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Ecological and biogeographic relationships of class Flavobacteria in the Southern Ocean

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Abstract

The abundance, spatial distribution and diversity of class Flavobacteria were investigated in the Southern Ocean euphotic zone across a latitudinal transect and in the ice pack off Eastern Antarctica. Surface seawater samples filter-fractionated into 0.8 μm particulate and 0.2 m planktonic fractions were investigated with different molecular techniques. The abundance of particle-associated Flavobacteria, ascertained with real-time PCR and DGGE band analysis using Flavobacteria-specific primers, was found to be significantly higher in Polar Front Zone (PFZ) and Antarctic Zone (AZ) water samples than in nutrient limited Temperate Zone (TZ) and Sub-Antarctic Zone (SAZ) waters. Abundance of particle-associated Flavobacteria correlated positively with seawater chlorophyll *a* and nutrient concentrations, suggesting that increased Flavobacteria abundance may relate to enhanced primary production in the PFZ and AZ. This is supported by comparison of DGGE profiles that demonstrated significant differences in the total Flavobacteria community structure and 16S rRNA gene diversity between samples from the PFZ and AZ and those from TZ and SAZ. Sequence analysis revealed a broad diversity amongst class Flavobacteria in the Southern Ocean with several Flavobacteria clades detected in PFZ and AZ waters not detected in TZ and SAZ waters that putatively represent psychrophilic taxa. Sequence data included a large, so far uncultivated, cosmopolitan phylogenetic clade (''DE cluster 2'') that is distributed throughout the Southern Ocean.

Keywords: Seawater; Southern Ocean; DGGE; Flavobacteria; Cytophaga–Flavobacterium–Bacteroides group; Biogeography; Psychrophilic bacteria

1. Introduction

Bacterial assemblages mineralize approximately one-half of the carbon fixed photosynthetically in the marine environment [1,2]. Bacterial biogeochemical cycling thus can significantly determine the potential productivity that can be supported within the marine habitat. Heterotrophic marine bacteria play an important role in pelagic foodwebs [3]; for example heterotrophic bacteria redisperse silica [4] and can produce iron-binding ligands [5] and can significantly affect proliferation of phytoplankton. Fluorescent in situ hybridisation (FISH) analyses indicate that within the surface ocean the bacterioplantonic community includes mostly members of the phylum Proteobacteria and the phylum ''Bacteroidetes'', more commonly referred to as the Cytophaga–Flavobacterium–Bacteroides (CFB) group [6].

Greater knowledge of the CFB group in seawater would help in improving understanding of marine foodwebs and carbon biogeochemistry, especially if specific associations between taxa and chemical and physical conditions can be established. The CFB group represents a major biomass component in both seawater and sea-ice, typically representing 10–40% of prokaryote cells as measured by FISH, while in clone libraries they

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usually represent 5–20% of clones [6,7]. Recent analyses of coastal waters off Plymouth, UK [8] and Delaware, USA [7], using different CFB-specific and CFB subgroup specific probes and PCR primers, revealed significant diversity within the phylum at a resolution not usually available if only bacterial universal primers were employed. These studies indicated that a very high proportion of CFB group taxa belong to the class Flavobacteria, mostly in family Flavobacteriaceae and in various lineages generally represented by the particulate-associated clone agg58 [8,9].

Flavobacteriaceae, the clone agg58 group and the recently described family Cryomorphaceae [10] and can be detected with a specific oligonucleotide probe [11]. FISH and clone library studies have consistently indicated that CFB group members are abundant in marine aggregates, which encompasses sinking organic detritus from a few microns to several mm in width [12]; in the water column [6–8]; sea-ice [13,14]; surface benthos [15,16]; and in epibenthic fauna [17]. In algal blooms CFB group members can constitute as much as 70% of the total bacteria possibly indicating an opportunistic response to the organic carbon present, which corresponds to their suspected role as major mineralizers of organic matter [18]. Recent mesocosm studies indicated CFB group diversity was highest at the mid-point of a primary productivity gradient, as found for aquatic animals and plants [19], while Proteobacteria responded differently or not at all [20]. This relationship may relate to shifting balances between competition for colonization sites, nutrient acquisition and predation.

The goals of this study were to examine the abundance, distribution and diversity of class Flavobacteria in the Southern Ocean and to explore relationships between the Flavobacteria community and various variables including particulate matter, temperature, nutrient availability and oceanographic water masses. A number of molecular techniques were used to examine these relationships, including fluorescent in situ hybridisation, denaturing gradient gel electrophoresis (DGGE), real-time PCR and sequence analysis.

2. Methods

2.1. Seawater sampling

Seawater samples were obtained from two separate transects across different Southern Ocean zones at 9 stations (no. 4–13, Fig. 1) in the ice pack off Eastern Antarctica (61.12–69.18° S 62.55–98.52° E, water temperature ranged from −1.4. to 0.7 °C) and from one station in the Polar Front Zone (PFZ) (station 14, 52.54° S 124.36° E, water temperature 5.6 °C) during December 2000. A latitudinal transect of the Southern Ocean was performed during November 2001 with sam-

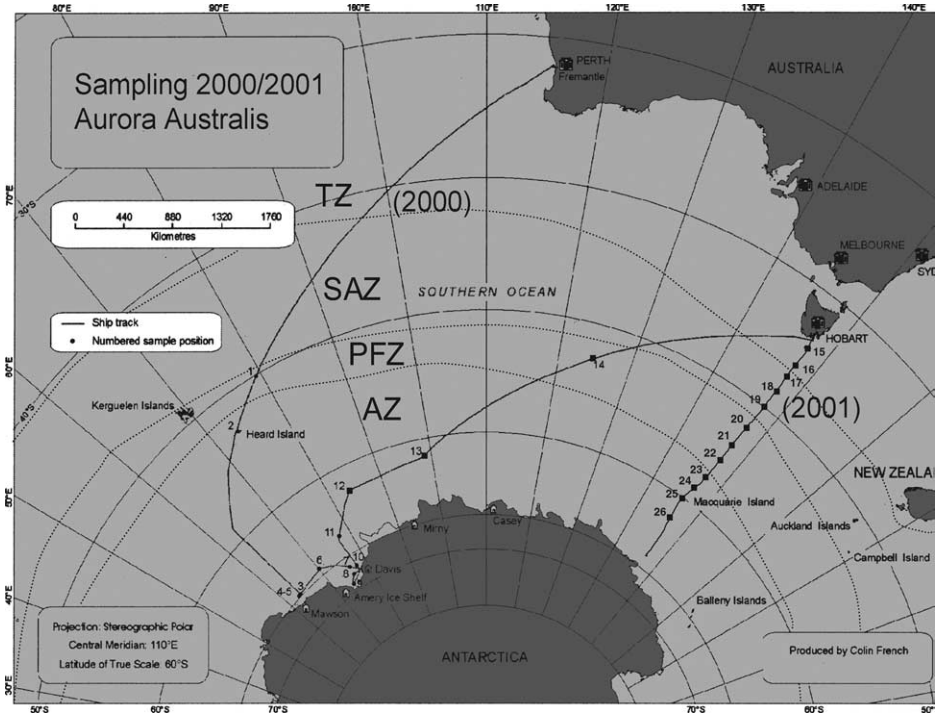

Fig. 1. Location of seawater sampling stations within the Southern Ocean over two different transects. Stations were assigned to different surface water masses based on hydrographic characteristics as defined by Deacon [49], including the Temperate Zone (TZ); the Sub-Antarctic Zone (SAZ), located between the Subtropical Convergence and the Polar Front; the Polar Front Zone (PFZ), location of the Antarctic Circumpolar Current; and the Antarctic Zone (AZ).

ples collected from 12 stations (no. 15–26, Fig. 1) between latitudes 44.7° S to 63.5° S along longitude 142 (±2)° E. Water temperature was 13–14 °C for samples collected from stations 15 and 16; 8–12 °C for stations 17–19, 2–6 °C for stations 20–22; and 1 to −1.4 °C for stations 23–26. Water samples were obtained from a depth of 10 m using an underway water sampler aboard the *RSV Aurora Australis* and were stored for less than 1 h in 25 l sterile carboys before processing. Biomass for DNA extraction was collected by filtering 1–5 l of water through a 0.8 μm pore size polycarbonate filter (Millipore) and then a 0.2 μm polycarbonate filter. Both the 0.8 and 0.2 μm filters were immediately stored at −20 °C before processing. To minimize the retention of free-living bacteria, the 0.8 μm filters were washed with approximately 1 l of filtered, autoclaved seawater using gentle filtration. Seawater chlorophyll *a*, nitrate, phosphate and silicate concentrations were measured as previously described [21].

