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Shewanella frigidimarina and *Shewanella livingstonensis* sp. nov. isolated from Antarctic coastal areas

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Three strains of psychrophilic bacteria isolated from Antarctic coastal marine environments were studied to determine their taxonomic position. These bacteria were Gram-negative rods, facultatively anaerobic and motile by means of a single polar flagellum. None of the bacterial isolates had an Na^+ requirement. Only one of the strains was capable of producing H_2S from thiosulfate. The DNA base content of these bacteria was 41–42 mol% G+C. DNA–DNA hybridization experiments showed that the isolates formed two related groups that exhibited about 70 and 24% DNA–DNA homology, respectively, with the type strain of *Shewanella frigidimarina*. The fatty acid profiles of the bacterial isolates were similar to the profiles of other *Shewanella* species. All the strains contained both ubiquinones and menaquinones, like *Shewanella* species. Methylmenaquinones were also found. 16S rRNA gene analysis confirmed that isolated strains belonged to the genus *Shewanella* and were phylogenetically related to the newly identified *Shewanella frigidimarina*. The results of the polyphasic taxonomic study assigned the three isolates to *Shewanella* and two of them specifically to *Shewanella frigidimarina*. The name *Shewanella livingstonensis* sp. nov. (type strain LMG 19866^T) is proposed for the third organism.

Keywords: Antarctica, *Shewanella frigidimarina*, *Shewanella livingstonensis*, polyphasic taxonomy

INTRODUCTION

The genus *Shewanella* was first described 14 years ago by MacDonell & Colwell (1985). The definition of *Shewanella* was based almost entirely on rRNA structure and included only the description 'straight or curved rods, Gram-negative, non-pigmented, motile by polar flagella, chemo-organotrophic, oxidase-positive, generally associated with aquatic or marine habitats. G+C mol% 44–47'. The species *Shewanella putrefaciens* (Derby & Hammer, 1931; Lee *et al*., 1977) and *Shewanella hanedai* (Jensen *et al*., 1980) were included in the new genus, as well as the new description of *Shewanella benthica*. Twelve species are included in the genus *Shewanella*, nine of which have been described in the last 3 years. Although the development of this bacterial group, from a taxonomic point of view, has taken place in the last decade, *Shewanella putrefaciens* has been studied since its first description as *Achromobacter putrefaciens* by Derby & Hammer (1931), because of its special interest in the areas of applied and environmental microbiology.

Shewanella putrefaciens, first isolated as *Achromobacter putrefaciens* from rancid butter, was classified in 1960 as *Pseudomonas putrefaciens* by Shewan *et al*. (1960) and, later, as *Alteromonas putrefaciens* by Lee *et al*. (1977). This micro-organism has been found to be associated with the spoilage of proteinaceous foods (Shewan, 1977). *Shewanella* species are widely distributed and have been isolated from diverse sources such as aquatic environments (Nealson *et al*., 1991), sediments (Myers & Nealson, 1988), oilfield fluids

Abbreviation: TMAO, trimethylamine *N*-oxide.

The EMBL accession numbers for the 16S rDNA gene sequence of strains NF12 and NF22^T are AJ300833 and AJ300834, respectively.

(Semple & Westlake, 1987) and, as mentioned above, spoilage of proteinaceous foods (Levin, 1972; Shewan, 1974; Parker & Levin, 1983; Stenstrom & Molin, 1990), and are considered opportunistic pathogens of humans (Debois *et al*., 1975; Nozue *et al*., 1992; Brink *et al*., 1995) and aquatic animals (Aguirre *et al*., 1994). This has been pointed out by many authors since the description of some *Pseudomonas*-like marine bacteria by Lee *et al*. (1977) through to the more recent description of *Shewanella pealeana* by Leonardo *et al*. (1999).

Venkateswaran *et al*. (1999) described the phylogenetic relationship between the described species of *Shewanella*, including *Shewanella putrefaciens*, *Shewanella hanedai*, *Shewanella benthica*, *Shewanella colwelliana* (Weiner *et al*., 1988; Coyne *et al*., 1989), *Shewanella algae* (Simidu *et al*., 1990; Nozue *et al*., 1992; Trüper & de'Clari, 1997), *Shewanella woodyi* (Makemson *et al*., 1997), *Shewanella gelidimarina* and *Shewanella frigidimarina* (Bowman *et al*., 1997), *Shewanella baltica* (Ziemke *et al*., 1998) *Shewanella amazonensis* (Venkateswaran *et al*., 1998), *Shewanella oneidensis* (Venkateswaran *et al*., 1999) and *Shewanella pealeana* (Leonardo *et al*., 1999).

Here we describe the isolation of three strains of the genus *Shewanella*, capable of growing anaerobically by dissimilatory Fe(III) reduction, from muddy soil, water and sediments collected in the Antarctic area of the South Shetland Islands. To elucidate the taxonomic position of these three isolates, conventional phenotypic and chemotaxonomic analyses were performed. The results obtained and the DNA–DNA hybridization and 16S RNA sequence analysis suggested that the organisms should be included in the genus *Shewanella*.

METHODS

Bacterial strains and isolation. The bacterial strains used in this study are listed in Table 1. Strain NF12 was isolated from mud collected in the Inlet Admiralty Bay (King George Island, South Shetland Islands, Antarctica), at the bottom of a glacier which is covered at high water. Strains NF22^T and NF24 were isolated from sediment and water, respectively, collected in Johnson's Dock (Livingston Island, South Shetland Islands, Antarctica).

Aliquots of samples were removed with a platinum loop and diluted in a saline solution containing (g l^{-1}, pH 7): NaCl, 0·56; KCl, 0·27; $CaCl_2$, 0·03; $NaHCO_3$, 0·01. Trypticase soy agar (TSA; ADSA) plates were inoculated with loopfuls of several sample dilutions using the streak plate method to obtain well isolated colonies. Petri dishes were incubated for 6 d at 15 °C. Isolates were maintained on TSA slopes at 4 °C. Bacteria were also stored at −20 °C in 50 % (v/v) glycerol. All media used in this study were sterilized at 121 °C for 20 min, unless otherwise indicated.

Morphology. Cell size and morphology were determined by scanning electron microscopy of cells grown in Trypticase soy broth (TSB; ADSA) at 15 °C. A Hitachi model S 3200 scanning electron microscope was used. Motility was determined by phase-contrast microscopy. The flagellar arrangement was examined with a Philips model 301 microscope and negative staining with 0·5 % (w/v) phosphotungstic acid adjusted to pH 6 with 1 M KOH was applied. TSB cultures, grown for 24 h, were used.

Physiological and biochemical characteristics. Oxidase activity was tested using the method of Kovács (1956). Catalase activity, nitrate reduction, ornithine, arginine and lysine decarboxylase activity were determined following Cowan & Steel (1993). The arginine dihydrolase reaction test was performed according to Thornley (1960), as modified by Lelliot *et al*. (1966). Acid production from carbohydrates was tested on the oxidation–fermentation medium of Leifson (1963) after incubation at 15 °C for 14 d.

Degradative tests were carried out at 15 °C. Tween 80 (1 %, v/v) was incorporated into Sierra's medium (Sierra, 1957) and plates were examined for opacity after 5 d. Hydrolysis of DNA was tested by the method of Jeffries *et al*. (1957). Hydrolysis of starch, aesculin, gelatin, casein, lecithin and chitin were determined following Cowan & Steel (1993). Haemolytic activity was tested on blood agar plates according to Cowan & Steel (1993).

API galleries (API 20E, API 20EN, ATB 32GN, API 20B, API ZYM; bioMérieux) were used to test additional biochemical characteristics and were prepared according to the manufacturer's instructions. The API strips were incubated for 5 d at 15 °C.

The pH range for the growth of each strain was determined in TSB with pH values of separate batches of medium adjusted to 4, 5, 6, 7, 8, 9 and 9·5 with 1 M HCl and 1 M NaOH. The test media were incubated at 15 °C for 14 d. The temperature range for growth was determined on TSA and TSA containing 3 % (w/v) NaCl, incubated for 14 d at 4, 11, 16, 20, 25, 30, 37 and 40 °C. Salt tolerance tests were performed on TSA with NaCl concentrations ranging from 0·6 to 20 % (w/v).

The sodium requirement was analysed in a medium containing (g l^{-1}, pH 7): K_2HPO_4, 5·0; $MgSO_4 . 7H_2O$, 0·5; $CaCl_2$, 0·05; $FeSO_4 . 7H_2O$, 0·028; NH_4Cl, 7; yeast extract, 5·0; agar, 20·0. Another medium with the same composition, except that it contained 1 g NaCl l^{-1}, was used as a positive control. A solution with all of the ingredients except $CaCl_2$ and $FeSO_4$ was sterilized by autoclaving it at 0·5 atm ($5{\cdot}065 \times 10^4$ Pa) for 30 min. $CaCl_2$ and $FeSO_4$ were added aseptically from sterile stock solutions. Plates were incubated for 14 d at 15 °C.

Carbon source utilization tests were performed in a mineral medium containing (g l^{-1}, pH 7): Na_2HPO_4, 10·0; KH_2PO_4, 3·0; K_2SO_4, 1·0; NaCl, 1·0; $MgSO_4 . 7H_2O$, 0·4; $CaCl_2$, 0·1; $FeSO_4 . 7H_2O$, 0·018, NH_4Cl, 3·0. The carbon source concentration used was 1 %, as described by Palleroni & Doudoroff (1972).

Dissimilatory iron reduction was tested on a defined medium (Myers & Nealson, 1988, 1990) supplemented with 30 mM lactate as carbon and energy source and 50 mM Fe(III) citrate as terminal electron acceptor (Lovley *et al*., 1992). Solid defined medium (DiChristina & DeLong, 1994) was prepared by adding Bacto Agar (Difco) to a final concentration of 1·5 % (w/v). Anaerobic growth, with 25 mM trimethylamine *N*-oxide (TMAO) (Saffarini *et al*., 1994) as sole terminal electron acceptor, was tested on the same solid defined medium in which Fe(III) citrate was omitted. The

Table 1. Strains used in this study

ACAM, Australian Collection of Antarctic Micro-organisms, University of Tasmania, Tasmania, Australia; ATCC, American Type Culture Collection, Manassas, VA, USA; CECT, Spanish Type Culture Collection (Colección Española de Cultivos Tipo), Burjasot, Valencia, Spain; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; NCTC, National Collection of Type Cultures, Public Health Laboratory, London, UK.

Strain	Other designations	Source
Shewanella putrefaciens LMG 2268^T	ATCC 8071^T	LMG
Shewanella putrefaciens LMG 2369		LMG
Shewanella algae CECT 331		CECT
Shewanella algae LMG 2265		LMG
Shewanella baltica CECT 323^T	LMG 2250^T, NCTC 10735^T	CECT, LMG
Shewanella frigidimarina LMG 19475^T	ACAM 591^T	LMG
Shewanella hanedai CECT 5017^T	ATCC 33224^T	CECT
NF12	LMG 19867, CECT 5932	Glacier mud, Admiralty Bay, Antarctica
NF22^T	LMG 19866^T, CECT 5933^T	Water, Johnson's Dock, Antarctica
NF24	LMG 19868, CECT 5934	Sediment, Johnson's Dock, Antarctica

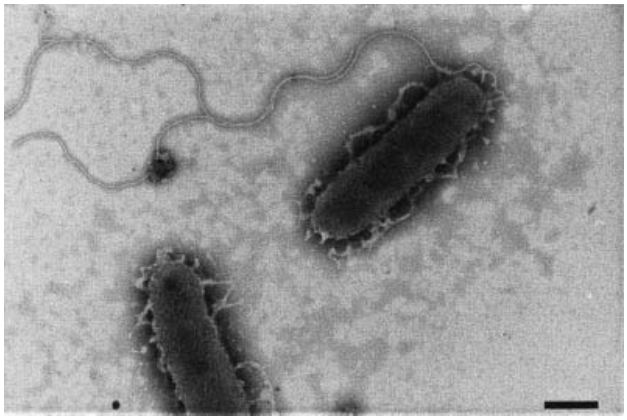

Fig. 1. Electron micrograph of negatively stained cells of NF24 from a liquid culture after 24 h incubation at 15 °C. Bar, 0·55 μm.

