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ORIGINAL ARTICLE

Winter diversity and expression of proteorhodopsin genes in a polar ocean

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Mixotrophy is a valuable functional trait used by microbes when environmental conditions vary broadly or resources are limited. In the sunlit waters of the ocean, photoheterotrophy, a form of mixotrophy, is often mediated by proteorhodopsin (PR), a seven helices transmembrane protein binding the retinal chromophore. Altogether, they allow bacteria to capture photic energy for sensory and proton gradient formation cell functions. The seasonal occurrence and diversity of the gene coding for PR in cold oligotrophic polar oceans is not known and PR expression has not yet been reported. Here we show that PR is widely distributed among bacterial taxa, and that PR expression decreased markedly during the winter months in the Arctic Ocean. Gammaproteobacteria-like PR sequences were always dominant. However, within the second most common affiliation, there was a transition from Flavobacteria-like PR in early winter to Alphaproteobacteria-like PR in late winter. The phylogenetic shifts followed carbon dynamics, where patterns in expression were consistent with community succession, as identified by DNA community fingerprinting. Although genes for PR were always present, the trend in decreasing transcripts from January to February suggested reduced functional utility of PR during winter. Under winter darkness, sustained expression suggests that PR may continue to be useful for non-ATP forming functions, such as environmental sensing or small solute transport. The persistence of PR expression in winter among some bacterial groups may offer a competitive advantage, where its multifunctionality enhances microbial survival under harsh polar conditions.
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Introduction

Mixotrophic strategies are important, often overlooked components of the microbial ecology in aquatic ecosystems (Eiler, 2006). Bacterial photoheterotrophy, a form of mixotrophy, combines the use of organic substrates with light energy and is increasingly thought to be a common strategy among bacteria in marine surface waters. The broad distribution of the proteorhodopsin (PR) gene suggests that PR may be a particularly widespread mechanism for facilitating photoheterotrophy (Béjà *et al.,* 2000, 2001). PR, together with its membrane-bound retinal chromophore, mediates light-activated proton pumping for both energy production and sensory function in bacteria. Rhodopsins have been associated with light-enhanced growth and survival for some cultivated strains (Gómez-Consarnau *et al.,* 2007, 2010), but not for others (Giovannoni *et al.,* 2005; Stingl *et al.,* 2007; Riedel and Tomasch, 2010; Riedel *et al.*, 2013). Although PR activity is commonly associated with photoheterotrophy, the gene likely possesses broader functional roles (Furhman *et al.*, 2008) and support for the diversity of PR functions has been reported (Mongodin *et al.,* 2005; Riedel *et al.*, 2013; Bamman *et al.*, 2014; Yoshizawa *et al.,* 2014).

PR has been identified in many oceans and seas (Béjà *et al.,* 2000, Cottrell and Kirchman, 2009; Riedel and Tomasch, 2010) and is phylogenetically diverse (Rusch *et al.,* 2007). Microbial rhodopsins are highly abundant in the photic biosphere. A metagenomic survey found them to be present on average in 48% of cells, and in some cases in all cells from both marine and terrestrial samples (Finkel *et al.,* 2013).

Despite the pervasive presence of PR genes in nature, there are limited data on PR expression. Only a few studies to date report PR transcripts from diverse natural communities, with most reports

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from short term, localized metatranscriptomic studies (Frías-López *et al.,* 2008; Poretsky *et al.,* 2009; Satinsky *et al.,* 2014). Patterns in PR expression would be expected to vary seasonally and covary as a function of light availability; however, such patterns have rarely been explored. Recently, a study using a quantitative PCR approach on SAR11 populations from North Atlantic waters showed a strong correlation between PR expression and photosynthetically active radiation; however, no obvious growth response was observed (Lami and Kirchman, 2014). In the Arctic ocean, although PR genes have been reported during both light-replete summer and light-limited winter at a coastal site (Cottrell and Kirchman, 2009), detailed long-term seasonal dynamics of PR's presence or expression in Polar oceans have yet to be described.

Bacteria in the upper Arctic Ocean remain active throughout the year, despite the lack of light, subzero temperatures and low substrate concentrations in winter (Garneau *et al.,* 2008; Kirchman *et al.,* 2009; Nguyen *et al.,* 2012). However,mechanisms for such persistence are unclear. These bacterial communities have some of the lowest growth efficiencies reported in marine environments, with mean seasonal values ranging from 2 to 7% (Kirchman *et al.,* 2009; Nguyen *et al.,* 2012) where lowest values are observed in winter. Such low bacterial growth efficiencies suggest that most of the available carbon is diverted toward energy generation rather than biomass synthesis, making PR-generated ATP a potentially precious commodity in the carbon economy of Polar Oceans. Alternatively, PR may contribute to bacterial survival in other ways, such as enhanced motility, sensing and active transport of small molecules (Fuhrman *et al.*, 2008). Indeed, PR could provide multiple functions for the same or different organisms over time and space, potentially providing bacteria possessing the gene an advantage over those that do not under varied conditions.

