Bacterioplankton communities in the Southern Ocean: composition and growth response to various substrate regimes

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ABSTRACT: To examine the adaptation of bacterioplankton communities in the Southern Ocean to various biopolymeric substrates, we carried out experiments at the Polar Front and in the Antarctic Coastal Current (CC) in which samples were enriched with agarose, starch, peptone and extracts of the green alga *Scenedesmus acutus* and diatoms from the Polar Front. Growth and metabolic activity were assessed based on leucine incorporation and turnover rates of glucose, dissolved free amino acids and protein. In both experiments, growth was highest on peptone and the diatom extract and lowest on agarose. There was a general trend of decreasing proportions of *Alphaproteobacteria* and increasing proportions of *Flavobacteria/Sphingobacteria* and *Gamma-proteobacteria*. The growth response and shift to *Flavobacteria/Sphingobacteria* was more pronounced in the CC experiment. A cluster analysis of denaturing gradient gel electrophoresis (DGGE) banding patterns showed that bacterial communities in each treatment of both experiments differed and that those in the treatments with peptone and the *Scenedesmus* and diatom extracts clustered together. Sequencing of excised bands revealed that *Gammaproteobacteria* comprised members of *Pseudoalteromonadaceae*, the *Colwellia* cluster of *Alteromonadaceae* and 1 other cluster. *Alphaproteobacteria* comprised only members of the *Roseobacter* clade and the *Bacteroidetes* phylum only of *Flavobacteria* and *Cytophagales*. The results show that the bacterioplankton communities in the Southern Ocean are able to adapt to various biopolymeric substrates. The most pronounced response to the additions of peptone and the diatom extract rich in protein is in line with previous observations that proteins are preferred bacterial substrates in this oceanic region.

KEY WORDS: Bacteria · Fluorescence *in situ* hybridization · DGGE · Cluster analysis · Biopolymers · Southern Ocean

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INTRODUCTION

During the last 2 decades, the Southern Ocean has been the focus of many research programs because of its sensitivity to climate effects, such as global warming and the impact of enhanced UV irradiation. This is one reason why biogeochemical processes of plankton communities and the heterotrophic picoplankton have been investigated intensively in this region (Lochte et al. 1997, Ducklow et al. 2001, 2012, Kirchman et al. 2009). The composition of the hetero-

trophic picoplankton community, including *Bacteria* and *Archaea*, has also been studied in the Southern Ocean and most intensely in the last decade. These studies have revealed that *Alpha-* and *Gammaproteobacteria*, *Sphingobacteria* and *Flavobacteria* of the *Bacteroidetes* phylum and in particular distinct subclusters of these groups are the major phylogenetic lineages (Simon et al. 1999, Abell & Bowman 2005a, West et al. 2008, Manganelli et al. 2009, Straza et al. 2010, Ducklow et al. 2011). These findings corroborate observations in other oceanic

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regions, but relevant subclusters of these major phylogenetic lineages in the Southern Ocean differ from other regions with higher water temperatures and other hydrographic regimes (Selje et al. 2004, Abell & Bowman 2005a, Giebel et al. 2009, Straza et al. 2010, Ghiglione & Murray 2012).

Despite a general understanding of the major phylogenetic lineages of the bacterioplankton in the Southern Ocean, little information is available on the substrate preferences and organic matter cycling of distinct phylogenetic lineages. Abell & Bowman (2005b) reported that subclusters of *Flavobacteria* were the dominant bacteria colonizing and decomposing diatom derived organic matter in various regions of the Southern Ocean. In a study around the western Antarctic Peninsula using microautoradiography and fluorescence *in situ* hybridization (MAR-FISH), Straza et al. (2010) found that specific subgroups of *Gammaproteobacteria*, SAR11 and of *Sphingobacteria/Flavobacteria* were the major bacterioplankton groups in organic matter consumption. Specific phylotypes of *Gammaproteobacteria* and *Sphingobacteria/Flavobacteria* were reported to be particularly responsive to glucose plus ammonium additions in a mesocosm experiment in the same region (Ducklow et al. 2011). To better understand organic matter cycling by bacterioplankton phylogenetic lineages in the Southern Ocean, more studies with various potentially important substrates are needed.

We studied the response of bacterioplankton communities to various substrate additions in 2 regions of the Southern Ocean, the Polar Front (PF) and the Antarctic coastal current (CC), in microcosm experiments. We tested whether biopolymers as single carbon (C) and as combined C and nitrogen (N) sources would result in different growth responses of the ambient bacterioplankton communities. Therefore, we carried out microcosm experiments in which various substrates, i.e. starch, agarose, peptone and an extract of a green alga and of a natural diatom community from the Polar Front, were added to ambient bacterioplankton communities. The growth response and composition of the bacterioplankton communities were analyzed by FISH and denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR) amplified 16S rRNA gene fragments, and the nucleotide sequences of excised bands were determined. The results show that distinct differences existed in the community composition and the growth response of the bacterioplankton to the various substrate additions and between the 2 locations.

MATERIALS AND METHODS

The present study was conducted in the Atlantic sector of the Southern Ocean during cruise ANT XVI/3 in austral fall 1999 with RV 'Polarstern'. It was embedded in a larger study that investigated the substrate turnover and biomass production of heterotrophic picoplankton in the Southern Ocean (Simon et al. 2004, Simon & Rosenstock 2007) and the community composition of the bacterioplankton (Giebel et al. 2009). Samples for the microcosm experiments were taken with 12 l Niskin bottles mounted on a rosette with a conductivity, temperature and depth profiler. Samples were collected from 40 m depth on 28 March 1999 in the Polar Frontal Zone (PF; 49° 20.64' S, 20° 01.50' E; Stn 157) (Giebel et al. 2009) and on 19 April 1999 in the Antarctic Coastal Current (CC) under pack ice (50% coverage) close to the shelf ice edge, which was ~30 km distant from the Antarctic continent (70° 11.29' S, 6° 22.16' W; Stn 182) (Giebel et al. 2009). Samples from 40 m depth were taken because this depth was still in the mixed layer but exhibited lower rates of bacterial production than at 20 m. *In situ* temperatures were 3.9°C at the PF and -1.8°C at the CC (Table 1).

