Published in final edited form as:

Metab Eng. 2019 July ; 54: 222–231. doi:10.1016/j.ymben.2019.04.010.

Cyanobacterial carboxysome mutant analysis reveals the influence of enzyme compartmentalization on cellular metabolism and metabolic network rigidity

Mary H. Abernathy^a, Jeffrey J. Czajka^a, Douglas K. Allen^{b,c}, Nicholas C. Hill^d, Jeffrey C. Cameron^{d,e,f,*}, Yinjie J. Tang^{a,*}

^aDepartment of Energy, Environmental and Chemical Engineering, Washington University in St. Louis, MO 63130, USA

^bDonald Danforth Plant Science Center, St. Louis, MO 63132, USA

^cUnited States Department of Agriculture, Agricultural Research Service, St. Louis, MO 63132, USA

^dDepartment of Biochemistry, University of Colorado Boulder, Boulder, CO 80309, USA

^eRenewable and Sustainable Energy Institute, University of Colorado, Boulder, CO 80309, USA

^fNational Bioenergy Center, National Renewable Energy Laboratory, Golden, CO 80401, USA

Abstract

Cyanobacterial carboxysomes encapsulate carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Genetic deletion of the major structural proteins encoded within the *ccm* operon in *Synechococcus* sp. PCC 7002 (*ccmKLMN*) disrupts carboxysome formation and significantly affects cellular physiology. Here we employed both metabolite pool size analysis and isotopically nonstationary metabolic flux analysis (INST-MFA) to examine metabolic regulation in cells lacking carboxysomes. Under high CO₂ environments (1%), the *ccmKLMN* mutant could recover growth and had a similar central flux distribution as the control strain, with the exceptions of moderately decreased photosynthesis and elevated biomass protein content and photorespiration activity. Metabolite analyses indicated that the *ccmKLMN* strain had significantly larger pool sizes of pyruvate (> 18 folds), UDPG (uridine diphosphate glucose), and aspartate as well as higher levels of secreted organic acids (e.g., malate and succinate). These results suggest that the *ccmKLMN* mutant is able to largely maintain a fluxome similar to the control strain by changing in intracellular metabolite concentrations and metabolite overflows under optimal growth conditions. When CO₂ was insufficient (0.2%), provision of acetate moderately promoted mutant growth. Interestingly, the removal of microcompartments may loosen the flux network and promote RuBisCO side-reactions, facilitating redirection of central metabolites to competing pathways (i.e., pyruvate to heterologous lactate production). This study

*Corresponding author. yinjie.tang@wustl.edu (Y.J. Tang). **Corresponding author. Department of Biochemistry, University of Colorado Boulder, Boulder, CO 80309, USA. jeffrey.c.cameron@colorado.edu (J.C. Cameron).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymben.2019.04.010>.

provides important insights into metabolic regulation via enzyme compartmentation and cyanobacterial compensatory responses.

Keywords

Compensatory response; INST-MFA; Microcompartment; Mixotrophic; Photorespiration; RuBisCO

1. Introduction

Cyanobacteria are promising production systems to meet renewable resource demands because they are photosynthetic, genetically tractable, and capable of rapid growth. Photosynthetic organisms assimilate carbon using ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). However, in addition to its slow carboxylation kinetics, RuBisCO also oxygenates ribulose-1,5-bisphosphate (RuBP) producing 2-phosphoglycolate (2PG), a compound that is toxic to cells at high concentrations (Kern et al., 2011). Therefore, cyanobacteria have evolved CO₂ concentrating mechanisms (CCM) around RuBisCO, encapsulating the enzyme within clathrate structures comprised of multiple proteins that form microcompartments referred to as carboxysomes. Carboxysomes result from protein-protein interactions between structural proteins (*ccmKLMNO*) and RuBisCO (*rbcL*, *rbcS*), which promotes the formation of an icosahedral shell that also encases carbonic anhydrase (CA, *cccA*) (Price et al., 1992; Cameron et al., 2013). The hierarchy of protein-protein interactions required for carboxysome assembly has previously been elucidated (Cameron et al., 2013). These polyhedral structures concentrate and increase CO₂ availability to RuBisCO (Cai et al., 2009; Dou et al., 2008), without saturating CA (Mangan and Brenner, 2014).