We tracked the seasonal prevalence and phylogenetic diversity of a marker for PR over 8 consecutive months, as part of the International Polar Year Circumpolar Flaw Lead study (Barber *et al.*, 2010). Our core objectives were to (1) follow seasonal changes in the PR signal from November to July through multiple-primer PCR amplifications, (2) characterize PR expression patterns using reverse transcribed PCRs and (3) look at the variations in winter diversity of bacteria possessing the PR gene via cloning and sequencing. We expected PR distribution and expression to follow seasonal cycles, where the gene would gradually disappear with decreased sunlight during winter and reappear in the spring with greatest prevalence under 24-h daylight during the Arctic summer. Unexpectedly, PR was recovered throughout the Arctic winter, along with evidence for its expression during this time suggesting other important functional roles of this gene that are not dependent on light availability.

Material and methods

Study site

The study was carried out onboard the CCGS Amundsen in the Amundsen Gulf of the Southeastern Beaufort Sea. Weekly samples were collected from November 2007 to June 2008. Detailed physical and chemical conditions during the International Polar Year Circumpolar Flaw Lead study are reported in Barber *et al.* (2010). Overall, 16 surface seawater samples were selected for this study (Supplementary Figure S1).

Sample collection and DNA preparation

Water samples were collected using a rosette system equipped with 24 12-l Niskin-type bottles. Three to six litres of water were sequentially filtered through 53-μm nylon mesh, 3-μm pore size polycarbonate filter (Millipore, Billerica, MA, USA), with samples for DNA collected in a 0.2-μm filter cartridge (Sterivex, Millipore) and preserved in buffer (40 mM EDTA; 50 mMTris pH = 8.3; 0.75 M Sucrose). Samples for RNA were collected onto 47-mm 0.22-μm pore size polycarbonate membrane filters (Millipore) and preserved in 2-ml cryovials with 600 μl of RLT buffer (Qiagen, Toronto, ON, Canada) and 1% beta-mercaptoethanol (Sigma–Aldrich, Oakville, ON, Canada). Filters were kept at $-80\,^{\circ}C$ and were extracted within 2 years after collection. Salt extraction of microbial DNA was adapted from Aljanabi and Martinez (1997) and included steps using lysozyme, proteinase K and sodium dodecyl sulfate (Terrado *et al.*, 2011). RNA was extracted as in Church *et al.* (2005), using an RNAeasy kit (Qiagen) and converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Burlington, ON, Canada) with random primers and an RNase inhibitor. Extraction of nucleic acids yielded concentrations ranging from 5.3 to 291 $ng\,\mu l^{-1}$ in DNA samples that were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The cDNA concentrations ranged from 0.28 to 2.1 $ng\,\mu l^{-1}$ and were measured using the PicoGreen (Molecular Probes Inc., Life technologies Inc., Burlington, ON, Canada) fluorometric method. As RNA was not quantified before reverse transcription, we assume cDNA concentrations were representative of the original RNA concentrations in samples.

PR Primers and PCR amplification

Degenerated PR primers were modified from Atamna-Ismaeel *et al.* (2008) and Koh *et al.* (2010), with an expected PCR product size of ~350 bp. An additional reverse primer (PR-R4) was created after *in silico* testing with the Fuzznuc web application (http://emboss.bioinformatics.nl/cgi-bin/emboss/fuzznuc). Consensus sequences were added to the 5′ ends of primers to increase amplification

efficiency (Bodaker *et al.,* 2012). Six primer pairs from Koh *et al.* (2010) were used in combination with a seventh pair using the additional reverse primer (Supplementary Information; Table 1). It should be noted that sequences representative of the actinorhodopsin-like LG1 and LG2 groups (Sharma *et al.*, 2008, 2009) present a TWXXYP region at the 3′ end and would not be amplified by our set of reverse primers that are based on GWXXYP (Atamna-Ismaeel *et al.*, 2008) and SWXFY regions. PCRs were performed with the hot start Platinum Taq DNA Polymerase (Invitrogen, Madrid, Spain). Optimal PCR amplification followed: 1 cycle of 95 °C for 3 min, 40 cycles of 94 °C for 30 s, 48.5 °C for 30 s and 72 °C for 1 min and a final cycle of 72 °C for 10 min. Target load of template for the PCR reactions was 10 ng for DNA. As cDNA concentrations were low we did not achieve that target load and a 1.5-ng cDNA template per reaction was used. An environmental sample from the Blanes Bay Microbial Observatory time series, where PR presence was confirmed by DNA sequencing was used as a positive control. Nanopure water was used as a negative control. Particular attention was given to ensure conditions were kept constant between all reactions throughout the amplification procedure. Although, precise information on the efficiency of each primer pair relative to one another is unavailable, we assume that within each primer pair, all other things being equal, the differences in signal intensity can be reasonably attributed to differences in initial target DNA and cDNA, which is the underlying assumption of fingerprinting techniques as well as high throughput sequencing studies (Schauer *et al.,* 2003; Díez *et al.*, 2004; Gilbert *et al.*, 2009).

