Shewanella gelidimarina sp. nov. and *Shewanella frigidimarina* sp. nov., Novel Antarctic Species with the Ability To Produce Eicosapentaenoic Acid (20:5 ω 3) and Grow Anaerobically by Dissimilatory Fe(III) Reduction

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A polyphasic taxonomic study was performed to characterize dissimilatory iron-reducing strains mostly isolated from Antarctic sea ice. The strains were isolated from samples of congelated (land-fast) sea ice, grease ice, and ice algal biomass collected from the coastal areas of the Vestfold Hills in eastern Antarctica (68°S 78°E). The strains were facultatively anaerobic, motile, and rod shaped, were capable of anaerobic growth either by fermentation of carbohydrates or by anaerobic respiration, and utilized a variety of electron acceptors, including nitrate, ferric compounds, and trimethylamine *N*-oxide. A phylogenetic analysis performed with 16S rRNA sequences showed that the isolates formed two groups representing novel lineages in the genus *Shewanella*. The first novel group included seawater-requiring, psychrophilic, chitinolytic strains which had DNA G+C contents of 48 mol%. The members of the second strain group were psychrotrophic and did not require seawater but could tolerate up to 9% NaCl. The strains of this group were also unable to degrade polysaccharides but could utilize a number of monosaccharides and disaccharides and had G+C contents of 40 to 43 mol%. The whole-cell-derived fatty acid profiles of the sea ice isolates were found to be similar to the profiles obtained for other *Shewanella* species. The omega-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA) (20:5 ω 3) was detected in all of the sea ice isolates at levels ranging from 2 to 16% of the total fatty acids. EPA was also found at high levels in *Shewanella hanedai* (19 to 22%) and *Shewanella benthica* (16 to 18%) but was absent in *Shewanella alga* and *Shewanella putrefaciens*. On the basis of polyphasic taxonomic data, the Antarctic iron-reducing strains are placed in two new species, *Shewanella frigidimarina* sp. nov. (type strain, ACAM 591) and *Shewanella gelidimarina* sp. nov. (type strain, ACAM 456).

The genus *Shewanella* includes facultatively anaerobic, gram-negative, motile, rodlike cells. The original member of the genus, now called *Shewanella putrefaciens*, was first isolated from rancid butter by Derby and Hammer (8), who designated it "*Achromobacter putrefaciens*." Long and Hammer (19) later renamed this taxon "*Pseudomonas putrefaciens*." The difference in DNA G+C contents between typical members of the genus *Pseudomonas* and "*Pseudomonas putrefaciens*" prompted Baumann et al. (1) to reclassify this species as "*Alteromonas putrefaciens*." Finally, the genus *Shewanella*, including the species *Shewanella putrefaciens*, *Shewanella benthica*, and *Shewanella hanedai*, was created by MacDonell and Colwell (22) following a 5S rRNA sequence analysis. *S. benthica* includes barophilic isolates obtained from marine fauna collected from the deep ocean (5–7). *S. hanedai*, a bioluminescent species, was isolated from marine sediment and was originally described as "*Alteromonas hanedai*" (15). More recent additions to the genus *Shewanella* include *Shewanella colwelliana* and *Shewanella alga*. *S. colwelliana* (4), which initially was classified as "*Alteromonas colwelliana*" (43), was isolated from the oyster *Crassostrea virginica*. A tetrodotoxin-producing strain isolated from red algae (*Jainia* sp.) was named *S. alga* (39), and the description of this taxon was later emended by Nozue et al.

(32). More recently, 16S rRNA sequence-based phylogenetic analyses of *Shewanella* species (12, 37) indicated that the genus was most closely related to [*Vibrio*] *marinus* (brackets indicate a generically misclassified species), *Ferrimonas balearica*, *Pseudoalteromonas* spp., and *Colwellia psychroerythrus*.

In this study a number of strains were isolated from sea ice obtained from Antarctic coastal sites and from cyanobacterial mats and waters of Antarctic meromictic lakes. These isolates were found to form two distinct phenetic groups (2) and were able to grow anaerobically by dissimilatory iron reduction. A polyphasic taxonomic analysis demonstrated that these groups represent distinct and novel species in the genus *Shewanella*, and we propose the names *Shewanella frigidimarina* sp. nov. and *Shewanella gelidimarina* sp. nov. for these taxa.

MATERIALS AND METHODS

Isolation and bacterial strains. Antarctic samples were collected during the 1992 to 1995 summer seasons from locations around the Vestfold Hill ice-free zone on the southern Prydz Bay coast in eastern Antarctica. Grease ice samples were collected from surface waters. Congelation (land-fast) sea ice cores were obtained by using a SIPRE ice drill (diameter, 7 cm) and on the same day were melted in sterile seawater at 4°C. Ice-derived algal material was collected by filtration through 0.8- μ m-pore-size nitrocellulose filters. Algal material derived from breakout from ice, accumulating at the shoreline of Ace Lake (Vestfold Hills), was also collected. All samples were plated onto marine 2216 agar (Difco Laboratories, Detroit, Mich.). Plates were incubated at either 4 or 25°C for up to 2 months. This work was performed as part of a general survey of the bacterial diversity of Antarctic sea ice (2), and in this investigation a number of strains with a phenotype similar to the phenotype of the genus *Shewanella* were isolated (Table 1). All strains investigated in this study were routinely grown on marine

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TABLE 1. Strains examined in this study

