Survival of *Prochlorococcus* in extended darkness

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Abstract

*Prochlorococcus* is the smallest oxygenic phototroph in the ocean, where it can be found in great abundance throughout the euphotic zone in mid-latitude waters. Populations of this picocyanobacterium have been observed below the euphotic zone, but the viability of these cells is unclear. To explore the tolerance of *Prochlorococcus* to extended light-deprivation, we subjected multiple strains of *Prochlorococcus* to varying periods of darkness and examined their ability to recover when placed back in the light. Some strains recovered after 35 h of darkness while others could not; this variability was not related to whether the strains were members of high- or low-light adapted ecotypes. The presence of a marine heterotroph, *Alteromonas macleodii* MIT1002, in the cultures extended their ability to survive prolonged darkness, in the most extreme case by 11 d. This could be attributed at least in part to the reduction of hydrogen peroxide in co-cultures, consistent with known roles of “helper bacteria” in detoxifying hydrogen peroxide, and this effect could be mimicked to some degree by the addition of a known hydrogen peroxide quencher, sodium pyruvate. The addition of glucose alone to the cultures provided marginal enhancement, but when both pyruvate and glucose were added together, all strains were able to survive longer in darkness than they were with only the heterotroph added. Thus, it appears that *Prochlorococcus* dark-survival depends on a multitude of factors. Limited analyses of *Synechococcus* suggest that its dark-survival capacity is longer than that of *Prochlorococcus*, for reasons that are not yet clear.

Found primarily throughout the ocean’s euphotic zone between 45°N and 40°S, *Prochlorococcus* is the smallest (< 1 μm) and most abundant oxygenic phototroph (Partensky et al. 1999). The *Prochlorococcus* genus is composed of multiple distinct phylogenetic groups that are physiologically and genetically diverse, known as ecotypes (Moore et al. 1995, 1998; Moore and Chisholm 1999; West and Scanlan 1999; Rocap et al. 2002; Huang et al. 2012). These ecotypes can be broadly classified as either high-light (HL) or low-light (LL)—adapted based on multiple physiological characteristics including the optimal light level for growth in culture (Moore et al. 1995; Moore and Chisholm 1999; Zinser et al. 2007). The distributions of *Prochlorococcus* ecotypes in the euphotic zone show that HL and LL variants have distinct distributions, with HL-adapted cells dominating the surface waters and LL-adapted cells restricted primarily to deeper waters. Cells belonging to one of the LL clades (LLI, also known as ecotype eNATL2A), are relatively better adapted to vertically mixed waters than other LL-adapted clades (Zinser et al. 2006, 2007; Johnson et al. 2006; Malmstrom et al. 2010). While they are non-motile and neutrally buoyant, *Prochlorococcus* and other cyanobacteria are subject to displacement by vertical mixing or attachment to sinking particles by phytodetritus (Lochte and Turley 1988; Vilibić and Santić 2008; Jiao et al. 2013). Thus, cyanobacterial cells likely get mixed below the euphotic zone for extended periods of time. Their ability to withstand extended dark exposure could have important consequences for their biogeography, population genetics, long-term evolution, and the biogeochemistry of ocean basins.

Eukaryotic species of phytoplankton have been studied vis-à-vis their ability to survive extended periods of darkness and when these cells can no longer rely on photosynthesis, they must acclimate or die; potential options include resting stage formation (Smetacek 1985), reduction of metabolic rates (Dehning and Tilzer 1989), or using heterotrophic modes of acquiring energy (White 1974). Autotrophic flagellates, ciliates, and cryptophyte species are able to survive by mixotrophy or phototrophy in the summer and solely heterotrophy in winter when the cells experience complete darkness under ice coverage (Roberts and Laybourn-Parry 1999; Laybourn-Parry 2002; Cottrell and Kirchman 2009).

There have been many observations of *Prochlorococcus* and *Synechococcus* cells (collectively known as marine picocyanobacteria)
in locations and conditions where extended darkness prevails. Prochlorococcus, for example, can be found in abundance far below the euphotic zone (DeLong et al. 2006; Shi et al. 2011; Martínez et al. 2012; Jiao et al. 2013; Shibl et al. 2014). Furthermore, the abundance of Synechococcus has been shown to change minimally from summer to winter in arctic waters (Cottrell and Kirchman 2009) suggesting they can survive continuous darkness for extended periods under ice coverage. Synechococcus has also been found to persist for several months after deep mixing events—down to 600 m—in the Adriatic Sea (Vilibić and Šantić 2008), and cells sampled from 300 m in the Suruga Bay (Sohrin et al. 2011) have been observed to maintained their cell density for 30 d in the dark.

