Flavobacterium gelidilacus sp. nov., isolated from microbial mats in Antarctic lakes

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Twenty-two isolates from microbial mats in eastern Antarctic lakes showed similar fatty acid compositions and were investigated further using a polyphasic taxonomic approach. Repetitive extragenic palindromic DNA-PCR fingerprinting of the 22 strains revealed three groups, and DNA–DNA hybridizations between representatives showed more than 87 % DNA–DNA reassociation with each other. 16S rRNA gene sequence analysis placed two representative strains, LMG 21477^T and LMG 21619, within the genus *Flavobacterium*, with 95.1 % sequence similarity to *Flavobacterium flevense*, 95.0 % to *Flavobacterium tegetincola*, less than 95 % to other *Flavobacterium* species and less than 90 % to representatives of other genera. The name *Flavobacterium gelidilacus* sp. nov. is proposed, with LMG 21477^T (= DSM 15343^T) as the type strain, and a description of the species is given on the basis of morphological, biochemical and physiological characteristics and fatty acid composition. The G + C content of the genomic DNA is 30.0–30.4 mol%.

Members of the genus *Flavobacterium* have been isolated from diverse habitats such as fresh water (*Flavobacterium aquatile*, *Flavobacterium flevense*, *Flavobacterium hibernum*, *Flavobacterium saccharophilum*), soil (*Flavobacterium johnsoniae*, *Flavobacterium pectinovorum*, *Flavobacterium xanthum*) and sea ice (*Flavobacterium gillisiae*); some are known as important fish pathogens (*Flavobacterium branchiophilum*, *Flavobacterium columnare*, *Flavobacterium psychrophilum*). They are abundant in freshwater and marine ecosystems, and these heterotrophic bacteria may have a specialized role in the uptake and degradation of the high-molecular-mass fraction of dissolved organic matter in these environments (Kirchman, 2002).

Several novel species, added to the genus since 1996, were derived from Antarctic habitats, and several new genera containing polar organisms have recently been described within the family *Flavobacteriaceae* (*Gelidibacter*, *Psychroserpens*, *Polaribacter*, *Psychroflexus*, *Salegentibacter*). So far, only one species, *Flavobacterium tegetincola*, has been isolated from a cyanobacterial mat, collected from the Antarctic saline Ace Lake, located in the Vestfold Hills (McCammon & Bowman, 2000).

During the MICROMAT project (November 1998 to February 2001), 746 bacterial strains were isolated under heterotrophic conditions from microbial mat samples, collected from 10 Antarctic lakes in the Vestfold Hills (lakes Ace, Druzhby, Grace, Highway, Pendant, Organic and Watts), the Larsemann Hills (Lake Reid) and the McMurdo Dry Valleys (lakes Hoare and Fryxell) (Van Trappen *et al.*, 2002). Numerical analysis of their fatty acid composition revealed 41 clusters, and 16S rRNA gene sequence analysis, performed on representative strains, showed that they belong to the α -, β - and γ -subclasses of the *Proteobacteria*, the high- and low-G + C-content Gram-positives and to the *Cytophaga*–*Flavobacterium*–*Bacteroides* branch (Van Trappen *et al.*, 2002). The results of fatty acid analysis and 16S rRNA gene sequence analysis showed that the diversity of heterotrophic bacteria in microbial mats from Antarctic lakes is very high. Moreover, many fatty acid clusters contain multiple taxa, as defined by repetitive extragenic palindromic DNA-PCR (rep-PCR) fingerprinting, a technique used to investigate the genomic diversity of each fatty acid cluster in more detail (Van Trappen *et al.*, 2001).

In the present work, we studied further the taxonomic relationships of 22 strains from fatty acid cluster 10 (as delineated by Van Trappen *et al.*, 2002), related to the genus *Flavobacterium*, by genomic and phenotypic characterization. Van Trappen *et al.* (2002) found less than 96 % 16S rRNA gene sequence similarity to the closest relatives within the genus *Flavobacterium*, indicating that these strains constitute a novel species (Stackebrandt & Goebel, 1994).

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Abbreviation: rep-PCR, repetitive extragenic palindromic DNA-PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 21619 and LMG 21477^T are AJ507151 and AJ440996.

Table 1. Strains investigated in this study

Numbers with an 'R-' prefix refer to strains from the research collection of the LMG, as used by Van Trappen *et al.* (2002).

