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Diversity and Association of Psychrophilic Bacteria in Antarctic Sea Ice

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The bacterial populations associated with sea ice sampled from Antarctic coastal areas were investigated by use of a phenotypic approach and a phylogenetic approach based on genes encoding 16S rRNA (16S rDNA). The diversity of bacteria associated with sea ice was also compared with the bacterial diversity of seawater underlying sea ice. Psychrophilic (optimal growth temperature, ≤15°C; no growth occurring at 20°C) bacterial diversity was found to be significantly enriched in sea ice samples possessing platelet and bottom ice diatom assemblages, with 2 to 9 distinct (average, 5.6 ± 1.8) psychrophilic taxa isolated per sample. Substantially fewer psychrophilic isolates were recovered from ice cores with a low or negligible population of ice diatoms or from under-ice seawater samples (less than one distinct taxon isolated per sample). In addition, psychrophilic taxa that were isolated from under-ice seawater samples were in general phylogenetically distinct from psychrophilic taxa isolated from sea ice cores. The taxonomic distributions of psychrotrophic bacterial isolates (optimal growth temperature, >20°C; growth can occur at ~4°C) isolated from sea ice cores and under-ice seawater were quite similar. Overall, bacterial isolates from Antarctic sea ice were found to belong to four phylogenetic groups, the alpha and gamma subdivisions of the *Proteobacteria*, the gram-positive branch, and the *Flexibacter-Bacteroides-Cytophaga* phylum. Most of the sea ice strains examined appeared to be novel taxa based on phylogenetic comparisons, with 45% of the strains being psychrophilic. 16S rDNA sequence analysis revealed that psychrophilic strains belonged to the genera *Colwellia*, *Shewanella*, *Marinobacter*, *Planococcus*, and novel phylogenetic lineages adjacent to *Colwellia* and *Alteromonas* and within the *Flexibacter-Bacteroides-Cytophaga* phylum. Psychrotrophic strains were found to be members of the genera *Pseudoalteromonas*, *Psychrobacter*, *Halomonas*, *Pseudomonas*, *Hyphomonas*, *Sphingomonas*, *Arthrobacter*, *Planococcus*, and *Halobacillus*. From this survey, it is proposed that ice diatom assemblages provide niches conducive to the proliferation of a diverse array of psychrophilic bacterial species.

Sea ice critically influences the productivity of the Southern Ocean, global energy budgets, and atmosphere-ocean interactions in the Antarctic zone (41, 60). Ship transects of the pack ice have shown that sea ice microbial communities have a major influence on various trophic levels of the oceanic food web (1, 50) and occur as surface layer populations, within ice floes, and concentrated near the sea ice-seawater interface. Sea ice has been described as an ecosystem in which both physical phenomena and biological activity help shape the overall habitat (18, 51). Sea ice is temporally and spatially highly variable, rarely lasting longer than a few seasons, with internal temperatures typically ranging from −1 to −15°C. The salinity of sea ice brine inclusions correlates to the local ice in situ temperature and ranges from <10 to >150‰ (19, 44). The ecological structure and biological rates in sea ice are essentially driven by the prevailing temperature and salinity (47, 50). Nevertheless, sea ice is a biologically dynamic habitat and is in general more productive than the underlying seawater zone (4, 16). Within brine inclusions of sea ice, microbiota may form assemblages consisting largely of low-light-adapted diatoms (irradiance is limited by the ice and snow cover [16]) as well as dinoflagellates, autotrophic and heterotrophic flagellates, and ciliates (24). Metazoa, including polychaetes, amphipods, copepods, euphausiids, and the larval stages of ice fish such as species of *Trematomus* and *Dissostichus*, seasonally colonize Antarctic sea ice (24, 50). Studies have found bacteria to occur throughout sea ice and, in particular, in thick annual pack ice associated with surface communities (40) or with deteriorating sea ice crack pool communities (25). Bacterial populations are tightly coupled to primary productivity levels in sea ice. The population, distribution, and activity of bacteria correlate with the level of chlorophyll *a* and algal biomass in sea ice cores (28, 39, 40). Remineralization of dissolved organic matter to inorganic nutrients in sea ice is thought to be carried out primarily through the action of heterotrophic bacteria (36).

Various studies show that bacteria in sea ice are generally enriched compared to those in open and underlying seawater, bacterial cell biovolumes are 5 to 10 times larger in sea ice, and a high proportion of cells are epiphytic or particle associated (14, 25, 28, 30, 39, 46, 50, 56). A substantial proportion of sea ice bacteria have been found to harbor plasmids (37). Evidence for high numbers of psychrophiles in sea ice collected from the Weddell Sea ice edge was shown by Delille (14). These ice samples were rich in particulate organic carbon and contained a high proportion of large, particle-associated bacterial cells which were shown to be mostly psychrophilic following most-probable-number analyses. Limited phenotypic and chemotaxonomic surveys of sea ice bacteria (14, 54, 59) indicate that sea ice bacteria are predominantly gram-negative, nonfermentative rods or filaments which are often pigmented; however, *Vibrio*-like strains were shown to predominate (14) in some of the more-organic-rich samples and gram-positive bacteria were also abundant (53). Gas vacuolate bacteria from a diverse

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range of phylogenetic branches have also been isolated from sea ice and the sea ice-seawater interface of both Antarctic and Arctic ice samples (26, 27, 35).

The potential for the biotechnological application of psychrophilic bacteria is receiving increasing attention (2). Certain psychrophilic bacteria can form omega-3 polyunsaturated fatty acids of dietary significance (47) and may represent a low-cost food source, the application of which has been recently investigated for the finfish mariculture industry (48). Psychrophilic bacteria are also potential sources of novel pigments (as food additives) and cold-adapted enzymes for industrial application (2, 20).

Although studies have, to some extent, demonstrated the activity of bacteria in sea ice microbial communities, virtually no information exists on what bacterial species actually reside in sea ice, the type of adaptations that allow them to thrive in sea ice habitats, and the specific interactions which may occur between bacteria and ice diatoms and other ice-associated microbiota and metazoa. In this study, the bacterial communities associated with land-fast ice (ice attached to the shoreline) samples were investigated. The goal was to establish the identity of bacterial species associated with sea ice by the use of phenotypic comparisons and sequence comparisons of genes encoding 16S rRNA (16S rDNA). The investigation also focused on determining whether psychrophilic bacteria preferentially establish themselves in sea ice samples with high chlorophyll levels (and thus high diatom populations).

In this investigation, the specific identification of sea ice bacteria was done for the first time and indicates that the Antarctic sea ice bacterial community is made up of mostly psychrophilic and taxonomically novel members, with many taxa related to well-established marine bacterial genera but usually representing novel species in these genera or adjacent, related novel genera. In addition, it was conclusively shown that psychrophilic bacteria are closely associated with sea ice diatom assemblages.

MATERIALS AND METHODS

Sea ice and water samples. Most sea ice strains were isolated in a previous study in which the bacterial biodiversity of sea ice samples (collected in October to December of 1992 and 1993) was assessed on the basis of phenotypic traits (10). Additional strains were also isolated from sea ice cores collected during October and November of 1994 and 1995. The cores were obtained from areas of the Vestfold Hills coast (68°S 78°E), eastern Antarctica, especially along the length of Ellis Fjord. The ice cores were collected with a SIPRE drill with care being taken not to disturb the platelet and bottom algal assemblage if present. Core sections were approximately 30 to 60 cm in length, with two cores collected to penetrate through to the ice-water interface. A Kemmerer bottle was utilized to collect water samples from below the ice through holes generated by the ice drilling-coring process. The depth of under-ice seawater sampled ranged from 2 to 5 m. Samples were immediately transported to the operations base (Davis Station) for further processing.

