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Diversity and cold-active hydrolytic enzymes of culturable bacteria associated with Arctic sea ice, Spitzbergen

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Abstract The diversity of culturable bacteria associated with sea ice from four permanently cold fjords of Spitzbergen, Arctic Ocean, was investigated. A total of 116 psychrophilic and psychrotolerant strains were isolated under aerobic conditions at 4°C. The isolates were grouped using amplified rDNA restriction analysis fingerprinting and identified by partial sequencing of 16S rRNA gene. The bacterial isolates fell in five phylogenetic groups: subclasses α and γ of Proteobacteria, the *Bacillus–Clostridium* group, the order Actinomycetales, and the Cytophaga–Flexibacter–Bacteroides (CFB) phylum. Over 70% of the isolates were affiliated with the Proteobacteria γ subclass. Based on phylogenetic analysis (<98% sequence similarity), over 40% of Arctic isolates represent potentially novel species or genera. Most of the isolates were psychrotolerant and grew optimally between 20 and 25°C. Only a few strains were psychrophilic, with an optimal growth at 10–15°C. The majority of the bacterial strains were able to secrete a broad range of cold-active hydrolytic enzymes into the medium at a cultivation temperature of 4°C. The isolates that are able to degrade proteins (skim milk, casein), lipids (olive oil), and polysaccharides (starch, pectin) account for, respectively, 56, 31, and 21% of sea-ice and seawater strains. The temperature dependences for enzyme production during growth and enzymatic activity were determined for two selected enzymes, α-amylase and β-galactosidase. Interestingly, high levels of enzyme productions were measured at growth temperatures between 4 and 10°C, and almost no production was detected at higher temperatures (20–30°C). Catalytic activity was detected even below the freezing point of water (at −5°C), demonstrating the unique properties of these enzymes.

Keywords Arctic · Cold-active hydrolytic enzymes · Psychrotolerant bacteria · Sea ice

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Introduction

In recent years, growing attention in research has been devoted to cold-adapted microorganisms. They successfully colonize cold habitats, which compose more than 80% of the earth's biosphere, and play a major role in the processes of nutrient turnover at low temperatures (Kottmeier and Sullivan 1990; Rivkin et al. 1989). Polar regions are of interest since they provide diverse terrestrial and marine habitats for psychrophilic microorganisms.

Whereas most of microbial ecological studies of polar ecosystems have focused on benthic (Bowman et al. 2000; Brambilla et al. 2001; Ravenschlag et al. 1999; Sahm et al. 1999), soil (Fridmann 1980), lake (Priscu et al. 1998), and glacier (Abyzov et al. 1998) communities, sea-ice ecosystems represent a relatively undescribed biological resource. Among sea-ice prokaryotes, members of eight phylogenetic groups—subclasses α, β, and γ of Proteobacteria, the Cytophaga–Flavibacterium–Bacterioides (CFB) phylum group, the high- and low-G+C Gram positives, and the orders

Verrucomicrobiales and Chlamydiales—have been detected by using the 16S rDNA approach (Brown and Bowman 2001; Petri and Imhoff 2001). Archaea associated with sea ice have not been found yet (Brown and Bowman 2001). Whereas culture-independent methods demonstrated the presence of the same phylogenetic groups in sea ice on both poles, cultivation-based studies provided rather disjunctive data on diversity and physiology of culturable sea-ice bacteria (Bowman et al. 1997; Brown and Bowman 2001; Junge et al. 2002). Diverse and novel members of α and γ Proteobacteria, CFB, and the Gram-positive branch have been successfully isolated and characterized from Antarctic sea ice (Bowman et al. 1997; Staley and Gosink 1999). In contrast, only a few strains belonging to γ Proteobacteria and the CFB phylum have been isolated from Arctic sea ice (Junge et al. 2002).

Plankton communities of polar oceans appeared to be more diverse than sea-ice bacterial communities. Archaea, δ and ϵ Proteobacteria, and green nonsulfur bacteria were detected in seawater in addition to the phylogenetic groups known from sea ice (DeLong et al. 1994; Massana et al. 1998). The main fraction of the polar plankton community was found to be nonculturable (Amann et al. 1995; Bianchi and Giuliano 1996).

Despite some progress in the understanding of phylogenetic composition of prokaryotic communities associated with sea ice, limited information is available on the functional roles and the physiological characteristics of bacteria in a sea-ice ecosystem. While mineralization of organic matter in temperate marine environments (Chrost 1991; Riemann et al. 2000) and Antarctic/Arctic sediments are well established (Knoblauch and Jorgensen 1999; Knoblauch et al. 1999; Reichardt 1988; Sahm et al. 1999), biopolymer decomposition in sea ice and underlying seawater remain poorly understood. Elucidation of these processes will undoubtedly depend on the identification and characterization of the enzyme producers. Generally, enzymes from psychrophiles are heat labile and display high specific activity and catalytic efficiency at low temperatures (Feller and Gerday 1997). Recent investigations have provided numerous structural and biochemical data on cold-active enzymes and significantly improved our knowledge regarding their functional and structural characteristics. Diverse combinations of several molecular adaptations observed in the case of best-studied cold-active enzymes are believed to result in the synergistic increase in flexibility of the active site, coupled with a decrease in stability at high temperatures (Russell 2000).

Cold-adapted enzyme producers are very valuable in regard to their potential for biotechnological applications. Running processes at low temperatures reduces the risk of contamination by mesophiles and saves energy. In addition, thermosensitive biocatalysts can be easily inactivated by mild heat treatment. It is expected that cold-active enzymes will find applications in various industrial processes, for example, in the detergent and food industries, in the synthesis of fine chemicals, and in bioremediation (Gerday et al. 2000).

In the present study, diversity of culturable heterotrophic bacteria associated with Arctic sea ice was investigated. Isolation and molecular phylogenetic analysis of Arctic strains were performed in order to expand our knowledge on culturable fraction of sea-ice microbial communities. In addition, efforts focused on screening for cold-active enzymes.

Materials and methods

Collection of samples

Sea-ice and seawater samples were collected from four permanently cold fjords (Woodfjord, Wijdefjord, Isfjord, and Bellsund) of Spitzbergen, Arctic Ocean, in autumn 1999. In situ temperatures of the sample sites varied from -25 to $+8.5$°C and the pH from 7.8–8.5. Samples were transported to the laboratory at temperatures between 2 and 10°C.

Enrichment, isolation, and cultivation

Sea-ice samples were melted at 4°C. Enough seawater was filtrated through a piece of a membrane filter (cellulose acetate, pore size 0.2 μm) to collect a 0.5-ml sample; the sample was used for inoculation of 10 ml complex marine medium in 20-ml tubes. Complex medium consisted of basal medium supplemented with a solution of different carbon sources, which was sterilized by filtration and contained [final concentration (g/l)]: sodium acetate (0.5), sodium succinate (0.5), DL-malate (0.5), sodium pyruvate (0.5), D-mannitol (0.5), and glucose (2.0). The basal medium contained (l^{-1}): NaCl (28.13 g), KCl (0.77 g), $CaCl_2 \times 2\ H_2O$ (0.02 g), $MgSO_4 \times 7\ H_2O$ (0.5 g), NH_4Cl (1.0 g), iron-ammonium-citrate (0.02 g), yeast extract (0.5 g), tenfold-concentrated trace element solution (DSM 141) (1 ml), tenfold-concentrated vitamin solution (DSM 141) (1 ml), KH_2PO_4 (2.3 g), and $Na_2HPO_4 \times 2\ H_2O$ (2.9 g). The pH was adjusted with NaOH to 7.2. After 2 months, stable enrichment cultures developed at 4°C and were serially diluted onto complex marine medium agar plates. Plates were incubated at 4°C for 2 months before colonies were selected on the basis of differing morphological differences. For the isolation of pure cultures, serial dilution and plating techniques were applied. Isolates were routinely cultivated on complex marine medium agar plates at 4°C.