The observations of picocyanobacteria deep in the euphotic zone made us wonder whether they, like the eukaryotes described above, have evolved strategies for surviving extended darkness. Picocyanobacterial capacity for mixotrophy—the ability of a phototroph to utilize organic carbon—is now well known (Zubkov et al. 2003; Malmstrom et al. 2005; Zubkov and Tarran 2005; Paoli et al. 2008) and appears to be widespread in the global oceans (Yelton et al. in press). More specifically, Prochlorococcus genomes contain genes encoding the ability to utilize organic compounds including amino acids (Zubkov et al. 2003, 2004; Rocap et al. 2003; Church et al. 2004; Michelou et al. 2007; Mary et al. 2008), as well as glucose transporters (Rocap et al. 2003; Gómez-Baena et al. 2008; Muñoz-Marín et al. 2013). Recent studies have also shown that Prochlorococcus is able to take up glucose and amino acids in both light and dark conditions—in laboratory cultures and in the wild (Zubkov et al. 2003, 2004; Church et al. 2004; Michelou et al. 2007; Gómez-Baena et al. 2008; Mary et al. 2008; Gómez-Pereira et al. 2013; Muñoz-Marín et al. 2013; Evans et al. 2015)—as well as dimethylsulfoniopropionate (Vila-Costa et al. 2006). Amino acids could be used as either a carbon, energy, or nitrogen source, but glucose is likely only utilized for carbon and/or energy.

The goal of the work reported here was to begin to explore the ability of marine picocyanobacteria to survive extended darkness, with a primary focus on Prochlorococcus. To this end, we subjected axenic strains grown on a diel light:dark cycle to additional intervals of darkness of varying length, simulating deep mixing events, and then determined if they could recover when placed in the light. We then extended this basic experimental design to examine the effects of co-culture with heterotrophs and the addition of organic compounds on the extent of dark-survival. Finally we carried out a limited number of similar experiments with Synechococcus to lay the foundation for further comparative studies.

**Methods**

**Cultures and culture conditions**

All Prochlorococcus and Synechococcus cells were grown in 0.2 μm filtered sterile Sargasso Sea water amended with Pro99 nutrients prepared as previously described (Moore et al. 2007). Prochlorococcus cells starting at a concentration of 5 × 10^6 cells mL⁻¹ to 1 × 10^7 cells mL⁻¹, were grown in triplicate in a 13:11 light-dark (L:D) cycle with simulated dawn and dusk (Zinser et al. 2009) at 24°C. This simulation creates gradual light transitions at sunrise by ramping light slowly up to mid-day, remaining at peak light for 4 h, and then decreasing light to sunset over the course of 13 h. This gradual increase was important for reducing light shock on the cultures transitioning from extended darkness back into 13:11 conditions. Near optimal peak light levels for maximizing growth rate were used for all Prochlorococcus strains involved and included the following combinations: MED4 (78 μmol photons m⁻² s⁻¹), MIT9312 (75 μmol photons m⁻² s⁻¹), MIT9301 (54 μmol photons m⁻² s⁻¹), MIT9202 (87 μmol photons m⁻² s⁻¹), AS9601 (75 μmol photons m⁻² s⁻¹), NATL1A (37 μmol photons m⁻² s⁻¹), NATL2A (37 μmol photons m⁻² s⁻¹), MIT9211 (33 μmol photons m⁻² s⁻¹), MIT9313 (29 μmol photons m⁻² s⁻¹), and MIT9303 (29 μmol photons m⁻² s⁻¹). Light levels for Synechococcus cultures were: WH8102 (29 μmol photons m⁻² s⁻¹), WH7803 (37 μmol photons m⁻² s⁻¹). To subject cells to extended darkness, we placed exponentially growing cultures into a 24°C dark incubator at the end of the 13:11 L:D cycle for varying durations. Including the 11 h of their last “natural” L:D cycle, these cultures were in the dark for a total of 35, 59, 83, 107, 179, or 275 h, which amounts to an additional 1, 2, 3, 4, 7, or 11 d of darkness. The cultures were then shifted back into the L:D incubator at “sunrise” to reduce light shock effects, and recovery was monitored via bulk chlorophyll fluorescence (10AU model, Turner Designs, Sunnyvale, California) and flow cytometry (see below). All dark sampling and measurements were done in green light using layered neutral density filters #736 and 740 (Lee Filters, Burbank, California) over a white light source, which causes minimal gene expression effects, and recovery was monitored via bulk chlorophyll fluorescence (10AU model, Turner Designs, Sunnyvale, California) and flow cytometry (see below). All dark sampling and measurements were done in green light using layered neutral density filters #736 and 740 (Lee Filters, Burbank, California) over a white light source, which causes minimal gene expression effects, and recovery was monitored via bulk chlorophyll fluorescence (10AU model, Turner Designs, Sunnyvale, California) and flow cytometry (see below).
did not influence Prochlorococcus' growth rate under normal diel conditions (data not shown).

Flow cytometry and metagenomics

Prochlorococcus cell abundance measurements by flow cytometry were prepared and processed as previously described (Zinser et al. 2006; Malmstrom et al. 2010). Samples were run on an Influx flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.) with 2 μm blue and red excited reference beads. Cells were excited with a blue 488 nm laser analyzed for chlorophyll fluorescence (692/40 nm), SYBR Green I stained DNA fluorescence content (530/40 nm), and size (forward scatter). Calculations for relative cell size and chlorophyll per cell were performed by normalizing forward scatter and red chlorophyll fluorescence per cell to 2 μm diameter Fluoresbrite beads (Polysciences Inc., Warrington, Pennsylvania, U.S.A.) as previously described (Olson et al. 1990). Co-culture samples were also stained with 1x SYBR Green I (Invitrogen, Grand Island, New York, U.S.A.) and incubated for 10 min in the dark prior to running. All flow cytometry files were analyzed using FlowJo version 7.6.5.