Strain isolation and characterization. Sea ice cores were melted in equal volumes of seawater at 2 to 4°C to avoid hypotonic shock of halophilic bacteria. The thawed ice and seawater samples were then diluted to a ratio of approximately 1:10 in marine 2216 liquid medium at 2°C and incubated for 1 to 2 days before being serially diluted onto marine 2216 medium or in some cases onto marine 2216 agar supplemented with 10% (wt/vol) NaCl. The initial incubation of samples in liquid medium appeared to increase the recovery of the more slowly growing psychrophilic strains on solid media. Plates were then incubated at 2 to 4°C for up to 2 months before colonies were subcultured for purification. Colonies were selected on the basis of differing morphotype to obtain the widest possible diversity from the restricted cultivation conditions employed. Isolates were routinely cultivated on marine 2216 agar at 10°C, except for a number of highly psychrophilic strains which were cultivated at 4 to 5°C.

Phenotypic characterization. Gram stain, oxidase, and catalase tests were performed as described previously (55). Motility was tested by microscopically examining cell wet mounts. Seawater requirement and tolerance to various NaCl concentrations (0 to 12% NaCl) were tested in R2A agar (Oxoid Ltd., Basingstoke, United Kingdom). Tests of hydrolysis of starch, Tween 80, egg yolk, esculin, and casein were done as described by Smibert and Krieg (55) with marine 2216 agar as the basal medium. DNA hydrolysis was tested with DNase test agar (Oxoid). Chitin hydrolysis was tested on the medium of West and Colwell (57) prepared with artificial seawater. Additional biochemical tests were performed with the API 32 ID test kit (BioMerieux, Lyons, France), which was prepared as described in the manufacturer's specifications except that bacterial strains were suspended in chilled artificial seawater. Testing for oxidative and fermentative acid production from carbohydrates used the medium of Leifson (42). Carbon and energy source utilization was investigated by use of a basal medium containing (per liter of artificial seawater) 2 g of ammonium chloride, 2 mM potassium phosphate buffer (pH 7.0), 2 ml of SL-10 trace element solution (49), 1 g of yeast extract, and 10 ml of a vitamin solution. The vitamin solution (per liter of distilled water) consisted of 10 mg of pyridoxine-HCl, 5 mg of riboflavin, 5 mg of niacinamide, 5 mg of thiamine-HCl, 5 mg of *p*-aminobenzoate, 5 mg of lipoate, 2 mg of folate, 2 mg of biotin, 2 mg of pyridoxal, 2 mg of ascorbate, and 0.1 mg of cyanocobalamin. 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). Carbon substrates were added at a concentration of 0.1% (wt/vol), except for carbohydrates, which were tested at a concentration of 0.2% (wt/vol). Media lacking a carbon source were prepared as negative controls to account for any background growth. The actual carbon sources tested and other details on phenotypic testing can be obtained from the study of Bowman et al. (9). Numerical taxonomic analysis employing simple matching coefficients used the Taxon version 1.0 program (CSIRO Division of Entymology, Canberra, ACT, Australia). The data set for the numerical taxonomic analysis included 147 test results per strain coded in a binomial format.

Phylogenetic analysis. The 16S rDNA gene was amplified with a PCR previously described by Bowman et al. (9) with 9-27f and 1492-1515r 16S rDNA primers. The amplicons were purified by the QIAquick purification kit (Qiagen, Chatsworth, Calif.). The 16S rDNA sequence reactions were then prepared with either the dye primer cycle sequencing ready reaction (−21 M13 forward and M13 reverse) kit or the PRISM ready reaction dideoxy termination cycle sequencing kit (Perkin-Elmer). Sequences were then obtained with an Applied Biosystems model 377A automated sequencer for electrophoresis and data collection.

Sequences were compared to the compilation of 16S rDNA genes available in the GenBank nucleotide library by BLAST searching (5) through the U.S. National Institutes of Health Internet site. Sequences were then aligned to their closest related sequences determined from the BLAST searches. In some cases, the alignment of potentially ambiguous hypervariable regions was confirmed by checking them against the secondary structures of relevant 16S rRNA molecules determined previously by Gutell (31). Subsequent phylogenetic analyses of the sequence data sets utilized PHYLIP (version 3.57c) (21). DNADIST was used to determine sequence similarities with the maximum likelihood algorithm option. Phylogenetic trees were constructed by the neighborliness method with the program NEIGHBOR. Outgroup sequences utilized were *Rhodospirillum rubrum* (alpha subdivision of the class *Proteobacteria*), *Xanthomonas campestris* (gamma subdivision), *Atopobium minutum* (gram-positive branch), and *Thermonema lapsum* (*Flexibacter-Bacteroides-Cytophaga* phylum). Unrooted trees were then created by use of DRAWGRAM.

Sequences of organisms used for phylogenetic trees. The closest-neighboring species and strains (and GenBank accession numbers) to the 16S rDNA sequences determined in this study are listed below and include members of four phylogenetic groups. In most cases, species type strains (if available) with nearly full length 16S rDNA sequences were used. In addition, species isolated from Antarctic habitats were included where relevant for comparison purposes. Genus names in brackets are generically misclassified.

(i) *Proteobacteria*—alpha subdivision. The alpha subdivision of *Proteobacteria* included *Caulobacter bacteroides* (M83796), dimethylsulfoniopropionate-degrading strain LMR (L15345), *Erythromicrobium ramosum* (X72909), gas vacuolate strain 307 (U14583), *Hirschia baltica* (X52909), *Hyphomonas jannaschiana* (M83806), *Paracoccus denitrificans* (X69159), *Rhizobium leguminosarum* (D14513), *Rhodobacter capsulatus* (D16428), *Roseobacter algicola* (X78315), *Sphingomonas adhaesiva* (D38430), *Sphingomonas subarctica* (X94102), and marine ultramicrobacterium strain RB2256 (U11041).

(ii) *Proteobacteria*—gamma subdivision. The gamma subdivision of *Proteobacteria* included *Alteromonas macleodii* (X82145), *Colwellia psychroerythus* (AF001375), *Escherichia coli* (JO1859), *Ferrimonas balearica* (X93021), gas vacuolate strain S-51w(gv)1 (U14581), gas vacuolate strain S-36w(gv)1 (U14584), gas vacuolate strain 90-P(gv)1 (U14582), *Halomonas aquamarina* (M93352), *Halomonas meridiana* (M93356), *Halomonas subglaciescola* (M93358), *Halomonas variabilis* (M93357), *Marinobacter hydrocarbonoclasticus* (X67022), *Pseudoalteromonas atlantica* (X82134), *Pseudoalteromonas carrageenavora* (X82136), *Pseudoalteromonas denitrificans* (X82138), *Pseudoalteromonas espejiana* (X82143), *Pseudoalteromonas haloplanktis* (X67024), *Pseudoalteromonas nigrefaciens* (X82416), *Pseudoalteromonas undina* (X82140), *Pseudomonas aeruginosa* (X06684), *Pseudomonas mucidolens* (D84017), *Pseudomonas synxantha* (D84025), *Psychrobacter immobilis* (U39399), *Psychrobacter glacincola* (U46145), *Shewanella alga* (X81622), *Shewanella benthica* (X82131), *Shewanella hanedai* (X82132), *Shewanella putrefaciens* (X81623), *Vibrio cholerae* (X74695), and [*Vibrio*] *marinus* (X82142).