16S rDNA amplification

Biomass from a single colony, or 1 μl, of cell pellet obtained by centrifugation of 1 ml of exponentially growing cultures was used directly as a template for the

amplification of 16S rDNA. The 16S rDNA gene was amplified with 9-27f and 1492-1515r 16S rDNA primers (Buchholz-Cleven et al. 1997). Each 100-µl PCR reaction mixture contained: 50 pmol of each primer, 1–50 ng template DNA, 200 µM dNTP each, 30 µg bovine serum albumin, 20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase (Gibco). Negative control without DNA template was included in every reaction set. All reaction mixtures were incubated in a thermal cycler (PerkinElmer Gene Amp System 2400) for 5 min at 94°C and then subjected to 30 amplification cycles of 1.5 min at 94°C, 1.5 min at 44°C, and 1.5 min at 72°C. The amplicons were separated on 1.0% (w/v) agarose gel stained with ethidium bromide.

Amplified rDNA restriction analysis

Amplified rDNA restriction analysis (ARDRA) was used to group Arctic isolates. PCR amplification of 16S rDNA was performed as described above. Unpurified PCR products (200–700 ng) were digested with 10 U of four-base-specific restriction endonuclease *Msp*I (MBI Fermentas) for 3 h at 37°C. The resulting fragments were separated by 8% PAGE and stained with ethidium bromide. The strains were grouped according to their digestion patterns.

Sequencing and phylogenetic analysis

16S rRNA gene of representatives of ARDRA pattern groups and alone-standing strains were partially sequenced. The *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) was used to directly sequence the purified PCR product (QIAquick PCR purification kit, Qiagen). Sequencing reactions were analyzed on the Applied Biosystems 373S DNA sequencer. Forward primers 341F and 518F (Buchholz-Cleven et al. 1997) were used for partial sequence analysis. For the complete sequence analysis, both strands of the amplification product were sequenced also using the primers 7F, 787F, 787R, 1175R, 1099F, and 1492R (Buchholz-Cleven et al. 1997). Primer nomenclature refers to the 5′ ends of respective target on the 16S rDNA according to *Escherichia coli* numbering of 16S rRNA nucleotides.

Obtained sequences were used for phylogenetic analysis. To determine the closest relatives of the new isolate, preliminary searches in the EMBL database were performed with the program FASTA3. Reference sequences utilized in construction of phylogenetic trees were retrieved from the EMBL database and aligned with the newly determined sequence of the new isolate by using Clustal X. Software from PHYLIP, version 3.57c (Felsenstein 1993), was used for phylogenetic and molecular evolutionary analysis. The DNADIST program, with the maximum-likelihood option, was employed to analyze sequence similarities, and NEIGHBOR software (Kimura two-parameter correction) was used to create a phylogenetic tree.

Enzyme assay

Hydrolytic activities of all strains were tested qualitatively on diffusion agar plates containing the basal medium and 0.1% (w/v) of one of the following substrates: chitin, Red Amylopectin, Red Pullulan, azocasein, azurine cross-linked (AZCL) pullulan, AZCL amylopectin, AZCL-hydroxyethyl cellulose, AZCL arabinan, AZCL arabinoxylan, AZCL curdlan, AZCL amylose, AZCL dextran, AZCL galactan, AZCL galactomannan, AZCL β-glucan, AZCL xylan, and AZCL xyloglucan. Substrate degradation was detected by clearing zones/color-diffusion halo around the colonies after a strain was grown at 4°C for 2–4 days. AZCL polymers were purchased from Megazyme (Bray, Ireland). Red Amylopectin and Red Pullulan were prepared as described previously (Jorgensen et al. 1997). Lipase activity was detected by using Rhodamin B plus olive oil agar plates, as described previously (Kouker and Jaeger 1987). Degradation of polygalacturonic acid and alginate was detected on basal agar medium supplemented with 0.1% of each substrate as described previously (Cleveland and Cotty 1991). Esterase activity was detected on basal agar medium supplemented with 0.01% $CaCl_2{\times}2H_2O$ and Tween 80 (Smilbert 1994).

β-Galactosidase activity was determined qualitatively by the appearance of blue-colored colonies after the strain was grown at 4°C for 2–4 days on agar plates containing basal medium supplemented with 0.1% lactose and 0.002% Xgal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). In addition, β-galactosidase activity was determined by measuring the amount of released *o*-nitrophenol spectrophotometrically at 420 nm when the enzyme sample was incubated with *o*-nitrophenyl-β-galactopyranoside (ONPG). To determine intracellular enzyme activity, cells were collected by centrifugation of the culture broth ($OD_{600} = 1.5$) at 4,600 rpm at 4°C for 8 min. Cell pellet (1 g) was washed with 50 mM sodium phosphate buffer, pH 7.0, resuspended in 15 ml of the same buffer, and disrupted by ultrasonification for 40 min on ice. The disrupted cells (50 µl) were added to 750 µl 9 mM ONPG dissolved in 50 mM sodium phosphate buffer, pH 7, and the samples were incubated for 30 min at desired temperatures. The reaction was stopped by the addition of 300 µl of 1 M sodium carbonate and centrifuged for 3 min at 13,000 rpm. The supernatant containing released *o*-nitrophenol was diluted threefold before measurement.

α-Amylase activity was determined by measuring the amount of reducing sugars liberated when the cell culture supernatant was incubated in 50 mM sodium acetate buffer with 0.1% starch, as described previously (Bernfeld 1955). The dependence of the enzymatic activity on pH was determined by using the protocol described above, but replacing phosphate buffer with

0.12 M universal buffer to obtain a pH range from 2.0–11.0; all of the assays were performed at 25°C. The pH of the buffers was adjusted at room temperature.

Results

Isolation and 16S rDNA sequence analysis

A total of 60 samples were collected from four locations of different physico-chemical characteristics (Spitzbergen, Norway). Bacteria were enriched using aerobic heterotrophic conditions at 4°C. Serial dilution and subsequent cultivation on agar plates at 4°C resulted in the isolation of 116 pure bacterial cultures (Table 1).

The isolates were compared applying ARDRA fingerprinting, which has been shown to discriminate strains at species and subspecies level. Strains with identical ARDRA profiles were grouped in clusters. A total of 19 clusters accommodating 2–16 strains were formed. Strains with unique fingerprinting profiles composed over 25% of sea-ice and seawater isolates. Representative strains of each cluster as well as all unique strains were subjected to partial 16S rDNA analysis to determine their phylogenetic affiliation with already described species. In cases of the clusters exceeding ten strains, two or three strains were sequenced to access the phylogenetic homogeneity of the group. Partial 16S rDNA sequences (stretch ranging from positions 350–1200) were obtained for a total of 65 strains. Based on phylogenetic comparisons, over 40% of sequenced isolates (28 strains) potentially represent novel species or genera sharing less than 98% sequence similarity to the closest validly described species.

Overall, the bacterial isolates fell in five phylogenetic groups: the α and γ subclasses of Proteobacteria, low and high G+C Gram positives, and the CFB group (Table 1). The diversity of Arctic strains is not evenly distributed among the major groups. Over 70% of the isolates are affiliated with the γ subclass of Proteobacteria. Over 10% of sea-ice and seawater isolates were found to belong to the Gram-positive branch.