Clone libraries

PCR products were cloned using the Agilent Technologies StrataClone PCR cloning kit using competent cells of *Escherichia coli* (Agilent Technologies, Madrid, Spain). Colonies were screened by PCR to verify clonal insert size and ~500 clones were retained for standard Sanger sequencing (Genoscreen, Lille, France) using a 3730XL DNA Analyzer (Applied Biosystems) to confirm proteorhodopsin amplification. Specific PR primers (Supplementary Table S1) were used during sequencing rather than the adjacent T7 or other plasmid sequencing primers to minimize sequencing non-PR clones. This restriction, however, resulted in PR fragments shorter (150–279 bp, for an ~20–37% coverage of the gene) than the expected target after trimming primers and poor 5′ ends. After manual removal of low quality sequences (those with multiple ambiguous bases and Ns), 188 PR-clones were kept for subsequent analysis following a basic local alignment search (BLAST) against the NCBI nr database. Nucleotide sequences were clustered into operational taxonomic units (OTUs) at a 6% dissimilarity threshold (Riedel and Tomasch 2010) using MOTHUR (Schloss, 2009).

PR gene sequence data have been submitted to the GenBank databases under accession numbers KJ937475-KJ937662.

PR phylogeny

The amino-acid sequences of PR clones were manually curated and aligned against a reference PR alignment with the seed function of the MAFFT algorithm (Katoh and Toh 2010). Cleaning and alignment of sequences against known PR sequences, showed our fragments were relatively short and reliable reads only began at positions 109–111. The fragments included site A178 involved in conformational changes in the E and F helices, but they did not include regions coding for spectral tuning (L105), electron donor (E108) or electron acceptor (D97) that were on the low quality start of the sequences. With the exception of five sequences were the E108 position was present and indicated the proton pumping conformation, this precluded us from carrying further analysis on the aforementioned sites. Bootstrap resampling and maximum likelihood-based phylogenetic analyses were carried out using RAxML (Stamatakis 2006; Stamatakis *et al.,* 2008) and based on a GAMMAWAGF evolutionary

Table 1 Distribution and affiliation of clone sequences in EW and LW periods, based on maximum likelihood simulations subdivided in respect to the respective primer sets

Primer set	*Flavo*		*α-Proteo*		*γ-Proteo*		*OMG*		*Unassigned*		*Total*	
	EW	*LW*	*EW*	*LW*	*EW*	*LW*	*EW*	*LW*	*EW*	*LW*	*EW*	*LW*
A	–	–	3	12	–	11	16	46	4	10	23	79
B	–	–	–	1	3	–	6	7	1	–	10	8
C	3	2	–	–	2	2	11	3	–	–	16	7
D	–	–	–	–	–	1	2	–	–	–	2	1
F	13	2	–	–	11	1	9	5	–	–	33	8
Total	16	4	3	13	16	15	44	61	5	10	84	103

Abbreviations: α-proteo, alphaproteobacteria; EW, early winter; Flavo, flavobacteria; γ-proteo, gammaproteobacteria; LW, late winter; OMG, oligotrophic marine group gammaproteobacteria.

model, selected using the ProtTest web application (http://darwin.uvigo.es/software/prottest2_server.html). Tree visualization and design was done using the Interactive Tree of Life (Letunic and Bork, 2011, www.itol.org).

Environmental variables
Temperature and salinity profiles were obtained on the downward casts of the rosette equipped with a conductivity temperature depth (CTD) profiler (Sea-Bird 911 + CTD) with a sensor for photosynthetically active radiation. Nutrient samples were collected directly from the Niskin-like bottles and concentrations of nitrate + nitrite ($NO_3^- + NO_2^-$) and nitrite (NO_2^-) were determined on board with a Bran and Luebbe Autoanalyzer 3 (Delavan, WI, USA) using routine colorimetric methods (Grasshoff *et al.,* 1999). As nitrite levels were low, $NO_3^- + NO_2^-$ is hereafter referred to as NO_3^-. Samples for chlorophyll *a* (Chl *a*) were filtered onto 25-mm diameter Whatman GF/F filters (nominal pore size 0.7 mm). Filters were extracted onboard in 90% acetone over 24 h at 5 °C in the dark. Chl *a* fluorescence was measured using a Turner Designs fluorometer model 10-AU before and after acidification (Parsons *et al.,* 1984). Chl *a* concentrations were then calculated using equations of Holm-Hansen *et al.* (1965).

Bacterial abundance and production
Water samples for total bacterial abundance were preserved for 1 h in (5% v/v) formaldehyde and filtered onto 0.2-μm pore diameter black polycarbonate filters after staining with 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI). Concentrations were determined using epifluorescence microscopy (Porter and Feig, 1980) and counts include both Archaea and Bacteria, but are referred to as bacteria for simplicity. Bacterial production was measured using ^{3}H–leucine incorporation (Smith and Azam, 1992) where rates of leucine incorporation were corrected for radioactivity adsorption using TCA-killed controls. Detailed procedures on cell counts and bacterial production are given in Nguyen *et al.* (2012).