Bacteria, as simple systems, are presumed to have little or no spatial subcellular organization, and thus the cytosol is assumed to be homogenous. However, evidence of metabolons (a multi-enzyme complex formed by non-covalent bonds between enzymes in a metabolic pathway) in bacteria exists (Ishikawa et al., 2004; Broddrick et al., 2016), indicating that enzymes may be more self-organized than previously suspected (Sweetlove and Fernie, 2018). Carboxysome compartmentalization and photosystem co-localization on cyanobacterial membranes are an obvious exception, and the advanced spatial organization confers enhanced growth and productivity. A small fraction of un-encapsulated RuBisCO is also localized outside of carboxysomes, often in association with the cell periphery near the thylakoid membranes in procarboxysome assembly centers (Agarwal et al., 2009; Cameron et al., 2013). It is possible that a cascade of enzymes proximal to carboxysomes might form metabolons that channel CO₂ fixation reactions. The direct passing of metabolites among cascade enzymes may uniquely contribute to metabolic regulation (Abernathy et al., 2017b). In eukaryotes, the formation of metabolons are associated with cellular organelles and membrane compartments, such as the mitochondria or peroxisomes (Graham et al., 2007). In prokaryotes, evidence suggests that enzyme proximity channeling may be widely present in central pathways (Abernathy et al., 2019). Cyanobacterial RuBisCO is the starting point for reduced carbon metabolism in the cell, and the encapsulating carboxysome may offer an

anchoring point for cascade enzymes. Removal of carboxysomes can change the cellular locations of both RuBisCO and downstream enzymes (Fig. 1A). Currently, it is unclear how the redistribution of enzymes in space affects the metabolic network.

Isotopic experiments are regularly used to investigate pathway activity (Antoniewicz et al., 2007; Boyle et al., 2017) and metabolite channeling (Shearer et al., 2005; Williams et al., 2011; Zhang et al., 2017). Isotopically nonstationary metabolic flux analysis (INST-MFA) uses time-dependent mass isotopomer distributions (MID) of free metabolites to estimate fluxes through photosynthetic metabolism (Young et al., 2011; Abernathy et al., 2017a; Hendry et al., 2017; Xiong et al., 2015a; Ma et al., 2014). The same labeling experiments can also identify heterogeneous intracellular environments (i.e. metabolically inactive pools of central metabolites) (Abernathy et al., 2017a). In this study, INST-MFA on a carboxysome (*ccmKLMN*) mutant offers a strategy to understand *Synechococcus* 7002 metabolism in the absence of an innate microcompartment. Specifically, a *ccmKLMN* mutant was generated (Gordan et al., 2016), eliminating the protein structure around RuBisCO (Fig. 1). Due to the lack of a carbon concentrating mechanism, the mutant was grown under 1% v/v CO₂ environments (hereby referred to as the high CO₂ condition). The mutant phenotypes were measured and compared to the control strain, including spatial locations of RuBisCO, flux organizations, metabolite concentrations, biomass compositions, labeling dynamics of cascade metabolites, and photosynthetic capabilities. The results obtained here provide holistic insights into the effect of microcompartments on cyanobacterial metabolism.

2. Materials and methods

2.1. Strains and cultivation conditions

The *Synechococcus* PCC 7002 control strain and *ccmKLMN* mutant were cultured and maintained in A+ medium without citrate (18 g/L NaCl, 0.6 g/L KCl, 1 g/L NaNO₃, 5 g/L MgSO₄•7H₂O, 0.05 g/L KH₂PO₄, 0.37 g/L CaCl₂, 0.03 Na₂EDTA•2H₂O, 1 g/L Tris HCl, 0.555 mM H₃BO₃, 2.3 μM ZnCl₂, 0.21 μM MoO₃, 3 μM NH₄Fe(SO₄)₂•12 H₂O, 22 μM MnCl₂, 0.012 μM CuSO₄, 0.05 CoCl₂, 0.0004 g/L vitamin B12) (Stevens et al., 1973). 100 μg/mL kanamycin or 25 μg/mL gentamicin were added to the *ccmKLMN* and control strain cultures, respectively. Concentrated glycerol, acetate, pyruvate, and sodium bicarbonate stocks were filtered and added to denoted cultures for studying carbon substrate effects. The same temperature (37 °C), shaking speed (200 rpm), and culture volume (20 mL of medium in 125 mL shake flasks) were used in all experiments. Cultivation occurred at either 0.2% v/v CO₂ or 1% v/v CO₂ with continuous light. In order to balance the minimal CO₂ fixation that occurs in the low CO₂ condition (0.2%), the light intensity was adjusted to 25 μmol of photons m⁻²•s⁻¹. To assess the metabolism in the high CO₂ condition (1%), light intensity was scaled linearly to 125 μmol of photons m⁻²•s⁻¹. During cell growth, optical density was measured at 730 nm (OD₇₃₀).

