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ORIGINAL ARTICLE

Response of *Prochlorococcus* to varying CO_2:O_2 ratios

Sarah C Bagby[1,3] and Sallie W Chisholm[1,2]
[1]*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA and* [2]*Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA*

Carbon fixation has a central role in determining cellular redox poise, increasingly understood to be a key parameter in cyanobacterial physiology. In the cyanobacterium *Prochlorococcus*—the most abundant phototroph in the oligotrophic oceans—the carbon-concentrating mechanism is reduced to the bare essentials. Given the ability of *Prochlorococcus* populations to grow under a wide range of oxygen concentrations in the ocean, we wondered how carbon and oxygen physiology intersect in this minimal phototroph. Thus, we examined how CO_2:O_2 gas balance influenced growth and chlorophyll fluorescence in *Prochlorococcus* strain MED4. Under O_2 limitation, per-cell chlorophyll fluorescence fell at all CO_2 levels, but still permitted substantial growth at moderate and high CO_2. Under CO_2 limitation, we observed little growth at any O_2 level, although per-cell chlorophyll fluorescence fell less sharply when O_2 was available. We explored this pattern further by monitoring genome-wide transcription in cells shocked with acute limitation of CO_2, O_2 or both. O_2 limitation produced much smaller transcriptional changes than the broad suppression seen under CO_2 limitation and CO_2/O_2 co-limitation. Strikingly, both CO_2 limitation conditions initially evoked a transcriptional response that resembled the pattern previously seen in high-light stress, but at later timepoints we observed O_2-dependent recovery of photosynthesis-related transcripts. These results suggest that oxygen has a protective role in *Prochlorococcus* when carbon fixation is not a sufficient sink for light energy.
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Introduction

The marine picophytoplankter *Prochlorococcus* is the most numerous oxygenic marine phototroph and has the smallest genome among them (Partensky *et al.*, 1999; Dufresne *et al.*, 2003). It maintains access to a very large *pan*-genome via horizontal gene transfer, with hotspots in genomic islands, some of which contain genes that clearly have a role in niche differentiation (Coleman *et al.*, 2006; Kettler *et al.*, 2007; Scanlan *et al.*, 2009). Its genomic diversity has allowed *Prochlorococcus* to thrive over a broad range of environmental conditions, with distinct lineages adapted to different light levels, temperatures, and nitrogen, phosphorus, oxygen, and iron availabilities (Moore *et al.*, 1995, 2002; Moore and Chisholm, 1999; Goericke *et al.*, 2000; Johnson *et al.*, 2006; Coleman and Chisholm, 2007; Kettler *et al.*, 2007; Lavin *et al.*, 2010; Malmstrom *et al.*, 2013). One constant across ecotypes is their relative fitness in oligotrophic waters (Partensky *et al.*, 1999; Dinsdale *et al.*, 2008), promoted by traits like high surface-to-volume ratio and low phosphorus demand (Dufresne *et al.*, 2003; Van Mooy *et al.*, 2006, 2009). In these waters, *Prochlorococcus* can account for as much as half of net primary production at a given location (Vaulot *et al.*, 1995).

In oxygenic phototrophs, intracellular oxygen can reduce the efficiency of carbon fixation, because the enzyme Rubisco can catalyze the reaction of ribulose-1,5-bisphosphate with either CO_2 or O_2 (Bowes *et al.*, 1971). Carboxylation leads to the Calvin cycle; the energetically wasteful oxygenation reaction typically leads to photorespiration to clear the cytotoxic product 2-phosphoglycolate from the cell. The cyanobacterial carbon-concentrating mechanism is thought to promote carboxylation by accumulating CO_2 and sequestering Rubisco in O_2-impermeable carboxysomes (Price *et al.*, 2008), yet, in light, some cyanobacteria may photorespire almost constantly (Eisenhut *et al.*, 2008). Recent work suggests that the *Prochlorococcus* carbon-concentrating mechanism, although minimal, is highly efficient (Hopkinson *et al.*, 2014), compensating for the comparatively weak CO_2 affinity of *Prochlorococcus* Rubisco ($K_M \sim 260–750\ \mu M$) (Scott *et al.*, 2007; Roberts *et al.*, 2012; Hopkinson *et al.*, 2014).

Carbon dioxide and oxygen are also linked in cyanobacteria by cellular redox poise. The health of the photosynthetic electron transport chain depends on the dynamic balance between oxidized and

Correspondence: SW Chisholm or SC Bagby, Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, 15 Vassar Street, Cambridge, MA 02139, USA.
E-mail: bagby@geol.ucsb.edu or chisholm@mit.edu
[3]Current address: Marine Science Institute, Webb Hall, University of California, Santa Barbara, CA 93106, USA.
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reduced cofactors. Increasingly, that balance appears to feed into cellular signaling in many cyanobacteria, contributing not just to control of photosynthesis-related gene expression but also to systems from the circadian clock to phototaxis (Li and Sherman, 2000; Wakabayashi *et al.*, 2011; Kim *et al.*, 2012); it is not known whether *Prochlorococcus*, which has only a partial circadian clock (Holtzendorff *et al.*, 2008; Axmann *et al.*, 2009), makes use of redox poise as a signal. Redox poise can be threatened by factors at both ends of the electron transport chain: either an excess of arriving photons or an inability to siphon off sufficient reducing equivalents through carbon fixation can create what amounts to an impedance mismatch. In such cases, absent other photoprotective strategies, excess electrons can flow to molecular oxygen to produce damaging reactive oxygen species (Niyogi, 1999). Remarkably, in the course of genome reduction, *Prochlorococcus* appears to have 'outsourced' reactive oxygen species decontamination to its neighboring heterotrophic bacteria (Morris *et al.*, 2011, 2012) and to have minimized its photoprotective strategies (Mella-Flores *et al.*, 2012), likely limiting its ability to handle acute changes in redox poise.

To explore the relationships between oxygen and carbon dioxide physiology in *Prochlorococcus*, we first tracked the growth and fluorescence of the high-light-adapted *Prochlorococcus* strain MED4 under CO_2 and O_2 levels ranging from atmospheric to near-zero, with CO_2:O_2 ratios ranging from ~1:5250 to >4:1 (ambient: ~1:550). We then performed whole-genome transcriptional profiling of the MED4 response to acute CO_2 and O_2 limitation over a 24-h time course. Although *Prochlorococcus* would not encounter absolute CO_2 limitation at the severe levels examined in this study, midday light intensities in tropical surface waters should be well above saturating cellular CO_2 fixation capacity (Hopkinson *et al.*, 2014), making a steep imbalance between photon flux and CO_2 fixation a regular occurrence. Previous studies have exposed *Prochlorococcus* to high-light shock and to high-peak irradiances in a day:night cycle (Steglich *et al.*, 2006; Zinser *et al.*, 2009; Mella-Flores *et al.*, 2012). The experiments reported here provide an opportunity to examine how *Prochlorococcus* responds when low CO_2 rather than high light causes a photosynthetic impedance mismatch, and how O_2 availability conditions this response.

Materials and methods

Growth experiment

Axenic *Prochlorococcus* MED4 was grown in Sargasso seawater-based Pro99 medium (Moore *et al.*, 2007) amended with 20-mM Na-HEPES pH 8.1. The addition of HEPES at this concentration did not affect growth rate (data not shown). Standing (non-sparged) cultures were maintained in 25-mm glass tubes at 21 °C with continuous illumination at ~60 μmol photons $m^{-2} s^{-1}$ for >30 generations before inoculation of experimental cultures. After inoculation, tubes were capped loosely and allowed to grow without bubbling for ~24 h (Shi and Xu, 2009). Tubes were then sampled, fitted with sterile cap-and-frit assemblies, and connected to gas lines. Parallel standing controls were left with their original caps. Gas mixtures were prepared and certified by Airgas. Gases were bubbled through Milli-Q water, then passed through a needle valve, a 0.2-μm filter, and, in the culture tube, a fritted glass tube (porosity C, Ace Glass, Vineland, NJ, USA). Sparging rates were maintained as steadily as possible at ~2 ml min^{-1} given the limitations of standard gas-cylinder regulators.