Species	Strain ^a	Source
<i>S. alga</i>	ACAM 541 ^T (= ATCC 51192 ^T)	Red calcareous alga (<i>Jania</i> sp.)
<i>S. benthica</i>	ACAM 543 (= ATCC 43991)	Digestive tract, amphipod (<i>Scopelocheirus shellengi</i>), Puerto Rico Trench
<i>S. benthica</i>	ACAM 544 ^T (= ATCC 43992 ^T)	Intestine, holothurian (<i>Psychrobates longicauda</i>), Walvis Ridge, South Atlantic Ocean
<i>S. hanedai</i>	ACAM 540 ^T (= ATCC 33224 ^T)	Marine sediment, Arctic Ocean
<i>S. hanedai</i>	ACAM 585	Congelation ice, Ellis Fjord, Antarctica
<i>S. putrefaciens</i>	ACAM 574 (= NCIMB 8768)	Machine oil emulsion
<i>S. putrefaciens</i>	ACAM 575 ^T (= ATCC 8071 ^T = NCIMB 10471 ^T)	Rancid butter
<i>S. putrefaciens</i>	ACAM 576 (= ATCC 8072 = NCIMB 10472)	Butter with surface taint
<i>S. putrefaciens</i>	ACAM 577 (= ATCC 8073 = NCIMB 10473)	Butter with surface taint
<i>S. gelidimarina</i>	ACAM 456 ^T	Congelation ice, Prydz Bay, Antarctica
<i>S. gelidimarina</i>	ACAM 586	Congelation ice, Ellis Fjord, Antarctica
<i>S. frigidimarina</i>	ACAM 122	Pycnocline, Burton Lake, Antarctica
<i>S. frigidimarina</i>	ACAM 584	Cyanobacterial mat, Ace Lake, Antarctica
<i>S. frigidimarina</i>	ACAM 587	Congelation ice, Prydz Bay, Antarctica
<i>S. frigidimarina</i>	ACAM 588	Congelation ice, Prydz Bay, Antarctica
<i>S. frigidimarina</i>	ACAM 589	Grease ice, Ellis Fjord, Antarctica
<i>S. frigidimarina</i>	ACAM 590	Congelation ice, Prydz Bay, Antarctica
<i>S. frigidimarina</i>	ACAM 591 ^T	Congelation ice, Prydz Bay, Antarctica
<i>S. frigidimarina</i>	ACAM 592	Grease ice, Ellis Fjord, Antarctica
<i>S. frigidimarina</i>	ACAM 593	Grease ice, Ellis Fjord, Antarctica
<i>S. frigidimarina</i>	ACAM 594	Grease ice, Ellis Fjord, Antarctica
<i>S. frigidimarina</i>	ACAM 595	Cyanobacterial mat, Ace Lake, Antarctica
<i>S. frigidimarina</i>	ACAM 596	Cyanobacterial mat, Ace Lake, Antarctica
<i>S. frigidimarina</i>	ACAM 597	Cyanobacterial mat, Ace Lake, Antarctica
<i>S. frigidimarina</i>	ACAM 598	Ice algal biomass, Ellis Fjord, Antarctica
<i>S. frigidimarina</i>	ACAM 599	Congelation ice, Prydz Bay, Antarctica
<i>S. frigidimarina</i>	ACAM 600	Congelation ice, Prydz Bay, Antarctica
<i>S. frigidimarina</i>	ACAM 601	Ice algal biomass, Ellis Fjord, Antarctica

^a ACAM, Australian Collection of Antarctic Microorganisms, Antarctic CRC, University of Tasmania, Hobart, Tasmania, Australia; ATCC, American Type Culture Collection, Rockville, Md.; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland, United Kingdom.

2216 agar. All of the Antarctic isolates and *S. hanedai* were incubated at 10°C. *S. benthica* strains were incubated at 4°C. *S. alga* and *S. putrefaciens* were cultivated at 25°C.

Phenotypic characterization. Gram stain, oxidase, and catalase tests were performed as previously described (41). Motility was determined by microscopically examining cell wet mounts. Seawater requirement and tolerance to various NaCl concentrations (0 to 12%) were tested on nutrient agar (Oxoid, Ltd., Basingstoke, United Kingdom). Starch hydrolysis test plates were prepared similarly, and hydrolysis activity was detected by flooding the plates with a 1% (wt/vol) iodine solution. Tween 80 hydrolysis, egg yolk hydrolysis, esculin hydrolysis, and casein hydrolysis were tested as described by Smibert and Krieg (41). DNA hydrolysis was tested by using DNase test agar (Oxoid). Chitin hydrolysis and alginate hydrolysis were tested as described by West and Colwell (44) in media prepared with artificial seawater. In tests for acid production from carbohydrates we used the oxidation-fermentation medium of Leifson (18). Dissimilatory iron reduction was tested in a seawater medium adapted from the medium described by Widdel and Bak (45) and prepared by using the Hungate technique (13). The medium contained 10 mM amorphous ferric oxide-neutralized ferric chloride or 10 mM soluble ferric oxide-ferric pyrophosphate (21) as the electron acceptors, and 10 mM sodium acetate or sodium DL-lactate was used as the electron donor. Uninoculated controls lacking the iron electron donor were also prepared. Iron reduction was confirmed by the appearance of a black precipitate (magnetite) and by the Merckoquant iron test (Merck). Anaerobic growth with 20 mM potassium nitrate and 10 mM trimethylamine *N*-oxide (TMAO) was tested in a medium containing (per liter of artificial seawater) 2 g of ammonium chloride and 2 ml of trace element solution SL-10 (33) and 2 mM potassium phosphate buffer (pH 7.0). The medium pH was adjusted to 7.0 with 1 M KOH, and the medium was solidified with 1.3% (wt/vol) purified agar (Oxoid). Sodium acetate and sodium DL-lactate were each added at a concentration of 20 mM. Plates were incubated in an anaerobic jar by using anaerospaks (Oxoid). Growth on the plates was compared with growth on control plates lacking electron acceptors. Additional biochemical tests were performed with an API 20E test kit (BioMerieux, Lyon, France), which was prepared according to the manufacturer's specifications except that bacterial strains were suspended in chilled artificial seawater. For carbon and energy source screening we added most of the test compounds at a concentration of 0.1% (wt/vol); the exceptions were the carbohydrates, which were tested at a concentration of 0.2% (wt/vol). The basal medium used was the same medium utilized for the nitrate and TMAO electron donor experiments described above. Media lacking a car-

