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Cultivation and biochemical characterization of heterotrophic bacteria associated with phytoplankton bloom in the Amundsen sea polynya, Antarctica

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ABSTRACT

Polynyas are a key ecosystem for carbon cycling in the Antarctic Ocean due to the intensive primary production. Most of the knowledge regarding the bacterioplankton community in the Antarctic Ocean that is responsible for re-mineralization of fixed carbon comes from metagenomic analyses. Here, the extinction-dilution method was used to obtain representative heterotrophs from a polynya in the Amundsen Sea, Antarctica, and their biochemical potential for carbon re-mineralization were assessed. All 23 strains have close relatives belonging to type strains within the following genera (number of strains; % 16S rRNA gene sequence similarity): *Bizionia* (4; >97.8%), *Leeuwenhoekiella* (1; 96.2%), *Pseudoalteromonas* (14; >98.5%), *Pseudomonas* (1; 99.4%) and *Sulfitobacter* (3; 100%), which were also observed in 454 pyrosequencing-based analysis of 16S rRNA gene sequences of the polynya. Although sequence reads related to *Polaribacter* were the most common, *Polaribacter* strains could only be obtained from colonies cultured on agar plates. The strain of *Leeuwenhoekiella* showed a prominent ability in hydrolyzing diverse esters, amides, and glycosides while the strains of *Pseudoalteromonas, Polaribacter,* and *Bizionia* showed extracellular enzyme activities only on a narrow range of amides. The strains of *Leeuwenhoekiella*, *Pseudoalteromonas*, and *Sulfitobacter* utilized various labile carbon sources: carbohydrates, organic acids, amino acids, and peptides. The most frequent isolates, strains of *Pseudoaltermonas*, showed marked differences in terms of their potential to utilize different types of labile carbon sources, which may reflect high genomic diversity. The strains of *Bizionia* and *Pseudomonas* did not utilize carbohydrates. Unique biochemical properties associated with extracellular hydrolase activities and labile carbon utilization were revealed for dominant culturable heterotrophs which gives insights into their roles in active re-mineralization of fixed carbons in polynya.

1. Introduction

Polynyas are areas of open sea surrounded by sea ice. These small oceans recur annually in many regions of the Antarctic Ocean (Barber and Massom, 2007). Polynyas are opened early during austral summer and closed later than the surrounding oceans. The mechanisms underlying the recurrent development of polynyas in specific areas are unclear, although winds and currents are thought to be contributing factors (Jenkins et al., 2010).

Nutrients that accumulate under the sea ice during austral winter become available for phytoplankton growth as melting sea ice exposes the ocean to sunlight. The phytoplankton in polynyas feed on these nutrients while the surrounding oceans remain dark, which contributes to their early and intensive growth. Thus, Antarctic polynyas are some of the most productive oceans on Earth (Arrigo and van Dijken, 2003).

Biogeochemical carbon cycles in the Antarctic Ocean are important because they affect the overall rate of global climate change and vice versa. In fact, the fate of the enormous amount of organic carbon (which is fixed by intensive primary production) needs to be appreciated if we are to understand the carbon pump in the Antarctic Ocean. Recently, it was suggested that large fractions of fixed carbon might be re-mineralized by bacterioplankton before they descend to

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the deep ocean interior (Williams et al., 2013). Thus, the contribution of fixed carbon to overall carbon sequestration in the Antarctic Ocean might be lower than expected during austral summer, despite intensive primary production.

Due to logistic constraints, studies on the microbial populations involved in carbon re-mineralization in polar oceans are limited. Cultivation-independent molecular techniques have been used in an attempt to examine the composition of bacterial communities in the Antarctic Ocean (Kim et al., 2013; Murray et al., 2011; Straza et al., 2010). Recently, meta-omics (metagenomics, metaproteomics and metatranscriptomics) studies have provided new insight into the functional roles of heterotrophic bacteria associated with austral phytoplankton blooms (Grzymski et al., 2012; Wilkins et al., 2013; Williams et al., 2013). Bacterial populations in the Antarctic Ocean (as well as in other oceans) are typically dominated by three bacterial clades: *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* (Abell and Bowman, 2005; Kim et al., 2013; Wilkins et al., 2013). It is assumed that members of the *Bacteroidetes* clade (particularly *Flavobacteria*) break down complex organic matters of phytoplankton cells and phytoplankton-derived detrital particles in the ecosystems of phytoplankton blooms by using extracellular enzymes (Cottrell and Kirchman, 2000; Gómez-Pereira et al., 2012; Teeling et al., 2012). Thus, the *Flavobacteria* abundance correlates with the concentration of chlorophyll-*a* (Williams et al., 2013). *Alphaproteobacteria* and *Gammaproteobacteria* are dominant in the Antarctic Ocean and harbor the genetic potential to utilize labile (small) organic substrates (Ivars-Martinez et al., 2008; Wilkins et al., 2013; Williams et al., 2013). However, few studies have attempted to identify the ecophysiological and biochemical properties of psychrophilic heterotrophs obtained from polynyas in the Antarctic Ocean, despite their important roles in carbon re-mineralization. Further studies should aim to characterize representative strains related to the clades commonly identified by cultivation-independent methods. Here, we attempted to isolate abundant psychrophilic heterotrophic bacteria from a polynya and examine their potential for their carbon re-mineralization with a view to gaining a better understanding of the biogeochemical carbon cycle in the highly productive Antarctic Ocean.