Total Prochlorococcus abundance depth profiles were characterized from samples taken from a Hawaii Ocean Time-series (HOT152) cruise in October 2003 using flow cytometry (methods described above) and metagenomics. Metagenomic DNA samples from 50, 600, and 1000 m depths were extracted from frozen filters (100 mL raw seawater filtered onto a 0.2 μm diameter filter) using a phenol:chloroform based method (Urra et al. 2010), and 1 ng of DNA was used to generate sequencing libraries using the Nextera XT DNA library prep kit (Illumina, San Diego, California, U.S.A.). Libraries were sequenced on the Illumina NextSeq platform at the MIT BioMicro center, yielding ~20–30 million 150 + 150 nt paired-end reads per library. Illumina adapters were removed from the raw sequence data using cutadapt (Martin 2011), overlapped using clc_overlap_reads (CLC bio, Aarhus, Denmark) and remaining low-quality regions were removed by clc_quality_trimm (CLC bio, Aarhus, Denmark) using default settings. To determine the Prochlorococcus and Synechococcus content of each library, all overlapped and trimmed reads were first searched against a custom database of all sequenced Prochlorococcus and Synechococcus genomes (Biller et al. 2014a) using UBLAST (Edgar, R.C., unpublished, http://drive5.com/usearch). Any reads that had a significant hit (e value <= 1e-4) against this database were then searched against the NCBI nr database (downloaded April 25, 2015) with UBLAST, using the following options: “-evalue 1e-4 -maxhits 1 -accel 0.5”. The number of reads whose best hit in NR was to either Prochlorococcus or Synechococcus (according to the NCBI taxonomy ID for that best hit) were counted using a custom Python script. The metagenomic datasets were deposited in the NCBI Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under accession SRP065727.

Viability

Viability was tested by adding 1 μM SYTOX Green (Invitrogen, Carlsbad, CA) to the cells for 10 min prior to flow cytometry (as per manufacturer’s instructions) on a Guava easyCyte HT flow cytometer (EMD Millipore, Billerica, MA). Glutaraldehyde-killed negative controls were created by adding glutaraldehyde (final conc. 0.125% v/v) to samples and incubating these in the dark at room temperature for 1 h. Cells were excited with a blue 488 nm laser and analyzed for chlorophyll fluorescence (692/40 nm) and size (forward scatter). Using events within the Prochlorococcus defined gate, dead cells were determined by examining SYTOX Green content (580/30 nm) and size (forward scatter) and the events that had values similar to a glutaraldehyde-killed control were defined as dead cells.

Extracellular hydrogen peroxide

Hydrogen peroxide measurements were obtained by using an Amplex Red kit (A22188, Life Technologies, Grand Island, NY). Directions from the manufacturer were followed with the exception of using Pro99 medium (instead of buffer) and reducing the Amplex concentration to 50 μM (instead of 100 μM as suggested). All Prochlorococcus cultures were grown in a medium buffered with 3.75 mM TAPS pH 8 to maintain enzymatic functionality of the Amplex Red. Samples were filtered through a 96 well multiscreen HTS vacuum manifold (MSVMHTS00, EMD Millipore, Billerica, MA) using a 0.22 μm Multiscreen HTS GV sterile filter plate (MSGVS2210, EMD Millipore, Billerica, Massachusetts, U.S.A.). The filtrate was collected and transferred to black walled plates with H2O2 standard curves (0–2.5 μM), which were made by adding known hydrogen peroxide concentrations to a seawater background to obtain the slope of the linear peroxide-fluorescence relationship. We made a no-H2O2 control by adding 1 mM sodium pyruvate (H2O2 scavenger) to the seawater background 30–60 min prior to the assay; this consumed all of the peroxide the seawater sample and thus allowed us to determine the intercept of the peroxide-fluorescence relationship. As pyruvate quenches peroxide at a 1 : 1 molar ratio (Holleman 1904; Bunton 1949), this concentration of pyruvate is vastly in excess of what is needed to remove previously measured seawater media peroxide levels. Peroxide treatment yields results similar to reactions done with catalase, a light sensitive chemical used in previous studies for creating no-H2O2 control in seawater (Rose et al. 2010). These data and results from another report that used Amplex Red and sodium pyruvate (Zhu et al. 2010), confirm that sodium pyruvate is not inhibitory to Amplex Red. Plates were read on a fluorometer (BioTek Synergy2, Winooski, VT), using 530/25 nm excitation and 590/20 nm emission filters.