bon source were prepared as negative controls to account for any background growth.

Growth rate analysis. The growth rates of strains ACAM 456^T and ACAM 591^T were determined in a temperature gradient incubator (Toyo, Inc., Tokyo, Japan). The temperatures used ranged from 0 to 40°C, and tubes containing 10 ml of marine 2216 broth were inoculated with 0.5 ml of cells taken from cultures in the late logarithmic growth phase. Growth was measured by determining the decrease in transmittance at 550 nm for up to 14 days. A culture was considered positive for growth if the culture transmittance increased by more than 20%. The data were fitted to the Ratkowsky square root temperature growth model (36), and cardinal growth temperatures were determined.

Whole-cell fatty acid analysis. Representative strains were grown on marine 2216 agar at 10°C for 2 to 4 days, harvested by scraping into a small amount of artificial seawater, and then washed by centrifugation in artificial seawater. The pellets were lyophilized prior to solvent extraction with a vacuum freeze drier (Dynavac, Melbourne, Victoria, Australia). Whole-cell fatty acid profiles were quantitatively determined by using gas chromatography and gas chromatography-mass spectrometry (30). The geometry and position of double bonds in monounsaturated fatty acids were confirmed by using dimethyl disulfide derivatization and by performing an analysis in which gas chromatography-mass spectrometry (31) was used. The double bond positions were numbered from the methyl (ω) end of the fatty acid.

Genotypic analysis. Genomic DNA was extracted and purified from cells (grown in the same way as the cells used for the fatty acid analysis were grown) by using the procedure of Marmur and Doty (23). The DNA G+C content was then determined from thermal denaturation profiles (40).

DNA-DNA hybridization. A spectrophotometric renaturation rate kinetic procedure as adapted by Huss et al. (14) was used to determine levels of DNA-DNA reassociation between genomic DNAs of different strains. Genomic DNA was sheared to an average size of 1 kb by sonication and dialyzed overnight at 4°C in 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate, pH 7.0), and the concentration was adjusted to approximately 60 µg/ml. Following denaturation of the DNA samples, hybridization was performed at the optimal temperature for renaturation (T_{OR}), which was 25°C below the DNA melting temperature and was calculated from the following equation: $T_{OR} = 48.5 + (0.41 \times G+C \text{ content})$ (24). The declines in absorbance over a 40-min interval for DNA mixtures and control DNA samples were used to calculate DNA hybridization values from the following equation (14): % DNA hybridization = $[44B - A - B/2\sqrt{(A \times B)}] \times 100\%$, where A and B are the changes in absorbance for the two DNA samples

being compared and ΔA is the change in absorbance for an equimolar mixture of A and B . DNA hybridization values equal to or less than 25% were thought to represent background hybridization and thus were not considered significant (14).

Phylogenetic analysis. The 16S rRNA sequence was determined for the following strains: *S. gelidimarina* ACAM 456^T; *S. frigidimarina* ACAM 122, ACAM 584, ACAM 587, ACAM 591^T, ACAM 593, and ACAM 600; and strain ACAM 586. The 16S rRNA genes from these strains were amplified by PCR by using primers 27fM13f (5'-TGTAACACGACGGCCAGTAGAGTTGATCCTGGC TCAG-3') and 1492rM13r (5'-TACGGYTACCTTGTTACGACTTCAGGAA ACAGCTATGACC-3'), in which the -21 M13 forward and M13 reverse sites were combined with 16S rRNA primers 9-27f and 1492-1512r, respectively. The conditions used for the PCR have been described by Dobson et al. (9). The amplicons were purified by using a QiaQuick PCR purification kit. The 16S rRNA sequences were then generated by using dye primer cycle sequencing ready reaction -21 M13 forward and M13 reverse kits and a model A377 automated DNA sequencer (Applied Biosystems, Foster City, Calif.). The sequence data were manually aligned with *Shewanella* 16S rRNA sequences and the 16S rRNA sequences of related species. Software from PHYLIP, version 3.57c (10), was used to further analyze the sequence data set. DNADIST, with the maximum-likelihood option, was employed to determine sequence similarities, and NEIGHBOR was used to create a phylogenetic tree. Bootstrap analysis (with 1,000 replicates) was used to determine the branching proportions within the phylogenetic tree by using the programs SEQBOOT and CONSENSE.

