Shewanella vesiculosa sp. nov., a psychrotolerant bacterium isolated from an Antarctic coastal area

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Two strains of psychrotolerant bacteria, designated M7^T and M5, isolated from Antarctic coastal marine environments were studied to determine their taxonomic position. The organisms comprised Gram-negative, rod-shaped, facultatively anaerobic cells that were motile by means of single polar flagella. Neither of the bacterial isolates had a requirement for Na^+. These two psychrotolerant strains grew at temperatures ranging from −4 to 30 °C. Both strains were capable of producing H_2S from thiosulfate and were able to use sodium nitrate and trimethylamine *N*-oxide as terminal electron acceptors during anaerobic growth. 16S rRNA gene sequence analysis placed M7^T and M5 within the genus *Shewanella*; the strains showed the highest similarity (99.9 and 99.2 % respectively) with respect to the type strains of *Shewanella livingstonensis* and *Shewanella frigidimarina*. However the levels of *gyrB* sequence similarity between strain M7^T and the type strains of *S. livingstonensis* and *S. frigidimarina* were 87.6 and 87.4 %, respectively. DNA–DNA hybridization experiments performed between the Antarctic isolate M7^T and *S. livingstonensis* LMG 19866^T and *S. frigidimarina* LMG 19475^T revealed levels of relatedness of 32 and 35 %, respectively. Strain M5 showed 100 % DNA relatedness with respect to strain M7^T. The DNA G+C content of these bacteria was 42 mol%. Several phenotypic characteristics, the cellular fatty acid compositions and the quinone content of strains M7^T and M5 served to differentiate them from related shewanellae. On the basis of the data from this polyphasic taxonomic study, M7^T and M5 constitute a single genospecies. They represent a novel species of the genus *Shewanella*, for which the name *Shewanella vesiculosa* sp. nov. is proposed. The type strain is M7^T (=LMG 24424^T =CECT 7339^T).

The genus *Shewanella* was first described by MacDonell & Colwell (1985). The definition 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'. Although the taxonomy of this group has been developed in the last decade, *Shewanella putrefaciens* has been studied since its first description as *Achromobacter putrefaciens* by Derby & Hammer (1931), because of its special importance in the areas of applied and environmental microbiology.

Currently, more than 40 species are assigned to the genus *Shewanella* on the basis of genetic and phenotypic analyses (http://www.bacterio.cict.fr/s/shewanella.html). The shewanellae constitute a diverse group of facultatively anaerobic bacteria. Their ability to utilize a variety of final electron acceptors in the absence of oxygen and their capacity to grow at low temperatures and various salt concentrations and barometric pressures allow them to survive in extreme environments, such as those of Antarctica (Bowman *et al.*, 1997; Bozal *et al.*, 2002). Because of their potential uses in bioremediation and energy-generating biocatalysis, shewanellae are being studied intensively, not only taxonomically, but also in terms of genomics, ecology and biotechnology (Venkateswaran *et al.*, 1999; Hau & Gralnick, 2007).

During the characterization of psychrotolerant organisms isolated from Antarctica, strains M7^T and M5 were recovered from marine sediments collected at Deception Island (South Shetland Islands). Sample aliquots were removed with a platinum loop and were diluted in Ringer solution (Oxoid). Marine agar (Difco) and tryptic soy agar

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains M7^T and M5 are AM980877 and AM980878, respectively, and those for the *gyrB* gene sequences of strains M7^T and *S. livingstonensis* NF22^T are EU702750 and EU702751, respectively.

Transmission electron micrographs of ultrathin sections of cells of strain M7^T and details of the cellular fatty acid compositions for strains M7^T and M5 are presented as supplementary material available with the online version of this paper.

(TSA; Oxoid) plates were inoculated with loopfuls of several sample dilutions by using the streak-plate method to obtain isolated colonies. Plates were incubated for 7 days at 15 °C. Growth was observed on both media. Isolates were maintained aerobically on TSA slopes at 4 °C and also at −80 °C on cryo-beads (AES Laboratoire).