Design of the substrate addition experiments

We examined how the bacterioplankton communities in the 2 regions respond to various substrate additions by monitoring the bacterial growth and the turnover rates of glucose, dissolved free amino acids (DFAA) and protein and by examining how the composition of the bacterioplankton community changed as a function of the added substrates over

Table 1. Environmental and biological properties of the locations of the experiments at the polar Front and the Coastal Current under pack ice (data adopted partly from Giebel et al. 2009). RCA: *Roseobacter* clade affiliated cluster

Parameter	Polar Front	Coastal Current
Temperature (°C)	3.9	-1.8
Chlorophyll a ($\mu\text{g l}^{-1}$)	0.3	0.1
Bacterial abundance (10^5 ml^{-1})	3.5	3.3
Leucine incorporation ($\text{pmol l}^{-1} \text{ h}^{-1}$)	5.5	5.1
Bacterial generation time (d)	9.8	11.9
Glucose turnover rate (d^{-1})	0.1	0.03
RCA (% <i>Bacteria</i>)	23 ^a	18 ^a
SAR 11 clade (% <i>Bacteria</i>)	7	9
^a 60 m depth		

time. Unfiltered water samples were filled into acid-rinsed 2 l Nalgene bottles. The following substrates were added at final concentrations of 500 $\mu\text{g dry weight l}^{-1}$ ($\sim 20 \mu\text{M C}$): agarose (agar grade A, BD Sciences), starch (Merck) and peptone (digest of casein; Roth). Agarose was chosen as a model polysaccharide consisting of D-galactose and 3,6-anhydro-L-galactose linked by a β -glycosidic bond and starch as a model polysaccharide consisting of glucose linked with an α -glycosidic bond. An extract of diatoms collected with a centrifuge in the PF and an extract of the green alga *Scenedesmus acutus* were added to another bottle, each at final concentrations of $\sim 800 \mu\text{g dry weight l}^{-1}$ ($\sim 30 \mu\text{M C}$). The frozen extracts were thawed and boiled for 10 min prior to use. One bottle without any additions was used as a control. Bottles were incubated in the dark at *in situ* temperature ($\pm 1^\circ\text{C}$) for 9 (PF) and 10 d (CC) and subsampled daily for enumerating prokaryotic cell numbers after DAPI staining by epifluorescence microscopy according to Porter & Feig (1980). The incubation time was close to the generation time of the ambient bacterioplankton communities (Table 1). As *Bacteria* constitute the prokaryotic community to a great extent (see 'Results') and *Archaea* constitute only low fractions of the prokaryotic community in the austral summer and fall (Murray et al. 1998), we use the terms bacteria or bacterioplankton hereafter. Subsampling for other parameters (see the next 2 sections) was done less frequently after Day 4 in the PF experiment. When the experiments were terminated, 500 ml of samples were filtered onto 0.2 μm Nuclepore membranes (47 mm diameter) and stored at -20°C for DGGE analysis (see 'PCR amplification, DGGE and cluster analysis'). Due to constraints in handling, it was not possible to carry out the experiments with replicate bottles.

Leucine incorporation and turnover rates of DFAA, glucose and protein

Leucine incorporation was determined from the incorporation of ^{14}C -leucine into bacterial biomass according to Simon & Azam (1989) and as described in detail by Simon & Rosenstock (2007). We added ^{14}C -leucine (specific activity $10.8 \text{ GBq mmol}^{-1}$, Hartmann Analytik) at a final concentration of 10 nM (saturating concentration) in triplicates and a Formalin-killed control to 10 ml of sample and stopped the incubation with Formalin after 4 to 6 h. Uptake was still linear after this incubation time. Fixed samples were filtered onto 0.2 μm nitrocellulose filters (Sartorius), rinsed with ice-cold particle-free seawater,

extracted with ice-cold 5% trichloroacetic acid and radioassayed by liquid scintillation counting. The coefficient of variation (CV; SD/mean) of the triplicate measurements was $<10\%$.

Turnover rate constants of DFAA and glucose were determined by measuring the incorporation of a mixture of $16 \text{ }^3\text{H}$ -DFAA (mean specific activity 1.97 GBq mat C , TRK440, Amersham) and ^3H -glucose (specific activity $429.0 \text{ GBq mmol}^{-1}$, Amersham) by heterotrophic picoplankton as described by Simon & Rosenstock (2007). Each radiolabel was added to triplicate 10 ml subsamples and a Formalin-killed control at a final concentration of 0.1 nM. Turnover of glucose was measured in a dual label approach together with ^{14}C -leucine incorporation. Turnover rate constants of dissolved protein were determined by measuring the incorporation of a ^{14}C -algal protein (specific activity $1.63 \text{ GBq mmol}^{-1}$, custom made by Amersham, see Rosenstock & Simon 2001) according to Simon & Rosenstock (2007). The radiolabel was added in triplicate to 10 ml subsamples and a Formalin-killed control at 2 nM final concentration (amino acid equivalent). Incubations for all 3 substrates lasted for 4 to 6 h, and samples were further processed as for leucine incorporation. The CV of the triplicate measurements was $<15\%$. Turnover rate constants of DFAA, glucose and protein were calculated from the incorporated radioactivity divided by the added radioactivity.