Denaturing gradient gel electrophoresis
Bacterial community changes over the seasons were first evaluated using denaturing gradient gel electrophoresis (DGGE) fingerprinting (Schauer *et al.,* 2003). Briefly, DGGE was carried out on a DGGE 2000 system (CBS scientific, Del Mar, CA, USA) where partial 16 s rRNA genes were amplified using the bacterial specific forward primer GC358F with a GC clamp, and the universal reverse primer 907RM. About 800 ng of the PCR product was loaded onto a 6% acrylamide gel containing a denaturing gradient of 40–80%, where 100% of the denaturing agent contained 7 M urea and 40% deionised formamide. Gels were run at 100 V for 17 h at 60 °C. Following staining with Sybr Gold (Invitrogen) and image capture, a matrix of the relative contribution of bands to the total intensity of each lane was built using the Quantity One software (Bio-Rad, Barcelona, Spain). From this matrix, a distance matrix was calculated using the Hellinger dissimilarity index. This matrix was used for UPGMA clustering. Multiscale bootstrap analyses were done using the pvclust package (Suzuki and Shimodaira, 2014) in the R statistical package (R Core Team, 2014) to test the stability of clusters.

Results

Environmental and bacterial dynamics
Strong seasonal differences were evident over the study from November 2007 to July 2008 (Figure 1). Temperature remained near the freezing point of seawater (−1.7 °C) for most of the study and increased after ice melt in the late spring (Figure 1a). Total surface solar irradiance and photosynthetically active radiation (Figure 1b) sharply decreased in November and rapidly increased in February, reaching maximum values in May and June. Phytoplankton biomass, estimated from Chl *a* concentrations (Figure 1d), roughly followed irradiance, increasing from February to April (slope = 0.01, $P < 0.0001$). Over May and June Chl *a* levels were on average higher, but with a large degree of scatter over the sampling region; values declined slightly in the second half of June (slope = −0.06, $P = 0.014$). NO_3^- (Figure 1c) accumulated under the ice from late February to April and decreased from May to July, likely as a function of higher phytoplankton uptake following increased light availability in summer; first indications of surface NO_3^- depletion occurred in June.

Bacterial heterotrophic activity (Figure 1f) was low but detected throughout winter and rapidly increased in late spring, parallel to the order of magnitude increase in Chl *a*. Surface bacterial abundance (Figure 1e) followed a similar pattern with more than a fivefold increase in cells from April to June. Additional details of bacterial activity and carbon demand during the CFL study are reported elsewhere (Nguyen *et al.,* 2012). Community composition of the bacterioplankton also changed over time (Figure 2). Large community changes detected using DGGE, targeting the 16S rRNA gene, occurred first in April when sea-ice melt began, then in May with a summer community in May and June. In contrast, based on the DGGE results, the winter community from November to March changed very little.

Presence and expression of the PR gene
To characterize seasonal patterns and potential diversity of PR in Arctic samples, we used seven

