Flavobacterium weaverense sp. nov. and *Flavobacterium segetis* sp. nov., novel psychrophiles isolated from the Antarctic

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Two psychrophilic flavobacteria, designated AT1042^T and AT1048^T, were isolated from terrestrial samples from the Antarctic. Results of 16S rRNA gene sequence analyses indicated a close relationship of these isolates to *Flavobacterium flevense* (96.9 % similarity for strain AT1042^T) and *Flavobacterium psychrolimnae* (97.0 % for strain AT1048^T). Cells were non-motile and non-gliding. Flexirubin-type pigments were absent. Both isolates were psychrophilic, with an optimum and maximum growth temperature of about 15 and 20 °C, respectively. The major isoprenoid quinone, predominant cellular fatty acids and DNA G + C contents (35–37 mol%) were consistent with the placement of the Antarctic isolates in the genus *Flavobacterium*. Phylogeny based on 16S rRNA gene sequences and several phenotypic characteristics could be used to differentiate these isolates from recognized *Flavobacterium* species. Despite high 16S rRNA gene sequence similarity (98.9 %) between strains AT1042^T and AT1048^T, they represented two distinct species as demonstrated by low genomic relatedness (34 %) and a number of differential phenotypic characters. The polyphasic data presented in this study indicated that the new isolates should be classified within two novel species in the genus *Flavobacterium*. The names *Flavobacterium weaverense* sp. nov. (type strain AT1042^T = IMSNU 14048^T = KCTC 12223^T = JCM 12384^T) and *Flavobacterium segetis* sp. nov. (type strain AT1048^T = IMSNU 14050^T = KCTC 12224^T = JCM 12385^T) are proposed for these Antarctic isolates.

Two psychrophilic bacterial strains, designated AT1042^T and AT1048^T, were isolated from terrestrial samples from the Antarctic and subjected to a taxonomic study according to the minimal standards for describing novel taxa of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). On the basis of the polyphasic evidence presented, the Antarctic strains are considered to represent two distinct novel species in the genus *Flavobacterium*.

Strains AT1042^T and AT1048^T were isolated from soil samples collected on the Weaver Peninsula (62° 14' 07.8" S 58° 46' 33.3" W) and in a penguin habitat near the King Sejong Station (62° 12' 17.6" S 58° 47' 40.2" W), respectively, on King George Island, Antarctica. Isolation was carried out using marine agar 2216 (MA; Difco) at 10 °C following enrichment for 2 days in marine broth 2216 at 4 °C. The isolates were cultured routinely on R2A (Difco) at 15 °C and maintained as a glycerol suspension (20 %, w/v, in distilled water) at –80 °C.

The 16S rRNA gene was enzymically amplified from a single colony. Primers, PCR conditions and sequencing methods were as described by Chun & Goodfellow (1995). The sequences of strains AT1042^T and AT1048^T were aligned manually with representative sequences of the family *Flavobacteriaceae* obtained from GenBank. Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). Resultant tree topologies were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the jPHYDIT program (Jeon *et al.*, 2005) and PAUP 4.0 (Swofford, 1998) as described by Chun *et al.* (2000) and Kim *et al.* (2005). Almost complete 16S rRNA gene sequences of strains AT1042^T (1402 bp) and AT1048^T (1395 bp) were obtained. Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that the new isolates were related closely to the genus *Flavobacterium*. The newly determined sequences were then aligned manually against representatives of *Flavobacterium* species using bacterial 16S

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains AT1042^T and AT1048^T are AY581114 and AY581115, respectively.

rRNA gene secondary structure. The regions available for all sequences (positions 48–1468; *Escherichia coli* numbering system), excluding positions likely to show ambiguous alignment (positions 76–94), were used to generate phylogenetic trees. 16S rRNA gene sequence similarity between strains AT1042^T and AT1048^T was 98.9%, and the two Antarctic isolates were consistently recovered as a monophyletic clade (100% bootstrap support) within the genus *Flavobacterium* in all tree-making methods used in this study. Strain AT1042^T showed highest 16S rRNA gene sequence similarity to *Flavobacterium flevense* ATCC 27944^T (96.9%), followed by *Flavobacterium psychrolimnae* LMG 22018^T (96.7%), *Flavobacterium limicola* ST-82^T (96.6%) and *Flavobacterium saccharophilum* NCIMB 2072^T (96.5%). Strain AT1048^T showed highest 16S rRNA gene sequence similarity with *F. psychrolimnae* LMG 22018^T (97.0%), followed by *F. flevense* ATCC 27944^T (96.9%), *F. saccharophilum* NCIMB 2072^T (96.7%) and *F. limicola* ST-82^T (96.6%). In the phylogenetic trees generated in this study, strains AT1042^T and AT1048^T represented a distinct phyletic line corresponding to novel species in the genus *Flavobacterium* (Fig. 1).