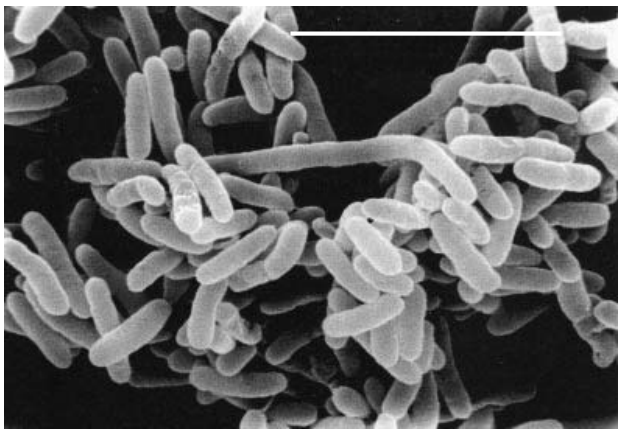

Fig. 2. Morphology of strain NF22^T by scanning electron microscopy. Cells were grown on TSB for 24 h at 15 °C. Bar, 5 μm.

inoculated plates [defined medium Fe(III) citrate or defined medium TMAO] were incubated under anaerobic conditions (Gas Pack system) for 10–14 d until the plates showed visible growth. Incubations were carried out in the dark at 15 °C for bacterial isolates and at room temperature (22 °C) for *Shewanella algae* CECT 331 and *Shewanella baltica* CECT 323^T. Before storage at 15 and 22 °C, each anaerobic jar was kept overnight at 4 °C to make sure that anoxic conditions had developed before allowing strain growth. Growth on the plates was compared with growth on two sets of defined medium control plates, one lacking electron acceptors and the other lacking organic carbon substrate.

Antibiotic sensitivities. Susceptibility to antibiotics was tested by using the method of Bauer *et al.* (1966). Disks (6·5 mm diam.) impregnated with antibiotics (bioMérieux) were laid on Müeller–Hinton agar (ADSA) plates which had been surface-inoculated with test strains.

Fatty acid composition. Fatty acids were prepared from 40 mg wet cell material harvested from a TSB agar (30 g TSB, 15 g agar; BBL) culture incubated for 5 d. Bacterial isolates were grown at 8 and 15 °C and *Shewanella algae* CECT 331 at room temperature (22 °C). Whole-cell fatty acids were isolated following the Microbial Identification System (MIS) guidelines (Microbial ID) and were analysed by GLC with a Hewlett Packard model HP5890A instrument. Fatty acids were identified and quantified by comparison with other micro-organisms, using MIDI System software, version 3.2. The relative amount of each fatty acid in a strain was expressed as a percentage of total fatty acids.

Quinone analysis. Strains NF12, NF22^T and NF24 were grown in 250 ml TSB medium and *Shewanella* species in 250 ml TSB medium with seawater. Cultures were incubated by shaking at 150 r.p.m. for 24 h at 15 °C for bacterial isolates and at room temperature (22 °C) for the reference strains. Biomass was harvested and lyophilized.

Table 2. Biochemical and physiological characteristics of strains NF12, NF22^T and NF24 isolated from Antarctic environments

All strains studied were straight polarly flagellated rods that were positive for catalase, oxidase and nitrate reduction, had no sodium requirement for growth and had negative Voges–Proskauer and indole reactions. The range of pH tolerance for growth was 6–9·5 for the three strains. They hydrolysed aesculin and Tween, but none hydrolysed starch. All of the strains were positive in tests for alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and α-glucosidase, but negative in tests for urease, DNase, lecithinase, α-galactosidase, trypsin, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-fucosidase, arginine dihydrolase, ornithine decarboxylase, arginine decarboxylase and lysine decarboxylase. The following carbon and energy sources were not used by any strain: L-arabinose, D-arabinose, gluconate, caprate, adipate, citrate, phenylacetate, salicin, D-melibiose, L-fucose, D-sorbitol, valerate, histidine, 2-ketogluconate, 4-hydroxybenzoate, α-L-rhamnose, itaconate, suberate, acetate, propionate, 5-ketogluconate, glycogen, 3-hydroxybenzoate, DL-malate, pimelate, D-fructose, sebacate, *m*-tartrate, DL-hydroxybutyrate and L-phenylalanine. All strains used D-glucose, maltose, cellobiose, sucrose, D-mannose, D-mannitol, D-galactose, D-xylose, *N*-acetylglucosamine, trehalose, malonate and succinate. All strains were capable of fermenting D-glucose, maltose, cellobiose, sucrose, D-mannose, D-mannitol, D-galactose, D-xylose, *N*-acetylglucosamine and trehalose. All strains were able to oxidatively produce acid from D-glucose, maltose and cellobiose, but not from L-arabinose, fructose, rhamnose, galactose, sorbitol, glycerol, *N*-acetylglucosamine or trehalose. All strains were susceptible to penicillin, 10 U per disk; tobramycin, 10 μg per disk; nalidixic acid, 30 μg per disk; chloramphenicol, 30 μg per disk; and tetracycline, 30 μg per disk. +, Positive; −, negative.

Characteristic	NF12	NF22^T	NF24
Cell length (μm)	1–2·5	1–3	1–3·7
Cell diameter (μm)	0·3–0·5	0·4	0·5
Temperature range of growth (°C)	4–30	4–20	4–20
Maximum NaCl concentration tolerated (%, w/v)	5·5	5	9
H_2S production (Kligler, TSI)	−	+	−
G+C content (mol%)	42	41	42
Hydrolysis of:			
Gelatin, casein	+	+	−
Chitin	+	−	+
Enzyme activity			
Lipase (C4), α-chymotrypsin	+	−	+
β-Galactosidase (ONPG)	−	+	−
α-Mannosidase	−	−	+
Oxidative acid production from:			
Sucrose, D-mannose	−	−	+
Starch	+	−	−
D-Mannitol	+	−	+
D-Galactose, D-xylose	−	+	−
Utilization of:			
L-Glutamate	+	−	+
3-Hydroxybutyrate, L-proline, inositol, sucrose, L-alanine, L-serine	−	−	+
D-Ribose, DL-lactate	−	+	+

Isoprenoid quinones were extracted from lyophilized ground cells by the method of Minnikin *et al.* (1984). HPLC was carried out to determine the isoprenoid quinone composition according to Tamaoka *et al.* (1983). The apparatus comprised a high-performance liquid chromatograph Phoenix 20 (Punts CE Instruments), a Kontron 535 UV detector (Kontron Instruments) and a reverse-phase type Spherisorb ODS2 (5 μm) column (4·6 mm i.d. × 150 mm; Waters). Samples were eluted with a mixture of acetonitrile/2-propanol (65:35, v/v) at a flow rate of 1 ml min^{-1}. Quinones were identified by MS (Platform Micromass) using a flow divisor 1/50 to apply the appropriate flow. The conditions for chemical ionization at atmospheric pressure (APCI) were: discharge needle, 3000 V; counter-electrode, 500 V; sampling cone, 40 V; photomultiplicator, 650 V; source temperature, 80 °C; tip temperature, 400 °C. The relative molar ratios of quinone homologues were determined by reference to the standard mixtures containing known amounts of MK-4 and Q-10.

Whole-cell protein profiles. To obtain whole-cell protein extracts, the Antarctic isolates and *Shewanella* species were grown on TSA plates for 5 d at 15 °C and at room temperature (22 °C), respectively. The bacterial growth on three Petri dishes was harvested and resuspended in 7 ml NaPBS buffer (0·2 M sodium phosphate buffer, pH 7·3, 8 g NaCl l^{-1}). The bacterial suspension was filtered through nylon gauze and centrifuged for 20 min at 5000 ***g***. The pellet was washed twice in the same buffer and 70 mg (wet wt) bacterial cells was transferred into an Eppendorf centrifuge tube. A 0·9 ml portion of sample treatment buffer (0·75 g Tris, 5 ml mercaptoethanol, 5 g sucrose and enough deionized water to bring the volume up to 100 ml, pH 6·8) was

added and the solution was mixed. Then 0·1 ml 20 % SDS was added. The mixture was incubated at 95 °C for 10 min, cooled on ice and centrifuged at 11000 ***g*** for 5 min in an Eppendorf centrifuge. Supernatants were stored at −20 °C. SDS-PAGE of whole-cell protein extracts was performed by the procedure of Sambrook *et al*. (1989) in a Miniprotean II electrophoresis cell (Bio-Rad) by using 12 % separation gels. The gels were stained with Coomassie blue.

Determination of DNA base composition. Cells from a culture of each isolate were harvested, washed and resuspended in 0·15 M NaCl/0·01 M sodium EDTA buffer (pH 8·0). The cells were lysed at 60 °C for 10 min by adding SDS to a final concentration of 1 % (w/v). The DNA was extracted and purified by the method of Marmur (1961). The G+C content was determined from the midpoint (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) obtained with a Perkin–Elmer model UV/Vis 551S spectrophotometer at 260 nm. T_m was determined by the method of Ferragut & Leclerc (1976) and the G+C content was calculated by using the equation of Owen & Hill (1979). The T_m of reference DNA from *Escherichia coli* NCTC 9001 in 0·1 × SSC (1 × SSC is 0·15 M NaCl, 0·015 M sodium citrate) was 74·6 °C (Owen & Pitcher, 1985).

DNA–DNA hybridization. Levels of DNA–DNA hybridization between bacterial isolates and type strains were determined spectrophotometrically by the initial renaturation method of De Ley *et al*. (1970).

16S rRNA sequence determination and analysis. Genomic DNA was extracted as described by Pitcher *et al*. (1989). The 16S rRNA gene was amplified by using the PCR method (Saiki *et al*., 1988). The forward amplification primer was 27F, 5′-AGAGTTTGATCCTGGCTCAG-3′ (corresponding to positions 8–27 in the 16S rDNA nucleotide sequence of *E. coli*), and the reverse amplification primer was 1522R, 5′-AAGGAGGTGATCCAGCCGCA-3′ (positions 1541–1522). The PCR products were purified by using the QIAquick PCR purification Kit (Qiagen). Purified PCR products were sequenced with an Applied Biosystems model 377 DNA sequencer, as recommended by the manufacturer (Perkin–Elmer), by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase. The following primers were used for sequencing: 358F, 5′-CTCCTACGGGAGGCAGCAGT-3′ (positions 339–358); 536F, 5′-CAGCAGCCGCGGTAATAC-3′ (519–536); 926F, 5′-AACTCAAAGGAATTGACGG-3′ (908–926); 1112F, 5′-AGTCCCGCAACGAGCGCAAC-3′ (1093–1112); 1241F, 5′-GCTACACACGTGCTACAATG-3′ (1222–1241); 339R, 5′-ACTGCTGCCTCCCGTAGGAG-3′ (358–339); 519R, 5′-GTATTACCGCGGCTGCTG-3′ (536–519); and 1093R, 5′-GTTGCGCTCGTTGCGGGACT-3′ (1112–1093).

The sequences were assembled by the program Auto Assembler (Applied Biosystems). Phylogenetic analysis was performed using the software package GeneCompar (Applied Maths), after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library at EMBL. This alignment was pairwise, calculated using an open gap penalty of 100 % and a unit gap penalty of 0 %. A similarity matrix was created by homology calculation with a gap penalty of 0 % after discarding unknown bases. A phylogenetic tree was constructed by applying the neighbour-joining method (Saitou & Nei, 1987).

