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Aerobic anoxygenic phototrophic bacteria in Antarctic sea ice and seawater

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Summary

Aerobic anoxygenic phototrophs are obligate aerobes with unusually high concentrations of carotenoids, low cellular contents of bacteriochlorophyll-*a* and they lack light-harvesting complex II. In this study, sea ice and seawater samples were collected from six different sites in the Ross Sea, Antarctica. Using a combination of primers for *puf*M (which encodes a pigment-binding protein subunit of the reaction centre complex), clone libraries of DNA and cDNA were created and a total of 63 positive clones were obtained from three sites, all clustering within the *α-Proteobacteria*. Fifty-three of these clones were from seawater. The remaining clones were from sea ice and all were found in the middle and bottom sections of the ice. These sea ice bacteria may favour the lower part of the ice matrix where irradiance is low. This report highlights the first findings of AAnPs in antarctic sea ice and seawater within the Ross Sea Region.

Introduction

Recent large-scale environmental metagenomic surveys have revealed a wide distribution and abundance of bacterial phototrophic genes throughout the world's oceans (Venter *et al*., 2004; Rusch *et al*., 2007; Yutin *et al*., 2007). Until 30 years ago when the first aerobic anoxygenic phototroph (AAnP) was reported (Shiba *et al*., 1979), these phototrophic bacteria had been thought to be strictly anaerobic. Since 1979, the AAnPs have been found to represent a functional group of bacteria, defined by their aerobic, heterotrophic and phototrophic abilities. AAnPs are obligate aerobes with unusually high concentrations of carotenoids, low cellular contents of bchl-*a* and they lack light-harvesting complex II (Kolber *et al*., 2001; Rathgeber *et al*., 2004). Given their larger cellular size and faster growth rates compared with the other heterotrophic bacteria (Schwalbach and Fuhrman, 2005; Jiao *et al*., 2007; Lami *et al*., 2007), AAnP might contribute up to 50% of the total bacterial production in oligotrophic waters and thus are a large sink for dissolved organic matter (Koblizek *et al*., 2007). In fact, we still do not understand their contribution to the carbon cycle, their true abundance and their distribution in the global marine environment (Kolber *et al*., 2000; 2001; Béjà *et al*., 2002; Jiao *et al*., 2010).

A part of the photosynthetic gene cluster in AAnPs is a 45 kilobase photosynthetic unit forming (*puf*) superoperon that codes for the reaction centre structural proteins (Béjà *et al*., 2002; Tank *et al*., 2009). This contiguous super-operon may be located on the bacterial chromosome or on extra-chromosomal elements (Pradella *et al*., 2004). In AAnP, the expression of the *puf* operon is insensitive to oxygen (Nishimura *et al*., 1996; Masuda *et al*., 2000). One of these genes, *puf*M has been frequently used as a marker in the investigation of diversity in AAnPs (Béjà *et al*., 2002; Lambeck *et al*., 2002; Hu *et al*., 2006; Waidner and Kirchman, 2008; Cottrell and Kirchman, 2009; Jiao *et al*., 2010).

During ice-covered seasons in the Southern Ocean, light levels in the water columns are governed by the thickness and transparency of sea ice (Becquevort *et al*., 2009). Microbes growing near the surface of sea ice experience very low temperatures, and high salinities and light levels that together comprise physical conditions close to the limits of life. Lower sections of sea ice are characterized by low light intensity (McMinn *et al*., 2003), stable temperatures and readily available nutrients (Thomas and Dieckmann, 2002; Lazzara *et al*., 2007; Ralph *et al*., 2007). The highest numbers of cells, both algal and bacterial, are therefore often found at the bottom 10 cm of the ice (McMinn *et al*., 1999; 2010; Lizotte, 2003; Ryan *et al*., 2011). The microbial communities in sea ice exhibit significantly higher single-cell metabolic activity than those in many other marine ecosystems despite the extreme environment (Junge *et al*., 2004; Martin *et al*., 2008; 2009), and they are highly diverse (Brown and Bowman, 2001; Brinkmeyer *et al*., 2003; Murray and Grzymski, 2007; Bowman *et al*., 2011). Very recently, the presence of

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Table 1. Primers used in this study.