Nucleotide sequence accession numbers. Sequences of the following organisms (with GenBank accession numbers in parentheses) were utilized in the phylogenetic analysis and were obtained directly from GenBank: *Cohwella psychroerythrus* ACAM 550^T (AF001365) (2), *F. balearica* DSM 9799^T (X93021) (38), *Pseudoalteromonas haloplanktis* ATCC 14393^T (X67024) (12), *S. benthica* ACAM 544^T (X82131) (9), *S. hanedai* ACAM 540^T (X82132) (12), *S. putrefaciens* ATCC 8071^T (X82123) (12), *Shewanella* (barophilic) sp. strain DB172R (D63824) (17), [*V.*] *marinus* NCIMB 1144^T (X82142) (12), and barophilic or barotolerant strains DB6705 (D21222), DB6906 (D21223), DSK1 (D21224), DSS12 (D21225), and DB5501 (D21229) (16). The 16S rRNA sequences of the following strains were obtained in this study and were deposited in GenBank (accession numbers are given in parentheses): *S. alga* ACAM 541^T (AF006669); *S. frigidimarina* ACAM 122 (U39399), ACAM 584 (U85902), ACAM 588 (U85905), ACAM 591^T (U85903), ACAM 593 (U85904), and ACAM 600 (U85906); *S. gelidimarina* ACAM 456^T (U85907); *S. hanedai* ACAM 585 (U85907); and *S. putrefaciens* ACAM 574 (AF006670), ACAM 576 (AF006671), and ACAM 577 (AF006672).

RESULTS

Isolation and growth characteristics. The isolates formed circular, tan-pigmented colonies 2 to 4 mm in diameter on marine 2216 agar following 3 to 5 days of incubation at 10°C. After approximately 7 days of incubation at 10°C, the colonies lost their opacity and became increasingly mucoid. The isolates could be readily divided into two groups based on their inherent ecophysiology and represent two novel species of the genus *Shewanella*, *S. gelidimarina* sp. nov. and *S. frigidimarina* sp. nov., which are described below.

S. frigidimarina strains (Table 1) were isolated either directly from sea ice or from cyanobacterial mats and the water columns and ice of marine salinity lakes, such as Ace Lake and Burton Lake. One strain (ACAM 122) was isolated from the pycnocline of Burton Lake during a previous study of that lake's microbiota (11). *S. frigidimarina* strains grew at temperatures between <0 and 27 to 28°C, while no growth occurred at 30°C. The optimal growth temperature was 20 to 22°C. This species does not require sodium ions or other seawater-derived cations for growth and tolerates a maximum NaCl concentration of 8 to 9% (wt/vol). The growth of the type strain, ACAM 591, was not stimulated by adding 1% (wt/vol) NaCl or seawater to sodium-free media.

S. gelidimarina included two strains, ACAM 456^T and ACAM 585 (Table 1), which were isolated from platelet ice layers of ice cores collected from the Ellis Fjord area of the Vestfold Hills coast (2). Both strains grew optimally at 15 to 17°C, and no growth occurred at temperatures above 23°C. Both strains required seawater for growth. The *S. gelidimarina* strains failed to grow on media containing less than 20% sea-

water or 1% NaCl but grew on marine agar prepared with up to 2× artificial seawater or on nutrient agar (Difco) supplemented with up to 6% NaCl. Neither *S. frigidimarina* nor *S. gelidimarina* required vitamins for growth, and both organisms were able to utilize inorganic nitrogen sources, such as ammonium chloride and potassium nitrate.

Phenotypic characterization. *S. frigidimarina*, *S. gelidimarina*, *S. alga*, and *S. putrefaciens* were able to grow anaerobically by reducing nitrate, TMAO, and ferric compounds while using DL-lactate or acetate as an electron donor. This was shown by positive growth or stimulation of growth when the strains were incubated anaerobically in media containing the electron acceptors compared to media which lacked the electron donors. TMAO and nitrate reduction but not iron reduction could be used for anaerobic growth by *S. benthica* ACAM 543 and *S. hanedai* ACAM 540^T and ACAM 585. Anaerobic growth by fermentation of carbohydrates was also observed with some *Shewanella* species. *S. frigidimarina*, *S. gelidimarina*, *S. hanedai*, and *S. benthica* could ferment D-glucose and/or N-acetylglucosamine. Neither *S. alga* nor *S. putrefaciens* can grow anaerobically via fermentation of carbohydrates. Fermentation of carbohydrates by *Shewanella* species was accompanied by acid production, but no gas was observed.

The Antarctic *Shewanella* isolates were strongly proteolytic and lipolytic, as are most other *Shewanella* strains (1, 42-44). Neither *S. frigidimarina* nor *S. gelidimarina* hydrolyzed starch, but *S. gelidimarina* was strongly chitinolytic (Table 2). This species, as well as *S. benthica*, could grow anaerobically by fermentation of chitin with concomitant acid production. *S. frigidimarina* was able to oxidatively produce acid from a broader range of carbohydrates than other *Shewanella* species (Table 2).

DNA base compositions. The overall DNA base composition range for *Shewanella* species ranges from 40 to 54 mol% (see Table 5). *S. frigidimarina* strains have G+C contents ranging from 40 to 43 mol% (average, 41.0 ± 1.6 mol%; $n = 11$), while *S. gelidimarina* ACAM 456^T and ACAM 586 have G+C contents of 48 mol% (Table 2). ACAM 585 has a G+C content of 43.8 mol%, which falls within the range that is typical of *S. hanedai* (1).

