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Flavobacterium frigidarium sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica

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A psychrophilic, aerobic bacterium designated *A2i*^{T} was isolated from marine sediment recovered from shallow waters surrounding Adelaide Island, Antarctica (67° 34′ S, 68° 07′ W). The organism exhibited xylanolytic and laminarinolytic activity and was halotolerant. Basic characterization showed that it was Gram-negative, non-motile, yellow-pigmented (*β,β*-carotene-3,3′-diol) and positive for oxidase and catalase synthesis. Analysis of the 16S rDNA sequence suggests that the organism belongs to the *Flexibacter–Cytophaga–Bacteroides* phylum. On the basis of its 16S rDNA sequence, the bacterium is 96·8% similar to *Flavobacterium columnare* ATCC 43622 – its closest relation. The genomic DNA G+C content was 35 mol%. Growth on xylan occurs optimally at 15 °C, though growth also occurs at 0 °C, and the doubling times are 9·6 and 34·8 h, respectively. The maximum growth temperature on xylan is at 24 °C. The bacterium is a neutrophile, growing across the pH range 5·6–8·4 and having an optimum at pH 7·5. Analysis of the 16S rDNA sequence, together with phenotypic characterization, suggests that the organism is a member of the genus *Flavobacterium*. DNA–DNA hybridization experiments have shown that it is a novel species; it is proposed, therefore, that the organism be designated as the type strain of *Flavobacterium frigidarium* sp. nov. (= ATCC 700810^T = NCIMB 13737^T).

Keywords: *Flavobacterium frigidarium*, psychrophilic, xylanolytic, laminarinolytic, halotolerant

INTRODUCTION

Psychrophilic bacteria have been isolated from environments such as Antarctica and deep-sea sediments (Feller *et al*., 1996). They are interesting because of their physiological adaptations, which enable them to survive at low temperatures (below 5 °C). These adaptations include cold-shock proteins, polyunsaturated branched-chain fatty acids in the cytoplasmic membrane (Russell & Nichols, 1999) and more efficient enzymes (Feller *et al*., 1996). The hydrolysis of organic polymers at low temperatures has received little attention until recently (Mountfort *et al*., 1997). This is despite the fact that carbohydrase-producing organisms must play an important role in the cycling of organic carbon in cold environments. To date, only one carbohydrase enzyme – an α-amylase from a psychrophilic bacterium – has been thoroughly characterized. This enzyme exhibited adaptive features, such as greater activity at 4 °C and increased thermolability relative to its mesophilic counterparts (Feller *et al*., 1994).

This study on the hydrolysis of cellulose, pectin, laminarin and xylan among psychrophilic marine bacteria has led to the isolation of a psychrophilic, xylanolytic and laminarinolytic bacterium (strain *A2i*^{T}). Xylan is the major hemicellulose in macroalgae and plant cell walls; it is hydrolysed by endo-*β*1,4-xylanases, which can be classified as either glycosyl hydrolase family 10 or family 11 enzymes (Charnock *et al*., 1997). Laminarin is a storage polymer of *Phaeophyta* (brown marine algae) and is an essentially

The GenBank accession number for the 16S rDNA sequence of *Flavobacterium frigidarium* is AF162266.

linear polymer of (1→3)-β-glucosidic linkages joining glucose residues, often terminating with D-mannitol.

Many mesophilic and thermophilic xylanases have been investigated and cloned, but there are few reports of xylanolytic activity in an aerobic psychrophilic bacterium. There have been no reports, to our knowledge, of an aerobic, laminarinolytic psychrophilic bacterium. The xylanase activity has the characteristic properties of cold active enzymes, e.g. a greater catalytic activity at temperatures below 20 °C and a much reduced thermostability at 37 °C and above, relative to the xylanases of the mesophile *Pseudomonas fluorescens* subsp. *cellulosa* (Humphry *et al.*, 1999). Enzyme activity analysis has also shown that the laminarinase has similar properties and so can be described as a psychrophilic enzyme (D. R. Humphry, unpublished data).

The isolate was examined on the basis of its phenotypic and genotypic properties. Phenotypic characteristics of the bacterium suggested that it was a member of the *Flavobacterium* genus. Sequence analysis and DNA–DNA hybridization of the 16S rDNA showed that its closest relation, *Flavobacterium columnare*, was too distant to be the same species. It is proposed that *A2i*^{T} be designated as the type strain of *Flavobacterium frigidarium* sp. nov.

METHODS

Sampling method. Samples of shallow-water marine sediment were collected off Adelaide Island, Antarctica (67° 34′ S, 68° 07′ W). Samples were prepared for transport to the UK by lyophilization (2 g) and sealed in an airtight container, and were imported to the UK under Ministry of Agriculture and Fisheries licence PHL 130/2653 (4/1998).

Methods for enrichment and isolation. Approximately 1 g lyophilized sediment was inoculated into a 50 ml volume of xylan minimal medium (XMM) in a 250 ml Erlenmeyer flask. XMM is minimal medium (MM) containing the following (w/v): 0·002% $FeSO_4$, 0·02% $MgSO_4$, 0·075% KNO_3, 0·05% K_2HPO_4 and 0·004% $CaCl_2$, plus 0·5% soluble xylan. The pH was adjusted to 7·2 with 1 M NaOH or 1 M HCl. The solid medium was prepared by the addition of 1·5% (w/v) Oxoid technical agar no. 3. The media were autoclaved at 121 °C for 15 min. Enrichment broths were incubated at 4 °C for 3 weeks on an orbital shaker (150 r.p.m.). The enrichment culture was streaked onto XMM agar plates, which were then incubated at 4 °C for 10 d. Individual colonies were picked off and inoculated into 50 ml XMM broth; this process was repeated until pure cultures were obtained.