Cultures were sampled daily by withdrawing 0.3 ml with a sterile transfer pipet. Experimental cultures were immediately resealed. Samples were protected from light and rapidly subsampled for bulk fluorescence measurements (data not shown) and flow cytometry. For flow cytometry, 10- or 100-μl samples were diluted to 1 ml with filtered seawater. Glutaraldehyde (5 μl, 25%) was added and samples were mixed, then incubated under foil for ~10 min. Fixed samples were flash-frozen in liquid nitrogen. Cells were counted using the blue (488 nm) laser on an Influx flow cytometer (Cytopeia, Seattle, WA, USA) with a 2-μm fluorescent bead standard. Quantification of flow cytometric results was performed in R (v. 2.12.0) (R Development Core Team, 2010) using the package curvHDR (v. 1.0-3) (Naumann *et al.*, 2010).

Transcriptional profiling

Axenic *Prochlorococcus* MED4 cultures were grown as described above at ~60 μmol photons $m^{-2} s^{-1}$ and 21 °C for >30 generations before the shock experiment. Three parallel 1.2-l cultures were then inoculated and grown to mid-log phase in clear 2-l bottles sparged with air at ~10 ml min^{-1}. At $t = -24$ h, 400-ml fresh medium was transferred to each of 12 clear 500-ml bottles for pre-equilibration with the experimental gases. Gases (Airgas, Cambridge, MA, USA) were 360 p.p.m. CO_2+<0.001% O_2 (balance N_2) for $-O_2$ shock; 40 p.p.m. CO_2+<0.001% O_2 (balance N_2) for $-CO_2/-O_2$ shock; and 40 p.p.m. CO_2+21% O_2 (balance N_2) for $-CO_2$ shock. Three bottles were sparged vigorously with each experimental gas, and three control bottles with air, for 24 h.

To start the experiment, each mid-log-phase 1.2-l culture was split into six and harvested by pelleting in sterile acid-washed 250-ml centrifuge bottles in a JA-14 rotor, spinning at 8000 r.p.m. for 10 min. Supernatants were immediately poured off and each pellet resuspended in 3-ml fresh medium. Cell suspensions were pooled and split evenly among four 500-ml bottles, each pre-equilibrated with a different gas. Bottles were swirled to mix, 21-ml

samples were immediately ($t = 0$) withdrawn into 30-ml centrifuge tubes, and sparging continued. A 1-ml subsample was immediately removed from each sample for bulk fluorescence and flow cytometry and protected from light; these subsamples were processed as described above. Meanwhile, the remaining 20 ml of each sample was pelleted by spinning at 12 500 r.p.m. and 4°C for 10 min in a JA-25.50 rotor. Supernatants were immediately discarded and each pellet resuspended in 500-µl resuspension buffer (200 mM sucrose, 10 mM NaOAc pH 5.2, 5 mM EDTA). Cell suspensions were transferred to 1.5-ml tubes and flash-frozen in liquid nitrogen. Sampling for each timepoint was completed within 20 min (from withdrawal of samples to flash freezing). Samples were taken at $t = 0$, 70 min, 3 h, 6 h, 9 h, 12 h, 18 h and 24 h.

For RNA extraction, cell suspensions were thawed in small batches and processed with the Mini RNA Isolation II kit (Zymo Research, Irvine, CA, USA). The manufacturer's protocol was followed except that cell lysis was allowed to continue for 20 min on ice. DNA was removed by treating 1 µg total nucleic acids with 2-µl Turbo DNAfree (Ambion, Foster City, CA, USA) in a 50-µl reaction for 1 h, followed by DNase inactivation according to the manufacturer's protocol. RNA was concentrated by ethanol precipitation and yields were quantified with Ribogreen (Molecular Probes, Carlsbad, CA, USA). RNA amplification (using the MessageAmp II—Bacteria kit (Ambion) with 200 ng input), labeling, and hybridization to the custom Affymetrix microarray MD4-9313 (Santa Clara, CA, USA) were performed by the MIT BioMicro Center (Cambridge, MA, USA). Amplification, labeling, and hybridization were performed for two biological replicates at timepoints $t = 0$, 70 min, 6 h, 12 h and 24 h.

Microarray data analysis was performed in R. Chip images were processed using the package Harshlight (Suárez-Fariñas *et al.*, 2005) before RMA normalization with the package affy (Bolstad *et al.*, 2003). Microarray data were deposited in the GEO database with accession number GSE65684. Genes showing significant differential expression were identified by Bayesian time-series analysis using the package betr (Aryee *et al.*, 2009), controlling for false positives at $\alpha = 0.02$ and setting the threshold for significance at $P(\text{differential expression}) > 0.98$ (that is, $P(\text{null}) < 0.02$). Clustering analysis of significantly differentially expressed genes was performed using the fuzzy c-means package Mfuzz (Futschik and Carlisle, 2005) with four cluster centers and 'fuzziness' parameter $m = 1.1$.

Gene functional categorizations are as annotated in CyanoBase (http://genome.kazusa.or.jp/cyanobase/) as of May 2009, except where updated gene annotation or comparison with KEGG pathways prompted manual reassignment. These exceptions are marked with asterisks in Supplementary Table S1, which gives both the original and reassigned gene categories.

Results and Discussion

Growth rates under different steady-state CO_2 and O_2 regimes

We first asked how growth is affected by CO_2 and O_2 availability by sparging cultures of *Prochlorococcus* MED4 with different gas mixtures. Three CO_2 concentrations (high, 0.036% (~atmospheric); moderate, 0.018%; and low, 0.004%) and three O_2 concentrations (high, 21% (atmospheric); moderate, 10%; and low, <0.001%) were examined in combination to provide nine growth conditions. We expected that, under moderate CO_2 limitation, competition between Rubisco's carbon fixation and oxygenation reactions would cause a greater reduction in growth rate at high O_2 than at moderate or low O_2, whereas severe CO_2 limitation would inhibit growth at any O_2 level. We used flow cytometry to measure cell counts and per-cell chlorophyll fluorescence, a proxy for cellular chlorophyll concentration (DuRand *et al.*, 2002).

Notably, growth and per-cell chlorophyll fluorescence of MED4 sparged with any combination of moderate and high CO_2 and O_2 was nearly indistinguishable from growth of parallel controls grown under lab air without sparging (Figure 1). By contrast, cultures grown at low O_2 and either moderate or high CO_2 began to show slower growth within 2 days, and approached stationary phase at lower cell densities. That is, far from releasing cells from photorespiratory pressure, suboxic conditions appeared to disrupt *Prochlorococcus* MED4 growth. With sufficient CO_2 for continuous carbon fixation, cells grown under constant light should produce a steady stream of O_2 at photosystem II (PSII). That exogenous O_2 limitation nonetheless limited growth suggests that the photosynthetic O_2 supply is insufficient to meet some cellular need, perhaps because of rapid diffusive loss to the suboxic medium.

Low CO_2 severely limited growth at all O_2 levels, with little or no cell division observed at $t > 1$ day (Figure 1a). Yet flow cytometry revealed O_2-dependent differences in chlorophyll fluorescence per cell among the low-CO_2 conditions, with a sharper drop under low O_2 than high or moderate O_2 (Figure 1b). Interestingly, in contrast to the pattern for growth, chlorophyll fluorescence per cell appeared to be more strongly influenced by low O_2 than low CO_2, as low O_2 caused a similarly steep decline at all CO_2 levels. These results suggest an indispensable role for O_2 under the growth conditions studied. We sought to investigate this role and its connection to CO_2 levels further by studying the response at the transcriptional level.