2. Materials and methods

2.1. Collection of samples and chlorophyll-a data

A sea water sample was collected from a polynya center station (112° 00′ W, 73° 30′ S) (Fig. 1A) in the Amundsen Sea, Antarctica, at the peak of a phytoplankton bloom (8 January, 2014). The peak of the bloom was estimated from satellite observation of chlorophyll-*a* levels (Fig. 1B). Moderate-Resolution Imaging Spectroradiometer Aqua-derived chlorophyll-*a* data were obtained from the Goddard Space Flight Center of NASA (http://oceandata.sci.gsfc.nasa.gov/) using Level 3 Standard Mapped Image data. The Korean icebreaker, Araon, (part of the Amundsen Sea project (2013/2014)) collected the sample at a depth of 10 m using a 10 l Niskin bottle mounted on a CTD rosette. The sample was kept in the dark and transported on ice to the laboratory, where psychrophilic heterotrophic bacteria were isolated. For extraction of metagenomic DNA, an aliquot of sea water sample (10 l) was immediately filtered through a 1.2 μm filter to eliminate particles and eukaryotes, and then again through a 0.22 μm pore-size filter to capture bacterial cells. The filter was then stored at −80 °C.

2.2. Microbial community analysis based on 454 pyrosequencing of 16S rRNA gene

Frozen filters were ground with sand beads to lyse the bacterial cells and metagenomic DNA was then extracted using phenol: chloroform (Massana et al., 1997). Preliminary measurements of DNA concentration were made using a spectrophotometer (Nanodrop Technologies, USA). PCR amplification of 16S rRNA genes was performed using the bar-coded primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAGT-3′) (Kim et al., 2013) and the following PCR conditions: 95 °C for 5 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, with a final extension for 5 min at 72 °C (Kim et al., 2013). 454 pyrosequencing was conducted using Roche GS FLX software (v 3.0) (Macrogen, Republic of Korea) and the data were analyzed as previously described (Kim et al., 2013). Briefly, both the proximal and distal primers were trimmed from the raw reads. Short reads (< 300 base pair (bp)) and reads longer than the expected PCR product size were removed. Chimeric sequences that were unassigned and/or related to non-bacterial sources (e.g., chloroplasts and mitochondria) were also removed to increase the quality of the analysis. A 5% dissimilarity level between sequences was used to define a genus. Sequence reads were compared with those in a reference database containing known bacterial 16S rRNA genes (Ribosomal Database Project (RDP)) and then assigned taxonomically based on the RDP classifiers (Cole et al., 2007).

2.3. Isolation of psychrophilic heterotrophic bacteria

Three different liquid media were used to increase the recovery of heterotrophic bacteria: Marine broth (MB; Difco, France), R2A (MBcell, Republic of Korea) and Sea Water Complete (SWC) medium (750 ml of sea water, 0.2 g of sodium acetate, 0.2 g of beef extract, 0.5 g of tryptone, 0.5 g of yeast extract and 250 ml of distilled water) (Irgens et al., 1989). All media were sterilized by autoclaving at 120 °C for 20 min. Single strains were isolated using the extinction-dilution method. The sea water sample was serially diluted 10-fold (from 10^{-1} to 10^{-9}) and 20 μl of dilution was inoculated into 96-well plates containing 180 μl medium in each well. For each medium one 96-well plate was used for each dilution. The inoculated plates were incubated at 10 °C to isolate psychrophiles. Single cells were isolated from the last plate showing turbidity in at least one well. Positive wells with turbidity were confirmed by measuring the optical density at 600 nm in a SpectraMax M2 microplate reader (Molecular devices, USA). Cultures from positive wells were streaked onto the corresponding solid medium (Marine agar, R2A agar, or SWC agar). The cultures showing identical colonies were picked and used to produce pure cultures.

SWC agar medium containing 15 g of agar was used to isolate *Polaribacter* colonies, which are often orange- or pink-coloured. Pigmented colonies were picked and screened by colony PCR using *Polaribacter*-specific PCR primers (see below) to identify strains of *Polaribacter*-specific clades. *Polaribacter*-specific PCR primers were designed as follows: 16S rRNA gene sequences from type strains within the genus *Polaribacter* were collected along with those from close neighbor genera. All sequences were aligned using BioEdit (Hall, 1999), and *Polaribacter*-specific regions were selected and checked using the RDP probe search program (Cole et al., 2007). PCR amplification was performed using the designed primers, 180 F (5′-TTT AGA AAT GAA GAT TAA TRC YC-3′) and 690 R (5′-CAT TCT ATC YAC TTC CAT ATG-3′). The PCR conditions were as follows: 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s, with a final extension for 5 min at 72 °C. The PCR amplicons were confirmed by sequencing.

All strains obtained by the extinction-dilution method grew on Marine agar (MA; Difco, France); the exception was *Polaribacter*, which grew only on SWC agar medium. Strains were maintained by periodic streaking on the corresponding agar medium.