Primer	Sequence (5′→3′)	Target gene	Reference
puf M.557F	CGCACCTGGACTGGAC		Achenbach *et al.* (2001)
puf M.750R	CCCATGGTCCAGCGCCAGAA		
puf M_uniF	GGNAAYYTNTWYTAYAAYCCNTTYCA	*puf*M	Yutin *et al.* (2005)
puf M_uniR	YCCATNGTCCANCKCCARAA		
puf M_WAW^R	AYNGCRAACCACCANGCCCA		

AAnPs were amplified from both DNA and cDNA extracts using *puf*M primers (Table 1), as recommended by Achenbach and colleagues (2001) and Yutin and colleagues (2005), and in combinations. Amplification conditions were at 94°C (5 min), 40 cycles of 94°C (45 s), 53.1°C (1 min) and 72°C (1 min), with a final extension at 72°C for 5 min.
F, forward; R, reverse.

Archaea has been reported in both the Arctic (Collins *et al.*, 2010) and Antarctic sea ice (Cowie *et al.*, 2011), and the newly described copiotrophic *Coraliomargarita* was found in sufficient abundance to suggest a niche occupation in sea ice (Bowman *et al.*, 2011). In fact, substantial research has been conducted on the ecology and biochemistry of sea ice (Krembs *et al.*, 2002; 2011; Collins *et al.*, 2008).

The majority of the sea ice bacterial research reported to date has focused on the heterotrophs (Delille, 2004; Martin *et al.*, 2009; Moreau *et al.*, 2010; Piquet *et al.*, 2011). Little attention has been placed on novel phototrophs such as AAnPs (Karr *et al.*, 2003); however, some other phototrophs have been found in Antarctic lakes and in the Southern Ocean (Giebel *et al.*, 2009) and we have recently described the presence of proteorhodopsin bacteria in sea ice communities (Koh *et al.*, 2010). Here, the *puf*M gene was used to describe for the first time the distribution of AAnP in the Antarctic seawater and ice environment.

Seawater was collected from the Ross Sea through the Scott Base water supply at ~ 5 m depth in a 20 l carboy, and transported back to New Zealand for filtration and further analyses. After thawing at 4°C, approximately 10 l of seawater was first filtered through a 3.0 μm nitrocellulose membrane filter (Millipore, USA) before passing the filtrate through a 0.22 μm mixed nitrocellulose acetate membrane filter (Pall Life Sciences, USA). This filtration was done in triplicates and both 3.0 and 0.22 μm filter papers were stored separately in a sterile 50 ml Falcon tube (BD Biosciences, USA) with sufficient 1 × Phosphate Buffer Solution (PBS) added to submerge the filter at the end of the filtration. The tubes were vortexed and 1 ml of suspension was transferred to a sterile microcentrifuge tube. The microcentrifuge tube was spun at 8000 *g* for 10 cycles of 5 min each (Eppendorf, Germany). The supernatant was then decanted before adding another aliquot of cell suspension. A total of 10 ml was taken from each tube to be used as starting material for subsequent DNA extraction using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Germany), according to the manufacturer's instructions.

Sea ice core samples were collected at five sites across the Victoria Land Coast. All procedures pertaining to samples collection, processing, nucleic acids preparation and extraction were carried out as previously described (Koh *et al.*, 2010).

In this study, *puf*M primers targeting the bchl-*a*-containing bacteria published by Achenbach and colleagues (2001) and Yutin and colleagues (2005) were used to screen the sample set (Table 1). These primers are specific to the *puf*M gene in the light-harvesting reaction centre of the phototrophic *Proteobacteria*, and they provide a qualitative estimate of the diversity of bacteria with this gene in the environment (Achenbach *et al.*, 2001).

Results and discussion

The goal of this study was to demonstrate the presence of bchl-a based phototrophy in the antarctic seawater and sea ice environment. No PCR products were detected after amplification with *puf*M primers from Yutin and colleagues (2005). Only the *puf*M primers from Achenbach and colleagues (2001) gave positive PCR amplicons and 30 plasmid clones from each sample were chosen for further EcoRI-digestion before sequencing. Sixty-three positive *puf*M clones from seawater (53) and sea ice (10) formed two clusters that showed phylogenetic relatedness to the α-*Proteobacteria* AAnP and none to the β- and γ-*Proteobacteria* (Fig. 1). This observation is similar to the Antarctic Lake Fryxell study by Karr and colleagues (2003) who also found an ice-bound community of AAnPs dominated by α-*Proteobacteria*. The similarity of the sea ice and seawater derived clones likely reflects the fact that sea ice is derived from seawater (Thomas and Dieckmann, 2002).