Fluorescence *in situ* hybridization

In situ hybridization with fluorescent rRNA-targeted oligonucleotide probes was done according to Glöckner et al. (1996). Subsamples of 2 to 5 ml were taken after 4 and 9 d from the PF microcosm and after 4 and 10 d from the CC microcosm, filtered onto 0.2 μm Nuclepore membranes (25 mm diameter) and fixed by overlaying the filters with 3 ml of a freshly prepared phosphate-buffered saline (pH 7.2) 4% paraformaldehyde solution for 30 min. Thereafter, the filters were washed with Milli-Q water and stored frozen at -20°C until hybridization. The hybridization protocol as described by Glöckner et al. (1996) was applied, and the following group-specific oligonucleotide probes were used: EUB338 for *Bacteria* (Amann et al. 1990), ALF968 for *Alphaproteobacteria* (Neef 1997), GAM42a for *Gammaproteobacteria* (Manz et al. 1992) and CF319a for the *Sphingobacteria/Flavobacteria* group of the *Bacteroidetes* phylum (Manz et al. 1996, Amann & Fuchs 2008). The probes were linked to the N-hydroxy-succinimide ester of the carbocyanine CY3 as the

fluorochrome. The hybridization procedure included a final staining with DAPI. Cells were viewed using an Axioplan epifluorescence microscope (Zeiss) and the filter sets Zeiss 01 for DAPI and Chroma HQ41007 for Cy3 (Chroma Technology). For each sample, 2 replicates were counted using 10 to 20 view fields with at least 500 DAPI-stained cells per sample. All counts were corrected by subtracting the counts obtained with a negative control (NON338), which never exceeded 1%.

Nucleic acid extraction

DNA of the bacterial biomass collected on the 0.2 µm Nuclepore membranes was extracted following a modified standard protocol of Zhou et al. (1996), which includes treatment with zirconium beads and a mixture of hot (60°C) phenol-chloroform-isoamyl alcohol in 100 mM sodium phosphate buffer at pH 8.3. The DNA was precipitated overnight at –80°C after addition of Na-acetate (3 M) and isopropanol (2.5 × vol). DNA extraction was checked on a 1% agarose gel. The stock of the extracted DNA was stored at –80°C and subsamples at –20°C until further analysis.

PCR amplification, DGGE and cluster analysis

The *Bacteria*-specific primers 341F and 907RM (Muyzer et al. 1998) were used to amplify 550 base pairs (bp) of 16S rRNA gene fragments, which were subsequently separated by DGGE. At the 5'-end of the GM5F primer, an additional 40 bp GC-rich nucleotide sequence (GC-clamp) was added to stabilize migration of the DNA fragments in DGGE (Muyzer et al. 1993). PCR amplifications were performed with an Eppendorf Mastercycler (Eppendorf) and using RedTaq™ DNA polymerase (Sigma) as described by Selje & Simon (2003). We used a touch down PCR program with a decreasing annealing temperature from 65 to 56°C (2 cycles at each temperature step) and additional 18 cycles at 55°C for a total of 38 cycles. The amplicons were examined on 2% agarose gels stained with ethidium bromide (1 µg ml⁻¹). DGGE was performed with the D-Code system (Bio-Rad Laboratories) as described by Selje & Simon (2003). After electrophoresis, the gels were stained with SYBR Gold (Molecular Probes) and visualized by a BioDoc Analyze Transilluminator (Biometra). A cluster analysis of the DGGE banding patterns was performed based on curves and, after

checking for normal distribution of the data, using Pearson correlations and the unweighted pair group method with arithmetic mean with the software GelCompare II, Version 2.5 (Applied Maths). This analysis takes into account the number and intensity of DGGE bands. For further details, see Selje & Simon (2003).

Cloning, sequencing and phylogenetic analysis

DGGE bands were excised by a sterile scalpel and suspended in 50 µl of water of PCR quality (Eppendorf). The accuracy of the bands and the position in the gel were double-checked on DGGE gels. Cloning was done with the Promega pGEM®-T-Easy Vector System II according to the manufacturer's advice. Further, we checked the sequences of bands at similar positions by randomly probing several clones of the same ligation reaction. Inserts were reamplified with primers M13F and M13R in two 100 µl batches using a standard PCR protocol with 30 cycles at an annealing temperature of 50°C. PCR products were purified with the Qiaquick PCR purification kit (Qiagen) and sequenced using a DNA Sequencer (Model 4200, LI-COR) as described by Selje & Simon (2003). Sequences were checked for chimera formation with Pintail (www.bioinformatics-toolkit.org/Pintail/) and compared to those in GenBank using the BLAST function of the NCBI server (<http://blast.ncbi.nlm.nih.gov/>). To increase the reliability of the results, the sequences of the primers were not included in the sequence analysis.

Phylogenetic trees were constructed using the ARB software package (Ludwig et al. 2004, see www.arb-home.de). The backbone tree was calculated with the bootstrap (2000') method using sequences with a minimum length of 1300 bp, including type strains of selected phylogenetic groups. For tree calculation, positions were excluded at which <50% of all sequences showed the same residues to avoid uncertain alignments. Sequences with <1300 bp were added to the backbone tree with the maximum parsimony method using the same filter. As outgroup, 16S rRNA gene sequences of 3 type strains belonging to *Cyanobacteria* were used.

Nucleotide sequence accession numbers

The sequences obtained in the present study are available from GenBank under accession no. JQ728958 to JQ728979.

RESULTS

The 2 locations of the microcosm experiments differed strongly with respect to water masses, temperature, pack ice cover and other parameters (Table 1). At the CC station, chlorophyll *a* concentrations, bacterial abundance and activity were lower than at the PF station. Further, as shown in Giebel et al. (2009) by DGGE analyses, the composition of the bacterioplankton community differed strongly between the PF and CC regions. At 60 m depth of the PF station, the *Roseobacter* clade affiliated (RCA) cluster and the SAR11 clade of *Alphaproteobacteria* constituted 23 and 7% of total eubacterial 16S rRNA genes, respectively, and at the CC station at the same depth, both phylogenetic lineages constituted 18 and 9%, respectively, assessed by quantitative (q)PCR (Giebel et al. (2009). qPCR data for 40 m depth and DGGE and FISH data for both stations are not available.