DNA-DNA hybridization. DNA-DNA hybridization data are shown in Table 3. Representative strains of *S. frigidimarina* had DNA reassociation values ranging from 68 to 96%. The level of DNA hybridization between *S. gelidimarina* ACAM 456^T and ACAM 586 was 84%. *S. frigidimarina* strains exhibited low DNA homology levels with *S. putrefaciens* ACAM 575^T (range, 27 to 43%; average, 37% ± 6%) (Table 3). *S. gelidimarina* likewise exhibited a low level of DNA hybridization with *S. benthica* ACAM 544^T (Table 3). Strain ACAM 585 had a DNA hybridization level of 70% with the genomic DNA of *S. hanedai* ACAM 540^T. Based on this level of DNA reassociation, ACAM 585 definitively is a member of the species *S. hanedai*.

Phospholipid fatty acid analysis. The major fatty acids of *Shewanella* species included i13:0, 14:0, i15:0, 16:1ω7c, 16:0, and 17:1ω8c (Table 4). The polyunsaturated fatty acid eicosapentaenoic acid (EPA) (20:5ω3) was also a significant constituent of the whole-cell fatty acids of *S. benthica*, *S. hanedai*, *S. gelidimarina*, and *S. frigidimarina* (Table 4). EPA was absent from the strains of *S. alga* and *S. putrefaciens* examined in this study.

16S rRNA phylogenetic analysis. The 16S rRNA genes of *S. frigidimarina* ACAM 122, ACAM 584, ACAM 587, ACAM 591^T, ACAM 593, and ACAM 600, *S. gelidimarina* ACAM 456^T and ACAM 585, *S. hanedai* ACAM 585, *S. alga* ACAM 541^T, and *S. putrefaciens* ACAM 574, ACAM 576, and ACAM 577 were 1,420 to 1,510 nucleotides long and extended from

TABLE 2. Phenotypic characteristics of *S. gelidimarina* sp. nov. and *S. frigidimarina* sp. nov.

Characteristic	<i>S. frigidimarina</i>	<i>S. gelidimarina</i>
Cardinal temp		
Optimum temp (°C)	20–22	15–17 ^a
Maximum temp (°C)	25–27	23 ^a
Minimum temp (°C)	–14	–26 ^a
Seawater requirement (stenohaline)	– ^b	+
Tolerance to 8% NaCl	+	–
Hydrolysis of:		
Chitin	–	+
DNA	+	v+
L-Tyrosine	v–	–
ONPG test	v–	–
Ornithine decarboxylase	v–	–
Arginine dihydrolase	v–	–
H ₂ S production (from thiosulfate)	v+	+
Simmons citrate tests	v+	–
Fermentation of:		
D-Glucose	+	–
N-Acetylglucosamine	–	+
Oxidative acid production from:		
Cellobiose, maltose, D-mannitol, sucrose	+	–
Trehalose	v+	–
D-Galactose, D-mannose, D-xylose	v–	–
Utilization of the following compounds as sole carbon and energy sources:		
L-Glutamate	v–	+
N-Acetylglucosamine	–	+
Cellobiose, D-glucose, maltose, sucrose, trehalose, mannitol, propionate, isobutyrate, succinate, fumarate, L-malate, oxaloacetate, L-leucine	+	–
Nonanoate, L-phenylalanine, L-proline	v+	–
D-Fructose, D-galactose, D-xylose, glycogen, caproate, L-glutamate, hydroxy-L-proline, γ -aminobutyrate	v–	–
G+C content (mol%) ^c	40–43	48

^a Data from reference 30.

^b –, 0 to 10% of the strains are positive; +, 90% or more of the strains are positive; v+, 11 to 89% of the strains are positive and the type strain is positive; v–, 11 to 89% of the strains are positive and the type strain is negative. The following tests were positive for all of the strains studied: growth at 0°C; growth with 0.5× to 2× seawater; tolerance to 0.0075% KCN and 5% ox bile salts; hydrolysis of gelatin, casein, and Tween 80; lecithinase activity (egg yolk reaction); catalase and cytochrome *c* oxidase activities; nitrate reduction; anaerobic growth by dissimilatory reduction of nitrate, TMAO, and Fe(III) with acetate and DL-lactate as electron donors; and utilization of acetate, butyrate, valerate, DL-lactate, and pyruvate as sole sources of carbon and energy. The following tests were negative for all of the strains studied: growth with 4× seawater; requirement for yeast extract and vitamin growth factors; lysine decarboxylase activity; L-phenylalanine and L-tryptophan deaminase activities; indole production from L-tryptophan; hydrolysis of alginate, esculin, urate, xanthine, urea, and starch; acid production from L-arabinose, L-rhamnose, lactose, D-melibiose, D-melezitose, D-raffinose, dextran, adonitol, *m*-inositol, D-sorbitol, and glycerol; and utilization of L-arabinose, lactose, D-raffinose, L-rhamnose, L-arabitol, *i*-erythritol, *m*-inositol, D-sorbitol, glycerol, α -glycerophosphate, D-gluconate, D-glucuronate, isovalerate, heptanoate, caprylate, malonate, adipate, glutarate, pimelate, azelate, *t*-aconitate, citrate, 2-oxoglutarate, DL-3-hydroxybutyrate, L-alanine, L-aspartate, L-asparagine, L-histidine, L-ornithine, L-serine, L-threonine, putrescine, and urate as sources of carbon and energy.