The morphology, size and shape of cells grown on TSA at 15 °C were determined by means of negative staining (uranyl acetate at 2 %, w/v) and transmission electron microscopy (JEOL 1010) of ultrathin sections after freeze fixation and freeze-substitution (Nevot *et al.*, 2006). Motility was investigated by means of phase-contrast microscopy. Oxidase, catalase and urease activities, nitrate reduction and hydrolysis of casein, lecithin, gelatin, DNA, starch and Tween 80 were determined according to Barrow & Feltham (1993). Utilization of carbohydrates, enzyme production and additional characteristics were determined by using API 50 CH, API ZYM and API 20NE strips (bioMérieux). NaCl tolerance was measured on nutrient agar (Cultimed) containing 0–10 % (w/v) NaCl; plates were incubated at 15 °C for 14 days. The temperature range for growth was determined on TSA incubated for 14 days at temperatures from −4 to 37 °C. The pH range for growth was established in tryptic soy broth (TSB; Difco) at pH 4.0–10.0 (using increments of 0.5 pH units) at 15 °C for 10 days. Anaerobic growth tests were performed in a modified YP liquid medium prepared according to Toffin *et al.* (2004). Trimethylamine *N*-oxide (10 mM) and sodium nitrate (3 mM) were tested as electron acceptors, with 10 mM lactate as the electron donor and carbon source. Anaerobic conditions were generated in anaerobic chambers (Oxoid) with AnaeroGen (Oxoid) and an anaerobic indicator (Oxoid) and plates were incubated at 15 °C for 10 days. Before storage at 15 °C, anaerobic jars were kept overnight at −4 °C to ensure that anoxic conditions had developed before strain growth could occur. Growth on the plates was compared with growth on control plates containing the same medium but without an organic carbon substrate.

The Antarctic bacterial isolates M7^T and M5 comprised Gram-negative, rod-shaped, non-spore-forming cells. On TSA incubated at 15 °C for 2 days, colonies were circular, smooth, convex, slightly mucous and 1–2 mm in diameter. After 1 week, colonies became more mucous and brownish-coloured. On marine agar, colonies were similar but orange in colour. Diffusible pigments and bioluminescence were not observed. Transmission electron microscopy of ultrathin sections of cells of strain M7^T showed that large numbers of outer-membrane vesicles were released (see Supplementary Fig. S1, available in IJSEM Online). Cells were motile by means of single polar flagella. Neither isolate required Na^+ for growth. The strains were chemo-organotrophic and capable of respiratory and fermentative metabolism. Both strains were positive for cytochrome oxidase and catalase. They reduced nitrate to nitrite, and nitrogen gas was not formed from nitrite. M7^T and M5 were able to grow anaerobically by reducing trimethylamine *N*-oxide or sodium nitrate while using DL-lactate as the electron donor.

The results of the morphological and phenotypic investigations are given in the species description and in Table 1. Phenotypic studies showed that the isolates displayed characteristics consistent with those for the genus *Shewanella*, but strain M7^T differed significantly from related species (*Shewanella livingstonensis*, *S. frigidimarina*, *S. hafniensis*, *S. putrefaciens*, *S. gaetbuli* and *S. morhuae*) in terms of several phenotypic properties, such us temperature requirements, NaCl requirements, enzyme activities and metabolism of certain sugars.

Cellular fatty acid and isoprenoid quinone compositions were determined for cell mass grown on TSB agar (30 g TSB, 15 g agar; Oxoid) for 4 days at 20 °C, as described previously (Bozal *et al.*, 2002). The most abundant fatty acids were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 25.41 % in strain M7^T), iso-$C_{13:0}$ (13.20 %), iso-$C_{15:0}$ (9.70 %), $C_{16:0}$ (9.73 %), $C_{17:1}\omega8c$ (7.84 %) and $C_{15:0}$ (5.73 %) (see Supplementary Table S1). The isolates had cellular fatty acid profiles that were similar to those of phylogenetically closely related type strains of *S. livingstonensis* and *S. frigidimarina*; however, strains M7^T and M5 had lower levels of monounsaturated fatty acids and increased levels of terminally branched saturated fatty acids and, unlike *S. frigidimarina*, strain M7^T did not produce eicosapentaenoic acid ($C_{20:5}\omega3$) (Nichols *et al.*, 1994; Bowman *et al.*, 1997; Venkateswaran *et al.*, 1999; Bozal *et al.*, 2002). Strains M7^T and M5 contained ubiquinones (Q-7, Q-8) and menaquinones (MK-7 and MMK-7) that are commonly found in *Shewanella* species (Bozal *et al.*, 2002; Bowman, 2005).