(iii) *Flexibacter-Bacteroides-Cytophaga* phylum. The *Flexibacter-Bacteroides-Cytophaga* phylum included *Cyclobacterium marinus* (M62788), *Cytophaga hutchinsonii* (D12663), [*Cytophaga*] *latercula* (D12665), [*Cytophaga*] *lytica* (D12666),

[*Cytophaga*] *marinoflava* (D12668), *Flavobacterium flevense* (D12662), [*Flavobacterium*] *gondwanense* (M92278), *Flavobacterium saccharophilum* (D12671), [*Flavobacterium*] *salegens* (M92279), [*Flectobacillus*] *glomeratus* (M58775), *Flectobacillus major* (M62787), *Flexibacter flexilis* (M62806), [*Flexibacter*] *maritimus* (D14023), gas vacuolate strain 23-P (M61002), gas vacuolate strain 301 (U14586), *Microscilla marina* (M58793), and *Polaromonas filamentus* (U73726).

(iv) Gram positives. The gram-positive species included *Arthrobacter agilis* (X80748), *Arthrobacter ilicis* (X83407), *Arthrobacter* (X80739), *Arthrobacter polychromogenes* (X80741), *Arthrobacter protophormiae* (X80745), *Halobacillus litoralis* (X94558), *Micrococcus luteus* (M38242), *Planococcus citreus* (X62172), *Planococcus kocurii* (X62173), and *Halobacillus halophilus* (X62174).

Nucleotide sequence accession numbers. The 16S rDNA sequences generated in this study were deposited in GenBank under the accession numbers listed in Table 1.

RESULTS

Ice samples. Platelet-associated and/or interstitial bottom diatom assemblages in a total of 22 of 39 ice core samples were observable as reddish-brown to dark-green discolorations of the ice. Although chlorophyll *a*-rich material was visibly distributed throughout the lower 20 to 30 cm of many of the ice cores, the bulk of the assemblage concentrated in a 2- to 10-cm layer at the very base of the core. Diatoms made up the bulk of the ice assemblages and were identified as *Thalassiosira antarctica*, *Fragiliopsis*-like species, *Nitzschia stellata*, *Entomoneis* sp., and *Pleurosigma* sp. The proportions of the various diatom species in the ice samples were not determined, but *Thalassiosira antarctica* was usually the most common on the basis of visual assessments.

Numerical taxonomic analysis of isolates from sea ice and under-ice seawater. A set of strains representing a sampling of different bacterial colony morphotypes had been previously investigated; these sea ice samples were collected along the Vestfold Hills coast during the spring and summer of 1992 and 1993 (10). To find additional colonial morphotypes for this study, isolations were also performed with ice collected during the summers of 1994 and 1995. However, the distribution, range, and appearance of bacterial colony morphotypes derived from sea ice bacteria isolated during the different collection times were quite similar to those of the 1992–1993 samples. For the purposes of comparing sea ice with pelagic bacterial communities, 96 strains were isolated from under-ice seawater samples taken from the same sites as those of the ice cores collected during the 1995 sampling series. The ice and under-ice seawater strains were compared by use of a variety of morphological, nutritional, and biochemical tests, with numerical taxonomic analysis utilized to group the strains into phenetic groups. Sea ice strains isolated from the 1992–1993 ice cores were also included in this analysis. An arbitrary simple matching coefficient cutoff of 80% was used to create each phenon. A simplified dendrogram comparing the sea ice bacterial taxa with taxa isolated from under-ice seawater is shown (Fig. 1). A total of 31 phena were formed in the analysis. Unclustered strains, which comprised 8% of the sea ice strains and about 11% of the seawater strains, were not included in the dendrogram (unless they were characterized by 16S rDNA sequence analysis). Representative strains within each phenon were characterized by 16S rDNA sequence analysis, and the results of this analysis were used to identify each of the clusters in the dendrogram. Sea ice isolates were represented in 29 of the 31 phena, while under-ice seawater isolates were represented in only 16 phena.

Phylogenetic analysis. The 16S rDNA sequences of representative strains from each of the phenetic clusters (Table 1) were obtained. In a number of cases, several strains of a phenon were sequenced to assess the phylogenetic homogeneity in the phenotypic group. For most strains, nearly complete (1,410- to 1,510-bp) sequences of the 16S rDNA gene, stretching from nucleotide positions 7 to 1510 (*Escherichia coli* equivalent), were obtained. The 16S rDNA sequences determined grouped into four bacterial phylogenetic branches, the alpha and gamma subdivisions of the *Proteobacteria*, the gram-positive branch, and the *Flexibacter-Bacteroides-Cytophaga* phylum (see Fig. 2 to 5). The nucleotide similarity of individual isolates to their closest phylogenetic neighbors and whether the strain was psychrophilic are shown in Table 1.

Alpha-subdivision (*Proteobacteria*) strains. Psychrotrophic isolates found to belong to the alpha-subdivision phylogenetic group were related to the genera *Hyphomonas* and *Sphingomonas* (Fig. 2). These strains were seawater-requiring (but not halotolerant), strictly oxidative psychrotrophs which possessed temperature optima of 20 to 25°C. The psychrophilic, seawater-requiring strains of phenon 29 (represented by IC141 and IC146) also belonged to the alpha subdivision. The closest sequence was found to be a gas vesicle-forming strain from Antarctic sea ice (26, 27) which has yet to be classified. The next-closest relative was *Roseobacter algicola*, with a similarity of 91.8 to 91.9%.

Gamma-subdivision (*Proteobacteria*) strains. Within the gamma subdivision, the isolates were found to form 10 groups which included two novel lineages (Fig. 3). The first novel group included strains IC067, IC059, IC085, and IC079, which are moderately related to *Alteromonas macleodii*, with a sequence similarity of 89.3 to 91.5% (Table 1). These strains are pale pink to magenta pigmented, nonmotile, rod shaped to filamentous, psychrophilic, and seawater requiring, possess a strictly oxidative metabolism, and form phenon 19 in the numerical taxonomic analysis (Fig. 1). The second novel group includes strains IC004, IC052, and IC047, which are yellow or nonpigmented, psychrophilic, seawater-requiring, facultative anaerobes which clustered adjacent to [*Vibrio*] *marinus* and the genera *Colwellia* and *Pseudoalteromonas* (similarity, 86.9 to 90.7%). Strains IC004 and IC047 are closely related to gas vacuolate strain 90-P isolated by Gosink and Staley (26) from the sea ice-seawater interface (similarities, 97.1 and 95.6%, respectively). None of these strains grouped in phena in the dendrogram. Other sea ice and under-ice seawater isolates falling into the gamma subdivision were identified as belonging to known genera, including *Colwellia* (phenon 20 and unclustered strains ACAM 179, IC035, and IC169), *Shewanella* (phena 1 and 19), *Pseudoalteromonas* (phena 2 and 3), *Marinobacter* (phena 8 and 24), *Pseudomonas* (phenon 14), *Psychrobacter* (phena 9 and 10), and *Halomonas* (phenon 13).