2.2. Fluorescent in situ hybridisation

Internal cellular rRNA levels in various seawater samples were augmented by the addition of an equal volume of a low nutrient seawater medium [16] supplemented with 50 mg l^{-1} chloramphenicol to samples within the filter manifold, followed by incubation for approximately 2 h at in situ temperatures (usually 0–10 °C). Subsequent microscopic analysis indicated negligible cell division during incubation. Following incubation, samples were filtered and washed with sterile phosphate-buffered saline solution (PBS; 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4; pH 7.0). Samples for FISH analysis were prepared in triplicate, with 50 ml of sample filtered through 47 mm 0.2 μm black isopore polycarbonate membrane filters (Millipore), which were fixed and stained with oligonucleotide probes as previously described [22]. Cy3-labelled oligonucleotide probes (Geneworks, Australia) used included Eub338 (Bacteria), Alf968 (α-proteobacteria), Gamm42a (γ-proteobacteria) [22] and Flavo1 (class Flavobacteria) [11]. Following hybridization, filters were washed and stained for 15 min at 48 °C on a heating block in 5 ml of wash buffer [11] containing 2 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI). The filters were dried, mounted on a glass slide with immersion oil and inspected, with digital pictures taken using a LDRMBE Leitz microscope (Leica, Heerbruug, Switzerland) fitted with a DC300F digital camera (Leica), equipped with a 50 W HBO mercury bulb (Osram, Regensburg, Germany) and specific filter sets for DAPI and Cy3 fluorophores. Fields were first viewed with the Cy3 filters before switching to the DAPI filters, to minimize photo-bleaching of the Cy3 fluorophore during DAPI examination. Cell counts for DAPI and each of the probes were determined from 10 to 15 random non-overlapping fields [23] and performed on at least two different hybridisations of filter sections. A range of bacterial pure cultures served as positive and negative controls. A number of samples had very poor FISH labeling, regardless of probe used, possibly due to extremely low cellular rRNA levels, and were excluded from the analysis.

2.3. DNA extraction and purification

Extraction of DNA from filtered samples followed a modification of a method employed by Furhman et al. [24]. Frozen filters were thawed, cut into small strips with a sterile razor blade and resuspended in 3 ml of STE buffer (100 mM NaCl, 10 mM Tris–HCl; 1 mM disodium EDTA; pH 8.0) in a sterile 15 ml centrifuge tube (Falcon, USA) and vortexed to detach biological material. To lyse cells, sodium dodecyl sulphate (SDS, final concentration 1% wt vol^{-1}) was added and the filter was held at 100 °C for 2 min., cooled on ice and then washed with an additional 1 ml of STE buffer and treated with SDS as before. The combined lysates were extracted twice with phenol: chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated by adding two volumes of cold absolute ethanol and 0.2 volumes of 3 M sodium acetate (pH 8.0) to the aqueous layer, storing overnight at −20 °C and centrifuging in a 50 ml centrifuge tube (Nalgene, Rochester, USA) for 30 min at 15,000*g* at 4 °C. The pellet was washed with 70% (vol./vol.) ethanol and centrifuged again. Residual liquid was removed and the air-dried pellet was resuspended in sterile water. DNA extracts were visualised on 1% (wt./vol.) agarose gels containing 10 μg ml^{-1} ethidium bromide using UV transillumination.

2.4. Real-time PCR

Real-time PCR reactions were prepared using the QuantiTect SYBR Green PCR kit (Qiagen, USA), in 200 μl flat lid PCR tubes (Axygen Scientific, Union City, USA) and a Rotorgene thermocycler (Corbett Research, Australia). Results were analysed with Rotorgene software (V. 4.6). Reactions (20 μl volumes) contained 10 μl of 2x QuantiTect SYBR Green PCR Master Mix, 200 μM of each primer, 0.1–10 ng of template DNA and UV-treated water. Primers used for amplification and detection of class Flavobacteria 16S rRNA genes included 558f (5′-ATT GGG TTT AAA GGG TCC-3′) [11] and 907r (5′-CCG TCA ATT CCT TTG AGT TT-3′) [25]. Assays were performed using a thermocycling program consisting of an initial 15 min, 95 °C step followed by 35 cycles consisting of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s with fluorescent acquisition, and a further fluorescent acquisition step at 80 °C. Total bacterial 16S rRNA gene amounts were estimated with primers 519f (5′ CAG CMG CCG CGG TAA TAC

3′) [25] and 907r. Fluorescence analysis was performed at a temperature at which all primer dimer had melted, but the specific product had not. Amplified products from mixed template samples only contained a single peak indicating that product length variability and G + C content did not have a significant effect on quantification. Positive control standards for the real-time PCR assay included 16S rRNA genes amplified with primers 558f/907r from *Gelidibacter algens* and *Cryomorpha ignava*. The controls were diluted in 10-fold series from 1 ng to 0.1 pg and analysed in parallel with seawater DNA samples. Negative controls included samples lacking template DNA and samples lacking primers. Primer concentrations used in the assay were determined by running a dilution series of primer including positive and negative controls (in the case of specific primers) and sample templates. The optimal primer concentration chosen was the minimum primer concentration required to achieve maximum end-point fluorescence. All real-time PCR products were examined using agarose gel electrophoresis to ensure products corresponded to the correct size and to ensure the absence of non-specific product. Experimental samples were diluted to three different concentrations and compared to the standard curve in the same run. Values were corrected for their dilution factor and averaged to calculate the total 16S rRNA amount in the starting material and then compared to the same sample analysed with the Flavobacteria specific primers to give a percent fraction of class Flavobacteria 16S rDNA to total bacterial 16S rDNA.

2.5. DGGE

Regions of the 16S rRNA gene were amplified using the universal primer set 907f (5′-AAA CTC AAA GGA ATT GAC GG-3′) [25] and a GC-clamped reverse primer 1392rc (5′-CGC CCG CCG CGC CCC CGC CCG GCC CGC CGC CCC CGC CCC ACG GGC GGT GTG TAC-3′) [26] and a Flavobacteria specific primer set, 558f and 1392rc. PCR amplification was performed using Advantage2 Taq (Clontech, Heidelberg, Germany) and 20 mM deoxynucleotide triphosphates (dNTPs), 10 ng genomic DNA and 25 pmol of both primers, in reactions made up to a volume of 50 μl with sterile MilliQ water. PCR was performed using a touch down protocol on a PTC-200 DNA Engine thermocycler (MJ Research, USA), with 21 0.5 °C steps from 65 to 55 °C, with each step including 2 min, 72 °C extension and a 1 min, 95 °C denaturation steps; and completed with a final 4 min, 72 °C extension step. Concentration of genomic DNA added to PCR reactions was varied to optimise the clarity of DGGE gels. For the FS-specific primer set, this often involved using significantly more template DNA (20–30 ng) to obtain clear banding patterns.