Within cluster A, 29 DNA clones from the sea water were grouped with the cDNA clones from sea ice and had 100% bootstrap support with minimal genetic distance between them (Fig. 1). Clones from TNB_Brine_CG200m65, on the other hand, were genetically distinct from the seawater DNA and other sea ice cDNA clones. Even within the TNB_Brine_CG200m65 clones, the

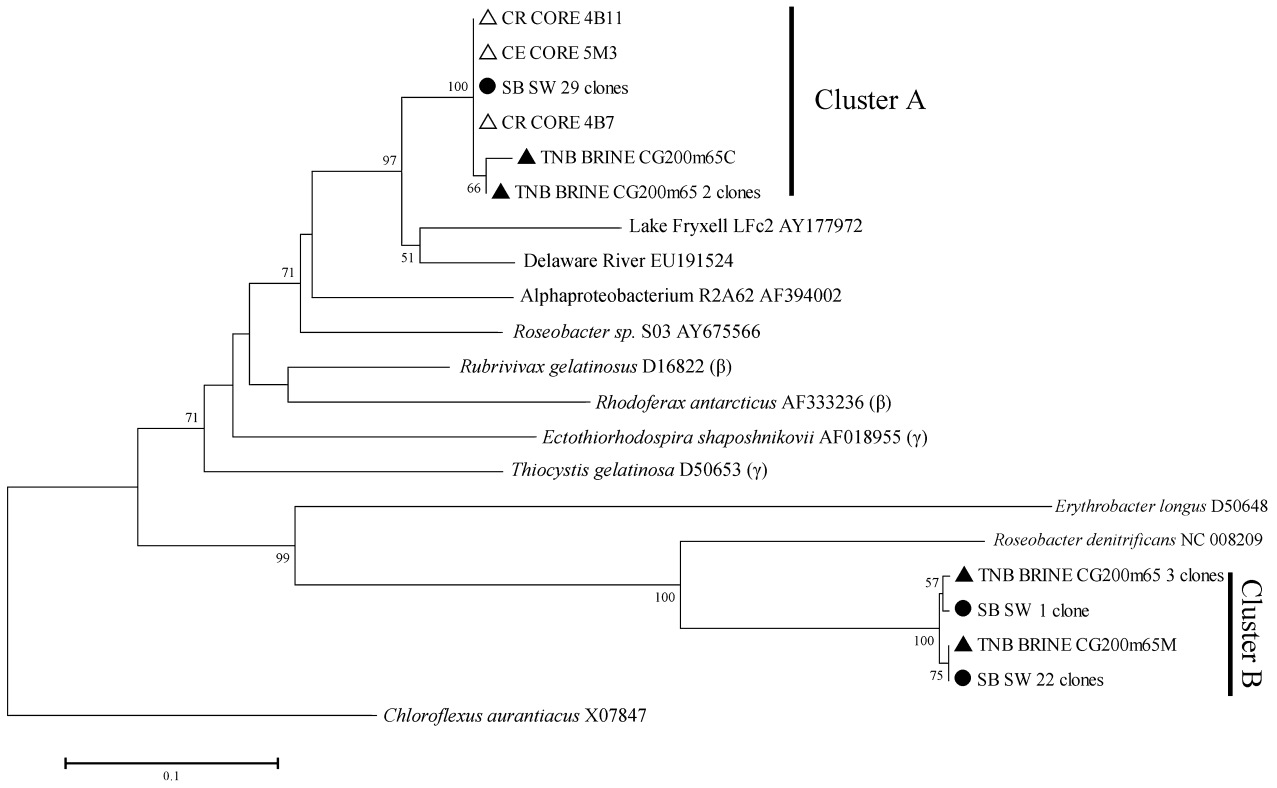

Fig. 1. Neighbour-joining tree based on the ~ 270 bp nucleotide sequence of the DNA fragment coding for the *puf*M gene. ● = seawater DNA; ▲ = DNA; △ = cDNA. 3 K ≥ 3.0 μm filter; 22 K and F ≤ 3.0 μm; CE = Cape Evans; CR = Cape Roberts; TNB = Terra Nova Bay; SB SW = Scott Base Seawater. CG200m65 = Brine collected at Campbell Glacier at 200 m distance 65 cm depth. The phylogeny of the DNA sequences was constructed in MEGA4.0 (Tamura *et al*., 2007) based on neighbour-joining algorithms with 1000 bootstrap replicates and *p*-distance as substitution model. Bootstrap values ≥ 50% are presented at the corresponding nodes. Green non-sulfur bacterium *Chloroflexus aurantiacus* (X07847) was used as out-group. The sequences reported in this study have been deposited in GenBank under the following accession numbers HQ142788-HQ142850.