Global transcriptional responses to acute changes in CO_2 and O_2 supply

For the transcription experiment, we focused on the first 24 h after cultures were split four ways for transfer to air-sparged medium and the growth

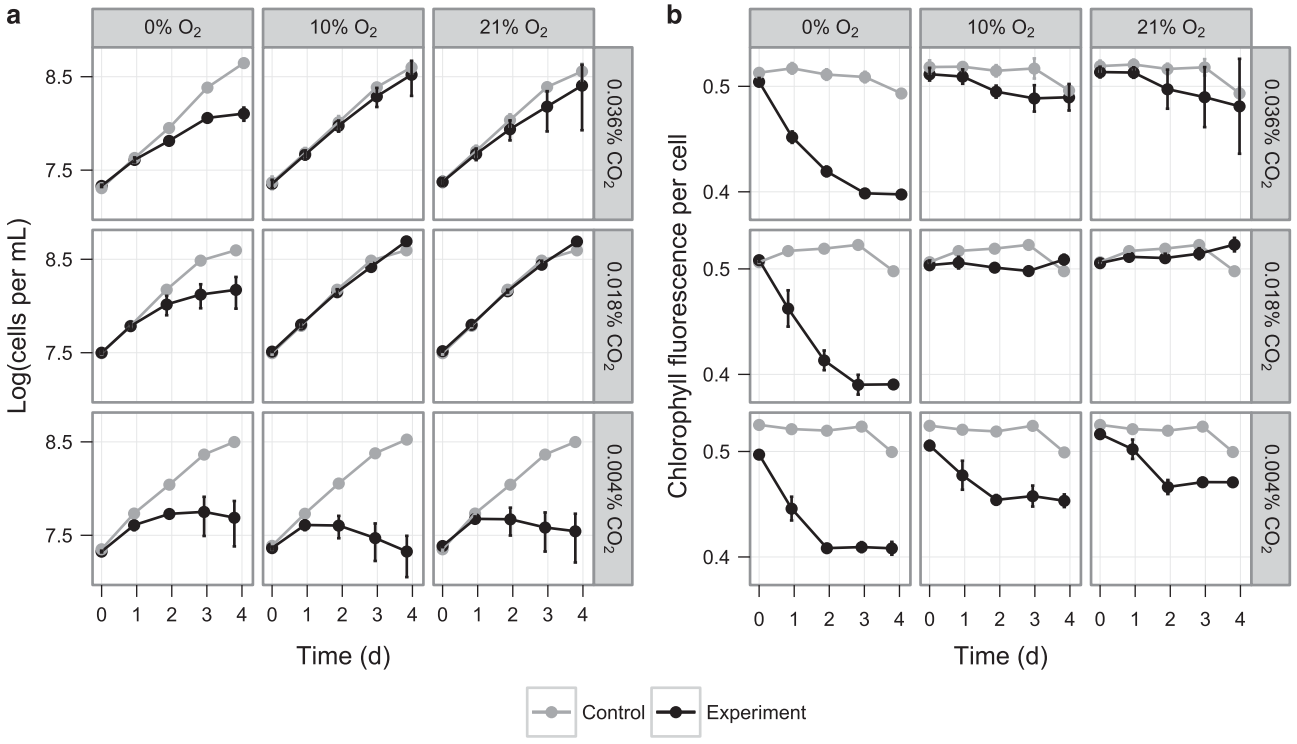

Figure 1 (**a**) *Prochlorococcus* MED4 cell concentration and (**b**) mean chlorophyll fluorescence per cell in cultures sparged with nine mixtures of O_2 and CO_2. All cultures were inoculated at $t=-1$ day. Experimental cultures (black) were sparged with the indicated gas mix (balanced with N_2), beginning at $t=0$ days; control cultures (gray) were inoculated and grown in parallel but not sparged. Data points are the mean of at least three cultures. Error bars indicate 1 s.d.; where error bars are not visible, they are smaller than the plotting symbol. The drop in chlorophyll fluorescence per cell in control cultures from $t=3$ days to $t=4$ days is expected for cultures entering stationary phase. In experimental cultures, the addition of CO_2 is expected to delay this transition (Moore *et al.*, 2007).

experiment's extremes: media sparged with 0.004% CO_2+21% O_2 (hereafter, $-CO_2$ shock), 0.004% CO_2+<0.001% O_2 ($-CO_2/-O_2$ shock) and 0.036% CO_2+<0.001% O_2 ($-O_2$ shock). We used an empirical Bayes approach to detect changes in expression (Aryee *et al.*, 2009), explicitly including the time dependence of samples within each condition to identify genes whose expression profiles over the time course differed significantly (*P*-value threshold, 0.98; false discovery rate, 0.02) from baseline. Within the air control, the baseline was expression at $t=0$; for the experimental conditions, the baseline was the full profile in air.

General patterns. Within the air-sparged control, we observed differential expression of 188 genes, including 67 hypothetical genes (Supplementary Table S1). These changes were typically small (Supplementary Table S1) and may reflect the stress of centrifugation immediately before the $t=0$ time-point. Differential expression with respect to air was minimal under $-O_2$ shock (4 of 2048 genes probed) but widespread under $-CO_2$ shock (296 genes) and $-CO_2/-O_2$ shock (261 genes) (Figure 2a, Supplementary Table S1). Although the latter two sets of genes overlap substantially, sharing 184 genes, clustering analysis of relative expression patterns in shock conditions with respect to air revealed an oxygen dependence to the expression profiles of many genes under carbon limitation (Figure 2b). Under $-CO_2/-O_2$ shock, sustained suppression of expression (cluster 4, 112 genes) was far more common than suppression with early recovery (cluster 3, 32 genes) among the differentially expressed genes; under $-CO_2$ shock, sustained suppression was slightly less common than early recovery (52 vs 66 genes), raising the possibility that oxygen availability partially mitigated the effects of carbon limitation on the timescale studied here.

As a first look at the relationship between the responses to upstream and downstream triggers of a photosynthetic imbalance, we examined the genes showing differential expression under acute high-light stress in earlier work (Steglich *et al.*, 2006, 2008) compared with those reported here for CO_2 limitation. There was very strong concordance in the direction of change: high light and $-CO_2/-O_2$ shock evoked congruent initial responses (increased high-light expression at $t=45$ min and gas shock cluster 1, or decreased high-light expression and gas shock cluster 2, 3 or 4; Figure 2b) in 35 of 41 shared genes, and high light and $-CO_2$ shock did so in 44 of 46 (Supplementary Table S2). Among genes that responded to $-CO_2/-O_2$ shock, the distribution of genes across clusters 2–4 was no different for genes that did respond to light stress than for those that did not ($P=0.387$, Fisher's exact test). By contrast, among all genes whose expression initially dropped

under $-CO_2$ shock, those that also responded to light stress were significantly more likely to recover rapidly even as CO_2 limitation continued (cluster 3; $P = 0.012$, Fisher's exact test). That is, access to oxygen appears to reduce the extent to which CO_2 limitation mimics high-light stress. As described below, this conclusion is borne out by closer examination of the groups of genes affected by $-CO_2$ and $-CO_2/-O_2$ shock.

