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ORIGINAL ARTICLE

The impact of elevated CO_2 on *Prochlorococcus* and microbial interactions with 'helper' bacterium Alteromonas

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Prochlorococcus **is a globally important marine cyanobacterium that lacks the gene catalase and relies on 'helper' bacteria such as** ***Alteromonas*** **to remove reactive oxygen species. Increasing atmospheric CO_2 decreases the need for carbon concentrating mechanisms and photorespiration in phytoplankton, potentially altering their metabolism and microbial interactions even when carbon is not limiting growth. Here,** ***Prochlorococcus*** **(VOL4, MIT9312) was co-cultured with** ***Alteromonas*** **(strain EZ55) under ambient (400 p.p.m.) and elevated CO_2 (800 p.p.m.). Under elevated CO_2,** ***Prochlorococcus*** **had a significantly longer lag phase and greater apparent die-offs after transfers suggesting an increase in oxidative stress. Whole-transcriptome analysis of** ***Prochlorococcus*** **revealed decreased expression of the carbon fixation operon, including carboxysome subunits, corresponding with significantly fewer carboxysome structures observed by electron microscopy.** ***Prochlorococcus*** **co-culture responsive gene 1 had significantly increased expression in elevated CO_2, potentially indicating a shift in the microbial interaction. Transcriptome analysis of** ***Alteromonas*** **in co-culture with** ***Prochlorococcus*** **revealed decreased expression of the catalase gene, known to be critical in relieving oxidative stress in** ***Prochlorococcus*** **by removing hydrogen peroxide. The decrease in catalase gene expression was corroborated by a significant ~6-fold decrease in removal rates of hydrogen peroxide from co-cultures. These data suggest** ***Prochlorococcus*** **may be more vulnerable to oxidative stress under elevated CO_2 in part from a decrease in ecosystem services provided by heterotrophs like** ***Alteromonas.*** **This work highlights the importance of considering microbial interactions in the context of a changing ocean.**

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Introduction

Prochlorococcus is the smallest and most abundant photosynthetic organism on the planet, having a crucial role in the marine carbon cycle, particularly in tropical and subtropical marine ecosystems (Buitenhuis *et al.*, 2012; Biller *et al.*, 2015). Atmospheric carbon dioxide is projected to double over this century, causing a decrease in pH and an increase in dissolved CO_2 in the ocean (Ciais and Sabine, 2013). Modeling studies suggest that the contribution of *Prochlorococcus* to the marine carbon cycle may be more negatively affected by the increase in carbon availability compared with other climate change impacts (Dutkiewicz *et al.*, 2015). To make better projections of the distribution and production of this globally important cyanobacterium, we need a mechanistic understanding of how CO_2 impacts *Prochlorococcus* growth, cellular processes and its interactions with other microbes.

Field experiments with natural communities of picoplankton from the subtropical North Atlantic under elevated CO_2 found a short-term increase in ^{14}C carbon fixation but no increase in net growth of *Prochlorococcus* over a few days (Lomas *et al.*, 2012), highlighting the need for simplified community experiments with the ability to acclimate strains in the lab for longer periods of time. Previous experiments with the high light I ecotype of *Prochlorococcus* (strain MED4) showed no significant differences in growth rate (Fu *et al.*, 2007) or expression of CCM genes with changes in CO_2 concentration (Hopkinson *et al.*, 2014; Bagby and

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Chisholm, 2015). Yet, significant expression changes of high light inducible (*hli*) genes resulted from shifts in O_2:CO_2 ratio (Bagby and Chisholm, 2015), leaving open the question of whether *Prochlorococcus* can sense and acclimate to changes in CO_2.

The *Prochlorococcus* high light II ecotype, exemplified by the strain MIT9312 and used as the model cyanobacterium in this work, is dominant in surface waters of the tropics and subtropics (Johnson *et al.*, 2006). Like all *Prochlorococcus* strains sequenced to date, MIT9312 lacks the catalase gene (Bernroitner *et al.*, 2009). Catalase is a key enzyme for removing hydrogen peroxide, a dangerous byproduct of metabolism and a ubiquitous oxidant in the surface ocean environment (Cooper *et al.*, 1988). *Prochlorococcus* strains are aided in detoxifying reactive oxygen species (ROS) such as hydrogen peroxide by 'helper' bacteria including *Alteromonas* sp. (Morris *et al.*, 2011, 2008). *Alteromonas* sp. are globally distributed heterotrophic bacteria (Ivars-Martínez *et al.*, 2008), known to stably associate with *Prochlorococcus* in culture and to inhibit or promote growth of the cyanobacterium depending on strain and relative abundance (Morris *et al.*, 2008; Avrani and Lindell, 2015; Aharonovich and Sher, 2016). Such studies have begun to investigate which factors drive these microbial interactions, yet there are many gaps in our understanding of these processes. Marine microbial interactions have the potential to greatly impact marine biogeochemical cycles and the structure of marine ecosystems including where and how quickly carbon is metabolized by the microbial loop (Azam and Malfatti, 2007). Given the biogeochemical importance of the relationship between *Prochlorococcus* and 'helper' bacteria like *Alteromonas*, it is vital to study the potential for changes in microbial interactions with rising CO_2.

Materials and methods

Cultures

Six clones of high light II *Prochlorococcus* VOL4, a streptomycin-resistant derivative of strain MIT9312 (Morris *et al.*, 2011), were isolated by dilution to extinction in Pro99 media. *Prochlorococcus* clones were pretreated with the helper bacterium *Alteromonas* sp. strain EZ55 as in Morris *et al.* (2011) that was originally isolated from another culture of high light II *Prochlorococcus*, strain MIT9215 (Morris *et al,* 2008). Before these experiments, six clonal isolates of EZ55 were obtained by streaking from a frozen glycerol stock onto yeast extract tryptone sea salts agar plates (per liter, 4 g tryptone, 2.5 g yeast extract, 15 g sea salts (Sigma-Aldrich, Carlsbad, CA, USA, product no. S9883), and 15 g agar), picking isolated colonies into fresh yeast extract tryptone sea salts medium and were cryopreserved in 20% glycerol at –80 °C. *Prochlorococcus* clones were made axenic by addition of streptomycin (Morris *et al.*, 2008) and cryopreserved by adding dimethylsulfoxide to a final concentration of 7.5% and flash-freezing in liquid nitrogen (Moore *et al.*, 2007). For co-culture experiments, each of the six replicate *Prochlorococcus* cultures were initiated from a single *Prochlorococcus* cell and a different EZ55 clonal population.