RESULTS AND DISCUSSION

Morphological and growth characteristics

The Antarctic bacterial isolates were Gram-negative, rod-shaped, non-spore-forming organisms. On TSA agar incubated at 15 °C, young colonies were circular, smooth, convex, slightly mucoid and red-brown, with a diameter of 1–2 mm. Neither diffusible pigments nor bioluminescence were observed. NF12, NF22^T and NF24 grew well at 15 °C in standard bacteriological nutrient media such as TSB or TSA. Cells were motile by means of a single unsheathed polar flagellum (Fig. 1). After 24 h incubation in liquid medium, larger cells appeared in the population and, sometimes, filamentous forms (Fig. 2) about 10 μm long were observed. None of the isolates required Na^+ to grow. The pH range for growth was 6–9·5 and the growth temperature range was 4–30 °C for NF12 and 4–20 °C for NF22^T and NF24.

Phenotypic characterization

The physiological and biochemical properties of the Antarctic isolates are summarized in Table 2. The strains were chemo-organotrophic and capable of respiratory and fermentative metabolism. All of the strains were positive for cytochrome oxidase and catalase. They reduced nitrate to nitrite and nitrogen gas was not formed from nitrite. NF12, NF22^T and NF24 were able to grow anaerobically by reducing TMAO and ferric compounds while using DL-lactate as electron donor. NF22^T was the only strain capable

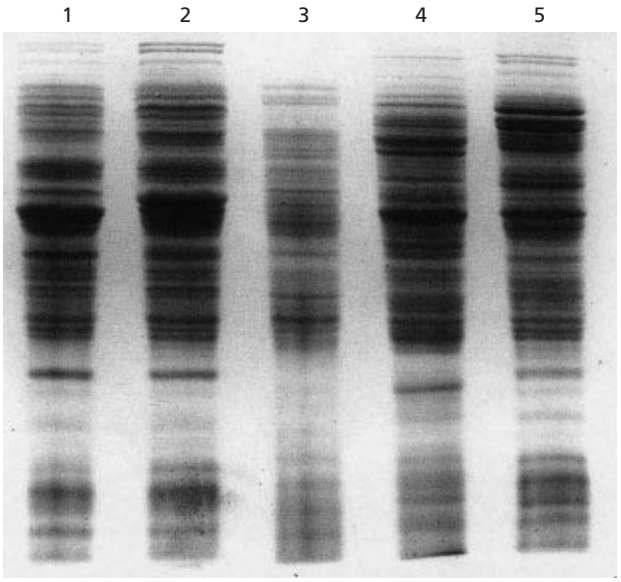

Fig. 3. SDS-PAGE gel of whole-cell proteins. Lanes: 1, NF24; 2, NF12; 3, NF22^T; 4, *Shewanella baltica* CECT 323^T; 5, *Shewanella algae* CECT 331.

Table 3. Fatty acid composition of bacterial isolates NF12, NF22^T, NF24 and *Shewanella algae* CECT 331*

n, Number of strains examined; TR, trace (< 1%); summed feature 1, fatty acids 14:1 *ω5c* and 14:1 *ω5t* could not be separated by GC by using the MIS software package and were considered together as summed feature 1; summed feature 2 consists of 13:0 3OH, 15:1 iso H and 15:1 iso; summed feature 3 consists of unknown 10·928 (fatty acid whose identity is unknown and whose equivalent chain-length is 10·928), 14:0 3OH and 16:1 iso I; summed feature 7 consists of 18:1 *ω7c*, 18:1 *ω9t* and 18:1 *ω12t* (not separated by MIS); summed feature 8 consists of 19:1 and unknown 18·756; summed feature 9 consists of unknown 18·846 and 18·858. In addition, small amounts (less than 3% of the total fatty acids) of one or more of the following fatty acids were found in the strains studied: 9:0, 10:0, 10:0 iso, 11:0, 10:0 3OH, unknown 11·798, 12:0 iso, 11:0 iso 3OH, unknown 12·487, 13:1 AT 12–13, 12:0 iso 3OH, 12:1 3OH, 15:1 iso, 15:0 anteiso, 14:0 iso 3OH, 16:0 iso, 16:1 *ω5c*, iso 17:1 *ω9c*, 15:0 3OH, 17:0 anteiso, 18:0 iso, 18:1 *ω5c*, 19:0 iso, 19:0 10Me and 20:0 iso.

Fatty acid	Percentage of total fatty acid at:						
	15 °C			Room temperature	8 °C		
	NF12 (*n* = 5)	NF22^T (*n* = 4)	NF24 (*n* = 6)	*Shewanella algae* (*n* = 6)	NF12 (*n* = 2)	NF22^T (*n* = 2)	NF24 (*n* = 1)
12:0	3·5–7·7	3·5–5	3–4·4	1·5–1·9	4·8	4	3·16
11:0 3OH	TR[c]	TR	TR	TR–1·7	TR	TR	TR
13:0 iso	7·5–16·7	8·9–10·1	8·8–17	4·8–9	7·8–8·4	6·8–7·1	8·7
13:0	TR–4	1–1·8	TR–2·4	TR–2	1·7–2·6	1·6–2	TR
12:0 3OH	1·4–4·7	2·4–2·6	1·5–2·4	2·1–2·7	2–2·6	2·2	2·1
14:0 iso	TR–1·8	TR	TR–2	TR	TR	1	TR
14:0	2·3–4·6	2·4–3·6	3·8–4·9	TR	3·3	2·7	3·7
13:0 iso 3OH	4·5–12	3·6–4·2	5·2–7	4·9–6·7	4·8–6·1	3·4	5·7
15:0 iso	6·7–9·1	7·6–10·3	8·4–10·7	17·3–28·8	5·6–6·6	5·9–6·7	10·6
15:1 *ω8c*	TR–2·6	TR–1·1	TR–1·4	TR	1·5–2·1	1·3–1·7	TR
15:1 *ω6c*	TR–2·5	TR	TR–1·4	TR	1·6–2	1·7	1
15:0	2·2–8·8	5·3–5·7	2·4–7	2·2–3·5	4·9–7·2	5·8–7·1	2·6
16:1 *ω9c*	1·1–1·9	TR	1·1–1·4	TR	1·2	TR	1
16:1 *ω7c*	24·9–35·1	23·7–24·8	23·9–36·6	6·7–12·8	32·3	27·2–28·4	36·6
16:0	4–9·1	9·7–11·4	5·4–13·4	3·7–6·3	6·4	6·7–6·9	7·1
15:0 iso 3OH	TR	TR	TR	TR–1·2	TR	TR	TR
17:0 iso	TR	TR	TR	2·5–4·4	TR	TR	TR
17:1 *ω8c*	2·6–6·4	6·9–8·4	2·8–6·4	14·7–20·5	6·9–7·5	10·7–11·5	4·7
17:1 *ω6c*	TR–1·2	1–1·3	TR	TR–1	1·2	2–2·2	1
17:0	TR	1·8–2·3	TR	3·5–4·4	TR	1·7	TR
18:1 *ω9c*	TR–2	1·2–1·6	TR–1·8	2·3–4·6	TR	TR–1	1·5
18:0	TR–2·1	TR	TR–1·1	TR	TR	TR	TR
Summed feature 1	TR	0–TR	TR	–	–	–	TR
Summed feature 2	TR–1·3	1·3–1·4	TR–1	2·2–3·9	1·1	1·4	TR
Summed feature 3	TR–2·4	1·3–1·5	TR–1	1–1·2	TR	TR	TR
Summed feature 7	TR–4·7	3·7–4·7	TR–4·1	3·7–6	2·5–3·6	4–4·8	–
Summed feature 8	–	–	–	TR	–	–	–
Summed feature 9	TR	0–TR	TR	–	–	–	–

of producing hydrogen sulfide from thiosulfate. Fermentation of carbohydrates by bacterial isolates was accompanied by acid production, but no gas was observed. The strains did not show haemolytic activity.

On the basis of the phenotypic characterization, it was found that the isolates belong to the genus *Shewanella* (Venkateswaran *et al.*, 1999). According to the phenotypic differentiation of *Shewanella* species reported by Venkateswaran *et al.* (1999), these bacterial isolates were phenotypically similar to *Shewanella frigidimarina* (Bowman *et al.*, 1997). NF12, NF22^T and NF24 could grow anaerobically by dissimilatory iron reduction, or by carbohydrate fermentation. The three isolates could ferment D-glucose and could grow at 20 °C. They were psychrophilic and halotolerant strains. Gelatinase was produced by NF12 and NF22^T. Venkateswaran *et al.* (1999), in their recent study on the polyphasic taxonomy of the genus *Shewanella*, point out that the inability to ferment glucose has traditionally been characteristic of shewanellae. However, Bowman *et al.* (1997) have reported glucose

Table 4. Isoprenoid quinone composition of the bacterial isolates and *Shewanella* strains

TR, Trace amounts (< 1%); Q-6, Q-7, Q-8 and Q-9, ubiquinones with six, seven, eight and nine isoprene units, respectively; MK-7 and MK-8, menaquinones with seven and eight isoprene units, respectively; MMK-7, methylmenaquinone with seven isoprene units.

Strain	Quinone composition (%) of:						
	Q-6	Q-7	Q-8	Q-9	MK-7	MK-8	MMK-7
Shewanella algae CECT 331	TR	9·4	36·6	TR	47·8	1·2	4
Shewanella baltica CECT 323^T	TR	63·8	11	–	TR	–	23·3
NF12	1	44·4	17	–	35·7	TR	1
NF22^T	TR	7·4	3·9	–	83·7	1	3·2
NF24	1·5	18	1·7	–	76·7	TR	1·7

Table 5. Levels of DNA relatedness among strains

Strain	Percentage DNA binding with:					
	Shewanella hanedai CECT 5017^T	*Shewanella frigidimarina* LMG 1947^T	*Shewanella algae* CECT 331	NF12	NF22^T	NF24
Shewanella putrefaciens LMG 2268^T				11	7	12
Shewanella putrefaciens LMG 2369				14	15	
Shewanella hanedai CECT 5017^T	100					
Shewanella frigidimarina LMG 19475^T		100		74		70
Shewanella baltica LMG 2250^T				20	11	
Shewanella algae CECT 331			100			
Shewanella algae LMG 2265				8	14	
NF12	10		12	100		
NF22^T	13	24	6	38	100	32
NF24	14		13	88		100

fermentation in *Shewanella benthica* and *Shewanella frigidimarina*.

SDS-PAGE protein profile patterns

The total-protein profile patterns of strains NF12 and NF24 were practically identical and similar to the profiles of *Shewanella algae* CECT 331 and *Shewanella baltica* CECT 323^T, respectively (Fig. 3). However, NF22^T was not so similar with respect to the protein profiles of the other isolates and *Shewanella* species.

Cellular fatty acid composition

The fatty acid composition of the bacterial isolates and *Shewanella algae* CECT 331 are shown in Table 3. The fatty acid profiles observed in the Antarctic isolates were in accordance with the profiles of other *Shewanella* species (Moule & Wilkinson, 1987; Matsui *et al*., 1991; Nichols *et al*., 1994; Bowman *et al*., 1997; Venkateswaran *et al*., 1998). In the three isolated bacteria the monounsaturated 16:1 ω7c was the major component. NF12 and NF24 presented high amounts of monounsaturated fatty acids (16:1 ω7c, 17:1 ω8c), followed by terminally branched saturates (13:0 iso, 13:0 iso 3OH, 15:0 iso) and straight-chain saturates (16:0, 15:0, 12:0) as occur in *Shewanella algae*, *Shewanella amazonensis*, *Shewanella gelidimarina* and *Shewanella oneidensis*. However, NF22^T had high amounts of monounsaturates, followed by straight-chain saturates and terminally branched saturates as found in *Shewanella frigidimarina*, *Shewanella benthica*, *Shewanella hanedai* and *Shewanella putrefaciens* (Venkateswaran *et al*., 1999).