2.2. *Synechococcus* PCC 7002 *ccmKLMN* mutant generation and imaging

The *Synechococcus* PCC 7002 mutant was generated previously (Gordan et al., 2016) and kindly provided by the Pfleger group (University of Wisconsin-Madison). Briefly, the

control strain was created by inserting RbcL-GFP in the SYNPEC7002_A2842 neutral site along with a gentamicin resistance cassette. The *ccmKLMN* mutant was generated by replacing *ccmKLMN* with a kanamycin resistance cassette along with RbcL-GFP (Gordan et al., 2016). The GFP marker enabled us to confirm the presence or absence of carboxysomes in the control and *ccmKLMN*, respectively (Fig. 1). Fluorescence microscopy images were acquired on a custom Nikon TiE inverted wide-field ORCA Flash 4.0 V2+ Digital sCMOS camera (Hamamatsu) using a Nikon CFI60 Plan Apochromat Lambda 100× oil immersion objective (1.45 N.A.) using an excitation wavelength of 488 nm (GFP) or 640 nm (Cy5/chlorophyll) with an LED source (Spectra X Light Engine, Lumencor, Beaverton, OR) and emission through standard GFP and Cy5 filter sets. Prior to imaging, cells were grown in ambient or high CO₂ conditions in an AL-41L4 Environmental Chamber (Percival Scientific, Perry, IA) maintained at 37 °C with continuous illumination (~150 μmol photons m⁻² s⁻¹) provided by cool white fluorescent lamps.

2.3. Dynamic labeling experiments

INST-MFA was conducted at 1% v/v CO₂ with 125 μmol of photons m⁻²•s⁻¹ to investigate phenotypic differences between the control and the mutant strains. For each experiment, cultures were inoculated from a seed culture to an OD₇₃₀ of 0.05 in A+ medium and grown to exponential phase. Prior to the labeling experiment, a 2 mL aliquot of saturated NaH¹³CO₃ (> 98% purity, Sigma Aldrich, St Louis) was injected into the medium for a final concentration of 4 g/L of NaH¹³CO₃. Following the ¹³C-pulse, the cultures were quenched at different time intervals (30, 60, 120, 180, 300, 150, 600, 900 s) with ice cold medium and a liquid nitrogen bath (Abernathy et al., 2017a). The time-courses samples were centrifuged for 5 min (8000×g, ~0 °C). Biomass pellets were then frozen at -80 °C until metabolite extraction and analysis. Biological duplicates were used to generate standard deviations for experimentally measured MID values. Relative metabolite pool sizes were analyzed by an MS isotopomer ratio approach using fully labeled cell extracts as internal standards, specifically, fully labeled *E. coli* K-12 (cultured with uniformly labeled ¹³C glucose and ¹³C sodium bicarbonate). The estimation of metabolite pool sizes was performed by mixing a known amount of ¹³C labeled *E. coli* biomass with unlabeled cyanobacteria (three biological replicates). Then, the mixtures were extracted for LC-MS analysis. The isotopic ratio of each metabolite (labeled vs. unlabeled) was normalized by the amount of *E. coli* and cyanobacterial biomass, respectively (Abernathy et al., 2017a).

2.4. Metabolite extraction and analysis

The pelleted samples were extracted with a chloroform-methanol method (Ma et al., 2014). Supernatant aliquots were taken for analysis by lyophilizing 5 mL of medium. All samples were re-suspended in 1 mL of ddH₂O for LC-MS/MS. Ion-pairing LC-MS/MS was performed at the Proteomics and Mass Spectrometry Facility, Donald Danforth Plant Science Center, St. Louis. Succinic acid in the supernatant was precisely quantified using an UV-method enzyme kit from R-Biopharm (Darmstadt, Germany) with A+ medium as controls (Table S1).