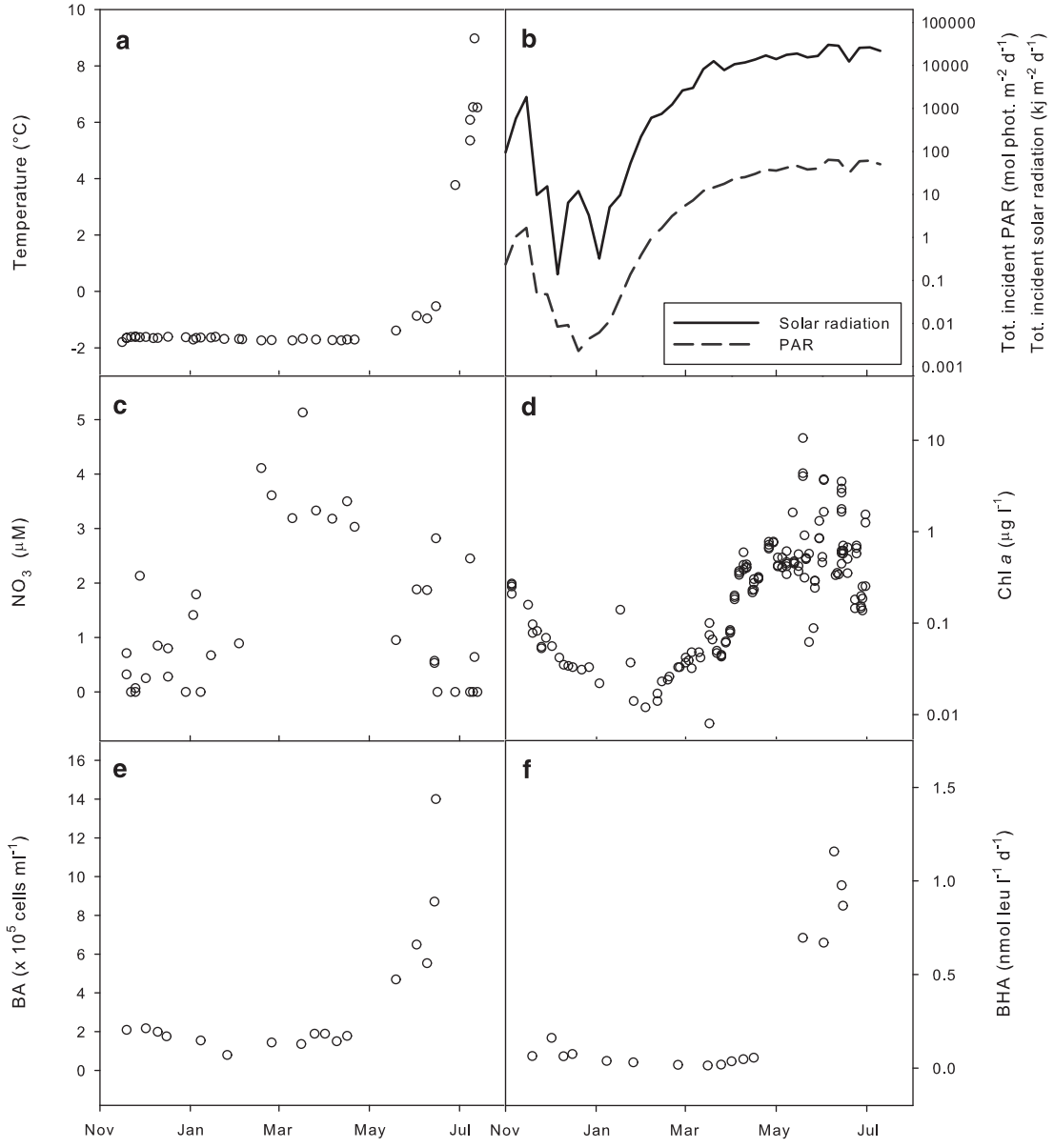

Figure 1 Temporal trends in surface water temperature (**a**), surface irradiance (**b**), nitrate concentrations (**c**), Chl *a* (**d**), bacterial abundance (**e**) and production (**f**) measured over the course of the International Polar Year Circumpolar Flaw Lead system study. Lines on the *x* axis represent the first day of each month.

combinations of degenerate primers (Supplementary Table S1). Two of the primer pairs that associated *in silico* to *Methylophilales* sp HTCC2181 and *Marinobacter* PR sequences did not yield PCR products (no visible bands) from any of the samples (Figure 3a, primers E and G). The other five combinations yielded positive PCR products and positive amplification of the PR gene (from DNA) was observed on all dates. Primer sets A and F resulted in the greatest amplification signals that remained relatively elevated throughout the sampling period, with only a slight decrease over the winter months (Figure 3a). Sets B and C amplification yields declined over winter, but only C appeared to regain relative intensity in late spring. Set D decreased during the winter months and only slightly increased toward the end of the study.

For primer pairs with positive DNA amplification of PR genes (A, B, C, D, F; Supplementary Table S1), we investigated the apparent gene expression using cDNA to gage PR transcript abundance in the Arctic Ocean. The intensity of the end-point PCR band was used as an estimate of that expression. All primer sets followed a similar pattern with at least one or more primer pairs with positive amplification of PR transcripts for all sampling dates (Figure 3b). Lower apparent expression was observed during winter, with a marked decrease from December to January. Expression then peaked in April at the time of increased irradiance and ice melt (Figure 1b). This increase

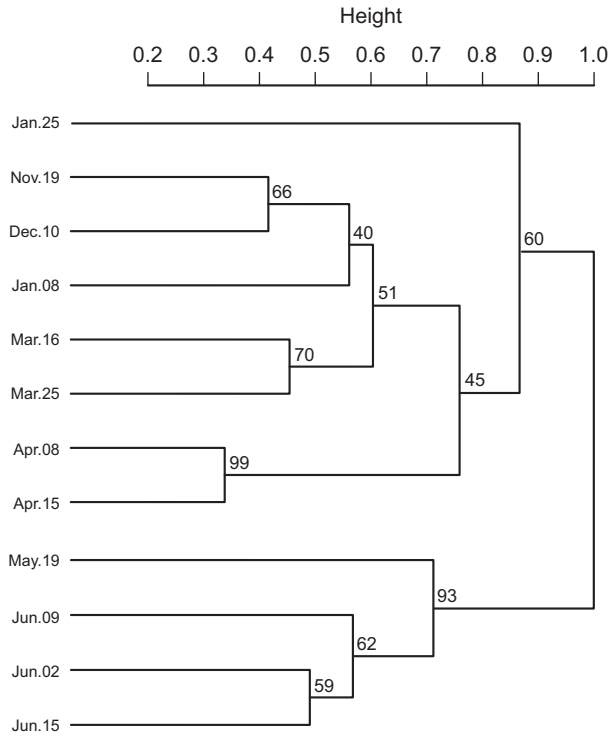

Figure 2 Patterns of diversity observed during this study illustrated as an UPGMA-based dendrogram showing seasonal variations in bacterial communities, based on cluster analysis of the DGGE fingerprints. Bootstrap probabilities (%) are shown on the dendrogram.

coincided with a change in the bacterial community (Figure 2). Later in May, when the community changed again, expression decreased based on the lower intensity of PCR bands. Apparent expression progressively increased toward the end of our study, with the onset of Arctic summer (Figure 3b).