^c G+C contents were determined by the thermal denaturation method.

nucleotide positions 5 to 1510 (*Escherichia coli* equivalent numbering). These sequences were compared with the sequences of other *Shewanella* species, closely related bacteria, such as *F. balearica*, [*V.*] *marinus*, *Pseudoalteromonas* spp., and

Colwellia psychroerythrus, and an assortment of barophilic or barotolerant strains examined in other studies (6, 16, 17). *S. frigidimarina* formed a clade which had levels of sequence dissimilarity ranging from 0.2 to 1.7%. The most divergent strain was the Burton Lake isolate, ACAM 122. The species most closely related to *S. frigidimarina* was *S. putrefaciens* (level of dissimilarity, 3.4% $\pm$ 0.3%), and these two species formed a common branch on the phylogenetic tree (bootstrap value, 100%) (Fig. 1). 16S rRNA sequences of strains representing the four DNA-DNA hybridization groups of *S. putrefaciens*, as determined by Owen et al. (34), were also obtained in this study to confirm that *S. frigidimarina* was a taxonomically distinct entity. *S. putrefaciens* ACAM 574 and ACAM 575^T (DNA hybridization group I), ACAM 576 (group II), and ACAM 577 (group III) were closely related to each other, exhibiting levels of dissimilarity of only 0.5 to 2.4%. DNA hybridization group IV of *S. putrefaciens* has been recognized previously as being equivalent to *S. alga* (32). The 16S rRNA sequence of *S. alga* ACAM 541^T is closely related to the *S. alga* FeRed sequence (37), exhibiting a level of dissimilarity of 1.5%, and *S. alga* clearly represents a distinct lineage within the genus *Shewanella* (Fig. 1). Barotolerant strain SC2A, which was isolated from California coastal waters (6) at a depth of about 2,000 m, is also closely related to *S. frigidimarina* (Fig. 1), suggesting that this species may be cosmopolitan and occur throughout cold marine ecosystems.

S. gelidimarina ACAM 456^T was most closely related to *S. benthica* and various barophilic strains closely related to *S. benthica* at dissimilarity values of 3.6% $\pm$ 0.2%. On average, the 16S rRNA of ACAM 456^T and the 16S rRNAs of other *Shewanella* species were 5.6% $\pm$ 0.5% dissimilar. The assumption that ACAM 585 is a strain of *S. hanedai* was supported by the similarity of its 16S rRNA sequence and the 16S rRNA sequence of *S. hanedai* ACAM 540^T (level of dissimilarity, 1.2%) (Fig. 1).

DISCUSSION

The genus *Shewanella* has been the focus of a variety of studies owing to the versatile metabolism of this genus and the

TABLE 3. DNA-DNA hybridization between Antarctic isolates and a number of *Shewanella* strains

Strain	% DNA-DNA hybridization with ^a :		
	<i>S. frigidimarina</i> ACAM 591 ^T	<i>S. gelidimarina</i> ACAM 456 ^T	<i>S. putrefaciens</i> ACAM 575 ^T
<i>S. frigidimarina</i> ACAM 122	76		38
<i>S. frigidimarina</i> ACAM 584	69		
<i>S. frigidimarina</i> ACAM 589	96		42
<i>S. frigidimarina</i> ACAM 590	65		
<i>S. frigidimarina</i> ACAM 591 ^T	100	16	38
<i>S. frigidimarina</i> ACAM 593	86		43
<i>S. frigidimarina</i> ACAM 598	94		36
<i>S. frigidimarina</i> ACAM 600	68		27
<i>S. gelidimarina</i> ACAM 456 ^T	16	100	11
<i>S. gelidimarina</i> ACAM 586		90	
<i>S. benthica</i> ACAM 544 ^T	19	36	
<i>S. hanedai</i> ACAM 540 ^{Tb}	9	28	20
<i>S. alga</i> ACAM 541 ^T	21	20	12
<i>S. putrefaciens</i> ACAM 575 ^T	38	11	100

^a The standard deviations for DNA-DNA hybridization values ranged from 5 to 15%. Triplicate analyses were performed.

^b *S. hanedai* ACAM 540^T and ACAM 585 have a DNA-DNA hybridization value of 70%.