DGGE was performed using the D-code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA) using parallel 6% polyacrylamide gels containing a 20–55% denaturing gradient. Approximately 25 μl of PCR product and 5 μl of gel loading dye were loaded and the gels run at 60 °C for 16 h. Gels were stained with 10,000 × SYBR-gold nucleic acid stain (Molecular Probes, Eugene, USA) and imaged using a UV transilluminator equipped with the MP4+ Instant Camera System (Polaroid, Waltham, USA) with an attached SyberGold filter. To reduce variation between individual gel runs, PCR and DGGE were performed twice for all samples and the data pooled [27]. For sequence analysis, two or more bands that had migrated to the same vertical positions on the gel were excised using sterile scalpel blades. DNA was eluted by soaking gel slices in 200 μl of sterile water for 30 min and then in 200 μl of STE buffer overnight at 37 °C. The DGGE band DNA was then reamplified using 558f/1392rc or 907f/1392rc primers with 1 μl of the band eluant used in the PCR reaction setup with the Hotstart PCR Kit (Qiagen). PCR was performed with an initial 15 min, 95 °C step, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, with a final 4 min, 72 °C step. A 10 μl volume of the amplified DNA was re-run on a DGGE gel and compared to the original DGGE band profile from which the band was excised to ensure correspondence to the original band, before elution of the band and reamplification as indicated above, purification and sequencing. Sequences were checked between bands migrating to the same gel positions from different samples and it was found that these differed by more than 0.1% in 12 cases and included mostly bands obtained by amplification with the bacteria-specific primers.

2.6. Analysis of DGGE banding patterns

DGGE analysis followed procedures described by Powell et al. [27]. DGGE banding patterns based on the Flavobacteria-specific primer set showed little variation between replicate gels ($p < 0.05$). For banding profile comparisons it was assumed single DGGE bands contained a single taxon and sequencing distinct DGGE bands revealed few examples of highly similar sequences (maximum likelihood distance <0.01) from different band locations within the gel. Bands migrating to the same distance (within ±1 mm) in different lanes of the same gel were also assumed to contain the same or very similar sequences (as explained above this was the case). DGGE bands were recorded in a presence/absence matrix by examining results of image analysis utilizing the Diversity Database program (Version 2.2.0, Bio-Rad) and by visual inspection of negative gel images. A band was scored positive if it exceeded minimum threshold intensity relative to the background of the whole gel

by at least 15% and corresponded to a band observable by eye. Samples producing faint DGGE profiles were reanalysed to increase band intensity. Duplicate DGGE data for each sample were pooled for analysis as described by Powell et al. [27]. DGGE banding patterns were analysed with the Primer5 v. 5.2.4 (Plymouth Marine Laboratory, UK) [28] program as detailed previously [27] using non-metric multidimensional scaling (nMDS) and one-way univariate analysis of similarity (ANOSIM) [29].

2.7. Sequencing and phylogenetic analysis

Sequencing of PCR products amplified from DGGE bands was carried out using CEQ DTSC Quick Start kits and the Beckman CEQ2000 automated DNA sequencer (Beckman–Coulter, Berkeley, USA). The electrophoretograms were manually checked and sequence data imported into a database using the BioEdit program [30]. Only sequences of greater than 301 bases in length were included in phylogenetic analyses. Bacteria and Flavobacteria primers generated sequences of 301–347 and 360–686 nucleotides, respectively. Sequences were compared to sequences in the GenBank database using Blast-n searches (http://www.ncbi.nlm.nih.gov/blast). Sequences from this study were then aligned to reference sequences obtained from GenBank using Bioedit and Clustal W [31]. Sequence maximum likelihood distance matrix and neighbor-joining analysis was performed using PHYLIP v. 3.6 [32] as previously described [16]. Distinct phylotype sequences from this study were deposited under GenBank accession codes AY457085–AY457140, AY496855–AY496860, AY575779 and AY661611–AY661641. Sequence phylotypes were defined as a collection of highly related sequences with a maximum likelihood distance of <0.01.

3. Results

3.1. Nutrient, chlorophyll *a* and abundance measurements

Chlorophyll *a* values were >1 $\mu g l^{-1}$ south of Tasmania (Figs. 1 and 2(a)) and rapidly declined at the Subtropical Convergence (at approximately 46–47°S), accompanied by very low nitrate, phosphate and silica levels (Fig. 2(a)). Further south, nutrient levels and chlorophyll *a* increased in concentration though chlorophyll *a* levels only increased slowly through the PFZ and the Antarctic Zone (AZ). From 9% to 32% of the DAPI direct count ($1.0–6.8 \times 10^5$ cells ml^{-1}) was detected by FISH with the bacteria-specific Cy3-labelled EUB338 probe even after chloramphenicol treatment [33]. In AZ samples (Fig. 1), 25–32% of cells bound to the EUB338 probe. α-Proteobacteria and γ-proteobacteria together represented 32–84% of the EUB338 cell count. The numbers of cells detected with the class Flavobacteria-specific (Flavo1) probe was highly variable, representing 2–35% of the EUB338 cell count. No correlation was observed between the FISH abundance estimates and any other variable measured. Cells with greatest fluorescence tended to occur in small aggregates and on the surfaces of phytoplankton.

Real-time PCR indicated that Flavobacteria comprised 1.0–25.8% of total 16S rRNA gene relative abundance in the 0.8 μm filter fractions, while this proportion ranged from 17.2% to 30.5% in the 0.2 μm filter fraction. In Temperate Zone (TZ) and Sub-Antarctic Zone (SAZ) samples (Fig. 1) particle-associated Flavobacteria appeared to represent a smaller portion of the total population (Fig. 2(b)). By comparison, particle-associated populations in the AZ samples appeared to be proportionally similar to the planktonic populations. Overall, the abundance of particle-associated Flavobacteria increased approximately 2.5-fold between the TZ and SAZ and the AZ (Fig. 2(b)). There was a positive correlation between increases in this abundance and an increase in chlorophyll *a* (Pearson's correlation coefficient, +0.607, $p < 0.05$) suggesting that abundance increases are linked at least in part to increases in primary production. Abundance was also positively correlated with nitrate, phosphate and silica concentration ($p < 0.01$) and negatively correlated with water temperature ($p < 0.05$) along the latitudinal transect (Fig. 1).

