Flavobacterium fryxellicola sp. nov. and *Flavobacterium psychrolimnae* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes

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Taxonomic studies were performed on seven strains isolated from microbial mats in Antarctic lakes of the McMurdo Dry Valleys. Phylogenetic analysis based on 16S rRNA gene sequences indicated that these strains are related to the genus *Flavobacterium*; sequence similarity values with their nearest phylogenetic neighbours ranged from 97.0 to 98.7%. Results of DNA–DNA hybridization and comparison of repetitive extragenic palindromic DNA-PCR fingerprinting patterns revealed that these strains are members of two distinct species. Genotypic results, together with phenotypic characteristics, allowed these species to be differentiated from related recognized *Flavobacterium* species. The isolates are psychrophilic, Gram-negative, chemoheterotrophic, rod-shaped cells. Their whole-cell fatty acid profiles are similar and include C_{15:0}, C_{15:0} anteiso, C_{15:0} iso, C_{15:1}ω6c, C_{16:0} iso, C_{16:0} iso 3-OH, C_{16:1} iso and summed feature 3 (which comprises C_{15:0} iso 2-OH and/or C_{16:1}ω7c) as the major fatty acid components. On the basis of these results, two novel species are proposed: *Flavobacterium fryxellicola* sp. nov., consisting of three strains with LMG 22022^T (= CIP 108325^T) as the type strain; and *Flavobacterium psychrolimnae* sp. nov., consisting of four strains with LMG 22018^T (= CIP 108326^T) as the type strain. DNA G + C contents of *F. fryxellicola* and *F. psychrolimnae* are 35.2–35.9 and 33.8–34.5 mol%, respectively.

Members of the phylum *Bacteroidetes* are abundant in freshwater and marine ecosystems and these bacteria may have a specialized role in the uptake and degradation of organic matter in cold, aquatic environments (Kirchman, 2002). Several novel species, added to the genus *Flavobacterium* since 1996, have originated from Antarctic habitats (Van Trappen *et al.*, 2003, 2004; and references cited therein).

During the MICROMAT project (November 1998–February 2001), 746 bacterial strains were isolated under

heterotrophic conditions from microbial mat samples that were collected from ten Antarctic lakes (Van Trappen *et al.*, 2002). Numerical analysis of the fatty acid composition of the isolates and 16S rRNA gene sequence analysis, performed on representative strains, showed that the diversity of heterotrophic bacteria in microbial mats from Antarctic lakes is very high. Moreover, many fatty acid clusters were shown to contain multiple taxa when tested 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.*, 2003, 2004). Several strains belonging to fatty acid clusters 5, 6 and 10 have previously been described as novel *Flavobacterium* species: *Flavobacterium gelidilacus*, *Flavobacterium degerlachei*, *Flavobacterium frigoris* and *Flavobacterium micro-mati* (Van Trappen *et al.*, 2003, 2004).

In the present work, the taxonomic relationships of 22 strains from fatty acid cluster 5 (as delineated by Van Trappen *et al.*, 2002) were studied. A group of 11 of these strains was identified as *Flavobacterium xanthum*, whereas another rep-cluster of four strains was identified as the recently described species *F. frigoris* (Van Trappen

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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 22018^T, LMG 22022^T, LMG 22020, R-9010 and R-7518 are AJ585428, AJ811961, AJ585427, AJ601392 and AJ601393, respectively.

Figures showing a rep-PCR profile and a phylogenetic tree, and tables giving the sources of strains, fatty acid composition data and phenotypic characteristics are available as supplementary material in IJSEM Online.

et al., 2004), based on 16S rRNA gene sequence analysis (see Fig. A, available as supplementary material in IJSEM Online) and DNA–DNA hybridizations (S. Van Trappen, unpublished results). These strains were not investigated further and are listed in Table A (available as supplementary material in IJSEM Online). Seven strains, listed in Table B (available as supplementary material in IJSEM Online), proved to belong to new taxa, and were further studied by polyphasic taxonomic analysis.

Unless otherwise mentioned, strains were cultivated routinely on R2A medium (Difco) at 20 °C for 48 h, except for *Flavobacterium pectinovorum* LMG 4031^T and *Flavobacterium saccharophilum* LMG 8384^T, which were cultivated on TSA medium (BBL) at 20 °C for 48 h, and *Flavobacterium xinjiangense* LMG 21985^T and *Flavobacterium omnivorum* LMG 21986^T, which were cultivated on R2A medium at 11 °C for 5 days.