Isoprenoid quinone composition

The quinone compositions are shown in Table 4. NF12, NF22^T and NF24 presented ubiquinones, menaquinones and small amounts of methylmenaquinones. Akagawa-Matsushita *et al*. (1992) pointed out that the presence of both ubiquinones and menaquinones could be a relevant differential characteristic among *Shewanella* species and other aerobic, motile

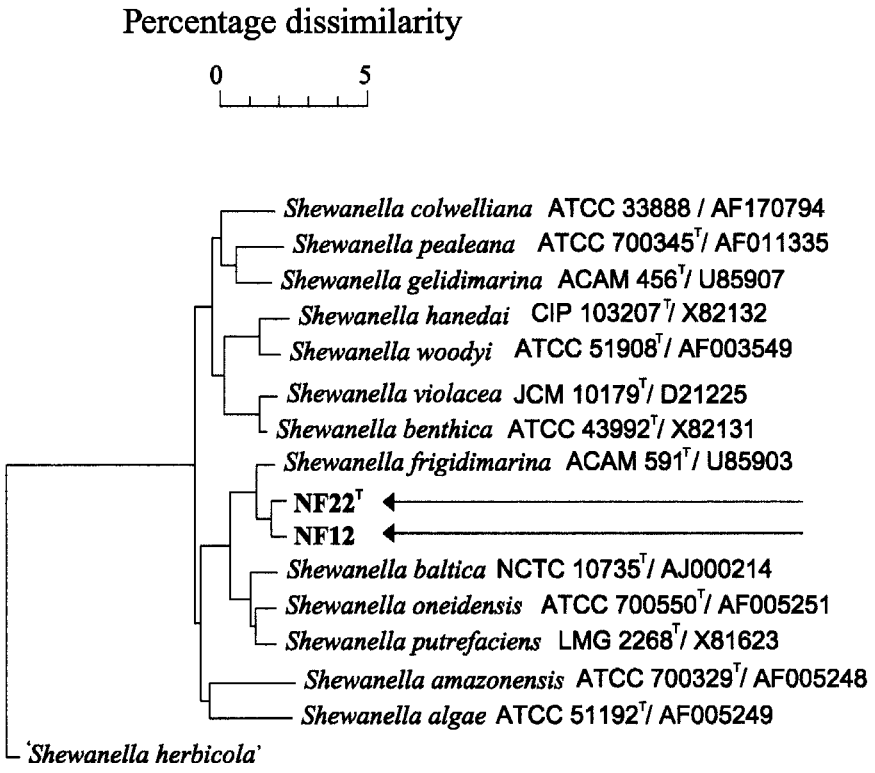

Fig. 4. Phylogenetic tree obtained by neighbour-joining analysis of 16S rRNA sequences showing the positions of the Antarctic isolates NF12 and NF22^T, and related organisms. Strain and accession numbers are indicated.

marine bacteria such as *Alteromonas*, *Pseudoalteromonas* and *Marinomonas*. The presence of methylmenaquinones in some strains of *Shewanella putrefaciens* has been reported by Collins & Jones (1981), Itoh *et al.* (1985), Moule & Wilkinson (1987), Akagawa-Matsushita *et al.* (1992) and Venkateswaran *et al.* (1999) (*Shewanella oneidensis*).

DNA base composition and DNA–DNA hybridization

The DNA G+C content of the Antarctic isolates was 41–42 mol% (Table 2), which agrees with the range described for the genus *Shewanella* (39–55 mol%; Venkateswaran *et al.*, 1999). Table 5 shows the levels of DNA–DNA relatedness among the strains studied. NF12 and NF24 presented a DNA reassociation percentage of 88%. The percentage similarity between these bacterial isolates and *Shewanella frigidimarina* LMG 19475^T was over 70%, which places the strains within the same species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). DNA–DNA similarity between NF12 and NF22^T was 38%. NF22^T and *Shewanella frigidimarina* LMG 19475^T showed a low level of DNA homology (24%). The genetic similarity of NF12, NF22^T and NF24 with the other type strains studied ranged from 6 to 20%.

16S rRNA phylogenetic analysis

16S rRNA phylogenetic studies showed similarities with the type strain of *Shewanella frigidimarina*, ACAM 591^T, of 98·7% to NF12 and 98·9% to NF22^T. The phylogenetic tree constructed by the neighbour-joining method is shown in Fig. 4. These results suggest that the isolates belong to the genus *Shewanella*. NF12 and NF24, with a G+C content of 42 mol%, belong to the species *Shewanella frigidimarina*. However, NF22^T occupies a separate position in the genus *Shewanella*. This Antarctic strain is proposed as a new species, designated *Shewanella livingstonensis* sp. nov.

Description of *Shewanella livingstonensis* sp. nov.

Shewanella livingstonensis (li.ving.sto.nen′sis. N.L. masc./fem. adj. *livingstonensis* pertaining to Livingston Island, Antarctica, where the organism was isolated).

Gram-negative, facultatively anaerobic, rod-shaped cells that are 0·3–0·5 μm wide and 1–3 μm long when the organism is grown in TSB. Cells occur singly or in pairs and after 24 h incubation in liquid medium filaments 10 μm long are observed. Endospores are not formed. Cells are motile by means of a single polar

flagellum. Growth occurs at temperatures from 4 to 20 °C. Colonies on TSA are circular, smooth, convex, slightly mucoid and red-brown in colour with a diameter of 1–2 mm after 5 d at 15 °C. Growth factors are not required. Sodium ions are not required for growth. Positive in oxidase, catalase, alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-galactosidase tests. Positive for hydrolysis of aesculin, Tween, gelatin and casein. Hydrogen sulfide is produced from thiosulfate. Cells are able to reduce nitrate to nitrite and grow anaerobically by reducing TMAO and ferric compounds with lactate as electron donor. Anaerobic growth occurs alternatively by fermentation of D-glucose, maltose, cellobiose, sucrose, D-mannose, D-mannitol, D-galactose, D-xylose, *N*-acetylglucosamine and trehalose. Growth occurs on D-glucose, maltose, cellobiose, sucrose, D-mannose, D-mannitol, D-galactose, D-xylose, *N*-acetylglucosamine, trehalose, malonate, succinate, D-ribose and DL-lactate. The main cellular fatty acids are monounsaturated acids (16:1 ω7*c*, 17:1 ω8*c*) followed by straight-chain saturates and terminally branched saturates. The G+C content of DNA is 41 mol%. Isolated from water collected from Johnson's Dock (Livingston Island, South Shetland Islands, Antarctica). Strain NF22^T has been deposited in the BCCM/LMG Bacteria Collection as LMG 19866^T and in the Spanish Type Culture Collection as CECT 5933^T.

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Characterization of several *Psychrobacter* strains isolated from Antarctic environments and description of *Psychrobacter luti* sp. nov. and *Psychrobacter fozii* sp. nov.

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Eleven psychrophilic bacteria isolated from Antarctic coastal marine environments were subjected to a polyphasic taxonomic study. The isolates were oxidase-positive, halotolerant, Gram-negative, non-motile coccobacilli with a strictly oxidative metabolism. The DNA G+C content ranged from 44 to 47 mol%. DNA–DNA hybridization experiments showed six homology groups, two of them related at the species level to the type strain of *Psychrobacter immobilis*, LMG 7203^T (70–83 %). The highest DNA relatedness of two other groups to known *Psychrobacter* species was found to the type strain of *Psychrobacter glacincola*, LMG 21282^T (51–57 %), and no significant similarity was found between *Psychrobacter* type strains and the last two groups. The predominant cellular fatty acids detected were typical of the genus *Psychrobacter* and included 18 : 1ω9*c*, 16 : 1ω7*c* and 17 : 1ω8*c*. 16S rRNA gene sequence analysis confirmed that the strains isolated belonged to the genus *Psychrobacter*. The results of the study assigned five isolates to *P. immobilis*, three isolates to *P. glacincola* and three isolates to novel *Psychrobacter* species. The names *Psychrobacter luti* sp. nov. (type strain NF11^T = LMG 21276^T = CECT 5885^T) and *Psychrobacter fozii* sp. nov. (type strain NF23^T = LMG 21280^T = CECT 5889^T) are proposed for these organisms.

INTRODUCTION

The genus *Psychrobacter* was created by Juni & Heym (1986) to accommodate a group of non-motile, oxidase-positive, non-pigmented, chiefly psychrotolerant, Gram-negative rods or coccobacilli isolated from the skin of fish and chickens and from various processed foods (Juni, 1991). These strains were referred to as '*Moraxella*-like' organisms (Shaw & Shewan, 1968) and the strains that were competent for genetic transformation (Juni & Heym, 1980) were grouped together as members of the genus *Psychrobacter* (Juni & Heym, 1986) which, at present, belongs to the family *Moraxellaceae* (Rossau *et al.*, 1991).

In the description of the genus *Psychrobacter*, Juni & Heym (1986) indicated the isolation of *Psychrobacter* organisms from a variety of sources including fish, poultry, meat products, clinical sources and sea water and also as contaminants on complex media. After the description of the first species of this genus, *Psychrobacter immobilis*, several species isolated from natural environments have been described from Antarctic ornithogenic soils, sea ice and krill, deep-sea environments and sea water of the Pacific Ocean, internal tissues of an ascidian collected in the Indian Ocean and a bioaerosol originating from pigeon faeces (Bowman *et al.*, 1996, 1997; Maruyama *et al.*, 2000; Denner *et al.*, 2001; Kämpfer *et al.*, 2002; Romanenko *et al.*, 2002). It is evident that cold environments constitute an ecological niche for *Psychrobacter* organisms and Antarctic environments are a source of *Psychrobacter* bacteria.

Several Gram-negative, oxidase-positive, non-motile, coccoid bacteria were isolated from samples collected in the South Shetland Islands (Antarctica) by a Spanish scientific expedition during the Antarctic summer of 1987–1988. These Antarctic isolates were assigned to the genus *Psychrobacter*. In this study, we establish the taxonomic position of these bacteria by using phenotypic, genotypic, chemotaxonomic and phylogenetic analyses. The results obtained enabled us to allocate some of them in known species, *P. immobilis* and *Psychrobacter glacincola*, and to describe two novel species, *Psychrobacter luti* sp. nov. and *Psychrobacter fozii* sp. nov.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains NF1, NF7, NF11^T and NF23^T are AJ430829, AJ430830, AJ430828 and AJ430827.

Detailed physiological and biochemical properties and fatty acid compositions of the Antarctic isolates, transformation assay results and DNA–DNA hybridization results are available as supplementary material in IJSEM Online.

Table 1. Bacterial strains used in this study

ACAM, Australian Collection of Antarctic Microorganisms, University of Tasmania, Tasmania, Australia; ATCC, American Type Culture Collection, Manassas, VA, USA; CECT, Colección Española de Cultivos Tipo, Burjasot, Valencia, Spain; CIP, Collection de l'Institut Pasteur, Paris, France; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium.

