**Flavobacterium lacus** sp. nov., isolated from a high-altitude lake, and emended description of *Flavobacterium filum*

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Two Gram-stain-negative, non-motile, rod-shaped bacterial strains, designated NP180^T^ and NR80, were isolated from water of Nam Co Lake, located in Tibet, China. Growth of strains NP180^T^ and NR80 occurred at 4–25 °C and at pH 6.5–10.0 (optima, 15–20 °C and pH 7.5–8.5). The 16S rRNA gene sequence similarity to the phylogenetically closest related strains, *Flavobacterium filum* EMB 34^T^, *F. ponti* GSW-R14^T^ and *F. gelidilacus* LMG 21477^T^, was 95.1, 94.8 and 94.6 %, respectively. The predominant fatty acids were iso-C_{15:0}, iso-C_{15:1} \(\omega_6\) c and 10-methyl C_{16:1} \(\omega_7\). The major menaquinone of the two strains was menaquinone 6 (MK-6). Phosphatidylethanolamine, one unidentified amino lipid and one unidentified lipid were the major polar lipids in both strains.

The G+C contents of the genomic DNA were 34.9 and 35.1 mol\%, respectively, for strains NP180^T^ and NR80. DNA–DNA relatedness between strains NP180^T^ and NR80 was 99 %, indicating that they belong to the same species. According to phylogenetic inference and phenotypic characteristics, a novel species, *Flavobacterium lacus* sp. nov., is proposed. The type strain is NP180^T^ (=CGMCC 1.12504^T^ =NBRC 109715^T^). An emended description of *Flavobacterium filum* is also provided.

At the time of writing, 122 species were included in the genus *Flavobacterium*, with *Flavobacterium aquatile* as the type species (http://www.bacterio.net/f/flavobacterium.html). Strains of the genus *Flavobacterium* occur in various habitats such as seawater (Yoon et al., 2011), marine sediment (Kaur et al., 2012), fresh water (Chun et al., 2013; Subhash et al., 2013), wastewater, soil (Lim et al., 2011; Liu et al., 2008), Antarctic lakes (McCammon et al., 1998; Van Trappen et al., 2003, 2004, 2005), glaciers (Dong et al., 2012; Xu et al., 2011; Zhang et al., 2006), salmonid fish (Kämpfer et al., 2012) and the guts of earthworms. Owing to the continuous exploration of extreme environments, the genus has expanded considerably, especially with psychrophilic and psychrotolerant species. Among published species of the genus *Flavobacterium*, 25 are cold-adapted organisms, of which 14 were isolated from the Antarctic (Humphry et al., 2001; McCammon & Bowman, 2000; McCammon et al., 1998; Van Trappen et al., 2003, 2004, 2005; Yi & Chun, 2006; Yi et al., 2005) and eight from glaciers in China (Dong et al., 2012; Xin et al., 2009; Xu et al., 2011; Zhang et al., 2006; Zhu et al., 2003, 2013).

Strains NP180^T^ and NR80 were isolated from Nam Co lake located in Tibet in the west of China, at an altitude of 4718 m (30° 30′ N 90° 16′ E); the annual mean water temperature is 10–15 °C. Samples of lake water were collected and 10-fold dilutions were plated on PYG agar (5.0 g Bacto peptone, 0.2 g yeast extract, 5.0 g glucose, 3.0 g beef extract, 0.5 g NaCl, 1.5 g MgSO_4_.7H_2O, 1.6 g KH_2PO_4, 1.2 g Na_2HPO_4_.12H_2O, 0.5 g MgCl_2_.6H_2O, 0.5 g CaCl_2, 0.2 g FeCl_3, 0.02 g MnCl_2 and 1 g Na_2SeO_3), and then incubated at 10 °C for 3 weeks under anaerobic conditions.
1000 ml sterile water, 15 g agar, pH 7.0). The plates were incubated at 8 °C for 1 month. Single colonies were picked and purified by blaking. All purified strains were preserved by lyophilization. Strains NP180T and NR80 were selected for taxonomic studies.

