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ORIGINAL ARTICLE

Degradation of hydrogen peroxide at the ocean's surface: the influence of the microbial community on the realized thermal niche of *Prochlorococcus*

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***Prochlorococcus*, the smallest and most abundant phytoplankter in the ocean, is highly sensitive to hydrogen peroxide (HOOH), and co-occurring heterotrophs such as *Alteromonas* facilitate the growth of *Prochlorococcus* by scavenging HOOH. Temperature is also a major influence on *Prochlorococcus* abundance and distribution in the ocean, and studies in other photosynthetic organisms have shown that HOOH and temperature extremes can act together as synergistic stressors. To address potential synergistic effects of temperature and HOOH on *Prochlorococcus* growth, high- and low-temperature-adapted representative strains were cultured at ecologically relevant concentrations under a range of HOOH concentrations and temperatures. Higher concentrations of HOOH severely diminished the permissive temperature range for growth of both *Prochlorococcus* strains. At the permissive temperatures, the growth rates of both *Prochlorococcus* strains decreased as a function of HOOH, and cold temperature increased susceptibility of photosystem II to HOOH-mediated damage. Serving as a proxy for the natural community, co-cultured heterotrophic bacteria increased the *Prochlorococcus* growth rate under these temperatures, and expanded the permissive range of temperature for growth. These studies indicate that in the ocean, the cross-protective function of the microbial community may confer a fitness increase for *Prochlorococcus* at its temperature extremes, especially near the ocean surface where oxidative stress is highest. This interaction may play a substantial role in defining the realized thermal niche and habitat range of *Prochlorococcus* with respect to latitude.**
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Introduction

Stress is a common state for microbes, and it is becoming clear that biological interactions can augment or ameliorate this condition. Apart from nutritional stress (Amin *et al.*, 2012), microbe–microbe interactions can mediate relief from a variety of abiotic environmental stresses, in a process generally referred to as facilitation. Facilitation is a widely (Bertness and Callaway, 1994; Brooker and Callaghan, 1998; Callaway *et al.*, 2002) though not universally (Maestre and Cortina, 2004) accepted principle in metazoan ecology, which posits that at environmental extremes positive interactions between organisms tend to dominate over negative ones (Callaway *et al.*, 2002; Brooker *et al.*, 2005). There are fewer investigations of facilitation for microbes, but examples include the bacterial-mediated cross-protection of phytoplankton from inorganic carbon limitation and oxygen toxicity (Chirac *et al.*, 1985; Ma and Eaton, 1992). Bacteria can also improve the thermotolerance of phytoplankton; in the case of the green alga *Chlamydomonas reinhardtii* bacteria provide vitamin B_{12} that is required for the thermotolerant isozyme of a key metabolic reaction (Xie *et al.*, 2013). Finally, microbes can also protect other microbes from oxidative stresses by the removal of the reactive oxygen species from the environment. Such beneficiaries include heterotrophic microbes (Ma and Eaton, 1992; Beliaev *et al.*, 2014; Morris *et al.*, 2014), Antarctic diatoms (Hunken *et al.*, 2008) and, as we demonstrated in prior studies (Morris *et al.*, 2008, 2011), the marine cyanobacterium *Prochlorococcus*.

Prochlorococcus is the smallest phytoplankter in the oligotrophic open ocean; they are also believed to be the most abundant photosynthetic autotroph on Earth (Partensky *et al.*, 1999; Flombaum *et al.*, 2013). Numerical success of *Prochlorocccus* in the nutrient-poor open ocean has been attributed to several evolutionary innovations, of which genome streamlining has been frequently cited (Strehl *et al.*, 1999; Dufresne *et al.*, 2005; Coleman and Chisholm, 2007;

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Scanlan *et al*., 2009). Streamlining can improve growth efficiency by reducing cellular nitrogen and/or phosphorus quotas, but the loss of genes can also result in deficiencies that must be otherwise satisfied if the streamlined organism is to take advantage of the improved efficiency. Prior work by our group has identified one such deficiency of *Prochlorococcus* resulting from genome streamlining: a diminished oxidative stress response (Morris *et al*., 2011). Unlike most cyanobacteria and other aerobes, *Prochlorococcus* lacks the hydrogen peroxide (HOOH)-degrading enzyme catalase, and consequently is highly sensitive to this oxidant. The ecological consequence for this loss is severe, in that *Prochlorococcus* growth at the ocean surface depends on the ability of the co-occurring microbes to degrade HOOH. The activity of the extant microbial community (Morris *et al*., 2016) maintains HOOH at concentrations permissive for *Prochlorococcus* growth (<200 nM) (Cooper *et al*., 1987; Miller and Kester, 1994; Hanson *et al*., 2001; Yuan and Shiller, 2001; Morris *et al*., 2011); in the absence of this community the HOOH concentrations in the sunlit mixed layer can rise to levels ($\geqslant 800$ nM) that can kill all ecotypes of *Prochlorococcus* found in the mixed layer (Morris *et al*., 2011). Hence, in the evolutionary history of *Prochlorococcus*, it may have taken advantage of the HOOH degradation capacity of the community as a 'public good', which made its own catalase dispensible and subject to loss via a 'Black Queen' evolutionary process (Morris *et al*., 2012).

In the natural world, multiple stresses can co-occur, and for photosynthetic microbes temperature stress is known to act synergistically with oxidative stress. Temperature extremes can generate excess reactive oxygen species (Smirnova *et al*., 2007), leading to oxidative stress (Lesser, 1996) and cell mortality (Davidson *et al*., 1996). In cyanobacteria, temperature extremes and HOOH both impact photosynthesis by interfering with a key step in the repair of damaged photosystem II (PSII). D1 is the reaction center chlorophyll-containing protein of PSII, and is rapidly damaged during photosynthetic electron flow. Rather than being repaired, damaged D1 is replaced, and both temperature and HOOH stress can interfere with the synthesis and/or post-processing steps involved in this replacement (Ma and Eaton, 1992; Nishiyama *et al*., 2001, 2006; Kojima *et al*., 2007). Given the potential for synergistic effects of oxidative and thermal stresses on *Prochlorococcus* growth and photosynthesis, we hypothesized that (1) temperature extremes sensitize *Prochlorococcus* to HOOH-mediated damage, and that (2) by reducing HOOH concentrations, the extant microbial community facilitates the survival and growth of *Prochlorococcus* at these temperature extremes.