Isolates related to the genus *Colwellia* were psychrophilic, motile, facultative anaerobes, formed off-white- to tan-pigmented colonies, and required seawater for growth. Most of the isolates belonging to the genus *Shewanella* were nonhalophilic psychrotrophs closely related to *Shewanella putrefaciens*. A few *Shewanella* strains were psychrophilic, required seawater, and were related to the species *Shewanella benthica* and *Shewanella hanedai*, which are also psychrophiles (12). *Psychrobacter* strains were isolated from virtually all ice and seawater samples. About 30% of ice-derived *Psychrobacter* strains were psychrophilic, moderately halotolerant with growth stimulated by seawater, and unable to form acid from carbohydrates. These strains were phenotypically distinct from other cold-adapted *Psychrobacter* species found in ornithogenic soils on the Antarctic continent (9). However, the isolates were closely related to a recently described species, *Psychrobacter glacincola*, which was isolated from the base of a 350-m-deep ice core taken from the Amery Ice Shelf (8). The remaining ice-derived *Psychrobacter* strains and all the *Psychrobacter* isolates from under-ice seawater were found to be phenotypically analogous to *Psychrobacter immobilis*. These strains were psy-

TABLE 1. Phylogenetic and ecophysiological relationships of Antarctic sea ice and under-ice seawater strains

Strain	GenBank no.	Phenon	16S rDNA identification (closest neighbor)[a]	Sequence similarity (%)	Psychrophilic growth[b]
Alpha subdivision of *Proteobacteria*					
SW54	U85838	7	*Sphingomonas adhaesiva*	93.8	−
SW47	U85839	6	*Hyphomonas jannaschiana*	96.1	−
IC141	U85840	29	*Roseobacter algicola*	91.8	+
IC146	AF001377	29	*Roseobacter algicola*	91.9	+
Gamma subdivision of *Proteobacteria*					
ACAM 607	U85847	—[d]	*Colwellia psychroerythrus*	95.9	+
ACAM 179	U85846	—	*Colwellia psychroerythrus*	95.5	+
ACAM 604	U85841	20	*Colwellia psychroerythrus*	99.0	+
IC064	U85842	20	*Colwellia psychroerythrus*	99.0	+
ACAM 605	U85843	20	*Colwellia psychroerythrus*	99.0	+
ACAM 459	U85845	20	*Colwellia psychroerythrus*	97.5	+
ACAM 606	U85844	20	*Colwellia psychroerythrus*	97.7	+
IC169	AF001376	—	*Colwellia psychroerythrus*	93.2	+
IC059	U85851	21	*Alteromonas macleodii*	90.8	+
IC085	U85852	21	*Alteromonas macleodii*	89.3	+
IC067	U85853	21	*Alteromonas macleodii*	90.2	+
IC079	U85854	21	*Alteromonas macleodii*	91.5	+
IC052	U85848	—	*Pseudoalteromonas* spp.	<90.3	+
IC004	U85849	—	*Pseudoalteromonas* spp.	<88.9	+
IC047	U85850	—	*Pseudoalteromonas* spp.	<87.8	+
MB8-11	U85855	3	*Pseudoalteromonas atlantica*	97.5	−
SW08	U85861	2	*Pseudoalteromonas nigrefaciens*	99.2	−
IC006	U85856	2	*Pseudoalteromonas nigrefaciens*	99.4	−
MB6-03	U85857	2	*Pseudoalteromonas nigrefaciens*	99.2	−
MB8-02	U85858	2	*Pseudoalteromonas nigrefaciens*	99.1	−
IC013	U85859	2	*Pseudoalteromonas nigrefaciens*	99.7	−
MB6-05	U86860	2	*Pseudoalteromonas nigrefaciens*	99.7	−
SW29	U85862	2	*Pseudoalteromonas nigrefaciens*	98.7	−
ACAM 585[c]	U85908	19	*Shewanella hanedai*	97.9	+
ACAM 456[c]	U85907	19	*Shewanella benthica*	96.4	+
ACAM 122[c]	U39398	1	*Shewanella putrefaciens*	96.1	−
ACAM 600[c]	U85906	1	*Shewanella putrefaciens*	96.9	−
ACAM 588[c]	U85905	1	*Shewanella putrefaciens*	97.1	−
ACAM 593[c]	U85904	1	*Shewanella putrefaciens*	96.5	−
ACAM 591[c]	U85903	1	*Shewanella putrefaciens*	96.9	−
ACAM 584[c]	U85902	1	*Shewanella putrefaciens*	96.6	−
IC022	U85863	8	*Marinobacter hydrocarbonoclasticus*	95.1	+
IC032	U85864	8	*Marinobacter hydrocarbonoclasticus*	95.1	+
IC180	U85865	24	*Marinobacter hydrocarbonoclasticus*	94.1	+
IC184	U85866	24	*Marinobacter hydrocarbonoclasticus*	94.1	+
IC065	U85867	8	*Marinobacter hydrocarbonoclasticus*	93.8	+
SW51	AF001374	24	*Marinobacter hydrocarbonoclasticus*	93.2	−
ACAM 213	U85868	14	*Pseudomonas mucidolens*	99.0	−
IC038	U85869	14	*Pseudomonas mucidolens*	99.3	−
A177	U85870	14	*Pseudomonas synxantha*	98.4	−
SW04, SW42[e]	U85871	13	*Halomonas variabilis*	98.8	−
SW32, SW33[e]	U85872	13	*Halomonas variabilis*	98.7	−
SW48	U85873	13	*Halomonas variabilis*	98.4	−
MB6-21	U85874	9	*Psychrobacter glacincola*	98.6	−
IC084	U85876	9	*Psychrobacter glacincola*	99.2	−
IC007	U85877	9	*Psychrobacter glacincola*	99.0	−
IC018	U85878	9	*Psychrobacter glacincola*	99.0	+
ICP9	U85879	9	*Psychrobacter glacincola*	99.0	+
IC008	U85875	9	*Psychrobacter glacincola*	98.5	+
IC040, SW23[e]	U85880	10	*Psychrobacter immobilis*	99.0	−
Flexibacter-Bacteroides-Cytophaga phylum					
ACAM 210	U85886	30	[*Cytophaga*] *lytica*	88.1	+
IC051	U85881	22	[*Flavobacterium*] *gondwanense*	97.7	+
IC068	AF001365	22	[*Flavobacterium*] *gondwanense*	98.0	+
IC076	U85882	22	[*Flavobacterium*] *gondwanense*	90.5	+
IC157	AF001370	28	[*Cytophaga*] *marinoflava*	90.7	+

Continued on following page

TABLE 1—*Continued*

Strain	GenBank no.	Phenon	16S rDNA identification (closest neighbor)[a]	Sequence similarity (%)	Psychrophilic growth[b]
IC148	AF001373	28	[*Cytophaga*] *marinoflava*	88.8	+
IC166	AF001366	4	[*Cytophaga*] *lytica*	91.5	−
ACAM 536[f]	U62914	26	[*Cytophaga*] *lytica*	89.5	+
ACAM 550[f]	U62915	26	[*Cytophaga*] *lytica*	89.4	+
ACAM 551[f]	U62916	26	[*Cytophaga*] *lytica*	89.7	+
IC147	AF001367	25	[*Cytophaga*] *lytica*	89.5	+
IC158	AF001369	25	[*Cytophaga*] *marinoflava*	88.8	−
IC164	AF001372	25	[*Cytophaga*] *marinoflava*	90.2	+
IC159	AF001371	25	[*Cytophaga*] *marinoflava*	88.2	+
SW17	AF001368	5	[*Cytophaga*] *marinoflava*	90.6	−
ACAM 188[f]	U62913	25	[*Cytophaga*] *latercula*	90.1	+
ACAM 181[f]	U62912	25	[*Cytophaga*] *latercula*	89.5	+
ACAM 167[f]	U62911	25	[*Cytophaga*] *latercula*	89.4	+
A103	U85887	27	*Flavobacterium flevense*	94.9	−
A265	U85888	27	*Flavobacterium flevense*	95.0	−
IC001	U85889	—	*Flavobacterium saccharophilum*	94.9	−
ACAM 123	U85890	—	*Flavobacterium saccharophilum*	95.0	+
IC063	U85885	25	*Polarobacter filamentus*	96.8	+
IC066	U85884	25	*Polarobacter filamentus*	97.0	+
IC025	U85891	31	*Cyclobacterium marinus*	81.9	+
Gram-positive branch					
MB6-12	U85892	16	*Arthrobacter polychromogenes*	92.6	−
MB9-0	U85893	15	*Arthrobacter nicotianae*	94.1	−
IC048	U85894	15	*Arthrobacter nicotianae*	96.6	−
IC044	U85895	18	*Arthrobacter agilis*	94.7	−
MB6-20	U85900	18	*Arthrobacter polychromogenes*	96.1	−
MB8-13	U85896	15	*Arthrobacter agilis*	99.4	−
MB6-07	U85897	17	*Arthrobacter ilicis*	99.2	−
MB6-16	U85898	11	*Planococcus kocurii*	96.7	−
IC024	U85899	12	*Planococcus citreus*	95.3	+
MB6-08	U85901	23	*Halobacillus litoralis*	92.0	−