Strain	Source
P. immobilis LMG 7203^T (=CECT 5008^T=ATCC 43116^T)	LMG, CECT
P. immobilis CECT 4646 (=ATCC 43117)	CECT
P. frigidicola LMG 21281^T (=ACAM 304^T)	ACAM
P. urativorans LMG 21283^T (=ACAM 534^T)	ACAM
P. glacincola LMG 21282^T (=ACAM 483^T)	ACAM
P. phenylpyruvicus LMG 5372^T (=ACAM 535^T=ATCC 23333^T)	LMG
P. proteolyticus LMG 21313^T (=CIP 106830^T=DSM 13887^T)	LMG
Moraxella nonliquefaciens CECT 465^T (=ATCC 19975^T)	CECT
Moraxella bovis CECT 468^T (=ATCC 10900^T)	CECT
NF1 (=LMG 21273=CECT 5882), NF7 (=LMG 21274=CECT 5883), NF8 (=LMG 21275=CECT 5884), NF11^T (=LMG 21276^T=CECT 5885^T)	Glacier mud, Admiralty Bay, Antarctica
NF18 (=LMG 21277=CECT 5886), NF19 (=LMG 21278=CECT 5887), NF20 (=LMG 21279=CECT 5888), EN1 (=LMG 21270=CECT 5879), EN2 (=LMG 21271=CECT 5880), EN4 (=LMG 21272=CECT 5881)	Water, Johnson's Dock, Antarctica
NF23^T (=LMG 21280^T=CECT 5889^T)	Sediment, Johnson's Dock, Antarctica

METHODS

Bacterial strains and isolation. Strains investigated in this study are listed in Table 1. Strains NF1, NF7, NF8 and NF11^T were isolated from mud collected in the inlet Admiralty Bay (King George Island, South Shetland Islands), at the bottom of a glacier that is covered at high water. Strains NF18, NF19, NF20, EN1, EN2 and EN4 were isolated from sediment collected in Johnson's Dock (Livingston Island, South Shetland Islands). Strain NF23^T was isolated from water collected in Johnson's Dock.

Aliquots of samples were removed with a platinum loop and diluted in a saline solution containing (g l^{-1}, pH 7): NaCl, 0·56; KCl, 0·27; $CaCl_2$, 0·03; $NaHCO_3$, 0·01. Trypticase soy agar (TSA; ADSA) plates were inoculated with loopfuls of several sample dilutions using the streak plate method to obtain well-isolated colonies. Subsequently, plates were incubated for 6 days at 15 °C. Isolates were maintained on TSA slopes at 4 °C and at −20 °C in 50 % (v/v) glycerol.

Morphology. Cell size and morphology were determined by scanning (Hitachi model S 3200) and transmission (Philips model 301) electron microscope observations of cells grown in trypticase soy broth (TSB; ADSA) at 15 °C. Motility was tested by using phase-contrast microscopy (Olympus model CHS).

Physiological and biochemical characteristics. Oxidase, catalase, nitrate reduction, hydrolysis of lecithin, aesculin, gelatin, starch, DNA, casein and Tween 80, pH and temperature ranges for growth, sodium requirement, salt tolerance and susceptibility to antibiotics were determined as described by Bozal *et al.* (2002). Acid production from carbohydrates and carbon- and energy-source utilization tests were performed as described by Bowman *et al.* (1996). Urease and phenylalanine deaminase activity were determined following Cowan & Steel (1993). Tolerance to 5 % (w/v) bile salts (Oxoid) was tested on nutrient agar (ADSA).

API galleries (API 20E, API 20NE, ATB 32GN, API 20B, API ZYM; bioMérieux) were used to test additional biochemical characteristics and were prepared according to the manufacturer's instructions, except that the API tests were incubated for 5 days at 15 °C.

Gram staining was performed according to Hucker & Conn (1923) and was confirmed by the L-alanine aminopeptidase assay (Manafi & Kneifel, 1990; Hernandez Molina *et al.*, 1991). Capsule staining was performed following the methods of Cowan & Steel (1993).

Determination of 2-keto-3-deoxyoctanoic acid (KDO) and LPS. 2-Keto-3-deoxyoctanoic acid (KDO) in LPS was determined according to protocols of Hanson & Phillips (1981), using cell-wall preparations obtained as described by Work (1971). LPS was obtained from whole-cell lysates using the methods of Hitchcock & Brown (1983) and Mandatori & Penner (1989). SDS-PAGE of whole-cell lysates was performed by the procedure of Sambrook *et al.* (1989) in a MiniProtean II electrophoresis cell (Bio-Rad) by using 12 % separation gels. Gels were silver-stained according to the method of Hitchcock & Brown (1983).

Transformation assay. Crude DNA samples of bacterial isolates, *Moraxella nonliquefaciens* CECT 465^T and *Moraxella bovis* CECT 468^T were prepared and assayed for their ability to transform a hypoxanthine- and thiamin-requiring mutant of *P. immobilis* ATCC 43117 to prototrophy, according to the transformation assay for psychrobacters described by Juni & Heym (1980).

Fatty acid composition. Fatty acids were prepared from 40 mg wet cell material harvested from a culture on TSB agar (30 g TSB, 15 g agar; BBL) incubated for 5 days at 15 °C. Whole-cell fatty acids were determined as described by Bozal *et al.* (2002).

Determination of DNA base composition. DNA was extracted from strains and purified by the method of Marmur (1961). The G+C content was determined as described by Bozal *et al.* (2002).

DNA–DNA hybridization and phylogenetic analysis. Genomic DNAs of bacterial strains were prepared by the procedure of Wilson (1987). DNA–DNA relatedness was measured fluorometrically by using the microplate hybridization method (Ezaki *et al.*, 1989). 16S rDNA sequences for the Antarctic *Psychrobacter* strains were determined and phylogenetic analyses carried out as described by Bozal *et al.* (2002).

RESULTS AND DISCUSSION

Morphological and growth characteristics

The 11 bacterial isolates were non-motile, Gram-negative rods or coccobacilli (Fig. 1a, c, e) and non-spore-forming. Diploforms were common. These strains formed non-pigmented colonies on TSA agar incubated at 15 °C. Colonies of NF1, NF7, NF8, NF23, EN1, EN2 and EN4 were circular, smooth, slightly convex and bright with a diameter of 2–4 mm, whereas colonies of NF11^T, NF18, NF19 and NF20 were smooth, opaque, non-circular and spread little throughout the growth medium, with similar dimensions. Neither diffusible pigments nor bioluminescence were observed. The cells of all bacterial isolates presented capsules and were about 0·4–1·8 by 0·4–0·8 μm in size. The strains were moderately halophilic and tolerated NaCl levels of about 9·5–12·5 %. Strains NF1 and EN4 required Na^+ at a concentration of 17 mM [0·1 % (w/v) NaCl]. The pH range for growth was 6–9·5 and the growth temperature range was 4–30 °C for all the strains isolated except NF1 and NF20, which grew at 4–25 °C. Bile salts (5 %) were not tolerated by the isolates.

Phenotypic characterization

The physiological and biochemical properties of the Antarctic isolates are summarized in Table A (available as supplementary material in IJSEM Online). All strains were oxidase- and catalase-positive and capable of oxidative

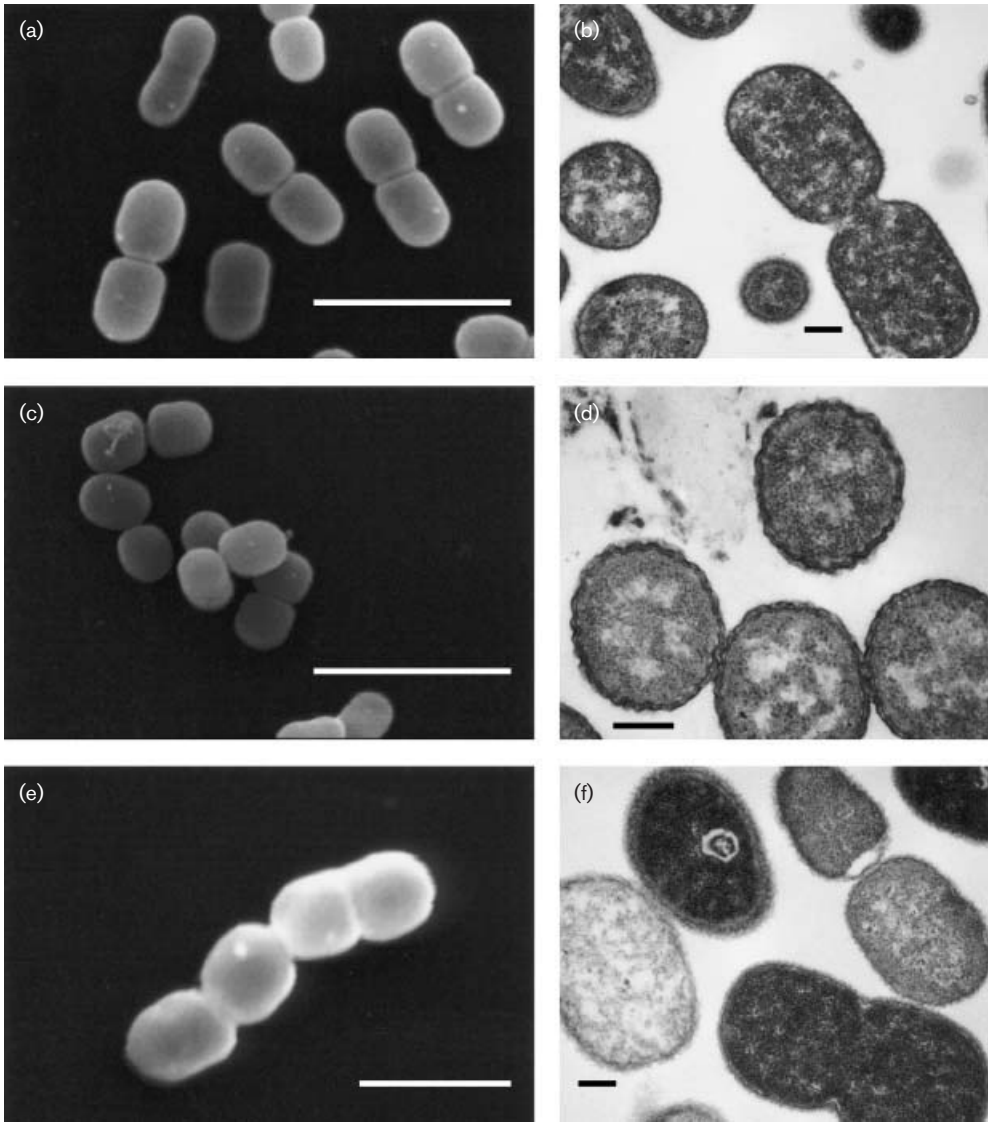

Fig. 1. Scanning electron micrographs (a, c, e) and transmission electron micrographs of ultrathin sections (b, d, f) of strains NF23^T (a, b), NF11^T (c, d) and NF1 (e, f). Cells were grown on TSB for 24 h at 15 °C. Bars, 2 μm (a, c), 1 μm (e) and 0·2 μm (b, d, f).