While multiple ecotypes of *Prochlorococcus* have been identified, the water column is typically dominated numerically by one of two ecotypes, eMED4 or eMIT9312 (also referred to as HL-I and HL-II, respectively (Rocap *et al*., 2002; Johnson *et al*., 2006; Zwirglmaier *et al*., 2008). These high-light adapted ecotypes partition the surface ocean by latitude, with eMIT9312 dominating the low latitudes (~ N30°–S30°), and eMED4 the high latitudes north and south of the equator (~ 30°–40°) (Johnson *et al*., 2006). Temperature was identified as the key environmental variable correlating with abundance of these two ecotypes (Johnson *et al*., 2006; Chandler *et al*., 2016). Consistent with the field data, studies with non-axenic cultures of isolated strains confirmed that while both ecotypes share a common temperature optimum (~24 °C), the eMED4 strains grew faster and at a broader range of temperatures below ~19 °C, compared to the eMIT9312 strains, while the opposite held true for cultures grown above the optimum (Johnson *et al*., 2006). Collectively, these studies suggested that the different physiological responses to temperature play a major role in establishing the distribution patterns of these recently diverged lineages in nature. However, as the culture experiments were performed at high cell densities and in the presence of heterotrophic bacterial contaminants (Johnson *et al*., 2006), the influence of the microbial community on the temperature range of *Prochlorococcus* was unconstrained, but could be significant due to its impact on the concentration of HOOH.

In this study we used low, ecologically relevant concentrations of representatives of the high- and low-temperature-adapted ecotypes of *Prochlorococcus*, grown in the presence or absence of a heterotrophic helper *Alteromonas* sp. EZ55, to directly test the hypothesis that HOOH acts synergistically with high or low temperatures to restrict growth. We show that HOOH is more deleterious to *Prochlorococcus* when growing at suboptimal temperatures, and that by elimination of HOOH, the activity of the community facilitates the growth of *Prochlorococcus* at its temperature extremes and may expand its meridional range in the open ocean.

Materials and methods

Prochlorococcus strains and culture conditions

Axenic streptomycin-resistant derivatives of *Prochlorococcus* strains MIT9312 and MED4 (VOL 4 and VOL 7, respectively (Morris *et al*., 2011)) were used in this study. These strains, referred to hereafter as MIT9312 and MED4 for simplicity, were cultured in filtered artificial seawater AMP-J medium (Morris *et al*., 2008; Morris and Zinser, 2013) (per L, 28.1 g NaCl, 6.9 g $MgSO_4 \times 7H_2O$, 5.49 g $MgCl_2 \times 6H_2O$, 0.67 g KCl, 1.47 g $CaCl_2$, 0.504 g $NaHCO_3$, with 2 ml 0.5 M TAPS pH 8.0, 1 ml 0.4 M $(NH_4)_2SO_4$, 2 ml 0.025 M NaH_2PO_4, pH7.5, 100 µl 10 000 X Pro99 Trace Metal Mix) with 40 µmol quanta $m^{-2} s^{-1}$ light on a 12:12 light:dark cycle using cool white fluorescent bulbs. Cultures were acclimated to the test temperature for at least three transfers (minimum 20 generations) at high cell concentration ($>10^7$

cells ml^{-1}), before inoculation at low cell concentration (30 000 cells ml^{-1}) for the assessment of temperature and HOOH impacts on growth. HOOH concentration was increased from basal levels in the medium (61–90 nM) where appropriate. Axenicity of *Prochlorococcus* cultures was monitored by purity test in YTSS and 1/10 ProAC heterotrophic growth media (Morris *et al.*, 2008).

The heterotrophic bacterium *Alteromonas* sp. EZ55 was grown for 48 h in 10 ml YTSS medium (Gonzalez and Moran, 1997) (per l, 4.0 g tryptone, 2.5 g yeast extract, 15 g sea salts, autoclaved) on a roller drum at 22 °C. Cells were then diluted 1:1000 into 10 ml minimal acetate medium (per 200 ml, 150 ml Pro99 (Morris *et al.*, 2008), 50 ml 18MΩ H_2O, 0.5% acetate, 100 µl 2000 X VA vitamin mix (Waterbury and Willey, 1988) and incubated for 24 h on the roller drum. Cells were harvested by centrifugation at 4500 *g* for 5 min, washed twice with sterile AMP-J medium and resuspended in sterile AMP-J medium. Resuspended cells were inoculated into AMP-J at a concentration of about 10^6 cells ml^{-1}, and incubated for 24 h at the appropriate temperature to precondition the media before the *Prochlorococcus* cells were added. *Altermonas* sp. EZ55 cells were left in the media throughout the *Prochlorococcus* growth experiment.

Quantification of Prochlorococcus and Alteromonas sp. EZ55

Concentration of *Prochlorococcus* was measured by flow cytometry with the Guava EasyCyte 8HT cytometer (Millipore, Billerica, MA, USA), using an established method (Morris *et al.*, 2011). Flow cytometric quantification of *Alteromonas* sp. EZ55 was performed following staining of the cells with SYBR green (Tripp *et al.*, 2008). Viable count assays for *Alteromonas* sp. EZ55 were performed by serial dilutions of the culture on YTSS plates. Plates were incubated overnight at room temperature and colonies were counted.

HOOH quantification and amendments to media

The HOOH concentration in the medium and cultures was measured by an Orion L Microplate Luminometer (Titertek Instruments Inc, Berthold Detection Systems, Pforzheim, Germany) based on an established method using acridinium ester (Morris *et al.*, 2011). Concentration of HOOH in the culture medium was adjusted as needed to 200, 400, or 800 nM, once the basal concentration of the AMP-J medium was determined. Rates of HOOH degradation k_{HOOH} were calculated as the slope of the regression of HOOH concentration over time, over at least 3 days.