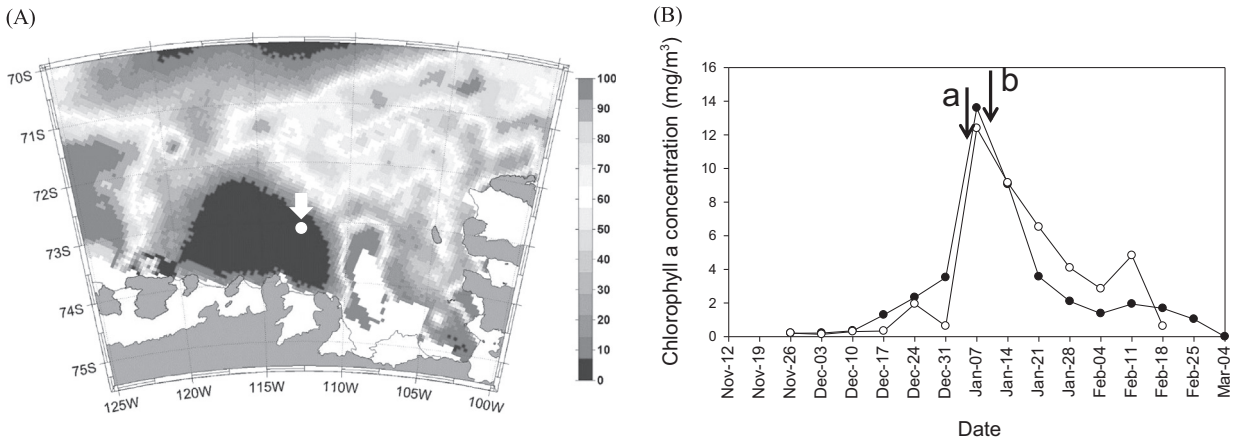

Fig. 1. (A) Sampling site (112° 00′ W, 73° 30′ S) at the center of the polynya in the Amundsen Sea. The scale bar indicates mean sea ice concentration (%) and the spot indicated by arrow points the position of the sampling station. Samples were taken on the 2013/2014 cruise. (B) Satellite-detected changes in chlorophyll-*a* concentration at the center of the polynya during the 2010/2011 (open circle) and 2013/2014 (closed circle) cruises. The arrows (labeled **a** and **b**) indicate the sampling date (2010/2011 and 2013/2014 cruises, respectively).

The total cell count in the sea water sample was measured using direct epifluorescence microscopic observation as described by Noble (1998). The sea water sample used for the count was fixed in 0.2 μm-filtered borate-buffered formalin (final concentration, 2%) and frozen at −80 °C before transportation to a land-based laboratory for total bacterial cell counting.

2.4. Phylogenetic analysis

Chromosomal DNA was extracted from strains using a commercial genomic DNA extraction kit (Cosmo Genetech, Republic of Korea). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer 27 F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492 R (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991) and the purified PCR product was sequenced by Cosmo Genetech Co. Ltd. The PCR conditions were as follows: 95 °C for 5 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, with a final extension for 5 min at 72 °C (Kim et al., 2013). The sequences (> 720 bp) were then used for classification and phylogenetic analysis.

The closest neighbors to the cultivated type strains were identified using EzTaxon-e (Kim et al., 2012). The 16S rRNA gene sequences of closely-related strains were downloaded from the GenBank database and multiple sequences were aligned and edited using the BioEdit program (Hall, 1999). A phylogenetic tree was generated using the Neighbor-Joining (Saitou and Nei, 1987) and maximum likelihood algorithms (Felsenstein, 1981), with 1,000 randomly-selected bootstrap replicates (MEGA 5 software; (Tamura et al., 2011)). Kimura's two-parameter model (Kimura, 1983) was used for the calculation of phylogenetic distances.

2.5. Phenotypic and physiological characterization of isolated strains

Growth was examined over a range of temperatures (0, 4, 10, 20, 25, 30 and 35 °C). Briefly, cells were suspended in artificial sea water (Berges et al., 2001) and then inoculated into MB (1% v/v). The *Polaribacter* suspension was inoculated into liquid SWC. Absorbance was then measured at 600 nm to determine both the temperature range suitable for growth and the optimal temperature for growth. Samples were incubated at each test temperature for 3 weeks.

The BIOLOG GN2 (BIOLOG, USA) and API ZYM (BioMérieux, France) were used for biochemical characterization, according to the manufacturer's instructions with slight modifications. Briefly, the BIOLOG GN2 test was performed as follows: cells from each strain were incubated on solid medium at the optimal growth temperature. Colonies were then suspended in GN/GP Inoculating Fluid (BIOLOG, USA) containing 3% sea salt (Sigma, USA) and dispensed into the wells of 96 well plates containing various carbon substrates. After 4 days of incubation, the reaction was read based on purple colour development. The API ZYM test was conducted as follows: cells from each strain were incubated on solid medium and colonies suspended in sterilized 0.85% saline solution. The suspension was dispensed into the wells of a strip containing substrates for different extracellular enzymes. After inoculation, the strip was incubated at the optimal growth temperature for each strain for 24 h before the addition of ZYM A and B reagents (BioMérieux, France) for determination of activity.

2.6. Accession numbers

The 16S rRNA gene sequences obtained in this study have been deposited in GenBank (NCBI) under accession numbers KP006517 to KP006530.

3. Results and discussion

3.1. Composition of the bacterial community in the polynya

The composition of the bacterial community in the polynya was examined by 454 pyrosequencing. Most of the bacterial sequence reads were associated with the phyla *Bacteroidetes* and *Proteobacteria* (*Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*) (see Fig. 2). The most abundant sequence reads (82.2%) covered only three clades (*Polaribacter*, AntF4D3 and *Pelagibacter*), whereas 14.3% of the sequences covered Unclassified Other Groups, and 3.6% were affiliated with many different microbial genera.