Results and discussion

Prochlorococcus below the euphotic zone

Adding to the growing body of evidence that Prochlorococcus is found well below the euphotic zone (DeLong et al. 2006; Shi et al. 2011; Martinez et al. 2012; Jiao et al. 2013; Shibli et al. 2014), we found Prochlorococcus and Synechococcus across a gradient of depths up to 1000 m at station ALOHA in the Pacific, as determined by both flow cytometric and metagenomic
approaches. Prochlorococcus and Synechococcus concentrations were relatively consistent between 600 m and 1000 m depth, at 3–4 × 10^4 cells mL⁻¹ and 2–6 × 10^2 cells mL⁻¹, respectively (Supporting Information Table S1). These deep populations were dominated by HL adapted Prochlorococcus ecotypes (Supporting Information Table S1) as was observed for deep water samples from the Luzon Straight off China (Jiao et al. 2013) and the Red Sea (Shibl et al. 2014). Subduction of surface waters in which HL adapted ecotypes are dominant is the likely explanation for their abundance in deep water but it is not clear how long they, or Synechococcus, might maintain a recognizable flow cytometric signature in the perpetual darkness at these depths.

Limits to Prochlorococcus culture survival in extended darkness

We wondered how “old” cells detected at 1000 m might be and to address this indirectly, we subjected a Prochlorococcus MED4 culture to complete darkness for 200 d, and measured its flow cytometry signature over this period (Fig. 1). Cell abundance remained roughly the same during the entire experiment (Fig. 1a), but the relative chlorophyll per cell and cell size (as inferred from forward scatter) decreased gradually (Fig. 1b,c). Flow cytometry dot plots after 197 d in the dark revealed a population that remained well above the noise level and recognizable as an intact population (Fig. 1d). We made attempts to revive cultures by returning them to the light conditions they were originally grown in, but none resumed growth (data not shown).

To determine the limit of “photon deprivation” beyond which the cells cannot recover in the absence of alternative sources of energy and whether LL-adapted ecotypes are better at surviving extended darkness, we took exponentially growing cultures of strains representative of the cultured high-light and low-light adapted Prochlorococcus clades and subjected them to 35, 59, and 83 h (1, 2, and 3 extra days of darkness beyond their 11 h night time dark cycle) of dark stress and then re-exposed to light to see if they could recover (Table 1). All but 3 of the 10 strains were able to recover after 35 h of darkness, but none were able to after 59 or 83 h in the dark. The three strains unable to tolerate even 35 h in the dark were members of the HL I (MED4), HL II (MIT9202), and LL II/III (MIT9211) clades. Thus there was no relationship between dark survival capacity and whether or not a strain was high or low-light adapted. Two Synechococcus strains (WH8102 and WH7803) were also examined for their dark survival times (Table 1) and both were able to recover even after 83 h of darkness, two days longer than other Prochlorococcus strains (further discussed below).

The role of co-cultured heterotrophs in dark survival

Evidence is mounting that the fitness of Prochlorococcus can be positively affected by the presence of heterotrophic bacteria when growing under continuous light or a diel light/dark cycle, due at least in part to the reduction of oxidative stress in the cultures (Morris et al. 2008, 2011; Sher et al. 2011). To examine if the presence of heterotrophs might also affect the ability of Prochlorococcus to survive the stress of extended darkness, we subjected cultures of MED4, NATL2A, and MIT9313 in co-culture with Alteromonas mac- leodii MIT1002 (hereafter referred to as Alteromonas), a heterotroph which was isolated from the original Prochlorococcus NATL2A culture, to periods of extended darkness and monitored their ability to regrow when re-introduced to the light. While the presence of Alteromonas did not influence the growth rate of these three strains in exponentially growing cultures grown on a diel cycle (Supporting Information Fig. S1), survival of MED4 (a HL—adapted strain) and NATL2A (a LL—adapted strain) in the dark was significantly enhanced.

Fig. 1. Changes in cell abundance (a), bulk chlorophyll per cell (b), bulk forward light scatter per cell (c), and chlorophyll fluorescence (d), in axenic Prochlorococcus MED4 cultures as a function of time in continuous darkness (data to the right of gray dotted vertical line). Flow cytometric signatures (d) of the cells at the beginning (before dark) and end (197 d of darkness) reveals that the population maintained its distinct flow cytometric signature, slightly shifted, even after 197 d in darkness. Populations in the upper right corner (d) are 2 μm diameter internal standard beads. Error bars represent the standard deviation from triplicate samples and are smaller than the data points when not visible.
by its presence (Fig. 2a,d and 2b,e respectively). When alone, Prochlorococcus MED4 was unable to survive even 35 h of continuous darkness (Fig. 2a), but in co-culture it could survive an additional 6 d (Fig. 2d). Similarly, Prochlorococcus NATL2A was able to survive an additional 10 d of darkness when with Alteromonas (Fig. 2e).