Media and culture conditions

All cultures were grown in PEv medium (artificial seawater amended with Pro99 nutrients at 1/25 standard concentrations). The artificial seawater medium (28.41 g NaCl, 7.21 g $MgSO_4$*7 H_2O, 5.18 g $MgCl_2$*6 H_2O, 1.58 g $CaCl_2$*2 H_2O, 0.789 g KCl per liter) was autoclaved, amended with sterile-filtered Pro99 nutrients (Andersen, 2005) and 2.325 mM (final concentration) sodium bicarbonate, then aseptically bubbled overnight with ambient air. Cultures were grown in 13 ml acid-washed conical bottom glass centrifuge tubes with screw caps. Cultures were prepared by placing 12.3 ml of fresh medium into a sterile tube, followed by amendments totaling 0.2 ml (either sterile milli-Q water for ambient CO_2 cultures, or a combination of sterile $NaHCO_3$, HCl, and milli-Q water for elevated CO_2 cultures) and 0.5 ml of a previous culture, leaving almost no headspace in the sealed tube. Inoculated tubes were placed on a tissue culture rotator (Thermo Fisher Scientific, Waltham, MA, USA) in a Percival growth chamber at 21 °C under 150 µmol photons m^{-2} s^{-1} on a 14:10 light–dark cycle. Preliminary tests with sterile media confirmed that the tubes were airtight and that elevated CO_2 conditions were stable in sterile media over at least a 1-week period.

Carbonate chemistry

Parameters of the carbonate system in culture media were determined by separately measuring pH and alkalinity and using these values to calculate dissolved inorganic carbon and CO_2 using the seacarb package in R (Gattuso and Lavigne, 2009). Alkalinity was assessed by titration with certified 0.1N HCl (Thermo Fisher Scientific) using a Mettler-Toledo automatic titrator (Dickson *et al.*, 2007). Medium pH was determined potentiometrically with a Mettler-Toledo electrode calibrated against standardized solutions of Tris-buffered seawater (Dickson *et al.*, 2007). After determination of the baseline carbonate chemistry of a batch of medium, the 'oa' function in seacarb was used to determine additions of calibrated sodium bicarbonate and hydrochloric acid stocks needed to adjust pCO_2 to approximately 800 p.p.m. Stability of media carbonate chemistry was assessed for test cultures with similar maximum biomass to experimental cultures (Supplementary Table 1). The pCO_2 slightly increased over the growth period in all cultures

indicating a shift toward net heterotrophy, likely supported by trace organic carbon in the media salts. Regardless, the treatments remained stably distinct between ambient and elevated CO_2 (Supplementary Table 1).

Growth experiments

Each of the six *Prochlorococcus* clones was grown in ambient or elevated CO_2 in co-culture with one of six *Alteromonas* clones in PEv media as described above. *Prochlorococcus* cell density was measured approximately every 2 days with a Guava Flow Cytometer (EMD Millipore, Darmstadt, Germany). *Alteromonas* cell density was determined by plating serial dilutions of cultures on yeast extract tryptone sea salts agar. Care was taken to minimize perturbations of the carbonate chemistry during subsampling by minimizing headspace and the time tubes were open. Briefly, a 10 µl subsample was quickly removed from each tube and diluted in 90 µl of artificial seawater. When cell densities exceeded 1.3×10^6 cells ml^{-1}, cultures were diluted 26-fold into fresh culture media. Cultures were allowed to acclimate to their target CO_2 conditions through one transfer (approximately five generations), and then growth rate was measured over at least two further transfers. To determine the impact of *Alteromonas* on *Prochlorococcus* growth, streptomycin was added to co-cultures to a final concentration of 100 µg ml^{-1}, rendering the streptomycin-resistant *Prochlorococcus* sp. strain VOL4 axenic by killing the streptomycin-sensitive *Alteromonas* cells. Axenic *Prochlorococcus* was transferred twice under ambient or elevated CO_2 or until cell densities failed to reach 10^6 cells ml^{-1} within 1 month. Axenic status of cultures was confirmed each time the culture was sampled for cell density, by spotting 10 µl of culture onto a yeast extract tryptone sea salts agar plate.

Four growth parameters were measured by manual inspection of at least two consecutive growth curves for each biological replicate. Realized growth rates m were calculated as the exponential change over time t in population density from the inoculation of the first culture (N_I) through the end of the final culture (N_E) after three or more (x) 26-fold transfers using the formula

$$m = 1/t * \ln\left(26^{x-1} * N_E/N_1\right)$$

Exponential growth rates μ were calculated as the slope of the regression of log cell density versus time for several measurements taken during the exponential growth phase of each individual culture; r^2 values for these estimates were generally >0.99. Lag phase duration (L) was calculated as the difference between the actual duration of each individual culture (d) and the time expected if the culture had been growing exponentially at rate (r) starting with its initial inoculation at cell density (N_0) until its final density (N_t):

$$L = d - (\ln(N_t) - \ln(N_0))/\mu$$

Finally, we observed that in most cases longer lag phases occurred along with a substantial decrease in cell densities after culture inoculation, suggesting a die-off of cells after exposure to fresh media. We calculated the magnitude of this die-off (D) by first calculating the ratio of the lowest cell density measurement (N_{min}) over the initial cell density measurement, and subtracting this value from 1 to yield a proportion:

$$D = 1 - N_{min}/N_0$$

The significance of differences in culture parameters was determined using linear mixed effects models with CO_2 concentration and presence/absence of *Alteromonas* as fixed effects and *Prochlorococcus* strain as a random effect. P-values were determined by comparing full models containing both fixed effects with null models omitting one or both. Models were fit in R v 3.3.1 using the *lme4* package (https://www.r-project.org/). Pairwise comparisons were performed using R packages *multcomp* and *lsmeans*.

RNA library prep and sequencing

Cultures for RNA preparations were prepared as for the growth experiments. Briefly, co-cultures of *Prochlorococcus* and *Alteromonas* or axenic *Alteromonas* cultures were transferred twice under ambient or elevated CO_2 in the same media and culture conditions described above and totaling approximately 10 generations before being harvested for RNA. After this acclimation step, co-cultures were split from a single tube to five technical replicate tubes, whereas axenic *Alteromonas* cultures were grown in 1 liter polycarbonate bottles with minimal headspace to increase biomass yield. To further reduce carbon chemistry perturbations for RNA experiments, growth was measured without opening the co-culture tubes by using a Trilogy fluorometer (Turner Designs, San Jose, CA, USA) fitted with a custom three-dimensional-printed cuvette designed to accept conical bottom centrifuge tubes. Co-culture fluorescence was measured daily, when at least three of these replicates crossed the cutoff value of about 5×10^6 cells ml^{-1}, all replicates were pooled to yield approximately 3×10^8 *Prochlorococcus* cells in 65 ml and harvested by gentle filtration ($\leqslant$30 kPa) on a 25 mm 0.2 µm polycarbonate filter. At the time of harvest for axenic *Alteromonas* cultures, carbonate chemistry was confirmed with pH measurements (Supplementary Table 1) and cell concentrations were determined with spot-titer plating, yielding approximately 3×10^8 *Alteromonas* cells in 200 ml, harvested by gentle filtration ($\leqslant$30 kPa) on a 25 mm 0.2 µm polycarbonate filter. Filters were flash frozen and stored at −80 °C until RNA extraction.