Genomic relatedness between strains AT1042^T and AT1048^T was further examined using slot-blot DNA–DNA hybridization (Chun *et al.*, 1998) as these isolates shared high 16S rRNA gene similarity (98.9%), corresponding to 16 nt differences. The two isolates shared a low DNA–DNA relatedness value of 34%, which is well below the threshold (70%) recommended for determining bacterial species (Wayne *et al.*, 1987). It is clear from DNA–DNA hybridization experiments that strains AT1042^T and AT1048^T belong to separate genomic species.

Chemotaxonomic characteristics were determined from cultures grown at 15 °C on R2A. Menaquinone was isolated from 7-day-old cells according to the method of Minnikin *et al.* (1984) and analysed by HPLC (Waters) as described by Collins (1985). DNA G+C content was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989), by using a reversed-phase column (Supelco). Fatty acid methyl ester analysis was performed by GLC according to the Microbial Identification (MIDI) System using 5-day-old cells. The chemotaxonomic properties of the strains were consistent with those of the genus *Flavobacterium* and are given in the species descriptions below. The major fatty acids of the new isolates (Table 1) were similar to those of phylogenetically related species, although in slightly different proportions (Bernardet *et al.*, 1996; Tamaki *et al.*, 2003; Van Trappen *et al.*, 2005).

Growth on several bacteriological media was tested using Anacker and Ordal agar [AOA; 10 g Bacto agar (Difco), 0.5 g Bacto tryptone (Difco), 0.5 g yeast extract (Difco), 0.2 g sodium acetate, 0.2 g beef extract (Difco), per litre of distilled water], cetrimide agar (Difco), MacConkey agar (Difco), MA, nutrient agar (NA; Difco), R2A, trypticase soy agar (TSA; Difco) and sea-salt-free Zobell's agar [15 g Bacto agar, 5 g Bacto peptone (Difco), 1 g yeast extract, 0.1 g ferric citrate, per litre of distilled water]. Maximum growth was observed on R2A. Abundant growth was also seen on AOA, MA, NA, TSA and sea-salt-free Zobell's agar. No growth was observed on cetrimide or MacConkey agar. Temperature ranges for growth were determined in a temperature-gradient incubator (TVS126MA; Advantec) using R2A broth in the range 5–25 °C. To determine cardinal temperatures, the resultant data were fitted to the Ratkowsky

Fig. 1. Phylogenetic position of strains AT1042^T and AT1048^T within the genus *Flavobacterium*, based on 16S rRNA gene sequences. The tree was created using the neighbour-joining method; numbers at nodes are percentage bootstrap support (>50%) from 1000 resampled datasets. Solid circles indicate that the corresponding nodes (groupings) were also recovered in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. *Cellulophaga lytica* ATCC 23178^T (M62796) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

Table 1. Cellular fatty acid composition (% of total) of *Flavobacterium weaverense* sp. nov. AT1042^T and *Flavobacterium segetis* sp. nov. AT1048^T

Fatty acids that amounted to <1% of the total fatty acids in both strains were not included.

Fatty acid	AT1042 ^T	AT1048 ^T
iso-C _{14:0}	5.1	3.6
iso-C _{15:1} G*	3.2	4.2
anteiso-C _{15:1} A*	1.1	2.0
iso-C _{15:0}	3.2	4.9
anteiso-C _{15:0}	3.7	8.5
C _{15:1} ω6c	14.4	8.9
C _{15:0}	8.1	5.1
iso-C _{14:0} 3-OH	1.1	0.9
iso-C _{16:1} H*	11.8	7.0
iso-C _{16:0}	8.6	4.8
Summed feature 3†	5.7	13.5
C _{16:0}	0.8	1.3
iso-C _{15:0} 3-OH	2.8	4.5
iso-C _{17:1} ω9c	1.0	1.7
C _{15:0} 3-OH	1.5	1.5
C _{17:1} ω6c	8.0	7.1
iso-C _{16:0} 3-OH	14.8	11.0
C _{16:0} 3-OH	0.5	1.0
iso-C _{17:0} 3-OH	2.1	3.5

*The double-bond position indicated by capital letters is unknown.

†Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

temperature growth model (Ratkowsky *et al.*, 1983) by non-linear regressions using the R 1.8.1 statistical package (R Foundation for Statistical Computing, 2003). Square-root growth rate – temperature plots showed that the notional minimum, optimum and maximum growth temperatures were –29.7, 15.3 and 19.9 °C, respectively, for strain AT1042^T and –29.7, 14.3 and 18.9 °C, respectively, for strain AT1048^T (Fig. 2). When tested on R2A (between 5 and 25 °C at 5 °C intervals), both strains failed to grow at 25 °C. Following the definition of Isaksen & Jørgensen (1996), the Antarctic isolates can be defined as psychrophiles. The minimum doubling times of strains AT1042^T and AT1048^T were about 2.9 and 6.8 h, respectively. Growth at different pH (between pH 4 and 12 at intervals of 1 pH unit) and NaCl concentrations [between 0 and 7% (w/v) at intervals of 1%] was determined using sea-salt-free ZoBell's medium. KOH and HCl (both at 6 M) were used to adjust the final pH. Growth was also assessed under anaerobic (with 4–10% CO₂) and microaerobic (with 5–15% O₂ and 5–12% CO₂) conditions, using GasPak Plus and CampyPak Plus systems (BBL) at 15 °C for up to 1 month. Detailed results of these growth experiments are given in the species descriptions.

Fig. 2. Fitted Ratkowsky model of growth versus temperature data for strains AT1042^T and AT1048^T. $\sqrt{r}$ is the square-root of growth rate.

Morphological and physiological tests were performed using R2A as the basal medium at 15 °C. Cellular morphology and motility were examined by scanning electron microscopy and phase-contrast microscopy using 3-, 5- and 10-day-old cells. Gliding motility was observed by direct microscopic examination of the edge of colonies in exponential phase on AOA, R2A and CY agar [3 g casitone (Difco), 1 g yeast extract, 1 g CaCl₂·2H₂O, 40 g sea salts (Sigma), 15 g Bacto agar, per litre of distilled water] plates, and motility was observed by the hanging drop technique with cells in exponential phase in R2A and CY broth. The presence of flexirubin-type pigments was determined by flooding the agar plate or biomass with 20% (w/v) KOH and was then confirmed by measuring the absorbance spectrum of an ethanol and alkaline-ethanol extract of lysed cells (Weeks, 1981). Congo red adsorption was tested by directly flooding colonies on agar plates with 0.01% aqueous Congo red solution.

Standard physiological and biochemical tests were performed at 15 °C as described by Smibert & Krieg (1994). Hydrolysis of alginate (0.5%, w/v), casein [50% skimmed milk (Difco), v/v], carboxymethylcellulose (CM-cellulose) [0.5% CM-cellulose (Sigma), w/v], chitin (0.5% colloidal chitin, w/v), egg yolk (5%, w/v), elastin (0.5%, w/v), starch (0.2%, w/v), Tween 80 (1%, v/v) and L-tyrosine (0.5%, w/v) was tested using R2A as the basal medium. PEK7 agar (Reichenbach, 1992) and DNase test agar (Difco) were used for pectinase and DNase assays, respectively. Production of H₂S was investigated using triple-sugar iron agar (Difco). Phenylalanine deaminase activity was determined on phenylalanine agar (Smibert & Krieg, 1994; 3 g yeast extract, 1 g L-phenylalanine, 1 g Na₂HPO₄, 5 g NaCl, 12 g Bacto agar, per litre of distilled water). Alkaline reaction on Christensen's citrate was tested on Christensen citrate agar (Christensen, 1949). Arginine dihydrolase and urease activities were determined using Thornley's semi-solid medium

(Thornley, 1960) and Christensen urea agar (Christensen, 1946), respectively. Acid production from carbohydrates was examined for up to 1 month using modified O/F agar plates (Leifson, 1963; 1.0 g casitone, 0.1 g yeast extract, 0.5 g ammonium sulfate, 0.5 g Tris base, 0.01 g phenol red, 15 g Bacto agar, per litre of distilled water, adjusted to pH 7.0). Nitrate and nitrite reduction, production of indole, aesculinase, gelatinase and β -galactosidase and assimilation of sole carbon sources (glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenylacetate) were tested using the API 20NE kit (bioMérieux) and other enzyme activities were determined using the API ZYM kit (bioMérieux). The results of morphological, biochemical and physiological tests are given in Table 2 and in the species descriptions.