For MIT9313 (LLV clade), the presence of Alteromonas extended the dark survival time from 35 h to 83 h, but could not mediate recovery for longer dark periods (Fig. 2c,f). Furthermore, its recovery was much slower relative to that of MED4 and NATL2A for the same period of darkness (Fig. 2d–f), consistent with observations of extended recovery lag periods in co-cultures of Alteromonas and Prochlorococcus relative to pairings with other heterotrophs (Sher et al. 2011). It is not clear what aspect of MIT9313 cell physiology would influence the “helping” function of the heterotroph vis a vis dark survival. One possibility is that it could be relatively more stressed upon reintroduction into the light as previous work has shown that MIT9313 is more sensitive to light shock than MED4 or NATL2A (Berta-Thompson 2015).

**Effect of oxidative stress on the survival of Prochlorococcus in the dark**

Heterotrophs play a key role in reducing oxidative stress of Prochlorococcus via the production of extracellular catalase-peroxidase (Morris et al. 2008). Prochlorococcus lacks the gene (katG) that encodes this enzyme, and co-culture with catalase-peroxidase producing heterotrophs has been shown to increase the fitness of Prochlorococcus both in culture and in the field (Morris et al. 2008, 2011). These observations have even led to a new theory about the evolution of dependencies and genome streamlining (Morris et al. 2012). While the production of hydrogen peroxide is typically thought to be due to photochemical reactions—either biologically or abiotically mediated—in sunlit ocean waters (Cooper and Zika 1983; Zepp et al. 1987; Johnson et al. 1989), biological production of H₂O₂ can also be significant in the dark (Palenik and Morel 1988; Yuan and Shiller 2004; Verityea et al. 2010). Thus we designed experiments to see whether detoxification of H₂O₂ could explain the ability of Alteromonas to partially mitigate the stress of prolonged darkness for Prochlorococcus.

We measured hydrogen peroxide levels in MED4 (Fig. 3a–d) and NATL2A (Fig. 3e–h) cultures during extended darkness and upon re-exposure to light, with and without additions of Alteromonas (Fig. 3a–d, blue and red lines). As before (Table 1 and Fig. 2), in the absence of other treatments MED4 cells were unable to survive even 35 h in the dark (Fig. 3a,b); NATL2A cells were able to tolerate 35 h of darkness but no longer (Fig. 3e,f). In all the untreated cultures that were unable to recover, hydrogen peroxide concentrations peaked 3–4 d after the cells were placed in the dark (Fig. 3e,f,h). In the untreated NATL2A culture subjected to 35 h of darkness (Fig. 3g) that did recover, hydrogen peroxide levels remained near media background levels throughout. In the 83 h dark treatment of both strains, hydrogen peroxide concentration increased before the cells were re-introduced into the light (Fig. 3f,h) indicating that its production was not due to light shock. Thus, for the three conditions tested in which the cells were unable to recover from dark stress, hydrogen peroxide concentrations reached levels 2–4 times those of media alone (Fig 3e–h, black dashed line).

When Prochlorococcus was co-cultured with Alteromonas, hydrogen peroxide concentrations stayed close to that of the media background, and all cultures recovered (Fig. 3a–h, red lines). From these data, we infer that the presence of Alteromonas reduced hydrogen peroxide concentrations in the cultures and promoted dark survival of Prochlorococcus. To try to explore this causality more directly we added sodium pyruvate, a reactive oxygen species (ROS) scavenger and organic carbon source, to cultures in the absence of Alteromonas (Fig. 3a–d green lines). Sodium pyruvate is commonly included in media used for Prochlorococcus isolations or purifications (Berube et al. 2015) and has no influence on its growth under standard conditions.

We were unable to use exogenous catalase as it is highly sensitive to light and degrades quickly, so it would not be effective in these long-term experiments. Sodium pyruvate, on the other hand, has a very quick and effective 1 : 1 molecule reaction with hydrogen peroxide (Holleman 1904; Bunton 1949)—reducing the concentrations of H₂O₂ in all the cultures to zero (Fig 3a–d, green lines).

The presence of pyruvate in the Prochlorococcus NATL2A cultures extended their dark survival times from 35 h to

**Table 1. Regrowth of axenic high-light (HL)- and low-light (LL)-adapted Prochlorococcus and Synechococcus cultures following extended darkness conditions. Cultures were placed into the dark for an additional 24, 48, or 72 h (for a total of 35, 59, or 83 h) before re-exposure to light. Cultures were monitored for regrowth based on increases in bulk chlorophyll fluorescence. Plus symbols (+) indicate that the cultures grew when placed back into the light, minus symbols (−) a lack of growth following dark stress.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clade</th>
<th>Growth following time in dark</th>
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<tr>
<td><strong>HL Prochlorococcus</strong></td>
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<tr>
<td>MED4</td>
<td>HLI</td>
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<td>MIT9202</td>
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<td>HLI</td>
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<tr>
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<td><strong>LL Prochlorococcus</strong></td>
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<tr>
<td>NATL2A</td>
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<tr>
<td>MIT9211</td>
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<tr>
<td>WH8102</td>
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<td>WH7803</td>
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Prochlorococcus *in extended darkness*
83 h (Fig. 3c,d), mimicking the effect of Alteromonas. While the addition of pyruvate allowed MED4 to survive 35 h of darkness—which it was unable to do in the no treatment control—the time MED4 took to recover was delayed relative to that in the presence of Alteromonas (compare red and green Fig. 3a). Furthermore, Alteromonas could facilitate MED4’s survival of 83 h of darkness, while pyruvate could not (Fig. 3b). This suggests that its role in promoting dark survival does not lie solely in \( \text{H}_2\text{O}_2 \) detoxification. One possibility is that organic exudates from Alteromonas can be used as sources of organic carbon by Prochlorococcus.