Xylanase activity was determined by staining of the XMM plates with 1% (w/v) Congo red and destaining with 1 M NaCl solution, which reveals haloes around xylanolytic colonies. Xylanase and laminarinase activities were quantified using the dinitrosalicylic acid assay (Miller, 1959) on supernatants from XMM and carboxymethyl-curdlan (Megazyme) MM *A2i*^{T} cultures, respectively.

Basic characterization. Standard bacteriological methods were use throughout. Colonies from XMM agar were Gram-stained and examined by light-microscopy. Production of hydrogen sulphide was tested on slopes and stabs of triple-sugar iron agar (CM277; Oxoid) and Kligler iron agar (CM33; Oxoid). DNase agar was made and DNase activity was tested for using the protocol described previously (Bridson, 1998). Egg-yolk agar was made using egg-yolk emulsion (SR47; Oxoid).

Acid- and gas production from glucose, fructose, xylose, mannitol and maltose were tested with incubation at 15 °C for 10 d and checks every day. Growth on MacConkey agar (CM7; Oxoid) was tested for at 4 and 15 °C. Colony morphology on Anacker–Ordal agar (AOA) was determined (Bernadet *et al.*, 1996), and extracellular galactosamine glycan production was detected using Congo red. Growth of isolate *A2i*^{T} on nutrient agar (NA) (CM3; Oxoid), trypticase soy agar (TSA) (CM 131; Oxoid), seawater agar (medium 246; DSMZ) and in seawater Lemco broth (medium 627; DSMZ) was tested. Growth on MacConkey agar, NA, TSA, in MM and XMM broths was also determined for the *Flavobacterium* species that were most similar on the basis of their 16S rDNA sequences.

The substrate-utilization profile of the bacterium was determined in triplicate at 2 and 10 °C in MM containing 0·5% (w/v) substrate. Certain substrates were added, after sterilization, by inoculating filter-sterilized stock solutions to the required concentration, to prevent caramelization during autoclaving. Carboxymethyl-cellulose (CM-cellulose) and soluble xylan were added as solids, as these could be autoclaved. Agar degradation was tested on MM agar at 1·5% (w/v). Pullulanase activity was tested for by incorporating 0·5% (w/v) red pullulan (Megazyme) and an identical concentration of purified soluble starch in to MM broth agar plates. Endo-1,3-β-glucanase (laminarinase) activity was tested for by adding 0·5% (w/v) Azcl-curdlan (Megazyme) to MM broth agar according to the manufacturer's instructions. The cultures were incubated at 2 and 10 °C for 2 weeks.

Duplicate API 20NE and API ZYM identification strips were set up as instructed by the manual and incubated at 15 °C for 3 d. API ZYM strips were set up for isolate *A2i*^{T} and for the closest valid *Flavobacterium* species. The flavobacteria tested were grown up on NA at 25 °C. *A2i*^{T} was grown up on NA and AOA at 15 °C for two sets of API ZYM tests. All of the strips were incubated at 15 °C for 15 h then developed and read.

Pigment characterization. Pigment analysis was performed using the KOH test (Fautz & Reichenbach, 1980) and confirmatory testing was done by pigment extraction (Jagannadham *et al.*, 1991), followed by TLC. The mobile phase was 2% (v/v) acetone and 98% light petroleum ether, the stationary phase was silica, and the bands were visualized in an iodine bath. For tentative identification of the pigment, it was necessary to do a spectrophotometric analysis for comparison with published λ_{max} values (Schmidt *et al.*, 1994).

Antibiotic-sensitivity tests. Duplicate antibiotic-sensitivity tests were done using filter-paper disks containing the following: chloramphenicol (25 μg), erythromycin (5 μg), fusidic acid (10 μg), methicillin (10 μg), novobiocin (5 μg), penicillin G (1 U), streptomycin (10 μg) (Mast Diagnostics), tetracycline (10 μg) and ampicillin (25 μg) (Sigma). Disks were placed on Luria–Bertani (LB) plates spread with *A2i*^{T} culture and were then incubated at 15 °C for 7 d.