[a] The most closely related validly described species, in terms of 16S rDNA sequence, to a given Antarctic isolate are given. Species shown in brackets are generically misclassified.
[b] Optimal growth temperature is ≤15°C.
[c] Sequence data from reference 12.
[d] —, strain not grouped into a phenon in Fig. 1.
[e] The 16S rDNA sequences for these strain pairs are identical.
[f] Sequence data from reference 11.

chrotrophic, broadly halotolerant (grow in concentrations of NaCl between 0 to 15% and the growth is not stimulated by seawater), and able to form acid from several carbohydrates. Strains IC040 and SW23, which represent this group, possessed identical 16S rDNA sequences that were 99.0% similar to that of *Psychrobacter immobilis* (Table 1).

Isolates related to the genera *Pseudoalteromonas*, *Pseudomonas*, and *Halomonas* were exclusively fast-growing psychrotrophs with an oxidative metabolism. The *Pseudoalteromonas* isolates were characteristically nonpigmented and halotolerant and required at least 1% NaCl for growth. Several representative strains of phenon 2 (Fig. 1) formed a very shallow phylogenetic clade along with several other nonpigmented *Pseudoalteromonas* species. Overall sequence similarity averaged approximately 99% (Table 1); however, phenotypic data suggest that the isolates were most similar to *Pseudoalteromonas nigrefaciens*. Phenon 3, which includes a small group of nonpigmented *Pseudoalteromonas* ice isolates, represented by strain MB811, was found to be distinct from the other nonpigmented *Pseudoalteromonas* strains of phenon 2, clustering at a similarity of 97.0 to 97.5%. *Pseudomonas* strains isolated from alga-rich sea ice samples (IC038 and A177) and under-ice seawater (ACAM 213) formed a pyoverdin-like green fluorescent pigment and were nonhalophilic, neither requiring sodium ions for growth nor tolerating NaCl levels greater than 6%. 16S rDNA sequences indicated a close relatedness with *Pseudomonas mucidolens* and *Pseudomonas synxantha*. *Halomonas* strains were almost entirely isolated from under-ice seawater samples, were able to grow over a broad range of NaCl concentrations (0 to >15%), and were nonpigmented and strictly oxidative. The strains isolated in this study were found to be most closely related to *Halomonas variabilis* (previously called *Halovibrio variabilis* [17]).

***Flexibacter-Bacteroides-Cytophaga* phylum.** Many pigmented sea ice isolates were found to belong to the *Flexibacter-Bacteroides-Cytophaga* phylum (Fig. 4) and were particularly concentrated in the [*Flexibacter*] *maritimus* rRNA branch of the family *Flavobacteriaceae* (7). Overall, a total of 11 phylogenetic lineages were identified among the sea ice and seawater isolates belonging to this phylum (Fig. 4). The strains belonging to this branch were psychrophilic, seawater requiring, yellow to red-orange pigmented, filamentous, strictly oxidative, and often able to glide (phena 4, 5, 22, 25 to 28, 30, and 31). A number of these strains were highly psychrophilic, not growing at temperatures above 12 to 15°C, and had estimated temperature optima of 4 to 8°C, including the representative strains IC051, IC168, IC076, IC063, and IC066. Strains which were unclustered in the numerical taxonomic analysis (ACAM 123 and