3.2. DGGE banding patterns

Differences in class Flavobacteria community structure between free-living and attached sample fractions and between different Southern Ocean water masses were studied by nMDS and ANOSIM analysis of DGGE banding patterns. The relatively high stress values (the "goodness of fit value" for the nMDS plots) obtained arose from DGGE band profile heterogeneity and suggested that, although the plots (Fig. 3) represent a potentially useful two-dimensional representation of the data, too much reliance should not be placed on the detail within the plot [28,29]. All samples from the AZ formed a common group which was distinct from samples from all other Southern Ocean water masses ($R = 0.321–0.628$, $p < 0.01$) (Table 1). The PFZ was also distinct from the TZ and SAZ samples ($R = 0.281–0.321$, $p < 0.05$) (Fig. 3(a) and Table 1). No significant difference was found between DGGE patterns for the 0.2 and 0.8 μm filter fractions obtained with the Flavobacteria specific primers ($R = 0.043$, $p > 0.05$) (Fig. 3(b)). The results indicated that the same Flavobacteria species occur planktonically as well as in and on particulate matter, but species distribution appears different in the waters of the PFZ and AZ compared to the lower latitude samples. No significant difference was found between profiles of samples from different Southern Ocean

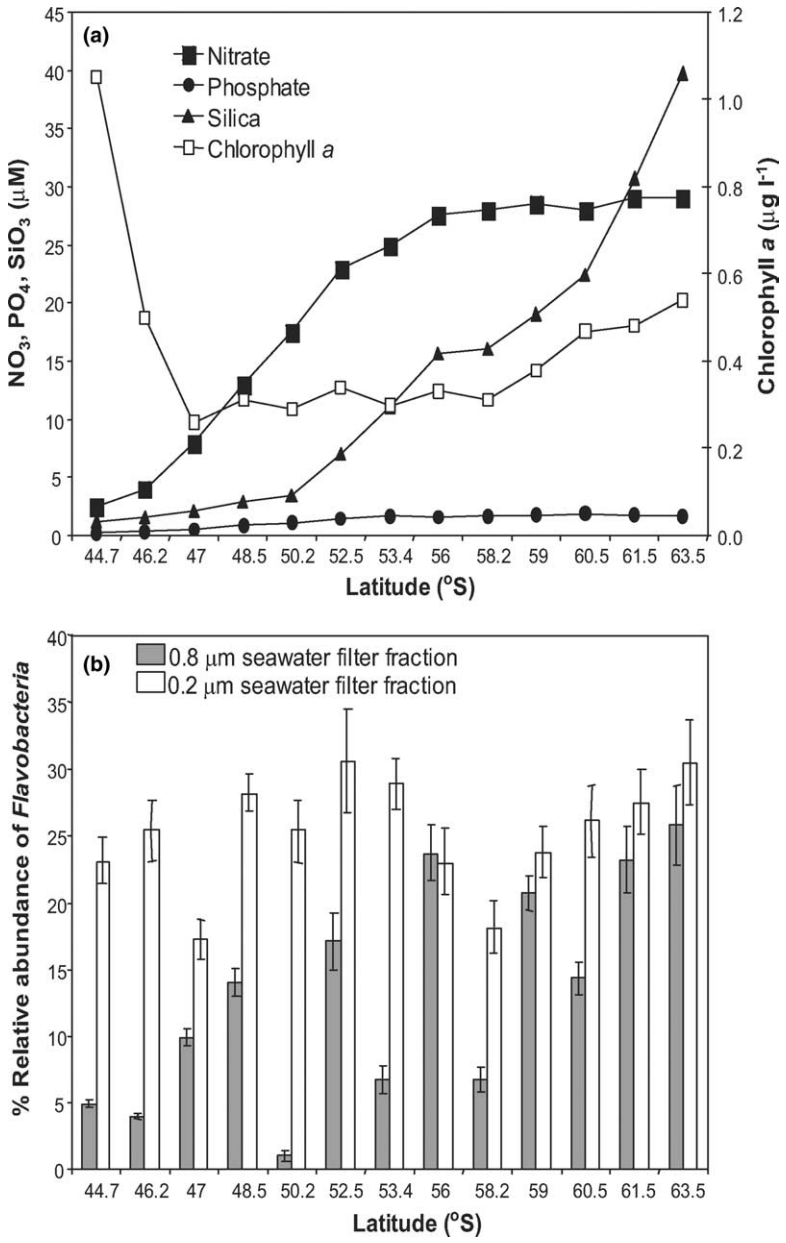

Fig. 2. (a) Nutrients, chlorophyll *a* and (b) relative abundance of class Flavobacteria (in relation to chlorophyll *a* concentrations) in Southern Ocean seawater filter fractions along a latitudinal transect.

zones or from different seawater filter fractions generated with the bacterial primer set (data not shown).

3.3. DGGE band sequence data

Sequences were derived from DGGE-PCR bands to identify the bacterial populations present in the 0.2 and 0.8 μm filter fractions of seawater from 22 sample stations (Fig. 1, Section 2.1). Gel bands (105) were sequenced from samples analysed using bacteria-specific primers. CFB sequences comprised 30% of total band sequences and 18 distinct phylotypes. Most other band sequences belonged to γ-proteobacteria (42% of bands) and α-proteobacteria (14% of bands). The remaining bands sequenced were from planctomycetes (2%), *Verrucomicrobia* (1%), the *Synechococcus–Prochlorococcus* clade (2%), Actinobacteria (2%), the genus *Bacillus* (1%), the uncultivated SAR406 group (1%) and chloroplast/plastid 16S rRNA genes from diatoms and other algae (5%). Non-CFB sequences were quite similar to cloned and DGGE band 16S rRNA gene sequences obtained from the Arctic and Southern Oceans (e.g. [34]) and included representatives of most major seawater bacterial clades (SAR11, SAR86, *Roseobacter* clade etc.; sequences deposited as GenBank No. AY661611–AY661641).

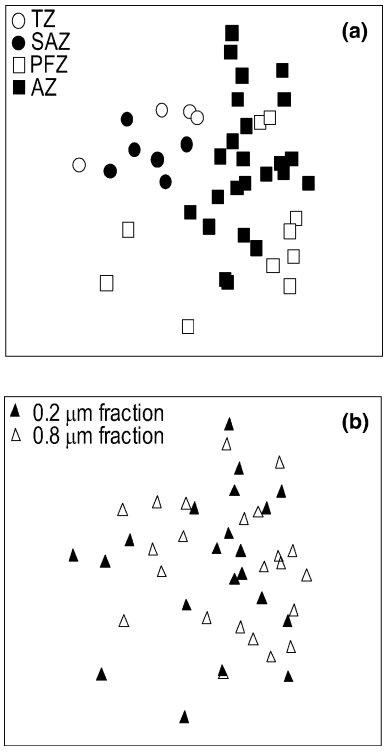

Fig. 3. (a) nMDS plot showing the relative similarities of class Flavobacteria community structure based on DGGE banding patterns between oceanic regions. Stress values for both plots were 0.22. (b) nMDS plot showing the relative differences between the 0.2 and 0.8 μm seawater filter fractions.

The Flavobacteria specific primer set was clearly able to sample Flavobacteria diversity more deeply than the bacterial-specific primers with 92 DGGE band sequences analysed grouping into 40 phylotypes. All phylotypes grouped in class Flavobacteria, including the family Flavobacteriaceae (69 sequences, 39 phylotypes) (Fig. 4) and the Cryomorphaceae/agg58 cluster (23 sequences, 11 phylotypes) (Fig. 5). Substantial overlap was found between phylotypes obtained with the two different primer sets. Of the 18 CFB phylotypes detected using universal bacterial primers, 11 identical or near identical (1–2 nucleotide mismatches) equivalent phylotypes were detected using the Flavobacteria specific primers. Of the seven phylotypes detected only with bacterial primers, two phylotypes grouped in the family "Saprospiraceae", which includes filamentous, sheathed, gliding species. These sequences possessed two nucleotide mismatches with the Flavobacteria specific primers, which had a highly reliable specificity for class Flavobacteria since the phylotypes amplified had either zero or one nucleotide mismatches to the 558f primer.

Table 1
Analysis of similarities (ANOSIM) of DGGE banding patterns of class Flavobacteria between different Southern Ocean zones

	TZ	SAZ	PFZ	AZ
Temperate Zone	–			
Sub-Antarctic Zone	0.375	–		
Polar Front Zone	0.291*	0.281*	–	
Antarctic Zone	0.618**	0.624**	0.321**	–

* Significance $p < 0.05$.
** $p < 0.01$.