Independently from the results of ARDRA fingerprinting, all Arctic isolates were tested for their ability to produce cold-active enzymes (Table 2). Strains, which shared identical DNA-fingerprinting profiles, generally demonstrated a different activity spectrum, proving the heterogeneity of ARDRA clusters. Differences in enzyme spectra were also detected between strains from different ARDRA groups, which are highly similar at 16S rDNA level (up to 99%).

α Subclass of Proteobacteria

Of 116 isolates, only one strain (which has a unique ARDRA profile) was found to belong to the α subclass of Proteobacteria (Table 1). The strain 1–5 isolated from sea ice fell in the *Rhodobacter*/*Rhodovulum* clade, but did not cluster with species of either genera (Fig. 1). The closest relatives were found to be a group of yet unclassified bacterium, QSSC1-20, isolated from Antarctic quartz stones (Smith et al 2000), sharing 94.7% similarity at 16S rDNA level. The closest validated relative is *Rhodobacter veldkampii* (Hiraishi and Ueda 1994), with a similarity of 93.6%. The new isolate is nonpigmented and psychrotolerant, showing no growth above 30°C. Good growth was observed at 10°C and optimal growth at 25°C. None of the substrates tested were hydrolyzed by the newly isolated strain.

γ Subclass of Proteobacteria

Within the γ subclass of Proteobacteria, Arctic isolates fell into the genera *Marinomonas*, *Colwellia*, *Psychromonas*, *Psychrobacter*, *Shewanella*, *Pseudomonas*, and *Pseudoaltheromonas* (Table 1; Fig. 1). Within the genus *Marinomonas*, novel lineage includes sea-ice strains 31-1-2 and 6-2 and seawater strain 2-2-1, all with identical ARDRA profiles. Partial 16S rDNA sequencing of 31-1-2 and 6-2 isolates as representatives of this small group revealed moderate relatedness to *M. communis*, with a sequence similarity of 94.5 and 94.0%, respectively. Both isolates are psychrotolerant, showing a broad growth temperature range from 10–37°C, with an optimum at 30°C. No growth was observed at temperatures above 37°C. Despite high similarity on 16S rDNA level and identical fingerprinting profile, the strains differ significantly in their hydrolytic activities. Whereas 2-2-1 and 6-2 are able to degrade lipids, only isolate 31-1-2 is proteolytic. In addition, strain 2-2-1 is able to degrade alginate and polygalactouronic acid.

Within the *Colwellia* genus, novel lineage consisted of only two strains, 2-2-2 and 42-2, with identical ARDRA profiles. Isolates possess sequence similarities of 95.2–96.6% to Antarctic sea-ice bacteria *C. maris* (Yumoto et al. 1998). Both *Colwellia* strains originated from sea ice and are psychrophilic, with an optimal growth temperature at 15°C and lysis at 30°C.

The third novel lineage was formed within the genus *Psychromonas* and contained strains of two different ARDRA profiles. Sea-ice isolate 39-3, which has an identical fingerprint pattern with strain 37-5, and a group of six strains, represented by sea-ice isolate Pull 5.3, possesses sequence similarity of 94.8–96.6% to *P. antarctica* (Mountfort et al. 1998). All Arctic *Psychromonas* isolates are nonpigmented, able to digest lactose, and grow well in a temperature range between 15 and 20°C. Starch-degrading enzymes are produced by five to six isolates.

One large ARDRA group consisting of 17 strains – represented by isolate 3-3 – and five strains with unique ARDRA profiles fall into the *Psychrobacter* genus. Although *Psychrobacter* isolates differ significantly from each other in their 16S rDNA sequences and growth characteristics, all of them clustered with or near

Table 1 Phylogenetic relationships of Arctic sea-ice and seawater isolates. *ARDRA* Amplified rDNA restriction analysis, *ND* not determined, *CFB* Cytophaga – Flexibacter – Bacteroides phylum

Strain[a]	Temperature for maximal growth rate	Maximal growth temperature	Sequence similarity (%)	16S rDNA identification (closest neighbor)[b]	Sampling site	GenBank accession no.	Strains showing identical ARDRA profile
Low-G + C Gram positives							
1-1	25	30	96.9	*Planococcus kocurii*	Wijdefjord	AF513385	
4-2	20	25	97.6	*Planomicrobium okeanokoites*	Wijdefjord	AF513386	
13-3	*ND*	*ND*	99.4	*Carnobacterium funditum*	Bellsund	AF513387	
17-4	*ND*	*ND*	98.8	*C. alterfunditum*	Isfjord	AF513388	14-2-1
11-1	20	25	97.4	*C. alterfunditum*	Wijdefjord	AF513389	17-2-1-2
22-6	20	25	95.6	*C. alterfunditum*	Woodfjord	AF513390	33-1
37-3-1	20	30	99.3	*C. piscicola*	Mushamna	AF513391	
High-G + C Gram positives							
5-1	20	30	95.8	*Agreia bicolorata*	Wijdefjord	AF513392	
37-4	25	30	95.9	*A. bicolorata*	Mushamna	AF513393	
17-2	20	25	96.9	*Arthrobacter nicotianae*	Isfjord	AF513394	
34-1	25	30	97.0	*A. sulfureus*	Mushamna	AF513395	1-4-1
Lact 1	10	20	99.4	*Rhodococcus erythropolis*	Wijdefjord	AF513396	Lact 4
Lact 5.2	25	37	98.1	*Brachybacterium faecium*	Wijdefjord	AF513397	
CFB							
4-3	*ND*	*ND*	91.6	*Gelidibacter algens*	Wijdefjord	AF513398	
13-4	*ND*	*ND*	91.6	*G. algens*	Bellsund	AF513399	
α Proteobacteria							
1-5	25	30	93.6	*Rhodobacter veldkampii*	Wijdefjord	AF513400	
γ Proteobacteria							
42-2	15	25	96.8	*Colwellia maris*	Woodfjord	AF513401	2-2-2
6-2	30	37	94.0	*Marinomonas communis*	Wijdefjord	AF513402	31-1-2, 2-2-1
7-1-2	*ND*	*ND*	99.1	*Pseudoalteromonas elyakovii*	Wijdefjord	AF513403	13 strains[c]
33-3	20	25	99.2	*P. elyakovii*	Woodfjord	AF513404	33-4, 34-3
38-4-2-1	20	25	97.3	*P. citrea*	Woodfjord	AF513405	38-4-2-2
39-2-1	20	25	98.9	*P. citrea*	Woodfjord	AF513406	
31-1-1	25	30	98.9	*P. citrea*	Mushamna	AF513407	37-1-2, 31-2
Pull 1	25	25	98.6	*P. citrea*	Wijdefjord	AF513408	
Amyl 2	25	25	99.7	*P. citrea*	Wijdefjord	AF513409	Amyl 3
Amyl 4	25	25	99.8	*P. elyakovii*	Wijdefjord	AF513410	Amyl 5, Prot 1
Prot 2	25	25	98.2	*P. citrea*	Wijdefjord	AF513411	
Prot 3	25	37	99.1	*P. elyakovii*	Wijdefjord	AF513412	Pect 2
Prot 4	25	30	99.8	*P. citrea*	Wijdefjord	AF513413	
Prot 5	25	37	99.1	*P. elyakovii*	Wijdefjord	AF513414	
Prot 6	25	25	99.2	*P. elyakovii*	Wijdefjord	AF513415	
1-7-1	20	25	98.8	*Psychrobacter glacincola*	Wijdefjord	AF513416	
1-2	25	37	95.8	*P. glacincola*	Wijdefjord	AF513417	
3-3	20	25	97.1	*P. glacincola*	Wijdefjord	AF513418	14 strains[d]
5-3-3	25	30	98.6	*P. glacincola*	Wijdefjord	AF513419	
12-1-3	20	30	96.8	*P. glacincola*	Wijdefjord	AF513420	
33-2	20	30	98.6	*P. glacincola*	Mushamna	AF513421	
39-3	15	25	96.6	*Psychromonas antarctica*	Mushamna	AF513422	37-5
Pull 5.3	20	25	94.8	*P. antarctica*	Wijdefjord	AF374385	5 strains[e]
4-1	*ND*	*ND*	98.0	*Shewanella frigidimarina*	Wijdefjord	AF513423	
12-1-2	25	25	98.0	*S. frigidimarina*	Wijdefjord	AF513424	15 strains[f]
14-1	20	25	98.7	*S. frigidimarina*	Bellsund	AF513425	
22-1	25	30	97.3	*S. putrefaciens*	Woodfjord	AF513426	
31-4	30	30	97.5	*S. putrefaciens*	Mushamna	AF513427	12-3
40-3	30	30	96.1	*S. putrefaciens*	Mushamna	AF513428	
40-2	20	25	98.1	*Pseudomonas fluorescens*	Mushamna	AF513429	
Pect 4	25	37	99.0	*P. fluorescens*	Wijdefjord	AF513430	
Pect 5	25	37	98.6	*P. fluorescens*	Wijdefjord	AF513431	
Lip 2	25	30	99.3	*P. fluorescens*	Wijdefjord	AF513432	
Lip 4	25	30	99.3	*P. fluorescens*	Wijdefjord	AF513433	