PR winter diversity

To gain a better understanding of seasonal distribution and diversity of PR-carrying clades, clone libraries for early (16 December) and late (25 March) winter were constructed using the five primer sets that successfully amplified PR from Arctic DNA. A total of 187 clones were sequenced, of which 118 were unique and aligned against 16 PR reference sequences from GenBank (Supplementary Figure S2). Phylogenetic analysis using maximum likelihood revealed four main PR clades (Supplementary Figure S2 and Table 1). Most PR sequences were associated with Gammaproteobacteria (73% of all clones), with over half (56%) of all clones falling into the subclade matching Gammaproteobacteria from the Oligotrophic Marine Group. Alphaproteobacteria and Bacteroidetes formed the other prominent clades. Finally, a small proportion of sequences remained unassigned (8% of all clones). All clades were represented at both dates, with Flavobacteria-like sequences being more abundant in early winter and more Alphaproteobacteria-like sequences in late

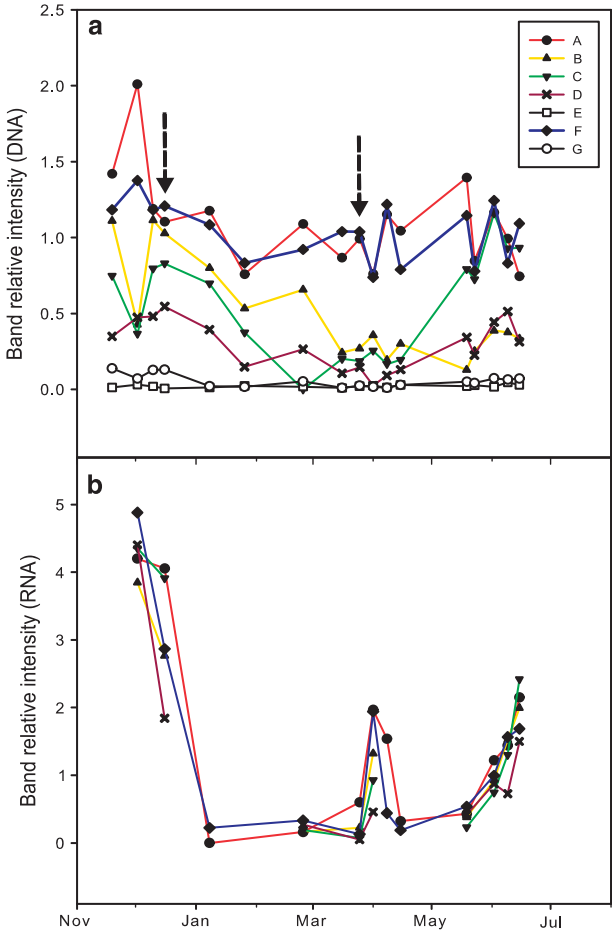

Figure 3 Change in the relative intensity of PCR bands for the multiple PR primer sets over time. (**a**) Relative intensity observed in DNA samples and (**b**) Relative intensity observed in RNA samples on each sampled date. The black arrows show the two dates selected for cloning and sequencing. Missing data points indicate dates where samples were either lost or in insufficient amount to allow testing of all primer sets. Lines on the *x* axis represent the first day of each month. An environmental sample from the Blanes Bay Microbial Observatory time series, where PR presence was confirmed by DNA sequencing was used as a positive control. Nanopure water was used as a negative control. Conditions were kept constant between all reactions throughout the amplification procedure. We assume that within each primer pair, all other things being equal, the differences in signal intensity can be reasonably attributed to differences in initial target DNA and cDNA.

winter (Table 1). With the exception of non-Oligotrophic Marine Group Gammaproteobacteria-like sequences, all clades had strong bootstrap support (>75%, Supplementary Figure S2).

The four major clades and primer pairs did not match precisely, but some distinctive patterns of association emerged (Table 1). Primer set A produced the largest number of clones (Figure 3a), and was associated with most of Alphaproteobacteria-like and Gammaproteobacteria-like clades. Primer set B showed similar clade associations as set A, but produced fewer clones. Sets C and F, which were meant to target Flavobacteria also amplified Gammaproteobacteria. The clones from set F were

the most abundant in early winter. Finally, set D was associated *in silico* to freshwater PR clades (Atamna-Ismael *et al.*, 2008), and only produced three clones. Results from operational taxonomic clustering and BLAST analysis showed, similarly to maximum likelihood placements, a predominance of Gammaproteobacteria-like PRs on both dates, with secondary dominance of Flavobacteria-like PRs in early winter, replaced by Alphaproteobacteria-like PRs in late winter (Supplementary Figure S3).