Sodium pyruvate scavenging HOOH

Final concentration of 1 mM sodium pyruvate (Sigma-Aldrich, St Louis, MO, USA) was used as a direct scavenger of HOOH in the medium (Giandomenico *et al.*, 1997; Kao and Fink, 2010). Sodium pyruvate was added to the medium at the same time as *Alteromonas* sp. EZ55 was added, which was 24 h before the addition of *Prochlorococcus* cultures. 1 mM sodium pyruvate has been shown to have the most similar HOOH scavenging ability to *Alteromonas* sp. EZ55 (Supplementary Figures S1 and S2); by 24 h the concentration fell below the limit of detection (10 nM) (Supplementary Figure S1).

Measurement of photosynthesis parameters Fv/Fm with lincomycin

Fast Induction and Relaxation (FIRe) fluorometer (Satlantic, Halifax, Nova Scotia, Canada) was used to measure the photophysiological parameters (initial fluorescence, Fo; maximal fluorescence, Fm; and variable fluorescence, Fv) by established methods (Johnson, 2004). Lincomycin (Sigma-Aldrich) was dissolved in water and added into tubes with a final concentration of 500 µg ml^{-1}. Higher concentrations had the same impact on Fv/Fm in trial studies (data not shown), providing confidence that maximum inhibition was achieved at this concentration. Flow cytometry verified that neither lincomycin nor high (800 nM) HOOH impacted cell counts over the 8 h incubation at 24 °C: the ratio of the change in cell number ($T_{0\,h}/T_{8\,h}$) for the lincomycin or high HOOH treatment versus no lincomycin, low (90 nM) HOOH control was 1.07 or 1.16 for MIT9312, and 1.22 or 1.22 for MED4, respectively.

Modeling of potential effect of HOOH to the distribution of Prochlorococcus

The potential range compression of surface communities of *Prochlorococcus* in the global ocean due to synergistic HOOH and temperature stressors was estimated by using an annual composite (Level 3 processing) surface temperature from 2013 quantified at 4 km resolution using the NASA Aqua MODIS 4 µ sensor. For the base case (no HOOH effect), *Prochlorococcus* is assumed to be present at all temperatures greater than 10 °C, while at 400 nM HOOH *Prochlorococcus* is only found between 22 and 24 °C.

Results

Growth of the high-temperature-adapted Prochlorococcus strain MIT9312 at different temperatures under oxidative stress

Prior work with dense, non-axenic cultures indicated strain MIT9312, of the high-temperature-adapted eMIT9312 (HL-II) ecotype, had a growth optimum at 24 °C (Johnson *et al.*, 2006), and we confirmed this optimum for axenic, ecologically relevant concentrations (inoculum $<10^5$ cells ml^{-1}) (Supplementary Figure S3). At 24 °C, 800 nM HOOH is lethal to strains of all ecotypes of *Prochlorococcus*

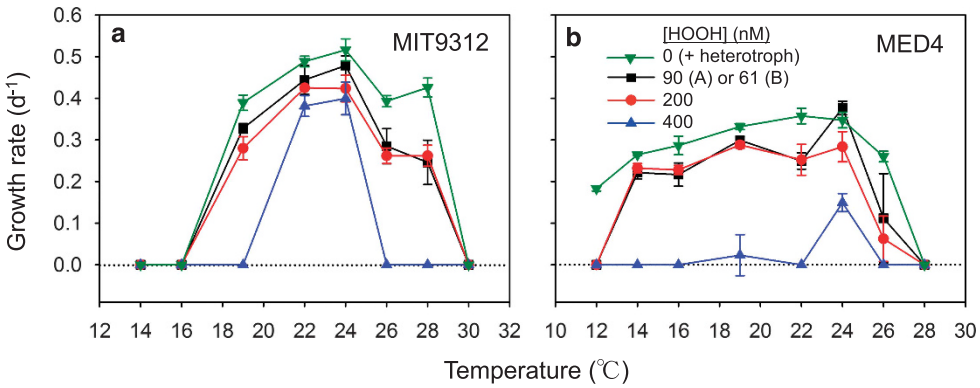

Figure 1 Growth of ecologically relevant concentrations of MIT9312 (**a**) and MED4 (**b**) at different temperatures and exogenous HOOH concentrations. Cells from temperature-acclimated dense ($>10^7$ cells ml^{-1}) mid-log cultures were inoculated into media containing ambient (90 or 61 nM for MIT9312 or MED4, respectively) or elevated (200 or 400 nM) HOOH, or in media pre-inoculated with the heterotroph Alteromonas sp. EZ55 (around 0 nM HOOH). Values on the black dotted line denotes zero or negative net growth over the incubation period. See Supplementary Figure S4 for plots of calculated negative growth rates. Error bars denote the standard deviation for three biological replicates; see Supplementary Tables S1 and S2 for statistical analysis of the data.

found in the mixed layer (Morris *et al*., 2011), but concentrations below this value (90–400 nM) had minimal impact on growth of MIT9312 (Morris *et al*., 2011, and Figure 1a). Results at 22 °C were similar to 24 °C, but for other temperatures within the growth range of this strain, sensitivity to HOOH was significantly increased (Figure 1a, Supplementary Table S1). At the unadjusted medium HOOH concentrations (90 nM), the permissive temperature range was 19–28 °C, similar to the prior study with dense non-axenic cultures (Johnson *et al*., 2006) (Supplementary Figure S3). Increases to 200 nM HOOH did not significantly impact growth over this temperature range relative to 90 nM (Supplementary Table S1), although the overall trend was a slight decrease in rate (Figure 1a). At 24 °C (its growth optimimum) and 22 °C, MIT9312 growth in 400 nM HOOH was significantly slower than 90 nM ($P<0.05$) but not 200 nM ($P>0.05$) (Figure 1a, Supplementary Table S1). Critically, 400 nM HOOH was lethal at all other temperatures tested (Figure 1a). At 19, 26 and 28 °C, a slow but steady decline in cell concentrations was observed over several days, whereas at the more extreme temperatures tested (14, 16, 30 °C), a more dramatic effect was observed (Supplementary Figure S4). At these extremes, no cells could be detected after only 24 h post-inoculation, indicating that the cells rapidly lost chlorophyll-based red fluorescence and/or cell integrity (see Discussion).