[a]Strains in *boldface* were isolated from sea ice

[b]The most closely related validly described species, in terms of 16S rDNA sequence, to a corresponding Arctic isolate are given

[c]Strains with identical ARDRA profile: **7-1-1**, **7-1-2**, 22-4-2, 22-5, **30-1-1**, **30-2-2**, **31-3**, **32-1**, **32-3**, 33-5, 34-2, **35-1**, **35-2**, and **41-1-2**

[d]Strains with identical ARDRA profile: **1-7-2**, **2-1**, 3-3, 4-4, **6-3**, 11-2-2, 14-2-2, 14-3, 17-2-1-1, 22-2, 34-4, **37-2**, **37-3-2**, **38-3-3**, and **41-3-2**

[e]Strains with identical ARDRA profile: **Pull 5.3**, **Pull 6.3**, **Pull 6.5**, Pull 7.2, Pull 15.3, Pull 16.2

[f]Strains with identical ARDRA profile: **1-3**, 5-3-1, 11-3, 12-1-2, 12-2, 13-2-1, 13-2-2, 14-4-2, 17-1, 17-3-2, 17-5, 17-6, 22-4-1, **37-6**, **41-3-1**, and **41-2**

Table 2 Hydrolytic activities of Arctic sea-ice and seawater isolates. Substrate hydrolysis was determined after cultivation of isolated bacteria for 2–5 days at 4°C on agar plates supplemented with dyed substrates. *AZCL* Azurine cross-linked, ++ strong positive, + positive, – negative

Strain[a]	Genus of the closest phylogenetic relative	AZCL arab-inan	AZCL arabin-oxylan	AZCL casein	AZCL HE cellulose	AZCL curdlan	AZCL amylose	AZCL dextran	AZCL galactan (lupin)	AZCL galactan (potato)	AZCL galacto-mannan	AZCL Beta-glucan	AZCL pullulan	AZCL xylan	AZCL Xylan-birch-wood	AZCL xylo-glucan	Tween 80	Polyga lactu-ronic acid	Alginate	Chitin	Red amylo-pectin	Red pullulan	X-gal	Skim milk	Olive oil + rhod-amin B
Low G + C Gram positives																									
1-1	*Planococcus*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
4-2	*Planomicrobium*	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
13-3	*Carnobacterium*	++	++	–	++	–	+	–	–	–	++	+	–	+	–	–	+	–	–	–	+	–	++	–	++
14-2-1	*Carnobacterium*	++	++	–	++	–	–	–	–	–	++	–	–	++	–	–	–	–	–	–	+	–	++	–	++
High G + C Gram positives																									
37-4	*Agreia*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++
1-4-1	*Arthrobacter*	–	–	++	–	–	++	–	–	–	–	–	++	–	–	–	–	+	+	–	++	++	–	+	–
17-2	*Arthrobacter*	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
34-1	*Arthrobacter*	–	–	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–	+	–	+	+	–	+	–
Lact 5.2	*Brachybacterium*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	–	+	–
CFB																									
4-3	*Gelidibacter*	++	++	–	+	–	–	–	–	–	++	++	–	++	–	–	+	–	–	–	–	–	+	+	++
γ Proteobacteria																									
2-2-1	*Marinomonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	++	–	–	–	–	–	+
6-2	*Marinomonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+
31-1-2	*Maronomonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Pull 1	*Pseudoalteromonas*	–	+	–	+	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	+	++	++	+	–
Pect 2	*Pseudoalteromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Amyl 2	*Pseudoalteromonas*	–	+	+	+	–	+	–	–	–	–	+	–	–	+	–	–	–	–	–	++	–	++	++	–
Amyl 3	*Pseudoalteromonas*	–	+	+	+	–	+	–	–	–	–	+	–	–	–	–	–	+	–	–	++	–	++	++	–
Amyl 4	*Pseudoalteromonas*	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	+	++	–
Amyl 5	*Pseudoalteromonas*	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	+	++	–
Prot 1	*Pseudoalteromonas*	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	++	–
Prot 2	*Pseudoalteromonas*	–	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	+	–
Prot 3	*Pseudoalteromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
Prot 4	*Pseudoalteromonas*	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	++	–
Prot 5	*Pseudoalteromonas*	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Prot 6	*Pseudoalteromonas*	–	+	+	–	–	+	–	–	–	–	+	–	+	+	–	–	–	–	–	+	–	++	++	–
7-1-1	*Pseudoalteromonas*	–	–	––	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
7-1-2	*Pseudoalteromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
22-4-2	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
22-5	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–	–	+	–
30-1-1	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	++	–	–	–	–	+	–
30-2-2	*Pseudoaletromonas*	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
31-3	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–	–	–	–	+	–
31-1-1	*Pseudoalteromonas*	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
31-2	*Pseudoalteromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
32-1	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	–	+	–
32-3	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
33-3	*Pseudoalteromonas*	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
33-4	*Pseudoalteromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–	–	–	+	–	–
33-5	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	+	–
34-3	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
34-2	*Pseudoaletromonas*	–	–	++	–	–	++	–	–	–	–	–	++	–	–	–	+	–	–	–	++	++	–	+	–
35-1	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	++	++	–	+	–	–	+	–
35-2	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–
37-1-2	*Pseudoaletromonas*	–	–	+	++	–	–	–	–	–	–	++	++	–	–	–	+	–	–	–	++	++	++	+	–
38-4-2-1	*Pseudoalteromonas*	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
39-2-1	*Pseudoalteromonas*	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
41-1-2	*Pseudoaletromonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–
Pect 4	*Pseudomonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Pect 5	*Pseudomonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Lip 2	*Pseudomonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	++
Lip 4	*Pseudomonas*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++