genetic substitution rate was at 0.03 and the branch was supported by a 66% bootstrap value. Clones in cluster A have 97% bootstrap values to non-cultured *puf*M relatives from the Delaware estuary (Waidner and Kirchman, 2008) and Lake Fryxell in Antarctica (Karr *et al*., 2003), and also well-supported bootstrap values to environmental *puf*M-containing *Roseobacter* (Oz *et al*., 2005) and *Alphaproteobacterium* R2A62 (Béjà *et al*., 2002).

Cluster B contained DNA clones from seawater and the same TNB_Brine_CG200m65 sample from Cluster A (Fig. 1). All of the sea-ice-derived sequences in this cluster were from brine collected in the middle portion of the sea ice. There was 100% bootstrap support to *Roseobacter denitrificans* and 99% to *Erythrobacter longus*, both of which are α-*Proteobacteria* AAnPs from cultured samples (Fig. 1), implying that clones in both clusters could be *Roseobacter*-clade affiliated (RCA). *Roseobacter* are well represented across diverse marine habitats (reviewed by Buchan *et al*., 2005; Wagner-Dobler and Biebl, 2006) and RCA bacteria in particular, have been observed to constitute about 20% of the total bacterial community in the Southern Ocean (Selje *et al*., 2004; Giebel *et al*., 2009; 2011). Biodiversity studies based on the 16S rDNA gene conducted on sea ice from other Antarctic study sites within our research group have also yielded clones with high similarities to *Roseobacter* (E.W. Maas, A.M. Simpson, A.R. Martin, K.G. Ryan and R. O'Toole, unpubl. data) and *Erythrobacter* sp. (R.O.M. Cowie, E.W. Maas and K.G. Ryan, unpubl. data).

The 10 positive clones from sea ice were extracted from three different sites separated by ~300 km: Cape Evans (CE; 1), Cape Roberts (CR; 2) and Terra Nova Bay (TNB; 7). Three of these were of cDNA origin (represented by open triangles in Fig. 1) and seven of DNA origin (filled triangles in Fig. 1). Only two clones were from the bottom sea ice at CR, while the rest were from the middle portion. No *puf*M clones were observed from the top portion of the sea ice from any location.

Most of the sea ice AAnPs were detected in mid sections of the ice, where the salinity was more than twice (~87‰) that of seawater. Only two clones were found in bottom sections of the ice where the salinity matches that

of the seawater. While some AAnP bacteria have been reported in Canadian hypersaline lakes (Csotonyi *et al*., 2008) and saline lakes on the Tibetan Plateau (Jiang *et al*., 2009; 2010), in other studies, the diversity of the *puf*M gene is inversely proportional to salinity (Jiang *et al*., 2009). Waidner and Kirchman (2008) and Jiang and colleagues (2010) have reported that high salinity reduced AAnP bacterial community diversity and composition, and Giebel and colleagues (2009) noted the inverse correlation of salinity and RCA cluster abundance from the subtropics to the Southern Ocean. The role of salinity in AAnP distribution is equivocal at present and more studies in hypersaline environments such as sea ice are needed.

One important factor that contributes to the successful competitive adaption of AAnP bacteria to the environment may be detritus particle attachment (Waidner and Kirchman, 2008; Lami *et al*., 2009; Cottrell *et al*., 2010), as observed in the Delaware estuary where AAnP attached to detritus particles accounted for > 10% of the total prokaryotic numbers (Waidner and Kirchman, 2008). In sea ice, detritus particles are distributed throughout the ice column (Sullivan and Palmisano, 1984), and these could serve as an attachment surface for AAnP. *Roseobacter* spp., for instance, are known to encode genes that support attachment (Slightom and Buchan, 2009). Thus, the sea ice and seawater clones in this study that clustered close to RCA sequences (Fig. 1) might favour a particle attachment lifestyle, via sea ice crystals and dendritus particles.