Flavobacteria phylotypes grouped into a series of yet uncultivated clades or into clades corresponding to known genera, as shown in Table 2. Most of these belonged to the family Flavobacteriaceae (Fig. 4), the remainder clustering within a large clade incorporating the family Cryomorphaceae, which contains various polar species [10] and the agg58 clone cluster (Fig. 5), a group associated with algal blooms and marine aggregates in seawater samples [8,9]. The distribution of individual clades was not affected by which filter fraction was analysed (Table 2), but clade distribution varied substantially between samples taken from different parts of the Southern Ocean. Only five Flavobacteria clades were detected in each of the TZ and SAZ samples (Table 2). In the PFZ and AZ samples, the number of detected clades ranged from 8 to 15. The number of different Flavobacteria clades detected in each sample correlated positively with particle-associated Flavobacteria relative abundance ($p < 0.01$), nutrient levels ($p < 0.01$) and chlorophyll *a* concentrations ($p < 0.05$).

4. Discussion

4.1. Flavobacteria abundance relationships

Abundance measurements and DGGE data provide good support for previous analyses that suggested bacterioplanktonic community structure changed, with an increase in the abundance of CFB group members, between the Subtropical Convergence (at about 45–47° S) and the Polar Front (at about 56–58° S) through to the Antarctic coast [35]. However, it is clear that class Flavobacteria also represents a major fraction of the bacterioplanktonic biomass (Fig. 2(b)) and, with α-proteobacteria and γ-proteobacteria, dominates the bacterial populations in the Southern Ocean. Similar results have been shown in other oceanic areas [6–10,18,22]. A high abundance of CFB group members (up to 70%, detected with the CF319a probe) in Southern Ocean waters [22,35] was found in samples collected from algal blooms, indicative of the strong connection between the CFB and primary production [6,13,20]. We observed, however, that in high chlorophyll, nutrient limited TZ and SAZ waters attached Flavobacteria abundance was low (Fig. 2(b)). These samples contained bacteria that bound poorly to the EUB338 probe (<20% of the DAPI count) suggesting that a generally inactive bacterial population was present. The particle-associated

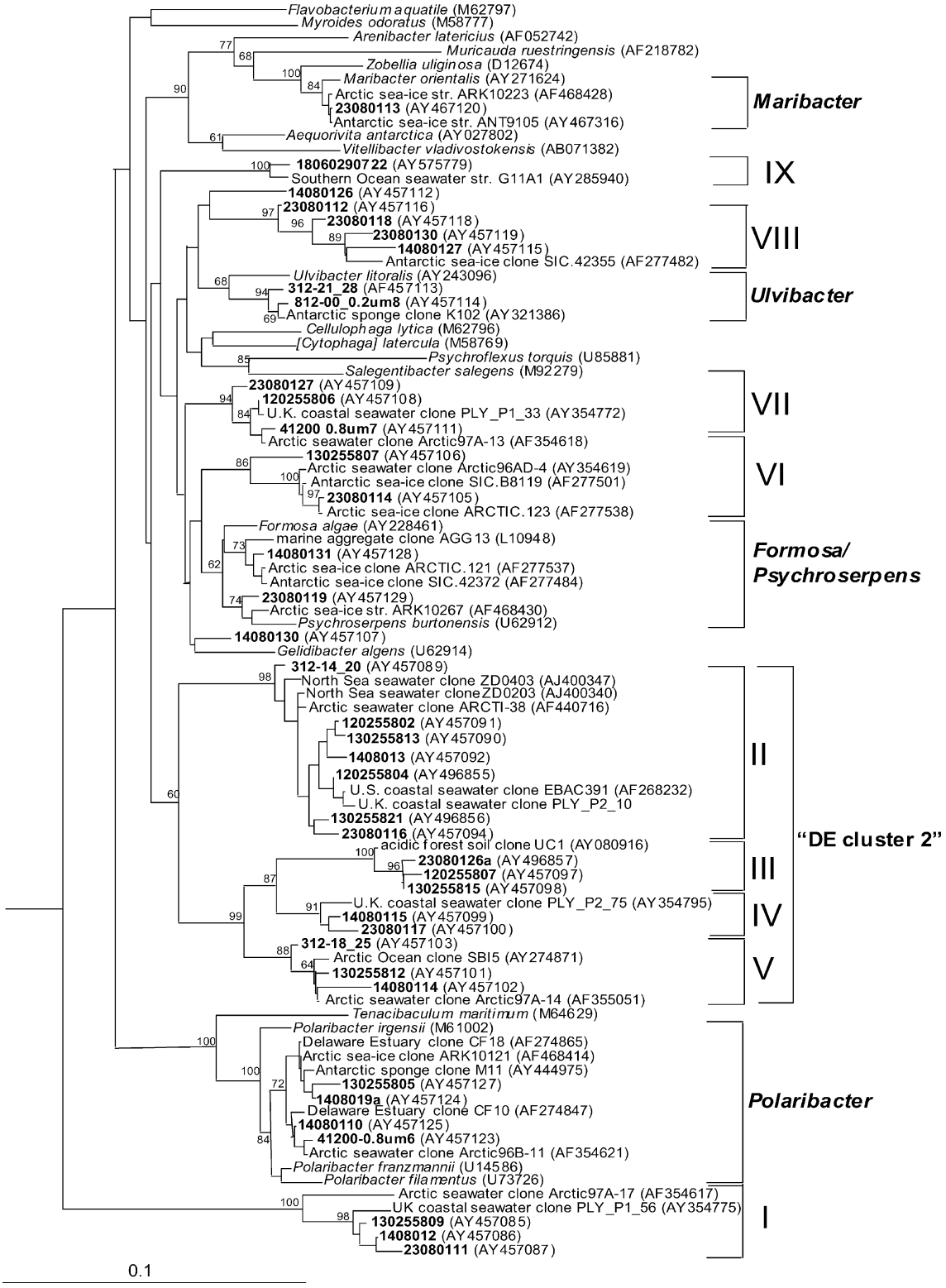

Fig. 4. A phylogenetic tree of 16S rRNA gene sequences of members of the family Flavobacteriaceae and phylotypes detected in the Southern Ocean using PCR-DGGE. *Rhodothermus marinus* was used as the outgroup species. Branching points with >60% bootstrap support (1000 replicates) have values shown at nodes. The scale bar indicates 0.1 changes per nucleotide.

community was dominated by α-proteobacteria, which appear to compete better than the CFB group under highly oligotrophic conditions [20]. This is suggested by the increase of class Flavobacteria relative abundance in the 0.8 μm seawater filter fractions, which includes cells mostly associated with particulate material.