1-7-2	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	−	−	−	−	−	+	+
1-7-1	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
2-1	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++
4-4	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+
5-3-3	*Psychrobacter*	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
6-3	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
11-2-2	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	++
12-1-3	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
14-2-2	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+
14-3	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	++
17-2-1-1	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	++
22-2	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
34-4	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++
33-2	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
37-2	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	+	−	−	−	−	−	++
37-3-2	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	+	−	−	−	−	−	+
38-3-3	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	+	++	−	−	−	−	−	+
41-3-2	*Psychrobacter*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
37-5	*Psychromonas*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	−	+	−	++	−	−
39-3	*Psychromonas*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Pull 5.3	*Psychromonas*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	++	++	−	−
Pull 6.3	*Psychromonas*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++	+	−	−
Pull 6.5	*Psychromonas*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−
Pull 7.2	*Psychromonas*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++	+	−	−
Lact 4	*Rhodococcus*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Lact 1	*Rhodococcus*	−	−	−	−	−	−	−	−	−	−	+	−		−	−	−	−	−	−	−	−	−	−	−
1-3	*Shewanella*	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
4-1	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++	−	−	−	−	+	+
5-3-1	*Shewanella*	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	+	++
11-3	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++
12-1-2	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
12-2	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−
12-3	*Shewanella*	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	+	+
13-2-1	*Shewanella*	−	−	++	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	++	−	+	+
13-2-2	*Shewanella*	−	−	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	++	−	+	+
14-1	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
14-4-2	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++
17-1	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
17-5	*Shewanella*	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	++	+	−	+	+
17-6	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	++	+	−	+	+
22-4-1	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	++
22-1	*Shewanella*	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
31-4	*Shewanella*	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	+	−
37-6	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
41-3-1	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	+	−
40-3	*Shewanella*	+		+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	−	+	−
41-2	*Shewanella*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	+	−

[a]Strains in *boldface* were isolated from sea ice. The following strains were not included in the table:**1-2**, **1-5**, **2-2-2**, 3-3, 5-1, **11-1**, 13-4, 17-2-1-2, 17-3-2, 17-4, 22-6, 33-1, **37-3-1**, **38-4-2-2**, 40-2 (no hydrolytic activity), 42-2, Pull 15.3, and Pull 16.2 (not tested)

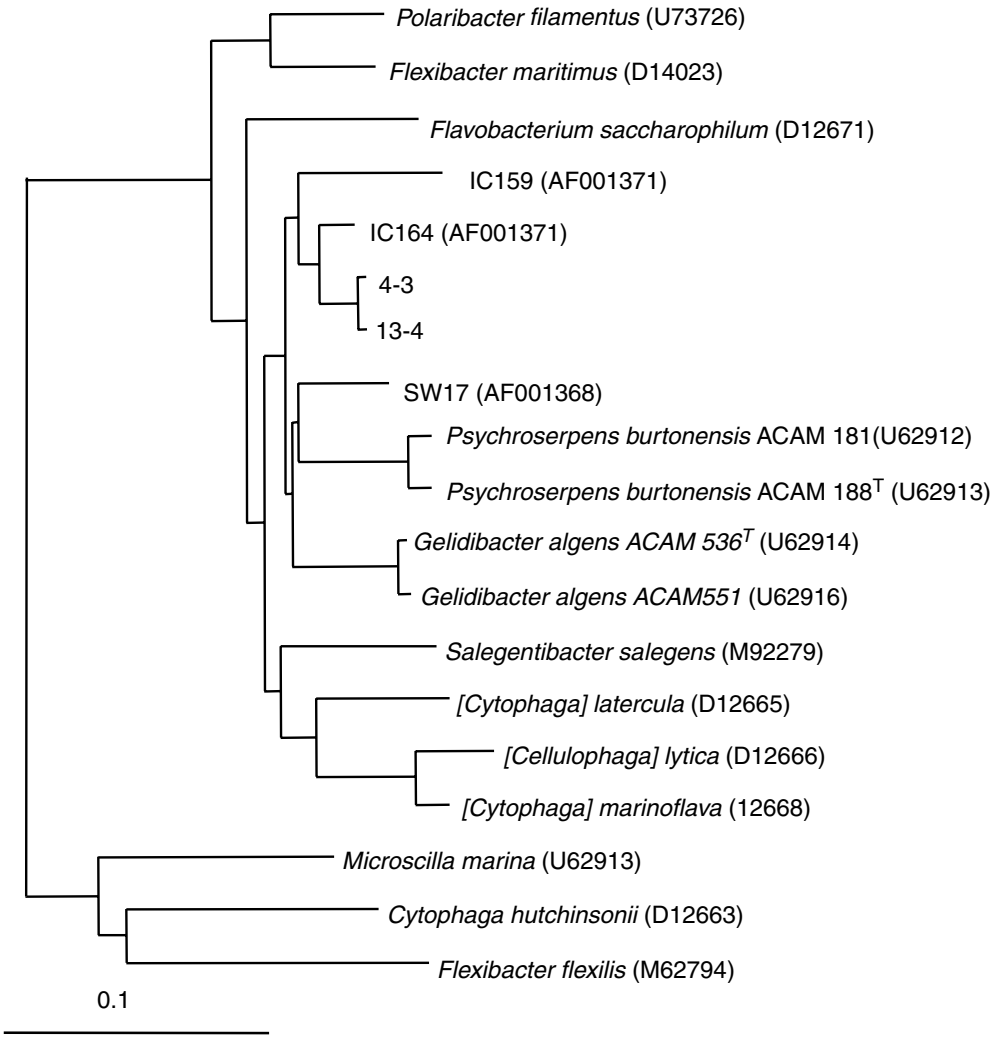

Fig. 1 Phylogenetic tree showing the affiliation of the isolated strains with selected sequences of the α and γ subclasses of Proteobacteria. Strains in *boldface* were isolated from sea ice. The tree is based on 850 bp (region 400–1200) and was calculated by neighbor-joining analysis. *Bacillus subtilis* (AF234850) was used as an outgroup (not shown). The *bar* represents 10% estimated sequence divergence

P. glacincola. Maximal growth temperature of *Psychrobacter* strains varies from 25–37°C, with optimal growth at 20–25°C. Over 80% of *Psychrobacter* isolates are lipolytic and 30% are able to hydrolyze skim milk.

Isolates falling into genus *Shewanella* formed one multistrain group and several ungrouped strains on the basis of ARDRA fingerprinting. All *Shewanella* isolates are tan-pigmented, psychrotolarant, and clustered near *S. putrefaciens* and *S. frigidimarina* species. Among a broad variety of enzymes produced by *Shewanella* isolates, protease (77% of isolates) and lipase (50% of isolates) were dominating. Over 30% of the strains are able to hydrolyze alginate, starch, and polygalacturonic acid.

Other sea-ice and seawater isolates of the γ subdivision were identified on the basis of high 16S rDNA similarity values. They belong to already known species, including members of *Pseudomonas* and *Pseudoalteromonas* genera. *Pseudoalteromonas* strains were isolated from virtually all seawater and sea-ice samples. They are nonpigmented and psychrotolerant. Protease and β-galactosidase producers accounted 99 and 40% of *Pseudoalteromonas* isolates, respectively. Strain 39-2-1, which represents a large ARDRA group, and several other strains with unique fingerprinting profiles possess 16S rDNA sequences that are 98.2–99.0% similar to that of *P. citera*. The remaining *Pseudoalteromonas* strains clustered with *P. elyakovii* and *P. distincta*. *Pseudomonas* isolates were isolated from algae-rich sea-ice (Lip 2, Lip 4) and seawater samples (40-2, Pect 4, Pect 5). All *Pseudomonas* strains demonstrated different ARDRA profiles and are most closely related to *P. fluorescens* with sequence similarities of 98.6–99.3%.