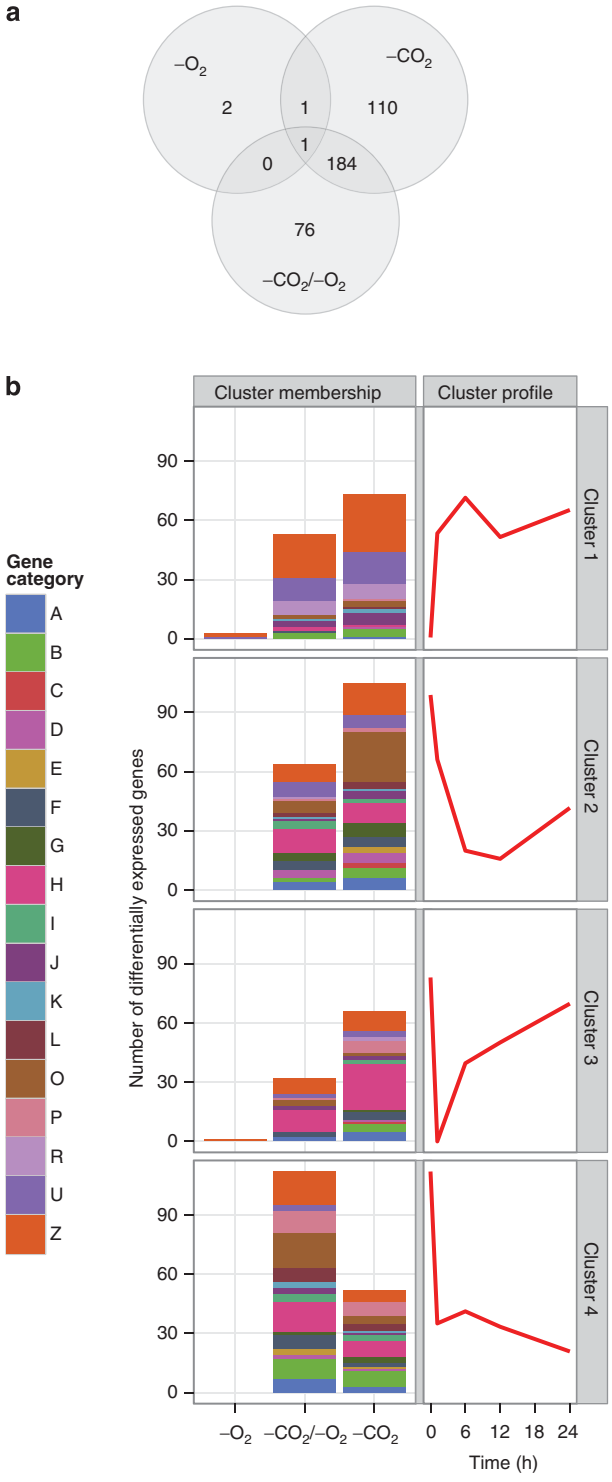

Figure 2 (**a**) Results of whole-genome transcriptional profiling in the gas shock experiment. Venn diagram of genes showing significant differential expression with respect to air in the three experimental conditions: $-O_2$ shock (0.036% CO_2, 0% O_2), $-CO_2$ shock (0.004% CO_2, 21% O_2) and $-CO_2/-O_2$ shock (0.004% CO_2, 0% O_2). (**b**) Clustering analysis of changes in gene expression under gas shocks relative to changes in air. Autoscaled cluster centroids are shown at right, in red. Clusters 2 and 3 are categorized as showing suppression and subsequent recovery. At left, bar plots give the number of genes in each cluster under each gas shock condition. Gene categories: A, amino-acid biosynthesis; B, biosynthesis of cofactors, prosthetic groups, and carriers; C, cell envelope; D, cellular processes; E, central intermediary metabolism; F, energy metabolism; G, fatty acid, phospholipid, and sterol metabolism; H, photosynthesis and respiration; I, purines, pyrimidines, nucleosides, and nucleotides; J, regulatory functions; K, DNA replication, recombination, and repair; L, transcription; O, translation; P, transport and binding proteins; R, RNA; U, other categories; Z, hypothetical proteins.

Hli gene family. The *hli* gene family encodes small proteins thought to interact with the photosystems under oxidative stress conditions (He *et al.*, 2001; Yao *et al.*, 2007). Named for their high-light inducibility, *hli* genes in MED4 have been induced by phage infection, N starvation, Fe starvation and a range of light shocks (Steglich *et al.*, 2006; Tolonen *et al.*, 2006; Lindell *et al.*, 2007; Thompson *et al.*, 2011), suggesting a more general role in stress response. We found that expression of six *hli* genes decreased after $t = 0$ in the air control (Figure 3), and interpret this drop as a recovery to baseline after upregulation due to sample-handling stresses (centrifugation and resuspension) just before the $t = 0$ timepoint. By contrast, expression of these *hli* genes remained at or above $t = 0$ levels throughout the $-CO_2/-O_2$ time course, suggesting that this condition did not permit recovery to a non-stressed state.

Under carbon limitation, the large majority of differentially expressed *hli* genes showed a persistent and strong increase in expression with respect to air ($-CO_2$, 11 of 12 differentially expressed *hli* genes in cluster 1; $-CO_2/-O_2$, 10 of 11), but *hli3* (PMM1482) was an intriguing exception. MED4's *hli* suite is dominated by multi-copy genes (Bhaya *et al.*, 2002; Lindell *et al.*, 2004), which respond to a variety of stimuli (Tolonen *et al.*, 2006; Steglich *et al.*, 2006; Lindell *et al.*, 2007; Thompson *et al.*, 2011). Their presence in genomic islands (Coleman *et al.*, 2006) and phage suggests that they are exchanged through horizontal gene transfer, forming part of the highly niche-dependent 'flexible' genome (Kettler *et al.*, 2007). By contrast, the five single-copy *hli* genes, including *hli3*, belong to the core genome and have never previously been seen to respond to a stress condition in MED4 (Steglich *et al.*, 2006; Tolonen *et al.*, 2006; Lindell *et al.*, 2007; Thompson *et al.*, 2011; Kettler, personal communication). Here, both carbon limitation conditions strongly

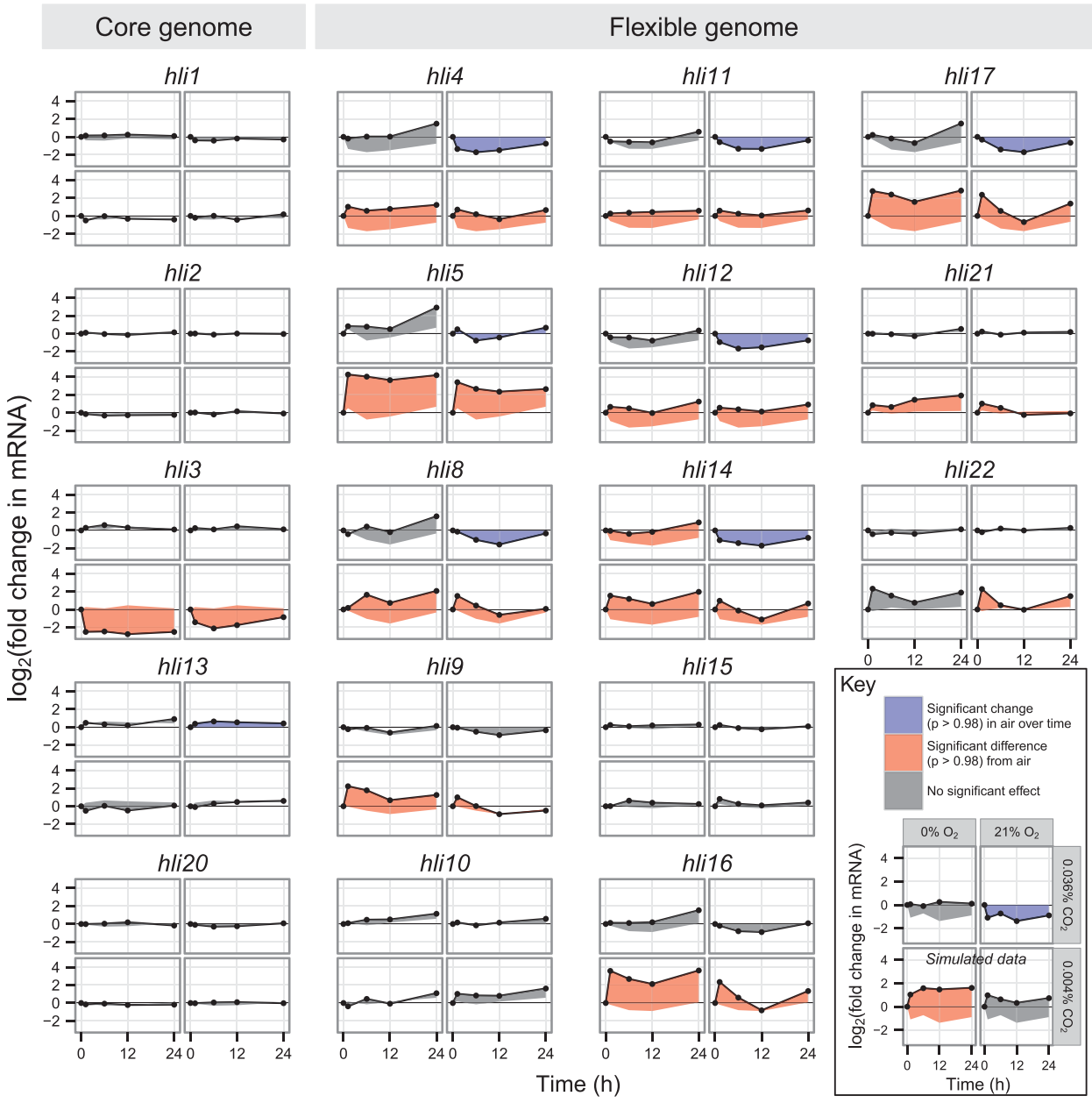

Figure 3 Expression of high-light-inducible (*hli*) genes of the core (left) and flexible (right) MED4 genome in the gas shock experiment. Data points are the geometric means of two biological replicates. Fold change is relative to $t = 0$ for each condition. All thumbnail plots share the same layout; for details, see sample plot at lower right. Data in sample plot (see Key) is simulated for illustration. The region between each time course and the baseline used for significance testing (for the air control, expression at $t = 0$; for experimental conditions, the parallel time course in air) is shaded according to the outcome of that test: a significant change in expression in air with respect to $t = 0$, blue; a significantly different time course in an experimental condition than in air, red; no significant effect, gray.