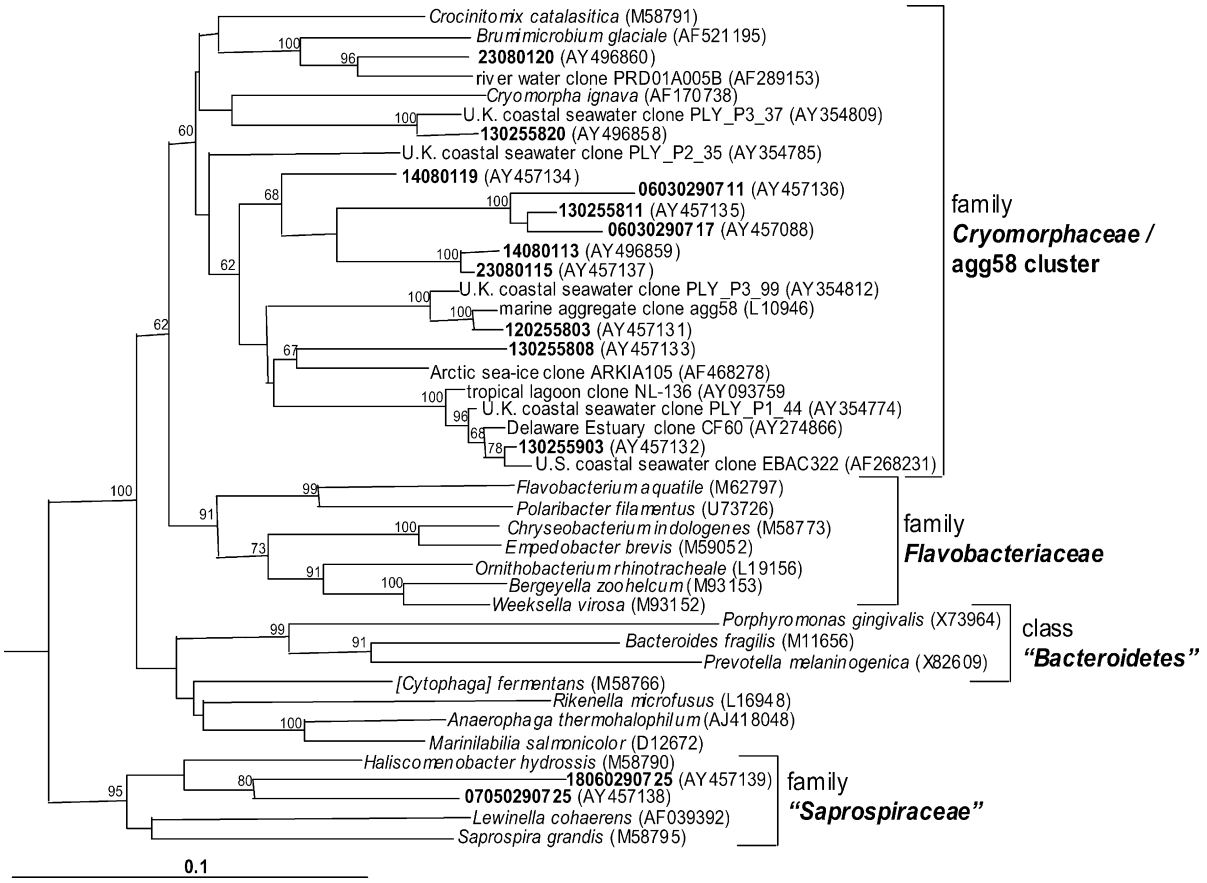

Fig. 5. A phylogenetic tree of 16S rRNA gene sequences of various members of the family Cryomorphaceae/agg58 cluster and the family "Saprospiraceae" and phylotypes detected in the Southern Ocean using PCR-DGGE. *R. marinus* was used as the outgroup species. Branching points with >60% bootstrap support (1000 replicates) have values shown at nodes. The scale bar indicates 0.1 changes per nucleotide.

A possible increase in abundance of Flavobacteria was observed in relation to chlorophyll *a* and nutrients suggesting that this segment of the microbial community is coupled to primary and secondary production even under oligotrophic situations. By comparison, no significant trend was observed in abundance of Flavobacteria in 0.2 μm seawater sample fraction, which contains mainly planktonic cells (Fig. 2(b)). From the DGGE banding pattern analysis no difference was found between the particle-associated and planktonic populations indicating that the same species were present. This similarity may arise from bacterial cells dispersing from particulates as they decompose and sink [3]. The planktonic populations have less access to nutrients and thus are not active, resulting in poor detection with the FISH probes [33]. However, bacterial cells associated with particulates, including Flavobacteria, are much more biologically active than cells in seawater [14].

The Southern Ocean south of the Subtropical Convergence is affected strongly by iron limitation [36,37], limiting primary production, which becomes relatively enhanced in the AZ [38], due to sea-ice melting distributing atmospherically deposited iron [36] and creating less surface water mixing [37], as suggested by the increases in chlorophyll *a* along the latitudinal transect (Fig. 2(a)). The class is known for its strong association with algae and is often regarded as having an epiphytic lifestyle, which is relevant to the exclusively chemoheterotrophic nature of the group [6]. The increase in utilizable carbon and aggregate formation derived from this primary production thus potentially provides increased opportunities for Flavobacteria growth. The high variability of abundance in particle-associated Flavobacteria (Fig. 2(b)) may derive from localized patchiness of phytoplankton populations in the Southern Ocean due to the effects of advection, iron and silica nutrient limitation [39], as well as competition with other bacteria, in particular Proteobacteria.

4.2. Structural aspects of the Flavobacteria community

A significant gradient in water temperature occurs across the PFZ, acting as an important physical barrier in the Southern Ocean, reducing water mass mixing and potentially affecting microbial community distribution [40,41]. There was a temperature differential of about

Table 2
Class Flavobacteria taxonomic groups in the Southern Ocean detected by DGGE, including detection relative to different primer sets utilized and seawater filter fraction as well as incidence of taxonomic groups in the different Southern Ocean zones

Clades[a]	GenBank accession numbers	Detection of phylotypes with different primer sets and seawater filter fractions				Incidence of taxonomic groups in Southern Ocean zones (combining both seawater filter fractions)		
		Bacterial primers	Flavobacteria primers	0.2 μm	0.8 μm	TZ, SAZ	PFZ	AZ
		Number of phylotypes detected out of total number of phylotypes detected for the corresponding clade:				Number of samples containing DGGE band of corresponding clade out of total number of samples		
I	AY457085- 457088	2/4	+[b]	+	1/4	1/5	1/4	5/13
II	AY457089– 457094 AY496855–496856	4/8	6/8	+	+	+	+	+
III	AY457097–457098, AY496855	1/3	+	+	+	+	2/4	7/13
IV	AY457099–457100	–	+	+	+	–	2/4	3/13
V	AY457101–457104	2/4	2/4	2/3	2/3	2/5	3/4	11/13
VI	AY457105–457106	1/2	+	1/2	+	–	2/4	+
–	AY457107	–	+	+	+	–	–	1/13
VII	AY457108–457111	2/4	+	1/4	+	–	+	+
Ulvibacter	AY457113– 457114	+	+	+	1/2	1/5	2/4	8/13
–	AY457112	–	+	+	+	–	1/4	3/13
VIII	AY457115–457118	–	+	3/4	+	–	3/4	5/13
IX	AY575779	+	–	–	+	–	1/4	6/13
Maribacter	AY457120	–	+	+	+	–	–	1/13
Polaribacter	AY457123–457127	+	+	2/5	+	–	2/4	10/13
Psychroserpens	AY457128–457129	–	+	1/2	+	–	1/4	3/13
Saprospiraceae	AY457138–457139	+	–	+	+	–	–	2/13
AGG58/Cryomorphaceae	AY457131–457137, AY496858,AY496859	3/11	9/11	10/11	9/11	–	2/4	5/13

[a] The phylogenetic position of the clades and identity of individual phylotypes are indicated in Figs. 4 and 5.

[b] +, All phylotypes were detected or all indicated Southern Ocean zone samples contained at least one DGGE band for the indicated clade; –, no phylotype detected or no sample from the indicated Southern Ocean zones contained bands of the indicated group.