Growth was tested against various dissolved antibiotics (see Results) in duplicate at four different concentrations. The appropriate volumes of antibiotic stocks (2 mg ml^{-1}) were

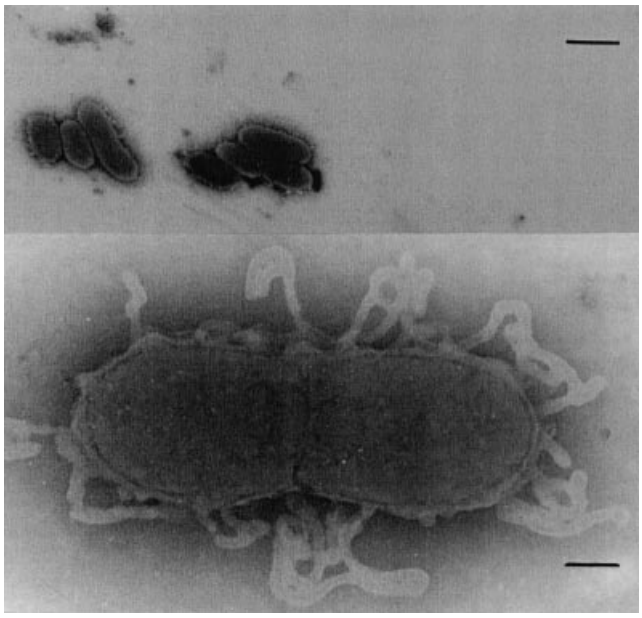

Fig. 1. Negative-stain transmission electron micrographs of *Flavobacterium frigidarium* cells in exponential growth phase at 10 °C: (top) a group of cells (bar, 1 μm); (bottom) dividing encapsulated cell (bar, 0·167 μm).

added to LB agar (pH 7·2) to give final volumes of 20 ml. A discrete *A2i*^{T} colony (grown at 2 °C on XMM agar), was then spread onto each plate. The cultures were incubated at 2 and 15 °C for 14 d.

Temperature, pH, and salinity limits. The specific growth rate of the bacterium was determined in XMM broth at between 0 and 25 °C. The optimal growth temperature was determined by obtaining the mean specific growth rates of three independent cultures grown at each temperature. Growth was monitored by optical density at 660 nm using a spectrophotometer (LKB).

The growth rate over the pH range 4·25–9·68 was determined, in duplicate, at a temperature of 15 °C in LB broth supplemented with an appropriate buffer. The buffers used were as follows: acetate buffer, pH 4·25–6; PIPES buffer, pH 5·5–7·5; Tris, pH 7–9; and Na_2CO_3 pH 8·5–9·68. The pH values of the growth media were checked both after cell inoculation and each time a sample was taken for absorbance testing. A 400 μl volume of bacterial cells was used to inoculate each culture; these were obtained from an XMM culture grown up at 2 °C and washed twice and resuspended in 50 mM buffer at the appropriate pH.

Growth in up to 10 % (w/v) sodium chloride was determined in triplicate, at 2 °C in xylose minimal medium (XeMM; MM broth containing 0·5 %, w/v, xylose) in Anacker–Ordal broth (AOB) and in LB broth.

16S rDNA gene PCR amplification method. The 16S rDNA primers used to obtain the initial PCR product were identical to the *Escherichia coli* 16S rDNA sequence, between positions 8 and 27 (forward) and 1509 and 1491 (reverse). These primers were 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 5′-GGH TAC CTT GTT ACG ACT T-3′, respectively. Each PCR reaction contained 1 × *Taq* buffer, 2·5 U *Taq* DNA polymerase, 0·2 mM of the four dNTPs (all from Life Technologies), 1 μg isolate genomic DNA and 0·5 μM each primer. The PCR was done in the presence of 1, 2, 3 and 4 mM $MgCl_2$ (Life Technologies). The PCR conditions used were as follows: one cycle at 95 °C for 3 min; 35 cycles at 95 °C for 2 min; 1 min at 35, 38, 42 or 46 °C (the specific primer annealing temperature); 4 min at 72 °C; then 20 min at 72 °C.

Cloning and sequencing of the PCR products. The PCR products from two of the 16 PCR reactions (for sequence comparison) were cloned into the pGEM-T 'easy' plasmid (Promega). Insert cloning efficacy was checked and plasmid DNA was extracted from two bacterial clones by using a micro-spin column (Qiagen). Each clone carried a PCR product originating from different PCR reactions. The two plasmid inserts were then sequenced completely, using a DNA sequencer (PE Biosystems). This was done by using the forward and reverse primers for the pGEM-T plasmid, initially; subsequently, oligonucleotide primers were built onto the ends of the resulting sequences. These oligonucleotide primers were 5′-GCC GCG TGC AGG ATG AC-3′, 5′-GAC ATG ACT CGC TGG CAA CT-3′ and 5′-GTG GCT AAG CGA AAG TGA TAA GTA-3′, 5′-GGC CGT TCC ACC GTT GAG C-3′, respectively. The sequences from the two clones matched, although they were in opposite orientations.

16S rDNA sequence analysis. The 16S rDNA sequence of the bacterium was 1391 bases long, with no non-base characters. Its seven closest relatives were individually aligned against it, all columns containing non-base characters were removed, and then the percentage similarities were calculated. The sequence was also aligned against 19 other sequences at once, using the CLUSTAL method in MEGALIGN program (DNAStar), and then all non-base characters were removed manually. This reduced the lengths of the sequences analysed by the PHYLIP and MEGALIGN programs to 1136 bases. The 19 16S rDNA sequences were taken from GenBank and the Ribosomal Database Project (RDP; http://www.cme.msu.edu/RDP/html/index.html). GenBank accession numbers or strain numbers are shown on the dendrogram (see Fig. 2).