Bacterial growth and substrate turnover

The various treatments resulted in pronounced differences in the response of bacterial growth and substrate turnover rates of both microcosm experiments. In the PF microcosm experiment, a response of most parameters in all treatments did not occur until Days 4 to 6 (Fig. 1). Thereafter, bacterial numbers in the control remained low, whereas leucine incorporation and glucose turnover rates in the control and starch treatment increased strongly, in contrast to the agarose treatment, in which these parameters remained low (Fig. 1A,C,E). Turnover rates of DFAA and protein in the treatments with agarose and starch were 2- to 3-fold higher than in the control until Day 6 and also higher than turnover rates of glucose (Fig. 1G,I). Toward the end, turnover rates of DFAA and protein in the control also increased and reached levels in the range of those of the treatments with agarose and starch. In the treatments with peptone and the diatom extract, bacterial numbers and leucine incorporation reached values 50 to 100% higher than in the treatments with agarose and starch (Fig. 1B,D). Turnover rates of glucose, DFAA and protein in these treatments simultaneously increased, but values were in the same range as in the other treatments (Fig. 1F,H,J). In the treatment with the *Scenedesmus* extract, respective values remained lower than in the treatments with peptone and the diatom extract and similar to those of the treatments with agarose and starch, except for DFAA turnover rates (Fig. 1B,D,F,H,J).

In the CC microcosm experiment, the response of bacterial growth and substrate turnover rates to the various substrate additions started earlier than in the PF microcosms, in most cases after 3 to 4 d, and responses were higher than in the PF experiment (Fig. 2). Whereas bacterial numbers in the control and the agarose treatment exhibited only minor changes, those in the treatments with starch and the *Scenedesmus* extract increased 3-fold and in those with peptone and the diatom extract even more than 4-fold (Fig. 2A,B). Rates of leucine incorporation and glucose turnover in the agarose treatment remained lower than in the other treatments, and highest responses occurred in the treatments with peptone and the diatom extract (Fig. 2C–F). Temporal patterns of the latter, however, differed strongly. Turnover rates of DFAA and protein in all treatments exhibited rather similar temporal patterns with an increase after Day 4 and lowest rates in the control (Fig. 2G–J). Protein turnover rates in the treatments with peptone and the *Scenedesmus* and diatom extracts reached values on Day 7 that were 5-fold higher than in the control and the other treatments. Values in the control remained lower than in the treatments with substrate additions but also increased after Day 4, as did leucine incorporation and glucose turnover in the control. Data of DFAA and protein turnover rates are only available until Day 7.

Bacterial community composition

The composition of the bacterial community in both experiments was analyzed by FISH on Day 4, the end of the first phase with little changes in growth and substrate turnover rates, and at the end of the experiments. In the PF experiment, 41 to 51% of DAPI cell counts were detected by the EUB probe in the control, agarose and starch treatments on Day 4 and 63 to 73% in the other treatments (Table 2). On Day 9, the detection efficiency in the various treatments was 73 to 92%, with lowest values in the control and agarose treatments. *Sphingobacteria/Flavobacteria* and *Alphaproteobacteria* constituted ~20% of DAPI cell counts in the control on Day 4, but in the treatments with agarose and starch, *Alphaproteobacteria* clearly dominated with 30 and 25% and the 2 other groups made up <17% (Fig. 3). In the other treatments, *Sphingobacteria/Flavobacteria* dominated and constituted 25 to 46%. In the treatments with peptone and the *Scenedesmus* extract, *Alphaproteobacteria* were the second most abundant group,

Fig. 1. (A,B) Bacterial abundance, (C,D) leucine incorporation, (E,F) turnover rates of glucose, (G,H) DFAA and (I,J) protein during the incubations of the treatments with additions of agarose, starch, peptone and extracts of *Scenedesmus* and diatoms and a control without any additions of the experiment at the Polar Front

Fig. 2. (A,B) Bacterial abundance, (C,D) leucine incorporation, (E,F) turnover rates of glucose, (G,H) DFAA and (I,J) protein in the experiment in the Coastal Current. See Fig. 1 for more detail

Table 2. Relative proportions of *Bacteria* (enumerated by probe EUB338; mean $\pm$ SD) on Day 4 and the final sampling on Days 9 or 10, in the microcosm experiments at the Polar Front and the Coastal Current in all treatments and the control

	Bacteria (% DAPI cell counts)	
	Day 4	Final sample
Polar Front		
		Day 9
Control	50.8 $\pm$ 13.2	74.0 $\pm$ 13.3
Starch	40.7 $\pm$ 8.5	80.6 $\pm$ 8.6
Agarose	46.7 $\pm$ 14.9	72.8 $\pm$ 16.0
Peptone	63.5 $\pm$ 15.9	92.4 $\pm$ 10.1
Diatoms	72.6 $\pm$ 10.1	88.2 $\pm$ 8.8
<i>Scenedesmus</i>	63.0 $\pm$ 13.8	81.4 $\pm$ 15.4
Coastal Current		
		Day 10
Control	31.1 $\pm$ 3.9	78.8 $\pm$ 14.0
Starch	66.2 $\pm$ 13.1	84.3 $\pm$ 8.6
Agarose	40.2 $\pm$ 8.9	72.8 $\pm$ 17.5
Peptone	60.4 $\pm$ 22.4	80.4 $\pm$ 8.6
Diatoms	74.1 $\pm$ 12.3	84.1 $\pm$ 16.6
<i>Scenedesmus</i>	63.6 $\pm$ 17.1	89.0 $\pm$ 9.9

whereas in that with the diatom extract, *Gammaproteobacteria* were the second most abundant. On Day 9, the composition had changed substantially because in the control and all treatments, except aga-

rose, *Sphingobacteria/Flavobacteria* strongly dominated and constituted 34 to 69% (Fig. 3). For the agarose treatment, only data for *Sphingobacteria/Flavobacteria* are available, constituting only 24%, and for the control, data on *Gammaproteobacteria* are missing. On Day 4, the 3 probes together constituted 46 to 100% of DAPI cell counts with the lowest fractions in the starch and peptone treatments and the highest fraction in that with the diatom extract. Respective cumulative proportions amounted to 60–100%, with highest values in the starch and peptone treatments.