Prochlorococcus lacks catalase but can, at slow rates, remove HOOH from the medium (Morris *et al*., 2011). While the degradation rates are too slow to dramatically improve tolerance to HOOH at ecologically relevant cell concentrations (Morris *et al*., 2011), it was nonetheless notable that the rates of HOOH degradation by MIT9312 varied as a function of temperature and initial concentration of HOOH (Supplementary Figure S5). There were significant effects of both temperature and initial HOOH concentration on the rate of HOOH degradation (k_{HOOH}) in axenic cultures. k_{HOOH} was significantly higher at 24 °C than at any other tested temperature (Supplementary Figure S5A), coinciding with the optimum growth temperature for this strain (Figure 1a). The influence of initial HOOH concentration was negative and significantly linear ($P=0.002$), with k_{HOOH} decreasing by about 0.0004 d^{-1} for each increase of 1 nM initial HOOH (Supplementary Figure S5B).

In a prior study we described how heterotrophic bacteria can protect *Prochlorococcus* from HOOH-mediated oxidative damage by removing HOOH from the medium (Morris *et al*., 2011). As this earlier work was performed exclusively at 24 °C, we investigated if this helping phenomenon occurs throughout the thermal range of *Prochlorococcus*, and whether co-culture with helpers might expand the permissive range for growth. The helper heterotroph *Alteromonas* sp. EZ55 was able to rapidly remove HOOH to below detectable limits at both high (28 °C) and low (19 °C) temperatures (Supplementary Figure S2), and thus could serve to help *Prochlorococcus* throughout its thermal growth range. While no range expansion was observed for MIT9312, significantly higher ($P<0.05$) rates of growth were observed at 26 and 28 °C in media pre-conditioned by the presence of *Altermonas* sp. EZ55, relative to the untreated control (Figure 1a, Supplementary Table S1). Similar results were found at 19 °C although they were only marginally significant ($P=0.052$) (Figure 1a, Supplementary Table S1). *Alteromonas* sp. EZ55-treated media had undetectable levels of HOOH (data not shown; limit of detection = 10 nM), whereas the control cultures had low but detectable HOOH (90 nM). The growth rate in *Alteromonas* sp. EZ55-treated media was significantly higher ($P<0.05$) than growth in 200 or 400 nM HOOH at all temperatures (Figure 1a, Supplementary Table S1). These data suggest that even 90 nM HOOH can harm *Prochlorococcus*, especially at elevated

temperature, consistent with the observation of a linear relationship between k_{HOOH} and initial HOOH concentration (Supplementary Figure S5B), and that this harm can be negated by the action of co-cultured heterotrophic bacteria.

To assess if the enhancing effect of the helper at suboptimal temperatures was due strictly to its HOOH degrading capacity, these results were compared to those using an alternative, abiotic means of removing HOOH from the medium. Pyruvate effectively scavenges HOOH (Mizunoe *et al.*, 1999; Long and Halliwell, 2009; Coe *et al.*, 2016) (Supplementary Figure S1) by an abiotic chemical reaction (Giandomenico *et al.*, 1997; Kao and Fink, 2010); such treatments have proven valuable in the cultivation of other microbes (Troxell *et al.*, 2014; Kim *et al.*, 2016) and for the prolonged survival of *Prochlorococcus* in darkness (Coe *et al.*, 2016). At 28 °C, the growth rate of MIT9312 in pyruvate-treated medium (~0 nM HOOH) was significantly higher ($P=0.0058$) than in the untreated control (90 nM) (Figure 2). Thus, by different chemistries the two treatments employed—live bacteria with enzymes and pyruvate—effectively decreased HOOH below detection, and both lead to an enhancement of MIT9312 growth post-treatment. These data support the prior conclusion that even 90 nM HOOH can negatively impact *Prochlorococcus* growth. Importantly, however, cultures given pre-treatment with a heterotrophic helper grew faster than those pre-treated with pyruvate (Figure 2). At a less extreme temperature of 26 °C the trends were similar but not significant (Figure 2).

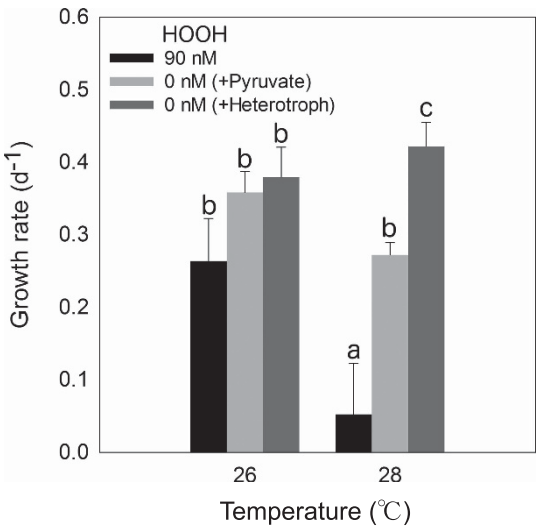

Figure 2 The effect of pyruvate as an alternative HOOH scavenger. Growth rates of MIT9312 in ambient (90 nM HOOH) medium or medium pre-treated with pyruvate or Alteromonas sp. EZ55 (both: 0 nM HOOH) at 26 or 28 °C. Letters above bars denote groups of treatments whose values were not statistically different ($P>0.05$). Error bars denote the standard deviation for three biological replicates.