Computer programs used for phylogenetic analysis included SEQBOOT (1000 replicates), DNADIST (Jukes & Cantor), NEIGHBOUR (neighbour-joining), CONSENSE (Felsenstein, 1993), DNAPARS (parsimony analysis) and TREEVIEW (Page, 1996). The conserved node frequency data were added to the dendrogram produced by NEIGHBOUR for the original molecular sequence data. Reliable nodes were also found by doing maximum-likelihood, Jin & Nei and Kimura two-parameter analyses followed by neighbour-joining (Saitou & Nei, 1987) on the original data, as well as by producing a consensus tree of 1000 replicates by using the normal parsimony method. Nodes that occurred in these trees and in the Jukes & Cantor dendrogram were marked (see Fig. 2).

Genomic DNA G+C content and DNA–DNA hybridizations. The genomic DNA of the bacterium was extracted using a microcentrifuge separation column (Qiagen). The G+C content was determined in triplicate, using a spectrophotometer (LKB) and a thermal denaturation method (Marmur & Doty, 1962). The DNA was isolated by chromatography (Cashion *et al*., 1977), then DNA–DNA hybridization was performed as described previously (De Ley *et al*., 1970), but with a modification (Huss *et al*., 1983; Escara & Hutton, 1980). The procedure was performed on a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Finally, renaturation rates were computed using the TRANSFER.BAS program (Jahnke, 1992).

Transmission electron microscopy. A sample of *A2i*^{T} culture in exponential growth phase was negatively stained with

0·7% sodium silicotungstate solution (Agar Scientific), as described by Brenner & Horne (1959), and then examined.

Membrane fatty acid identification. Fatty acid methyl esters were prepared from freeze-dried cells (grown up in XMM broth at 15 °C) by transmethylation (Komagata & Suzuki, 1987). They were analysed by GC (UNICAM 610) using a 25 m BPX70 (SGE) column and a temperature programme of 100–240 °C. Split injection was used along with a flame-ionization detector; the injector and the detector were maintained at 280 °C. The analyses were performed in triplicate on three independent cultures. Individual fatty acids were identified by using authentic standards, and identities were confirmed by GC-MS.

RESULTS

Morphological properties

Colonies of *A2i*^{T} were bright yellow, raised and had an entire edge on XMM agar; the colonies on AOA, however, were flat, round and entire. Micro-morphological properties included the presence of a capsule and pili but no flagella (Fig. 1). The cells are 0·8–2 μm long and 0·5–0·7 μm wide, they occur singly or in pairs and they are non-motile. Cell morphology ranged from coccoid-like cells to fat, medium-length rods. The cells stained Gram-negative and no evidence of spores was detected on any growth medium.

Physiological characteristics

Isolate *A2i*^{T} was a psychrophilic, neutrophilic, aerobic, xylanolytic and laminarinolytic bacterium. It was also capable of growth in AOB containing from 0 to 5% (w/v) NaCl, inclusive, in XeMM containing from 0 to 4% (w/v) NaCl, inclusive, and in LB containing from 0 to 9% (w/v) NaCl, inclusive. Catalase and cytochrome oxidase tests on distinct colonies were positive. There was no nitrate reduction, no indole production and no arginine dihydrolase, urease, DNase or *β*-galactosidase activity was detected. The bacterium

Table 1. Some of the phenotypic characteristics of *A2i*^{T}

+, All strains positive; −, all strains negative; (+), weakly positive; v, variable among strains; V, variable among references; ND, not determined. For ease of comparison with other validly published *Flavobacterium* species, the data are presented in the same format as that in Bernadet *et al*. (1996).

Characteristic	Isolate *A2i*^{T}
Anacker–Ordal agar colony morphology	Flat, round, with entire margins
Congo red absorption*	+
Growth on seawater media	+
Growth on nutrient agar	+
Growth on trypticase soy agar	+
Gliding motility	−
Flexirubin pigment type†	−
Glucose used as sole carbon and energy source	+
Acid produced aerobically from carbohydrates	−
Degradation of:	
Gelatin	+
Casein	+
Starch	−
CM-cellulose	−
Agar	−
Pectin	−
Chitin	−
Aesculin	+
Deoxyribonucleic acid	−
Tyrosine	−
Brown diffusible pigment produced on tyrosine agar	−
Precipitate formed on egg-yolk agar	−
β-Galactosidase activity‡	−
Hydrogen sulphide production	−
Cytochrome oxidase activity	+
Nitrate reduction	−

* Congo red absorption revealed the production of an extracellular galactosamine glycan.

† Flexirubin pigments were detected by using a 20% (w/v) KOH aqueous solution.

‡ Determined by the ONPG test.

Table 2. API ZYM profiles and other test results for isolate *A2i*^{T} and the strains of the validly described *Flavobacterium* species that appear to be most related on the basis of 16S rDNA sequence analysis

The values given are API ZYM scores: 0, no activity; 1, least activity; 5, most activity. +, Growth; −, no growth; NA, not applicable. For all strains, scores were 5 with 2-naphthyl-phosphate, and 0 with 2-naphthyl-butyrate, 2-naphthyl-myristate, *N*-benzoyl-DL-arginine-2-naphthylamide, naphthol-AS-BI-*β*-D-glucoronide, 6-Br-2-naphthyl-α-D-mannopyranoside and 2-naphthyl-α-L-fucopyranoside. All strains grew on nutrient agar and trypticase soy agar.