Many of the *Bacteroidetes* sequences (45.2%) were related to those of the genus *Polaribacter* within the *Flavobacteria*. The major bacterial sequences for *Proteobacteria* were affiliated with those of the Ant4FD3 cluster (uncultivated *Oceanospirillaceae*) within the class *Gammaproteobacteria* (31.7%). Sequences related to the AntF4D3 cluster were abundant in the Antarctic Ocean during austral summer (Murray and Grzymski, 2007); they were also abundant at the surface of the same polynya station in the similar bloom phase during austral summer in 2010/2011 (see Fig. 1B) (Kim et al., 2013). SAR11 (*Alphaproteobacteria*) clade sequences, which were predominant in oligotrophic sea water (i.e., 20% of the population in coastal oceans during austral winter (Grzymski et al., 2012) and 30% of the population in deep ocean polynyas

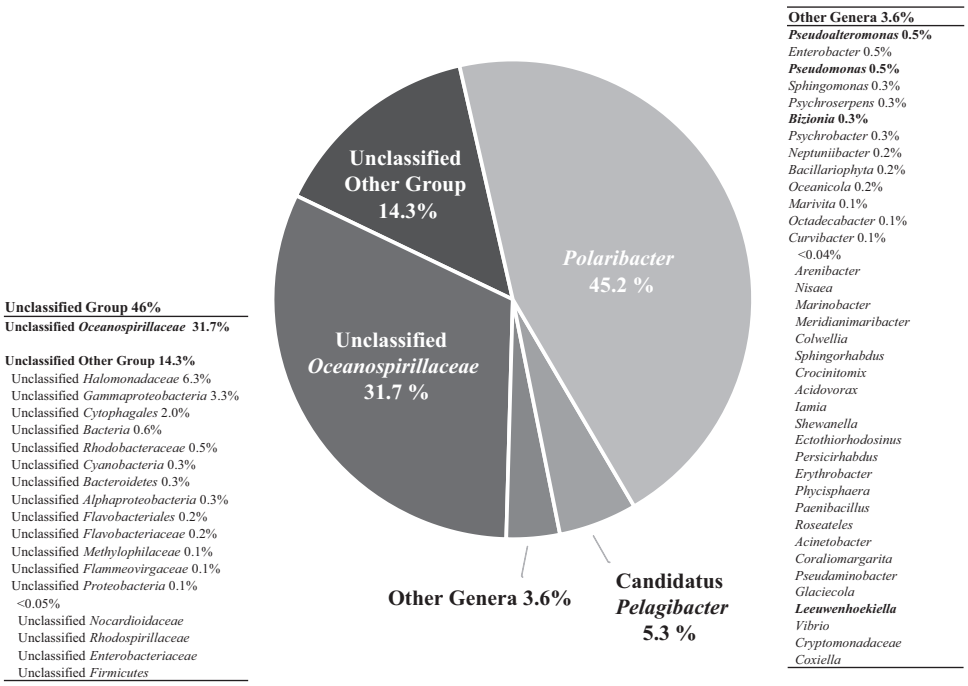

Fig. 2. Composition of the bacterial community present in the polynya in the Amundsen Sea based on 16S rRNA gene sequences obtained by 454 pyrosequencing. The genera cultivated in the study are indicated in bold.

(Kim et al., 2013), were only 5.3% in the polynya sample. While *Flavobacteria* are frequently associated with phytoplankton blooms in Antarctic sea water, the number belonging to the *Pelagibacter* clade is inversely proportional to the chlorophyll-*a* concentration (Williams et al., 2013). Despite the overall similarity in composition, the greater abundance of *Polaribacter* over *Pelagibacter* and the absence of SUP05 groups (Marshall and Morris, 2012) mean that the community composition during austral summer in 2013/2014 is different from those observed in 2010/2011 (Kim et al., 2013). Fig. 1B shows that the samples were taken at near-peak chlorophyll-*a* concentrations during the 2010/2011 and 2013/2014 cruises. Thus, dynamic changes around the peak of the initial polynya bloom, or factors other than primary production, might play a role in the development of the bacterial community.

3.2. Cultivation of heterotrophic bacteria using the extinction-dilution method

The total bacterial cell count in the sea water sample (as measured by epifluorescence microscopy) was about 2.0×10^6 cells/ml. Depending on the culture medium used (MB, 7.6×10^2; R2A, 7.2×10^2; or SWC, 3.9×10^2), the number of viable cells in the sample ranged from 10^2 to 10^3 cells/ml (Table 1), whereas real-time PCR quantification of the 16S rRNA gene suggested $10^5 - 10^6$ copies/ml (Kim et al., 2013). Bussmann et al. (2001) suggested that marine bacteria are difficult to cultivate. The lower counts of cultivable heterotrophic cells suggest that most of cells in this eutrophic polynya might be non-culturable (Oliver, 2005). Based on 16S rRNA gene sequence analysis, all 23 strains have close relatives belonging to type strains within the following genera (number of strains; % 16S rRNA gene sequence similarity): *Bizionia* (4; > 97.8%), *Leeuwenhoekiella* (1; 96.2%), *Pseudoalteromonas* (14; > 98.5%), *Pseudomonas* (1; 99.4%) and *Sulfitobacter* (3; 100%) (Table 1). Strains showing a close relationship with *Leeuwenhoekiella* and *Pseudomonas* were obtained after culture in SWC. Strains with a close relationship to the genus *Sulfitobacter* were obtained after culture in MB and SWC, and strains closely related to the genera *Bizionia* and *Pseudoalteromonas* were obtained after culture in all media. The high prevalence of *Pseudoalteromonas* strains observed in this study is intriguing considering the low number of sequence reads obtained for this clade when performing 454 pyrosequencing. Taken together, these results indicate that many bacteria identified by 16S rRNA gene sequence analysis could not be cultured successfully.