RNA extractions were performed with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with a small modification to the lysis procedure. Lysis was performed by adding 0.6 ml Buffer RLT and ~250 µl zirconium/silica beads (0.5 mm) and vortexing for 5 min at 250 r.p.m. Genomic DNA was removed by an on-column DNase digestion with RNase-free DNase I (Qiagen). Ribosomal RNA was removed with the Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, San Diego, CA, USA). After rRNA depletion, samples were purified and concentrated using the RNeasy MinElute cleanup kit (Qiagen). Post-digested RNA was assessed for quantity and quality with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). The mRNA libraries were prepared for Illumina Hi-seq 2500 paired-end sequencing (PE100) with TruSeq RNA sample Prep Kit v2 (Illumina, San Diego, CA, USA). Fragmentation size target was 100 bp, paired-ends were non-overlapping with an insert size of approximately 300 bp. Samples were barcoded for multiplex sequencing and run in a single lane by the Sulzberger Columbia University Genome Center (CUGC) (New York, NY, USA). Sequence files are available from the National Center for Biotechnology Information (NCBI) through BioProject (accession: PRJNA377729) and Sequence Read Archive (SRA) accession numbers SRR5320558-SRR5320569 and SAMN07374746 – SAMN07374757.

Sequence read processing and alignment

Sequence reads were processed at the sequencing facility using the default setting from bcl2fastq, coupled with adaptor trimming to remove barcodes. The subsequent fastq data release were additionally assess with FastQC (www.bioinformatics.babraham.ac.uk/projects/). All sequencing reads passed this quality control and required no further trimming. Reads were aligned to both the MIT9312 gene models (accession: NC_007577.1) and the draft EZ55 genome (Genbank accession SRX022631) using bowtie2 with sensitive end-to-end mode (Langmead and Salzberg, 2012). Reads aligning to coding sequences in the MIT9312 gene models were counted with samtools idxstats (Li *et al*., 2009). Reads aligning to the coding regions of EZ55 draft genome were counted with HT-seq count (Anders *et al*., 2015) because of the ability of HT-seq to count by region without requiring alignment to gene models, which were not available for the draft genome.

Differential gene expression and gene set enrichment analyses

Read counts were normalized using trimmed mean of M-values (Robinson and Oshlack, 2010) to estimate transcript abundances corrected for any differences in sequencing library preparation. Differential expression probabilities and fold change of gene expression in elevated CO_2 compared with ambient were calculated from normalized transcript abundances with edgeR (McCarthy *et al*., 2012) using a general linear model to estimate the tag-wise common dispersion with a paired-sample design to minimize the impact of strain–strain variability. Genes with significant differential expression ($P<0.05$, exact test, edgeR) were tested for gene set enrichment as follows: gene set categories were chosen from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways except those noted as keyword searches from annotations, then gene set enrichment probabilities were calculated with a hypergeometric test (phyper, stats, R) for genes with increased (log2 fold change >0) and decreased (log2 fold change <0) expression that fell into pre-defined categories compared with the total number genes in the same category within the genome.

Cryogenic electron microscopy (cryo-EM)

Co-cultures of *Prochlorococcus* (VOL4, MIT9312) and *Alteromonas* (EZ55) were acclimated to target CO_2 conditions as described above before being used to inoculate approximately 1.2 liter of PEv medium in clear polycarbonate bottles. These bottles were filled, leaving very little headspace and sealed with parafilm before incubation. When cultures exceeded approximately 10^6 cells ml^{-1} they were collected by gentle filtration on a 47 mm, 0.2 µm polyethersulfone membrane filter and resuspended in 1 ml of a sterile mixture of artificial seawater and milli-Q water (1:1). The resuspended cells were concentrated 50-fold by centrifugation (10 min, ~18 000x*g*). The resulting preparation contained between 10^{10} and 10^{12} cells ml^{-1}, and was kept in a cool dark box during transport to the cryo-EM facility where cells were immediately cryogenically frozen in preparation for microscopy.

For cryo-EM, 3 µl of the *Prochlorococcus* suspension were applied to glow-discharged 200 mesh Quantifoil R 2/1 Nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and vitrified in liquid ethane using an FEI Vitrobot Mark IV (Thermo Fisher Scientific). The grids were observed on an FEI Tecnai F20 electron microscope (Thermo Fisher Scientific) operated at 200 kV with typical magnification ×38 000 and 4–10 µm defocus. Images were collected under low-dose conditions using a Gatan Ultrascan 4000 CCD camera. Only images where internal cell structures were sufficiently visible were included in further analysis. Carboxysomes were visible as dark inclusions within cells, generally with a visible hexagonal shape, and were counted manually for each image. Statistical tests were performed in R using the wilcox.test function (package stats).

Hydrogen peroxide degradation rates

Concentrations of H_2O_2 were determined using the protocol of Morris *et al*. (2011) modified for use in a

Synergy H1 plate reader (BioTek, Winooski, VT, USA). In brief, 2 ml samples were removed from cultures and sterile H_2O_2 was added to approximately 250 nM starting concentration. Duplicate 200 µl samples were then removed into black 96-well plates every 15 min for 1 h for H_2O_2 determination by sequential injections of 50 µl 2 M Na_2CO_3 (pH 11.3), and 3.5 mM acridinium NHS ester (pH 3, Cayman Chemical) followed by integration of chemiluminescent light emission for 1 s. Absolute determination of H_2O_2 concentration was achieved by comparison against a standard curve produced by adding H_2O_2 from 0 to 500 nM to artificial seawater media. Before calculations, chemiluminescent output of all samples was adjusted by subtracting the readings for a true blank to which 10 µM sodium pyruvate had been added to eliminate any incidental H_2O_2. Degradation rate constants were calculated as the regression of log H_2O_2 concentration versus time and normalized to *Alteromonas* cell concentration determined from spot-titer plating.

Results and Discussion

The impact of rising CO_2 on ocean ecosystems is a topic of increasing interest, however, little is known about the influence of rising CO_2 on the microbial interactions that underpin these ecosystem dynamics. Here, a strain of the cyanobacterium *Prochlorococcus* (VOL4, MIT9312) was cultured with a heterotrophic bacterial isolate *Alteromonas* (EZ55) to investigate the potential for CO_2-driven changes in *Prochlorococcus* physiology and the microbial interaction with *Alteromonas* (Figure 1). Six single-cell isolates of *Prochlorococcus* and *Alteromonas* were co-cultured under ambient (~400 p.p.m.) or elevated (~800 p.p.m.) CO_2 (Supplementary Table 1) at low cell densities ($< 5 \times 10^6$ cells ml^{-1} per culture) to prevent the alteration of carbonate chemistry, and to more closely mimic *in situ* cell densities.

Prochlorococcus in co-culture with *Alteromonas* had a realized growth rate (m) 47% slower under elevated CO_2 compared with ambient CO_2 (Figure 1a, Table 1). There was no significant difference between CO_2 treatments in the true exponential growth rates (µ), similar to the findings of Fu *et al.* (2007). Instead, the observed difference in realized growth rate was due to a significantly longer lag phase duration ($P = 1.48 \times 10^{-5}$, linear mixed effects model) (Table 1). Longer lag durations (L) were often coupled with substantial die-offs (D) during the days after inoculation. The magnitude of these die-offs was significantly greater under elevated CO_2 than ambient conditions ($P = 8.46 \times 10^{-5}$) (Table 1). Previous experiments have shown that die-offs in *Prochlorococcus* cultures are often attributable to increased oxidative stress, which can be mitigated by the presence of helper heterotrophic bacteria such as *Alteromonas* (Morris *et al.*, 2011; Coe *et al.*, 2016). These observations suggest that *Prochlorococcus* may experience greater oxidative stress under elevated CO_2 even in the presence of the 'helper' bacterium *Alteromonas*, and highlights the importance of accounting for microbial interactions in evaluating changes in growth rate and mortality under future CO_2 scenarios.