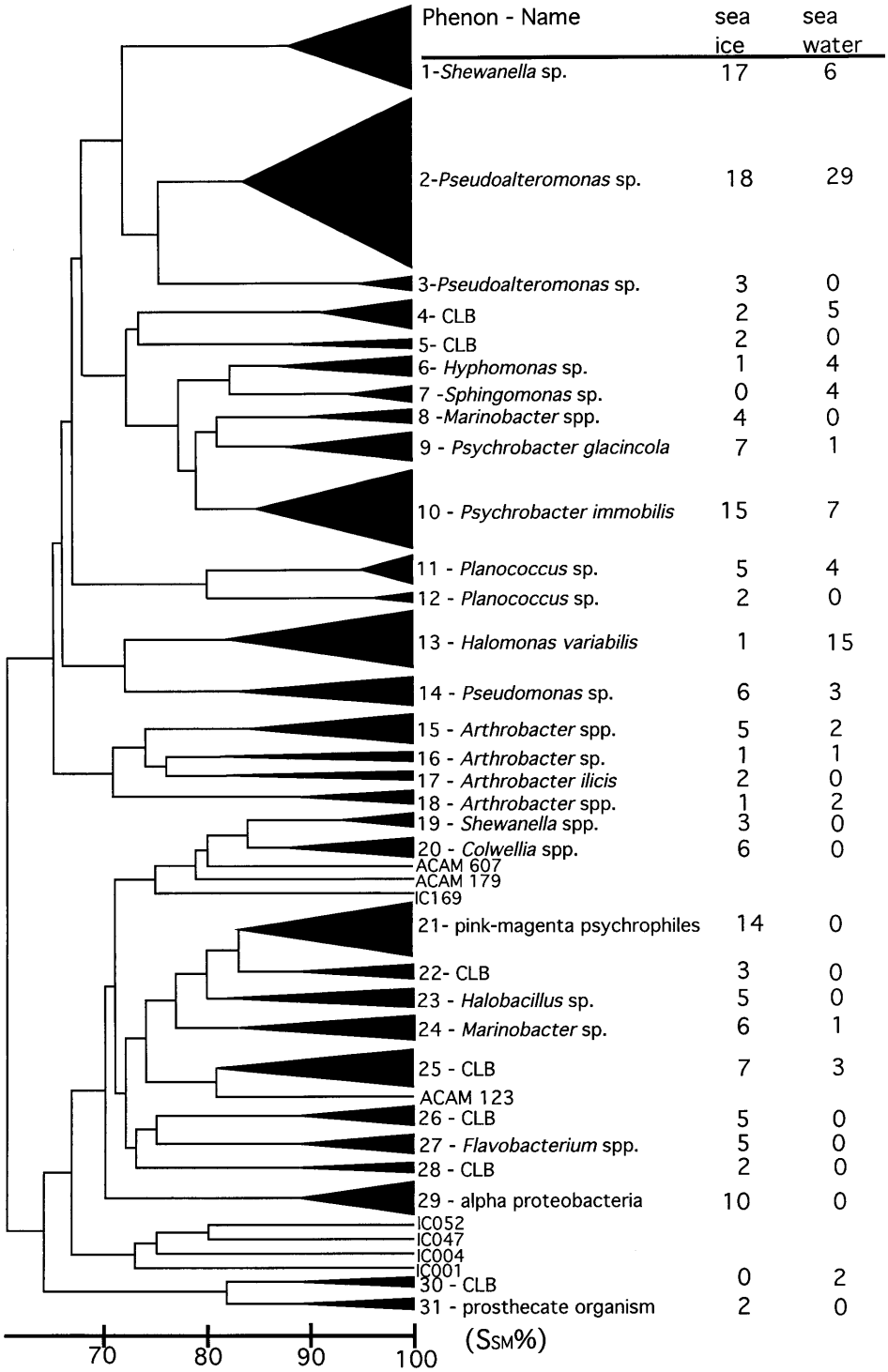

FIG. 1. Dendrogram based on unweighted pair group average linkage of simple matching (S_{SM}) coefficients of phenotypic data of sea ice and seawater strains. The phenon number is followed by the 16S rDNA-based genus and/or species identification. The twin columns of numbers indicate the number of strains isolated from sea ice and from under-ice seawater, respectively. CLB, *Cytophaga*-like bacteria.

IC001) and strains of phena 27 (represented by A103 and A265) belonged to the genus *Flavobacterium*. Most of the *Flavobacterium* strains could grow without added sodium ions and had a temperature optimum at 20 to 25°C; this was not so for one *Flavobacterium* isolate (ACAM 123), which was psychrophilic and slightly halophilic. Strain IC025 (phenon 31) formed an isolated branch in subdivision II of the *Flavobacterium-Cytophaga* complex, with the most closest relative being *Cyclobacterium marinus* at 81.9% similarity. This strain has a *Prosthecobacter*-like morphology (33) but, unlike members of the genus *Prosthecobacter*, possesses a much lower DNA G+C content (35 to 37 mol% [10]) and a fatty acid profile rich in monounsatu-

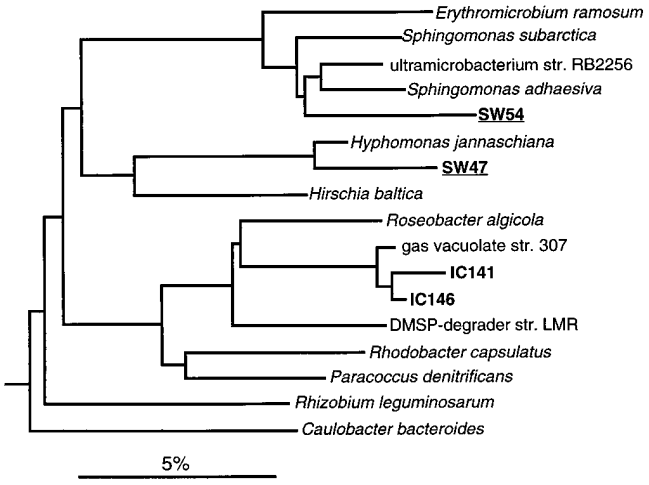

FIG. 2. Phylogenetic tree derived from 16S rDNA sequence data for members of the alpha subdivision of the *Proteobacteria. Rhodospirillum rubrum* was used as the outgroup organism. Bar, 5% sequence dissimilarity. The lengths of the vertical lines are not significant. Strains in bold type were isolated from sea ice, while strains shown underlined were isolated from under-ice seawater. DMSP, dimethylsulfoniopropionate.

rated branched-chain fatty acids (13). Bacteria with a morphology similar to that of *Prosthecobacter fusiformis* have been previously observed colonizing certain ice diatoms (56).

Gram-positive branch. Gram-positive strains isolated from the ice and seawater habitats were all pigmented and usually spherical in shape, strictly oxidative, halotolerant, and psychrotrophic. 16S rDNA sequences of representative isolates fell into two distinct phylogenetic clades (Fig. 5). Micrococci which were yellow or red pigmented clustered within or near the genus *Arthrobacter* (phena 15 to 18), which is part of the high-G+C gram-positive branch. Orange-pigmented micrococci (phena 11 and 12) belonged to the genus *Planococcus*, which is part of the low-G+C gram-positive branch. A few *Planococcus* isolates (i.e., strain IC024, phenon 12) isolated from ice differed in that they were psychrophilic and required seawater for growth. In addition, sporulating, psychrotrophic, moderately halophilic strains (phenon 23), which grew best with 6 to 8% NaCl, were found to cluster with the genus *Halobacillus*, which is related to the genera *Planococcus* and *Bacillus.*

DISCUSSION

16S rDNA-based analysis was utilized as a means of conclusively identifying sea ice isolates. By comparison, phenotypic analysis (with accompanying DNA base composition data) usually only succeeded in identifying ice isolates to the genus level (10). In addition, the sequencing of 16S rDNA of bacterial isolates was used rather than direct analysis of samples by 16S rDNA clone library characterization. Psychrophilic bacteria (known to date), unlike thermophilic bacteria and some other physiologically unusual bacteria, appear to be closely related to species which have a mesophilic temperature range for growth. This was demonstrated in studies of novel species from Antarctic lakes which were usually found to belong to known genera but represented marine species that had evolved to become more cold adapted (22).

It is clear that sea ice represents a rich source of psychrophilic, heterorophic bacterial biodiversity. This has been already highlighted in a study concentrating on gas vacuolate bacteria from the sea ice-seawater interface area of fast ice of McMurdo Sound (27). The overall phylogenetic distribution of the gas vacuolate bacteria (27) was very similar to what was found in this study. In addition, a number of isolates in this study were closely related to those found in McMurdo Sound, including the novel gas vacuolate bacterium *Polarobacter filamentus* (26), which has been isolated from both Arctic and Antarctic sea ice samples.

In this study, 26 phylogenetic groups putatively equivalent to bacterial genera were identified in Antarctic sea ice samples, including a number apparently representing novel genera. Of the total sea ice bacterial strain set, 45% were psychrophilic, and 14 phena (and several unclustered strains) were isolated only from sea ice and not from under-ice seawater samples. However, there is still insufficient information to confirm if any of these psychrophilic or psychrotrophic taxa are strictly specific to sea ice habitats. Molecular method-based investigations of bacterial microbiota associated with metazoa, phytoplankton, and particulate organic detritus, all of which could conceivably support predominantly sessile, psychrophilic bacterial populations, would be needed to confirm this. For example, psychrophiles have been found to be quite common in the waters of Burton Lake (23). However, the psychrophiles from Burton Lake (including ACAM 123, ACAM 167, ACAM 179, ACAM 181, ACAM 188, and ACAM 210) are for the most part phenotypically and phylogenetically distinct from those isolated from the ice samples investigated in this study. This, however, is only one example, and further surveys are required to ascertain the uniqueness of Antarctic and Arctic sea ice bacterial communities.