Total DNA for complete 16S rRNA gene sequence analysis was prepared according to the protocol of Niemann *et al.* (1997). A total of 1495 nt from the 16S rRNA gene were sequenced, as described previously by Bozal *et al.* (2002). A phylogenetic analysis was performed using the software package BioNumerics (Applied Maths). Distance analysis and clustering was done using the neighbour-joining method (Saitou & Nei, 1987). For strain M7^T and *S. livingstonensis* LMG 19866^T, the *gyrB* gene was PCR-amplified with universal primer sets, as described by Yamamoto & Harayama (1995), and subsequently sequenced. The identity of a given PCR product was verified by means of bidirectional sequencing analysis. Multiple alignment, distance matrix analysis and phylogenetic analysis of *gyrB* sequences obtained in this study and others available in the public databases were performed using MEGA, version 4.0 (Tamura *et al.*, 2007). A phylogenetic tree was constructed using the neighbour-joining method (with Kimura's two-parameter model and the pairwise deletion option) and its topological robustness was evaluated by means of a bootstrap analysis of 1000 replicates. For DNA–DNA hybridizations and determination of G+C content, total DNA was prepared according to a modification of the procedure of Wilson (1987). The

G+C content was determined by using the HPLC technique, as described by Mesbah *et al.* (1989). DNA–DNA hybridizations were performed at 47 °C according to a modification (Goris *et al.*, 1998; Cleenwerck *et al.*, 2002) of the method described by Ezaki *et al.* (1989).

The 16S rRNA gene phylogenetic studies confirmed that the Antarctic isolates (strains M7^T and M5) were members of the genus *Shewanella*. The highest levels of 16S rRNA gene sequence similarity were found with *S. livingstonensis* LMG 19866^T (99.9 %) and *S. frigidimarina* ACAM 591^T (99.2 %); lower levels of similarity (97.0–97.5 %) occurred with strains of other *Shewanella* species with validly published names (Fig. 1). Strain M5 showed 100.0 % 16S rRNA gene sequence similarity with respect to M7^T, indicating that these strains probably belong to the same species. On the basis of *gyrB* gene sequences, the nearest neighbours of strain M7^T were again the type strains of *S. livingstonensis* and *S. frigidimarina* (Fig. 2), but with sequence similarities of 87.6 and 87.4 %, respectively. These similarity values are lower than the 90 % species cut-off value proposed for *Shewanella* by Venkateswaran *et al.* (1999), and the *gyrB* sequence of strain M7^T formed a monophyletic branch in the tree shown in Fig. 2, suggesting that this strain represents a novel *Shewanella* species. To verify the taxonomic position of strain M7^T, DNA–DNA hybridizations were performed with *S. livingstonensis* LMG 19866^T and *S. frigidimarina* LMG 19475^T. The low DNA–DNA reassociation values (32 and 35 % with respect to *S. livingstonensis* LMG 19866^T and *S. frigidimarina* LMG 19475^T, respectively) and the results of the 16S rRNA and *gyrB* gene sequence analyses indeed demonstrated the distinct position of strain M7^T within the genus *Shewanella* (Wayne *et al.*, 1987). Strain M5 showed 100 % DNA relatedness with respect to M7^T and it can be concluded that M5 and M7^T belong to the same genospecies. Their DNA G+C content (42 mol%) lies

Table 1. Differential characteristics for strains M7^T and M5 and their closest phylogenetic neighbours

Strains/species: 1, M7^T (strain M5 showed identical results with the exception that it did not grow with 7 % NaCl); 2, *S. livingstonensis* (data from Bozal *et al.*, 2002); 3, *S. frigidimarina* (Bowman *et al.*, 1997); 4, *S. hafniensis* (Satomi *et al.*, 2006); 5, *S. putrefaciens* (Venkateswaran *et al.*, 1999); 6, *S. gaetbuli* (Yoon *et al.*, 2004); 7, *S. morhuae* (Satomi *et al.*, 2006). All species are Gram-negative rods that are motile by means of single polar flagella and all are positive for growth at 4 °C at 3 % NaCl and for catalase and oxidase activities. +, Positive; −, negative; ND, no data available; V−, 11–89 % of strains negative, type strain negative.

