Flavobacterium antarcticum sp. nov., a novel psychrotolerant bacterium isolated from the Antarctic

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A yellow-pigmented, Gram-negative and aerobic bacterial strain, designated AT1026^T, was isolated from a terrestrial sample from the Antarctic. Results of 16S rRNA gene sequence analysis indicated that the Antarctic isolate belonged to the genus *Flavobacterium*, with the highest sequence similarity to *Flavobacterium tegetincola* (96.4%). Cells were non-motile, non-gliding and psychrotolerant, with optimum and maximum temperatures of about 20 and 25 °C. Flexirubins were absent. The major isoprenoid quinone (MK-6), predominant cellular fatty acids (iso-C_{15:1} G, iso-C_{15:0} and a mixture of C_{16:1} ω7c and/or iso-C_{15:0} 2-OH) and DNA G + C content (38 mol%) of the Antarctic isolate were consistent with those of the genus *Flavobacterium*. In contrast, several phenotypic characters can be used to differentiate this isolate from other flavobacteria. The polyphasic data presented in this study indicated that this isolate should be classified as a novel species in the genus *Flavobacterium*. The name *Flavobacterium antarcticum* sp. nov. is therefore proposed for the Antarctic isolate; the type strain is AT1026^T (= IMSNU 14042^T = KCTC 12222^T = JCM 12383^T).

A yellow-pigmented bacterial strain, designated AT1026^T, was isolated from a terrestrial sample from the Antarctic and was the subject of taxonomic study according to the minimal standards for describing new taxa of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). On the basis of polyphasic evidence, the Antarctic strain represents a novel species in the genus *Flavobacterium*, for which the name *Flavobacterium antarcticum* sp. nov. is proposed.

A soil sample was collected from a penguin habitat near the King Sejong Station on King George Island, Antarctica (62° 14' 01.2" S 58° 46' 47.4" W). 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 isolate was cultured routinely on R2A (Difco) at 15 °C and maintained as a glycerol suspension (20%, w/v) at –80 °C.

16S rRNA genes were enzymically amplified from a single colony. Primers, PCR conditions and sequencing methods were described elsewhere (Chun & Goodfellow, 1995). The

sequence of strain AT1026^T was 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, 1972) 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 (available at <http://chunlab.snu.ac.kr/jphydit>) and PAUP 4.0 (Swofford, 1998) as described previously (Chun *et al.*, 2000). An almost complete 16S rRNA gene sequence of strain AT1026^T (1406 bp) was obtained. Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that our isolate was related closely to the genus *Flavobacterium*. The newly determined sequence was then aligned manually against representatives of *Flavobacterium* species using bacterial 16S rRNA secondary structure. The regions available for all sequences (positions 46–1477; *Escherichia coli* numbering system), excluding positions likely to show ambiguous alignment (positions 76–94), were used to

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Fig. 1. Phylogenetic position of strain AT1026^T within the genus *Flavobacterium*, based on 16S rRNA gene sequences. The tree was created using the neighbour-joining method; percentages at nodes are levels of 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 nt substitution per position.

construct the phylogenetic trees. Based on 16S rRNA gene sequence similarity, the Antarctic isolate shared 91.3–96.4% similarity with members of the genus *Flavobacterium* and the closest bacterial relatives with validly published names were *Flavobacterium tegetincola* (96.4%), *Flavobacterium flevense* (95.9%), *Flavobacterium micromati* (95.6%) and *Flavobacterium xinjiangense* (95.5%). Strain AT1026^T and *F. tegetincola* formed a robust (96% bootstrap value) monophyletic clade (Fig. 1) in all trees inferred in this study, and formed a further monophyletic clade with *F. flevense* and *Flavobacterium johnsoniae* in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. On the basis of our phylogenetic analysis, it is evident that our isolate is a member of the genus *Flavobacterium* and represents a novel genomic species.

Growth on several bacteriological media was tested; Anacker and Ordal agar [AOA; Bacto agar (Difco), 10 g; Casitone (Difco), 0.5 g; yeast extract (Difco), 0.5 g; sodium acetate, 0.2 g; beef extract (Difco), 0.2 g; distilled water, 1 l], cetrimide agar (Difco), MacConkey agar (Difco), MA, nutrient agar (NA; Difco), R2A, tryptic soy agar (TSA; Difco) and sea-salt-free Zobell's agar [Bacto agar, 15 g; Bacto peptone (Difco), 5 g; yeast extract, 1 g; ferric citrate, 0.1 g; distilled water, 1 l]. Maximum growth was observed on R2A and abundant growth was observed on AOA, MA, NA, TSA and sea-salt-free Zobell's agar. No growth was observed on cetrimide or MacConkey agar. The temperature range for growth was determined in a temperature-gradient incubator (TVS 126MA; Advantec) using R2A broth in the range 5–35 °C (5.0, 9.7, 12.4, 15.1, 17.4, 19.6, 21.8, 24.1, 26.6, 28.9, 31.9 and 35.0 °C). To determine cardinal temperatures, the resultant data were fitted to the Ratkowsky temperature growth model (Ratkowsky *et al.*, 1983) by non-linear regression using the R 1.8.1 package (R