DNA was prepared according to the method of Pitcher *et al.* (1989) and rep-PCR fingerprinting (based on primers targeting the repetitive extragenic palindromic sequence) was performed on all strains of FAA cluster 5 (75 strains) of Van Trappen *et al.* (2002) using the primer GTG₅ (Versalovic *et al.*, 1991), as described previously (Van Trappen *et al.*, 2003). Seven of these strains, listed in Table B, could be divided into two different clusters according to their profile type (see Fig. B, available as supplementary material in IJSEM Online) and numerical analysis was carried out using the BIONUMERICS software package (Applied Maths), as described by the same authors. These clusters are hereafter referred to as rep-PCR profile type I (comprising three strains) and type II (with four strains) (see Table B).

Almost-complete 16S rRNA gene sequences (1466–1479 nt) of strains LMG 22022^T, LMG 22018^T, LMG 22020, R-9010 and R-7518 were determined as described previously (Van Trappen *et al.*, 2004). The most closely related sequences were found using the program FASTA; sequences from reference strains were aligned, and editing of the alignment and reformatting was performed with the programs BIOEDIT (Hall, 1999) and FORCON (Raes & Van de Peer, 1999). Evolutionary distances were calculated by using the Jukes–Cantor evolutionary model and a phylogenetic tree (Fig. A, available as supplementary material in IJSEM Online) was constructed by using the neighbour-joining method (Saitou & Nei, 1987) with the program TREECON (Van de Peer & De Wachter, 1994).

The seven novel Antarctic strains form two distinct branches within the genus *Flavobacterium*, supported by high bootstrap values, and they belong to a clade of the phylogenetic tree which consists almost exclusively of recently described *Flavobacterium* species from cold environments, such as *Flavobacterium gillisiae*, *F. degerlachei*, *F. frigoris*, *F. xinjiangense*, *F. xanthum*, *F. omnivorum*, *Flavobacterium frigidarium*, *F. gelidilacus*, *Flavobacterium limicola*, *Flavobacterium tegetincola* and *F. micromati*. Other

psychrophilic *Flavobacterium* species, such as *Flavobacterium hibernum* and *Flavobacterium psychrophilum*, do not belong to this clade and form separate branches.

The 16S rRNA gene sequence of the representative strain of rep-PCR profile type I (LMG 22022^T) showed 98.5 % similarity to *F. omnivorum*, 98.1 % to *F. frigidarium*, 97.7 % to *F. xinjiangense*, 97.6 % to *F. limicola*, 97.5 % to *F. micromati*, 97.4 % to *F. xanthum* and less than 97.4 % to other *Flavobacterium* species. A FASTA search indicated that the highest similarity for the LMG 22022^T sequence is 99.9 % with '*Flavobacterium* aff. *xylanivorum*' A1/C-aer/OIV (GenBank/EMBL/DBJ accession no. AJ297440), which was also isolated from a microbial mat from Lake Fryxell. Sequences of the representative strains of rep-PCR profile type II (LMG 22018^T and LMG 22020) are identical and showed a sequence similarity of 98.7 % to *F. limicola*, 98.4 % to *F. omnivorum*, 97.9 % to *F. xinjiangense*, 97.7 % to *F. degerlachei*, 97.6 % to *F. frigoris*, 97.5 % to *F. gillisiae* and 97.3 % to *F. xanthum* and less than 97.0 % to other *Flavobacterium* species. Sequences of strains LMG 22018^T and LMG 22022^T showed a sequence similarity of 98.0 % to each other.