Test	Isolate *A2i*^{T}	*F. columnare* ATCC 43622	*F. johnsoniae* NCIMB 10150	*F. saccharophilum* NCIMB 2072^T	*F. hydatis* ATCC 29551^T
Substrate for hydrolysis					
2-Naphthyl-caprylate	2	2	1	1	1
L-Leucyl-2-naphthylamide	5	5	3	2	5
L-Valyl-2-naphthylamide	5	5	1	1	5
L-Cystyl-2-naphthylamide	1	3	1	0	3
N-Glutaryl-phenylalanine-2-naphthylamide	1	0	0	0	0
2-Naphthyl-phosphate	5	5	4	5	5
Naphthol-AS-BI-phosphate	5	4	3	2	5
6-Br-2-naphthyl-α-D-galactopyranoside	0	0	2	0	0
2-Naphthyl-*β*-D-galactopyranoside	0	0	1	1	0
2-Naphthyl-α-D-glucopyranoside	0	3	3	1	5
6-Br-2-naphthyl-*β*-D-glucopyranoside	0	0	3	0	0
1-Naphthyl-*N*-acetyl-*β*-D-glucosaminide	0	1	2	1	5
Degradation of xylan	+	−	+	+	−
Growth on MacConkey agar	−	+	+	−	+
Growth at 25 °C	−	+	+	+	+
16S rDNA similarity to *A2i*^{T} (%)*	NA	96·8	96·2	95·9	95·9
Genomic DNA homology to *A2i*^{T} (%)	NA	21·9	29·5	33·2	35·7

* 16S rDNA sequence similarities for *A2i*^{T} against *F. columnare*, *F. johnsoniae*, *F. saccharophilum* and *F. hydatis* were calculated for aligned sequences containing no spaces or undetermined base characters; the lengths of the *A2i*^{T} comparison sequence alignments were 1382, 1374, 1243 and 1377 bp, respectively.

could hydrolyse aesculin (*β*-glucosidase activity) and gelatin (protease activity). The Voges–Proskauer test was negative, and there was no hydrogen sulphide production under any circumstances.

No precipitate was produced on egg-yolk agar, brown pigment was not secreted on tyrosine agar and there was no growth on MacConkey agar at any temperature. There seemed to be absorption of Congo red by *A2i*^{T} colonies, indicating the presence of extracellular galactosamine glycan; this may be the capsular substance around the cell (Fig. 1, bottom). The bacterium did not produce any acid or gas from glucose, fructose, xylose, mannitol or maltose. The main phenotypic properties of *A2i*^{T} are given in Table 1. The results of the API ZYM strips for *A2i*^{T} and the closest valid *Flavobacterium* species, on the basis of 16S rDNA sequence similarity, are displayed in Table 2.

Growth occurred on xylan, xylose, glucose, fructose, maltose, glycerol, tryptone, succinate, mannose, mannitol, laminarin, gelatin and casein as sole carbon and energy sources. However, there was no growth on CM-cellulose, pectin, starch, chitin, agar, acetate, phenylacetate, arabinose, *N*-acetylglucosamine, gluconate, caprate, adipate, malate, citrate, tyrosine, galactose, inositol, lactose, pyruvate, raffinose, ribose, sucrose or trehalose. There was no evidence of growth under anaerobic conditions.

The doubling times in XMM broth at 0, 5, 10, 15 and 20 °C were measured and found to be 34·8, 28·1, 10·9, 9·6 and 13·1 h (±10%), respectively. The optimum growth temperature was 15 °C in XMM and the maximum growth temperature was 24 °C. The doubling times in LB broth at 15 °C and at pH values of 5·8, 6·1, 6·6, 7·1, 7·5, 7·8 and 8·1 were determined and found to be 12·5, 7·7, 6·9, 4·4, 4·3, 10·8 and 13·3 h (±10%), respectively. The isolate had optimal growth at pH 7·5 but could not grow at pH 5·5 or pH 8·5 in LB broth.

Pigment characterization

The KOH test was negative, indicating that the pigment was not flexirubin. The pigment was extracted and compared with carotenoid standards, using TLC on silica, and also with published R_F values. Spectrophotometric analysis determined that its λ_{max} value in acetone (453 nm) matched the published value for zeaxanthin (*β*,*β*-carotene-3,3′-diol).