Discussion

The Arctic Ocean, with its strong seasonal changes in irradiance, ice cover and primary productivity, provided the perfect setting to follow *in situ* PR gene dynamics over time, and assess the potential implication of environmental factors in PR presence, expression and diversity. Overwinter access to the Arctic Ocean enabled the collection of this unique dataset covering 8 consecutive months of sampling. PR was widely distributed and expressed throughout the entire study, although the relative intensity of expression decreased over the winter. Variations in expression levels coincided with the temporal succession of bacterial communities and lagged changes in environmental conditions. Although Gammaproteobacteria-like sequences accounted for most of the diversity, the change in dominance of the secondary clade in early to late winter from Flavobacteria-like to Alphaproteobacteria-like sequences may have been related to changes in local carbon dynamics. Overall, patterns in the clones' relative abundance (Table 1) followed relative intensity patterns of apparent expression from PCRs (Figure 3b).

Seasonal trends in PR presence and expression

Possession of PR would not represent an obvious *a priori* competitive advantage during Polar darkness given the absence of light (Béjà *et al.,* 2001), and the photon requirements of the PR pathway. However, positive amplification for all of the PR primer combinations in all of our Arctic DNA samples from November to June suggests otherwise. Whereas the PR gene is often reported from the photic biosphere (Finkel *et al.,* 2013), aphotic regions appear to be undersampled. The positive PR amplification for all primer pairs throughout the sampling period suggests that the gene's presence was not influenced by seasonally driven environmental conditions including light availability. A previous study that detected PR genes in Polar winter samples off the coast of Barrow, Alaska, assumed PR provided some advantage to carriers (Cottrell and Kirchman, 2009); however, no specific associated function was identified.

Although higher levels of gene transcripts in spring and summer suggest gene regulation by light, the low expression detected during winter darkness would be consistent with constitutive expression, regulation by other factors and possibly changes in community structure. In any case, the metabolic costs of PR synthesis appear to be minimal (Kirchman and Hanson, 2013). If light was the primary factor promoting expression, potential sources of light during Polar darkness are bioluminescence and the Moon. Most deep-sea animals and many bacteria are bioluminescent and high levels of bioluminescence have been detected in the deep Mediterranean for periods lasting several months (Tamburini *et al.,* 2013). This process also occurs in the Arctic, but the reported photic levels (Berge *et al.,* 2012) are lower than those observed in the Mediterranean. When a full Moon occurs at high latitudes, it provides light over prolonged periods. Some moonlight could be reflected and scattered in snow and ice rather than absorbed and reach the upper waters. Photic flux from moonlight in air is known (Raven and Cockell, 2006), but the actual quantity of moonlight that could penetrate the snow and ice cover of the Arctic Ocean during winter is not. If we assume the moonlight attenuation is similar to what is known for solar radiation (Petrich and Eicken, 2009), the photons reaching the surface are likely insufficient for significant energy conservation via PR proton pumping. The same applies for photons generated via bioluminescence. Perhaps a differential dependence on bioluminescence or moonlight, which emit in the green to blue and visible spectrum, respectively, could be inferred from the spectral tuning properties of PR in our samples; however, our fragments were too short to assess this possibility. Given that PR is a relatively simple protein compared with bacterial photosynthetic pigments (for example, BChl *a*; Kirchman and Hanson, 2013), thus the cost of expressing PR over winter may be minimal even when little or no ATP gains are possible. However, even when photons are limited, PR could provide indirect gains if used as a spatial or temporal biosensor (Fuhrman *et al.,* 2008).

Even minimal light could provide an advantage to some PR bacteria, as a few key sites of rhodopsin genes have been shown to be diagnostic of sensory functions (Spudich and Jung 2005). The more recently described site A178 (Yamada *et al.,* 2010) is of particular interest. In addition to a shift toward the red spectrum, a mutation at this position can induce a conformational change in the E and F helixes, resulting in the photocycle lasting 10 times longer (Bamann *et al.,* 2014). In proteins with this conformational change, the binding pocket would thus remain open much longer, supporting sensory rather than ATP-generating functions, particularly in low-light environments. We found that most of the sequences (79%) contained this «long photocycle» variant at site A178, and only 36 sequences had the more usual type (Ala, Cys or Thr).