Genomic DNA of the two isolates was extracted using the Genomic DNA Rapid Isolation Kit for bacterial cells (BioDev-Tech). 16S rRNA gene sequences were amplified using the universal bacterial primers 27F and 1492R (Lane, 1991). PCR products were cloned using the pGEM-T Easy vector and sequenced. The 16S rRNA gene sequences (1477 bp for strains NP180T and NR80) were submitted to GenBank (http://www.ncbi.nlm.nih.gov/) and aligned with available sequences of related taxa in GenBank and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). Sequences of related strains were downloaded and multiple alignments were performed using BioEdit (Hall, 1999). Phylogenetic trees were reconstructed with MEGA 5.0 by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) algorithms. Bootstrap values were calculated based on 1000 replications in order to evaluate confidence levels of the nodes. The topology of the neighbour-joining phylogenetic tree (Fig. 1) revealed an affiliation with the genus Flavobacterium, and strains NP180T and NR80 constituted a cluster together with Flavobacterium filum EMB 34T. The maximum-parsimony and maximum-likelihood trees showed the same topology. Strains NP180T and NR80 exhibited 100% 16S rRNA gene sequence similarity with each other and 95.1, 94.8 and 94.6% sequence similarity with F. filum EMB 34T, F. ponti GSW-R14T and F. gelidilacus LSM 21477T, respectively. Sequence similarity with other type strains of the genus Flavobacterium was <94% (93.1% with F. aquatile DSM 1132T).

For morphological studies, strains NP180T and NR 80 and F. filum KCTC 12610T were grown on PYG at 20 °C for 3 days and observed by transmission electron microscopy (JEM1400; JEOL) (Fig. S1, available in the online Supplementary Material) and light microscopy. Colony morphology was examined after 7 days. Growth was tested on R2A, tryptic soy agar, nutrient agar, marine agar 2216 (Difco) and Anacker and Ordal’s agar (Bernardet & Bowman, 2006). Gliding motility was tested by using the hanging drop technique (Bernardet et al., 2002). The presence of a capsule was assessed using wet negative staining (Fig. S2) and the Hiss staining method (Beveridge et al., 2007). The temperature range for growth was assessed on PYG slants at 4, 8, 15, 20, 25, 30, 35, 37 and 40 °C. Growth was assessed in PYG broth adjusted to pH 4.0–10.0 using 100 mM acetate buffer (for pH 4.0–5.0), 100 mM phosphate buffer (pH 6.0–8.0) or 100 mM NaHCO3/Na2CO3 buffer (pH 9.0–10.0) (Breznak & Cos-tilow, 2007; Yumoto et al., 2004) and sterilized by filtration. NaCl tolerance was tested in PYG broth supplemented with 0 to 5.0% (w/v) NaCl (at 0.5% intervals). Strains were incubated in an anaerobic jar in order to test the requirement for oxygen using the Atmosphere Generation System (Oxoid). Catalase and oxidase activities were assessed using 3% (v/v) H2O2 and 1% (w/v) N,N,N′,N′-tetramethyl p-phenylenediamine, respectively. Production of flexirubin-type pigments and Congo red absorption were assessed following the methods of Bernardet et al. (2002). Hydrolysis of casein, starch, chitin, tyrosine, gelatin, Tween 20, 60 and 80, aesculin, urea, pectin and CM-cellulose was evaluated according to Smibert & Krieg (1994) and Reichenbach (1992). Hydrolysis of dextrin was investigated on PYG agar containing 1% (w/v) dextrin. The DNase assay was carried out on the DNase test agar (Merck). Hydrolysis of egg yolk was tested on PYG agar supplemented with 50% egg yolk emulsion. Acid production from carbohydrates, enzyme activities and additional biochemical characteristics were tested in API 50CH, API 20NE, API 20E and API ZYM strips (bioMérieux), while oxidation of substrates was evaluated using the GN3 MicroPlate system (Biolog), both according to the manufacturers’ recommendations. Sensitivity to antibiotics was tested on PYG plates by using antibiotic discs (Beijing TianTan Biological Products) containing ampicillin (10 μg), amikacin (30 μg), azithromycin (15 μg), cefaclor (30 μg), cefazolin (30 μg), cefoperazone (75 μg), cefotaxime (30 μg), cefazidime (30 μg), ceftriaxone (30 μg), cefuroxime sodium (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clarithromycin (15 μg), clindamycin (2 μg), doxycycline (30 μg), erythromycin (15 μg), fleroxacin (5 μg), lomefloxacin (10 μg), minocycline (30 μg), metilimcin (30 μg), nitrofurantoin (300 μg), oxacillin (1 μg), penicillin G (10 IU), piperacillin (100 μg), rifampicin (5 μg), sulfamethoxazole (1.25 μg), tetracycline (30 μg), tobramycin (10 μg) and vancomycin (30 μg). The morphological, cultural and physiological properties of strain NP180T are given in Table 1 and in the species description.