The determination of psychrophilic-psychrotrophic bacterial populations in marine waters has been open to question because of the inadequacy of cultivation methods and the difficulty in accurately interpreting results from these methods (36). However, the enrichment of psychrophilic bacteria in sea ice has been observed previously in ice samples collected from the Weddell Sea ice edge and pack ice during the summer (14) and winter (34). In this study, most psychrophiles were isolated from ice samples which possessed a well-developed diatom interstitial or platelet ice-associated assemblage (Fig. 6) but were much less common in ice samples lacking such assemblages. In the case of ice cores containing an observable diatom assemblage, two to nine (5.6 ± 1.8 taxa [mean $\pm$ standard deviation], $n = 22$) different psychrophilic taxa (taxa being equivalent to the phena in Fig. 1) were isolated on marine 2216 agar plates at 2°C. By comparison, psychrophilic strains were isolated much less often from ice cores which lacked an observable assemblage. The average number of psychrophilic taxa in these samples was 0.6 (± 0.5, $n = 17$), and the psychrophiles isolated were usually strains of *Psychrobacter glacincola* (8). Psychrophilic bacteria were not successfully isolated from seawater underlying sea ice in this study; however, several psychrophiles examined originated from the pycnocline of Burton Lake (23). Other studies have also found that psychrotrophic bacteria vastly predominate in Southern Ocean seawater samples, with psychrophilic bacteria being rarely isolated (15, 34, 59). These results indicate that psychrophilic bacterial populations are closely associated with sea ice assemblages.

Psychrophilic bacteria in sea ice are ostensibly commensals of sea ice diatoms and other biota; however, stronger, more mutualistic arrangements must also be considered (28). Since sea ice assemblage bacterial heterotrophy is tightly coupled to primary productivity, psychrophilic bacteria would then conceivably carry out a large proportion of the secondary productivity and thus provide a flow of inorganic nutrients to sustained primary production. This has been previously suggested by [^{3}H]thymidine and $H^{14}CO_3$ incorporation rates of diatom

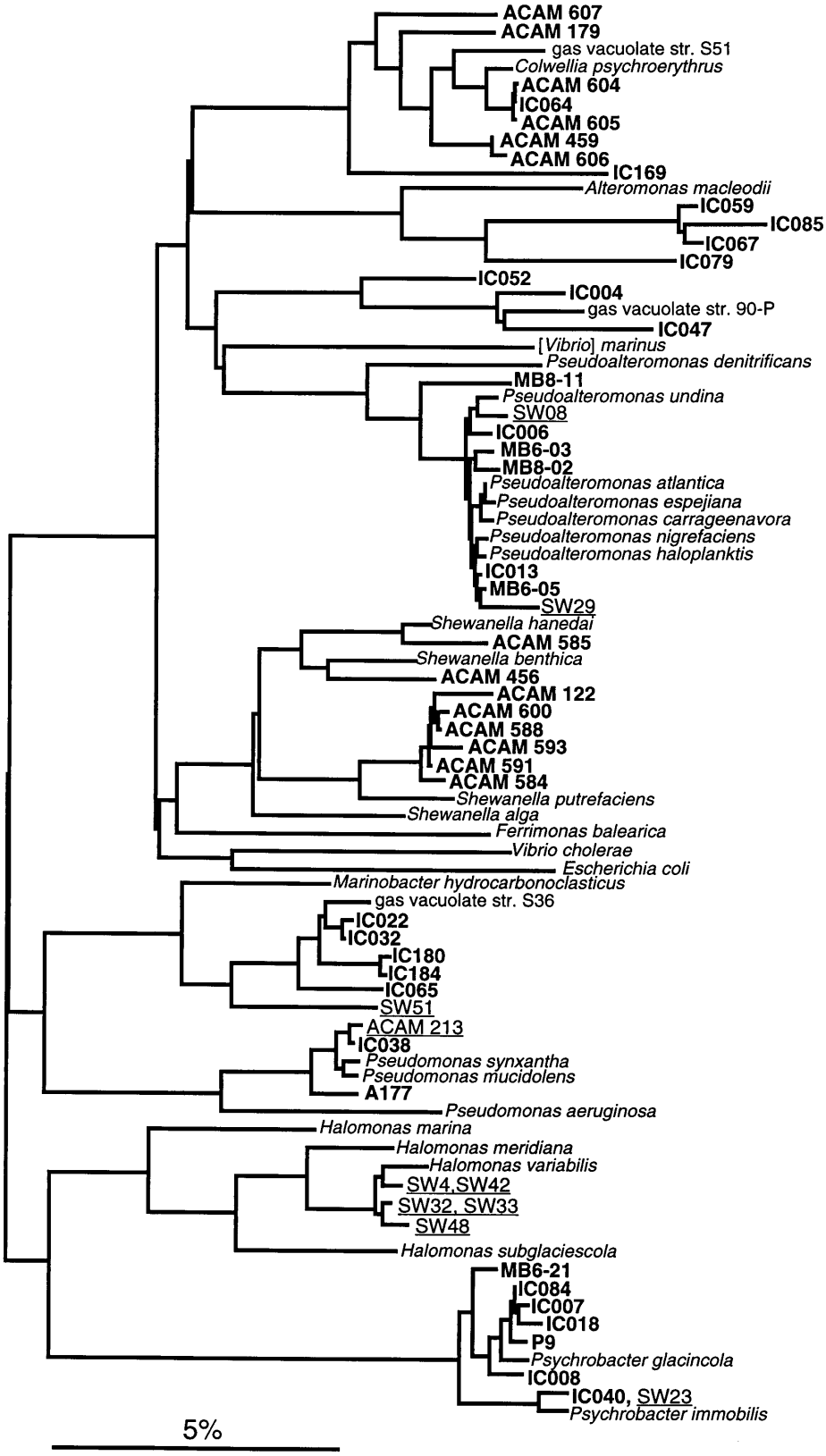

FIG. 3. Phylogenetic tree derived from 16S rDNA sequence data for members of the gamma subdivision of the *Proteobacteria*. *Xanthomonas campestris* was used as the outgroup organism. Bar, 5% sequence dissimilarity. The lengths of the vertical lines are not significant. Strains in bold type were isolated from sea ice, while strains shown underlined were isolated from under-ice seawater.