suppressed *hli3* expression (Figure 3). The direction of this change substantiates the idea that *hli* genes in the core and flexible genomes have fundamentally different roles (Zinser *et al.*, 2009).

Regulatory genes. Under both $-CO_2$ and $-CO_2/-O_2$ shocks, RNA polymerase genes (*rpoABC1C2*) were significantly downregulated, likely contributing to a broad suppression of transcription (Figure 1, Supplementary Figure S1). The translational machinery was likewise substantially suppressed under both conditions, with roughly half the 30S and a third of 50S ribosome genes showing lowered expression (Supplementary Figure S2, Supplementary Table S1). Consistent with these changes, *groEL* and *groEL2*, components of the GroEL chaperone for newly synthesized proteins, were also expressed at lower levels (Supplementary Table S1). Despite these broad similarities, we observed some distinct regulatory responses to the stresses. The type II alternative sigma factor encoded by PMM1289 has previously been found to show differential responses

to nutrient stresses, with transcript abundance increasing in the early hours of N starvation but not in response to P starvation (Martiny *et al.*, 2006; Tolonen *et al.*, 2006). Here, PMM1289 was significantly upregulated in $-CO_2$ stress only (Supplementary Figure S1), suggesting that CO_2 limitation in the presence of oxygen triggers invocation of a different transcriptional program than does co-limitation of CO_2 and O_2.

Mean expression of the Crp-family gene at locus PMM0806 showed a >4-fold increase in expression under $-CO_2$ shock, while dropping by nearly 4-fold under both $-O_2$ shock and $-CO_2/-O_2$ shock (Figure 4a). Although these decreases failed to meet our conservative differential expression threshold with respect to air, the difference between expression under $-CO_2$ shock and under the oxygen limitation conditions was highly significant ($P=1$, false discovery rate controlled at 0.001). Crp-family proteins are transcriptional regulators, typically activators, controlled by a range of signals including cAMP, redox potential, and O_2 (Körner *et al.*, 2003). The highly oxygen-dependent transcriptional response we observe, together with previous experiments demonstrating PMM0806 induction under iron limitation (Thompson *et al.*, 2011), strongly

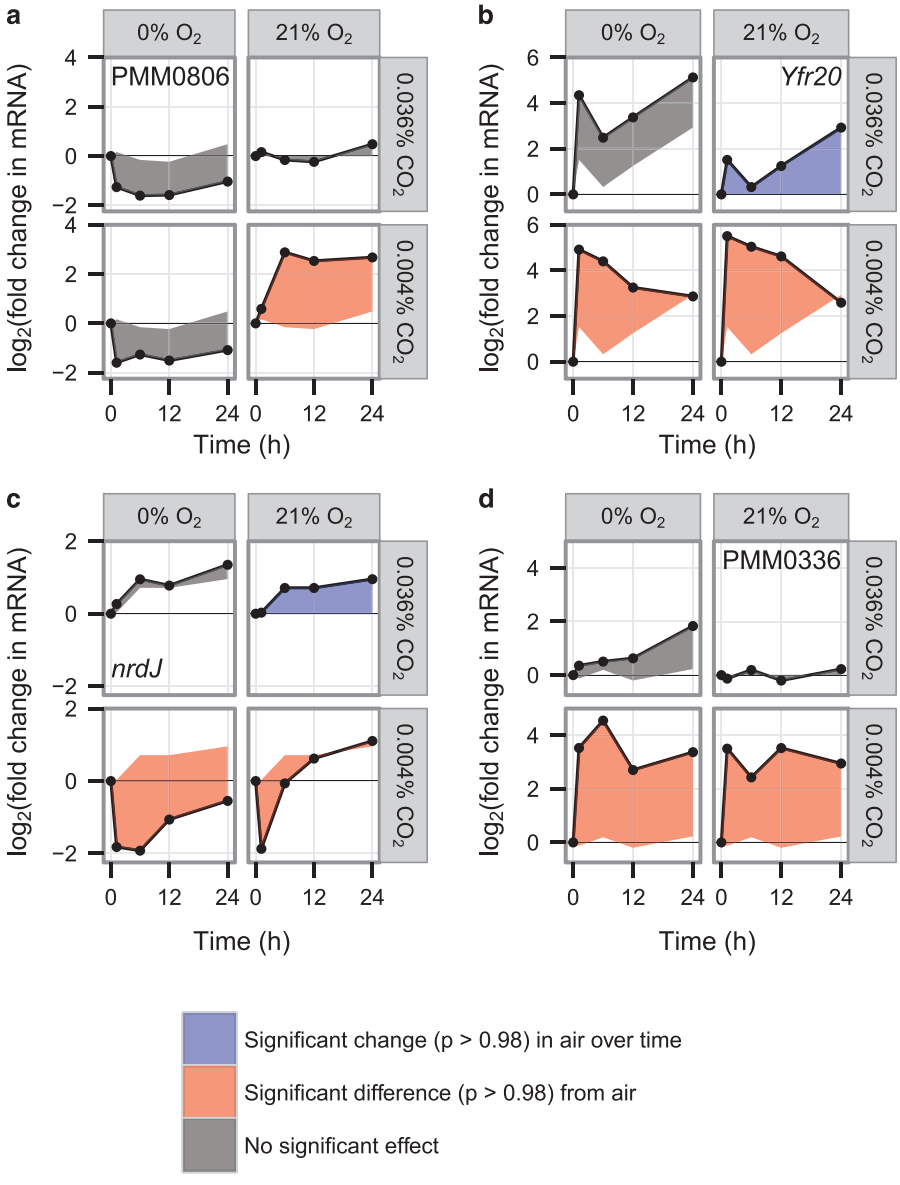

Figure 4 Expression of (**a**) the putative CRP-family regulatory gene encoded by MED4 locus PMM0806, (**b**) Yfr20, (**c**) *nrdJ*, and (**d**) the PTOX locus PMM0336 in the gas shock experiment. Data points are the geometric means of two biological replicates. Fold change is relative to $t=0$ for each condition. The region between each time course and the baseline used for significance testing (for the air control, expression at $t=0$; for experimental conditions, the parallel time course in air) is shaded according to the outcome of that test: a significant change in expression in air with respect to $t=0$, blue; a significantly different time course in an experimental condition than in air, red; no significant effect, gray.

suggests that PMM0806 expression responds to a redox-related signal.

Although genome streamlining has left *Prochlorococcus* with a minimal complement of regulatory proteins, its complement of non-coding and antisense RNA genes is comparable to those of well-studied enterobacteria (Steglich *et al.*, 2008) and is expected to have a major role in regulation. Here, a few antisense RNA genes were differentially expressed, but these patterns showed little clear relation to the expression of their target genes (Supplementary Figure S3). Strikingly, however, the strongest response observed for any gene was the ~30-fold upregulation of the non-coding RNA Yfr20 under both $-CO_2$ and $-CO_2/-O_2$ shock (Figure 4b). Yfr20 is common to high-light *Prochlorococcus* strains and has previously been observed to be very strongly induced by high-light stress (Steglich *et al.*, 2008).