2.5. Isotopically nonstationary ^{13}C -MFA

A cyanobacterial metabolic network was constructed with carbon atom transitions, which included the Calvin cycle, the oxidative pentose phosphate pathway, the TCA pathway, anaplerotic pathways, and a grouped photorespiration pathway (Table S2). A lumped biomass equation based on biomass composition analysis and biochemical equations was used to fit the model to biomass production. The INCA platform used a custom MATLAB ODE solver for a least squares regression to fit the experimentally determined free metabolite labeling data for *Synechococcus* PCC 7002 control and the *ccmKLMN* mutant (Young, 2014). INCA provided a goodness of fit via a Chi square statistical test as well as 95% confidence intervals of all estimated parameters by evaluating the sensitivity of the sum of squared residuals to parameter variations. Dilution parameters from INCA were used to account for labeling dilutions due to metabolically inactive pools (Young et al., 2011).

2.6. Biomass composition analysis

The cells were harvested during exponential growth by centrifugation, washed with a 0.9% NaCl solution and then freeze-dried. Protein and amino acid compositional analyses were performed by the Molecular Structure Facility, University of California (Davis, CA). Total carbohydrate was measured using the Anthrone Method, as described in our previous paper (Abernathy et al., 2017a). Fatty acid content and profile analyses were performed by Microbial ID (Newark, USA). Chlorophyll, pigments, RNA, DNA, and inorganics were estimated from previously published literature (Hendry et al., 2017). The biomass measurements were used to fine-tune the biomass equation in the INCA model. *p* values were calculated using a two-tailed student's *t*-test.

2.7. GC–MS analysis of proteinogenic amino acids from mixotrophic cultures

To assess the capability of *Synechococcus* 7002 to uptake glycerol, acetate, or pyruvate, unlabeled seed cultures were grown at 1% CO_2 and used to inoculate A+ medium (5% v/v ratio). The A+ medium was supplemented with either 25 mM glycerol-2- ^{13}C (second position labeled), 25 mM fully labeled acetate- $^{13}\text{C}_2$, or 25 mM pyruvate-3- ^{13}C (third position labeled). The ^{13}C -cultures were transferred to either 1% CO_2 or 0.2% CO_2 conditions and grown until mid-log phase. The labeling in proteinogenic amino acids was then analyzed using a TBDMS method (You et al., 2012).

2.8. Measuring photosynthetic parameters and oxygen evolution

Cells were harvested during log growth and adjusted based on cell numbers. The chlorophyll fluorescence parameter, maximum quantum yield (F_V/F_M) of PSII, was measured using a fluorescence fluorometer, FL-200 (Photon Systems Instruments, Brno, Czech Republic). Cells were dark adapted for 90 s before F_0 and F_M were measured using red LED measuring light. Using a custom-built Clark-type electrode, light induced oxygen evolution was measured for 2 min at 37 °C following the addition of 10 mM NaHCO_3 as described by a previous protocol (Ungerer et al., 2018). For respiratory O_2 uptake, O_2 consumption was measured for 2 min under dark conditions. Chlorophyll *a* content was measured by removing one milliliter of cell culture, extracting with methanol, and measuring the resulting absorbance at 665 and 652 nm as determined by a previous method (Porra et al., 1989).

3. Results

3.1. Growth comparison between the control strain and *ccmKLMN* mutant

The *Synechococcus* control strain and the *ccmKLMN* mutant were both grown at high CO₂ (1% v/v) and under continuous light (125 μmol photons m⁻²·s⁻¹) (Fig. 1 B&C). The control and *ccmKLMN* mutant growth rates in the exponential phase were $0.11 \pm 0.005 \text{ h}^{-1}$ and $0.085 \pm 0.005 \text{ h}^{-1}$, respectively. In the high CO₂ concentration, the addition of NaHCO₃ reduced mutant growth while there was no growth effect on the control strain. Under low CO₂ conditions (0.2% v/v CO₂), inclusion of 50 mM NaHCO₃ reduced growth rate for the control strain. When the CO₂ concentration was 0.2% or below, the *ccmKLMN* mutant exhibited little growth and the addition of 50 mM NaHCO₃ was harmful to mutant survival. Under high CO₂ conditions, neither the control strain nor the *ccmKLMN* strain utilized glycerol to promote cell growth. The mutant showed slight growth with glycerol under low CO₂ circumstances. These observations confirmed the deleterious impact on cyanobacterial growth if the cyanobacterial ability to concentrate CO₂ in microcompartments is compromised.