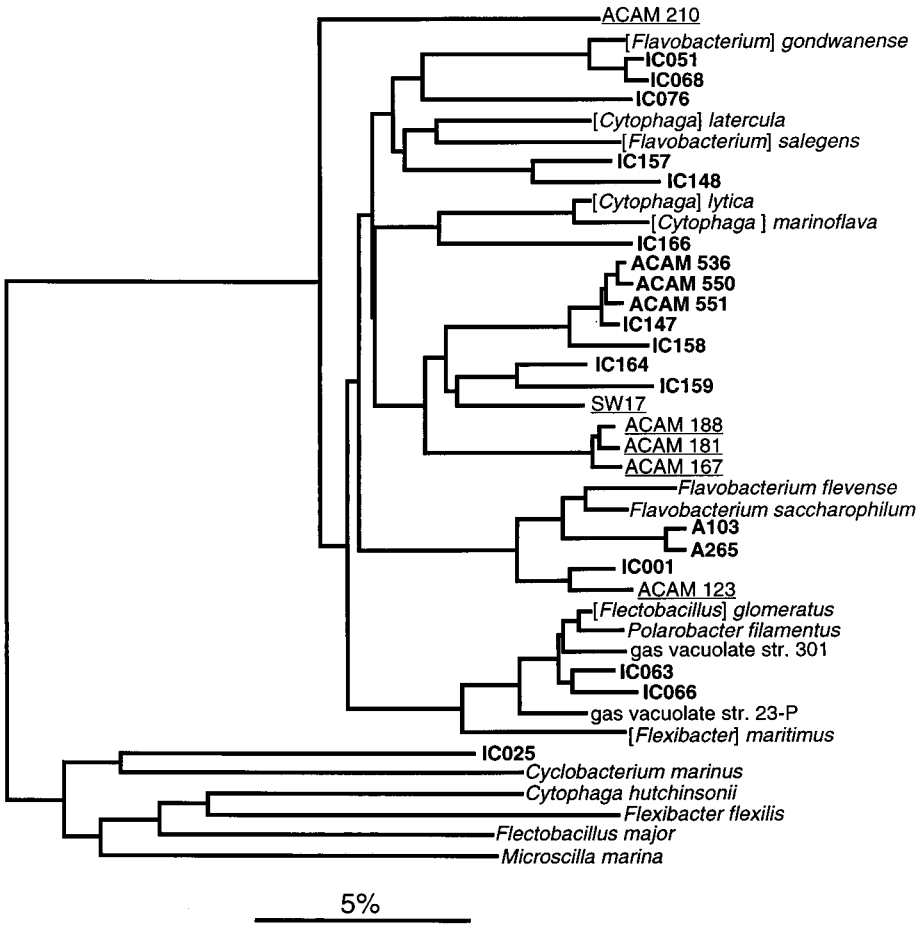

FIG. 4. Phylogenetic tree derived from 16S rDNA sequence data for members of the *Flexibacter-Bacteroides-Cytophaga* phylum. *Thermonema lapsum* was used as the outgroup organism. Bar, 5% sequence dissimilarity. The lengths of the vertical lines are not significant. Strains in bold type were isolated from sea ice, while strains shown underlined were isolated from under-ice seawater.

assemblages in McMurdo sea ice (38), both of which show a distinct psychrophilic adaptation. Additionally, a large proportion of the ice-derived psychrophilic isolates were either nonmotile or capable of gliding motility; examples include *Psychrobacter glacincola* (phenon 9), the strains of phenon 21, and many isolates of the *Flexibacter-Cytophaga-Bacteroides* phylum (phena 4, 5, 22, 25, and 26 to 28). These bacterial groups, based on the incidence of colonies during isolation, appear to be common in sea ice. Since gliding motility requires a solid matrix, this suggests that gliding bacteria in sea ice are epiphytes of sea ice diatoms or associated with aggregates of particulate organic carbon. Nonmotile strains may form holdfasts of polysaccharide and attach themselves to diatom surfaces (56). Diatoms commonly associated with interstitial ice assemblages are often thickly colonized by a wide diversity of bacterial morphotypes, including rods, cocci, filaments, and *Prosthecobacter*-like cells, all types of which are represented in this biodiversity survey (3, 45, 56). Microscopic examination has shown approximately 30% of the total bacterial population in congelation sea ice assemblages to be epiphytic (50).

The enrichment of psychrophiles may be initiated early in ice formation. As diatoms are recruited into consolidating ice, they may already be colonized to some extent by epiphytic and phycospheric free-living bacteria (6), which like the diatoms are well adapted to and possibly prefer sympagic habitats. Simultaneously, pelagic bacterial populations are suppressed

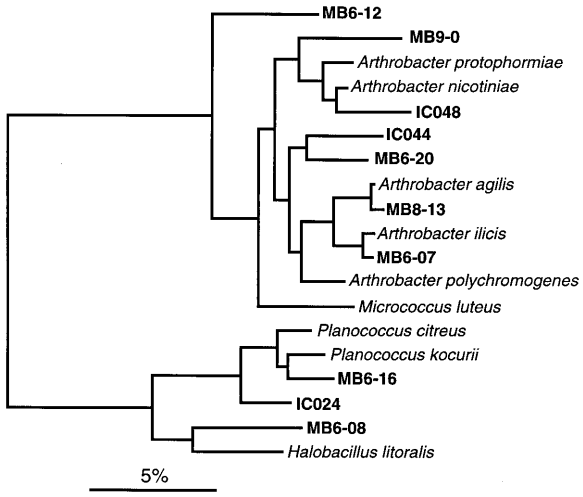

FIG. 5. Phylogenetic tree derived from 16S rDNA sequence data for members of the gram-positive phylum. *Apotobium minutum* was used as the outgroup organism. Bar, 5% sequence dissimilarity. The lengths of the vertical lines are not significant. Strains in bold type were isolated from sea ice.

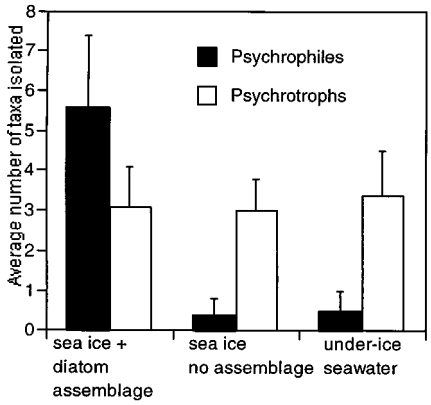

FIG. 6. Average number of psychrophilic and psychrotrophic bacterial taxa (equivalent to phenetic groups shown in Fig. 1) isolated from individual sea ice core cores in which diatom ice assemblages were present compared to ice cores lacking diatom assemblages and under-ice seawater samples.

during the ice formation process (29, 30). With increasing ice consolidation, psychrophilic bacteria appear to become more common (34). Ice-active, protein-like substances with an antifreeze capacity have been shown to be produced by the ice diatoms *Nitzschia stellata* and *Porosira pseudodenticulata* (52), and it is theorized that these substances allow better attachment of diatoms onto sea frazil. This is fundamentally a more active process of recruitment compared to previous theories, which suggested that diatoms are recruited within the frazil matrix simply due to their inherent adhesive properties or by physical entrapment (18). Whether ice bacteria have the same capacity to form ice-active substances is unknown. Owing to the large difference between the productivity of the lower sections of sea ice and that of underlying seawater, it can be seen that this process of entrapment, active and/or passive, would be beneficial to psychrophilic bacteria, and this has perhaps promoted the commensalism (and possibly mutualism) that is apparent between sea ice bacteria and ice diatoms. Spring blooms of ice diatoms, resulting in increased levels of exopolymer and free amino acids (43, 47), correlate with pronounced increases in sea ice bacterial growth rates and metabolic activity (28). The combination of a number of environmental factors, including initial selection during sea ice formation (30), diatom surface colonization, enhanced substrate level (58), and more rapid growth at temperatures of less than 4°C (32), provides the best evidence for why psychrophiles predominate in sea ice. Psychrotrophic bacteria appear to form a background, mostly free-living population within sea ice and seawater, the survival of which depends on their ability to rapidly respond to the presence of available nutrients, utilize a wide range of carbon substrates, and tolerate a broad range of salinities. Certain psychrotrophs, for example, *Halomonas variabilis*, appear to be much more common in seawater than in ice samples based on the incidence of isolation. This may stem from poor survival and competition for nutrients in the sea ice habitat. In addition, Antarctic ornithogenic soils harbor high populations of certain bacterial species, in particular, *Psychrobacter immobilis*, nonhalophilic *Planococcus* strains, and *Arthrobacter* strains (10, 53, 54), which have also been isolated from sea ice and are probably transported into the ocean during the summer glacial melt.

In this study, it was shown that Antarctic sea ice diatom assemblages are habitats which have become colonized by a prolific number of often-novel psychrophilic and psychrotrophic bacteria. Sea ice is thus a potentially rich resource for biotechnology, with psychrophiles possibly becoming sources of novel natural products, including lipids, pigments, pharmaceuticals, and enzymes. The improved understanding of the nature of the bacterial community in sea ice developed in this study will also help provide a better foundation for more-detailed studies of the ecophysiology and ecology of sea ice bacteria and for the study of psychrophilic bacteria in general.

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