Foundation for Statistical Computing, 2003). Square-root growth rate–temperature plots showed that the notional minimum, optimum and maximum growth temperatures were respectively –14.5, 21.2 and 25.1 °C (Fig. 2). When tested on R2A (between 5 and 35 °C at 5 °C intervals), strain AT1026^T grew at 25 °C, but not at 30 °C. From the definition of Isaksen & Jørgensen (1996), our Antarctic isolate can be defined as a psychrotolerant bacterium. The minimum doubling time of strain AT1026^T was about 4.9 h. Growth at different pH (between pH 4 and 12 with an interval of 1) and NaCl concentrations [between 0 and 7% (w/v) at 1%

Fig. 2. Fitted Ratkowsky model of growth versus temperature data for strain AT1026^T. $\sqrt{r}$ is the square-root of growth rate. The minimum, optimum and maximum temperatures are respectively –14.5, 21.2 and 25.1 °C.

intervals] was determined using sea-salt-free ZoBell's medium. KOH and HCl (both at 6 M) were used to adjust the final pH. Anaerobic and microaerophilic growth was checked 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. The results of tests for growth conditions are given in the species description.

Morphological and physiological tests were performed using R2A as the basal medium at 15 °C. Cellular morphology and motility were examined by SEM 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 [casitone (Difco), 3 g; yeast extract, 1 g; CaCl₂·2H₂O, 1 g; sea salts (Sigma), 40 g; Bacto agar, 15 g; distilled water, 1 l] plates, and motility was observed by the hanging drop technique for 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 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 previously (Smibert & Krieg, 1994). Hydrolysis of alginate (0.5 %, w/v), casein [50 % skimmed milk (Difco), v/v], carboxymethyl cellulose [0.5 % carboxymethyl 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, 1991) and DNase test agar (Difco) were respectively used for pectinase and DNase assays. Production of H₂S was investigated using triple-sugar iron agar (Difco). Phenylalanine deaminase activity was determined on phenylalanine agar (Smibert & Krieg, 1994; yeast extract, 3 g; L-phenylalanine, 1 g; Na₂HPO₄, 1 g; NaCl, 5 g; Bacto agar, 12 g; distilled water, 1 l). 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; casitone, 1.0 g; yeast extract, 0.1 g; ammonium sulfate, 0.5 g; Tris base, 0.5 g; phenol red, 0.01 g; Bacto agar, 15 g; distilled water, 1 l; adjusted to pH 7.0). Nitrate and nitrite reduction, indole production, aesculinase, gelatinase, 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 enzymic activities were determined using the API ZYM kit

Table 1. Characteristics that differentiate strain AT1026^T from other related species

Species: 1, *F. antarcticum* sp. nov. AT1026^T; 2, *F. tegetincola*; 3, *F. flevense*. +, Positive; –, negative; C, clear zone formed on egg-yolk agar. Data from this study and from Bernardet *et al.* (1996) and McCammon & Bowman (2000). All taxa are positive for growth on sea-water media and formation of acid from carbohydrates.

Characteristic	1	2	3
Gliding	–	+	+
Glucose utilization	–*	+	+
Degradation of:			
Gelatin	+	–	–
Casein	+	–	–
Agar	–	–	+
Pectin	–	–	+
Aesculin	–	–	+
DNA	+	–	–
Precipitate formed on egg-yolk agar	C	–	–
β -Galactosidase activity	–	–	+

*On API 20NE test strip.

(bioMérieux). The results of morphological, biochemical and physiological tests are given in Table 1 and the species description.

Chemotaxonomic and genotypic characteristics were determined from cultures grown at 15 °C on R2A. Menaquinone was isolated from 7-day-old cells according to 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), using a reverse-phase column (Supelco). Fatty acid methyl esters analysis was performed by GLC according to the Microbial Identification (MIDI) System using 5-day-old cells. The chemotaxonomic properties of the test strain are consistent with those of the genus *Flavobacterium* and are given in the species description. The major fatty acid composition of our isolate is similar to those of phylogenetically related species, but differs somewhat from them in quantities (Bernardet *et al.*, 1996; McCammon & Bowman, 2000).