3.2. Biomass composition analysis

The biomass compositions (Fig. 2) were measured to reveal the differences in carbon partitioning between the control strain and the *ccmKLMN* mutant. The fatty acid profile was compositionally similar between the two strains, with the exception of statistically significant (p value < 0.05) increases of 18:2 *cis*-9,12 and 18:1 *cis*-11 fatty acids and a decrease of 18:1 *cis*-9 fatty acid in the *ccmKLMN* mutant. Protein-based amino acid profiles were also similar, with the exceptions of slightly elevated levels of leucine and lysine and slightly reduced levels of serine and threonine in the mutant. The biomass of the *ccmKLMN* strain exhibited a higher protein fraction (p value < 0.01), $51.2 \pm 1.7 \text{ wt\%}$ protein of dry cell weight (DCW), compared to the control strain ($43.7 \pm 0.4\%$). The total glycogen/carbohydrate and lipid content were comparable with the *ccmKLMN* mutant comprised of $10.4 \pm 3.1 \text{ wt\%}$ carbohydrate and $7.3 \pm 3.0 \text{ wt\%}$ lipids and the control strain containing $12.3 \pm 3.4 \text{ wt\%}$ carbohydrate and $6.8 \pm 4.9 \text{ wt\%}$ lipids. The remaining components were expected to be chlorophyll and other pigments, soluble metabolites, and ashes.

3.3. Flux topology in PCC 7002 control and *ccmKLMN* mutant strains

Using the biomass constraints as a sink reaction in the metabolic model, MFA was performed with the control and *ccmKLMN* mutant strains under the high CO₂ and high light conditions. Fig. 3 represents a simplified flux network and is scaled with net fluxes normalized to a CO₂ uptake of 100 units using a network derived from annotated genes found in the KEGG database. The full model can be found in Table S2. The INST-MFA model produced statistically acceptable sum-of-squared-residual fits for both the *ccmKLMN* mutant and the control strain (Tables S3 and S4, respectively).

Based on the INST-MFA analysis, both the control and mutant strains showed similar flux topology under high light and high CO₂ conditions. The Calvin cycle in PCC 7002 made use of ~110 mmol CO₂. Due to the malic shunt (Malate → Pyruvate + CO₂) and pyruvate

dehydrogenase (Pyruvate $\rightarrow$ AcCoA + CO₂), ~15% of the fixed carbon was lost. The resulting biomass synthesis was 2.5 gDCW for *ccmKLMN* mutant and 2.8 gDCW for the control strain. Nearly 11% of the carbon flux from CO₂ was directed to citrate synthesis for initiating the TCA pathway, while major carbon flux was cycled through the CBB cycle to regenerate RuBP. The relative fluxes through most central metabolic pathways (including the glycolysis, the TCA and the Calvin cycle pathways) were statistically indistinguishable between the *ccmKLMN* strain and the mutant (despite small differences in fluxes directed towards biomass synthesis and extracellular organic acids). This result indicated that, under sufficient light/CO₂, *ccmKLMN* largely maintains a similar flux network to the control strain.

Compared to the other model cyanobacterium strains, *Synechocystis* 6803 (Young et al., 2011) and *Synechococcus* 7942 (Jazmin et al., 2017), PCC 7002 showed subtle metabolic network differences. In the TCA cycle, PCC 7002 lacks fumarate hydratase which interconverts malate and fumarate such that fumarate is solely generated as a byproduct of purine biosynthesis (Fig. 3). PCC 7002 also lacks the typical γ -aminobutyric acid shunt to close the TCA cycle. However, the TCA cycle can be functionally connected via the succinic semialdehyde bypass route (Zhang et al., 2016). Under our growth condition, the flux from AKG to succinate was close to zero and thus the TCA pathway was mainly used for biosynthesis. Furthermore, phosphoketolase, phosphate acetyltransferase, and acetate kinase are not present in the PCC 7002 genome annotation. Thus PCC 7002 may not be able to generate acetyl-CoA from the pentose phosphate pathway. In contrast, *Synechocystis* 6803 has a phosphoketolase shunt that splits xylulose-5-phosphate to acetyl phosphate and G3P (note: acetyl phosphate can be converted into acetyl-CoA or acetate to promote bio-production) (Knoop et al., 2013; Xiong et al., 2015b; Chwa et al., 2016; Moriyama et al., 2014). As INST-MFA is limited in its ability to distinguish between these pathways, we were unable to determine any non-annotated homologues or new enzymes in PCC 7002 central pathways. These enzymes may further fine-tune the flux network.