To test the importance of *Alteromonas* in mitigating the negative impacts of high CO_2, we attempted to render co-cultures axenic with the addition of the antibiotic streptomycin. Under ambient CO_2, all six *Prochlorococcus* cultures that were successfully rendered axenic were able to survive at least one growth cycle (Figure 1b), but survivorship was much lower under elevated CO_2, with only a quarter of

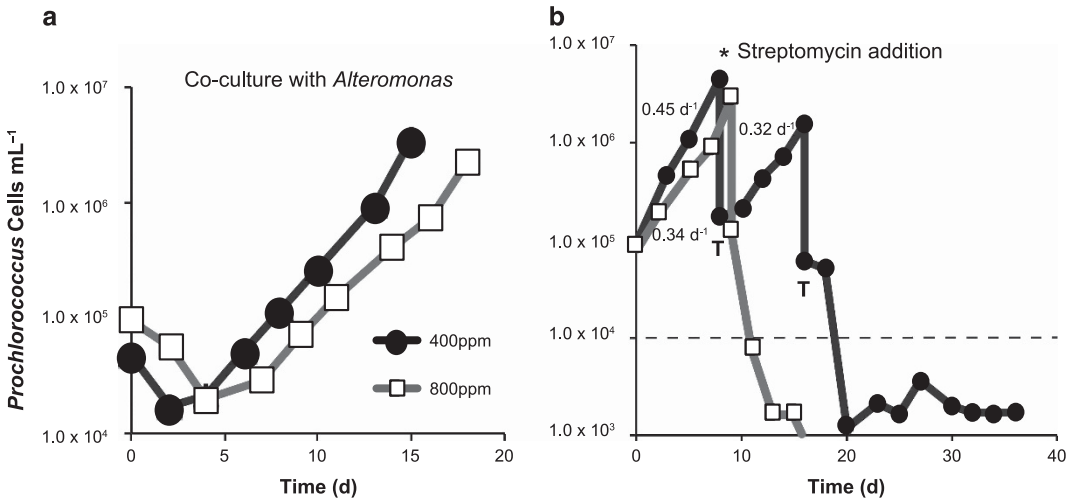

Figure 1 Representative growth curves of *Prochlorococcus* under ambient and elevated CO_2. (**a**) Cell density (cells ml^{-1}) versus time (days) of *Prochlorococcus* (VOL4, MIT9312) in co-culture with *Alteromonas* (EZ55) at 400 p.p.m. (black circles) or 800 p.p.m. (white squares) CO_2. Growth statistics are in Table 1. (**b**) Cell density of *Prochlorococcus* (VOL4, MIT9312) over consecutive transfers (indicated by 'T') in co-culture with *Alteromonas* (EZ55). The asterisk indicates addition of streptomycin rendering *Prochlorococcus* axenic, the dashed line indicates the limit of detection and the numbers are exponential growth rate constants (day^{-1}) calculated from growth curves.

Table 1 Growth parameters of *Prochlorococcus* with or without *Alteromonas* under ambient (~400 p.p.m.) or elevated (~800 p.p.m.) CO_2 regimes

CO_2 treatment	*m (day^{-1})*	*μ (day^{-1})*	*L (day)*	*D*
Ambient	0.353 ± 0.040	0.440 ± 0.023	2.21 ± 1.38	0.223 ± 0.165
Elevated	0.187 ± 0.041	0.426 ± 0.071	10.14 ± 3.53	0.688 ± 0.164
Ambient axenic	0.048 ± 0.060	0.219 ± 0.098	9.73 ± 10.82	0.539 ± 0.286
Elevated axenic	0.027 ± 0.086	0.095 ± 0.124	4.57 ± 10.93	0.699 ± 0.443

Values are means of replicate cultures ± 95% confidence intervals ($n = 6$, non-axenic; $n = 7$, ambient axenic; $n = 4$, elevated axenic). *m*, realized growth rate over at least two consecutive batch cultures; *μ*, exponential growth rate of individual cultures; *L*, lag phase duration; *D*, die-off proportion.

successfully purified cultures surviving two growth cycles. Where axenic cultures survived, they had significantly reduced growth rates ($P < 0.001$) compared with co-cultured *Prochlorococcus* in both ambient and elevated CO_2 (Table 1). This supports the findings of previous studies on the toll of oxidative stress on axenic *Prochlorococcus* in the absence of a 'helper' heterotroph (Morris *et al.*, 2011), and suggests that the impact of natural oxidative stress is at least partially mitigated by the presence of *Alteromonas* under both CO_2 treatments. Interestingly, *Alteromonas* seemed more resistant to streptomycin at high CO_2: only 4 of 12 attempts were successful in creating an axenic culture compared with 7 of 9 under ambient CO_2. We hypothesize that a change in *Alteromonas* metabolism may have reduced sensitivity to streptomycin at elevated CO_2, either as a result of changing microbial interactions or in response to acidification. Of the four axenic *Prochlorococcus* cultures at elevated CO_2, only two cultures survived the first growth cycle and none survived beyond the second, so differences in growth parameters could not be statistically tested (Table 1), nor could changes in gene transcription be evaluated for axenic *Prochlorococcus*.

To investigate how elevated CO_2 impacts the metabolic functions of *Prochlorococcus* and interactions with 'helper' bacterium *Alteromonas*, whole transcriptomes of the co-cultures under ambient and elevated CO_2 were sequenced. Among transcripts aligning to the *Prochlorococcus* (MIT9312) genome, only 35 genes were differentially expressed ($P < 0.05$, fold change > 2) under elevated CO_2 (Figure 2, Supplementary Table 2). There were significant decreases in expression of high light inducible (*hli*) genes from genomic islands in response to elevated CO_2 (Figure 2a). Decreased expression of *hli* genes from the genomic islands (non-core genome) was previously observed for increasing CO_2:O_2 ratio in the high light I MED4 strain of *Prochlorococcus* (Bagby and Chisholm, 2015). The functions of these genes are not known, but are potentially involved in photorespiration or the oxidative stress response. Notably, there was an approximately twofold decrease in transcripts of an apparent carbon fixation operon (Figure 2b). Not all genes in the carbon fixation operon met our threshold for significant differential expression, including the Rubisco subunits; however, decreased expression of the carboxysome subunits *csoS1D*, *csoS1*, and *csoS2* and *csoSCA* were statistically significant ($P < 0.05$) (Figure 2b). Decreased expression of carboxysome subunits of a similar magnitude was observed under elevated CO_2 experiments with MED4, but the changes were not statistically significant because of large uncertainties (Hopkinson *et al.*, 2014).