Growth of the low-temperature-adapted strain MED4 under temperature and oxidative stresses

Strain MED4 of the low-temperature-adapted ecotype eMED4 (HL-I) exhibited similar growth responses to HOOH, temperature, and helper treatments as the high -temperature-adapted MIT9312, although some strain–strain differences were noted (Figure 1b, Supplementary Table S2). MED4 cultures grew between 12 and 26 °C, which was cold-shifted relative to MIT9312 (16 to 28 °C) and its temperature versus growth rate profile was similar to that reported for dense non-axenic cultures (Johnson *et al.*, 2006) (Supplementary Figure S3). Importantly, dilute MED4 could only grow at 12 °C when in the presence of heterotrophs (Figure 1b), showing that heterotrophic bacteria can expand the permissive temperature range for this strain of *Prochlorococcus*, and also suggesting that even 61 nM HOOH (the unadjusted medium concentration) can be lethal to *Prochlorococcus* under certain conditions. Like MIT9312, growth of pure cultures of MED4 was highly restricted at 400 nM HOOH, in this case occurring only at the growth optimum, 24 °C, and at a significantly lower rate ($P<0.001$) than at ambient HOOH (Figure 1b). The kinetics of cell loss at the non-permissive temperatures was slow enough that rates of cell loss could be calculated, except at 12 °C, when all cells (without helpers) disappeared by 24 h (Supplementary Figure S4). In general, the rate of cell loss at the non-permissive temperatures increased as a function of HOOH and extremity of temperature (Supplementary Figure S4). Pre-treatment with heterotroph helpers increased the growth rate of MED4 at 26 °C, and decreased the death rate at 28 °C (Figure 1b), showing a similar trend to MIT9312 where the helping effect was maximal at the temperature extremes. Unlike MIT9312, MED4 did not show faster HOOH decay at its 24 °C growth optimum relative to other temperatures (Supplementary Figure S5C), but like MIT9312 did show a negative and significantly linear ($P=0.0002$) influence of initial HOOH concentration, with k_{HOOH} decreasing by about 0.00023 d^{-1} for each increase of 1 nM initial HOOH (Supplementary Figure S5D).

Impacts of temperature and HOOH on photosynthetic efficiency

Our prior work on *Prochlorococcus* at 24 °C showed that HOOH can strongly diminish Fv/Fm, a fluorescence-based proxy for PSII efficiency and health (Morris *et al.*, 2011). To assess the combined contributions of temperature and oxidative stress on *Prochlorococcus* PSII health, we quantified Fv/Fm under various temperature and HOOH conditions. We first examined the influence of temperature on Fv/Fm during growth, keeping the HOOH at low, ambient concentrations (90 nM). A previous study of MED4 found a small but significant increase in Fv/Fm over the 16–24 °C range (Kulk *et al.*, 2012), and

our cultures of this strain grown with 90 nM HOOH showed similar trends (Supplementary Figure S6). Growth at more extreme 14 or 26 °C temperatures had minimal influence on Fv/Fm, but a shift to the lethal 12 °C temperature resulted in a significant decline (Supplementary Figure S6). Similarly, MIT9312 maintained an Fv/Fm value of approximately 0.6 for the 19–28 °C range, but growth at 16 °C, its low temperature lethal limit, resulted in a significant decline of Fv/Fm (Supplementary Figure S6).

To assess the combined influence of temperature and oxidative stress, we challenged the temperature-acclimated cultures with exposure to elevated concentrations of HOOH. As *Prochlorococcus* can potentially mitigate damage from HOOH over longer incubations via a slow depletion of external HOOH (Supplementary Figure S5), we performed our analysis during short incubations (up to 8 h). Exposure to ambient concentrations of HOOH (90 nM) did not lead to decreases of Fv/Fm over the 8 h incubation (Supplementary Figures S7 and S8), nor did exposure to 400 nM HOOH (Supplementary Tables S5 and S6). However, exposure to 800 nM HOOH—the lethal concentration at 24 °C (Morris *et al.*, 2011)—resulted in declines of Fv/Fm that varied by temperature and strain (Figure 3). The response was significant (*t*-test, $P<0.05$) and most pronounced at the low temperature extreme for both strains: 16 and 14 °C for MIT9312 and MED4, respectively (Figure 3, Supplementary Tables S3 and S4). While not statistically significant (Supplementary Table S3), the overall trend for MIT9312 over the rest of the temperature range was a decrease in Fv/Fm with the 800 nM exposure versus the ambient low HOOH concentration (Figure 3). A similar trend was observed for MED4 (Figure 3), except that the differences were significant at 14, 16 and and 19 °C (Supplementary Table S4).

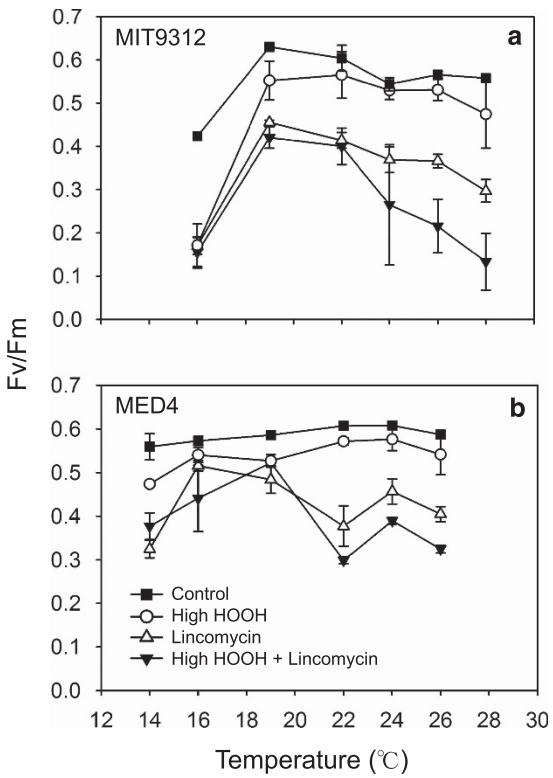

Figure 3 Photosynthetic efficiency of MIT9312 (**a**) or MED4 (**b**) as a function of temperature, HOOH and translation inhibitor lincomycin. Cultures grown at high cell density were diluted to low density and incubated for 8 h in the presence of 800 nM HOOH (high HOOH), lincomycin, both, or neither (control) treatment. Fv/Fm values after the 8 h incubation are reported. Error bars denote the standard deviation for three biological replicates.

