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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.

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