The decrease in PR expression over winter, suggests some regulation in gene expression rather

than being exclusively constitutive. Examples of both constitutive and regulated expression of PR have been reported; Gómez-Consarnau *et al.* (2007) showed increased growth and expression in response to light, whereas Riedel and Tomasch (2010) did not, even though both studies used a marine flavobacterium (*Dokdonia* MED134 and PRO95, respectively). However, this may have been influenced by substrate availability: the former study used low organic carbon concentrations in their experiments, whereas the latter used concentrations that were two orders of magnitude higher. Recent studies show that nutrient availability is also important in regulating PR expression (Akram *et al.,* 2013). Under oligotrophic conditions, increased PR expression and growth was observed in some PR carriers undergoing starvation (Gómez-Consarnau *et al.,* 2010) and also in unamended seawater samples exposed to light–dark cycles (Lami *et al.,* 2009). However, a recent study (Riedel *et al.*, 2013) did not detect a measurable growth increase in the Flavobacterium PRO95 under nutrient-poor conditions mimicking oligotrophic environments and an additional requirement of stress or starvation may be a prerequisite for measurable PR enhanced growth (Riedel *et al.*, 2013). Although evidence for decreased respiration from light-mediated ATP production in starved SAR11 isolates (Steindler *et al.,* 2011) and significant light-driven proton pumping in marine Flavobacteria (Yoshizawa *et al.,* 2012) supports the notion that PR supplements bacterial energy requirements, PR's precise physiological role and impact on carbon cycling is likely very different in different bacteria.

Quantitative PCR assays in the coastal North Atlantic showed a tight correlation between SAR11 PR expression and photosynthetically active radiation, whereas growth-related gene activity did not (Lami and Kirchman, 2014), suggesting that energy collected from PR may be directed toward other functional responses, besides growth. Altogether, the presence of PR during the Polar darkness seems to involve several mechanisms and more functional complexity than reported from experiments.

Winter patterns in PR diversity

During this study, we retrieved and sequenced 187 PR clones. This is an important contribution to the understanding of PR diversity in polar waters as to our knowledge, previous polar studies yielded fewer clones (<50; Cottrell and Kirchman, 2009; Koh *et al.,* 2010). This difference is likely a consequence of our use of seven pairs of degenerate primers versus only three used in previous Arctic studies. We identified four main PR-carrying clades (Supplementary Figure S2) and highlighted changes in diversity from early and late winter, with almost half of the OTUs being found on one sampling date (Supplementary Figure S3). Although degenerate primers can capture a wide range of PRs, other types of microbial rhodopsins may not be amplified (see Materials and Methods section). For example, our primers did not target a diverse group of rhodopsins, the actinorhodopsins, associated with Actinobacteria (Sharma *et al.*, 2008, 2009).

Actinorhodopsins are typically associated with freshwater (Sharma *et al.*, 2008) or brackish aquatic systems (Salka *et al.,* 2014) and are not expected to be abundant at our marine sites. However, diverse Actinobacteria assemblages have recently been found in Arctic sediment samples (Zhang *et al.,* 2014) and are considered to be underestimated in some marine regions (Ghai *et al.*, 2013). Xanthorhodopsins can contribute to microbial rhodopsin diversity in sea-ice (Vollmers *et al.,* 2013), and may have been present, but would not be detected with our PR primers. In general, the relative abundance of the different major groups should be treated with caution. Alphaproteobacteria especially SAR11 are common in Arctic waters (Kirchman *et al.,* 2010; Ghiglione *et al.,* 2012) and are known carriers of PR (Grote *et al.,* 2012) and would be expected to be better represented in our libraries. However, in terms of presence or absence our results reflect earlier pyrosequencing 16 s rRNA gene surveys where Gammaproteobacteria, Alphaproteobacteria and Flavobacteria (Kirchman *et al.,* 2010) were the three most abundant groups.

Conclusions

Our work shows PR was widely distributed in Arctic bacterial communities and expressed even during the polar winter. Although involvement in light-activated proton pumping may be the gene's most well-characterized function, low but sustained expression of the PR gene over winter suggests other functional roles. This study indicates that polar bacterial communities modulate PR expression on a timescale of weeks to months in response to environmental changes. Although significant benefits from PR-synthesized ATP are unlikely overwinter, its role in environmental biosensing may contribute to bacterial survival. PR could facilitate a bacterium's ability to seek out and access-limited resources. The gene persisted throughout the study despite seasonal changes in the bacterial community structure, with relative expression patterns co-occurring along with changes in the communities. Changes in PR's function between the winter and spring could potentially explain this apparent synchrony. When winter PR diversity was evaluated over time, Gammaproteobacteria-like PR sequences dominated, but a transition from Flavobacteria-like to Alphaproteobacteria-like PRs occurred from early to late winter, concomitant with changes in organic matter lability and availability observed during the CFL study. These observations suggest a linkage between local carbon dynamics and PR-diversity

patterns in Polar Oceans. Further assessment of PR's non-ATP forming pathways, which need very low levels of photic energy, is required if we are to understand the impact of this gene on marine microbial dynamics and carbon economy. These will need to be paired with metabolic rates of carbon consumption by PR carriers if we are to quantify the potential impacts of PR on C cycling.

Conflict of Interest

The authors declare no conflict of interest.

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