The combined influences of (low) temperature and (high) HOOH on PSII health described above are consistent with the findings in other studies (Murata *et al.*, 2007; Takahashi *et al.*, 2009) that these multiple stressors prevent PSII repair by blocking synthesis and/or processing of nascent D1 protein. To calibrate the extent to which temperature and HOOH may block this repair, we compared their impacts to that of lincomycin, an effective inhibitor of translation in *Prochlorococcus* and *Synechococcus* (Six *et al.*, 2007). For MIT9312, exposure to lincomycin or HOOH at 16 °C led to the same dramatic decline of Fv/Fm over the 8 h incubation (Figure 3, Supplementary Figure S7). At higher temperatures, the decline in Fv/Fm for lincomycin treatment exceeded that of high HOOH (Figure 3 and Supplementary Table S3). For MED4, significant differences in Fv/Fm with high HOOH exposure versus lincomycin treatment were found at 14, 22, 24 and 26 °C, but not at 16 and 19 °C (Figure 3, Supplementary Figure S8, Supplementary Table S4). Notably, for both strains at multiple temperatures, the degree to which Fv/Fm dropped when cells were challenged with both lincomycin and high HOOH exceeded that for either treatment individually (Figure 3). This higher drop in the combined treatment was significant at 19, 26 and 28 °C for MIT9312, and all temperatures examined except 19 °C for MED4 (Supplementary Tables S3 and S4).

Discussion

The results of this study support the hypothesis that growth at temperature extremes heightens the sensitivity of *Prochlorococcus* to hydrogen peroxide. Three major interactive effects were observed between temperature and HOOH. First, the range of permissive temperature decreases with elevated HOOH. Even as little as 61 nM HOOH was sufficient to constrain the permissive temperature range for growth, as evidenced by MED4 at 12 °C, while higher concentrations (400 nM) constrained the permissive temperature to the growth optimum or just below. Second, within the permissive temperature range, growth rate tended to decrease as a function of HOOH concentration through the assayed range of 0–400 nM. Third, for the 400 nM treatment, the death rate (measured as the loss of detectable cells) at the

lethal temperatures increased with increasing divergence from optimal temperature (24 °C). These three effects were observed in both MIT9312 and MED4, chosen as representatives of the two numerically dominant ecotypes of *Prochlorococcus* in the ocean (Rocap *et al.*, 2002), and at ecologically relevant concentrations of cells and HOOH. The final major conclusion of this study is that this heightened sensitivity to temperature is tempered significantly by the presence of HOOH-consuming bacteria. With all due caution in extending results from culture-based studies into natural populations, these results demonstrate that temperature extremes and high HOOH act as synergistic stresses on *Prochlorococcus*, and suggest that *Prochlorococcus* is more dependent on the microbial community at its temperature extremes.

Damage mechanisms of oxidative and thermal stress in Prochlorococcus

Photosynthetic efficiency (Fv/Fm) is used as a diagnostic of photosynthetic health of natural phytoplankton communities, including the open ocean where *Prochlorococcus* contributes significantly to chlorophyll and primary production (Behrenfeld *et al.*, 2006; Johnson *et al.*, 2010; Ryan-Keogh *et al.*, 2013; Wilhelm *et al.*, 2013; Lin *et al.*, 2016). Prior studies showed that as a single stressor, temperature had only a minor influence on photosynthetic efficiency of *Prochlorococcus* (Fu *et al.*, 2007; Kulk *et al.*, 2012). Our results are in general agreement, but we also add that Fv/Fm drops significantly ($P = 0.0001$) at extreme low temperature, as observed in both strains at their respective temperature limit. While temperature alone may not impact photosynthetic efficiency in *Prochlorococcus*, except at the aforementioned cold-temperature limit, we found that it sensitizes this genus to co-occurring oxidative stress, to which it is already highly sensitive even under optimal temperature (Morris *et al.*, 2011; Mella-Flores *et al.*, 2012). This was evident in the Fv/Fm measurements for 8-h exposures to 800 nM HOOH; lower concentrations did not show any effects, however. The relatively short timeframe was chosen to limit the ability of *Prochlorococcus* to influence the HOOH concentration during the exposure (Morris *et al.*, 2011, and Supplementary Figure S5) and thus mitigate the threat to its photosystems (it was also chosen to minimize the influence of global transcription arrest in the Fv/Fm assays involving lincomycin treatments, discussed below). We suspect that the trends found for the acute 800 nM HOOH exposures will reflect those of chronic exposures to lower concentrations of HOOH, and future studies, where HOOH concentrations can be effectively stabilized over days of *Prochlorococcus* growth, should be performed to test this hypothesis.

The increased sensitivity to HOOH at the temperature extremes is partially explained by a decrease in the innate capacity of *Prochlorococcus* to degrade HOOH (Supplementary Figure S5). Notably, growth at low temperature also sensitizes *Prochlorococcus* strain MED4 to excessive ultraviolet and photosynthetically available radiation (Neale and Thomas, 2017), likely through generation of singlet oxygen (Vass, 2011), an reactive oxygen species agent to which photosystem II of *Prochlorococcus* is especially vulnerable (Murphy *et al.*, 2017). Collectively, these observations suggest that the impacts of oxidative stressors on the photosynthetic health and activity of *Prochlorococcus* depend on the temperature at which they are growing in the ocean, which can vary widely by latitude and season.

In freshwater cyanobacteria, HOOH and temperature block D1 replacement at translation and post-translation stages, respectively (Ma and Eaton, 1992; Nishiyama *et al.*, 2001, 2006; Chaloub *et al.*, 2003), and our results suggest similar blocks may be occurring in *Prochlorococcus* that contribute to the decline in photosynthetic efficiency of PSII. Comparisons to lincomycin-treated cultures indicated that the combined HOOH and temperature treatments may only partially block D1 replacement (except at the low-temperature limit) (Figure 3). However, although D1 is the most highly turned over protein and thus most susceptible to translation inhibition by lincomycin, the inability to synthesize other proteins over the 8 h incubation may have also contributed to the loss in Fv/Fm of the lincomycin-treated cultures. Thus, further investigation of D1 translation and post-translational processing is warranted to assess the full extent of the block by HOOH and temperature. With this caveat in mind, we noted a curious result: the combination of high HOOH and lincomycin could diminish Fv/Fm more than lincomycin alone (Figure 3). This suggests that HOOH may have additional impacts than D1 replacement (and translation, generally). These non-mutually exclusive mechanisms may include damaging D1 directly (Vass *et al.*, 1992; Keren *et al.*, 1997), or electron carriers downstream of PSII (Morris *et al.*, 2011), or the diunsaturated fatty acids present in *Prochlorococcus* (Biller *et al.*, 2014) that compose the photosynthetic membranes.