In the CC microcosm experiment, cumulative proportions of the 3 probes were 72% of DAPI cell counts in the agarose treatment and at least 85% in all other treatments and the control (Fig. 3). On Day 4, *Alphaproteobacteria* dominated in the control and the agarose treatment with 58 and 36%, respectively, but on Day 10, *Sphingobacteria/Flavobacteria* accounted for significantly higher percentages than *Alphaproteobacteria*. In the other treatments, *Sphingobacteria/Flavobacteria* constituted highest percentages already on Day 4, ranging from 46 to 59%, and further increased until Day 10, accounting for 72 to 88% of DAPI cell counts (Fig. 3). *Gammapro-*

Fig. 3. Bacterial numbers (% of DAPI cell counts) of *Sphingobacteria/Flavobacteria* (*Sphingo/Flavo*), *Alphaproteobacteria* (*ALF*) and *Gammaproteobacteria* (*GAM*) at the Polar Front and the Coastal Current on Day 4 and at the final sampling of the experiment (Polar Front on Day 9; Coastal Current on Day 10) in the treatments with additions of agarose, starch, peptone (Pept) and extracts of *Scenedesmus* (Scen) and diatoms and a control without any additions

Table 3. Number of DGGE bands at the final sampling of the microcosm experiment at the Polar Front and the Coastal Current in all treatments and the control

	Polar Front	Coastal Current
Control	9	15
Starch	14	14
Agarose	9	14
Peptone	9	14
Diatoms	10	16
<i>Scenedesmus</i>	11	13

teobacteria were the second most abundant group in these treatments, with fractions of 23 to 40% on Day 4 and of 28 to 53% on Day 10. Fractions of *Alphaproteobacteria* decreased from Days 4 to 10 in the control and all treatments and finally constituted <14%.

The DGGE and cluster analysis further substantiated that pronounced differences occurred between the bacterial communities in both experiments and among the various treatments. The number of bands at the termination of the PF experiment ranged from 9 to 14, with lowest numbers in the control and the agarose and peptone treatments and highest numbers in the starch treatment (Table 3, Fig. 4A). In the CC experiment, the number of bands ranged between 13 and 16 at the final sampling, with lowest numbers in the treatment with *Scenedesmus* extract and highest numbers in that with diatom extract. The starch addition yielded almost similar numbers of bands in both experiments. In the control and the other treatments, the number of bands in the CC experiment was consistently higher than in the PF experiment. A cluster analysis showed that the banding patterns differed strongly between both experiments (Fig. 4B). In the PF experiment, the treatments with starch, peptone and the diatom and *Scenedesmus* extract clustered together, with a similarity of the banding patterns of >70%. Banding patterns of the control and the agarose treatment differed strongly from those of the other treatments and also from each other.

Banding patterns of the various treatments in the CC experiment exhibited 2 subclusters, one with a similarity of >65%, including peptone and the *Scenedesmus* and diatom extract, and another with the other treatments and a similarity of >70%.

A total of 22 bands were excised and sequenced. Six sequences affiliated to *Flavobacteria* (ANT-PF1, -PF18, -CC6, -CC7, CC16 and -CC20), 2 to *Cytophagales* of the *Bacteroidetes* phylum (ANT-PF19 and -CC8) and 5 to the *Roseobacter* clade of *Alphaproteobacteria* (ANT-PF2, -PF-3, -PF9, -PF10 and -PF22). Further, 9 sequences affiliated to *Gammaproteobacteria*, of which 4 belonged to the *Pseudoalteromonadaceae* (ANT-PF4, -PF13, -PF14 and -PF15), 3 to the *Colwellia* cluster of *Alteromonadaceae* (ANT-PF5,

Fig. 4. (A) DGGE banding patterns and (B) cluster analysis of bacterial communities at the final sampling of the experiment at the Polar Front (P) on Day 9 and in the Coastal Current (C) on Day 10 in the treatments with additions of agarose, starch, peptone and extracts of *Scenedesmus* (Scen) and diatoms and a control without any additions. Std: standard for the DGGE analysis. * and numbers indicate excised bands that were sequenced

Table 4. Clone ID, origin, sequence length and accession number of the phylotypes retrieved from the DGGE bands of the 2 experiments and treatments and the next related sequence's name, accession number, similarity (%), of the retrieved phylotypes, affiliation and location. Numbers after ANT-PF/CC refer to numbers of the excised bands in the DGGE banding patterns of Fig. 4. Uncult.: uncultured