15 °C between the TZ and AZ that appears significant in shaping the Flavobacteria community structure (Fig 3(a), Table 1). DGGE analyses described a distinct trend in scale, with Flavobacteria communities not resolved at the microscale [42], as shown by comparisons of the filter fractions (Fig. 4(b)) to still significant but more constrained heterogeneity in different Southern Ocean water masses (Fig. 3(a)). These findings are analogous to reports on the distributions of *Roseobacter* phylotypes and Archaea in water masses north and south of the PFZ [40,41]. The predominance of psychrophilic species in Antarctic waters and sea-ice is well established [43] and the trait occurs amongst members of class Flavobacteria (e.g. [44,45]). Thus, it is logical that temperature selection plays an important role in shaping this segment of the microbial community, by selecting for psychrophilic species. These more cold adapted species would not likely compete well in warmer lower latitude waters and thus could be contributing to differences in the community DGGE profiles. No equivalent difference in DGGE profiles was found using bacteria-specific primers. This could be due to the large variability and diversity in the structure of the overall bacterial community being sufficient to obscure any community differences.

4.3. Biogeographical aspects of Flavobacteria in the Southern Ocean

No obvious difference was found between the TZ, SAZ, PFZ and AZ samples in terms of bacterial 16S rRNA sequence diversity (data not shown) suggesting that the DGGE sequence analysis only included the more abundant species. However, particle-associated Flavobacteria diversity was also found to be high in the Southern Ocean and, the distribution of phylotypes and clades varied considerably between different water masses (Table 2). The incidence of the different Flavobacteria clades was found to correlate significantly ($p < 0.01$) between PFZ and AZ samples but not with TZ and SAZ samples ($p > 0.05$), indicating both the physical barrier of the Polar Front and water temperature, appearing to select for different communities of Flavobacteria as also shown by DGGE patterns (Fig. 3(a)). TZ and SAZ samples had relatively low Flavobacteria 16S rRNA sequence diversity compared with PFZ and SAZ samples, with band sequences grouping only in five different clades (Table 2), including clades I–V and genus *Ulvibacter* (Fig. 4). All of these clades had widespread distribution and are present in PFZ and AZ samples (Table 2). Clades II–V, which comprised most of the Flavobacteria 16S rRNA gene diversity in the TZ/SAZ can be combined in a larger clade (Fig. 4), equivalent to a clade designated DE cluster 2 [7], named for clones obtained from the Delaware Estuary, coastal USA and of the Chukchi Sea, Arctic Ocean. By application of a specific FISH probe, DE cluster 2 was found to make up to 10% of total prokaryotes in those samples [7]. The clade is widely distributed in temperate to polar waters as it has been also detected off the coast of England [8], the North Sea [46], the US west coast [47] and the Arctic Ocean [34] (Fig. 4). Clones derived from either Arctic or Antarctic sea-ice, for which there is now extensive data [13,14], did not cluster in DE cluster 2, suggesting that the taxa belonging to this clade are mostly limited to seawater.

The diversity of 16S rRNA gene sequences from PFZ and AZ samples was substantially higher than in TZ and SAZ samples (Table 2). Clades VI–IX, genus *Polaribacter*, genus *Psychroserpens* (Fig. 4), the agg58/Cryomorphaceae cluster (Fig. 5) and some more rarely detected phylotypes detected by DGGE appeared to be restricted to colder waters of the AZ and PFZ (Table 2). These clades could thus represent psychrophilic taxa well adapted to cold AZ and PFZ waters. The genera *Polaribacter* and *Psychroserpens* are already known to contain exclusively psychrophilic species [44,45]. These clades and genera, however still may be present in the TZ and SAZ owing to the inherent detection limits of the DGGE-PCR, estimated to be at approximately 0.1% of the population [48]. More sensitive methods and larger sample numbers are required to confirm the complete distributions of these groups between the different Southern Ocean water masses.

It is possible that the difference in Flavobacteria 16S rRNA gene diversity and community structure found between the SAZ/TZ and PFZ/AZ samples could be due to the influence of primary production since the number of different Flavobacteria clades detected in samples positively correlated chlorophyll *a* levels, particle-associated Flavobacteria abundance and nutrients. Mesocosm studies [20] demonstrated that CFB 16S rRNA gene diversity increased with increased primary productivity (estimated by chlorophyll *a* fluorescence) to a maximum, whereupon diversity declined in the presence of high productivity levels, far above those encountered in samples collected in this study. Here a similar but incomplete relationship was observed, as production in the Southern Ocean regions sampled did not appear to reach levels high enough to hinder further expansion of Flavobacteria diversity.