The role of helper bacteria in protecting Prochlorococcus from interactive thermal and oxidative stresses

In prior work we demonstrated that co-cultured bacteria can protect *Prochlorococcus* from oxidative stress (Morris *et al.*, 2008, 2011), and suggested that this beneficial interaction contributes to the evolutionary history (Morris *et al.*, 2012) and ecological success (Morris *et al.*, 2011) of this cyanobacterial genus. This work confirms that bacteria can protect *Prochlorococcus* from oxidative stress, and further demonstrates that this interaction can facilitate improved responses to temperature stress. This was

perhaps most evident in MED4 growing at its cold limit: this strain was unable to grow at all at 12 °C unless heterotrophic bacteria were present. In addition to expanding the permissive range of MED4, the helper also improved the growth rate of both strains within their permissive range. At high temperatures, even 60–90 nM HOOH was sufficient to slow the growth of *Prochlorococcus*, relative to 0 nM HOOH (Figures 1 and 2). As such concentrations can be found in surface waters at these temperatures, even in the presence of HOOH-degrading community (Cooper *et al.*, 1987; Miller and Kester, 1994; Hanson *et al.*, 2001; Yuan and Shiller, 2001; Morris *et al.*, 2011), this indicates that HOOH may limit growth under these conditions, and suggests that the community may not perfectly protect *Prochlorococcus* under all situations in the natural world. Another benefit of the heterotrophic helpers was observed for MED4 at lethal high temperatures, where these helpers slowed the death kinetics of MED4 relative to the unamended control. This latter effect may be important for *Prochlorococcus* survival during acute exposures to lethal concentrations of HOOH, which can occur during rainfall events (Cooper *et al.*, 1987; Hanson *et al.*, 2001).

There are several means by which one species can protect another from thermal stress, including shading (Bertness *et al.*, 1999; Maestre *et al.*, 2003; Bulleri, 2009) and provision of a co-factor for a heat-resistant isozyme that catalyzes an essential reaction in the beneficiary (Xie *et al.*, 2013). The findings in this study are consistent with a third mechanism of thermal cross-protection: elimination of co-occurring oxidative stress. Stresses often co-occur in nature, and the interactive deleterious effects of high reactive oxygen species and temperature extremes have been well described in both photoautotrophic (Wise, 1995; Lesser, 1996; Thomas *et al.*, 1999; Kocsy *et al.*, 2001; Kranner *et al.*, 2005; Almeselmani *et al.*, 2006; Allakhverdiev *et al.*, 2008) and heterotrophic (Davidson *et al.*, 1996; Kranner *et al.*, 2005; Chen *et al.*, 2013) systems. Lichens (Kranner *et al.*, 2005), anemones (Richier *et al.*, 2005) and terrestrial plants (Redman *et al.*, 2002) have all exhibited better responses to temperature stress when cross-protected by microbes from oxidative stress, and our results suggest that *Prochlorococcus* can be added to this list.

That pyruvate was able to help *Prochlorococcus* grow at higher temperatures, but not to the extent that live heterotrophic bacteria can, suggests that the bacteria are providing thermal benefits in addition to removal of HOOH. Live *Alteromonas* helpers similarly outperformed pyruvate in improving the survival of *Prochlorococcus* in extended darkness (Coe *et al.*, 2016). The lower growth rate in the pyruvate- versus helper-treated media could be a negative consequence of a high concentration of pyruvate on *Prochlorococcus* growth. We believe this to be unlikely, however, as the growth rates of MIT9312 and MED4 at 24 °C, a temperature where the HOOH-consuming helper does not improve growth (Figure 1), are not decreased by the addition of pyruvate; pyruvate-treated cells grow as fast as helper-treated or un-treated cells (Supplementary Figure S9). Thus, the ability of *Alteromonas* to protect *Prochlorococcus* from diverse stressors is not completely explained by the removal of HOOH from culture media. In our earlier work we were able to rule out the importance of facilitation phenotypes found in other algae:helper systems (for example, metabolisms that deplete oxygen, replenish inorganic carbon, stabilize pH, and/or provide an essential vitamin or other growth factor (Schiefer and Caldwell, 1982; Chirac *et al.*, 1985; Keshtacher-Liebson *et al.*, 1995; Mouget *et al.*, 1995; Croft *et al.*, 2005; Geng and Belas, 2010; Xie *et al.*, 2013)). However, those studies were performed at optimal temperature (Morris *et al.*, 2011), and might be important for *Prochlorococcus* growth at temperature extremes or under other mixed stress regimes, and should be addressed in future studies.

Ecological implications

The meridional range for *Prochlorococcus* in the oligotrophic ocean is approximately N40°–S40°; survival and growth at higher latitudes are restricted by temperature (Johnson *et al.*, 2006; Flombaum *et al.*, 2013). Within the permissive range for *Prochlorococcus*, the concentration of HOOH in the open ocean mixed layer varies but rarely exceeds 100 nM, except during rainfall events (Cooper *et al.*, 1987; Miller and Kester, 1994; Hanson *et al.*, 2001; Yuan and Shiller, 2001; Morris *et al.*, 2011). The microbial community maintains this concentration at this low value; in absence of microbes the HOOH concentration exceeds the lethal limit for all known *Prochlorococcus* ecotypes (mean daily accumulation of 800 nM) (Morris *et al.*, 2011). By reporting the maximum growth rate for either MED4 or MIT9312 as a proxy for the growth performance of *Prochlorococcus* as a genus, we note a striking observation (Figure 4a). While the ability of the microbial community to maintain the concentration of HOOH below 800 nM facilitates *Prochlorococcus* growth at or near its temperature optimum, the ability of the community to maintain HOOH below 400 nM is essential for growth of this genus at all other temperatures.