Strain	Isolation source
Rep-PCR profile type I	
LMG 21477 ^T (=DSM 15343 ^T =R-8899 ^T), R-8897, R-8908, R-8969, R-8972, R-9283	Ace Lake, Vestfold Hills
R-9024	Lake Watts, Vestfold Hills
R-11278	Pendant Lake, Vestfold Hills
LMG 21618 (=R-12566)	Lake Reid, Larsemann Hills
Rep-PCR profile type II	
LMG 21619 (=R-8885), R-8888, R-8898, R-9104, R-9110, R-9158	Ace Lake, Vestfold Hills
LMG 21621 (=R-9330), R-9004, R-9019	Lake Watts, Vestfold Hills
R-11078, R-11277, R-8983	Pendant Lake, Vestfold Hills
Rep-PCR profile type III	
LMG 21620 (=R-9056)	Pendant Lake, Vestfold Hills

The isolates investigated, together with their sources, are listed in Table 1. The strains were routinely cultivated on R2A medium (Difco) at 20 °C for 48 h or, for strain LMG 8328^T, on TSA medium (BBL) at 20 °C for 48 h, except where mentioned otherwise.

DNA was prepared according to the method of Pitcher *et al.* (1989), and rep-PCR fingerprinting was performed on all strains of fatty acid analysis cluster 10 of Van Trappen *et al.* (2002), using the primer GTG₅ (Versalovic *et al.*, 1991), as described by Rademaker & de Bruijn (1997) and Rademaker *et al.* (2000). The 22 strains listed in Table 1 showed similar profiles (data not shown). Numerical analysis (data not shown) was carried out using the BIONUMERICS software package (Applied Maths), as described by the same authors, and these 22 strains could be divided into three clusters according to their profile type, hereafter referred to as rep-PCR profile type I (with 9 strains), rep-PCR profile type II (with 12 strains) and rep-PCR profile type III (containing the single strain LMG 21620) (Table 1). Versalovic *et al.* (1994) have shown that strains with the same rep-PCR profile are always closely related, and this has been confirmed by several authors (e.g. Rademaker & De Bruijn, 1997).

Five strains (LMG 21477^T, LMG 21618, LMG 21619, LMG 21620 and LMG 21621) representing the three rep-PCR profile types and chosen on the basis of their isolation source were used for DNA–DNA hybridizations to investigate their genomic relatedness. DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 30 °C and reciprocal experiments were performed for every pair of strains. The DNA–DNA binding values among the five strains were high, ranging from 87 to 97 %, and differences between reciprocal experiments were less than 13 %. These DNA–DNA binding values confirm that the 22 strains belong to a single species (Wayne *et al.*, 1987).

The G + C content of the DNAs from strains LMG 21477^T, LMG 21618, LMG 21619, LMG 21620 and LMG 21621 was determined using an HPLC method. DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters Symmetry Shield C8 column thermostatted at 37 °C. The solvent was 0.02 M NH₄H₂PO₄, pH 4.0, with 1.5 % acetonitrile. Non-methylated λ -phage DNA (Sigma) was used as the calibration reference. The G + C contents of the novel strains were 30.0–30.4 mol%, which is slightly below the range (32–37 mol% G + C) mentioned by Bernardet *et al.* (1996) for the genus *Flavobacterium*.

Almost-complete 16S rRNA gene sequences (1467–1468 bp) of strains LMG 21477^T (rep-profile type I) and LMG 21619 (rep-profile type II) were obtained as described previously (Mergaert *et al.*, 2001). The most closely related sequences were found using the FASTA program. Phylogenetic analysis was performed using the BIONUMERICS software package, taking into account homologous nucleotide positions after discarding all unknown bases and gaps. A neighbour-joining dendrogram (Saitou & Nei, 1987) with the nearest phylogenetic relatives was constructed on the basis of global alignment of the sequences, using the same software package (Fig. 1). Dendrograms obtained using maximum-parsimony and maximum-likelihood analyses showed essentially the same topography. The 16S rRNA gene sequences of strains LMG 21477^T and LMG 21619 differed by only one base, and showed 95.1 % similarity to that of *F. flevense*, 95.0 % to that of *F. tegetincola*, less than 95 % to sequences of other *Flavobacterium* species and less than 90 % to sequences of other genera, indicating that they belong to a novel *Flavobacterium* species.