Carbon transport and fixation. *Prochlorococcus* has few known transporters for inorganic carbon (Ci) (Scanlan *et al.*, 2009). Like most open-ocean cyanobacteria, MED4 lacks inducible high-affinity Ci uptake systems, likely reflecting the relative homogeneity of Ci levels in pelagic waters as compared with coastal and lacustrine systems (Badger *et al.*, 2006; Scanlan *et al.*, 2009). Instead, MED4's Ci acquisition is expected to depend primarily on the Na^+/HCO_3^- symporter BicA (PMM0214) (Badger *et al.*, 2002, 2006; Price *et al.*, 2004; Hopkinson *et al.*, 2014). Although minimal, this Ci uptake machinery contributes to a highly efficient carbon-concentrating mechanism; its half-saturation point for uptake has recently been measured at 80–130 μM HCO_3^- (Hopkinson *et al.*, 2014). With pH controlled at 8.1, both CO_2 limitation conditions should give $[HCO_3^-] \sim 213$ μM, theoretically within the saturation regime. That we observe marked growth limitation and a substantial transcriptional stress response at this CO_2 level suggests either that the half-saturation point is higher under our culture conditions or that, even at moderate cell densities ($\sim 5 \times 10^7$ cells ml^{-1}), photosynthesis renders bubbling insufficient to maintain pCO_2 at 40 μatm (Shi and Xu, 2009) and HCO_3^- at saturating concentrations. Whatever the mechanism of the limitation, its effect on MED4's carbon-concentrating mechanism is remarkable: expression of *bicA* dropped rapidly and remained suppressed under both $-CO_2$ and $-CO_2/-O_2$ shock (Supplementary Table S1). No other candidate cryptic Ci transporters (Harano *et al.*, 1997; Palinska *et al.*, 2002; Shibata *et al.*, 2002; Scanlan *et al.*, 2009) were induced by CO_2 limitation. It is difficult to rationalize this strategy if we assume that MED4 is directly sensing Ci availability, but given MED4's extensive genome streamlining that assumption is likely false: the environmental Ci concentrations *Prochlorococcus* encounters should consistently be well above saturating for Ci uptake, such that direct Ci sensing is unlikely to offer a selective advantage.

Both carbon limitation conditions also caused broad transcriptional suppression of the machinery necessary for carbon fixation. Fixation places a heavy demand for ATP production on the cell, high enough that ATP synthase expression during daytime carbon fixation exceeds expression during nighttime aerobic respiration (Zinser *et al.*, 2009). Under both carbon limitation conditions, expression of 9 of the 10 ATP synthase complex genes (at loci PMM1438–1439 and PMM1450–1457) was significantly decreased (Supplementary Table S1), with similar time course profiles across the two conditions. We further observed decreased expression of genes encoding Calvin cycle enzymes (Figure 5), several carboxysome components (*csoS1* (PMM0549), *csoS2* (PMM0552), *csoSCA* (PMM0553), and *csoS4B* (PMM0553); Supplementary Table S1), and glycogen synthesis enzymes (*glgABC*, encoded by PMM0609, PMM0584 and PMM0769, respectively; Supplementary Table S1) under CO_2 limitation. However, the availability of oxygen appears to modulate this response: while Calvin cycle gene expression was reduced under both carbon limitation conditions, the effect was generally stronger and more sustained under $-CO_2/-O_2$ shock than under $-CO_2$ shock (Figure 5). This disparity is most striking in the Rubisco genes *rbcLS*. As discussed below, the minimal disruption of Rubisco expression under $-CO_2$ shock suggests that, with carbon fixation throttled, the availability of molecular oxygen allows *Prochlorococcus* to buffer light stress via Rubisco's oxygenation reaction.

Photosystems. Halting carbon fixation slows NADPH consumption, threatening the photosynthetic electron transport chain with over-reduction. Two-thirds of the genes encoding proteins in the light-harvesting complex and PSI and PSII were differentially expressed under carbon limitation ($-CO_2/-O_2$ shock, 15 of 34 genes; $-CO_2$ shock, 19; total, 22). Of these, all were initially suppressed relative to air (Supplementary Table S1); but whereas just over half the photosystem genes suppressed under $-CO_2/-O_2$ shock remained suppressed (cluster 4; Figures 2b and 6), nearly all photosystem genes affected by $-CO_2$ shock recovered sharply and substantially (cluster 3; Figures 2b and 6). The same pattern marked cytochrome b_6f and nicotinamide nucleotide transhydrogenase (*pntA1*, *pntA2* and *pntB*), which couples the proton gradient to the transfer of reducing equivalents between the NAD(H) and NADP(H) pools (Figure 6b). The expression profiles seen throughout the photosynthetic electron transport chain—sustained suppression under $-CO_2/-O_2$, suppression with recovery under $-CO_2$—strongly suggest that, in carbon-limited cells, access to oxygen affects the health of this system.

Cyanobacterial iron physiology is closely tied to photosystem status (Thompson *et al.*, 2011), and