Pelagibacter and *Polaribacter* strains could not be obtained using the extinction-dilution method, even though 16S rRNA gene sequence analysis suggested that they are the most dominant members in the polynya in this study and Kim et al. (2013), and have cultivated relatives. The culture media used in the present study may be too rich for oligotrophic bacteria, such as those belonging to the genus *Pelagibacter* (Giovannoni et al., 2005). However, we did not expect that we would fail to cultivate any *Polaribacter* strains because it is known that *Polaribacter* strains can be selectively cultivated and isolated in SWC. To isolate *Polaribacter*-related strains, sea water samples were spread on SWC agar plates, and orange and pink colonies were selected (Gosink et al., 1998). The *Polaribacter*-specific PCR primer (designed specifically for this study) amplified three *Polaribacter* strains. Thus, it may be that (1) a large fraction of *Polaribacter* cells in the polynya might be in a non-cultivable state (Oliver, 2005), or (2) PCR-based analysis of 16S rRNA gene sequences over-represented the number of *Polaribacter*-related sequences.

3.3. Phylogenetic analysis of the strains

EzTaxon-e software showed that all 26 strains were clearly affiliated to the corresponding genera within the phyla *Bacteroidetes* and *Proteobacteria* (*Alphaproteobacteria* and *Gammaproteobacteria*) (Fig. 2) (see above). Analysis of 16S rRNA gene sequences showed that all *Polaribacter* strains (PB1, PB2 and PB3) were closely related to Antarctic seawater isolates, *P. irgensii* 23-P^T and *P. glomaratus* ATCC43844^T (> 982% sequence similarity). The sequences of *Polaribacter* strains were also related to NGS sequences of *Polaribacter* clade (see Supplementary Fig. 1). Four strains (BB1, BB2, BB3 and BB4) belonging to the genus *Bizionia* had identical 16S rRNA gene sequences and were most closely related to *B. saleffrena* HFD^T (> 97.8% similarity) (Bowman and Nichols, 2005). Strain LB was related to *Leeuwenhoekiella marinoflava* ATCC19326^T (Nedashkovskaya

Table 1
Strains obtained from a polynya in the Amundsen Sea.

Strain (isolation medium)	Closest type strain (accession no.; sequence similarity (%); isolation source)	Growth temperature range (optimum)°C
	Flavobacteria	
	Polaribacter	
PB1 (SWC[a])	*P. irgensii* 23-P^T (M61002; 99.2; Sea water, Antarctic Ocean)	0 – 10 (10)
PB2 (SWC[a])	*P. irgensii* 23-P^T (M61002; 99.7; Sea water, Antarctic Ocean)	
PB3 (SWC[a])	*P. irgensii* 23-P^T (M61002; 98.2; Sea water, Antarctic Ocean)	
	Bizionia	
BB1 (MB)	*B. saleffrena* HFD^T (AY694005; 98.0; Costal fast sea-ice brine, East Antarctica)	
BB2 (MB)	*B. saleffrena* HFD^T (AY694005; 98.0; Costal fast sea-ice brine, East Antarctica)	
BB3 (SWC)	*B. saleffrena* HFD^T (AY694005; 98.0; Costal fast sea-ice brine, East Antarctica)	0 – 30 (25)
BB4 (R2A)	*B. saleffrena* HFD^T (AY694005; 98.0; Costal fast sea-ice brine, East Antarctica)	
	Leeuwenhoekiella	
LB (SWC)	*L. marinoflava* ATCC 19326^T (AF203475; 96.2; North Sea off Aberdeen, Scotland, UK)	0 – 30 (25)
	Gammaproteobacteria	
	Pseudoalteromonas	
PA1 (R2A)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA2 (MB)	*P. marina* mano4^T (AY563031; 99.9; Tidal flat, Yellow Sea, Korea)	0 – 30 (25)
PA3 (R2A)	*P. marina* mano4^T (AY563031; 99.9; Tidal flat, Yellow Sea, Korea)	
PA4 (SWC)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA5 (SWC)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA6 (R2A)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA7 (MB)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA8 (R2A)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA9 (R2A)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA10 (SWC)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA11 (SWC)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
PA12 (SWC)	*P. translucida* KMM 520^T (AY040230; 99.6; Sea of Japan)	0 – 30 (25)
PA13 (MB)	*P. prydzensis* ACAM 620^T (U85855; 98.5; Sea ice, Antarctica)	0 – 30 (25)
PA14 (SWC)	*P. nigrifaciens* NCIMB 8614^T (X82146; 100; Mussel, Troitza Bay, Japan)	
	Pseudomonas	
PD (SWC)	*P. sabulinigri* J64^T (EU143352; 99.4; Black sand, Jeju island, Korea)	0 – 35 (25)
	Alphaproteobacteria	
	Sulfitobacter	
SB1 (MB)	*S. litoralis* Iso 3^T (DQ097527; 100; East sea, Korea)	
SB2 (MB)	*S. litoralis* Iso 3^T (DQ097527; 100; East sea, Korea)	0 – 30 (25)
SB3 (SWC)	*S. litoralis* Iso 3^T (DQ097527; 100; East sea, Korea)	