Clone ID	Origin	Sequence length (bp)	Accession no.	Next relative	Accession no.	% similarity	Affiliation	Location
ANT-PF1	Control	390	JQ728968	<i>Polaribacter</i> sp. IC063	U85885	98	<i>Bacteroidetes</i>	Antarctic sea ice
ANT-PF2	Control	541	JQ728969	Uncult. bacterium clone F9P2610_S_P23	HQ672235	99	<i>Alphaproteo</i>	Subarctic Pacific
ANT-PF3	Control	406	JQ728970	Uncult. bacterium clone SH100_100m_clone_48	HQ173757	97	<i>Alphaproteo</i>	Continental shelf of Oregon
ANT-PF4	Control	549	JQ728971	<i>Pseudoalteromonas</i> sp. BS120577	DQ492776	99	<i>Gammaproteo</i>	Arctic sea ice
ANT-PF5	Control	482	JQ728972	Uncult. gammaproteobacterium	AB496536	97	<i>Gammaproteo</i>	Deep sea hydrothermal vent field
ANT-PF13	Diatom	535	JQ728973	<i>Pseudoalteromonas</i> sp.	JN864014	99	<i>Gammaproteo</i>	Arctic sediments
ANT-PF14	Diatom	534	JQ728974	<i>Pseudoalteromonas</i> sp.	JF273879	99	<i>Gammaproteo</i>	Sponge-assoc. microbial Antarctic communities
ANT-PF15	Diatom	533	JQ728975	<i>Pseudoalteromonas</i> sp.	HM771249	99	<i>Gammaproteo</i>	Arctic invertebrate gut
ANT-PF18	Agarose	543	JQ728976	<i>Polaribacter</i> sp. BSW20012b	FJ196065	99	<i>Bacteroidetes</i>	Arctic sea water
ANT-PF19	Agarose	534	JQ728977	Uncult. bacterium clone SS1_B_01_16	EU050905	99	<i>Bacteroidetes</i>	Arctic sediment
ANT-PF27	Starch	535	JQ728978	<i>Colwellia</i> sp. BSs20151	EU365514	99	<i>Gammaproteo</i>	Arctic sediments
ANT-PF28	Starch	581	JQ728979	Uncult. bacterium clone SF-Oct-45	HQ203828	97	<i>Gammaproteo</i>	Seawater
ANT-CC6	Control	536	JQ728958	Uncult. <i>Polaribacter</i> sp.	JN177606	99	<i>Bacteroidetes</i>	Arctic Ocean, 10 m depth
ANT-CC7	Control	533	JQ728959	Uncult. marine bacterium	GU235590	99	<i>Bacteroidetes</i>	Antarctic sea water
ANT-CC8	Control	337	JQ728960	Uncultured bacterium clone 2D_F10	EU734060	98	<i>Bacteroidetes</i>	Phytoplankton bloom Scripps Pier
ANT-CC9	Control	503	JQ728961	Antarctic seawater bacterium COL-68	HQ534354	99	<i>Alphaproteo</i>	Antarctic sea water
ANT-CC10	Control	540	JQ728962	Uncult. <i>Loktanella</i> sp.	JN177605	99	<i>Alphaproteo</i>	Arctic Ocean
ANT-CC11	Control	377	JQ728963	Uncult. marine bacterium SHFB994	GU235324	98	<i>Gammaproteo</i>	Antarctic sea water
ANT-CC12	Control	553	JQ728964	Uncult. gammaproteobacterium NABOS_AGGBact20	EU544853	98	<i>Gammaproteo</i>	Aggregate, arctic seawater
ANT-CC16	Agarose	481	JQ728965	<i>Polaribacter irgensii</i> strain ANT9243	AY167325	99	<i>Bacteroidetes</i>	Antarctic pack ice
ANT-CC20	Peptone	533	JQ728966	Uncultured bacterium clone 44F2	JF827412	98	<i>Bacteroidetes</i>	Pacific oyster, Tasmania
ANT-CC22	Peptone	538	JQ728967	Antarctic seawater bacterium COL-68	HQ534354	99	<i>Alphaproteo</i>	Antarctic sea water

-PF27 and -PF28) and 2 to other lineages of *Gammaproteobacteria* (ANT-CC11 and -CC12) (Table 4, Fig. 5). In most cases, the next related phylotypes originate from Antarctic, Arctic, subarctic, upwelling and deep sea habitats.

Several bands appeared in all treatments of a given experiment, such as in the PF experiment bands ANT-PF1 and -PF18 and in the CC experiment bands ANT-CC7, -CC16, ANT-CC9, -CC22 and ANT-CC10, although with different intensities (Fig. 4A). Other bands were present only in a few treatments, such as band ANT-PF4 in the control and the treatment with diatom extract. Similar bands in both experiments appeared only in the treatment with the diatom extract, with bands ANT-PF13, -PF14 and -PF15.

DISCUSSION

Response of bacterial growth and substrate turnover to biopolymer additions

Micro- or mesocosm experiments are a well established approach to examine the response in growth, substrate turnover and composition of bacterioplankton communities to altered substrate regimes relative to ambient conditions. In most cases, either a phytoplankton bloom was experimentally induced by the addition of inorganic nutrients or by inoculation with distinct phytoplankton species (e.g. Lebaron et al. 1999, Riemann et al. 2000, Murray et al. 2007) or selected simple inorganic nutrients and/or organic substrates, such as glucose and/or DFAA, were applied (e.g. Church et al. 2000, Massana et al. 2001, Ducklow et al. 2011, Gómez-Consarnau et al. 2012). More than 2 treatments with different organic compounds were rarely applied, such as dissolved protein and starch (Pinhassi et al. 1999) or other simple

Fig. 5. Phylogenetic trees of *Gamma*- and *Alphaproteobacteria* as well as *Flavobacteria* and the *Cytophagales* cluster of the *Bacteroidetes* phylum calculated with bootstrap (2000') based on 16S rRNA gene fragments. Sequences obtained in the present study in **bold**. The scale bar indicates the percentage of the sequence divergence

organic acids and amino acids (Gómez-Consarnau et al. 2012). To examine how bacterioplankton communities in the Southern Ocean adapt to various biopolymers potentially available to these communities and in higher concentrations than monosaccharides and DFAA (Simon & Rosenstock 2007), we applied agarose, starch, peptone and extracts of *Scenedesmus* and phytoplankton from the PF dominated by diatoms. The growth response in the CC experiment was more pronounced than in the PF experiment, as reflected in bacterial numbers, leucine incorporation and substrate turnover rates, despite the lower temperature. The conditions controlling bacterioplankton growth appeared to favor bacterial growth and substrate turnover more in the CC with lower phytoplankton biomass than at the PF. These conditions may have included protozoan grazing, phage infection and limitation by inorganic nutrients, which were not assessed during the experiments.

Massana et al. (2001) examined the growth response of bacterioplankton communities to additions of acetate and glucose at various locations in the Southern Ocean, including the polar frontal region and ice edge, but found little difference among the various locations. However, strong differences of the growth response of bacterioplankton communities to additions of glucose and DFAA were reported between the Subtropical and Polar Front by Church et al. (2000). At the Subtropical Front, bacterial growth was strongly enhanced by these substrates, but growth was not enhanced at all at the PF.