CFB

Two marine strains, 4-3 and 13-4, isolated from Wijdefjord and Bellsund, respectively, were found to belong to the CFB phylum (Fig. 2). Both strains form an isolated branch with yet unnamed strains IC 159 and IC 164, isolated from Antarctic sea ice and seawater (Bowman et al. 1997a, b), in the family Flavobacteriaceae. *Gelidibacter algens*, the closest validated relative,

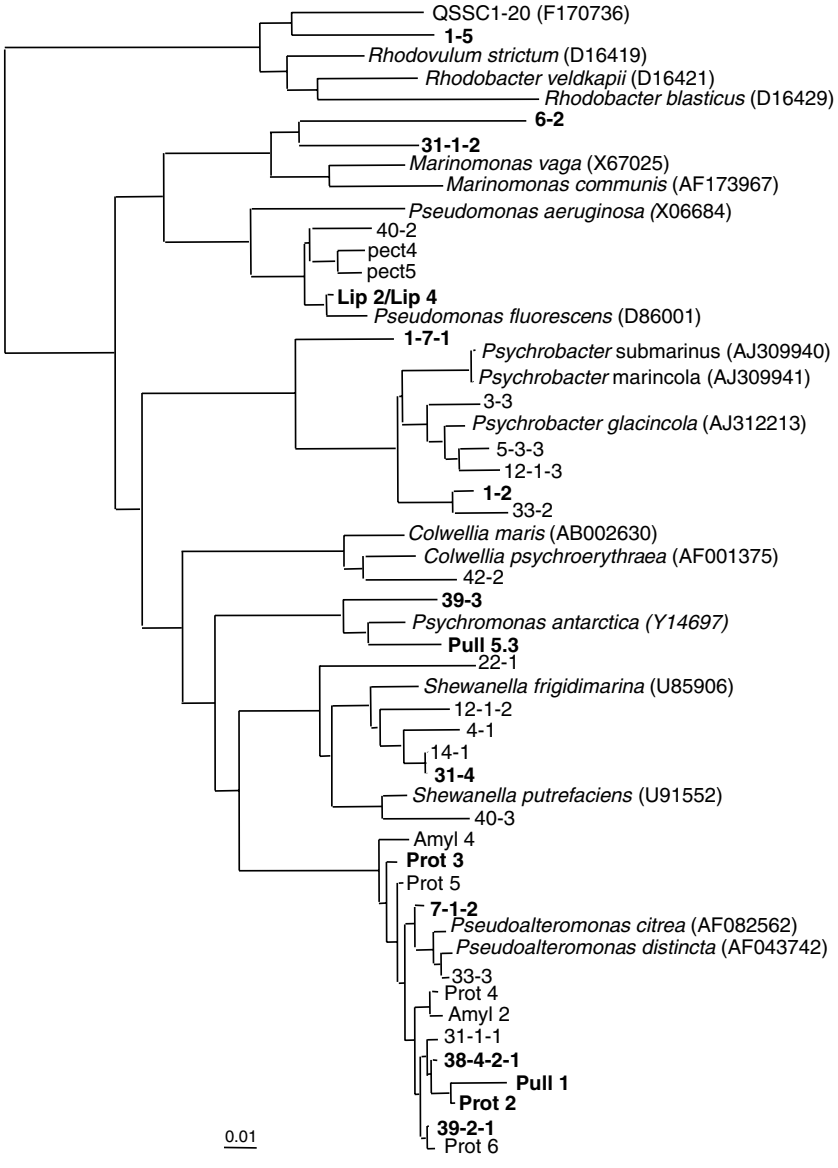

Fig. 2 Phylogenetic tree showing the affiliation of the isolated strains with selected sequences of the Cytophaga–Flexibacter–Bacteroides group. The tree is based on almost complete 16S rDNA sequences (1,400 bp) and was calculated by neighbor-joining analysis. *Escherichia coli* (AB035923) was used as an outgroup (not shown). The *bar* represents 1% estimated sequence divergence

isolated from Antarctic ice core, shares 91.6% similarity at the 16S rDNA level (Table 1). Despite high sequence similarity to each other at the 16S rDNA level (98.5%), strains 4-3 and 13-4 differ significantly in their enzyme profile. Whereas 4-3 produces a variety of hydrolytic enzymes, no enzymatic activity was detected for 13-4 (Table 2).

Gram-positive branch

Within the low G + C Gram-positive branch, 16S rDNA sequences of the representative isolates fell into two distinct phylogenetic clades (Fig. 3). Wijdefjord sea-ice strain 1-1 and seawater strain 4-2 were found to cluster with genera *Planomicrobium* and *Planococcus*, showing 96.0 and 97.0% similarities on the 16S rDNA level, respectively. In addition to their ability to hydrolyze proteins, the former strain *Planomicrobium* also hydrolyzes galactan and lactose. Strains of the second cluster in low G + C Gram-positive group fall into the *Carnobacterium* genus and are most closely related to *C. funditum*, *C. alterfunditum*, and *C. piscicola*. From all *Carnobacterium* isolates, only two strains (13-3 and 14-2-1) are able to produce various hydrolytic enzymes. Lipase, β-galactosidase, α-amylase, cellulase, and xylanase were the common enzymes produced by both strains.

Strains of six different ARDRA profiles were found to belong to the order Actinomycetales, which is part of the high G + C Gram-positive branch (Fig. 3). Seawater strains 5-1 and sea-ice strain 37-4, with unique ARDRA profiles, formed an isolated lineage in family Microbacteriaceae and were moderately related to *Agreia bicolorata*, with a sequence similarity of 95.5–95.9%. Among a variety of tested substrates, only olive oil is degraded by isolate 37-4. The yellow-pigmented strains