Characteristic	1	2	3	4	5	6	7
Cell length (μm)	1.5–3	1–3	1.0–2.5	1.0–1.2	ND	1.5–3.0	1.0–1.2
Cell diameter (μm)	0.5	0.4	0.5–0.8	0.5–0.7	ND	0.5–0.7	0.5–0.7
Growth at/with:							
30 °C	+	−	−	+	+	+	+
37 °C	−	−	−	−	+	+	−
0 % NaCl	+	+	+	+	+	−	ND
7 % NaCl	+	−	+	−	−	+	−
Reduction of nitrate to nitrite	+	+	+	+	+	−	+
Production of H_2S	+	+	+	+	+	−	+
Enzyme activities							
Lipase (C14)	+	−	ND	ND	ND	−	ND
α-Chymotrypsin	+	−	ND	ND	+	+	ND
β-Galactosidase (ONPG)	+	+	V−	ND	ND	−	ND
N-Acetyl-β-glucosaminidase	+	−	ND	ND	−	+	ND
Cystine arylamidase	−	+	ND	ND	ND	−	ND
DNase	+	−	+	+	+	ND	+
Lecithinase	+	−	+	ND	ND	ND	ND
Gelatinase	+	+	+	+	−	+	+
Utilization of:							
D-Galactose	+	+	V−	ND	+	−	ND
Glucose	+	+	+	+	−	ND	−
Sucrose	+	+	+	−	−	−	−
D-Xylose	−	+	V−	ND	ND	ND	ND
N-Acetylglucosamine	+	+	−	+	+	ND	+
Trehalose	−	+	+	ND	ND	ND	ND
Malate	+	−	+	+	+	−	+
D-Ribose	−	+	ND	ND	ND	ND	ND
DNA G+C content (mol%)	42	41	40–43	47	43–47	42	44

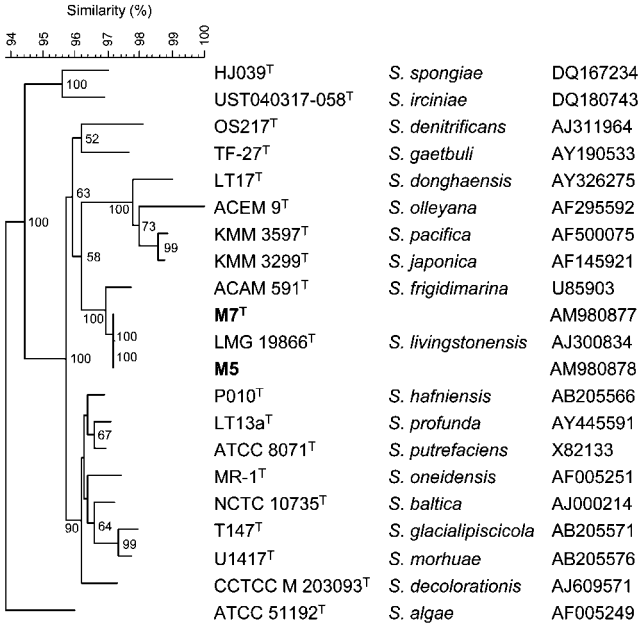

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of Antarctic isolates M7^T and M5 among neighbouring species of the genus *Shewanella.* Bootstrap percentages >50 % (based on 1000 replications) are shown at branch points.

within the range described for members of the genus *Shewanella.*

Description of *Shewanella vesiculosa* sp. nov.

Shewanella vesiculosa (ve.si.cu.lo′sa. L. fem. adj. *vesiculosa* full of blisters, vesiculous).