In the phylogenetic trees, our Antarctic isolate clearly belongs to the genus *Flavobacterium* and forms a distinct phyletic line with low 16S rRNA gene sequence similarity (96.8 %), which indicates that it represents a novel genomic species (Stackebrandt & Goebel, 1994). Moreover, a number of phenotypic characteristics (Table 1) readily differentiate the Antarctic strain from other related *Flavobacterium* species. The polyphasic data obtained in this study clearly show that the test strain merits novel species status within the genus *Flavobacterium*. The name *Flavobacterium antarcticum* sp. nov. is therefore proposed for strain AT1026^T.

Description of *Flavobacterium antarcticum* sp. nov.

Flavobacterium antarcticum (ant.arc'ti.cum. L. neut. adj. *antarcticum* southern, and, by extension, pertaining to Antarctica).

Gram-negative, oxidase- and catalase-positive and psychrotolerant. Cells are rod-shaped with rounded ends, approximately $0.5\text{--}1.3 \times 0.3\text{--}0.4\ \mu\text{m}$ and non-motile. Colonies are convex, translucent, glistening, butyrous, yellow, circular with entire margins and becoming mucoid after prolonged incubation on R2A and AOA. Does not glide or adhere to agar plates. Flexirubin-type pigment is 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. Growth is aerobic. Grows weakly under microaerobic conditions (with about 5–15 % O_2 and 5–12 % CO_2 created by CampyPak Plus system) and poorly under anaerobic conditions (with about 4–10 % CO_2 created by GasPak Plus system). Growth occurs at pH 6–10 (optimum pH 7) and 0–4 % NaCl (optimum 0 %). Grows at $5\text{--}24.1\ ^\circ\text{C}$, with notional minimum, optimum and maximum growth temperatures of -14.5 , 21.2 and $25.1\ ^\circ\text{C}$. Minimum doubling time is 4.9 h. Decomposes Tween 80, but not alginate, chitin, carboxymethyl cellulose, elastin, starch or tyrosine. Produces brown pigment weakly on tyrosine agar. Positive reaction for arginine dihydrolase. Negative reactions for nitrate reduction, urease, L-phenylalanine deaminase, H_2S production, indole production and alkalization on Christensen's citrate agar. 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. Alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive; trypsin, α -glucosidase and N-acetyl- β -glucosaminidase are weakly positive; esterase (C4), lipase (C14), cystine arylamidase, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase are negative in API ZYM kits. Cannot assimilate any of the compounds contained in API 20NE kits as sole carbon sources. Other physiological and biochemical characteristics are given in Table 1. Maximum absorption peak of pigment is at 452 nm and the next shoulder peak is at 479 nm. Major isoprenoid quinone is MK-6. Predominant cellular fatty acids are iso- $\text{C}_{15:1}$ G (15.3 %; the double bond position is unknown), iso- $\text{C}_{15:0}$ (15.8 %) and $\text{C}_{16:1}\omega 7c$ and/or iso- $\text{C}_{15:0}$ 2-OH (10.9 %; the two fatty acids cannot be separated by GLC with the MIDI system). Smaller amounts of iso- $\text{C}_{14:0}$ (2.6 %), $\text{C}_{14:0}$ (2.1 %), anteiso- $\text{C}_{15:1}$ A (3.3 %; double bond position unknown), anteiso- $\text{C}_{15:0}$ (6.7 %), $\text{C}_{15:1}\omega 6c$ (1.5 %), $\text{C}_{15:0}$ (8.2 %), iso- $\text{C}_{16:1}$ H (3.2 %; double bond position unknown), iso- $\text{C}_{16:0}$ (4.1 %), iso- $\text{C}_{15:0}$ 3-OH (6.2 %), iso- $\text{C}_{17:1}\omega 9c$ (1.7 %), anteiso- $\text{C}_{17:1}\omega 9c$ (1.4 %), iso- $\text{C}_{16:0}$ 3-OH (4.5 %), $\text{C}_{16:0}$ 3-OH (2.2 %), $\text{C}_{18:1}\omega 5c$ (1.0 %) and iso- $\text{C}_{17:0}$ 3-OH (3.0 %) are also present. DNA G+C content is 38 mol%.

The type strain, AT1026^T (=IMSNU 14042^T=KCTC 12222^T=JCM 12383^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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