3.4. Photorespiration and photosynthesis

Carboxysome knock-out may impact photorespiration and photo-system activities. Photorespiration occurs when RuBisCO oxygenates RuBP to 2PG and 3-phosphoglycerate (3PGA). 2PG can be metabolized to glyoxylate and then to glycine, serine, and glycerate. In the control strain, photorespiration can be repressed by the carboxysomes which provide an efficient CO₂ concentrating mechanism. The flux map showed (Fig. 3) that the control strain produced 4.5 mol of 2PG per 100 C-moles of biomass under the high light/high CO₂ condition. Best estimates of photorespiratory flux for the *ccmKLMN* mutant indicated a net flux of 7.6 mol of 2PG per 100 C-moles of carbon fixation. This increase in photorespiration is consistent with increased free RuBisCO without a carboxysome's physical protection against O₂ (Cameron et al., 2013). To investigate the functions of the photosystems, chlorophyll fluorescence (F_V/F_M) under high CO₂ conditions was measured for both strains (Fig. 4a). The F_V/F_M ratio determines the quantum efficiency of photosystem II (PSII). Here, the variable fluorescence (F_V) was increased in the control strain compared to the *ccmKLMN* mutant while the maximum fluorescence (F_M) remained similar. Thus, there was a 15% higher F_V/F_M ratio in the control strain (i.e., higher

photosynthetic efficiency in the control strain). Furthermore, photosynthesis activity is associated with O₂ evolution. The *ccmKLMN* mutant exhibited a lower net O₂ evolution than the control strain ($397 \pm 68 \mu\text{mol O}_2/\text{mg chl } a/\text{hr}$ vs $496 \pm 74 \mu\text{mol O}_2/\text{mg chl } a/\text{hr}$). But respiratory O₂ uptake by the control and the mutant strains was identical under dark condition (Fig. 4b), which accounted for $30 \pm 7\%$ of the O₂ evolution in the *ccmKLMN* strain and $20 \pm 3\%$ in the control strain. In cyanobacteria, respiratory O₂ uptake is due to photorespiration, oxidative phosphorylation (Wan et al., 2017), and the Mehler reactions (formation of H₂O₂ by photosynthesis) (Allahverdiyeva et al., 2011). Therefore, photorespiration/photosynthesis analyses suggested that removal of carboxysomes moderately increased cyanobacterial photorespiration and decreased the photosystem efficiency under high CO₂ growth conditions.

3.5. Change in pool sizes in response to carboxysome knockout

Metabolite concentrations impact reaction free energy and flux organization (Park et al., 2016). This study further investigated *ccmKLMN* compensatory responses at metabolite levels (Heise et al., 2015). Pool sizes were measured using fully ¹³C labeled *E. coli* cell extracts as an internal standard (Fig. 5a). The *ccmKLMN* mutant had significantly increased levels of intracellular pyruvate compared to the control (18-folds higher). The mutant also had significantly higher levels of intracellular UDPG (2.1-fold), aspartate (2.5-fold), and glycerate (1.6-fold) and reduced levels of ADPG (0.5-fold). UDPG was also more rapidly labeled in the *ccmKLMN* mutant (Fig. S1), which may be beneficial for sucrose synthesis (Qiao et al., 2018). The metabolite pool size of RuBP was lower and the levels of 2PG and 3PGA were slightly decreased compared to the control. The *ccmKLMN* mutant also had an increased pool size and labeling rate of glycerate (a downstream photorespiration intermediate), which provides further evidence for the elevation of photorespiration flux. In the *ccmKLMN* mutant, the flux from PEP to OAA (aspartate precursor) was expected to decrease in comparison to the control strain (Hackenberg et al., 2012). However, free aspartate accumulated in the mutant, possibly due to a slower anabolism for utilizing free amino acids. Increased expression of *nblA1*, involved in degradation of the phycobilisome light-harvesting apparatus, has been previously observed in the cyanobacteria lacking carboxysomes (Hackenberg et al., 2012). This suggests that protein turnover could also influence metabolite pools in the *ccmKLMN*. Overflow metabolites in the supernatant were also detected by LC-MS. The *ccmKLMN* mutant excreted a small amount of organic acids (e.g., succinate and malate) (Fig. 5b). We utilized a quantitative enzyme-based spectroscopic assay and found that the *ccmKLMN* mutant produced extracellular succinate up to $2.8 \pm 0.3 \text{ mg/L}$ in the late growth phase (Table S1).

Intriguingly, ¹³C-pulse experiments indicated that central metabolites in the mutant strain had high ¹³C-enrichments at the end of tracing experiments (Fig. 3 and Fig. S1). For example, ¹³C-enrichments in ASP, UDPG, and glycerate from the mutant reached > 80% at isotopic steady state while enrichment in same metabolites from the control strain was between 40 and 60% (see discussion section for further explanations).