Cells are rod-shaped (0.5 μm wide and 1.5–3 μm long), Gram-negative, facultatively anaerobic and non-spore-forming. Cells are motile by means of single polar flagella. After 48 h incubation at 15 °C on TSA, colonies are 1–2 mm in diameter, smooth, round with regular edges, slightly mucous and slightly brownish. The growth temperature ranges from −4 to 30 °C (optimum 15–20 °C), the pH range for growth is 6.0–8.5 (optimum approx. pH 7.5) and sodium ions are not required for growth. Optimal growth occurs in the presence of 2 % NaCl (w/v); no growth in the presence of >7 % NaCl. Positive for oxidase, catalase, alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, β-galactosidase and *N*-acetyl-β-glucosaminidase, but negative for cystine arylamidase, trypsin, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase. Positive for hydrolysis of aesculin, Tween 80, gelatin, casein and lecithin, but negative for hydrolysis of urea and starch. Hydrogen sulfide is produced from thiosulfate. Cells are able to grow anaerobically by reducing trimethylamine *N*-oxide and sodium nitrate with lactate as the electron donor. With the API 50 CH test, growth is positive for D-glucose, D-galactose, D-mannitol, *N*-acetylglucosamine, cellobiose, maltose, melibiose, malate and sucrose. The most abundant fatty acids are summed feature

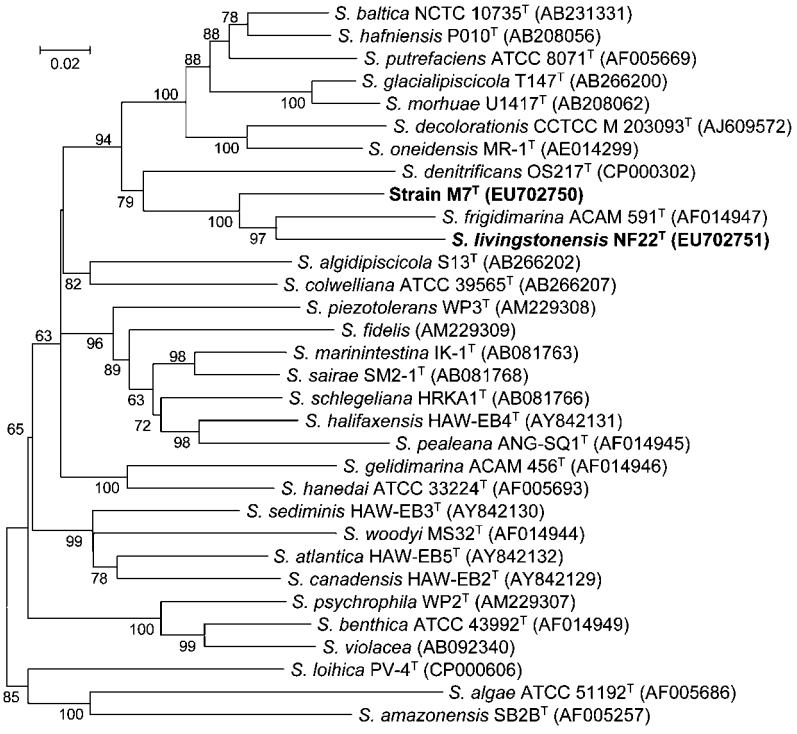

Fig. 2. Neighbour-joining phylogenetic tree based on *gyrB* sequences of strains M7^T and *S. livingstonensis* NF22^T (determined in this study) and strains of the genus *Shewanella.* Bootstrap percentages >50 % (based on 1000 replicates) are shown. Bar, 2 % sequence divergence.

3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), iso-$C_{13:0}$, iso-$C_{15:0}$, $C_{16:0}$, $C_{17:1}\omega8c$ and $C_{15:0}$. The quinone composition of the type strain is Q-7 (50.5 %), Q-8 (25.3 %), MK-7 (14.4 %) and MMK-7 (9.8 %). The DNA G+C content of the type strain is 42 mol%.

The type strain, M7^T (=LMG 24424^T =CECT 7339^T), was isolated from marine sediments collected from Deception Island (South Shetland Islands, Antarctica).

Acknowledgements

This paper is dedicated to the memory of Jesús Guinea Sánchez, who isolated these Antarctic strains. We would like to thank Josefina Castellví for providing Antarctic samples. We gratefully acknowledge the assistance of F. García (Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Spain) with the fatty acid analysis. We are grateful to I. Casals for help with quinone identification and to C. López-Iglesias for transmission electron microscopy support (Serveis Científico Tècnics de la Universitat de Barcelona). We acknowledge the BCCM/LMG Identification Service (Universiteit Gent, Ghent, Belgium) for performing hybridization analyses and 16S rRNA gene sequence analysis. This research was supported by the Government of Spain (CICYT project CTQ 2007-60749/PPQ) and by the Autonomous Government of Catalonia (grant 2005SGR00066).

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