Genomic relatedness between the novel Antarctic strains (representing the two different rep-PCR profile types and their most closely related phylogenetic neighbours) was determined by DNA–DNA hybridizations. DNA–DNA hybridizations were carried out with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 BioAssay reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 30 °C and reciprocal experiments were performed for every pair of strains. Differences between reciprocal experiments were less than 11 %. The hybridization level between strains LMG 22022^T, LMG 22023 and LMG 22024 of rep-PCR profile type I was 79.0–93.3 %, indicating that these three strains belong to a single species (Wayne *et al.*, 1987). Hybridization values of LMG 22022^T with its nearest phylogenetic neighbours (*F. omnivorum* LMG 21986^T, *F. frigidarium* LMG 21010^T and *F. xinjiangense* LMG 21985^T) were in the range of 16–54 % and the hybridization level between strains LMG 22018^T and LMG 22022^T was also low, with a mean value of 34 %. These results indicate that the strains from rep-PCR profile type I represent a novel *Flavobacterium* species, for which the name *Flavobacterium fryxellicola* sp. nov. is proposed. Hybridization results between strains LMG 22018^T and LMG 22020 of rep-PCR profile type II (96.5 %) showed that the strains of rep-PCR profile type II belong to a single species. It is now well established that similar rep-PCR profiles correlate with high genomic DNA–DNA hybridization values (Versalovic *et al.*, 1994; Rademaker & De Bruijn, 1997; Rademaker *et al.*, 2000; Van Trappen *et al.*, 2003, 2004). The low hybridization level (21.9–48.8 %) between LMG 22018^T and its nearest phylogenetic neighbours (*F. limicola* LMG 21930^T, *F. omnivorum* LMG 21986^T, *F. xinjiangense* LMG 21985^T, *F. degerlachei* LMG 21915^T, *F. frigoris* LMG 21922^T,

F. gillisiae LMG 21422^T and *F. xanthum* LMG 8372^T) revealed that the four strains of rep-PCR profile type II constitute a novel species, for which the name *Flavobacterium psychrolimnae* sp. nov. is proposed. These results clearly show that the novel Antarctic isolates are genotypically distinct from related *Flavobacterium* species, although the novel isolates share more than 97% (up to 98.7%) 16S rRNA gene sequence similarity with their closest phylogenetic neighbours.

DNA G+C contents of the Antarctic strains were determined using an HPLC method, as described by Van Trappen *et al.* (2003). The DNA G+C contents of *F. fryxellicola* strains LMG 22022^T, LMG 22023 and LMG 22024 are 35.2, 35.9 and 35.5 mol%, respectively; those of *F. psychrolimnae* strains LMG 22018^T, LMG 22019, LMG 22020 and LMG 22021 are 34.5, 33.9, 34.1 and 33.8 mol%, respectively. These values are consistent with G+C contents of the genus *Flavobacterium*, which range from 30 to 37 mol% (Bernardet *et al.*, 1996; Van Trappen *et al.*, 2003).

Cellular fatty acid patterns of the Antarctic strains are based on data generated by Van Trappen *et al.* (2002) and are very similar (see Table C, available as supplementary material in IJSEM Online). The major constituents include C_{15:0}, C_{15:0} iso, C_{15:1}ω6c, C_{16:0} iso 3-OH and summed feature 3 (which comprises C_{15:0} iso 2-OH and/or C_{16:1}ω7c). Hydroxylated fatty acids and iso- and anteiso-branched fatty acids were present as minor components. Strains of *F. psychrolimnae* also possessed relatively large amounts of C_{16:0} iso. The fatty acid profiles resemble those determined for other *Flavobacterium* species (Bernardet *et al.*, 1996).

Morphological, physiological and biochemical tests were performed as described previously (Van Trappen *et al.*, 2003). The strains show the typical morphological characteristics of *Flavobacterium* species (Bernardet *et al.*, 2002). Their physiological and biochemical characteristics are given in the species descriptions. *F. fryxellicola* and *F. psychrolimnae* can be clearly differentiated from each other and related *Flavobacterium* species by several phenotypic characteristics (see Table D, available as supplementary material in IJSEM Online); *Flavobacterium* species not given in the table also differ from these novel species.

Description of *Flavobacterium fryxellicola* sp. nov.

Flavobacterium fryxellicola [fry.xel.li'co.la. N.L. n. *Fryxellum* (or *Fryxellus*) Lake Fryxell; L. suffix *-cola* an inhabitant; N.L. n. *fryxellicola* inhabitant of Lake Fryxell].

Cells are Gram-negative, short rods (1.0–1.5 × 3.0–4.0 μm) that often form short chains. Gliding motility is not observed. Grows at 5–25 °C, with optimal growth at 20 °C; no growth occurs at 30 °C. Yellow–orange, convex, translucent colonies with entire margins and a diameter of 1–3 mm are formed on R2A plates after 6 days incubation.