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References

[1] Fenchel, T. (1988) Marine plankton food-chains. Annu. Rev. Ecol. Syst. 18, 19–38.
[2] Cole, J.J, Findlay, S. and Pace, M.L. (1988) Bacterial production in fresh and saltwater ecosystems – a cross-system overview. Mar. Ecol. Prog. Ser. 43, 1–10.
[3] Azam, F. (1998) Microbial control of oceanic carbon flux: The plot thickens. Science 280, 694–696.
[4] Bidle, K.D., Brzezinski, M.A., Long, R.A., Jones, J.L. and Azam, F. (2003) Diminished efficiency in the oceanic silica pump caused by bacteria-mediated silica dissolution. Limnol. Oceanogr. 48, 1855–1868.
[5] Brown, G.E. and Parks, G.A. (2001) Sorption of trace elements on mineral surfaces: Modern perspectives from spectroscopic studies, and comments on sorption in the marine environment. Int. Geol. Rev. 43, 963–1073.
[6] Kirchman, D.L. (2002) The ecology of Cytophaga–Flavobacteria in aquatic environments. FEMS Microbiol. Ecol. 39, 91–100.
[7] Kirchman, D.L., Yu, L.Y. and Cottrell, M.T. (2003) Diversity and abundance of uncultured Cytophaga-like bacteria in the Delaware Estuary. Appl. Environ. Microbiol. 69, 6587–6596.
[8] O'Sullivan, L.A., Fuller, K.E., Thomas, E.M., Turley, C.M., Fry, J.C. and Weightman, A.J. (2004) Distribution and culturability of the uncultivated 'AGG58 cluster' of the Bacteroidetes phylum in aquatic environments. FEMS Microbiol. Ecol. 47, 359–370.
[9] DeLong, E.F., Franks, D.G. and Alldredge, A.L. (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol. Oceanogr. 38, 924–934.
[10] Bowman, J.P., Mancuso Nichols, C.A. and Gibson, J.A.E. (2003) *Algoriphagus ratkowskyi* gen. nov., sp. nov., *Brumimicrobium glaciale* gen. nov., sp. nov., *Cryomorpha ignava* gen. nov., sp. nov., *Crocinitomix catalasitica* gen. nov., sp. nov., novel flavobacteria isolated from various polar habitats. Int. J. Syst. Evol. Microbiol. 53, 1343–1355.
[11] Weller, R., Glöckner, F.O. and Amann, R. (2000) 16S rRNA-targeted oligonucleotide probes for the in situ detection of members of the phylum Cytophaga–Flavobacterium–Bacteroides. Syst. Appl. Microbiol. 23, 107–114.
[12] Ploug, H., Grossart, H.P., Azam, F. and Jorgensen, B.B. (1999) Photosynthesis, respiration, and carbon turnover in sinking marine snow from surface waters of Southern California Bight: implications for the carbon cycle in the ocean. Mar. Ecol. Prog. Ser. 179, 1–11.
[13] Brown, M.V. and Bowman, J.P. (2001) A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). FEMS Microbiol. Ecol. 35, 267–275.
[14] Brinkmeyer, R., Knittel, K., Jurgens, J., Weyland, H., Amann, R. and Helmke, E. (2003) Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. Appl. Environ. Microbiol. 69, 6610–6619.
[15] Llobet-Brossa, E., Rossello-Mora, R. and Amann, R. (1998) Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microbiol. 64, 2691–2696.
[16] Bowman, J.P. and McCuaig, R.D. (2003) Diversity and biogeography of prokaryotes dwelling in Antarctic continental shelf sediment. Appl. Environ. Microbiol. 69, 2463–2484.
[17] Webster, N.S., Negri, A.P., Munro, M.M.H.G. and Battershill, C.N. (2004) Diverse microbial communities inhabit Antarctic sponges. Environ. Microbiol. 6, 288–300.
[18] Cottrell, M.T. and Kirchman, D.L. (2000) Natural assemblages of marine proteobacteria and members of the Cytophaga–Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. 66, 1692–1697.
[19] Mittelbach, G.G., Steiner, C.F., Steiner, S.M., Gross, K.L., Reynolds, H.L., Waide, R.B., Willig, M.R., Dodson, S.I. and Gough, L. (2001) What is the observed relationship between species richness and productivity?. Ecology 82, 2381–2396.
[20] Horner-Devine, C.M., Leibold, M.A., Smith, V.H. and Bohannan, B.J.M. (2003) Bacterial diversity patterns along a gradient of primary productivity. Ecol. Lett. 6, 613–622.
[21] Kopczynska, E.E., Dehairs, F., Elskens, M. and Wright, S. (2001) Phytoplankton and microzooplankton variability between the Subtropical and Polar Fronts south of Australia: Thriving under regenerative and new production in late summer. J. Geophys. Res.-Oceans 106, 31597–31609.
[22] Glöckner, F.O., Fuchs, B.M. and Amann, R. (1999) Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl. Environ. Microbiol. 65, 3721–3726.
[23] Lebaron, P., Servais, P., Troussellier, M., Courties, C., Vives-Rego, J., Muyzer, G., Bernard, L., Guindulain, T., Schafer, H. and Stackebrandt, E. (1999) Changes in bacterial community structure in seawater mesocosms differing in their nutrient status. Aquat. Microb. Ecol. 19, 255–267.
[24] Fuhrman, J.A., Comeau, D.E., Hagstrom, A. and Chan, A.M. (1988) Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Appl. Environ. Microbiol. 54, 1426–1429.
[25] Lane, D.J. (1991) 16S/23S rRNA sequencing In: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt, E. and Goodfellow, M., Eds.), pp. 115–175. John Wiley & Sons, New York.
[26] Ferris, M.J., Muyzer, G. and Ward, D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. 62, 340–346.
[27] Powell, S.M., Bowman, J.P., Snape, I. and Stark, J.S. (2003) Microbial community variation in pristine and polluted nearshore Antarctic sediments. FEMS Microbiol. Ecol. 45, 135–145.
[28] Clarke, K.R. and Warwick, R.M. (2001) Change in marine communities: an approach to statistical analysis and interpretation, 2nd ed. Primer-E Ltd., Plymouth, UK. (http://www.pml.a-c.uk/primer/).
[29] Schiffman, S.S. and Reynolds, M.L. (1981) Introduction to Multidimensional Scaling. Theory, Methods and Applications. Academic Press, London, UK.
[30] Hall, T.A. (1999) A user-friendly biological sequence alignment editor for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, pp. 95–98.
[31] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Clustal-W- improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.
[32] Felsenstein, J. (1993) PHYLIP, phylogenetic inference package. http://evolution.genetics.washington.edu/phylip.html.
[33] Ouverney, C.C. and Fuhrman, J.A. (1997) Increase in fluorescence intensity of 16S rRNA in situ hybridization in natural samples treated with chloramphenicol. Appl. Environ. Microbiol. 63, 2735–2740.
[34] Bano, N. and Hollibaugh, J.T. (2002) Phylogenetic composition of bacterioplankton assemblages from the Arctic Ocean. Appl. Environ. Microbiol. 68, 505–518.
[35] Simon, M., Glöckner, F.O. and Amann, R. (1999) Different community structure and temperature optima of heterotrophic

picoplankton in various regions of the Southern Ocean. Aquat. Microb. Ecol. 18, 275–284.
[36] de Baar, H.J.W., Buma, A.G.J., Nolting, R.F., Cadee, G.C., Jacques, G. and Treguer, P.J. (1990) On iron limitation of the Southern-Ocean – experimental-observations in the Weddell and Scotia seas. Mar. Ecol. Prog. Ser 65, 105–122.
[37] Timmermans, K.R., van Leeuwe, M.A., de Jong, J.T.M., McKay, R.M.L., Nolting, R.F., Witte, H.J., van Ooyen, J., Swagerman, M.J.W., Kloosterhuis, H. and de Baar, H.J.W. (1998) Iron stress in the Pacific region of the Southern Ocean: evidence from enrichment bioassays. Mar. Ecol. Prog. Ser. 166, 27–41.
[38] Vincent, W.F. (1988) Microbial Ecosystems of Antarctica. Cambridge University Press, Cambridge, UK.
[39] Hence, I., Zimmermann, R., Beckman, A. and Batman, U.V. (2003) Regional ecosystem dynamics in the ACC: simulations with a three-dimensional ocean-plankton model. J. Mar. Syst. 42, 31–51.
[40] Murray, A.E., Wu, K.Y., Moyer, C.L., Karl, D.M. and De Long, E.F. (1999) Evidence for circumpolar distribution of planktonic Archaea in the Southern Ocean. Aquat. Microb. Ecol. 18, 263–273.
[41] Selje, N., Simon, M. and Brinkhoff, T. (2004) A newly discovered *Roseobacter* cluster in temperate and polar oceans. Nature 427, 445–448.
[42] Long, R.A. and Azam, F. (2001) Microscale patchiness of bacterioplankton assemblage richness in seawater. Aquat. Microb. Ecol. 26, 103–113.
[43] Bowman, J.P., McCammon, S.A., Brown, M.V., Nichols, D.S. and McMeekin, T.A. (1997) Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl. Environ. Microbiol. 63, 3068–3078.
[44] Bowman, J.P., McCammon, S.A., Brown, J.L., Nichols, P.D. and McMeekin, T.A. (1997) *Psychroserpens burtonensis* gen. nov., sp. nov. *and Gelidibacter algens* gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. Int. J. Syst. Bacteriol. 47, 670–677.
[45] Gosink, J.J., Woese, C.R. and Staley, J.T. (1998) *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov., *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the Cytophaga–Flavobacterium–Bacteroides group and reclassification of ''*Flectobacillus glomeratus*'' as *Polaribacter glomeratus* comb. nov. Int. J. Syst. Bacteriol. 48, 223–235.
[46] Zubkov, M.V., Fuchs, B.M., Archer, S.D., Kiene, R.P., Amann, R. and Burkill, P.H. (2002) A population of the alpha-proteobacteria dominates the bacterioplankton and dimethylsulphoniopropionate uptake after an algal bloom in the North Sea. Deep-sea Res. II, Top. Stud. Oceanogr. 49, 3017–3038.
[47] Beja, O., Suzuki, M.T., Koonin, E.V., Aravind, L., Hadd, A., Nguyen, L.P., Villacorta, R., Amjadi, M., Garrigues, C., Jovanovich, S.B., Feldman, R.A. and DeLong, E. (2000) Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. Environ. Microbiol. 2, 516–529.
[48] Casamayor, E.O., Schafer, H., Baneras, L., Pedros-Alio, C. and Muyzer, G. (2000) Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: Comparison by microscopy and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 66, 499–508.
[49] Deacon, G.E.R. (1984) The Antarctic Circumpolar Ocean. Cambridge University Press, Cambridge.