With respect to the individual substrate additions in our experiments, starch supported growth better than agarose at both locations, as reflected by bacterial numbers, leucine incorporation and glucose turnover. However, turnover rates of DFAA and protein did not co-vary with the former parameters and thus appear not to reflect the carbohydrate metabolism of the bacteria but rather their amino acid metabolism. Agarose and starch differ with respect to their glycosidic bond and monosaccharide building blocks. The trait of cleaving the α -glycosidic bond of starch and taking up the cleaved glucose appears to be more widespread in the bacterioplankton communities in the Southern Ocean than that of cleaving the β -glycosidic bond of agarose and taking up its building block galactose. Galactose constitutes only 9 to 22 mol% of dissolved combined monosaccharides in the Southern Ocean as compared to glucose with at least 35 mol% but often 45 to 73 mol% (Kirchman et al. 2001, Simon & Rosenstock 2007). It appears to be a more general phenomenon that bacterioplankton prefer glucose relative to galactose as

turnover rates of glucose are 2- to 3-fold higher than those of galactose (Bunte & Simon 1999).

With respect to the complex substrate additions, peptone and the diatom extract supported bacterial growth much better than the *Scenedesmus* extract, starch or agarose in both experiments, as reflected by bacterial abundance, leucine incorporation and glucose turnover but not by that of DFAA and protein, except for the latter in the PF experiment. The growth response to the addition of the *Scenedesmus* extract was similar to that of starch. A possible reason for the lower bacterial growth yield on the *Scenedesmus* extract may be that the C:N ratio of this alga was presumably rather high. The C:N ratio varies between 7 and 25, depending on the N-limitation of the alga (Adams & Sterner 2000). Our extract was from the stationary phase of a culture growing under N-limitation, thus being of lower quality, i.e. C and N supply was not in balance for protein synthesis. In contrast, the C:N ratio of the peptone and diatom extract presumably was lower and closer to the requirements for protein synthesis. The C:N ratio of peptone is ~ 3 (Zapata-Vélez & Trujillo-Roldán 2010), and that of particulate organic matter from the PF dominated by diatoms is ~ 7 (Rubin 2003). Our observation of an enhanced growth response of the bacterioplankton to the additions of peptone and the diatom extract relative to starch and agarose is in line with results of Pinhassi et al. (1999). These authors found that dissolved protein (bovine serum albumin) supported bacterioplankton growth in the Southern California Bight much better than starch. Polysaccharides, such as starch and agarose, are only a C source for bacteria and need a complementation of an N source, such as ammonium, for amino acid and protein synthesis, provided that the bacteria are capable of cleaving the glycosidic bond and taking up the mono- and/or oligosaccharides. Our observations of the enhanced bacterial growth response to the addition of peptone and the diatom extract relative to the other substrate additions is also consistent with findings of Simon & Rosenstock (2007). These authors found that bacterioplankton production in the Southern Ocean is much more closely correlated to concentrations and incorporation of dissolved protein than to concentrations of dissolved carbohydrates. Peptone and dissolved proteins are basically identical substrates, and 45 to 60 % of the biomass of diatoms consists of protein (Renaud et al. 1999). Our results do not provide any evidence that the enhanced concentrations of the added extracts of diatoms and *Scenedesmus* favored growth more than the other substrates. The diatom extract and peptone

additions yielded the highest response, whereas the *Scenedesmus* extract yielded a lower response similar to that of starch.

We note that in the control of both experiments, the leucine incorporation and substrate turnover rates increased toward the end, and the composition of the bacterial community changed relative to Day 4. This indicates that even without substantial changes in bacterial numbers, the metabolic activities and structure of the bacterial communities underwent alterations, possibly induced by mortality, such as grazing and phage infection, and by recycling of the nutrients released by these cell lysing processes.

The experiments were run without replicates because of various constraints. However, the general outcome of both experiments was similar. Further, in other comparable experiments, including those carried out in the Southern Ocean, replicates usually agreed fairly well (Pinhassi et al. 1999, Riemann et al. 2000, Massana et al. 2001, Ducklow et al. 2011). Therefore, we assume that the pronounced differences among the various treatments in our experiments are sound and much greater than just among replicates of a single treatment.

Response of the bacterial community composition to biopolymer additions

Enrichments with simple and/or complex substrates have been applied for more than a decade to examine how bacterioplankton communities respond structurally to growth conditions differing from the ambient situation. Massana et al. (2001) and Ducklow et al. (2011) found that the addition of glucose and acetate favored growth of distinct populations of *Flavobacteria* and *Gammaproteobacteria* in the Southern Ocean. Abell & Bowman (2005b) examined the decomposition of diatom-derived detritus by bacterial communities in the Southern Ocean at the Polar Front, the ice edge and in pack ice and reported that *Flavobacteria* were the main decomposers at the Polar Front, whereas *Gammaproteobacteria* dominated at the other locations. *Flavobacteria* and *Sphingobacteria* were most responsive during an experimentally induced diatom bloom in the Southern California Bight of the Pacific (Riemann et al. 2000). At the same location, populations of *Cytophagales* and *Flexibacteriales* were the most responsive bacterial lineages of the entire community when grown on protein (Pinhassi et al. 1999), whereas starch did not support growth of these bacterial communities at all.