On the basis of 16S rRNA gene sequence analyses, the two Antarctic isolates are considered to represent an independent novel phyletic line in a clade encompassing different members of the genus *Flavobacterium*. These strains are further differentiated by a low level of genomic relatedness (34 %) and a number of phenotypic characters, namely colonial pigmentation, production of aesculinase, DNase, β -galactosidase, urease, arginine dihydrolase and α -fucosidase

Table 2. Characteristics that differentiate strains AT1042^T and AT1048^T from related *Flavobacterium* species

Taxa: 1, *F. weaverense* sp. nov. AT1042^T; 2, *F. segetis* sp. nov. AT1048^T; 3, *F. flevense*; 4, *F. psychrolimnae*; 5, *F. limicola*; 6, *F. saccharophilum*. +, Positive; –, negative; w, weakly positive; v, variable; ND, not determined; C, clear zone formed on egg-yolk agar. Data are taken from this study and from Bernardet *et al.* (1996), Tamaki *et al.* (2003) and Van Trappen *et al.* (2005).

Characteristic	1	2	3	4	5	6
Growth at 25 °C on agar	–	–	+	+	+	+
Congo red absorption	–	–	–	–	+	–
Growth on seawater media	+	+	+	–	–	–
Gliding motility	–	–	+	–	–	+
Flexirubin-type pigments	–	–	–	–	–	+
Acid from carbohydrates	+	+	+	–	–	ND
Degradation of:						
Gelatin	–	–	–	–	+	+
Casein	+	+	–	+	+	+
CM-cellulose	–	–	–	–	–	+
Agar	–	–	+	–	+	+
Pectin	–	–	+	–	–	+
Aesculin	–	+	+	+	+	+
DNA	–	+	–	–	–	–
Tyrosine	w	w	–	–	+	+
Brown pigment on tyrosine agar	w	w	–	–	+	–
Precipitate formed on egg-yolk agar	C	–	–	–	–	–
β -Galactosidase activity	–	+	+	+	–	+
H ₂ S production	–	–	–	–	–	+
Nitrate reduction	–	–	v	–	–	+

and acid production from lactose, D-trehalose, sucrose and D-cellobiose. Therefore, we conclude that strains AT1042^T and AT1048^T should be assigned to the genus *Flavobacterium* as the type strains of two separate novel species.

Description of *Flavobacterium weaverense* sp. nov.

Flavobacterium weaverense (wea.ver.en'se. N.L. neut. adj. *weaverense* pertaining to the Weaver Peninsula, the geographical origin of the type strain).

Gram-negative, oxidase- and catalase-positive and psychrophilic. Cells are non-motile long rods during the exponential phase (approx. 1.6–12.5 × 0.3–0.5 µm) and become short rods during the stationary phase (approx. 0.7–1.5 × 0.3–0.4 µm). Colonies are convex, translucent, glistening, viscid, yellow, circular with entire margins and become mucoid after prolonged incubation on R2A or AOA. Does not glide or adhere to agar plates. Flexirubin-type pigments are absent. Congo red is not adsorbed. Spores are not formed. Growth occurs on R2A, AOA, MA, NA and TSA, but not on cetrimide or MacConkey agar. Grows well under aerobic conditions, weakly under microaerobic conditions (with 5–15 % O₂ and 5–12 % CO₂, created by use of the CampyPack Plus system) and poorly under anaerobic conditions (with 4–10 % CO₂, created by use of the GasPack Plus system). Growth occurs at pH 6–12 (optimum pH 6–7) and with 0–3 % NaCl (optimum 1 %). Grows at 5.0–19.9 °C with notional minimum, optimum and maximum growth temperatures of –29.7, 15.3 and 19.9 °C, respectively. Minimum doubling time is 2.9 h. Decomposes starch and Tween 80, but not alginate, chitin or elastin. Positive reaction for urease. Negative reactions for L-phenylalanine deaminase, indole production and alkalization on Christensen's citrate agar. Test for arginine dihydrolase is positive on Thornley's semi-solid medium, but negative with the API 20NE kit. Produces acid from D-glucose and maltose, but not from D-cellobiose, D-fructose, D-mannitol, D-raffinose, D-salicin, D-trehalose, D-xylose, lactose, L-arabinose, L-rhamnose or sucrose. In API ZYM kits, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase, *N*-acetyl- β -glucosaminidase and α -fucosidase activities are present; weak cystine arylamidase and trypsin activities are present; and esterase (C4), lipase (C14), α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase and α -mannosidase activities are absent. Assimilates glucose, mannose, *N*-acetylglucosamine and maltose as sole carbon sources in API 20NE kits, but not arabinose, mannitol, gluconate, caprate, adipate, malate, citrate or phenylacetate. Other physiological and biochemical characteristics are given in Table 2. Maximum absorption peak of pigment is at 451 nm and the next shoulder is at 479 nm. Major isoprenoid quinone is MK-6. Predominant cellular fatty acids are C_{15:1}ω6c, iso-C_{16:1} H and iso-C_{16:0} 3-OH; the full fatty acid profile is given in Table 1. DNA G + C content of the type strain is 37 mol%.