Marine γ-*Proteobacteria* with AAnP genes have also been reported recently (Hu *et al*., 2006; Cho *et al*., 2007; Fuchs *et al*., 2007; Spring *et al*., 2009). However, no *puf*M-containing clones from the γ-*Proteobacteria* group were found in this study, although we have found other γ- and α-*Proteobacteria* phototrophs such as proteorhodopsin-bearing bacteria in antarctic sea ice in a separate study (Koh *et al*., 2010). These findings suggest that phototrophic *Proteobacteria* may have varied strategies to obtain energy for cellular needs, either using bchl-*a* (Shiba and Harashima, 1986; Kolber *et al*., 2000) or proteorhodopsin (Béjà *et al*., 2000; Koh *et al*., 2010) to supplement their heterotrophic existence.

Metabolic gene probes, such as the *puf*M gene, have several advantages over rDNA-based probes, in that they provide an association to an exclusive physiology in a phylogenetically diverse group. And when coupled with mRNA detection techniques, phototrophic metabolism can be inferred. Reverse-transcription PCR was used to produce cDNA from mRNA transcripts stored in RNAlater to determine whether *puf*M was being transcribed at the time of sampling. In this study, only three cDNA transcripts from cluster A were apparently actively transcribing *puf*M in the sea ice at the time of sampling. Given that the *puf* operon of AAnP is strongly repressed by high irradiance (Rathgeber *et al*., 2004; Yurkov and Csotonyi, 2009), and that our samples were collected near the mid-austral summer, it is possible that many sea ice AAnP bacteria were not active at the time of collection, and instead have later or earlier onset of photosynthetic activity when ambient light levels are lower. In addition, this could explain why *puf*M cDNA transcripts were only detected from the mid-bottom ice matrix and not near the surface where irradiance can be up to a thousand-fold higher than at the bottom of the ice (Buckley and Trodahl, 1987; Harashima *et al*., 1987; Yurkov and Beatty, 1998). In addition, the higher amount of nutrients present in the underlying seawater (Thomas and Dieckmann, 2002) may further support AAnP growth. More abundance data from polar marine environments of AAnP are urgently required.

Table 2. Distribution of brine, sea ice and seawater clones within each clonal group.

Clonal group	Seawater DNA	Sea ice cDNA	Brine	Total
A	30	3	3	36
B	23	0	4	27
Total	53	3	7	63

All clones derived were derived from amplification with *puf*M primers reported by Achenbach and colleagues (2001). The seawater analysed in this study was collected from the sea water supply line at Scott Base (SB SW). DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Germany), according to the manufacturer's instructions (S1). The collection and DNA extraction of sea ice and brine were carried out as documented in Koh and colleagues (2010).

Schwalbach and Fuhrman (2005) reported that in the Western Antarctic, AAnP only comprised ~ 0.05% of the total bacterial abundance, while Cottrell and Kirchman (2009) reported up to 8% in the Arctic Ocean. In this study, we observed a higher number of clones from seawater than from the sea ice (Table 2) and this may indicate that at least in ice-covered regions of the Ross Sea region, AAnPs are more abundant in the open water than in the ice although further quantitative work is needed to confirm this observation.

Conclusion

Using a combination of standard and reverse-transcription PCR to screen antarctic sea ice and seawater samples collected over 300 km apart in the Ross Sea region, we present the first report of AAnP bacteria in antarctic seawater and sea ice. The seawater clones were closely grouped to previously identified environmental clones and to *Roseobacter dentrificans*, with the latter suggesting presence of the RCA cluster in the Ross Sea region. The sea ice clones, on the other hand, exhibited stronger grouping to environmental clones than to cultured representatives. Not only did we find that the majority of the bchl-*a*-bearing bacteria were found in the middle section of

the sea ice and within the water column, we also observed cDNA regenerated from *puf*M-RNA in sea ice bacterial cells, which suggests that the cells are actively synthesizing the bchl-*a* gene within this extreme ecosystem.

Bacteria in sea ice are highly metabolically active (Martin *et al*., 2008) and perhaps bacterial phototrophy provides a selective advantage in such extreme conditions. Microbial energetics within the sea ice is still poorly understood and the ecological importance of the AAnPs in the sea ice environment needs further verification to provide a better understanding of their functions and their contributions to the sea ice 'microbial loop'.

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