One of the significantly differentially expressed carboxysome subunit genes, *csoSCA,* also functions as a carbonic anhydrase (So *et al.*, 2004; Ting *et al.*, 2015). The observed decreased expression of *csoSCA* supports the hypothesis that under elevated CO_2, phytoplankton downregulates CCM genes because of increased diffusive supply of CO_2, or pH-induced transcriptional regulation. There was no significant change in expression of the bicarbonate transporter genes (Supplementary Table 2) in agreement with a previous study on MED4 (Hopkinson *et al.*, 2014). Our data suggest that carboxysome and bicarbonate transporter genes are regulated by separate mechanisms, a conclusion supported by the existence of different conserved sequence motifs for carboxysome subunits and bicarbonate transporters in *Prochlorococcus* strains (Ting *et al.*, 2015). The lack of a significant shift in expression of energy-consuming bicarbonate transporter genes may indicate that there would not be a significant energetic savings for *Prochlorococcus* under elevated CO_2. However, a shift in the expression of carboxysome subunit genes would be expected to have a significant impact on the morphology or number of carboxysomes per cell. To investigate this possibility, cell cultures were imaged with cryo-EM to determine whether the transcriptome changes resulted in a change in cell morphology. Under ambient CO_2, there were typically two to three carboxysome structures per *Prochlorococcus* cell (Figures 3a and c), with as many as ten observed in one cell. Exposed to elevated CO_2, *Prochlorococcus* cells frequently contained no apparent carboxysomes (Figure 3b), with typical cells containing two carboxysomes and rarely more (Figure 3d). The number of carboxysomes per cell was significantly greater in ambient (mean 2.76) compared with elevated CO_2 (mean 1.73) treatments ($P < 0.01$, Mann–Whitney *U*-test). There were a higher proportion of large diameter (>125 nm)

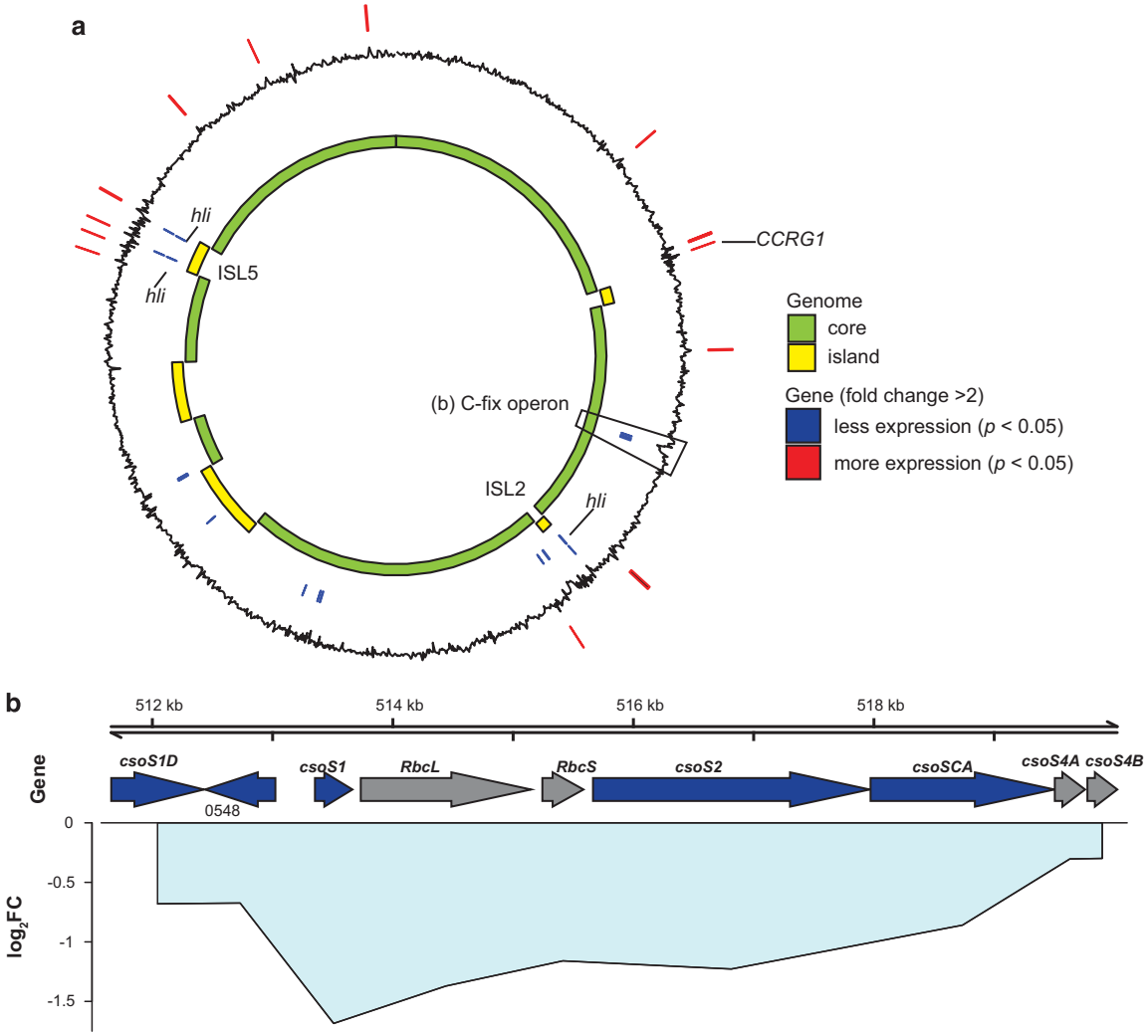

Figure 2 *Prochlorococcus* differential gene expression under elevated CO_2. (**a**) Whole-genome view of differentially expressed genes ($n = 35$) in relation to core and flexible genome (that is, islands) for *Prochlorococcus*. Black line indicates log2 fold change in expression, with positive (outward) indicating genes with increased expression, and negative (inward) values indicating decreased expression under elevated CO_2. Positions of significantly differentially expressed genes (pairwise exact test, $P < 0.05$, $n = 6$, with fold change > 2) are indicated by lines on the outer ring (red) for genes with increased expression, and the inner ring (blue) for genes with decreased expression. (**b**) Region containing the putative operon with genes for carbon fixation (C-fix operon): Rubisco (*RbcL, RbcS*), carboxysome subunit genes (*csoS1D, csoS1, csoS2, csoSCA, csoS4A* and *csoS4B*). Arrows indicate location and strand of genes, primary colors indicate significant differential expression ($P < 0.05$, $n = 6$, blue = decreased, gray = no significant difference) under elevated CO_2, lines indicate the mean $\log_2$ fold change (log2FC) of each gene, centered on each gene's position in the genome, and are filled with light blue (decreased expression) to indicate cohesiveness of fold change across multiple genes.

carboxysomes in *Prochlorococcus* cells exposed to elevated CO_2 (Supplementary Figure 1); however, the differences in diameter (mean ~80 nm) were not statistically significant and were similar to the range (~90–130 nm) previously observed for *Prochlorococcus* (Ting *et al.*, 2007).