The type strain, AT1042^T (=IMSNU 14048^T=KCTC 12223^T=JCM 12384^T), was isolated from a soil sample from the Weaver Peninsula, King George Island, Antarctica.

Description of *Flavobacterium segetis* sp. nov.

Flavobacterium segetis (se.ge'tis. L. gen. n. *segetis* of the soil).

Gram-negative, oxidase- and catalase-positive and psychrophilic. Cells are rod-shaped with rounded ends, approximately 1.1–2.3 × 0.2–0.3 µm and non-motile. Colonies are convex, translucent, glistening, butyrous, orange, circular with entire margins and become mucoid after prolonged incubation on R2A or AOA. Does not glide or adhere to agar plates. Flexirubin-type pigments are absent. Congo red is not adsorbed. Spores are not formed. Growth occurs on R2A, AOA, MA, NA and TSA, but not on cetrimide or MacConkey agar. Grows well under aerobic conditions, weakly under microaerobic conditions (with 5–15 % O₂ and 5–12 % CO₂ created by use of the CampyPack Plus system) and poorly under anaerobic conditions (with 4–10 % CO₂ created by use of the GasPack Plus system). Growth occurs at pH 6–11 (optimum pH 7) and with 0–3 % NaCl (optimum 0 %). Grows at 5.0–21.8 °C with notional minimum, optimum and maximum growth temperatures of –29.7, 14.3 and 18.9 °C, respectively. Minimum doubling time is 6.8 h. Decomposes starch and Tween 80, but not alginate, chitin or elastin. Negative reactions for arginine dihydrolase, urease, L-phenylalanine deaminase, indole production and alkalinization on Christensen's citrate agar. Produces acid from D-cellobiose, D-glucose, D-trehalose, maltose and sucrose, but not from D-fructose, D-mannitol, D-raffinose, D-salicin, D-xylose, L-arabinose or L-rhamnose. Produces acid from lactose after prolonged incubation (4 weeks). In API ZYM kits, alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and N-acetyl-β-glucosaminidase activities are present; weak esterase lipase (C8), cystine arylamidase, trypsin and β-glucosidase activities are present; and esterase (C4), lipase (C14), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase activities are absent. Assimilates glucose, mannose, N-acetylglucosamine (weakly) and maltose as sole carbon sources in API 20NE kits, but not arabinose, mannitol, gluconate, caprate, adipate, malate, citrate or phenylacetate. Other physiological and biochemical characteristics are given in Table 2. Maximum absorption peak of pigment is at 472 nm and the next shoulder is at 452 nm. Major isoprenoid quinone is MK-6. Predominant cellular fatty acids are iso-C_{16:0} 3-OH and summed feature 3 (containing C_{16:1}ω7c and/or iso-C_{15:0} 2-OH); the full fatty acid profile is given in Table 1. DNA G + C content of the type strain is 35 mol%.

The type strain, AT1048^T (=IMSNU 14050^T=KCTC 12224^T=JCM 12385^T), was isolated from a soil sample of a penguin habitat near the King Sejong Station on King George Island, Antarctica.

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