Table 2. Phenotypic characteristics of strains NF11^T, EN4 and NF23^T and other *Psychrobacter* species

Species/strains: 1, *P. luti* sp. nov. NF11^T; 2, *P. fozii* sp. nov. EN4; 3, *P. fozii* sp. nov. NF23^T; 4, *P. immobilis* (data from Bowman *et al.*, 1996); 5, *P. glacincola* (Bowman *et al.*, 1997); 6, *P. frigidicola* (Bowman *et al.*, 1996); 7, *P. urativorans* (Bowman *et al.*, 1996); 8, *P. phenylpyruvicus* (Bowman *et al.*, 1996); 9, *P. pacificensis* (Maruyama *et al.*, 2000); 10, *P. proteolyticus* (Kämpfer *et al.*, 2002; Denner *et al.*, 2001); 11, *P. faecalis* (Kämpfer *et al.*, 2002); 12, *P. submarinus* (Romanenko *et al.*, 2002); 13, *P. marincola* (Romanenko *et al.*, 2002). All taxa were positive for oxidase and catalase. All taxa were negative for growth at 40 °C (determined at 45 °C for *P. faecalis*), glucose fermentation, indole and H_2S production, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrolysis of aesculin, starch and DNA (not determined for *P. faecalis*) and utilization of adipate, *myo*-inositol, D-melibiose, L-rhamnose, sucrose and D-sorbitol (not determined for *P. proteolyticus*). For strains NF11^T, EN4 and NF23^T, results are scored as positive or negative. For other species, results are scored as: +, 90–100 % of strains positive; −, 0–10 % of strains positive; V+, 11–89 % of strains positive, type strain positive; V−, 11–89 % of strains positive, type strain negative; (+), weak reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Activity of:													
Urease	−	+	+	V+	V−	−	V+	+	+	+	−	−	−
Phenylalanine deaminase	+	−	−	+	−	+	−	+	−	ND	ND	−	−
Tryptophan deaminase	−	−	−	V−	−	+	−	−	−	−	ND	−	−
Nitrate reduction	+	−	−	V−	V+	−	V−	−	−	−	ND	−	−
Tolerance of 5 % bile salts	−	−	−	+	+	−	−	+	ND	−	+	ND	ND
Growth in NaCl (w/v):													
0	+	−	+	+	+	+	+	+	−	ND	ND	−	−
1 %	+	+	+	+	+	+	+	+	V−	ND	+	+	+
8 %	+	+	+	+	+	+	+	+	−	+	+	+	+
10 %	−	+	+	V+	+	−	−	−	−	+	+	+	+
12 %	−	−	+	V+	V+	−	−	−	−	+	+	+	+
15 %	−	−	−	−	−	−	−	−	−	ND	−	+	+
Growth at:													
4 °C	+	+	+	+	+	+	+	+	+	+	+	+	−
25 °C	+	+	+	+	−	−	+	+	+	+	+	+	+
30 °C	+	+	+	+	−	−	V+	+	+	ND	+	+	+
35 °C	−	−	−	V+	−	−	−	+	+	−	+	+	+
37 °C	−	−	−	−	−	−	−	+	+	−	ND	−	−
Hydrolysis of:													
Lecithin	+	−	−	V+	+	−	−	−	ND	ND	ND	ND	ND
Gelatin	−	−	−	−	−	−	−	−	−	+	ND	−	−
Casein	+	−	−	−	−	−	−	−	−	+	ND	−	−
Tween 80	+	−	−	+	+	−	−	+	ND	(+)	ND	+	+
Acid phosphatase	−	+	−	−	−	−	−	+	ND	+	ND	ND	ND
Alkaline phosphatase	+	+	+	V−	−	−	−	+	ND	+	ND	ND	ND
Esterase (C4)	+	+	+	V+	−	−	−	−	ND	+	ND	ND	ND
Lipase (C14)	+	−	−	V+	+	−	−	−	ND	−	ND	ND	ND
Cystine arylamidase	−	−	+	−	−	−	−	−	ND	−	ND	ND	ND
Leucine arylamidase	+	+	+	V+	+	−	−	+	ND	+	ND	ND	ND
Valine arylamidase	−	−	−	−	−	−	−	−	ND	(+)	ND	ND	ND
Acid from carbohydrates	−	−	−	+	−	−	−	−	+	(+)	−	+	−
Utilization of:													
Trehalose	−	−	−	−	−	−	−	−	ND	ND	−	+	−
Citrate	+	−	−	V−	V+	−	−	+	−	+	+	−	−
Acetate	+	−	+	+	+	+	V+	+	V−	+	+	−	−
L-Malate	+	+	+	+	−	+	V+	+	+	−	+	−	−
Propionate	−	+	+	V+	+	−	−	+	−	+	(+)	−	−
L-Histidine	+	+	+	+	V+	−	−	−	+	+	+	−	−
L-Proline	+	+	+	+	+	+	+	+	+	ND	−	−	−
L-Alanine	−	+	+	+	V+	−	−	+	V−	+	+	−	−
Suberate	−	−	−	V−	ND	+	−	−	V−	ND	−	ND	ND
L-Hydroxyproline	+	+	+	V−	+	−	−	−	ND	ND	ND	ND	ND
D-Glucose, L-arabinose	−	−	−	−	−	−	−	−	−	−	(+)	−	−

Table 2. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
D-Mannitol	−	−	+	−	−	−	−	−	−	ND	−	−	−
Lactose	−	−	−	−	−	−	−	−	−	ND	ND	−	−
D-Xylose, cellobiose, glycerol	−	−	−	−	−	−	−	−	ND	ND	−	−	−
D-Fructose	−	−	−	−	−	−	−	−	ND	ND	−	ND	ND
Putrescine	+	+	+	V+	−	−	V−	+	ND	ND	−	ND	ND
L-Phenylalanine	−	−	−	+	−	+	V+	+	ND	−	−	ND	ND
L-Ornithine	+	−	+	V−	−	−	−	−	ND	ND	(+)	ND	ND
N-Acetylglucosamine	−	−	−	−	−	−	−	−	−	−	+	−	−
DNA G+C content (mol%)	45	46	44	44–47	44	41–42	44	43	44–45	43·6	ND	46·7	50·7

metabolism. Strains EN1 and EN2 formed acid aerobically from sugars, whereas the other isolates failed to oxidize carbohydrates. Except for strain NF11^T, all the strains possessed urease activity. Strains NF11^T, NF18, NF19 and NF20 reduced nitrate to nitrite. Strains NF1, NF8, NF11^T, NF18 and NF19 deaminated phenylalanine. None of the strains was capable of deaminating tryptophan. Strains NF7, NF8, NF11^T, NF18, NF19 and NF20 were sensitive to penicillin.

Gram stain tended to be retained in all the strains. The presence of a Gram-negative cell-wall structure was demonstrated by electron microscopy examinations of ultrathin sections (Fig. 1b, d, f) and the presence of KDO and LPS. Except for strains NF7 and NF8, all isolates possessed L-alanine aminopeptidase, again indicating their Gram-negative character.

On the basis of the standard bacteriological characteristics (Gram-negative, oxidase-positive, coccoid morphology, lack of motility, growth a 4 °C, considerable halotolerance and strictly oxidative metabolism), the Antarctic bacterial isolates can be assigned to the genus *Psychrobacter* (Juni, 1991). Differences between strains NF11^T, EN4 and NF23^T and known *Psychrobacter* species are shown in Table 2.

Identification by transformation

DNA samples from all of the Antarctic isolates were able to transform an auxotrophic mutant of *P. immobilis* ATCC 43117 to prototrophy. The appearance of transformant colonies on a medium M9A plate (Juni & Heym, 1980) confirmed unequivocally that the isolates were members of the genus *Psychrobacter* (Juni, & Heym, 1986; Juni, 1991). The results of the transformation assay are shown in Fig. A, available as supplementary material in IJSEM Online.

Cellular fatty acid composition

The results of the fatty acid analysis are summarized in Table B (available as supplementary material in IJSEM Online). Whole-cell fatty acid profiles were found to be similar to those of species of genus *Psychrobacter*, with 18 : 1ω9*c*, 17 : 1ω8*c* and 16 : 1ω7*c* as the predominant components. The unsaturated fatty acid 18 : 1ω9*c* (oleic acid) accounted for 41–63 % of the total content, as reported for *P. immobilis* (Moss *et al.*, 1988), *Psychrobacter frigidicola* (Bowman *et al.*, 1996), *P. glacincola* (Bowman *et al.*, 1997), *Psychrobacter pacificensis* (Maruyama *et al.*, 2000), *Psychrobacter proteolyticus* (Denner *et al.*, 2001), *Psychrobacter faecalis* (Kämpfer *et al.*, 2002), *Psychrobacter submarinus* and *Psychrobacter marincola* (Romanenko *et al.*, 2002). The fatty acid profiles of strains NF23^T and EN4 presented some differences with respect to the other isolates. These strains contained higher levels of 12 : 0 3-OH and 10 : 0 and less than half of the total content was oleic acid. The predominant components of the novel isolates NF11^T, EN4 and NF23^T (with respective contents in parentheses) were 10 : 0 (2·2, 5·9 and 5·8 %), 12 : 0 3-OH (2·4, 6·5 and 7·1 %), 16 : 1ω7*c* (16, 23·1 and 21 %), 16 : 0 (1·4, 2·1 and 2·3 %), 17 : 1ω8*c* (9·7, 8·8 and 12·6 %) and 18 : 1ω9*c* (60·1, 45 and 41·3 %).

DNA base composition and DNA–DNA hybridization

The DNA G+C contents of the Antarctic isolates were 44 (NF23^T), 45 (NF7, NF8, NF11^T, NF18 and NF19), 46 (NF1, EN1, EN2 and EN4) and 47 (NF20) mol%, which agree with the range described for the genus *Psychrobacter* (44–46 mol%; Juni, 1991). Levels of DNA–DNA relatedness among the strains studied are shown in Table C (available as supplementary material in IJSEM Online). Strains EN1 and EN2 shared 98 % DNA–DNA reassociation, clearly above the level of 70 % accepted as the limit for species relatedness (Wayne *et al.*, 1987). Other strain groups were defined with similarities above 70 %: NF7 and NF8; NF18, NF19 and NF20; and EN4 and NF23^T. The DNA relatedness of NF7 and NF8 with NF1 was 57–59 %, whereas NF11^T and strains EN4 and NF23^T showed relatedness values of 30–40 % with respect to the other isolates. Relatedness at the borderline of species level was found between strains EN1 and EN2 and strains NF18, NF19 and NF20, showing relatedness values between 63 and 68 %. The latter group shared DNA relatedness above 83 % with *P. immobilis* LMG 7203^T, which places these strains within this species. EN1 and EN2 showed relatedness values of 63–70 % to *P. immobilis* LMG 7203^T. The phenotypic traits of isolates EN1 and EN2, such as the

capacity to produce acid aerobically from some carbohydrates, suggests that EN1 and EN2 also belong to *P. immobilis*. The loosely related groups strain NF1 and strains NF7 and NF8 have common biochemical and physiological characteristics that place these isolates in the same species. The highest DNA relatedness of the latter three isolates to known *Psychrobacter* species was to *P. glacincola* LMG 21282^T (51–57 %). No significant similarities were found between the *Psychrobacter* type strains and isolate NF11^T or the group constituted by EN4 and NF23^T. The highest DNA relatedness of these isolates was found to *P. immobilis* LMG 7203^T (36–39 %).

Phylogeny

16S rRNA phylogenetic studies confirmed that strains NF1, NF7, NF11^T and NF23^T are members of the genus *Psychrobacter* (Fig. 2). Similarities significant for possible species relatedness (over 97 %; Stackebrandt & Goebel, 1994) were found between the four strains, as follows: strains NF11^T–NF23^T (99·1 %), NF1–NF7 (99·0 %), NF7–NF23^T (98·9 %), NF1–NF11^T (98·8 %), NF7–NF11^T (98·5 %) and NF1–NF23^T (98·3 %). The similarities shown by NF1 and NF7 to *Psychrobacter* type strains were respectively 98·9 and 99·0 % to *P. glacincola* LMG 21282^T, 97·8 and 98·8 % to *P. immobilis* LMG 7203^T, 97·8 and 97·7 % to *P. proteolyticus* LMG 21313^T and 97·8 and 97·4 % to *P. faecalis* DSM 14664^T. Strain NF1 showed similarities of 97·3 % to *P. submarinus* DSM 14161^T and 97·1 % to *P. marincola* DSM 14160^T. The highest similarity of NF11^T to a defined type strain was to *P. glacincola* LMG 21282^T (98·4 %) followed by *P. immobilis* LMG 7203^T (98·2 %), *P. submarinus* DSM 14161^T (98·0 %) and *P. proteolyticus* LMG 21313^T, *P. marincola* DSM 14160^T and *P. faecalis* DSM 14664^T (97·8 %). The relatedness values shown by NF23^T were 99·0 % to *P. immobilis* LMG 7203^T, 98·8 % to *P. glacincola* LMG 21282^T, 98·2 % to *P. proteolyticus* LMG 21313^T and 97·0 % to *P. submarinus* DSM 14161^T and *P. marincola* DSM 14160^T.