The finding that *Prochlorococcus* had a 37% decrease in carboxysomes per cell at elevated compared with ambient CO_2 agrees remarkably well with the ~50% decrease in expression of carboxysome genes. Despite this measurable difference in cell structures associated with carbon concentration and fixation, exponential growth rates did not change significantly (Table 1). We infer that Rubisco in carboxysome structures was not the limiting step for carbon fixation under these conditions, similar to the findings of Hopkinson *et al.* (2014) for MED4. However, assuming that the efficiency of carbon fixation via carboxysomes remains the same, fewer carboxysomes per cell indicates that the maximum capacity for carbon fixation via carboxysomes declined in response to elevated CO_2.

In addition to changes in cell morphology, the *Prochlorococcus* transcriptome data revealed a possible shift in the interspecies interaction with *Alteromonas*. A *Prochlorococcus* gene of unknown function, termed co-culture responsive gene 1 (CCRG1) by Aharonovich and Sher (2016), had the highest fold increase under elevated CO_2 (Supplementary Table 2). This gene was previously

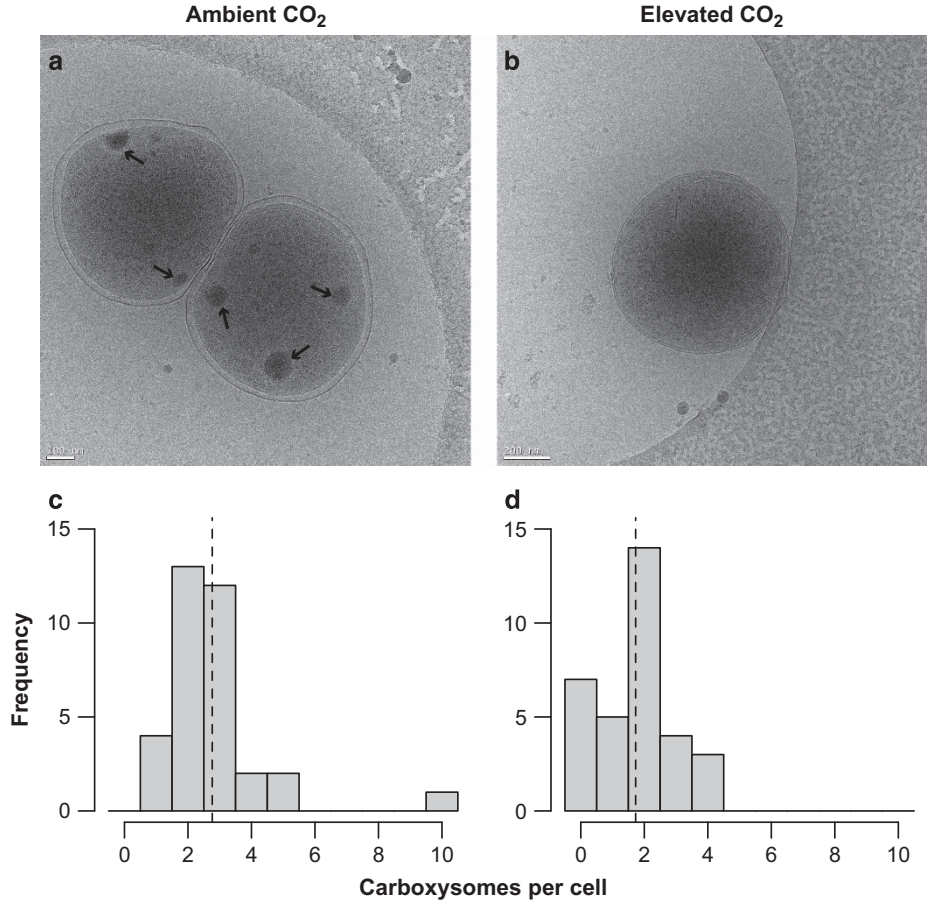

Figure 3 Carboxysome content per *Prochlorococcus* cell with example cryo-electron micrographs. Cryo-electron microscopy (cryo-EM) images of *Prochlorococcus* cells acclimated to (**a**) ambient (~400 p.p.m.) CO_2 and (**b**) elevated (~800 p.p.m.) CO_2 (**b**). Arrows indicate location of carboxysome structures. Histograms of carboxysome structures per cell acclimated to (**c**) ambient or (**d**) elevated CO_2 ($n = 34$ and 33 cells, respectively). Dashed lines represent the mean number of carboxysomes per cell in each treatment.

characterized by its significantly increased expression in a low light IV *Prochlorococcus* strain co-cultured with a different strain of *Alteromonas*. Although CCRG1 is not completely conserved, it is found in strains belonging to both high light and low light *Prochlorococcus* ecotypes (Aharonovich and Sher, 2016). The increased expression of CCRG1 under elevated CO_2 is unanticipated because the concentration of *Alteromonas* remained relatively constant across all measured time points at $5.34 \pm 1.2 \times 10^6$ cells ml^{-1} at ambient CO_2 and $4.48 \pm 1.27 \times 10^6$ cells ml^{-1} at elevated CO_2 (mean ± 95% confidence interval), and these values were not significantly different between CO_2 treatments (*t*-test, $df = 122$, $P = 0.34$). In low light IV *Prochlorococcus* (strain MIT9313), CCRG1 expression was associated with production of a largely intracellular protein whose function remains unknown, despite phylogeny suggesting it originated before the diversification of the *Prochlorococcus* lineage (Aharonovich and Sher, 2016).

In light of these changes in *Prochlorococcus* gene expression, transcriptome sequences of the 'helper' bacterium *Alteromonas*, with and without *Prochlorococcus* under elevated and ambient CO_2, were used to interrogate the potential shift in the nature of the interspecies interaction. Indeed, there were over 400 genes with significant differential expression between CO_2 treatments ($P < 0.05$, fold change > 2) (Figure 4, Supplementary Table 3) in *Alteromonas* co-cultured with *Prochlorococcus*. Even after correcting for its larger gene complement, *Alteromonas* had a greater gene expression response than *Prochlorococcus* (8.5% of *Alteromonas* versus 1.8% of *Prochlorococcus* genes were significantly differentially expressed). *Alteromonas* cultures without *Prochlorococcus* had a smaller gene expression response to elevated CO_2 relative to co-cultures with 128 genes ($P < 0.05$, fold change > 2) (Supplementary Table 4). These results suggest a more complex change in the interaction with *Prochlorococcus* under elevated CO_2 rather than a direct response of *Alteromonas* to elevated CO_2.

