T\)** were extracted from 100 mg freeze-dried cells using the two-stage method described by Tindall (1990a, b). Polar lipids were separated by two-dimensional TLC on silica gel (no. 818 135; Macherey–Nagel). The first direction was developed with chloroform : methanol : water (65 : 25 : 4, v/v) and the second direction with chloroform : methanol : acetic acid : water (80 : 12 : 15 : 4, v/v). Detection was performed using 5 % ethanolic molybdophosphoric acid for the total lipids, molybdenum blue for phospholipids, ninhydrin for aminolipids, the periodate-Schiff reagent for z-glycols and z-naphthol sulphuric acid for glycolipids (Tindall et al., 2007). One spot that reacted with ninhydrin and molybdenum blue showed 2D migration rate (R\(_g\)) values very similar to those of phosphatidylserine (PS) used as a reference on the control TLC plate. Though the R\(_g\) value of the first dimension was exactly the same as the reference, there was a small deviation in the R\(_g\) value of the second dimension. Therefore, the spot was considered an unidentified aminophospholipid (APL7) instead of PS.

The only isoprenoid quinone of strain MK3**\(^T\)** was MK-6, in line with all other members of the family *Flavobacteriaceae* (Bernardet & Bowman, 2011). The major polar lipids of strain MK3**\(^T\)** were phosphatidylethanolamine, one unidentified aminophospholipid and two unidentified phospholipids (Fig. 2). Minor amounts of one unidentified aminolipid and other unidentified aminophospholipids and phospholipids were also detected. The polar lipid profile *F. ceti* CECT 7184**\(^T\)** was similar, except that the major phospholipid in strain MK3**\(^T\)** (PL4) was only present in minor amounts and that three additional unidentified aminolipids were present.

For determination of the G+C content of strain MK3**\(^T\)**, genomic DNA was extracted and purified using the method of Marmur (1961), and the G+C content was determined from the midpoint value (T\(_m\)) of the thermal denaturation profile (Mandel & Marmur, 1968). The DNA of *Escherichia coli* K-12 was used as standard. The DNA G+C content of strain MK3**\(^T\)** was 38.6 mol%, a value consistent with that of members of the genus *Flavobacterium* (Bernardet & Bowman, 2011).

Hence, on the basis of the phylogenetic distance from all recognized species of the genus *Flavobacterium* and the combination of unique phenotypic characteristics (Table 1), strain MK3**\(^T\)** represents a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium anatoliense* sp. nov. is proposed. In addition, an emended description of *Flavobacterium ceti* is proposed on the basis of new data obtained in this study.

**Description of *Flavobacterium anatoliense* sp. nov.**

*Flavobacterium anatoliense* (a.na.to.li.en’se. N.L. neut. adj. anatoliense of or belonging to Anatolia).

Cells are Gram-staining-negative, strictly aerobic, non-motile, non-spore-forming rods approximately 0.2–0.4 µm
in diameter and 0.8–1.2 μm in length. Optimal growth occurs on Degryse 162 agar. Colonies are convex, circular and dark yellow with regular edges. Good growth also occurs on TSA and NA, and weak growth occurs on LB agar. No growth occurs on MacConkey agar. Growth occurs at 6–40 °C (optimum, 28 °C) but no growth occurs 4 °C and 45 °C. Optimal pH for growth is pH 7.0; growth occurs at pH 5.5–10.0. Growth occurs in the absence of NaCl and in the presence of up to 6% (w/v) NaCl, but not with 7% and 10% (w/v) NaCl. Flexirubin pigments are produced (KOH test-positive) and Congo red is not absorbed by colonies. Catalase and oxidase tests are positive. Casein is hydrolysed but starch, aesculin, chitin, DNA, carboxymethylcellulose and Tweens 20 and 80 are not. Tyrosine is degraded and a brown pigment is also produced on tyrosine agar. In the API 20E strip, OPNG activity is present; citrate utilization, hydrogen sulfide and indole production and lysine and ornithine decarboxylases, nitrate reduction, hydrolysis, production of urease, tryptophan deaminase and arginine dihydrolase and the Voges–Proskauer reaction are positive. Acid is not produced from D-glucose, D-mannitol, L-malate, L-lactate and succinate. Also in the VITEK 2 GNI System: http://ijs.sgmjournals.org 2079

The DNA G+C content of the type strain is 38.6 mol%.

**Emended description of Flavobacterium ceti Vela et al. 2007**

The description is as given by Vela et al. (2007) with the following amendment. The major polar lipids are phosphatidylethanolamine, two unidentified aminophospholipids and one unidentified phospholipid. Significant amounts of unidentified aminolipids, unidentified phospholipids and unidentified aminophospholipids are also present.

**References**