Colonies on agar of Anacker & Ordal (1955) are flat, round with entire margins and 0.5–1.0 mm in diameter after 14 days incubation. Growth also occurs on trypticase soy agar (weak growth) and nutrient agar; no growth is observed on marine agar. Colonies do not adhere to the agar. Growth occurs in 0–2% NaCl, but not in 5–10% NaCl. Aesculin is degraded. Catalase and oxidase tests are positive. Growth is observed (API 20NE) on glucose and maltose, whereas no growth is detected on arabinose, mannitol, mannose, *N*-acetylglucosamine, gluconate, caprate, adipate, malate, citrate or phenylacetate. Acids are not produced from carbohydrates (API 20E). Agar, alginate, pectin, chitin, casein, carboxymethyl-cellulose, DNA, gelatin, starch, tyrosine and urea are not degraded. Congo red is not absorbed and no flexirubin-type pigments are present. Brown diffusible pigment is not produced on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, nitrate reduction and hydrogen sulfide production are negative. Voges–Proskauer reaction is positive for all strains. None of the strains has arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E), lipase (C14), α-chymotrypsin, trypsin, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase (API ZYM) activities. Weak enzymic activity is observed for esterase lipase (C8), cystine arylamidase and α-galactosidase, medium activity is observed for esterase (C4) and α-glucosidase, and strong activity is observed for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (API ZYM). Cells contain the fatty acids C_{15:0}, C_{15:0} iso, C_{15:1}ω6c, C_{16:0} iso, C_{16:0} iso 3-OH and summed feature 3 (which comprises C_{15:0} iso 2-OH and/or C_{16:1}ω7c) as the main constituents. DNA G+C content is 35.2–35.9 mol%. Isolated from microbial mats from Lake Fryxell (fresh/brackish) in the McMurdo Dry Valleys, Antarctica.

The type strain is LMG 22022^T (=CIP 108325^T).

Description of *Flavobacterium psychrolimnae* sp. nov.

Flavobacterium psychrolimnae (psy.chro'lim.nae. Gr. adj. *psychros* cold; Gr. fem. n. *limna* lake; N.L. gen. n. *psychrolimnae* of the cold lake).

Cells are Gram-negative, short rods (0.5 × 2.0 μm). Gliding motility is not observed. Grows at 5–25 °C, with optimal growth at 20 °C; weak growth is observed at 30 °C and no growth occurs at 37 °C. Yellow, convex, translucent colonies with entire margins and a diameter of 1–3 mm are formed on R2A plates after 6 days incubation. Colonies on agar of Anacker & Ordal (1955) are flat, round with entire margins and 0.5–1.0 mm in diameter after 14 days incubation. Growth also occurs on trypticase soy agar and nutrient agar; no growth is detected on marine agar. Colonies do not adhere to the agar. Growth occurs in 0–2% NaCl, but not in 5–10% NaCl. Aesculin, casein

and starch are degraded. Catalase and oxidase tests are positive. Growth is observed (API 20NE) on glucose, mannose and maltose, whereas no growth is detected on arabinose, mannitol, *N*-acetylglucosamine, gluconate, caprate, adipate, malate, citrate or phenylacetate. Acids are not produced from carbohydrates (API 20E). Agar, alginate, pectin, chitin, carboxymethyl-cellulose, DNA, gelatin, tyrosine and urea are not degraded. Congo red is not absorbed and no flexirubin-type pigments are present. Brown diffusible pigment is not produced on L-tyrosine agar and no precipitate is formed on egg-yolk agar. Tests for indole production, citrate utilization, nitrate reduction, Voges-Proskauer reaction and hydrogen sulfide production are negative. None of the strains has arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase (API 20E) and lipase (C14), α -galactosidase, β -galactosidase, β -glucuronidase, α -mannosidase or α -fucosidase (API ZYM) activities. Weak enzymic activity is observed for esterase (C4), esterase lipase (C8), cystine arylamidase, α -chymotrypsin, trypsin and β -glucosidase, medium activity is observed for *N*-acetyl- β -glucosaminidase, acid phosphatase, α -glucosidase and naphthol-AS-BI-phosphohydrolase, and strong activity is observed for alkaline phosphatase, leucine arylamidase and valine arylamidase (API ZYM). Cells contain the fatty acids C_{15:0}, C_{15:0} iso, C_{15:1} ω 6c, C_{16:0} iso, C_{16:0} iso 3-OH, C_{16:1} iso and summed feature 3 (C_{15:0} iso 2-OH and/or C_{16:1} ω 7c) as the main constituents. DNA G+C content is 33.8–34.5 mol%. Isolated from microbial mats from the freshwater lakes Fryxell (fresh/brackish) and Hoare in the McMurdo Dry Valleys, Antarctica.

The type strain is LMG 22018^T (=CIP 108326^T).

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