Gene set enrichment analysis was performed to determine which categories of genes were significantly skewed toward lower or higher expression in *Alteromonas* in co-culture with *Prochlorococcus* under elevated CO_2 (Figure 4b, Supplementary Table 5). Gene sets with lower expression under elevated CO_2 included carbon, fatty acid and amino-

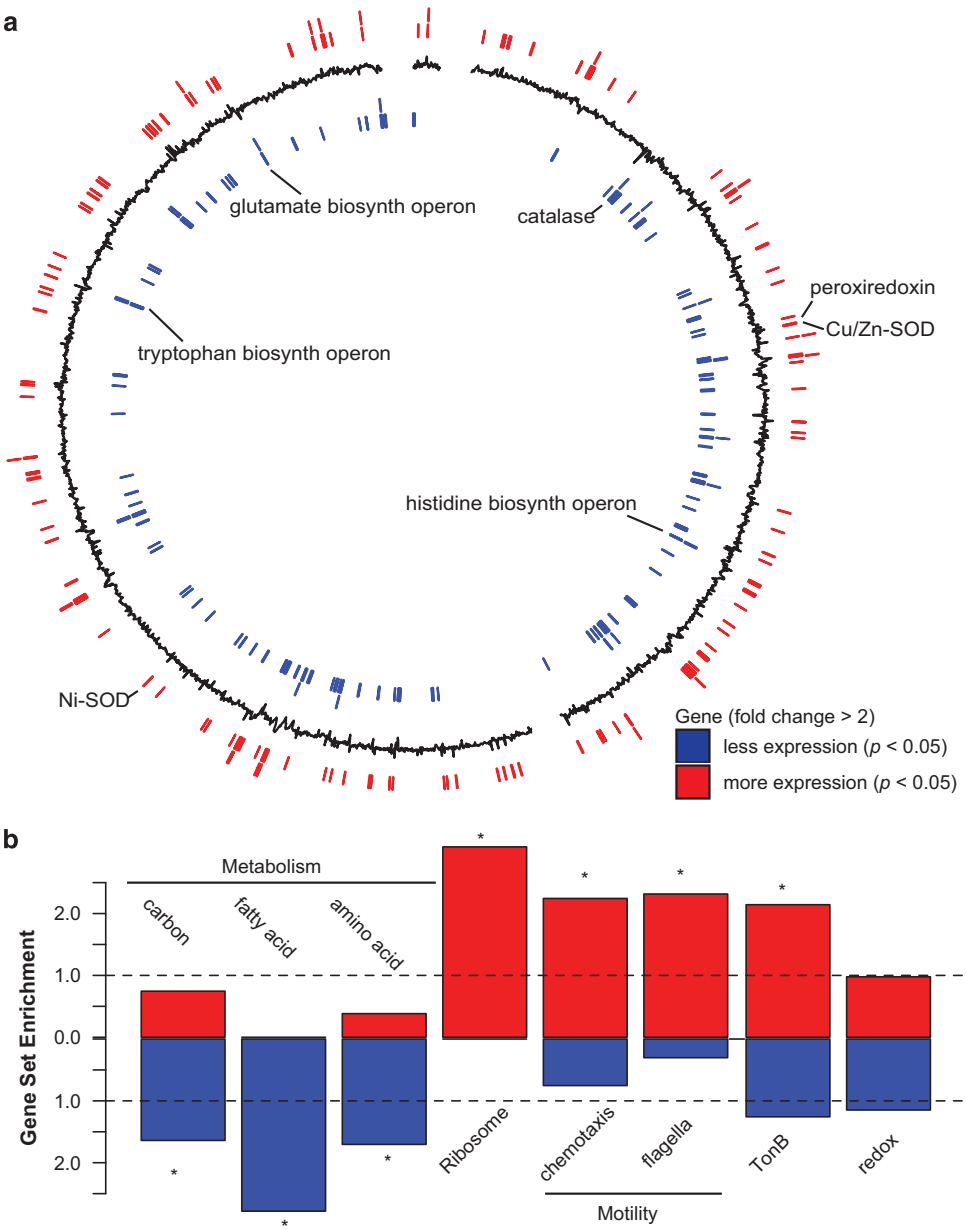

Figure 4 *Alteromonas* differential gene expression under elevated CO_2. (**a**) Whole-genome view of differentially expressed genes for *Alteromonas* (strain EZ55). Black line indicates log2 fold change in expression, with positive (outward) indicating genes with increased expression, and negative (inward) values indicating decreased expression under elevated CO_2. Positions of significantly differentially expressed genes (pairwise exact test, $P<0.05$, $n=6$, with fold change >2) are indicated by lines on the outer ring (red) for genes with increased expression, and the inner ring (blue) for genes with decreased expression. Select genes and putative operons are labeled. (**b**) Gene set enrichment analysis of differentially expressed genes ($P<0.05$, $n=6$), with increased expression (red) or decreased expression (blue) for KEGG categories of carbon, amino-acid and fatty acid metabolism, ribosome, chemosynthesis, and flagellar genes, as well as categories defined by annotation keyword: TonB and redox (Supplementary Table 5). Dashed line indicates expected ratio of genes in a set based on the number of genes within the same set in the genome, asterisk indicates the gene set is significantly enriched (hypergeometric test, $P<0.05$) compared with the expected value of random selection from the genome.

acid metabolism (Figure 4b). However, only certain amino-acid metabolic pathways in *Alteromonas* had lower expression, including putative tryptophan, histidine and glutamate biosynthesis operons (Figure 4a). Ribosomal genes, chemotaxis-related genes and TonB-dependent transporters were over-represented in genes with significantly increased transcript abundance under elevated CO_2 (Figure 4b, Supplementary Table 5). Similar expression changes in flagellar and chemotaxis genes have been observed in response to low pH in *Escherichia coli* (Maurer *et al*., 2005), potentially as a pH homeostasis strategy, however, a similar expression pattern was not observed in axenic *Alteromonas* exposed to elevated CO_2 (Supplementary Tables 3 and 4). None of the previously characterized pH homeostasis

genes identified in a mesocosm experiment from marine heterotrophic bacteria (Bunse *et al*., 2016) had significantly increased expression in *Alteromonas* under elevated CO_2. However, several dehydrogenase genes, which encode enzymes that produce hydrogen ions, had significantly decreased expression in both co-cultured and axenic *Alteromonas* under elevated CO_2 (Supplementary Tables 3 and 4), suggesting another potential strategy to decrease hydrogen ion concentrations within the cell.

In order to elucidate how ecosystem services provided by *Alteromonas* might change in the future ocean, we examined the response of genes that alter concentrations of ROS. Redox-related genes were found to have both higher and lower relative transcript abundance in co-cultured *Alteromonas* under elevated CO_2 with no significant enrichment compared with the genome (Figure 4b). The gene primarily responsible for catalyzing the removal of hydrogen peroxide, catalase, had significantly lower expression in *Alteromonas* in co-culture with *Prochlorococcus* under elevated CO_2 (Figure 4a, Supplementary Table 3), but not in *Alteromonas* alone. In both co-cultured and axenic *Alteromonas*, there was a significant decrease in the expression of the Ohr family peroxiredoxin (Supplementary Tables 3 and 4), which is thought to degrade organic peroxides. In addition, two superoxide dismutase genes (Cu/Zn SOD and Ni SOD) responsible for the conversion of superoxide to hydrogen peroxide (Figure 4a, Supplementary Table 3) had significantly increased expression in *Alteromonas* in co-culture with *Prochlorococcus*. This suggests that *Alteromonas* may be producing hydrogen peroxide at a greater rate and/or degrading it more slowly, thereby increasing oxidative stress in *Prochlorococcus* under elevated CO_2 compared with ambient CO_2. Previous evidence points to removal of ROS as a cornerstone of the microbial interaction between *Prochlorococcus* and marine bacteria such as *Alteromonas* (Morris *et al*., 2011), so shifts in expression of these genes is evidence of a potential shift in the microbial relationship of these ecologically important marine bacteria. To test whether these gene expression changes resulted in changes in *Alteromonas'* ability to protect *Prochlorococcus* from oxidative stress, the degradation rates of hydrogen peroxide were measured under ambient and elevated CO_2 for co-cultures of *Prochlorococcus* and *Alteromonas* as well as axenic *Alteromonas* (Table 2). There was more than a sixfold decrease in the degradation rate of hydrogen peroxide at elevated CO_2 in co-cultures of *Prochlorococcus* and *Alteromonas* (Table 2). The average degradation was also lower in axenic *Alteromonas* cultures under elevated CO_2, but the difference was not statistically significant (Table 2). These results strengthen the hypothesis that the catalase gene has a key role in eliminating ROS such as hydrogen peroxide. The change in hydrogen peroxide degradation also helps explain the higher mortality rate observed for *Prochlorococcus* under elevated CO_2 (Table 1), apparently because of a decline in the expression of catalase in the 'helper' bacterium *Alteromonas* in co-culture with *Prochlorococcus*.