For fatty acid analysis, strains NP180T and NR80 and F. filum KCTC 12610T were grown on TSA at 20 °C and cells were harvested at the exponential growth phase (48 h for the two former strains and 45 h for the latter). Cellular fatty acids were methylated, separated and identified according to the standard procedure of the Microbial Identification System (MIDI 6.0 version; Agilent 6890) (Sasser, 1990). Peaks were identified using the TSBA6.0 database.

Polar lipids were extracted from the three strains and analysed by two-dimensional TLC following the procedures of Minnikin et al. (1984) and Komagata & Suzuki (1987); 5% ethanolic molybdophosphoric acid, molybdenum blue, ninhydrin and α-naphthol were sprayed onto the plates to detect total polar lipids, phospholipids, aminolipids and glycolipids, respectively. The quinones of strains NP180T and NR80 and F. filum KCTC 12610T were extracted and purified according to Collins (1985) and analysed by HPLC (Wu et al., 1989).

The DNA G+C contents of the two isolates were determined by HPLC (Mesbah et al., 1989) using DNA of bacteriophage lambda as a reference (49.8 mol%). DNA–DNA
hybridization of strains NP180^T and NR80 was carried out according to De Ley et al. (1970), using a Lambda 35 UV/Vis spectrometer and a temperature programme controller (Perkin-Elmer).

Strains NP180^T and NR80 contained mainly iso-C\(_{15}:0\) (29.1 and 27.7 %, respectively), iso-C\(_{15}:1\) G (19.8 and 17.3 %), iso-C\(_{17}:0\) 3-OH (8.1 and 8.4 %) and summed feature 9 (iso-C\(_{17}:1\)\(^\text{cis-9}\) and/or 10-methyl C\(_{16}:0\) 8.1 and 7.6 %). The fatty acid profiles of strains NP180^T and NR80 were nearly identical and similar to that of \(F.\) \textit{filum} KCTC 12610^T, although containing smaller proportions of iso-C\(_{15}:0\) and iso-C\(_{15}:1\) G, and larger proportions of summed feature 9 (iso-C\(_{17}:1\)\(^\text{cis-9}\) and/or 10-methyl C\(_{16}:0\)) (Table 2). MK-6 was the predominant respiratory quinone in strains NP180^T and NR80 (97 and 96 %, respectively); they also

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**Fig. 1.** Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationship of strains NP180^T and NR80 and members of other \(F.\) \textit{lacus} species. Chryseobacterium gleum CCUG 14555^T was used as an outgroup. Bootstrap values are shown at branching points (percentages of 1000 resamplings; only values above 70 % are shown). Filled circles indicate nodes that were also recovered by using the maximum-likelihood and maximum-parsimony methods. Bar, 0.02 substitutions per nucleotide position.
Table 1. Characteristics that differentiate strain NP180T from F. filum KCTC 12610T

All data shown were obtained in the present study. +, Positive; (+), weakly positive; −, negative. The two strains are positive for hydrolysis of aesculin, dextrin, pectin, casein, Tween 60 and gelatin [an opposite result was reported for gelatin by Ryu et al. (2007)] and negative for hydrolysis of DNA, starch, urea, chitin, tyrosine and CM-cellulose, production of flexirubin-type pigments and absorption of Congo red.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NP180T</th>
<th>F. filum KCTC 12610T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony pigmentation on R2A agar</td>
<td>Orange</td>
<td>Pale yellow</td>
</tr>
<tr>
<td>Gliding motility</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Presence of a capsule</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>30 °C</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Optimum NaCl concentration (%, w/v)</td>
<td>1.0–1.5</td>
<td>0–1.5</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Hydrolysis of Tweens 20 and 80</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Enzyme activities (API ZYM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esterase (C4)</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>N-Acetyl-β-D-glucosaminidase</td>
<td>+</td>
<td>( + )</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>( + )</td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from: (API 50CH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Oxidation of: (GN3 MicroPlate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