**Use of alternative carbon and energy sources as a means to survive darkness**

As discussed previously, Prochlorococcus has the genomic capacity for mixotrophy, and is known to take up glucose and other organic compounds. To test whether non-ROS scavenging organic compounds could enhance dark survival, we grew Prochlorococcus MED4 with 5 mM glucose and subjected cultures to 59, 83, or 107 h of darkness (Fig. 4a–d). While this is a very high concentration of glucose relative to that found in the wild, so is the concentration of cells in our experiments; thus proportionally it is not that high. Since we know that this amount of glucose does not inhibit or enhance growth rates under standard growth conditions in the light (data not shown), we opted to use this concentration to insure that there would be a sufficient supply available to the cells to determine whether it might have an effect in the dark. That is, our intention was simply to see whether saturating levels of glucose might extend dark-survival. In the absence of any treatment, MED4 cells are unable to recover after 59 or more hours of darkness (Fig. 

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**Fig. 2.** Response of axenic Prochlorococcus high-light (HL)-adapted MED4 (a) and low-light (LL)-adapted NATL2A (b) and MIT9313 (c) to extended darkness compared with the same strains cocultured with the heterotrophic bacterium Alteromonas macleodii (d, e, f) under otherwise identical conditions. Cultures were inoculated with A. macleodii at the beginning of the experiment and placed into the dark (gray dotted vertical line) for an additional 24, 48, 72, 168, or 264 h beyond the normal “night” (for a total of 35, 83, 179, or 275 h of darkness) and then re-exposed to light (represented by colored ticks on axis). Experimental cultures were monitored using bulk chlorophyll fluorescence for over 100 d to detect whether they could be revived when placed back in the light; cultures which did not regrow during this timeframe were monitored visually for an additional 2 months, but no growth was observed. Note that both axes are on a log scale.
with glucose they can withstand 59 h in the dark, but no longer (Fig. 4b). When we added pyruvate to the cultures in addition to the glucose, the cells recovered even after 83 h of darkness (Fig. 4d). MED4 cells kept in the dark for 197 d in the presence of glucose and pyruvate, however, did not resume growth when returned to their original light conditions; the cell abundance, bulk chlorophyll per cell, and bulk forward light scatter per cell results were similar to untreated MED4 shown in Fig. 1a–d (data not shown). It is important to note that there was no population growth in the dark when glucose and pyruvate are supplied, suggesting that organics can play a role in maintenance metabolism.

**Fig. 3.** Changes in cultures of two *Prochlorococcus* strains after extended dark treatment, as measured by bulk chlorophyll fluorescence (a–d) and hydrogen peroxide concentration (e–h); data are shown in the presence and absence of either *Alteromonas* (red) or sodium pyruvate (green). Cultures of high-light (HL)-adapted MED4 (a, b, e, f) and low-light (LL)-adapted NATL2A (c, d, g, h) were grown either alone, with *Alteromonas*, or with 5 mM sodium pyruvate and were placed into the dark (indicated by gray shading) for 35 (a, c, e, g) or 83 (b, d, f, h) h before re-exposure to light. "*Prochlorococcus only*" cultures (blue) were not amended with sodium pyruvate or *Alteromonas*. Peroxide measurements (panels e–h) were made in the cultures alongside a media control (black dashed line) and were taken during the critical time frame for the experiment (before extended dark, throughout the extended darkness, and after re-exposure).
and reduction of oxidative stress which facilitates survival and recovery after extended darkness. In an independent experiment, we examined whether results seen in Fig. 4 with MED4 were consistent with another Prochlorococcus strain, NATL2A, and indeed the combination of glucose and pyruvate enabled all cultures to recover (Supporting Information Fig. S2). We suggest that Alteromonas might play this dual role in its interactions with Prochlorococcus.

Cell viability and intra-population variability in response to stress
In the experiments described above we used bulk culture fluorescence as a measure of culture status. That is, we interpret exponential increases in fluorescence when cells are placed in light after prolonged darkness to reflect the growth—or at least the metabolic activity—of surviving cells. It is well established, however, that bulk fluorescence does not reflect cell number when a culture is not in steady state exponential growth (Falkowski and Kiefer 1985; Olson et al. 1988; Thompson et al. 2011). Thus we explored what was happening to individual Prochlorococcus cells in a limited set of the dark-stressed cell populations using flow cytometry. Where relevant, we also monitored the population dynamics of Alteromonas in the co-cultures. Examining the effects of 83 h of darkness on MED4 alone and in co-culture with Alteromonas, we found that while bulk culture fluorescence plummeted after re-exposure to light in both the axenic and cocultures (Fig. 5a,b, blue line), the total Prochlorococcus cell abundance remained constant over the whole experiment (Fig. 5a,b, black line); however, only Prochlorococcus in the co-cultures with Alteromonas recovered (Fig. 5b). The abundance patterns of Alteromonas mimicked that of Prochlorococcus, at 10-fold lower concentration throughout dark and recovery time periods (Fig. 5b, red line)—likely due to the fact that Alteromonas is unable to grow in Pro99 medium alone and is dependent on the organic compounds produced by Prochlorococcus (Biller et al. 2014b).