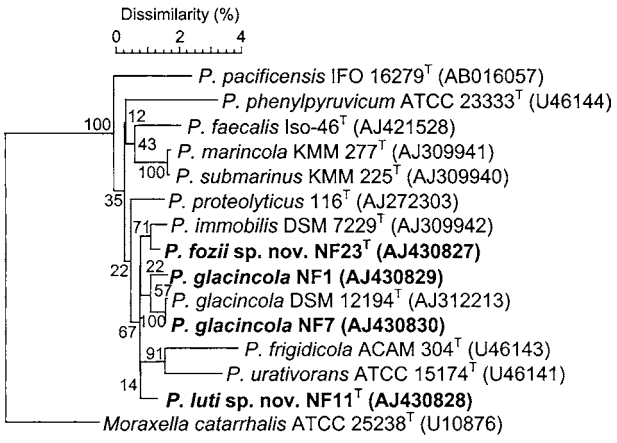

Fig. 2. Phylogenetic tree obtained by neighbour-joining analysis of 16S rRNA gene sequences, showing the positions of the Antarctic isolates NF11^T, NF23^T, NF1 and NF7 within the genus *Psychrobacter*.

On the basis of DNA–DNA hybridizations and 16S rDNA analysis, strains NF7 and NF1 were placed in the same group and their closest relative was *P. glacincola*. Some biochemical traits were found to differ between the type strain of *P. glacincola* and isolates NF7 (and NF8) and NF1, but not enough to consider these Antarctic isolates as distinct taxa. However, the results obtained for isolates NF11^T and NF23^T (and EN4) suggested that they belonged to novel, distinct species in the genus *Psychrobacter*, designated as *Psychrobacter luti* sp. nov. and *Psychrobacter fozii* sp. nov.

Description of *Psychrobacter luti* sp. nov.

Psychrobacter luti (lut′i. L. masc. gen. n. *luti* of mud, referring to the isolation of strains from Antarctic glacier mud).

Cells are Gram-negative, non-motile, non-pigmented, non-spore-forming coccobacilli, 0·4–1·8 μm long and 0·4–0·6 μm wide. Growth occurs at 4–30 °C. Colonies on TSA are about 2 mm in diameter, smooth, opaque and non-circular, and spread little throughout the growth medium after 5 days at 15 °C. Able to grow in the absence of NaCl and can tolerate 9·5 % (w/v) NaCl. Strictly aerobic; oxidase and catalase tests are positive. Acid is not produced from carbohydrates. Cells are able to reduce nitrate to nitrite. Urease and tryptophan deaminase are not produced. Positive in the following biochemical tests: phenylalanine deaminase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase and lecithinase. Positive for hydrolysis of casein and Tween 80. Growth occurs on L-histidine, L-proline, L-hydroxyproline, L-malic acid, sodium succinate, L-arginine, L-glutamine, Tween 80, DL-phenylalanine, putrescine, sodium acetate, L-ornithine, sodium citrate, 1-butanol and L-asparagine. The main cellular fatty acids are 18 : 1ω9*c*, 16 : 1ω7*c* and 17 : 1ω8*c*. The G+C content of DNA of the type strain is 45 mol%.

The type strain, strain NF11^T (=LMG 21276^T=CECT 5885^T), was isolated from muddy soil collected from the inlet Admiralty Bay on King George Island, South Shetland Islands, Antarctica.

Description of *Psychrobacter fozii* sp. nov.

Psychrobacter fozii (fo′zi.i. N.L. gen. n. *fozii* of Foz, named after Amadeo Foz, a Spanish physician who was an early pioneer in Spanish brucellosis).

Cells are Gram-negative, non-motile, non-pigmented, non-spore-forming coccobacilli, 0·4–1·8 μm long and 0·4–0·6 μm wide, and occur in pairs or in short chains. Growth occurs at 4–30 °C. Colonies on TSA are circular, smooth, slightly convex and bright with a diameter of 2–4 mm after 5 days at 15 °C. Halotolerant, able to grow in the presence of 10–12·5 % (w/v) NaCl. Strictly aerobic.

Acid is not produced from carbohydrates. Oxidase, catalase and urease tests are positive. Nitrate reduction and tryptophan deaminase are negative. Positive in the following biochemical tests: alkaline phosphatase, esterase (C4), esterase lipase (C8) and leucine arylamidase. Growth occurs on ethanol, L-alanine, D-alanine, L-histidine, L-proline, L-hydroxyproline, L-malic acid, propionic acid, sodium succinate, sodium pyruvate, L-arginine, L-glutamine, Tween 80, DL-phenylalanine, L-asparagine and putrescine. The type strain also uses D-mannitol, laevulose, 1-butanol, sodium acetate and L-ornithine. The main cellular fatty acids are $18:1\omega9c$, $16:1\omega7c$ and $17:1\omega8c$. The G+C content of DNA is 44–46 mol%.

The type strain, strain NF23^T (=LMG 21280^T=CECT 5889^T), was isolated from sediment collected in Johnson's Dock, Livingston Island, South Shetland Islands, Antarctica.

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Paenibacillus antarcticus sp. nov., a novel psychrotolerant organism from the Antarctic environment

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An endospore-forming strain, 20CM^T, was isolated from Antarctic sediment and identified as a member of the genus *Paenibacillus* on the basis of phenotypic and phylogenetic analyses. The organism stained Gram-variable and was facultatively anaerobic. Strain 20CM^T was psychrotolerant, growing optimally at 10–15 °C. Like other *Paenibacillus* species, it contained anteiso-$C_{15:0}$ as the major cellular fatty acid. The DNA G+C content was 40·7 mol%. 16S rRNA gene sequence analysis placed strain 20CM^T within the *Paenibacillus* cluster, with a similarity value of 99·5 % to *Paenibacillus macquariensis* DSM 2^T. DNA–DNA hybridization experiments between the Antarctic isolate and *P. macquariensis* DSM 2^T revealed a reassociation value of 47 %, indicating that strain 20CM^T and *P. macquariensis* DSM 2^T belong to different species. Based on evaluation of morphological, physiological, chemotaxonomic and phylogenetic analyses, a novel species, *Paenibacillus antarcticus* sp. nov., is proposed; the type strain is 20CM^T (=LMG 22078^T=CECT 5836^T).

Based on 16S rRNA analysis, Ash *et al.* (1993) proposed the genus *Paenibacillus* to accommodate a group of aerobic or facultatively anaerobic rod-shaped, endospore-forming bacteria. Many *Bacillus* species were transferred to the genus *Paenibacillus* based on a comparison of their 16S rRNA gene sequences with other members of the *Bacillaceae* (Ash *et al.*, 1993; Heyndrickx *et al.*, 1995, 1996a, b; Shida *et al.*, 1997a; Pettersson *et al.*, 1999). Traditionally, Gram-positive, rod-shaped, endospore-forming bacteria have been classified in the genus *Bacillus* (Claus & Berkeley, 1986). However, in recent years, the genus *Bacillus* has been separated into several distinct genera, such as *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Aneurinibacillus* and *Brevibacillus* (Shida *et al.*, 1996), *Halobacillus* (Spring *et al.*, 1996), *Paenibacillus* (Ash *et al.*, 1993), *Amphibacillus* (Niimura *et al.*, 1990), *Filobacillus* (Schlesner *et al.*, 2001), *Geobacillus* (Nazina *et al.*, 2001), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Gracilibacillus* and *Salibacillus* (Wainø *et al.*, 1999) and *Ureibacillus* (Fortina *et al.*, 2001). Antarctica is a source of novel *Bacillus* species (Logan *et al.*, 2000, 2002, 2004a); novel members of the genus *Paenibacillus* have also been described recently in this environment (Logan *et al.*, 2004b).

According to Ash *et al.* (1993), members of the genus *Paenibacillus* produce ellipsoidal endospores in swollen sporangia. The cell wall shows structures typical of Gram-positive bacteria, but usually stains negatively. The DNA G+C contents range from 40 to 54 mol% and anteiso-$C_{15:0}$ is the major cellular fatty acid. Some members of the genus produce antibacterial compounds (Slepecky & Hemphill, 1991) and iturin-like antifungal antibiotics (Chung *et al.*, 2000). One distinctive characteristic of the genus *Paenibacillus* is the ability to excrete a wide variety of enzymes that degrade natural biopolymers such as alginate, chondroitin, chitin, curdlan, starch (Kanzawa *et al.*, 1995; Nakamura, 1987; Chung *et al.*, 2000; van der Maarel *et al.*, 2000) and other polysaccharides (Priest *et al.*, 1988).

In this study, the taxonomic status of strain 20CM^T was investigated using a combination of phenotypic characterization, sequencing of the 16S rRNA gene, DNA base composition, DNA–DNA hybridization and cellular fatty acid composition analysis. Strain 20CM^T is proposed as a representative of a novel species, *Paenibacillus antarcticus* sp. nov.

Strain 20CM^T was isolated from sediment collected in Chlorite Lake on the Byers Peninsula of Livingston Island (South Shetland Islands, Antarctica). Sample aliquots were

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The GenBank/EMBL/DDBJ accession number for the16S rRNA gene sequence of *Paenibacillus antarcticus* 20CM^T is AJ605292.

A table giving fatty acid composition (Table A) and figures showing electron micrographs and a phylogenetic tree (Figs A and B, respectively) are available as supplementary material in IJSEM Online.

removed with a platinum loop and diluted in a saline solution containing (g l^{-1}, pH 7): NaCl, 0·56; KCl, 0·27; $CaCl_2$, 0·03; and $NaHCO_3$, 0·01. Trypticase soy agar (TSA; ADSA) plates were inoculated with loopfuls of several sample dilutions using the streak-plate method to obtain well-isolated colonies. Plates were incubated for 4 days at 15 °C. Isolates were maintained aerobically on TSA slopes at 4 °C and also at −80 °C on cryo-beads.

Morphology, cell size and shape of spores were determined by scanning (Hitachi model H 2300) and transmission (Hitachi model H 600AB) electron microscope observations of cells grown in trypticase soy broth (TSB; ADSA) at 15 °C. Motility was determined by phase-contrast microscopy (Olympus model CHS). Gram staining was performed according to Hucker & Conn (1923). Two alternative methods, the KOH test and the L-alanine aminopeptidase assay (Manafi & Kneifel, 1990), were also used. Oxidase, catalase and urease activities, methyl red reaction, Voges–Proskauer, nitrate reduction, indole production, citrate utilization, and hydrolyses of casein, lecithin, gelatin, DNA, tyrosine, starch and Tween 80 were determined following Cowan & Steel (1993). Dihydroxyacetone production, phenylalanine deamination and growth in the presence of lysozyme (0·1 and 0·001 %, w/v) were determined as described by Claus & Berkeley (1986). Acid production from carbohydrates and additional tests were determined using the API 50CH and API 20E system (bioMérieux). Tolerance to NaCl was measured on nutrient agar (ADSA) containing 0–10 % (w/v) NaCl. Plates were incubated at 15 °C for 30 days. The temperature range for growth was determined on TSA incubated for 14 days at 4, 10, 15, 20, 25, 30, 31, 32, 33 and 37 °C. Anaerobic growth was determined on TSB plus agar-agar (1·5 %; ADSA) by incubation in an anaerobic chamber at 15 °C for 5 days.

