***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 H₂S 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; CaCl₂, 0.03; NaHCO₃, 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): K₂HPO₄, 5.0; MgSO₄ · 7H₂O, 0.5; CaCl₂, 0.05; FeSO₄ · 7H₂O, 0.028; NH₄Cl, 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 CaCl₂ and FeSO₄ was sterilized by autoclaving it at 0.5 atm (5.065 × 10⁴ Pa) for 30 min. CaCl₂ and FeSO₄ 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): Na₂HPO₄, 10.0; KH₂PO₄, 3.0; K₂SO₄, 1.0; NaCl, 1.0; MgSO₄ · 7H₂O, 0.4; CaCl₂, 0.1; FeSO₄ · 7H₂O, 0.018; NH₄Cl, 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
<i>Shewanella putrefaciens</i> LMG 2268 ^T	ATCC 8071 ^T	LMG
<i>Shewanella putrefaciens</i> LMG 2369		LMG
<i>Shewanella algae</i> CECT 331		CECT
<i>Shewanella algae</i> LMG 2265		LMG
<i>Shewanella baltica</i> CECT 323 ^T	LMG 2250 ^T , NCTC 10735 ^T	CECT, LMG
<i>Shewanella frigidimarina</i> LMG 19475 ^T	ACAM 591 ^T	LMG
<i>Shewanella hanedai</i> 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 μ g per disk; nalidixic acid, 30 μ g per disk; chloramphenicol, 30 μ g per disk; and tetracycline, 30 μ g per disk. +, Positive; –, negative.

Characteristic	NF12	NF22 ^T	NF24
Cell length (μ m)	1–2.5	1–3	1–3.7
Cell diameter (μ m)	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 ₂ S 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 μ m) column (4.6 mm i.d. $\times$ 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; photomultiplier, 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 Miniprotein 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'-CAGCAGCCGCGTAATAC-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'-GTTGCGCTCGTTGCGGGA-CT-3' (1112–1093).

The sequences were assembled by the program Auto Assembler (Applied Biosystems). Phylogenetic analysis was performed using the software package GeneCompass (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 ω 5*c* and 14:1 ω 5*t* 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 ω 7*c*, 18:1 ω 9*t* and 18:1 ω 12*t* (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 ω 5*c*, iso 17:1 ω 9*c*, 15:0 3OH, 17:0 anteiso, 18:0 iso, 18:1 ω 5*c*, 19:0 iso, 19:0 10Me and 20:0 iso.

Fatty acid	Percentage of total fatty acid at:						
	15 °C			Room temperature <i>Shewanella algae</i> (n = 6)	8 °C		
	NF12 (n = 5)	NF22 ^T (n = 4)	NF24 (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 ω 8 <i>c</i>	TR–2.6	TR–1.1	TR–1.4	TR	1.5–2.1	1.3–1.7	TR
15:1 ω 6 <i>c</i>	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 ω 9 <i>c</i>	1.1–1.9	TR	1.1–1.4	TR	1.2	TR	1
16:1 ω 7 <i>c</i>	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 ω 8 <i>c</i>	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 ω 6 <i>c</i>	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 ω 9 <i>c</i>	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 frigidi-*

marina (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
<i>Shewanella algae</i> CECT 331	TR	9.4	36.6	TR	47.8	1.2	4
<i>Shewanella baltica</i> 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:					
	<i>Shewanella hanedai</i> CECT 5017 ^T	<i>Shewanella frigidimarina</i> LMG 1947 ^T	<i>Shewanella algae</i> CECT 331	NF12	NF22 ^T	NF24
<i>Shewanella putrefaciens</i> LMG 2268 ^T				11	7	12
<i>Shewanella putrefaciens</i> LMG 2369				14	15	
<i>Shewanella hanedai</i> CECT 5017 ^T	100					
<i>Shewanella frigidimarina</i> LMG 19475 ^T		100		74		70
<i>Shewanella baltica</i> LMG 2250 ^T				20	11	
<i>Shewanella algae</i> CECT 331			100			
<i>Shewanella algae</i> 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 ω 7c was the major

component. NF12 and NF24 presented high amounts of monounsaturated fatty acids (16:1 ω 7c, 17:1 ω 8c), 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 methylmen-aquinones 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 ω 7c, 17:1 ω 8c) 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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