Examination of the flow cytometry dot plots of Prochlorococcus from this experiment revealed complex intra-population dynamics (Fig. 5c–j). We observed flow cytometric evidence for Prochlorococcus differentiation into subpopulations which we defined as “High Fluorescence Prochlorococcus” (red circle) and “Total Prochlorococcus” (black outline), the latter a combination of the High Fluorescence population and the Prochlorococcus populations that decrease in fluorescence over time. In the Prochlorococcus-alone cultures, which were unable to recover (Fig. 5a), the mean fluorescence per cell of the entire population decreased steadily upon reintroduction to the light (Fig. 5c–f), even though total cell numbers remained constant (black line, Fig. 5a). In the presence of Alteromonas the population trajectory was similar to that of Prochlorococcus-alone cultures for 14 d after reintroduction to the light, but in this case a population of Prochlorococcus cells appeared after 38 d with fluorescence and light scatter values identical to those observed after 3 d in the dark (compare Fig. 5g,j, circled). We hypothesize that a very small subpopulation of cells began growing upon re-exposure to light and only became detectable after 38 d. It appears that this small subpopulation was the seed population that grew exponentially between days 50 and 65 and is evident in the bulk culture fluorescence values (Fig. 5b). Thus, we argue, the “rebound” of this culture has its origins in a small subset of the cells. If we assume this subpopulation began growing exponentially immediately upon re-exposure to light, the size of this “inoculum” is estimated to be around 83 cells mL$^{-1}$—or 0.00015% of the cells that were placed in the dark.

We note that the cell number data for Alteromonas reported in Fig. 5b were determined by staining a replicate set of

**Fig. 4.** Response of Prochlorococcus high-light (HL)-adapted MED4 to the presence of glucose, sodium pyruvate, or both, during extended dark exposure. Cultures were placed into the dark (grey dotted line) for 59 h or 83 h before re-exposure to light (colored ticks on axis). Cultures were monitored using bulk chlorophyll fluorescence to detect whether they could be revived when placed back in the light. Note that the x axis is a linear scale, unlike that of Figs. 2a–f, 3a–d.
samples, not from the exact same populations identified in Fig. 5g–k. *Alteromonas* cells in the latter display red fluorescence under these conditions, as a by-product of glutaraldehyde staining. This same strain does not autofluoresce when grown in the organic media (ProMM) in which it is maintained and it does not influence the results presented here, as this population can be clearly separated from *Prochlorococcus*.

To this point, we have been defining dark-survival as the ability to grow upon re-exposure to light. One wonders, however, whether cells held in the dark for long periods of time might indeed be “viable,” but we have simply not found the optimal conditions to allow them to actually grow and replicate while in the dark. A common way to address this type of question is through the use of a vital stain to determine what fraction of the population remains “viable”—i.e., alive—in the
To examine the viability of Prochlorococcus in the dark, we treated cultures with the nucleic acid stain SYTOX Green—an asymmetrical cyanine dye with three positive charges that is typically excluded from live eukaryotic and prokaryotic cells. This compound has been previously shown to identify viability in Prochlorococcus cultures (Hughes et al. 2011; Morris et al. 2011) and we found that it was effective in separating live and dead (glutaraldehyde-treated) cells in cultures grown on the standard 13:11 L:D cycle (Supporting Information Fig. S3a); as expected, glutaraldehyde-treated cells took up the stain while live ones did not. Surprisingly, after extended darkness, this assay failed to identify glutaraldehyde-treated cells as “dead” (Supporting Information Fig. S3b); the glutaraldehyde-killed cells excluded the stain, as would be expected for live cells (Supporting Information Fig. S2b, solid line). As a consequence of this intriguing phenotype, we were not able to use the SYTOX stain to differentiate “viable” and “dead” Prochlorococcus cells under extended darkness.

While it is possible that the extended-darkness cells were not easily killed by glutaraldehyde, we doubt this. It is possible, however, that extended darkness induces extensive physiological changes in Prochlorococcus that could influence the entry of the dye. It is also possible that the DNA in these cells has degraded and does not stain with this dye, as has been previously reported with other stressed cell cultures (Lebaron et al. 1998). We also tried other treatments as alternatives to dead-cell controls such as treating with glutaraldehyde for less time (10 min), or heat shocking under various conditions (60–80°C for 15 min, and 80°C for 5 min), but none of these methods were as effective at killing exponentially growing Prochlorococcus as the one hour glutaraldehyde treatment. Although our attempts at determining viability were inconclusive, Prochlorococcus have been isolated into culture from untreated euphotic zone seawater samples that were held in the dark for up to 21 d (Biller et al. 2014b); this illustrates the potential for relatively long term survival of these phototrophs in the dark and recovery upon reintroduction into the light—though only when embedded in their indigenous microbial community.