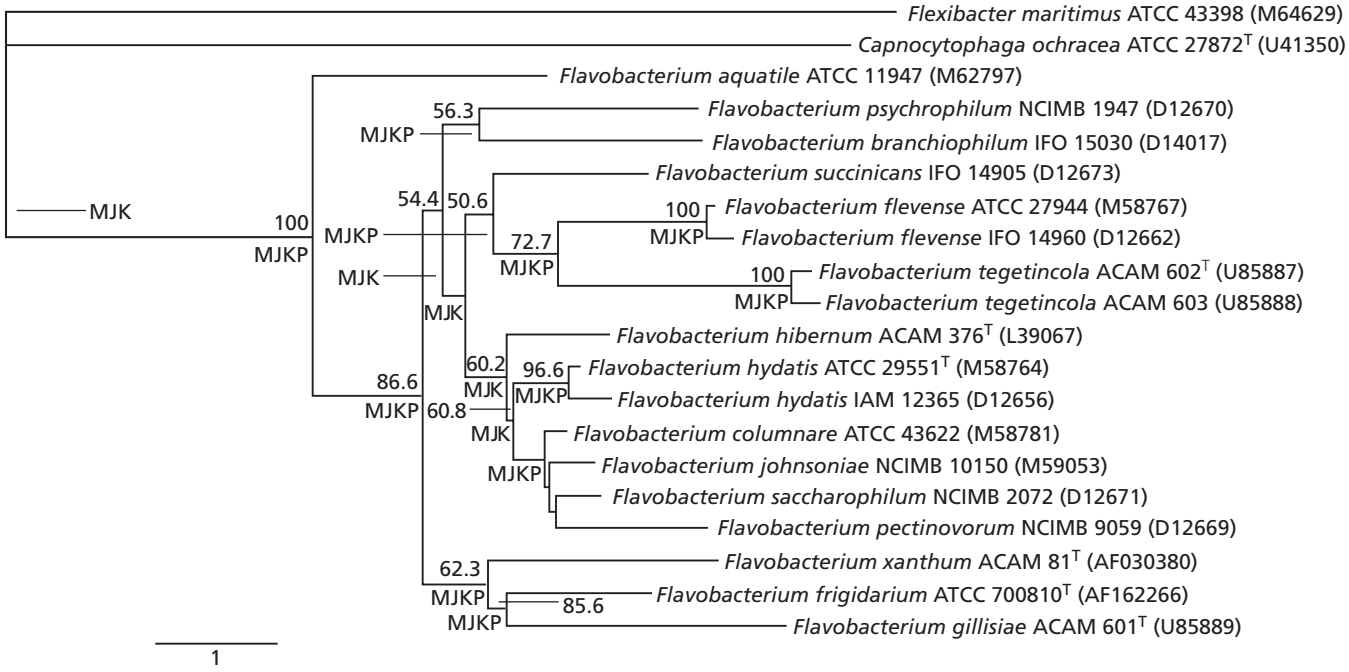

Fig. 2. Unrooted phylogenetic dendrogram for comparing the aligned 16S rDNA sequences (1136 bases long) of the valid *Flavobacterium* species with *F. frigidarium* (see Methods). Bootstrap-confidence percentages are shown on the nodes if they occurred in more than 50% of the trees. 'M', 'J', 'K' and 'P' denote nodes that also occurred in maximum-likelihood, Jin & Nei, Kimura two-parameter analyses (all with neighbour-joining) and an ordinary parsimony consensus tree of 1000 replicates, respectively. Bar, one nucleotide substitution per 100 nucleotides. Sequence accession numbers are in parentheses.

Antibiotic susceptibility

Isolate *A2i*^{T} was resistant to 10 μg ampicillin ml^{-1}, 200 μg neomycin ml^{-1}, 1 mg kanamycin ml^{-1}, 200 μg gentamicin ml^{-1} and 200 μg streptomycin ml^{-1}. It showed sensitivity to 10 μg erythromycin ml^{-1}, 50 μg chloramphenicol ml^{-1}, 10 μg tetracycline ml^{-1} and 10 μg rifampicin ml^{-1}. Filter-paper disks containing the following antibiotics showed zones of inhibition greater than 8 mm: fusidic acid, chloramphenicol, tetracycline, erythromycin and ampicillin. The novobiocin, penicillin G, methicillin and streptomycin disks did not cause any growth inhibition.

DNA G+C content and DNA–DNA hybridization

The G+C content of the genomic DNA of isolate *A2i*^{T} was determined to be 35 mol%. The genomic DNA of isolate *A2i*^{T} was 21·9, 29·5, 33·2 and 35·7% similar to the genomic DNA of *F. columnare* ATCC 43622, *F. johnsoniae* NCIMB 10150, *F. saccharophilum* NCIMB 2072 and *F. hydatis* ATCC 29551^T, respectively.

16S rDNA analysis

Phylogenetic analysis has shown that isolate *A2i*^{T} groups with members of the genus *Flavobacterium* (Fig. 2) as recently reorganized (Bernadet *et al*., 1996). The closest 16S rDNA sequence belongs to *F. columnare* ATCC 43622, whose sequence is 96·8% similar over 1382 bases. The second, third and fourth closest bacteria, respectively, were *Flavobacterium johnsoniae* NCIMB 10150, *Flavobacterium saccharophilum* NCIMB 2072 and *Flavobacterium hydatis* ATCC 29551^T (Table 2). The fifth, sixth and seventh closest bacteria were *Flavobacterium flevense* ATCC 27944, *F. hydatis* IAM 12365 and *Flavobacterium pectinovorum* NCIMB 9059, being 95·8, 95·4 and 95·4 similar, respectively. The lengths of the sequences (after alignment against the *A2i*^{T} 16S rDNA gene and removal of non-base characters) for each of these bacteria were 1356, 1238 and 1231 bases, respectively.

Membrane composition

The plasma membrane of *A2i*^{T} was composed of the following fatty acids: 3·66% branched C_{14}; 1·6% C_{14}; 8·8% iso-C_{15}; 15·1% anteiso-C_{15}; 2·2% branched C_{15}:1; 4·9% C_{15}; 1% C_{15}:1; 8·9% iso-C_{16}; 1·6% anteiso-C_{16}; 1·4% branched C_{16}:1; 3·1% C_{16}; 43·5% C_{16}:1*cis*; 3·13% unknown; 1% 16:0 2-OH and 1·7% 16:0 3-OH.