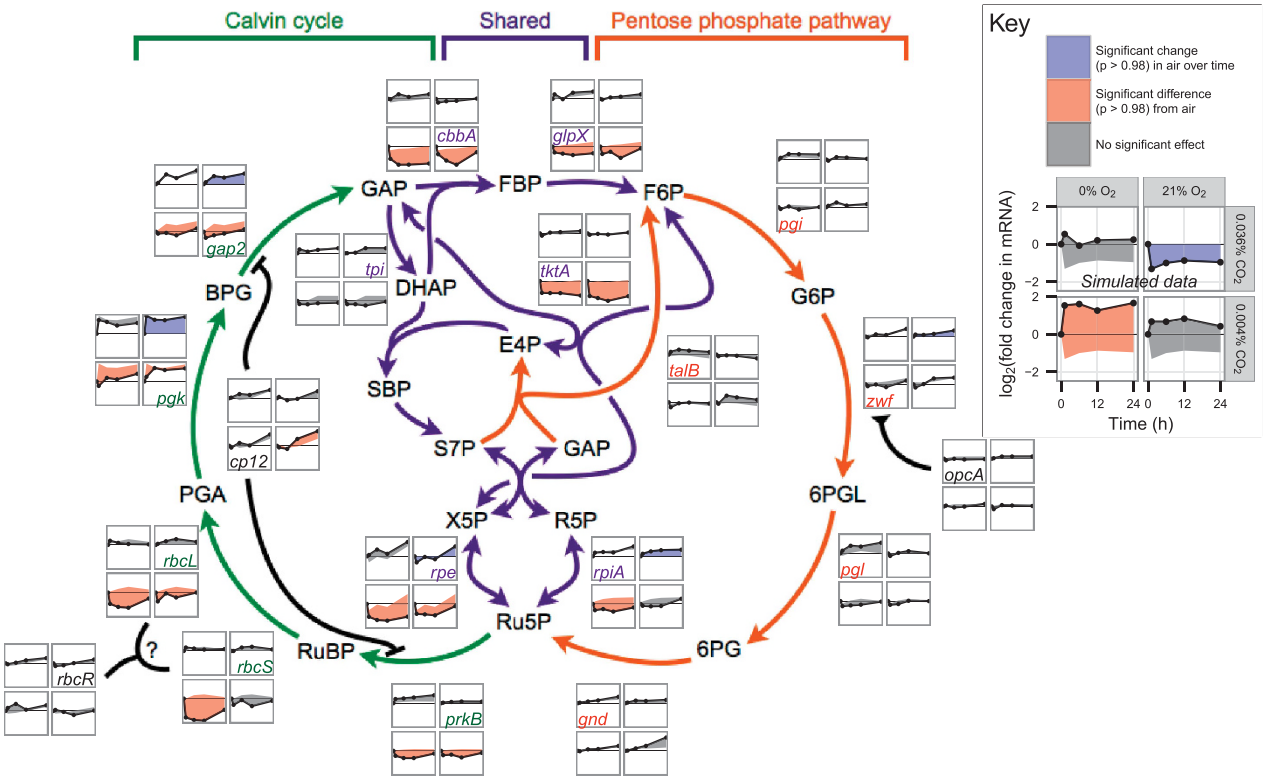

Figure 5 Expression of Calvin cycle and pentose phosphate pathway genes. The Calvin cycle (green) and pentose phosphate pathway (orange) interlock; because their shared reactions (purple) run in opposite directions, the cycles cannot productively run at the same time. Gas shock expression data (thumbnail plots) for each gene in these pathways suggests that, as expected, CO_2 starvation broadly downregulates the carbon-fixing Calvin cycle, while leaving the reductive pentose phosphate pathway largely intact. Data points are the geometric means of two biological replicates. Fold change is relative to $t=0$ for each condition. All thumbnail plots share the same layout and axis scales; for details, see sample plot at lower right. Data in sample plot (see Key) is simulated for illustration. The region between each time course and the baseline used for significance testing (for the air control, expression at $t=0$; for experimental conditions, the parallel time course in air) is shaded according to the outcome of that test: a significant change in expression in air with respect to $t=0$, blue; a significantly different time course in an experimental condition than in air, red; no significant effect, gray. Genes: *cbbA*, fructose-1,6-bis-P/sedoheptulose-1,7-bis-P aldolase; *cp12*, regulator CP12; *gap2*, glyceraldehyde-3-phosphate dehydrogenase; *glpX*, fructose-1,6/sedoheptulose-1,7-bisphosphatase; *gnd*, 6-phosphogluconate dehydrogenase; *opcA*, putative glucose-6-phosphate dehydrogenase effector; *pgi*, P-glucose isomerase; *pgk*, P-glycerate kinase; *pgl*, 6-P-gluconolactonase; *prkB*, P-ribulokinase; *rbcLS*, ribulose-1,5-bis-P carboxylase/oxygenase large and small subunits; *rbcR*, putative Rubisco transcriptional regulator; *rpe*, ribulose-5-P epimerase; *rpiA*, ribulose-5-P isomerase; *talB*, transaldolase; *tktA*, transketolase; *tpi*, triose-P isomerase; *zwf*, glucose-6-phosphate dehydrogenase. Substrates: BPG, 2,3-bis-P-glycerate; DHAP, dihydroxyacetone P; E4P, erythrose-4-P; FBP, fructose-1,6-bis-P; F6P, fructose-6-P; GAP, glyceraldehyde-3-P; G6P, glucose-6-P; PGA, 3-P-glyceric acid; R5P, ribose-5-P; RuBP, ribulose-1,5-bis-P; Ru5P, ribulose-5-P; SBP, sedoheptulose-1,7-bis-P; 6PG, 6-P-gluconate; 6PGL, 6-P-gluconolactone; S7P, sedoheptulose-7-P; and X5P, xylulose-5-P.

again, we observe divergent responses under $-CO_2$ and $-CO_2/-O_2$ shocks. Expression of the gene encoding the iron sequestration protein ferritin (PMM0804) and of one ferric uptake regulator (Fur) gene (PMM1030) increased significantly only under $-CO_2$, whereas the other Fur gene (PMM0637) was upregulated more strongly under $-CO_2$ shock than $-CO_2/-O_2$ shock (Supplementary Table S1). Unusually for regulatory proteins, Fur is typically expressed at high copy number; each subunit binds one Fe(III) ion, so upregulation contributes directly to iron sequestration (Andrews *et al.*, 2003). Oxygen-dependent differences in the expression of these genes under carbon limitation are consistent with distinct patterns of photosystem remodeling under $-CO_2$ and $-CO_2/-O_2$ shocks.

DNA synthesis. One gene related to DNA synthesis also showed oxygen-dependent recovery under carbon limitation: the class II ribonucleotide reductase *nrdJ* (PMM0661) (Figure 4c). Ribonucleotide reductases convert ribonucleotides to deoxyribonucleotides, preparing the cell for DNA replication and cell division. The strong initial decrease in *nrdJ* transcript abundance under both $-CO_2$ and $-CO_2/-O_2$ shocks was consistent with the expectation that severe carbon limitation would halt cell division. But why should *nrdJ* expression then fully recover under $-CO_2$ shock? Intriguingly, in the obligate aerobe *Streptomyces coelicolor*, *nrdJ* activity is essential for rapid recovery after oxygen starvation (Borovok *et al.*, 2004); ongoing deoxyribonucleotide synthesis may prepare the cell to begin DNA

synthesis as soon as environmental conditions permit. If the same is true in *Prochlorococcus*, cultures released from $-CO_2$ shock would be primed for recovery, whereas those released from $-CO_2$/$-O_2$ shock would not.

Opening safety valves under carbon limitation: PTOX and 2-phosphoglycolate

When energy cannot flow from photons to reducing equivalents to carbon–carbon bonds, the cell risks widespread damage. Of the protective strategies thought to be available to *Prochlorococcus* (Scanlan *et al.*, 2009), no mechanism is yet known for non-photochemical quenching, and we find no indication at the transcriptional level that carbon limitation modulates cyclic electron flow or the Mehler reaction, with 13 of 14 *ndh* genes, *flv1* (PMM0042), and *flv3* (PMM0043) unchanged under $-CO_2$ and $-CO_2/-O_2$ shocks. Instead, carbon-limited MED4 appears to use two photoprotective strategies, both relying on oxygen (Figure 7). These strategies may explain why light-stress-related genes whose expression initially responded to CO_2 limitation were disproportionately likely to recover in cultures with access to exogenous O_2.

First, PSII can pass electrons to oxygen instead of cytochrome b_6f via the plastoquinol terminal oxidase (PTOX, PMM0336), a strategy demonstrated in *Synechococcus* WH8102 cultures and observed in an iron-limited open-ocean cyanobacterial population dominated by *Prochlorococcus* (Bailey *et al.*, 2008; Mackey *et al.*, 2008). Previous *Prochlorococcus* transcriptome experiments have shown PTOX upregulation when iron limitation induced PSI downregulation (Thompson *et al.*, 2011) and when light shocks threatened PSII and the plastoquinone (PQ) pool with over-reduction (Steglich *et al.*, 2006). By contrast, when the PSII inhibitor DCMU was used to trap PQ in an oxidized state, PTOX transcription was significantly repressed (Steglich *et al.*, 2006). Here, PTOX expression increased >11-fold under both $-CO_2$ and $-CO_2/-O_2$ shocks (Figure 4d). Interpretation of this result is difficult for $-CO_2/-O_2$ shock: PSII may have continued to generate molecular oxygen, but given the steep gradient for diffusive loss to the 0% O_2 medium, little of that oxygen may have been