The fatty acid compositions are based on the data generated by Van Trappen *et al.* (2002), or were determined as described by the same authors. The 22 novel strains yielded very similar fatty acid profiles. The mean composition was 4 % 14:0 iso, 10 % 15:0, 1 % 15:0 3-OH, 8 % 15:0 anteiso,

Fig. 1. Neighbour-joining dendrogram based on 16S rDNA sequences showing the estimated phylogenetic relationships of *Flavobacterium gelidilacus* sp. nov., other *Flavobacterium* species and *Polaribacter franzmannii* (outgroup). Bootstrap values are shown as percentages of 1000 replicates, if higher than 95%. Bar, 5% sequence divergence.

12 % 15:0 iso, 6 % 15:0 iso 3-OH, 1 % 15:1 anteiso, 10 % 15:1 iso, 6 % 15:1 ω 6c, 8 % 16:0 iso, 10 % 16:0 iso 3-OH, 4 % 16:1 iso, 6 % 17:0 iso 3-OH, 3 % 17:1 ω 6c, 2 % 17:1 iso ω 9c, 1 % 18:1 ω 5c and 2 % 15:0 iso 2-OH and/or 16:1 ω 6c. Other fatty acids each accounted for less than 1 %. The fatty acid profiles of the novel strains resemble those determined for other *Flavobacterium* species (Bernardet *et al.*, 1996), but differ in terms of the relative amounts of 15:0 anteiso, 15:0 iso, 16:0 iso and 16:0 iso 3-OH.

The following morphological, physiological and biochemical tests were performed. Colony morphology was determined on R2A medium after 6 days. In addition, growth and adherence of colonies on marine and nutrient agars, TSA and Anacker & Ordal's agar (Anacker & Ordal, 1955) were tested after 14 days growth. Cells were tested for their reaction to the Gram stain and for catalase and oxidase activity. Tests in the commercial systems API ZYM, API 20NE and API 20E (bioMérieux) were performed according to the instructions of the manufacturer. API ZYM tests were read after 4 h incubation at 20 °C; other API tests were read after 48 h at 20 °C. Congo red absorption (Bernardet *et al.*, 2002), production of flexirubin-type pigments (Reichenbach, 1989), the presence of gliding motility, degradation of casein and chitin (Reichenbach & Dworkin, 1981), alginate (West & Colwell, 1984), DNA (using DNA agar from Difco, supplemented with 0.01 % toluidine blue from Merck), pectin (Paton, 1959), starch and L-tyrosine (Barrow & Feltham, 1993), the production of a brown diffusible pigment on L-tyrosine agar and the precipitation of egg-yolk agar (Barrow & Feltham, 1993) were also investigated; reactions were read after 5 days. Hydrolysis of carboxymethylcellulose was tested in Anacker & Ordal's broth

gelified with 3 % carboxymethylcellulose sodium salt (high viscosity; Sigma). This medium was stab-inoculated, and liquefaction of the medium within 7 days was scored as a positive reaction. Growth at different temperatures was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1–10 % NaCl after 14 days incubation.

The strains showed morphological characteristics typical of *Flavobacterium* (Bernardet *et al.*, 2002) and were almost identical in their physiological and biochemical characteristics (see Description). The novel species can be clearly differentiated from other *Flavobacterium* species by means of several phenotypic characteristics (Table 2).

The results of the polyphasic analysis support the recognition of a novel species within the genus *Flavobacterium*, for which the name *Flavobacterium gelidilacus* sp. nov. is proposed.

Description of *Flavobacterium gelidilacus* sp. nov.

Flavobacterium gelidilacus (ge.li.di.la'cus. L. adj. *gelidus* ice-cold; L. n. *lacus* lake; N.L. gen. n. *gelidilacus* of the ice-cold lake, referring to the isolation source, microbial mats in Antarctic lakes).

Gram-negative rods, $<1 \times 2\text{--}4\ \mu\text{m}$, that exhibit gliding motility on nutrient-poor medium (R2A), except for strains LMG 21477^T and LMG 21619, for which no gliding motility is detected. Strains grow at 5–25 °C, with optimal growth at 20 °C; no growth at 30 °C. Yellow to orange, convex, translucent colonies, 1–4 mm in diameter and with entire margins, are formed on R2A plates after 6 days at 20 °C. Colonies on Anacker & Ordal's agar are flat, round with entire margins and 0.5–1 mm in diameter after 14 days incubation. Growth also occurs on TSA, nutrient agar and marine agar, and colonies do not adhere to the agar. Degrades casein and starch. Gelatinase activity is observed, except in the case of strain LMG 21619. Catalase- and oxidase-positive. No growth is observed on glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate or phenylacetate. Acid is not produced from glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose. Agar, alginate, pectin, chitin, aesculin, carboxymethylcellulose, DNA, tyrosine and urea are not degraded. Congo red is not absorbed and no flexirubin-type pigments are present. There is no production of a brown diffusible pigment on L-tyrosine agar and no precipitate is formed on egg-yolk agar. The Voges–Proskauer reaction and tests for indole production, citrate utilization, nitrate reduction and hydrogen sulfide production are negative. None of the strains shows activity for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, lipase (*C*₁₄), α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -mannosidase or α -fucosidase. Weak enzymic activity is observed for cystine arylamidase,