DISCUSSION

Isolate *A2i*^{T} was isolated from marine sediment recovered from shallow waters surrounding Adelaide Island, Antarctica (67° 34′ S, 68° 07′ W). The isolation

of psychrophilic Antarctic bacteria was undertaken to investigate the enzymes that these microbes use to hydrolyse plant and algal cell wall polysaccharides. To date, there are few taxonomic or physiological data available concerning psychrophilic bacteria in the Antarctic habitat and which are involved in the degradation of complex polymers and carbon-cycling. *A2i*^{T} was isolated during a systematic search for xylanolytic, laminarinolytic, pectinolytic and cellulolytic bacteria in Southern Ocean near-shore sediments. In comparison to thermophiles, relatively few psychrophilic xylanolytic organisms have been described. The psychrophilic xylanolytic organisms that have been described include the anaerobic prokaryote *Clostridium vincentii* (Mounfort *et al*., 1997); the aerobic prokaryote *Hymenobacter roseosalivarius* (Hirsch *et al*., 1998) and the eukaryotes *Cryptococcus* TAE85 (Gerday *et al*., 1997) and *Cryptococcus adeliae* (Petrescu *et al*., 2000).

The phenotypic and genotypic features of isolate *A2i*^{T} suggest that it is a member of the genus *Flavobacterium*. This genus was originally defined only vaguely and included Gram-negative and Gram-positive species as well as aerobic and facultatively anaerobic species (Bergey *et al*., 1923). However, the genus has been extensively revised over the past 20 years. The first major revision limited it to Gram-negative, aerobic, non-motile rods with a G+C content of 31–42 mol% (Holmes *et al*., 1984). The second revision used DNA–rRNA hybridization experiments to allocate 10 species to the genus and to reorganize the family *Flavobacteriaceae* (Bernadet *et al*., 1996). This family contains a further 10 genera, including *Flexibacter* and *Capnocytophaga* (Jooste & Hugo, 1999). DNA–DNA hybridization tests showed that several other Antarctic isolates (*Flavobacterium hibernum*, *F. gillisiae*, *F. tegetincola* and *F. xanthum*) are new species of the genus (McCammon & Bowman, 2000; McCammon *et al*., 1998).

Comparative studies indicated that the bacterium was most closely related to *F. columnare* (96·8% similarity). The 16S rDNA sequence of isolate *A2i*^{T} also had $>$ 93% similarity to the other 13 valid *Flavobacterium* species; this was demonstrated for an alignment 1136 bases long with no non-base characters. The 16S rDNA analysis does not reveal any other genera that could be sufficiently closely related to accommodate this isolate. The G+C content of the genomic DNA (35 mol%) fits into the range of the genus (32–37 mol%). This supports the hypothesis that *A2i*^{T} is a member of the genus *Flavobacterium* as defined by Bernadet *et al*. (1996). In addition, the DNA–DNA hybridization tests comparing *A2i*^{T} with the four closest valid *Flavobacterium* species are sufficiently low (below 70%) to allow the isolate to be designated as a new species with the name *Flavobacterium frigidarium*.

The bacterium shares some key phenotypic characteristics with many other members of the *Flavobacterium* genus (Table 1 and Bernadet *et al*., 1996). There is growth on NA and TSA, which is also seen for the four closest strains of the valid *Flavobacterium* species. No growth occurs on MacConkey agar, but *F. saccharophilum* NCIMB 2072 and *Flavobacterium aquatile* also do not grow on this medium (Holmes *et al*., 1984). The bacterium shows no gliding motility and cannot hydrolyse starch, CM-cellulose, agar, pectin or chitin, whereas *F. johnsoniae*, *F. saccharophilum*, *F. hydatis*^{T} and *F. flevense* can hydrolyse some of these (Table 1 and Bernadet *et al*., 1996). It also has an API ZYM substrate-hydrolysis profile similar to those of the four closest *Flavobacterium* species (Table 2). Therefore, it seems appropriate that the isolate be placed in the *Flavobacterium* genus. The bacterium has a carotenoid pigment tentatively identified as zeaxanthin, which has also been seen in members of the *Flavobacteriaceae* (Schmidt *et al*., 1994).

The fatty acid complement of *A2i*^{T} is similar to those of the other *Flavobacterium* species. The main fatty acid in the membrane of *A2i*^{T} is C_{16}:1*cis* (43·5%); however, in most of the other *Flavobacteria*, iso-C_{15}:0 predominates (Bernadet *et al*., 1996). *F. hibernum* ACAM 376^T has the most similar fatty acid profile; its main fatty acid is iso-C_{15}:0 (18·6%), the second most common one is C_{16}:1 (17·7%), and anteiso-C_{15}:0 (7·5%) is also present in higher concentrations than in nine of the other *Flavobacterium* species (McCammon *et al*., 1998). In addition, *A2i*^{T} does not seem to produce iso-C_{17}:0 3-OH, C_{17}:1ω6*cis* and iso-C_{17}:1ω9*cis*, which are commonly found in the other *Flavobacterium* species. There is no evidence of polyunsaturated fatty acids in the fatty acid profile, although these have been isolated from related bacteria such as *Flavobacterium gondwanense* (Bowman *et al*., 1998). It has been suggested that polyunsaturated fatty acid production has evolved in bacteria subjected to constant low temperatures and high pressures (Nichols *et al*., 1995). However, numerous monounsaturated and branched fatty acids, particularly 16:1*cis* (the most abundant cytoplasmic fatty acid in *A2i*^{T}) are present. This profile is consistent with those of several other psychrophilic bacteria, in which such fatty acids contribute to the maintenance of membrane fluidity at low temperatures (Nichols *et al*., 1993).