NA: not applicable
[a] agar

et al., 2005) (96.2% 16S rRNA gene sequence similarity). Fourteen strains belonging to the genus *Pseudoalteromonas* formed three clusters (Fig. 3) and the strains in the largest cluster with 11 strains were closely related to each other (> 98.8% 16S rRNA gene similarity) and to the following type strains (> 99.6% similarity): *P. nigrifaciens* NCIMB 8614^T, *P. haloplanktis* ATCC 14393^T (Gauthler et al., 1995), *P. elyakovii* KMM 162^T (Sawabe et al., 2000) and *P. translucida* KMM520^T (Ivanova et al., 2002), respectively. *Pseudomonas* strain PD was closely related to *Pseudomonas sabulinigri* J64^T (Kim et al., 2009), showing 99.4% 16S rRNA gene sequence similarity. Three strains of *Sulfitobacter* had 100% identical 16S rRNA gene sequences, which were also 100% identical to that of *S. litoralis* Iso 3^T (Park et al., 2007). Most of close relatives of the strains examined in this study are strains that were isolated from polar oceans, which indicates that the strains are typical psychrophilic heterotrophs in Antarctic Oceans. We selected the following representative strains from each genus for further characterization: *Polaribacter* (PB1), *Pseudoalteromonas* (PA2, PA12 and PA13), *Sulfitobacter* (SB2), *Leeuwenhoekiella* (LB), *Bizionia* (BB3) and *Pseudomonas* (PD).

3.4. Phenotypic and physiological characteristics of the strains

All strains obtained by the extinction-dilution method grew over a wide temperature range (0–30 °C). Although the strains were isolated at 10 °C, most had an optimum growth temperature of 25 °C; thus they are classified as psychrotolerant bacteria. Because all of the representative strains were able to grow at 0 °C, they may play a role in carbon re-mineralization in polynyas. The only obligate psychrophilic strain was strain PB1 of *Polaribacter*, which grew at 0–10 °C (optimum 10 °C). This temperature range is similar to that favored by polar strains of *Polaribacter* (*P. filamentus* 215^T, *P. franzmannii* 301^T and *P. irgensii* 23-P^T) (Gosink et al., 1998). By contrast, relatives of strain PB1, such as *P. butkevichii* KMM 3938^T, *P. dokdonensis* DSW-5^T, *P. gangjinensis* K17-16^T and *P. glomeratus* ATCC 43844^T, which were isolated from temperate oceans, preferred higher growth temperatures (4–30 °C) (Nedashkovskaya et al., 2013). This implies that the psychrophilic *Polaribacter* strains have adapted to Antarctic sea water and may play an important role in the carbon cycling within Antarctic polynyas.

3.4.1. Extracellular enzyme activity

Polymers released by phytoplankton in polynyas are major carbon substrates for heterotrophic bacteria. These polymers are hydrolyzed to smaller, labile carbon substrates, which are then assimilated by heterotrophic bacteria. As *Flavobacteria* play an important role in polymer degradation by secreting extracellular enzymes, *Leeuwenhoekiella* strain LB, *Bizionia* strain BB3 and *Polaribacter* strain PB1 (all *Flavobacteria*) isolated in the present study showed extracellular enzyme activity against a wide range of test substrates. However, only strain LB showed various extracellular enzyme activities associated with polysaccharide, protein,

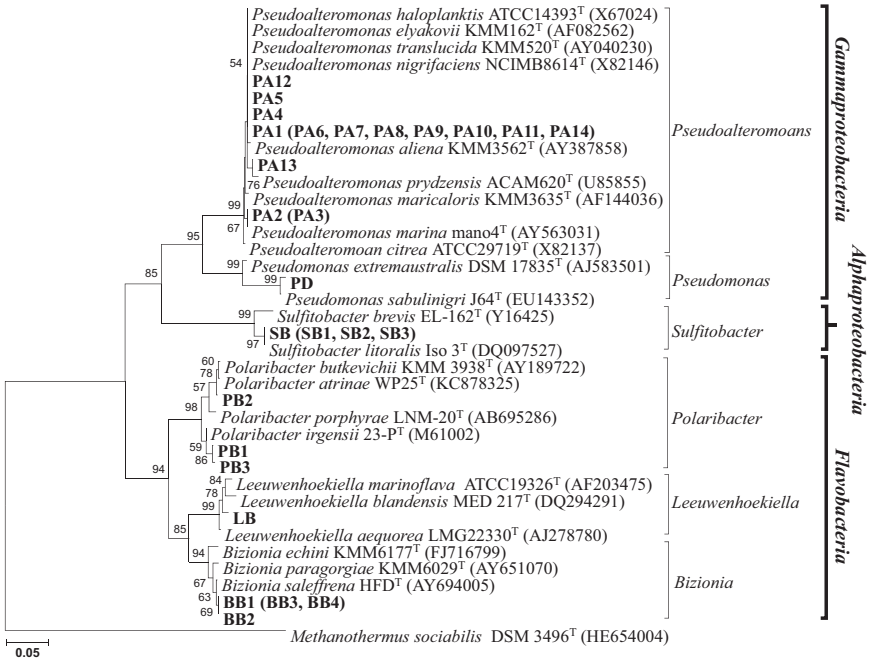

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains obtained from the polynya in the Amundsen Sea and the close relatives. Bootstrap values ≥50% (based on 1000 replicates) from maximum likelihood method are indicated at branch points.The tree is rooted by using the type strain of *Methanothermus sociabilis* as an outgroup. GenBank accession numbers are shown in parentheses. Bar, 0.05 substitutions per nucleotide position.