TABLE 4. Fatty acid compositions of *Shewanella* species

Fatty acid	% in:					
	<i>S. frigidimarina</i> (n = 8) ^a	<i>S. gelidimarina</i> (n = 2) ^b	<i>S. benthica</i> (n = 2)	<i>S. hanedai</i> (n = 2)	<i>S. putrefaciens</i> (n = 3)	<i>S. alga</i> (n = 1)
12:0	0–1.8	0.3–0.7	0.2–1.4	0.1–0.4	0.1–2.6	0.1
i13:0	3.1–8.3	5.4–15.8	2.8–8.9	5.1–7.7	1.0–3.6	0.5
13:0	0–0.4	tr–1.3 ^c	0–0.3	0–0.3	0–0.5	0.1
i14:0	0–1.0	0.1–0.6	0.7–1.0	0.1–0.4	tr–0.3	0.8
14:1 ω 7c	0.4–1.8	0.3–0.8	0.1–0.5	0–tr	0–tr	tr
14:0	2.7–4.1	3.8–4.4	11.0–11.9	7.6–10.1	1.5–5.9	0.7
i15:0	5.6–10.7	8.3–11.6	4.4–6.2	7.9–8.2	8.8–24.2	17.8
15:0	1.7–2.9	1.6–6.7	0.4–0.9	2.9–3.5	1.4–7.5	4.0
15:1 ω 8c	0.3–1.3	0.7–1.7		0–0.1	0–0.7	tr
15:1 ω 6c	0–2.2	0.4–1.7	0–0.2	0–0.5	0–0.3	tr
i16:0					0–tr	1.1
16:1 ω 9c	0–4.0	0–0.7			0–2.4	
16:1 ω 7c	37.6–55.1	27.4–37.0	24.0–43.9	25.0–33.1	25.2–31.4	16.3
16:1 ω 7t	0–1.4	0–0.1			1.0–1.7	0.2
16:0	4.5–16.9	6.4–11.1	10.6–11.6	12.2–15.0	27.9–31.0	13.3
i17:0	0–0.6	0.1–0.4	0.1–1.5	0.8–2.4	tr–1.6	0.5
17:1 ω 8c	1.7–3.7	1.9–4.2	1.6–6.6	0.5–1.5	2.7–3.7	14.7
17:1 ω 6c		0.8–0.9	0–1.2	0.3–0.5	0–0.4	1.4
17:0	0.2–2.0	0.4–0.5	0.5–8.1	0.6–1.0	0.6–1.6	4.6
18:1 ω 9c	0.2–2.8	0.3–0.9	0–0.2	0.2–0.8	1.1–3.6	6.0
18:1 ω 7c	3.1–6.6	1.0–8.4	0–1.7	2.2–3.7	2.4–3.9	7.2
18:1 ω 7t	0–1.2					0.2
18:0	0–0.2	0.1–0.2	0–0.5	0–0.4	0.5–3.2	2.7
18:2 ω 6					0.1–2.2	
18:4 ω 3	0–1.1	tr–0.3				
20:4 ω 3	0–0.4	0.3–0.8				
20:5 ω 3	1.7–6.8	11.7–16.0	15.8–18.5	19.2–22.2		
Other	0.2–0.8	0–0.2		0–0.3	0–0.8	2.7

^a n is the number of strains examined.^b Includes data from reference 30.^c tr, trace (<0.1%).

diversity of niches from which *Shewanella* species have been isolated. For example, *S. putrefaciens* has been found to be exceptionally versatile in the range of electron acceptors which it can use for anaerobic respiration; these electron acceptors include ferric compounds, manganese oxide, molybdenum ox-

ide, sulfur compounds, and nitrate (20, 21, 26). Dissimilatory iron reduction has also been observed in *S. alga* and the phylogenetically closely related organism *F. balearica* (37, 38). *Shewanella* strains have the ability to anaerobically dehalogenate chlorinated compounds, such as tetrachloroethane (35),

FIG. 1. Unrooted phylogenetic tree based on 16S rRNA comparisons, showing the taxonomic positions of *S. frigidimarina* and *S. gelidimarina* within the genus *Shewanella*. The branching pattern was generated by neighbor-joining methods, and bootstrap values (shown at the nodes) were calculated from 1,000 trees. Bar = sequence dissimilarity of 3%.

TABLE 5. Characteristics that differentiate *Shewanella* species^a

Species	Growth at:		Growth in the presence of 8% NaCl	Seawater required	Production of:			Fermentation of:		Dissimilatory Fe(III) reduction	Synthesis of EPA	G+C content (mol%)
	25°C	30°C			Chitinase	Amylase	Ornithine decarboxylase	D-Glucose	N-Acetylglucosamine			
<i>S. gelidimarina</i>	—	—	—	+	+	—	—	—	+	+	+	48
<i>S. frigidimarina</i>	+	—	+	—	—	—	v	+	—	+	+	40–43
<i>S. benthica</i>	—	—	—	+ ^b	+	—	—	+	+	v	+	47
<i>S. hanedai</i>	—	—	—	+ ^c	+	+	—	—	—	—	+	43–44
<i>S. putrefaciens</i>	+	+	—	—	—	—	+	—	—	+	—	44–47
<i>S. alga</i>	+	+	+	+	—	—	+	—	—	+	—	53–54
<i>S. colwelliana</i>	+	+	—	+	—	+	—	—	—	ND ^d	ND	46

^a Data from references 1, 2, 4, 22, 32, 39, 42, and 43 and this study.^b *S. benthica* requires a vitamin(s) for growth.^c *S. hanedai* requires yeast extract for growth.^d ND, no data available.

are major spoilers of food (25, 42), and occasionally are found to cause bacteremias in humans (3). 16S rRNA-based phylogenetic analysis indicates that the psychrophilic, barophilic or barotolerant, deep-sea bacteria (6, 7, 16, 17) are closely related to *S. benthica* or [*V.*] *marinus* (Fig. 1).

In the present study, it was found that strains which were isolated from Antarctic sympagic and lacustrine environments and were phenotypically similar to *Shewanella* species (2) could grow anaerobically by dissimilatory iron reduction, as well as by carbohydrate fermentation. Phenotypic analysis also showed that these isolates formed two distinct ecophysiological groups, one which was psychrotrophic and nonhalophilic and one which was psychrophilic and slightly halophilic. Phenotypically, these groups did not closely resemble any of the previously recognized *Shewanella* species (Table 2) and could be differentiated from other species of the genus *Shewanella* by a number of characteristics, as shown in Table 5.

Genotypic analysis demonstrated that these groups represent distinct taxa since only low levels of DNA reassociation were found between the Antarctic strains and other *Shewanella* species (Table 3). This conclusion was also supported by 16S rRNA phylogenetic studies, which showed that *S. frigidimarina* and *S. gelidimarina* form distinct lineages within the genus *Shewanella* (Fig. 1).