Table 2. Characteristics that differentiate between *F. gelidilacus* sp. nov. and other *Flavobacterium* species

Species: 1, *F. gelidilacus* sp. nov.; 2, *F. frigidarium*; 3, *F. hibernum*; 4, *F. xanthum*; 5, *F. gillisiae*; 6, *F. tegetincola*; 7, *F. flevense*; 8, *F. aquatile*; 9, *F. branchiophilum*; 10, *F. columnare*; 11, *F. psychrophilum*; 12, *F. hydatis*; 13, *F. johnsoniae*; 14, *F. pectinovorum*; 15, *F. saccharophilum*; 16, *F. succinicans*. Data were taken from Bernardet *et al.* (1996), McCammon *et al.* (2000), Humphry *et al.* (2001) and this study. +, Positive; (+), positive, weak or delayed response; –, negative; v, results vary between strains of species or between references; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Growth on:																
TSA	+	+	+	+	+	+	+	(+)	–	–	–	+	+	+	+	+
Nutrient agar	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	+
Growth at 25 °C on agar	+	–	+	(+)	(+)	(+)	+	+	+	+	–	+	+	+	+	+
Flexirubin-type pigment	–	–	+	–	–	–	–	–	–	+	+	+	+	+	+	–
Congo red absorption	–	+	–	–	–	–	–	–	–	+	–	–	v	–	–	–
Glucose utilization	–	+	+	+	+	+	+	ND	ND	–	–	+	+	+	+	+
Acid from carbohydrates	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+	+
Degradation of:																
Casein	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+
Starch	+	–	+	+	+	–	ND	v	+	–	–	+	+	+	+	+
Carboxymethylcellulose	–	–	–	–	–	–	–	–	–	–	–	ND	+	+	+	ND
Agar	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–
Alginate	–	–	–	–	–	–	–	ND	ND	ND	–	–	+	+	ND	ND
Pectin	–	–	–	–	–	–	+	ND	ND	ND	–	+	+	+	+	ND
Chitin	–	–	–	–	+	–	–	–	–	–	–	(+)	+	+	–	–
Aesculin	–	+	+	+	+	–	+	v	–	–	–	+	+	+	+	+
DNA	–	–	+	–	–	–	–	–	–	+	(+)	+	+	+	–	+
Tyrosine	–	–	+	–	–	–	–	v	+	–	v	+	+	+	+	–
Precipitate on egg-yolk agar	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–
β -Galactosidase activity	–	–	+	–	–	–	+	ND	+	–	–	+	+	+	+	+
H ₂ S production	–	–	–	+	–	–	–	–	–	+	–	–	–	ND	+	+
Nitrate reduction	–	–	+	+	–	–	ND	ND	–	ND	–	+	+	+	+	v
Mean G + C content (mol%)	30	35	36	36	32	32	35	33	33	32	35	34	34	35	33	36

medium activity is found for acid phosphatase, esterase lipase (C₈), phosphohydrolase and α -glucosidase and strong activity is found for alkaline phosphatase, leucine arylamidase and valine arylamidase. No β -glucosidase or *N*-acetyl- β -glucosaminidase activity is detected, except for strain LMG 21621. Different reactions are obtained for esterase (C₄) and trypsin. The cells contain the fatty acids 15:0 iso, 16:0 iso 3-OH, 15:1 iso, 15:0, 15:0 anteiso and 16:0 iso as the main constituents. Growth occurs in the absence of NaCl and in the presence of 1–5 % NaCl, but not 10 % NaCl, indicating that the strains are not halophilic but merely halotolerant. The G + C content is 30.0–30.4 mol%.

The type strain is LMG 21477^T (=DSM 15343^T). Twenty-two strains were isolated from microbial mats from freshwater and saline lakes in eastern Antarctica (Table 1).

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