Bernadet & Grimont (1989) report antibiotic-sensitivity and NaCl-tolerance results for *F. columnare* and *F. psychrophilum*, as well as *F. flevense* temperature data. The antibiotic-sensitivity pattern of isolate *A2i*^{T} is similar to those of *F. columnare* and *F. psychrophilum*, and *A2i*^{T} is resistant to very high concentrations of kanamycin. In contrast to *F. columnare*, *F. psychrophilum* and *F. flevense*, *A2i*^{T} could grow in AOB with up to 5% NaCl (w/v). *F. flevense* was also incapable of growth at 15 °C, in contrast to *A2i*^{T}, for which this was the optimal growth temperature. *F. columnare* and *F. psychrophilum* are major fish pathogens (Bader & Shotts, 1998); although the former has not been isolated in the Southern Hemisphere and has been isolated only in North America, Europe and Japan, the latter has been isolated in Tasmania (Chakroun *et al*., 1997).

Description of *Flavobacterium frigidarium*

Flavobacterium frigidarium (fri.gid.ar.i′um. L. n. *frigidarium* one who bathes in cold water).

Non-sporulating, non-motile rods, 0·8–2 μm long and 0·5–0·7 μm wide, with a thick capsule and pili. Aerobic, psychrophilic, halotolerant, xylanolytic and laminarinolytic. Gram-negative, non-flagellated and show no gliding motility. Produces the bright yellow carotenoid pigment zeaxanthin. Colonies on AOA are flat, round and entire. The G+C content of the genomic DNA is 35 mol%. Growth occurs on xylan at 0–24 °C; the minimum growth temperature on rich media is below 0 °C. Grows at pH 5·6–8·4, with an optimum of pH 7·5. Grows in AOB containing 0–5% (w/v) NaCl, in XeMM containing 0–4% NaCl, and in LB broth containing 0–9% NaCl. Grows on NA, TSA and seawater agar but not on MacConkey agar. Nitrate is not reduced, hydrogen sulphide is not produced under any conditions, and there is no indole production, no arginine dihydrolase, no urease, no DNase and no β-galactosidase. Hydrolyses aesculin and gelatin. Voges–Proskauer-negative. Does not produce any acid or gas from glucose, fructose, xylose, mannitol or maltose. No precipitate was produced on egg-yolk agar and no brown pigment was secreted on tyrosine agar. Resistant to the following in LB agar: 10 μg ampicillin ml^{-1}, 200 μg neomycin ml^{-1}, 1 mg kanamycin ml^{-1}, 200 μg gentamicin ml^{-1} and 200 μg streptomycin ml^{-1}. Sensitive to 10 μg erythromycin ml^{-1}, 50 μg chloramphenicol ml^{-1}, 10 μg tetracycline ml^{-1} and 10 μg rifampicin ml^{-1}. The following antibiotic disks caused zones of inhibition of more than 8 mm on LB agar: 10 μg fusidic acid, 25 μg chloramphenicol, 10 μg tetracycline, 5 μg erythromycin and 25 μg ampicillin. Disks of novobiocin (5 μg), penicillin G (1 IU), methicillin (10 μg) and streptomycin (10 μg) did not cause any growth inhibition. Chemo-organotrophic and utilizes xylan, laminarin, xylose, glucose, fructose, maltose, glycerol, tryptone, succinate, mannose, mannitol, gelatin and casein as sole carbon and energy sources. Does not utilize CM-cellulose, pectin, starch, chitin, agar, acetate, phenyl-acetate, arabinose, *N*-acetyl-glucosamine, gluconate, caprate, adipate, malate, citrate, tyrosine, galactose, inositol, lactose, pyruvate, raffinose, ribose, sucrose or trehalose. When grown up on NA, the substrates shown in Table 2 were or were not hydrolysed; when grown up on AOA, it additionally hydrolyses *N*-benzoyl-DL-arginine-2-naphthylamide and 6-Br-2-naphthyl-β-D-glucopyranoside. Plasma membrane is composed of the following fatty acids: 3·66% branched C_{14}; 1·6% C_{14}; 8·8% iso-C_{15}; 15·1% anteiso-C_{15}; 2·2% branched C_{15}:1; 4·9% C_{15}; 1% C_{15}:1; 8·9% iso-C_{16}; 1·6% anteiso-C_{16}; 1·4% branched C_{16}:1; 3·1% C_{16}; 43·5% C_{16}:1*cis*; 3·13% unknown; 1% 16:0 2-OH and 1·7% 16:0 3-OH. The single strain *A2i*^{T} (= ATCC 700810^T = NCIMB 13737^T type strain) was isolated from shallow-water Southern Ocean sediment recovered near Adelaide Island, Antarctica (67° 34′ S, 68° 07′ W). The GenBank accession number for the 16S rDNA sequence of *Flavobacterium frigidarium* is AF162266.

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