The G + C contents of the genomic DNA of strains NP180T and NR80 were 34.9 and 35.1 mol%, respectively, in accordance with that of the reference strain and within the range reported for species of the genus Flavobacterium (Bernardet & Bowman, 2011). DNA relatedness between strains NP180T and NR80 was 99 %, which indicated that the two strains belonged to the same genomic species. As the 16S rRNA gene sequence similarity between strain NP180T and strains of all species of the genus Flavobacterium with validly published names was well below 97.0 % (Stackebrandt & Ebers, 2006; Tindall et al., 2010), DNA–DNA hybridization between them were not performed.

The results of physiological, biochemical and genetic analyses support the recognition of a novel species within the genus Flavobacterium, for which the name Flavobacterium lacus sp. nov. is proposed. In addition, an emended description of Flavobacterium filum is also proposed on the basis of new data obtained in this study.

Emended description of Flavobacterium filum

Ryu et al. 2007

The description is as given by Ryu et al. (2007) with the following amendments. Oxidase-positive. Gelatin is hydrolysed. The major polar lipids are phosphatidylethanolamine, one unidentified aminolipid and one unidentified lipid; several unidentified lipids are also present. MK-6 is the only respiratory quinone.

Description of Flavobacterium lacus sp. nov.

Flavobacterium lacus (la’cus. L. gen. n. lacus of a lake).

Cells are Gram-stain-negative, strictly aerobic and non-motile rods, approximately 0.4–0.6 μm in diameter and 1.0–1.8 μm long. A thick capsule is present. Colonies are orange, convex, circular, 1–4 mm in diameter with entire margins and a sticky consistency on PYG agar after 7 days at 15 °C. Growth also occurs on TSA, NA, R2A and Anacker and Ordal’s agar, but not on marine agar 2216. Broth cultures are viscous. Growth does not occur under anaerobic conditions. Growth occurs at 4–25 °C (optimum, 20 °C), in PYG broth supplemented with 0–2.0 % NaCl (optimum,
Table 2. Whole-cell fatty acid compositions of strain NP180\textsuperscript{T} and *F. filum* KCTC 12610\textsuperscript{T}

Data are from this study. Fatty acids amounting to $<1\%$ of the total fatty acids in both strains are not shown. TR, Trace ($<1\%$). Values for strains NP180\textsuperscript{T} and NR80 are means ± SD.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>NP180\textsuperscript{T} and NR80</th>
<th>KCTC 12610\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-C$_{15}$:0</td>
<td>24.8 ± 1.0</td>
<td>38.1</td>
</tr>
<tr>
<td>anteiso-C$_{15}$:0</td>
<td>4.6 ± 1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>iso-C$_{16}$:0</td>
<td>2.3 ± 0.1</td>
<td>4.5</td>
</tr>
<tr>
<td>C$_{16}$:0</td>
<td>1.2 ± 0.1</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C$_{15}$:0 3-OH</td>
<td>5.7 ± 0.2</td>
<td>7.2</td>
</tr>
<tr>
<td>iso-C$_{16}$:0 3-OH</td>
<td>1.8 ± 0.1</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C$_{17}$:0 3-OH</td>
<td>8.3 ± 0.2</td>
<td>7.9</td>
</tr>
<tr>
<td><strong>Unsaturated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-C$_{15}$:1 G</td>
<td>18.6 ± 1.8</td>
<td>22.0</td>
</tr>
<tr>
<td>anteiso-C$_{15}$:1 A</td>
<td>1.2 ± 0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>C$_{15}$:106c</td>
<td>3.0 ± 0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>iso-C$_{16}$:1 H</td>
<td>1.7 ± 0.3</td>
<td>1.1</td>
</tr>
<tr>
<td>C$_{17}$:108c</td>
<td>1.1 ± 0.1</td>
<td>TR</td>
</tr>
<tr>
<td>C$_{17}$:106c</td>
<td>2.5 ± 0.6</td>
<td>TR</td>
</tr>
<tr>
<td>C$_{18}$:105c</td>
<td>1.0 ± 0.1</td>
<td>TR</td>
</tr>
<tr>
<td><strong>Summed features</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.5 ± 0.4</td>
<td>3.2</td>
</tr>
<tr>
<td>9</td>
<td>7.9 ± 0.3</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*As indicated by Montero-Calasanz et al. (2013), summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI System and include both peaks with discrete ECLs as well as those where the ECLs are not reported separately. Summed feature 3 was listed as C$_{16}$:107c and/or C$_{16}$:106c; summed feature 9 was listed as iso-C$_{17}$:109c and/or 10-methyl C$_{16}$:0.