Cells were Gram-variable, rod-shaped (0·7 × 2·5 μm) and motile by means of peritrichous flagella (see Fig. Aa, b; available as supplementary material in IJSEM Online). Strain 20CM^T produced ellipsoidal spores in swollen sporangia in the subterminal or terminal region of the cell (see Fig. Ac, d, e; available as supplementary material in IJSEM Online). Colonies grown on TSA at 15 °C were non-pigmented, circular, slightly convex, bright and cream coloured with a diameter of 1·0–1·5 mm. Cell wall structure was Gram-positive, as demonstrated by electron microscope examinations of ultra-thin sections (see Fig. Af, available as supplementary material in IJSEM Online), although results of the KOH test and the L-alanine aminopeptidase assay indicated a Gram-negative character. The isolate was facultatively anaerobic and grew at 4–31 °C. Growth was optimal at 10–15 °C. It grew in the presence of 4 % (w/v) NaCl and in 0·001 % (w/v) lysozyme, but not in 0·1 % (w/v) lysozyme. The final pH in Voges–Proskauer broth after 7 days incubation at 15 °C was less than pH 6. The isolate was negative for production of acetylmethylcarbinol, but positive for the methyl red reaction. Strain 20CM^T did not decompose tyrosine. Phenotypic characteristics of the Antarctic isolate and the closest phylogenetic relatives were compared (Table 1). Of the organisms compared, only strain 20CM^T was positive for oxidase production. Phenotypic studies showed that the Antarctic isolate displayed characteristics consistent with those of the genus *Paenibacillus* (Ash *et al.*, 1993).

Fatty acids were prepared from 40 mg wet cell material harvested from a TSB agar (30 g TSB l^{-1}, 15 g agar l^{-1}; BBL) culture incubated for 4 days at 15 °C. The whole-cell fatty acids were determined as described previously by Bozal *et al.* (2002). Fatty acid analysis of 20CM^T (Table A, available as supplementary material in IJSEM Online) revealed that anteiso-$C_{15:0}$ (55·32 %), iso-$C_{15:0}$ (15·04 %) and $C_{16:1}\omega11c$ (7·72 %) were predominant. This fatty acid profile was in accordance with that given in the description of the genus *Paenibacillus* (Ash *et al.*, 1993). anteiso-Branched saturated $C_{15:0}$ is the predominant fatty acid found in all members of the genus *Paenibacillus* (Shida *et al.*, 1997a).

Genomic DNA was prepared according to the method of Gevers *et al.* (2001). The G+C content was determined by HPLC as described by Mesbah *et al.* (1989). DNA–DNA relatedness was measured fluorometrically using the microplate hybridization method described by Ezaki *et al.* (1989). Determination of the 16S rRNA gene sequence of strain 20CM^T and phylogenetic analyses were carried out as described previously by Bozal *et al.* (2002).

Phylogenetic studies based on 16S rRNA gene sequences confirmed that strain 20CM^T is a member of the genus *Paenibacillus* (Fig. 1; Fig. B, available as supplementary material in IJSEM Online). The 16S rRNA gene sequence of strain 20CM^T showed 99·5 % similarity to that of *Paenibacillus macquariensis* DSM 2^T, which is significant enough to suggest possible species relatedness. Stackebrandt & Goebel (1994) suggested that a sequence similarity value greater than 97 % indicated conspecificity of the strains involved. The similarity values shown by 20CM^T to other *Paenibacillus* type strains were under 97 % (*Paenibacillus borealis* DSM 13188^T, 95·5 %; *Paenibacillus odorifer* LMG 19079^T, 94·7 %). To further verify the taxonomic position of isolate 20CM^T, DNA–DNA hybridizations were performed with *P. macquariensis* LMG 6935^T (Marshall & Ohye, 1966). The low DNA–DNA reassociation value of 47 % between these two strains and the 16S rRNA gene sequence analysis confirmed the distinct position of strain 20CM^T within the genus *Paenibacillus*. The G+C content of strain 20CM^T was 40·7 mol%, which lies within the range observed for members of the genus *Paenibacillus* (Shida *et al.*, 1997a).

Morphological, physiological, chemotaxonomic and phylogenetic data showed that strain 20CM^T belongs to the genus *Paenibacillus*. DNA–DNA hybridization analysis clearly distinguished strain 20CM^T from *P. macquariensis* (Marshall & Ohye, 1966). Based on polyphasic evidence, it is proposed that strain 20CM^T be assigned as the type strain of a novel species in the genus *Paenibacillus*, *Paenibacillus antarcticus* sp. nov.

Table 1. Phenotypic characteristics that differentiate *P. antarcticus* 20CM^T from its closest relatives in the genus *Paenibacillus*

Species: 1, *P. antarcticus* (data from this study); 2, *Paenibacillus graminis* (Berge *et al.*, 2002); 3, *Paenibacillus azotofixans* (Seldin *et al.*, 1984; Seldin & Penido, 1986); 4, *P. macquariensis* (Marshall & Ohye, 1966; Shida *et al.*, 1997a, b; Elo *et al.*, 2001); 5, *P. borealis* (Elo *et al.*, 2001); 6, *P. odorifer* (Berge *et al.*, 2002). All species produced acid from galactose, D-glucose, D-fructose, amygdalin, salicin, cellobiose, maltose, melibiose, sucrose, trehalose, D-raffinose, β-gentiobiose and D-turanose. None of the species produced acid from erythritol, D-arabinose, L-xylose, adonitol, L-sorbose, rhamnose, dulcitol, inositol, L-arabitol, 2-ketogluconate or 5-ketogluconate. All species were negative for growth at 42 °C. For *P. antarcticus*, results are scored as positive or negative. For other species, results are scored as follows: +, >90 % strains positive; −, <10 % strains positive; V, 11–89 % strains positive; ND, not determined.

Characteristic	1	2	3	4	5	6
Oxidase	+	−	−	−	−	−
Voges–Proskauer test	−	ND	+	−	−	ND
Nitrate reduction	−	+	−	−	−	+
Production of dihydroxyacetone	−	ND	−	−	−	ND
Casein hydrolysis	−	ND	−	−	+	ND
Starch hydrolysis	+	ND	−	+	−	ND
Gelatin liquefaction	−	ND	−	−	−	ND
Acid production from:						
Glycerol	−	+	−	−	+	V
L-Arabinose, D-xylose, methyl β-D-xyloside, *N*-acetylglucosamine, lactose, starch	+	+	−	+	+	+
Ribose	+	−	−	+	−	+
D-Mannose	+	+	+	+	+	V
Mannitol, melezitose	−	+	+	+	+	−
Sorbitol	−	−	+	−	V	−
Methyl α-D-mannoside	−	−	−	+	−	−
Methyl α-D-glucoside	+	+	+	+	V	+
Arbutin, glycogen	−	+	−	+	+	+
Inulin	−	V	+	−	+	+
Xylitol, D-lyxose, D-tagatose	−	−	−	−	V	−
D-Fucose	−	V	−	−	−	−
L-Fucose	−	−	−	+	−	V
D-Arabitol	−	−	−	−	+	−
Gluconate	−	V	−	+	−	−
Growth at (°C):						
0	−	−	−	+	−	−
5	+	V	−	+	+	+
10	+	+	−	+	+	+
31	+	+	+	−	+	+
32	−	+	+	−	+	+
35	−	+	+	−	+	+
37	−	V	+	−	+	−
40	−	V	V	−	−	−
Growth in the presence of:						
Lysozyme (0·001 %)	+	ND	−	−	−	ND
NaCl (5 %)	−	ND	−	−	−	ND
G+C content (mol%)	40·7	52·1	51·6	39	53·6	44·0

Description of *Paenibacillus antarcticus*

Paenibacillus antarcticus (ant.arc′ti.cus. L. masc. adj. *antarcticus* of the Antarctic environment, where the organism was isolated).

Cells are rod-shaped (0·7 × 2·5 μm) and motile by means of peritrichous flagella. Subterminal or terminal ellipsoidal spores are formed in swollen sporangia. Colonies grown on TSA are non-pigmented, circular, slightly convex, bright and cream coloured. Cells are facultatively anaerobic and stain Gram-variable. Growth is not inhibited by the presence of 4 % NaCl or 0·001 % lysozyme. Growth occurs at 4 and

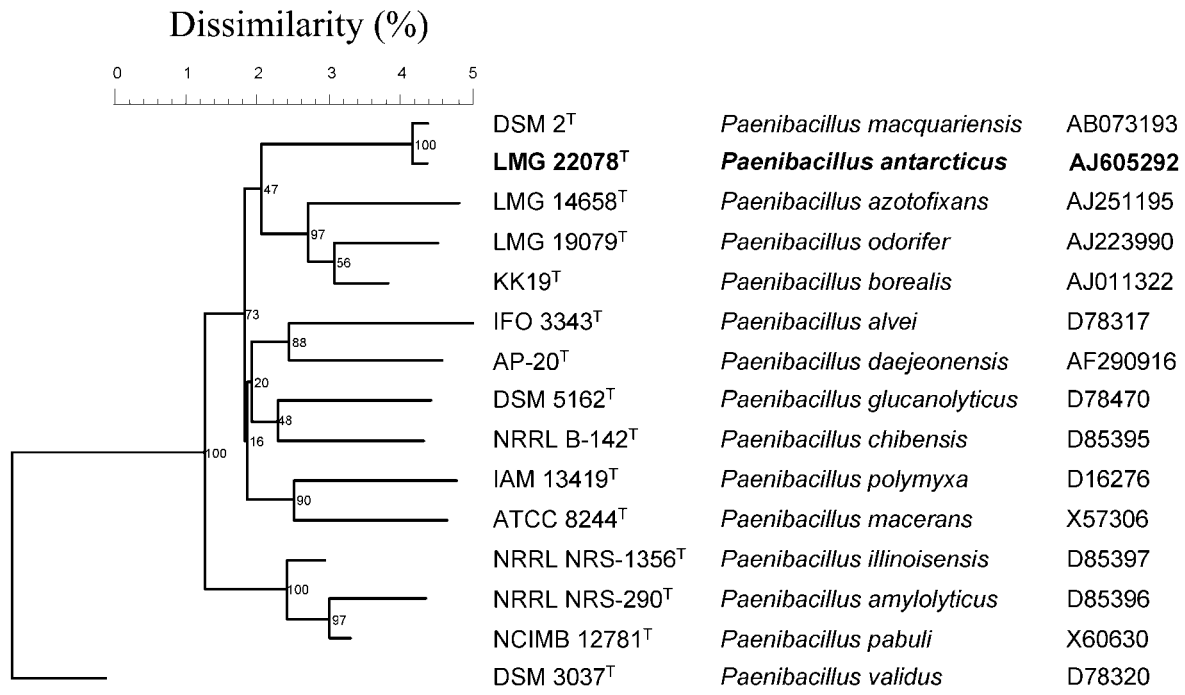

Fig. 1. Phylogenetic position of strain 20CM^T among neighbouring species of the genus *Paenibacillus*, based on 16S rRNA gene sequence analysis. Bootstrap values are indicated.

31 °C, but not at 0 or 32 °C; optimal growth occurs at 10–15 °C. Oxidase, catalase, urease and methyl red reactions are positive. Nitrate reduction, Voges–Proskauer reaction, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, dihydroxyacetone production, indole production, H_2S production, phenylalanine deamination and tryptophan deaminase are negative. Aesculin, starch and Tween 80 are hydrolysed. Does not hydrolyse casein, lecithin, gelatin, DNA or tyrosine. With API systems, acid is produced from L-arabinose, ribose, D-xylose, methyl β-D-xyloside, galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, D-raffinose, starch, β-gentiobiose and D-turanose. Acid is not produced from glycerol, erythritol, D-arabinose, L-xylose, adonitol, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, arbutin, inulin, melezitose, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. The predominant fatty acid is anteiso-$C_{15:0}$ (55·32 %).

The type strain is 20CM^T (=LMG 22078^T=CECT 5836^T); its G+C content is 40·7 mol%.

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