The FISH and DGGE data and the cluster analysis of our results show that the bacterial communities at the PF and in the CC under pack ice underwent changes during growth on the various substrates and exhibited distinctly different community structures as a result of the response to different substrate regimes. In both experiments and all treatments, there was a general trend to decreasing proportions of *Alphaproteobacteria* during the incubation and to a dominance of *Bacteroidetes* and more specifically *Flavobacteria* and *Cytophagales*, followed by *Pseudoalteromonadales* and members of the *Colwellia* cluster of *Gammaproteobacteria*. This dominance was most pronounced in the CC experiment and the treatments with the most active growth and highest bacterial numbers, i.e. all treatments except the agarose addition and the control. Despite this general similarity between both experiments, they exhibited distinct differences with respect to the detected bacterial populations of the 3 major phylogenetic groups. The number of DGGE bands in the PF experiment was consistently lower than in the CC experiment, and the detected phylogenotypes of all 3 major phylogenetic groups clustered well separately for both experiments except in 2 cases of *Flavobacteria* and *Cytophagales*. The PF experiment exhibited a higher diversity, i.e. number of DGGE bands, of *Gammaproteobacteria* compared to the CC experiment, whereas the latter exhibited a higher diversity of *Flavobacteria* and *Cytophagales*. These results of distinct adaptations are in line with previous observations from mesocosm experiments with additions of simple and complex substrates but provide a much more detailed picture with respect to the polymeric substrates applied and the bacterial community structure.

Flavobacteria and *Sphingobacteria* are known to be able to consume polymeric substrates (Kirchman 2002) and to be superior to *Gamma*- and *Alphaproteobacteria* in consuming protein (Cottrell & Kirchman 2000). The former phylogenetic lineages constitute high proportions of the bacterioplankton in the Southern Ocean, often exceeding *Gamma*- and *Alphaproteobacteria* (Simon et al. 1999, Abell & Bowman 2005a, Straza et al. 2010). Straza et al. (2010) showed that *Flavobacteria* and *Sphingobacteria*, together with *Gammaproteobacteria*, dominate the consumption of dissolved protein around the Antarctic Peninsula. Hence, the results of our study are in line with these observations but extend them by showing that members of *Flavobacteria* and *Cytophagales* and of *Pseudomonadales* and the *Colwellia* cluster of *Gammaproteobacteria* can grow on a great variety of complex substrates in the

Southern Ocean, although with different growth rates and yields. Our observations shed light on the great flexibility of these phylogenetic lineages in their substrate spectra and help to explain their success in this globally coldest oceanic region. Most bands in the DGGE banding patterns were present in all treatments of a given experiment, and the number of bands did not systematically decrease relative to the control. These results are in contrast to those of Ducklow et al. (2011), who found that in glucose amended mesocosms, the bacterial diversity became reduced to <50% of the number of phylogenotypes of the ambient bacterioplankton community after 10 d near Palmer station at the Antarctic Peninsula. We are aware of the fact that DGGE only detects phylogenotypes with a proportion of ~1% of the bacterial community (Muyzer et al. 1993), thus missing a considerable fraction of the less abundant members of the community. Two recent studies, carried out in the Southern Ocean, compared the DGGE approach with approaches of higher sensitivity in bacterial community analysis, such as pyrosequencing (Ghiglione & Murray 2012), length heterogeneity polymerase chain reaction and capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) (Ducklow et al. 2011). In both studies, cluster analyses of the DGGE and the other approaches yielded similar results, thus indicating that DGGE is a valid approach to analyze the bacterioplankton community composition.

Under conditions of low substrate availabilities, e.g. non-phytoplankton bloom conditions, *Alphaproteobacteria* and in particular members of the SAR11 and *Roseobacter* clades, such as the RCA and NAC11-7 clusters, can constitute proportions of similar or even higher magnitude than *Flavobacteria* and *Gammaproteobacteria* (West et al. 2008, Giebel et al. 2009). Such conditions existed at both locations where samples for the experiments were collected, as the SAR11 clade and the RCA cluster constituted ~30% of *Bacteria* in the ambient bacterioplankton community (Giebel et al. 2009). These conditions were still reflected in the PF experiment in the control and the treatments with agarose and starch and in the CC experiment in the control and the treatment with agarose on Day 4, when bacterial numbers were still low, as *Alphaproteobacteria* constituted similar or higher proportions than the other 2 phylogenetic groups.

Grazing and phage infection may have also affected dynamics of bacterial growth and community composition in the various treatments of both experiments because we used unfiltered water

samples. A detectable impact of grazing and phage infection in a microcosm approach requires a very active initial bacterial community, a growth response of the bacterial community or, if selective mortality occurs, mortality of the bacterial target groups. The initial bacterial communities had a bulk generation time of 9 to 10 d and thus cannot be considered as very active. *Flavobacteria* and *Sphingobacteria* were most responsive in both experiments, followed by *Gammaproteobacteria*, which increased in relative abundance in all treatments of both experiments except in treatments with the diatom extract. In contrast, *Alphaproteobacteria* decreased in relative abundance in all treatments between Day 4 and the end. These findings are in line with other experimental and field observations (see section 'Response of the bacterial community composition to biopolymer additions'). Therefore, we assume that grazing and phage infection did not affect our general results on the bacterial community composition even though we cannot rule out a minor influence on the bacterial growth response, in particular toward the end of both experiments.

CONCLUSIONS

Our experiments showed that bacterioplankton communities at the PF and in the CC exhibited distinct responses to the addition of various biopolymers, thus supporting our hypothesis of different growth responses of these communities to simple (C only) and complex (C + N) biopolymeric substrate additions. In both experiments, complex substrates and in particular peptone and a diatom extract supported growth best, whereas agarose supported growth least. *Flavobacteria* and *Cytophagales* of the *Bacteroidetes* phylum and *Pseudomonadales* and the *Colwellia* cluster of *Gammaproteobacteria* were the major phylogenetic lineages proliferating on these biopolymeric substrates. However, even in the treatment with agarose and the lowest growth response and in the control, shifts in the bacterial community structure toward the mentioned phylogenetic lineages occurred. These results indicate that bacterioplankton communities in the Southern Ocean are able to adapt to a great variety of substrates. Our findings shed further light on observations that distinct lineages of *Flavobacteria* and *Cytophagales* and of *Gammaproteobacteria* are the main players in organic matter decomposition during events of high inputs of polymeric substrates, such as during phytoplankton blooms and pack ice melting.

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