Placing this physiolgical information in a global ecological context, we see that the ability of the community to lower the HOOH concentration has a dramatic influence on the oceanic abundance and distribution of *Prochlorococcus*. Temperature (Figure 4b) sets the high latitude limits for *Prochlorococcus* in the open ocean surface mixed layer in the presence of a mixed microbial community that maintains the HOOH concentration below 200 nM (Figure 4c). In total absence of the community, the HOOH concentration reaches a mean 800 nM (Morris *et al.*, 2011), which would eliminate

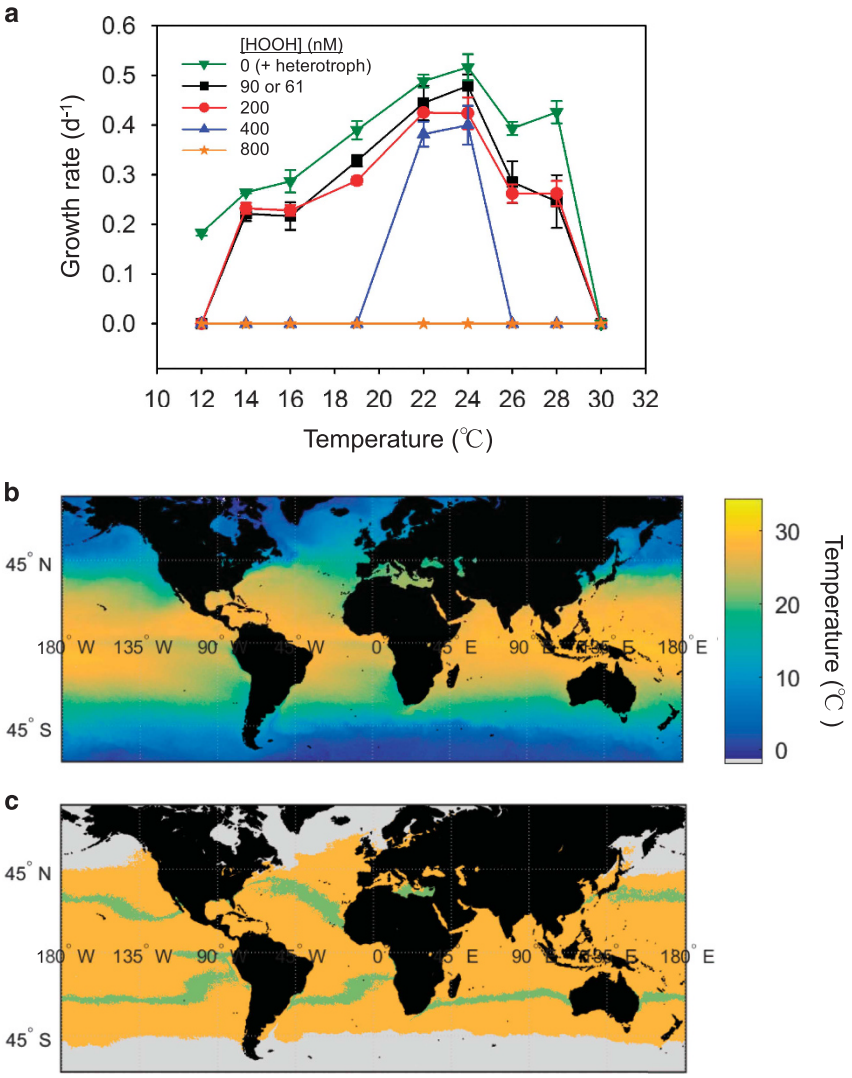

Figure 4 Genus-level relationships between *Prochlorococcus* growth and oceanic distribution as a function of temperature and HOOH. (**a**) Values were taken as the maximum growth rate for MED4 or MIT9312, which ever was highest under the given condition. The growth rates of *Prochlorococcus* with 800 nM HOOH were set to zero, based on prior studies at the optimal temperature, 24 °C (Morris *et al*., 2011). (**b**) False-color image of the 2013 yearly composite of sea surface temperature in the oceans. (**c**) Sea surface distributions for *Prochlorococcus* under natural (non-axenic) conditions (yellow + green) and under hypothetical conditions where the concentration of HOOH was elevated to 400 nM (green). See Discussion for details.

Prochlorococcus from the global mixed layer entirely. If the community was only half as effective as it currently is, resulting in a 400 nM standing stock of HOOH, the *Prochlorococcus* habitat range in the surface mixed layer (3.04×10^7 km^2) would be only 11% as large as it is currently, and would occur in isolated bands in the northern and southern hemispheres (Figure 4c). Survival below the mixed layer, where HOOH concentrations are very low, would still be possible, but the contribution of this genus to global elemental cycling would be dramatically reduced. Caution must certainly be made when extrapolating the results of two strains in culture to model the impacts in natural communities. With that caveat in mind, this model suggests that *Prochlorococcus* owes its existence within its full permissive temperature range for growth, and consequently its full latitudinal range in the oceans, due to the magnitude of the HOOH-degradation activity of the microbial community; were this magnitude to decrease, the range of *Prochlorococcus* would consequently shrink. Thus, with respect to temperature, the co-existing community functions to expand the realized niche of *Prochlorococcus*, effectively matching its physiologically constrained fundamental niche (Hutchinson, 1957).

Conclusions

Prochlorococcus is unusually sensitive to HOOH, and this sensitivity is increased when growing at suboptimal temperatures. By alleviating oxidative stress, co-cultures of heterotrophs—serving as a

proxy for the natural microbial community—effectively increase the temperature range at which *Prochlorococcus* can grow, and in more extreme scenarios slows the death kinetics, through the reduction of co-occurring oxidative stress. This study contributes to the growing body of evidence that facilitation, where one organism protects another from environmental stress, can expand the realized niche of microorganisms.

Conflict of Interest

The authors declare no conflict of interest.

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