The lipid profiles of the Antarctic strains closely resemble the lipid profiles of other *Shewanella* species (27) except for the levels of EPA, the synthesis of which appears to be species specific. EPA and other polyunsaturated fatty acids are believed to maintain the homeoviscosity of cellular membranes, making them more permeable in cold and high-pressure environments (29). In the case of the genus *Shewanella*, the synthesis of EPA appears to be more prevalent in species with low temperature optima, particularly *S. hanedai* and *S. benthica* (optimum temperatures, ~10°C). With increasing temperature optima EPA levels decline, and *S. frigidimarina* strains (optimum temperatures, 20 to 22°C) produce the lowest levels (Table 4). The fatty acid profiles of EPA-producing barophilic strains isolated from deep-sea locations (5) are similar to those of some *Shewanella* species, and these strains may be strains of *S. benthica*. No data are available for *S. colwelliana*, and *S. putrefaciens* and *S. alga*, which have mesophilic temperature optima (>30°C), do not form EPA.

In conclusion, in this study we demonstrated that sea ice and other Antarctic habitats harbor two novel species of the genus *Shewanella*. The proposed names of these species are *S. frigidimarina* sp. nov. and *S. gelidimarina* sp. nov.

Description of *Shewanella frigidimarina* sp. nov. *Shewanella frigidimarina* (fri.gid.i.ma.ri'na. L. adj. *frigidus*, cold; L. fem.

adj. *marina*, of the sea; M. L. adj. *frigidimarina*, of the cold sea). *S. frigidimarina* cells are rod shaped and either curved or straight (length, 1.0 to 2.5 µm; width, 0.5 to 0.8 µm) and occur singly or in pairs. Cells are motile. Facultatively anaerobic chemoheterotroph. Anaerobic growth occurs by fermentation of D-glucose or alternatively by anaerobic respiration with ferric compounds, nitrate, or TMAO as the electron acceptor and either sodium lactate or sodium acetate as the electron donor. Psychrotrophic. Growth occurs at temperatures from <0 to 27 to 28°C. Optimal growth occurs at 20 to 22°C. Nonhalophilic. Tolerates up to 9% NaCl. Growth factors are not required. Uses inorganic and organic nitrogen sources. Catalase and oxidase positive. Proteolytic and lipolytic. DNase is formed. May hydrolyze esculin and produce hydrogen sulfide from thiosulfate. A few strains show weak positive reactions in the *o*-nitrophenyl-β-D-galactopyranoside (ONPG), arginine dihydrolase, and ornithine decarboxylase tests. Amylase and chitinase are not produced. Acid is formed oxidatively from cellobiose, D-glucose, maltose, D-mannitol, and sucrose. Acid may also be formed from D-galactose, D-mannose, trehalose, and D-xylose. No acid is produced from L-arabinose, N-acetylglucosamine, D-adonitol, glycerol, *m*-inositol, or D-sorbitol. The following carbon sources are utilized by all strains: cellobiose, D-glucose, maltose, sucrose, trehalose, D-mannitol, acetate, propionate, butyrate, isobutyrate, valerate, succinate, fumarate, DL-lactate, L-malate, pyruvate, oxaloacetate, and L-leucine. Strains may also use D-fructose, D-galactose, D-xylose, caproate, nonanoate, L-glutamate, L-phenylalanine, hydroxy-L-proline, L-proline, and γ-aminobutyrate. Produces EPA. The G+C content of the DNA ranges from 40 to 43 mol% (as determined by the thermal denaturation method). Strains have been isolated from sea ice, sea ice algal biomass, cyanobacterial mats, and the water columns of marine salinity meromictic lakes in Antarctica. The type strain is ACAM 591, which was isolated from Antarctic sea ice.

Description of *Shewanella gelidimarina* sp. nov. *Shewanella gelidimarina* (ge.lid.i.ma.ri'na. L. adj. *gelidus*, ice-cold; L. fem. adj. *marina*, of the sea; M. L. adj. *gelidimarina*, of the ice-cold or icy sea). Cells are rod shaped and either curved or straight (length, 1.5 to 2.5 µm; width, 0.5 to 0.8 µm) and occur singly or in pairs. Cells are motile. Facultatively anaerobic chemoheterotroph. Anaerobic growth occurs by fermentation of N-acetylglucosamine and chitin (but not D-glucose) or alternatively by anaerobic respiration with ferric compounds, nitrate, or TMAO as the electron acceptor and either sodium lactate or sodium acetate as the electron donor. Psychrophilic. Growth occurs at temperatures from <0 to 23°C. Optimal growth occurs at 15 to 17°C. Requires seawater for optimal growth.

Growth occurs in media supplemented with NaCl at concentrations ranging from 1 to 6%. Growth factors are not required. Uses inorganic and organic nitrogen sources. Catalase and oxidase positive. Chitinolytic, proteolytic, and lipolytic. Hydrogen sulfide is formed from thiosulfate. May produce DNase. Esculin and starch hydrolysis, ONPG, arginine dihydrolase, and ornithine decarboxylase tests are negative. Acid is formed oxidatively from *N*-acetylglucosamine. No acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, sucrose, trehalose, D-xylose, D-adonitol, glycerol, *m*-inositol, D-mannitol, or D-sorbitol. The following carbon sources are utilized: *N*-acetylglucosamine, acetate, propionate, butyrate, valerate, DL-lactate, pyruvate, and L-glutamate. Produces EPA. The G+C content of the DNA is 48 mol% (as determined by the thermal denaturation method). The type strain is ACAM 456, which was isolated from Antarctic sea ice.

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