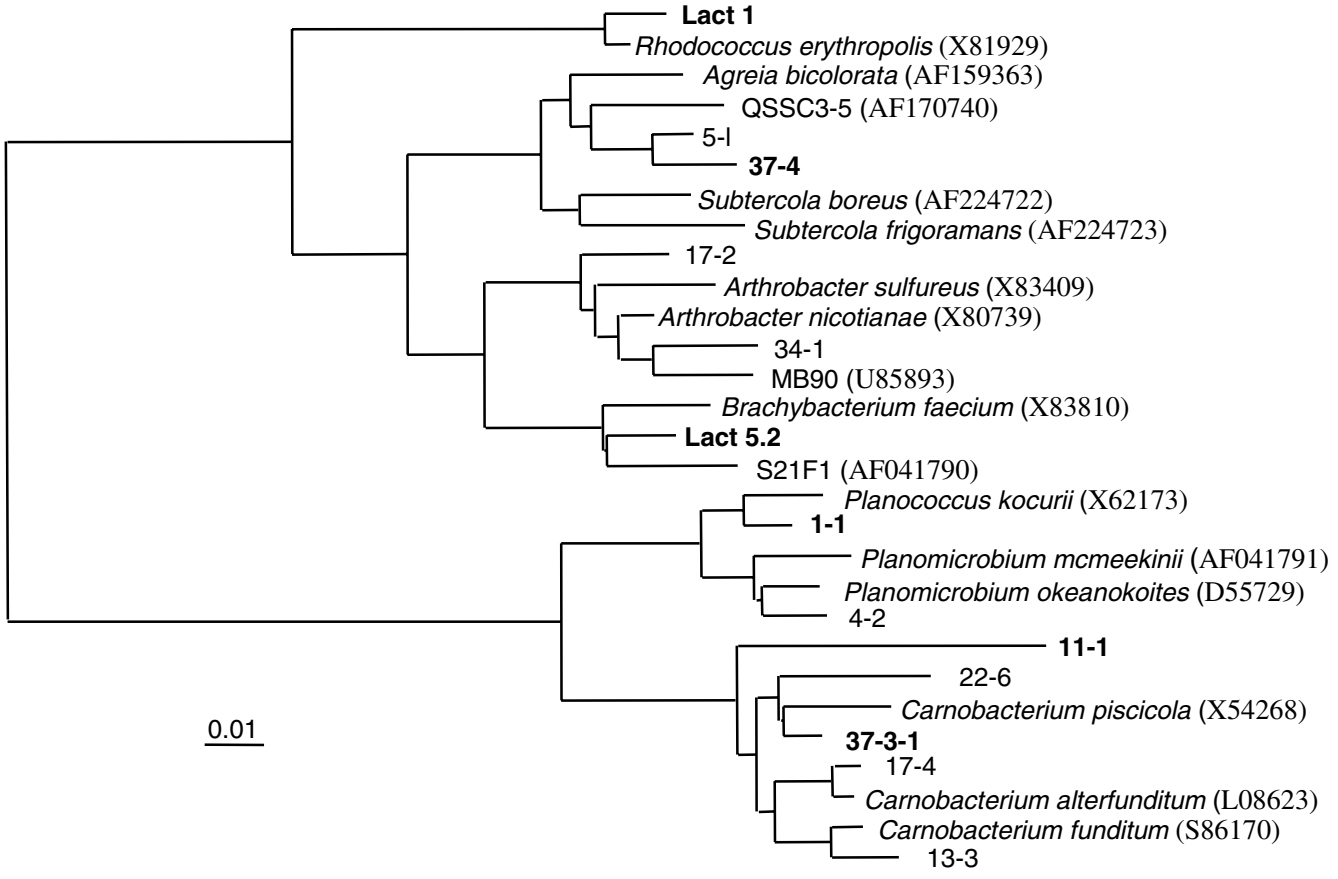

Fig. 3 Phylogenetic tree showing the affiliation of the Gram-positive isolates with selected sequences. Strains in *boldface* were isolated from sea ice. The tree based on 850 bp (positions 400–1250, according to *E. coli* numbering) and was calculated by neighbor-joining analysis. *E. coli* (AB035923) was used as an outgroup (not shown). The *bar* represents 1% estimated sequence divergence

(17-2, 1-4-1 and 34-1) of two different ARDRA profiles fall into the genus *Arthrobacter*. They cluster near *A. nicotianae* and *A. sulfureus* species, sharing 96.9 and 97.0% sequence similarities, respectively. Whereas all *Arthrobacter* isolates are able to produce protease, only two strains (1-4-1, 34-1) were also able to degrade starch and alginate.

Two nonpigmented strains, Lact 1 and Lact 4, fall into the genus *Rhodococcus* and possess almost identical 16S rDNA sequences to *R. erythropolis*. Lact 1 differs from Lact 4 by its ability to degrade starch and an extremely low optimal growth temperature of 10°C. The sea-ice isolate Lact 5.2, with unique ARDRA profile, fell into the genus *Brachybacterium*, sharing 98.1% of 16S rDNA sequence similarity to *B. faecium*. From the variety of polysaccharides tested, Lact 5.2 is able to degrade proteins and starch.

Growth patterns of the Arctic isolates

The majority of the strains, which were tested for their optimum growth temperature (a total of 47 isolates), were psychrotolerant with an optimal growth temperature between 20 and 25°C (Table 1). Active growth at 4°C and cell lysis at 30°C confirmed their psychrotolerant nature. Only three isolates (Lact 1, 42-2, 39-3) are psychrophilic showing an optimal growth at 10–15°C and no growth at 20–25°C.

Two different growth patterns were observed among the isolates at a maximal growth temperature of 30°C. Slow growth and low final cell yield at 30°C were characteristic for *Psychrobacter* strains. Most of the isolates, on the other hand, demonstrated relatively high growth rates at maximal growth temperatures, which were comparable to those at optimal growth temperature. However, rapid growth was followed by a dramatic decrease in cell density after a few hours and complete cell lysis after 10–20 h of cultivation.

Screening for cold-active enzymes

Skim milk, casein, olive oil, amylopectin, and pullulan were the most preferred substrates (Table 2). Isolates that are able to degrade proteins, lipids, and starch accounted for, respectively, up to 56, 31, and 21% of sea-ice and seawater isolates. Skim milk appeared to be the more favorable substrate for protease producers than AZCL casein. Other biopolymers, including alginate and polygalacturonic acid, were hydrolyzed by about 18% of the isolates. More than 10% of Arctic isolates

were able to digest lactose and Tween 80. No hydrolysis was observed on curdlan, dextran, galactan, xyloglycan, or chitin.

The broad screening investigations indicate that the production of certain enzymes is not restricted to a single group of the isolates. Whereas *Pseudoalteromonas* and *Shewanella* isolates make up a significant fraction of protease producers, accounting for 51 and 25% of the protolytic strains, *Psychrobacter* and *Shewanella* isolates dominated among lipolytic strains, composing 45 and 30% of lipase producers, respectively. The ability to degrade starch (amylopectin, pullulan) was almost equally distributed among the representatives of γ Proteobacteria and the Gram-positive branch. Polygalacturonic acid and alginate were almost exclusively hydrolyzed by the members of γ Proteobacteria (*Pseudoalteromonas*, *Psychrobacter*, *Shewanella*, *Marinomonas*, *Pseudomonas*).

The extracellular α-amylase from Lact 5.2 and the intracellular β-galactosidase from strain Pull 5.3 were selected for further characterization. The production of the enzymes tested was maximal at the cultivation temperatures from 0–10°C, and almost no production was detected at higher cultivation temperatures (20/30°C). In vitro, the enzymes were active over a broad temperature range of −5 to 60°C, with a maximal hydrolytic activity at 30°C. Interestingly, the temperature profiles of the tested enzymes were independent of the optimal growth temperature of the microorganisms. Optimal α-amylase and β-galactosidase activities were detected in vitro at 30/35°C, which is well above the upper growth limit (20/25°C) of the enzyme-producing strains. Surprisingly, both enzymes showed catalytic activity even at −5°C, and more than 20% of relative activity was still detected at 0°C (Fig. 4).

Discussion

The overall phylogenetic distribution of the strains isolated in this study shows similarity to the results obtained by analysis of the Arctic sea-ice/seawater 16S rDNA clone libraries (Bano and Hollibaugh 2002; Brown and Bowman 2001). However, the phylogenetic affiliation of the Arctic strains isolated in this work is slightly different from that of Arctic clone sequences.

Comparison of seawater isolates with Arctic bacterioplankton clone sequences (Bano and Hollibaugh 2002) indicates a moderate relationship. Similarity values ranging from 89.9–99.0% were observed between Arctic seawater clones and our isolates within α and γ Proteobacteria and the CFB group. In some of the cases, similarities to Arctic clone sequences exceed that of any validly described microorganisms (e.g., isolate 4-3/clone 96AD-4, isolate 38-4-2-1/clone 96AD-16). A similar tendency was observed by comparison of sea-ice isolates with Arctic sea-ice clones, though analysis was restricted to a limited number of clone sequences (13 clone sequences are available from a single sample). A direct comparison of Gram positives from Arctic sea-ice/seawater libraries and this study was not possible due to the lack of sequence information. Nevertheless, isolation of members of the *Bacillus–Clostridium* group and the order Actinomycetales from Arctic sea ice does not seem surprising, since Gram positives were successfully isolated previously from Antarctic sea-ice environments (Bowman et al. 1997a, b).