4. Discussion

4.1. Compensatory response in cyanobacteria conserves flux networks

Carboxysomes are key microcompartments in cyanobacteria for carbon concentration and assimilation. The loss of carboxysomes significantly impairs cell growth under atmospheric and low CO₂ conditions. Under high CO₂ concentration (1% v/v), the *ccmKLMN* mutant maintains flux ratios in central pathways similar to the control strain. In previous reports, quantitative RT-PCR and microarrays were used to monitor the abundance of gene transcripts in both *ccmM* and wild type cyanobacterial cells grown in high CO₂ conditions (Emlyn-Jones et al., 2006; Hackenberg et al., 2012). Despite large transcriptional changes seen in genes involved in photosynthesis, high-light stress, and N assimilation in the *ccmKLMN* mutant, little-to-no transcriptional change was detected in its carbon uptake (i.e., genes encoding carbon transporter systems and transcriptional regulators), the Calvin Cycle, and other central metabolic pathways. These reports indicate that these central metabolic pathways are likely post-transcriptionally regulated (Hackenberg et al., 2012). The loss of the CCM impairs the carbon supply and energy-balance, leading to significant accumulations of intracellular metabolites (i.e., UDPG, glycerate, and pyruvate) as well as secretion of succinate and malate. The CCM is also closely tied to allosteric metabolites (Burnap et al., 2015), and a loss of carboxysomes may cue metabolite flux shifts towards free amino acids (i.e. aspartate). Macromolecule partitioning in biomass was also changed and the *ccmKLMN* mutant contains a higher protein content. Potentially, the *ccmKLMN* mutant may accumulate more stress proteins than the wild type strain (Hackenberg et al., 2012).

Free RuBisCO was reported to produce a small level of pyruvate instead of 3PGA via beta elimination of a phosphate ion and oxidative stress conditions (e.g., H₂O₂) increased this RuBisCO based pyruvate formation (Andrews and Kane, 1991). It has also been proposed that the loss of the carboxysome limits cyanobacterium ability to dissipate excess light energy. The resulting stresses (e.g., production of reactive oxygen species) on *ccmKLMN* mutant may lead to a significant increase of intracellular pyruvate accumulation through this RuBisCO side-reaction. Additionally, high-expression of *nblA1*, involved in degradation of the phycobilisome light-harvesting apparatus, was also observed in the cyanobacterial strain lacking carboxysomes (Hackenberg et al., 2012). This suggests that protein turnover in the *ccmKLMN* can be another source of pyruvate as well as free aspartate. Furthermore, the *ccmKLMN* mutant had lower [ATP]/[AMP] than the control strain (Fig. S2). An elevated ratio of [NADPH]/[NADP] was also observed in the *ccmKLMN* mutant (Fig. S2), which may indicate saturated terminal electron acceptors for photosynthesis and thus lead to lower net O₂ evolution (Wang et al., 2016). In summary, *ccmKLMN* mutant demonstrated a suboptimal balance between the rates of photosynthesis and carbon fixation after spatial re-organizations of RubisCO. However, the compensatory responses at different cellular processes as well as metabolite overflow were able to restore a conserved flux network under high CO₂ conditions (Cano et al., 2018).

4.2. Using carboxysome-deficient mutants to redirect carbon fluxes

Cyanobacterial species are promising photobiorefineries (Yu et al., 2015). Often, cyanobacterial engineering efforts target pathways that act as carbon sinks (Xiong et al., 2015a). For example, blocking the synthesis of glycogen is considered a useful strategy for rerouting carbon flux towards bioproduction (Ungerer et al., 2018; Hendry et al., 2017). However, such a strategy does not necessarily increase product titers (Li et al., 2014). In this study, the *ccmKLMN* mutant was able to increase UDPG pool sizes (by two-fold) as well as UDPG synthesis rates (Fig. 5 and Fig. S1). As UDPG is the precursor for sucrose and other valuable polysaccharides like heparosan (Sarnaik et al., 2019), the *ccmKLMN* mutant can potentially be utilized for production of value-added products. Additionally, the *ccmKLMN* mutant under high CO₂ growth conditions accumulates pyruvate and organic acids, while largely maintaining a similar growth rate as the control strain. Common metabolic engineering strategies for increasing pyruvate concentration typically include N-deprivation and knocking out glycogen synthesis, but both methods cause significant growth inhibition (Benson et al., 2016; Jackson et al., 2015). Thus, knocking out carboxysomes may represent an alternative strategy to increase intracellular pyruvate (Figs. 3 and 5). For example, the removal of carboxysomes from a lactate producing PCC 7002 strain was recently shown to increase productivity by 24% and to increase titer by 125% (Clark et al., 2018). As carboxysome-free cyanobacterial mutants offer a unique method of for biocontainment of genetically engineered cell factories, these findings offer additional benefits for using a *ccmKLMN* mutant as a phototrophic platform.