Putting these results together, we propose a new conceptual model for how primary production and microbial interactions may change with rising CO_2 in

Table 2 Hydrogen peroxide degradation rates of *Prochlorococcus* in co-culture with 'helper' bacterium *Alteromonas* and axenic *Alteromonas*

Culture	*Ambient CO_2 (modern)*	*Elevated CO_2 (year 2100)*	P-*value*
Co-cultures	2.57 ± 1.86	0.42 ± 0.23	0.03
Axenic *Alteromonas*	1.09 ± 1.00	0.77 ± 0.30	NS

Normalized degradation rates of hydrogen peroxide (10^{-9} min^{-1} *Alteromonas* $cell^{-1}$) mean ± s.d. ($n = 4$ for co-culture and $n = 6$ for axenic *Alteromonas*) with *P*-value for differences between ambient and elevated CO_2 (*t*-test, one-tailed), NS indicates not significant.

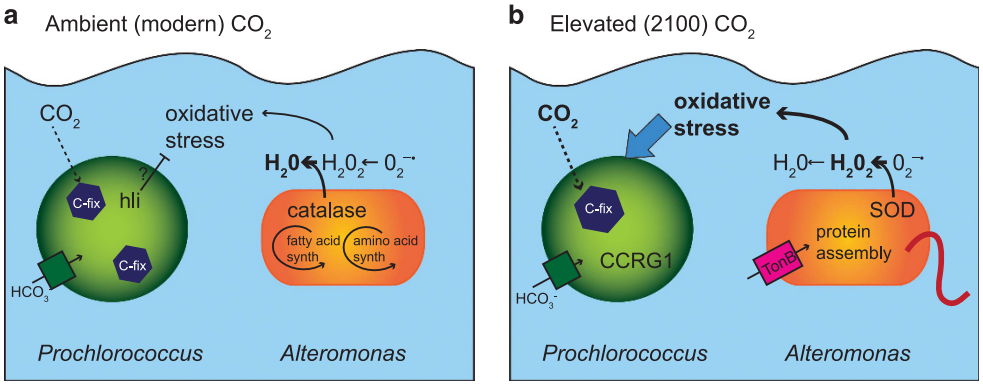

Figure 5 Conceptual model of the interaction of *Prochlorococcus* and *Alteromonas* under ambient and elevated CO_2. *Prochlorococcus* cell in green and *Alteromonas* cell in orange under (**a**) ambient, modern CO_2 and (**b**) elevated CO_2 predicted for 2100. Arrows indicate putative fluxes or transformations of metabolites, both passive (dashed), active (solid), and inhibitory (flat head arrow). Relative increases in fluxes or transformations indicated by bold arrows and text. The thick red line on *Alteromonas* represents flagellar and chemotaxis gene expression. high light induced (hli), CCRG1, superoxide dismutase (SOD), TonB-dependent transporters and related genes (tonB).

the tropical and subtropical oceans where *Prochlorococcus* dominates (Figure 5). At ambient CO_2, *Prochlorococcus* fixes inorganic carbon using a bicarbonate transporter and carboxysome structures to concentrate CO_2 for the enzyme Rubisco (Figure 5a). In addition, *hli* genes expressed at higher levels under ambient CO_2 may have a role in protecting *Prochlorococcus* from oxidative stress because of high light or unbalanced growth. *Alteromonas* uses the enzyme catalase to efficiently rid the cell and surrounding environment of hydrogen peroxide, benefiting the whole-microbial community, and allowing minimal genome organisms like *Prochlorococcus* to thrive (Morris *et al*., 2012). At elevated CO_2 predicted for 2100, *Prochlorococcus* responds to a change in CO_2 or pH by downregulating carboxysome genes, resulting in fewer carboxysome structures (Figure 5b). The cyanobacterium also experiences a higher level of oxidative stress, potentially because of decreased *hli* gene expression or from a shift in microbial interaction with *Alteromonas*. *Prochlorococcus* upregulates the CCRG1 gene potentially as a signaling or defense mechanism against bacterial attack. Concurrently, *Alteromonas* downregulates production of amino acids while upregulating TonB-dependent transporters, ribosomes for protein assembly, and chemotaxis and flagellar genes for motility (Figure 5b), perhaps in response to a higher abundance of dead and dying *Prochlorococcus* or the exchange of metabolites. In addition, *Alteromonas* downregulates the enzyme catalase and upregulates superoxide dismutase. The net result of these changes in enzyme levels is a decrease in the rate of hydrogen peroxide removal, exacerbating the oxidative stress of *Prochlorococcus* under elevated CO_2. These changes in *Prochlorococcus* and *Alteromonas* gene expression and physiology suggest that key microbial interactions may shift in elevated CO_2 from mutualism toward antagonism, potentially inhibiting the dominant primary producer in the tropical and subtropical oceans.

With the global importance of *Prochlorococcus* and its limited gene complement for acclimating to environmental change, it is vital to explore how microbial partnerships may shift to sustain or inhibit this dominant primary producer. Several lines of evidence point to fundamental changes in the microbial interaction between *Prochlorococcus* and *Alteromonas* under elevated CO_2, which may underpin changes in *Prochlorococcus* ecophysiology. These changes include an apparent increase in dependence of *Prochlorococcus* on 'help' from other bacteria, increased expression of CCRG1 in *Prochlorococcus* and decreased expression of the catalase gene in *Alteromonas* with a concomitant decrease in hydrogen peroxide degradation. In a more complex natural ecosystem, another bacterium may provide the ecosystem services that decreased in *Alteromonas*. Our experiments were done in a simplified co-culture, whereas the web of microbial interactions in natural ecosystems are much more complex and merit further study in the light of these findings. Regardless, these changes in gene expression and physiology in both *Prochlorococcus* and the 'helper' bacterium *Alteromonas* underscore that the increase in CO_2 expected for the end of this century can rapidly alter microbial partnerships at the base of the marine food web. Changes in interspecies interactions could in turn alter ecosystem dynamics and should be considered in future modeling efforts.

Conflict of Interest

The authors declare no conflict of interest.

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