and lipid hydrolysis (Table 2). A North Sea strain, *L. marinoflava* ATCC 19326^T, and an Antarctic Ocean strain, *Leeuwenhoekiella aequorea* LMG 22550^T (both of which are close relatives of strain LB) showed both cellulase and chitinase activity (Nedashkovskaya et al., 2009). *Polaribacter* strain PB1 and *Bizionia* strain BB3 showed enzyme activities only on a narrow range of amide substrates. This was unexpected because the genome of *P.* strain MED152 harbors putative genes responsible for the degradation of polymers, including polysaccharides (González et al., 2008). Furthermore, the abundance of *Polaribacter* is thought to be linked to its ability to digest polymers released by phytoplankton blooms in polar oceans (Gómez-Pereira et al., 2012). Comparative genome sequence analyses of polar strains of *Polaribacter* will provide insights into the ecological niches occupied by Antarctic *Polaribacter* and the roles they play in the carbon cycling in Antarctic polynyas. Although strains of *Bizionia* from Antarctic Oceans show amidase enzyme activity against a relatively broad range of substrates (Bercovich et al., 2008), strain BB3 (isolated in the present study) showed only a arylamidase activity. A previous study shows that the genome of *B. argentinensis* JUB59^T contains 24 putative extracellular peptidase genes (Lanzarotti et al., 2011); however, no information is available regarding the extracellular enzyme activity of other Antarctic *Bizionia* spp.

Strains of *Proteobacteria* showed extracellular enzyme activity against a limited range of test substrates. For example, *Pseudoalteromonas* PA strains showed amidase and chymotrotrypsinase activity, although Ivanova et al. (2002) reported that an Antarctic strain, *Pseudoalteromonas prydzensis* ACAM 620^T, showed both amylase and chitinase activity. *Sulfitobacter* strain SB showed only phosphatase activity, although *Sulfitobacter litoralis* Iso3^T had esterase and amidase activity. *Pseudomonas sabulinigri* J64^T showed lipase, phosphatase and esterase activity, whereas *Pseudomonas* strain PD showed only esterase activity. *Pseudomonas sabulinigri* J64^T and *Sulfitobacter litoralis* Iso3^T were both isolated from temperate oceans (Kim et al., 2009; Park et al., 2007) and thus are not directly comparable. Few studies have examined the extracellular enzyme activities of Antarctic bacterial strains; therefore, the results presented herein may help us to better understand bacterial traits related to polymer degradation in polynyas.

3.4.2. Utilization of labile carbon sources

Bizionia strain BB3 used various amino acids and peptides while carbohydrates and organic acids were not utilized, which indicates its adaptation to peptide utilization (Table 2). *Polaribacter* within polynya environments are fastidious, since none of the culture conditions used for BIOLOG GN2 test supported the growth of strain PB1; thus no positive activity was detected in the BIOLOG GN test. It is interesting that strain LB of *Leeuwenhoekiella* showed utilization of a wide range of labile carbon substrates in addition to activities of various extracellular enzymes. Various carbohydrates, organic acids, amino acids, and peptides were utilized by strain LB.

Strains of *Proteobacteria* also utilized various labile carbon substrates. In particular, the three strains of *Pseudoalteromonas* (*Gammaproteobacteria*) assimilated different types of labile carbon substrates. For example, strain PA12 showed a relatively narrow spectrum of labile carbon substrate assimilation with no amino acids or peptides utilization. Strain PA2 and PA13 showed utilization of wide range of carbohydrates and amino acids. Only strain PA13 could utilize the amino sugar N-acetyl-D-glucosamine and N-acetyl-D-glucosamine, which are constituents of many complex polysaccharides as *Leeuwenhoekiella* strain LB. This result suggests that strains of *Pseudoalteromonas* have high levels of genomic and biochemical plasticity, despite the high similarity of their 16S rRNA gene sequences (>99%) (Al Khudary et al., 2008; Ivars-Martinez et al., 2008). This ability to utilize various labile carbon substrates is consistent with the finding that *Pseudoalteromonas* is a typical copiotroph and presents in oceans worldwide (Holmström and Kjelleberg, 1999). *Sulfitobacter* strain SB2 also showed the ability to utilize a wide range of carbon substrates as *Leeuwenhoekiella* strain LB and *Pseudoalteromonas* strains PA2 and PA3. The highest number of organics acids was utilized by strain SB2. Metagenomics analyses

Table 2
Extracellular enzyme activity and labile substrate utilization by the different strains.