**Fig. 6.** Summary of the factors influencing survival and regrowth of Prochlorococcus MED4ax cultures (green ovals) after 83 h dark exposure. On the left are conditions that did not result in regrowth upon reintroduction to the light. On the right are shown the experimental treatments that result in regrowth, including the results in co-culture with Alteromonas (brown oval). H2O2 scavengers (red) indicate extracellular quenching of H2O2. Solid arrows represent functions either imposed by the experimental design, or for which there is experimental evidence, and dotted lines indicate hypotheses based on this work. Results indicate that if extracellular H2O2 concentrations are lowered by the addition of pyruvate, and a source of carbon (glucose) is supplied, Prochlorococcus is able to regrow after 83 h of darkness; it is unable to recover with either of these treatments on its own, or in the control with no treatment. Co-culture with Alteromonas yields similar results to those of the combined glucose and pyruvate additions, suggesting that Alteromonas could be providing Prochlorococcus with an alternative energy source.

**Differences between Prochlorococcus and Synechococcus**

Since Prochlorococcus always co-occurs with Synechococcus, we thought it of interest to compare the dark-survival times of the two genera in a limited set of experiments. Two Synechococcus strains (WH8102 and WH7803) were examined simply for their dark survival times in the absence of any treatments. WH8102 is typically found in oligotrophic open-ocean waters whereas WH7803 is a widely distributed oceanic clade (Scanlan et al. 2009). Both were able to recover even after 83 h of darkness, two days longer than any Prochlorococcus strains in the absence of any treatments (Table 1). While data is too limited for strong inference, the difference in survivability in the dark between Prochlorococcus and Synechococcus might be rooted, at least in part, in the differences between their circadian clock networks. Prochlorococcus has an incomplete circadian clock (Holtzendorff et al. 2008; Axmann et al. 2009; Mullineaux and Stanewsky 2009); while its cell division and gene expression synchronizes tightly to a diel light dark
cycle (Zinser et al. 2009; Waldbauer et al. 2012) when switched to continuous light it loses the rhythmicity quickly (Holtzendorff et al. 2008), suggesting that it needs light to reset its rudimentary circadian clock (Axmann et al. 2009). By contrast, Synechococcus has a complete circadian clock and displays free-running rhythmicity for several days when switched to continuous light (Holtzendorff et al. 2008). Perhaps its complete circadian clock allows Synechococcus to keep cellular functions coordinated over a few days in the dark, thus increasing their probability of survival when re-introduced into the light.

Another difference between Prochlorococcus and Synechococcus that may play a role in their relative abilities to survive extended darkness is their sensitivity to oxidative stress. While the catalase-peroxidase gene, katG, is absent in all Prochlorococcus genomes sequenced thus far (Regelsberger et al. 2002; Scanlan et al. 2009; Morris et al. 2012; Biller et al. 2014a), it is present in most strains of Synechococcus (Scanlan et al. 2009). That said, only one (WH7803) of the two Synechococcus strains we examined has katG yet both had similar dark survival characteristics —longer than any of the Prochlorococcus strains. Thus the presence or absence of katG cannot be the primary factor in the differences observed between these two genera.

Conclusions

We have shown that all axenic strains of Prochlorococcus tested can survive up to 35 h of darkness, but not beyond. Survival capacity was unrelated to whether or not a strain was high- or low-light adapted. In the presence of the heterotroph Alteromonas macleodii MIT1002, survival of Prochlorococcus in darkness is significantly enhanced by 3–11 d. This enhancement effect appears to be due at least in part to the reduction of oxidative stress by the heterotroph under extended darkness. Addition of glucose to the media also extends dark survival, and the combined effects of added glucose and pyruvate (which quenches hydrogen peroxide) mimics the effect of co-culture with Alteromonas. Two strains of Synechococcus were able to survive up to, but not limited to, 83 h of darkness in the absence of any treatments, longer than any Prochlorococcus strains examined under these conditions. Thus in addition to strain to strain variability, it is clear that one needs to consider the impact of biotic and abiotic milieu, the residence time in dark, the affect of their surrounding microbial community, and the unpredictable conditions of their reintroduction to the light in assessing the real limits of picocyanobacterial survival over extended dark periods.

While these results do not fully resolve one of the questions that motivated this study—i.e., whether or not Prochlorococcus cells observed below the euphotic zone are viable—they do point us in interesting directions vis-a-vis the role of oxidative stress in dark survival and the role of metabolic exchange with heterotrophs that promote the survival under this particular stress condition (Fig. 6). Fruitful directions to pursue in future studies would be to identify the mechanisms through which co-culture with Alteromonas prolongs dark survival and to further explore the differences between Prochlorococcus and Synechococcus and their ability to tolerate extended darkness.

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