Interestingly, several strains obtained in this study were more closely related phylogenetically to Antarctic sea-ice isolates (Bowman et al. 1997a, b) than to those isolated from the Arctic (Junge et al. 2002). For example, isolate 4-3 from the Arctic was more closely related to Antarctic strain SW 17 (96.1%) than to strain AWS-4U1 (95.6%) from the Arctic. In addition, some of our isolates were also related to strains from terrestrial Antarctic environments (Smith et al. 2000).

Studies to date indicate that similar representatives of few genera (*Octadecabacter*, *Polaribacter*, "*Iceobacter*") occur at both poles; however, cosmopolitan species have not been discovered yet (Brown and Bowman 2001; Staley and Gosink 1999). Our 16S rDNA analysis of the isolated strains confirmed Staley and Gosink's (1999) opinion that genera, but not species, would most likely exhibit a cosmopolitan distribution and be found at both poles. In agreement with 16S rDNA clone data

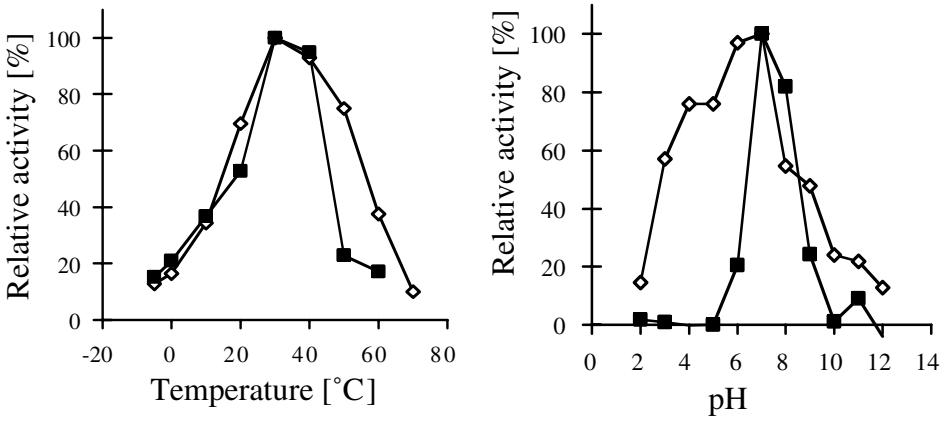

Fig. 4 Effect of temperature and pH on the activity of β-galactosidase and α-amylase produced by psychrotolerant strains Pull 5.3 and Lact 5.2. *Open diamond* β-Galactosidase produced by Pull 5.3, *filled square* amylase produced by Lact 5.2. To determine the temperature optimum, 50 µl of enzyme (crude-cell extract or supernatant) was incubated in 50 mM sodium phosphate buffer, pH 7.0, for 30 min. To determine the pH optimum, the enzyme was incubated at 25°C for 30 min

(Brown and Bowman 2001), cultivation results revealed similarities in diversity of sea-ice microbial communities at both poles. However, phylogenetic affiliation of Arctic/Antarctic isolates was slightly different, and none of the Arctic strains shared identical 16S rDNA sequences with strains that have been previously isolated from Antarctic.

Since cultivation-based biodiversity surveys reveal community members that are readily cultured (Eilers et al. 2000; Giuliano et al. 1999; Schafer et al. 2000), the prevalence of *Pseudoalteromonas*, *Shewanella*, and *Psychrobacter* isolates most probably reflect their ability to successfully compete under heterotrophic conditions. Although other bacterial groups (including the CFB group, high- and low-G+C Gram positives, and α Proteobacteria) are recognized to be common in sea ice or brine (Bowman et al. 1997a, 1997b), some strains isolated in this study could have terrestrial/sediment origin and have been transported into the ocean during the summer glacial melt.

A large number of the strains demonstrated good growth in a wide temperature range of 4–20°C. This can be considered as an advantage in survival strategy in low-temperature biotopes with changing conditions. According to a commonly accepted definition for cold-adapted bacteria (Morita 1975), most Arctic isolates are not strict psychrophiles but psychrotolerant microorganisms. Other studies have also shown that psychrotolerant bacteria numerically dominate in seawater, with psychrophilic bacteria being rarely isolated (Bowman et al. 1997; Helmke and Weyland 1995). The generation time of the newly isolated strains, which ranges from 0.07–0.75 h^{-1}, is comparable to that of other psychrophilic/psychrotolerant and mesophilic microorganisms (Feller and Gerday 1997).

The ability of the newly isolated psychrotolerant strains to produce a broad spectrum of cold-active enzymes is of great interest for both fundamental research and industrial applications. The high number of protease-producing bacteria among the isolated strains (Table 2) could be related to the natural habitat from which the microorganisms were isolated. Many strains were isolated from alga-rich seawater samples or ice-alga assemblages where diatoms are known to dominate (Palmisano and Garrison 1993). Most of the organic nitrogen in the diatom assembly is primarily produced in the form of high-molecular-weight organic material, which cannot be directly assimilated by bacteria. Hydrolysis of these macromolecules through the action of extracellular enzymes is a first and often limiting step of organic nitrogen utilization by microorganisms (Hoppe 1991). The ability of many isolates to grow on polysaccharides with α-1-4 linkages and utilize lipids suggests that they play a primary role in the degradation of phytoplankton detritus and colonization of diatom ice assemblages. A vast variety of cold-active biopolymer-degrading enzymes detected in this study indicate that isolated microorganisms can contribute significantly to the hydrolysis of the major organic constituents (α- and β-linked polysaccharides, proteins, esters) in sea ice and underlying seawater.

Temperature has been identified as a factor controlling secretion of extracellular enzymes and their activity in psychrophilic bacteria (Barbaro et al. 2001; Buchon et al. 2000; Huston et al. 2000). In agreement with previously reported data, maximal production of the enzymes was observed at temperature close to their natural environment (0–4°C), which is well below the optimal growth temperature of the isolates (20–25°C). This thermoregulation effect is known to involve alterations in the secretory pathway in the upper range of temperatures and losses due to the thermoinactivation of the enzyme (Feller et al. 1992; Gugi et al. 1991). Similar to the results reported for proteolytic strains isolated from Alpine habitats and Antarctic sediments, optimal temperature of the hydrolytic enzymes did not correlate with the optimal growth temperature of Arctic isolates, but was always above maximal growth temperature of enzyme producer (Reichardt 1988; Schinner et al. 1992). It is supposed that reduced activity of enzymes at the environmental temperature had to be compensated by an increase in production and specificity of the enzymes to enhance biopolymer degradation.

Cold-active enzymes including proteases, lipases, cellulase, and α-amylases produced by Arctic strains may find applications in various industries (food, detergents, etc.). The possible applications of enzymes, which are active at low temperature (4°C), are numerous in the food industry and offer many advantages, e.g., risk of contamination is reduced, and flavor is not destroyed at elevated temperatures. In the dairy industry, cold-adapted β-galactosidase will reduce the lactose content of milk at low temperatures. Diverse starch-modifying enzymes, xylanases, and proteases detected in this study can be used to reduce dough fermentation time and improve the properties of dough in bakeries. In addition, cold-adapted lipases are of considerable interest as flavor-modifying enzymes in the production of fermented food, cheese manufacture, beer treatment, and biotransformation reactions in fine chemical processes.

The data obtained in this study on the biodiversity of culturable bacteria from Arctic sea ice and seawater expand our knowledge on the extent of bacterial diversity in the psychrophilic realm and improve our understanding of the biogeographic distribution of prokaryotes in the polar regions.

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