Strain	Extracellular enzyme	Substrate
Bizionia		
BB3	Leucine arylamidase Alkaline phosphatase	α-keto butyric acid, urocanic acid L-alaninamide, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-leucine, L-ornithine, L-proline, L-serine
Leeuwenhoekiella		
LB	α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase	α-cyclodextrin, dextrin, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arabinose, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, glycerol, glucose-L-phosphate
	Leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin	Mono-methyl-Succinate, acetic acid, D-galacturonic acid, D-glucuronic acid, α-keto valeric acid, succinic acid, bromo succinic acid
	Esterase (C4), esterase lipase (C8)	L-alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy-L-proline, L-proline, L-serine, L-threonine
	Alkaline phosphatase, acid phosphatase, naphtol-AS-BI-phosphohydrolase	Tween 40
Polaribacter		
PB1	Leucine arylamidase, valine arylamidase Alkaline phosphatase, acid phosphatase, Naphtol-AS-BI-phosphohydrolase	NA
Pseudoalteromonas		
PA2	Leucine arylamidase alkaline phosphatase, acid phosphatase	α-cyclodextrin, dextrin, D-cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, maltose, D-mannitol, D-mannose, D-melibiose, sucrose, D-trehalose Methyl pyruvate, mono-methyl-succinate, cis-aconitic acid, citric acid, D-gluconic acid L-alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-proline, uridine, glycerol Tween 40, Tween 80
PA12	Leucine arylamidase Alkaline phosphatase,	D-mannitol Methyl pyruvate, D-gluconic acid Tween 40, Tween 80
PA13	α-chymotrypsin alkaline phosphatase, acid phosphatase	α-cyclodextrin, dextrin, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arabinose, D-cellobiose, D-fructose, gentiobiose, α-D-glucose, maltose, D-raffinose, sucrose, D-trehalose Methyl pyruvate, mono-methyl-succinate, acetic acid, cis-aconitic acid, citric acid, D-galacturonic acid, α-keto butyric acid, propionic acid, succinic acid L-alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-glutamic acid, glycyl-L-glutamic acid, inosine, uridine Tween 40, Tween 80
Pseudomonas		
PD	Esterase	Methyl pyruvate, acetic acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, D,L-lactic acid, sebacic acid D-alanine, L-alanine, L-asparagine, L-glutamic acid, L-proline, putrescine Tween 40, Tween 80
Sulfitobacter		
SB2	Alkaline phosphatase, acid phosphatase	α-cyclodextrin, glycogen, D-fructose, D-mannitol, D-mannose, turanose Methyl pyruvate, mono-methyl-succinate, acetic acid, cis-aconitic acid, citric acid, D-glucuronic acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, α-keto glutaric acid, D,L-lactic acid, quinic acid, succinic acid, bromo succinic acid, succinamic acid L-alaninamide, D-alanine, L-alanine, L-alanyl-L-glycine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-proline, L-serine, L-threonine, γ-amino butyric acid, glycerol, D,L-α-glycerol phosphate

NA: not available.

show that members of *Rhodobacterales* harboring *Sulfitobacter* have various ATP-binding cassette transporters that enable uptake of labile substrates (Williams et al., 2013). *Sulfitobacter* also plays an important role in the sulfur cycle by converting dimethylsulfidepropionate to dimethylsulfide, which is a typical climate-changing gas (Curson et al., 2008). Taken together, these results support the suggestion by Grzymski et al. (2012) and Teeling et al. (2012) that *Pseudoalteromonas* (*Gammaproteobacteria*) and *Sulfitobacter* (*Alphaproteobacteria*) are typical microbes that assimilate labile organic substrates in polar oceans. Indeed, a metagenomics study showed that these strains are frequently observed in Antarctic Oceans during phytoplankton blooms (Kim et al., 2013). The strains of *Gammaproteobacteria* and *Alphaproteobacteria* isolated in the present study might be representatives of psychrophilic heterotrophs that utilize labile carbon substrates generated during phytoplankton blooms in Antarctic polynyas.

4. Conclusions

The cultivation-dependent (extinction-dilution method) and -independent (454 pyrosequencing of 16S rRNA gene sequences) analyses performed herein show that *Flavobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* represent the dominant classes of psychrophilic heterotrophs in an Antarctic polynya. These three bacterial clades are frequently abundant in eutrophic oceans, including the Antarctic Ocean during austral summer. Although many of the clades identified in the cultivation-independent study were not isolated by the

extinction-dilution method, we did obtain representatives of each clade. *Leeuwenhoekiella* strain LB (*Flavobacteria*) showed extracellular enzyme activities on most of the substrate groups: amides, lipids, and glycosides while *Polaribacter* and *Bizionia* strains (*Flavobacteria*) showed enzyme activities only on a limited range of amides. This corresponds with the activity of labile carbon source utilization: *Leeuwenhoekiella* strain LB utilized most of labile organic carbons while *Bizionia* strain BB3 utilized only amino acids and peptides. Extracellular enzyme activities of *Proteobacteria* were limited: only *Pseudoalteromonas* (*Gammaproteobacteria*) showed amidase activities. However, *Sulfitobacter* (*Alphaproteobacteria*) and *Pseudoalteromonas* strains had capability to utilize various carbohydrates, organic acids, amino acids, and peptides. Further genomic and biochemical characterization of the representative strains obtained in this study may provide insight into the ecophysiological features and carbon remineralization potential of psychrophilic heterotrophs present in polynyas within the Antarctic Ocean.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dsr2.2015.04.027.

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