1.0–1.5\% and at pH 6.5–10.0 (optimum, pH 7.5–8.5). Catalase and oxidase activities are present. Nitrate is not reduced to nitrite. H$_2$S and indole are not produced, but citrate is utilized. The Voges–Proskauer test is weakly positive. Hydrolyses gelatin, casein, aesculin, dextrin, pectin and Tween 20 and 60, but not tyrosine, DNA, Tween 80, starch, urea, chitin or CM-cellulose. No brown pigment is produced on tyrosine agar. Flexirubin-type pigments are not produced and Congo red is not absorbed by colonies. No precipitate is formed on egg yolk agar. The following activities are detected in the API ZYM strip: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, χ-chymotrypsin, acid phosphatase, χ-glucosidase, naphthol-AS-Bl-phosphohydrolase (weak) and N-acetyl-β-glucosaminidase (weak). The following activities are not detected: χ-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, χ-mannosidase and χ-fucosidase. In the API 50CH strip, acid is produced from D-galactose, D-glucose, aesculin, starch and glycogen. Acid production from lactose is negative (type strain) or weakly positive, and acid is not produced from the other substrates in the API 50CH strip.

In the GN3 MicroPlate, the following substrates are oxidized: acetoacetic acid, acetic acid, L-arginine, L-aspartic acid, citric acid, dextrin, D-galacturonic acid, gelatin, χ-D-glucose, L-glutamic acid, glycyl L-proline, D-lactic acid methyl ester, D-malic acid, malteose, muscic acid, pectin, L-serine, Tween 40, D-aspartic acid (weak), D-glucuronic acid (weak) and methyl pyruvate (weak). The other substrates in the GN3 MicroPlate are not oxidized. Resistant to chloramphenicol (30 μg), fleroxacin (5 μg), oxacillin (1 μg), sulfamethoxazole (1.25 μg) and tobramycin (10 μg), but sensitive to amikacin (30 μg), ampicillin (10 μg), azithromycin (15 μg), cefaclor (30 μg), cefazolin (30 μg), ceftoperazone (75 μg), cefoxatine (30 μg), cefazidime (30 μg), ceftriazone (30 μg), cefuroxime sodium (30 μg), cephalothin (30 μg), ciprofloxacin (5 μg), clarithromycin (15 μg), clindamycin (2 μg), doxycycline (30 μg), erythromycin (15 μg), lomefloxacin (10 μg), minocycline (30 μg), metillicin (30 μg), nitrofurantoin (300 μg), penicillin G (10IU), piperacillin (100 μg), rifampicin (5 μg), tetracycline (30 μg) and vancomycin (30 μg). The predominant (>$7\%$) fatty acids are iso-C$_{15}$:0, iso-C$_{15}$:1 G, iso-C$_{17}$:0 3-OH and summed feature 9 (iso-C$_{17}$:109c and/or 10-methyl C$_{16}$:0). MK-6 is the major respiratory quinone. Phosphatidylethanolamine, one unidentified aminolipid and one unidentified lipid are the major polar lipids.

The type strain is NP180\textsuperscript{T} (=CGMCC 1.12504\textsuperscript{T}=NBRC 109715\textsuperscript{T}), isolated from water of Lake Nam Co in Tibet, China. The DNA G+C content of the type strain is 34.9\%.

Acknowledgements

We thank Professor J. P. Euzéby for kind assistance with Latin nomenclature. This work was supported by the National Natural Science Foundation of China (NSFC, no. 187 31070001), and the Knowledge Innovation Program of the Chinese Academy of Sciences (grant no. 188 KSCX2-YW-Z-0937).

References