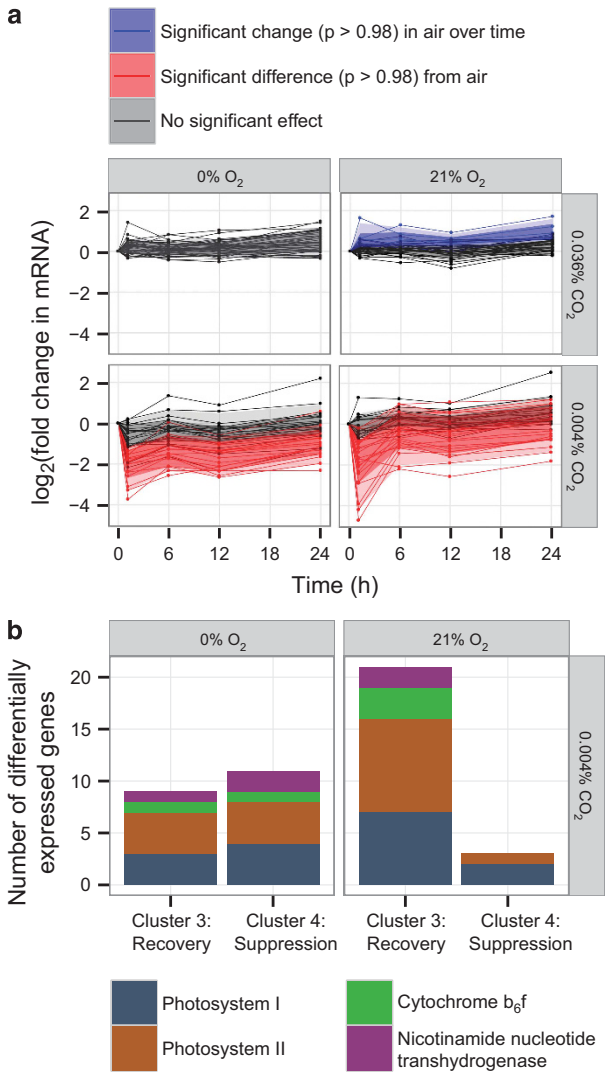

Figure 6 (**a**) Expression of MED4 genes encoding PSI and PSII, cytochrome b_6f and *pnt* genes in the gas shock experiment. Data points are the geometric means of two biological replicates. Fold change is relative to $t=0$ for each condition. Shock conditions causing significant differential expression in the air control with respect to time 0 are highlighted in blue; shock conditions causing significant differential expression over the time course with respect to air are highlighted in red. Ninety percent of data points lie within the outer (light) shaded bands; 70% lie within the inner (dark) bands. (**b**) Oxygen dependence of the cluster affiliations of the genes in panel **a** under carbon limitation. No differentially expressed genes in this set fell into cluster 1 (increased expression) or 2 (delayed recovery). See Figure 2b for cluster profiles.

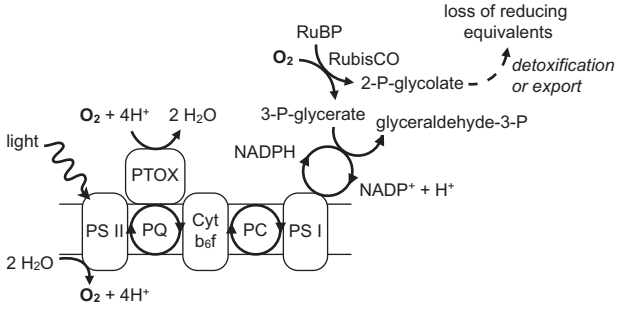

Figure 7 Proposed 'safety valves' for carbon-limited *Prochlorococcus*. Light energy drives the photosynthetic electron transport chain, transferring reducing power from PSII via PQ to cytochrome b_6f (Cyt b_6f); from there via plastocyanin (PC) to PSI; and from there to the NADP(H) pool. In the presence of CO_2, carbon fixation provides a final sink for these reducing equivalents; in its absence, the electron transport chain becomes over-reduced. However, so long as oxygen is still present, the electron transport chain can divert excess electrons from PQ to O_2 via the PQ terminal oxidase (PTOX), or ribulose-1,5-bisphosphate (RuBP) can be oxygenated, forcing the consumption of reducing equivalents to process the products.

available to PTOX. With transcriptional data alone, we cannot say whether continued PTOX expression under $-CO_2/-O_2$ shock represented maintenance of a successful strategy or the futile result of signaling from an electron transport chain under continuing stress. But the broader patterns of O_2-dependent transcriptional recovery suggest that any PTOX activity achieved under $-CO_2/-O_2$ shock was insufficient. If, like *Synechococcus elongatus*, *Prochlorococcus* uses PQ redox poise as a signaling input (Kim *et al.*, 2012), different rates of PTOX activity in the presence and absence of exogenous O_2 may have had a major role in triggering the different transcriptional programs observed under $-CO_2$ and $-CO_2/-O_2$ shocks.

Second, *Prochlorococcus* can exploit Rubisco's oxygenation reaction to produce one 3-phosphoglycerate and one 2-phosphoglycolate. The former can be recycled through the Calvin cycle, consuming NADPH directly; the latter must be either detoxified via photorespiration or expelled. Although photorespiration appears to be essential in some cyanobacteria (Eisenhut *et al.*, 2008), MED4, like most sequenced *Prochlorococcus* strains, lacks some components of the known detoxification pathways (Scanlan *et al.*, 2009); moreover, it is known to secrete substantial quantities of glycolate even under exponential growth (Bertilsson *et al.*, 2005). Whereas PTOX may retain some activity under $-CO_2/-O_2$ shock through use of the photosynthetic O_2 generated nearby, the sequestration of Rubisco within carboxysomes makes any use of this safety valve highly unlikely when cellular O_2 is low. Consistent with this, *rbcLS* were strongly (~4-fold) downregulated under $-CO_2/-O_2$ shock; but under $-CO_2$ shock, *rbcS* was not significantly affected, and *rbcL* expression dropped but rapidly recovered (cluster 3, Figures 2b and 5).

We find no evidence for increased expression of reactive oxygen species scavengers under carbon limitation (data not shown), leaving cells without photoprotection vulnerable to mounting oxidative damage. In some heterotrophs, reactive oxygen species defense during starvation is necessary for recovery thereafter (McDougald *et al.*, 2002). The effect may be the same in *Prochlorococcus*: the expression pattern of *nrdJ*, discussed above, suggests that only MED4 cells with O_2 access could prepare for recovery from CO_2 starvation. But why should MED4 need the ability to recover from CO_2 starvation at all, when it is unlikely ever to encounter such low CO_2 levels in the oligotrophic ocean? The concordance between the CO_2 limitation and light stress responses, including their shared extremely strong expression of the non-coding RNA Yfr20, points to the conclusion that, on short (<1 day) timescales, the relevant factor is not the absolute CO_2 concentration but the balance between CO_2 and irradiance.

Threats to this central balance would be part of daily life for *Prochlorococcus* populations in tropical surface waters, so the ability to recover after a few hours' exposure to a photosynthetic impedance mismatch may be highly adaptive. If this is true, access to oxygen and the safety valve machinery should be most important in surface waters; conversely, exogenous oxygen may be dispensable at the very low irradiances that penetrate to oxygen minimum zones. Notably, although PTOX homologs are widespread in high-light-adapted *Prochlorococcus*, the only low-light-adapted *Prochlorococcus* ecotype known to carry a PTOX homolog is the LLI clade, whose members are abundant in some deeply mixed surface waters (Zinser *et al.*, 2007; Scanlan *et al.*, 2009).

Though a photosynthetic impedance mismatch may be a regular environmental stress for *Prochlorococcus*, a mismatch lasting continuously for >1 day is an impossibility, and the ability to withstand such a stress will not have been under selection. Here, the timing of the shifts observed in the growth experiment (Figure 1) bears further examination. From $t=0$ to 1 day, MED4 cells at 0.004% CO_2 and 21% O_2 tracked closely with the controls in both growth and per-cell chlorophyll fluorescence. Beyond 1 day in constant light, cell density increased no further, and per-cell chlorophyll fluorescence fell off. Although constant, the irradiance used in this study was too low (~60 µmol photons $m^{-2} s^{-1}$) to provoke photoinhibition in carbon-replete and oxygen-replete MED4 (Moore and Chisholm, 1999); but lack of exogenous O_2 provokes a substantial drop in per-cell chlorophyll fluorescence at all CO_2 levels within 1 day of constant light, and a slowing of growth within 2 days. Oxygen-dependent safety valves may thus be essential not only for recovery from severe but transient imbalances between irradiance and carbon fixation capacity but also for the maintenance of normal photosynthetic electron transport in carbon-replete cells in moderate light.

Although the carbon limitation used in these experiments is far more extreme than *Prochlorococcus* would ever experience in nature, our results suggest that the resulting general stress is a familiar one, perhaps even daily for marine populations in low latitudes. Whether the photosynthetic electron transfer chain is threatened by increasing irradiance or throttling carbon fixation, the cell must dissipate an excess of reducing equivalents to survive. For *Prochlorococcus* MED4, the cell's capacity to respond and recover appears to hinge on oxygen. To explore this response, future work should directly measure NAD(P)/NAD(P)H balance, pigment content, and the maximum photosynthetic quantum yield (Fv/Fm) in the presence and absence of exogenous O_2 across a range of CO_2 levels and irradiances. The recovery phase will be of particular interest: for severely CO_2-limited cells, could recovery be initiated simply by turning out the lights?

Conflict of Interest

The authors declare no conflict of interest.

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