4.3. Impact of carboxysome loss on photomixotrophic metabolism

To probe photomixotrophic metabolism in the WT and carboxysome mutant, labeled glycerol, acetate, and pyruvate were used for isotopic experiments (Fig. 6). Both strains incorporated acetate and resulted in labeled Leu (derived from acetyl-CoA) and Glu (derived from ketoglutarate), which indicates that organic carbons are incorporated to metabolites of the TCA cycle. The incorporation of the ¹³C-carbon from acetate labeling into leucine but not isoleucine suggests PCC 7002 uses a threonine dependent pathway rather than the citramalate pathway for isoleucine synthesis (Wu et al., 2010). Under the high CO₂ conditions, a mixotrophic environment had minimal impact on *ccmKLMN* mutant growth. Under the low CO₂ condition, acetate supplementation rescued growth of the *ccmKLMN* mutant and resulted in the incorporation of acetate carbons into the biomass (revealed by isotopic labeling of amino acids). The increase of acetate usage benefitted photomixotrophic metabolism when cyanobacterial carboxysomes were disrupted. Glycerol was also found to be incorporated into biomass in the *ccmKLMN* mutant under the low CO₂ condition. Literature has indicated that glycerol increased NAD(P)H in PCC 7002 cells growing at atmospheric conditions (Ludwig and Bryant, 2012). Here, neither strain utilized significant amounts of labeled glycerol under high CO₂ conditions. Finally, exogenous pyruvate can be co-utilized by both strains for synthesis of some amino acids (such as leucine, isoleucine and glutamate). However, pyruvate impaired *ccmKLMN* mutant survival under low light and low CO₂ conditions.

4.4. Insights into microcomponent based flux regulation

Presence of bacterial metabolons or metabolite channeling may cause a heterogeneous mixture of intracellular metabolites. In INST-MFA, dilution parameters are typically introduced to aid in model fitting due to observations that metabolites never reach fully isotopic enrichment. These fitted dilution parameters for multiple metabolites represent unlabeled biomass reflux or heterogeneous metabolite pools that arise from enzyme localization and/or metabolite channeling. Removal of carboxysomes resulted in higher steady state ^{13}C enrichment in central metabolites (particularly sugar phosphate metabolites) and smaller dilution parameters from INST-MFA. This indicates that metabolite pools become more homogeneous after disruption of metabolons. Recent literature has focused on metabolite channeling via enzyme clusters as a way for cells to regulate fluxes and cell homeostasis (Zhang et al., 2017; Sweetlove and Fernie, 2018; Abernathy et al., 2017b). In cyanobacteria, the Calvin cycle enzymes may channel intermediates through enzyme co-localization, as evidenced through ultra-centrifugation and immunoelectron microscopy studies (Agarwal et al., 2009). Disruption of native channels and redistribution of enzymes may decrease inactive metabolite pools, leading to more ^{13}C enrichment in some metabolites at the end of pulse-tracing experiments. The channeling concept offers a new method for loosening metabolic rigidity and for flux redirection towards products. Deciphering channeling associated flux regulation can improve microbial feedstock utilization and biomanufacturing prospects (Abernathy et al., 2017b).

5. Conclusions

Carboxysome removal in PCC 7002 alters the spatial organization of CO_2 assimilation. Enzyme organization and protein-protein interactions affect flux networks, but the quantitative details remain poorly understood. The information provided here on cell metabolons/channeling can improve INST-MFA modeling results and minimize the isotopic dilution effect. The outcomes from this study also offers insights into micro-compartment based metabolic regulations. Future studies are needed to elucidate the rules dictating the formation of metabolons or metabolite channeling in central pathways, and as such will enable bio-designers to add an extra level of metabolic control to current methods that rely mostly on gene copy number, transcriptional and translational regulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Department of Energy, DESC0012722, the USDA-ARS, and the National Science Foundation, NSF-MCB #1616820 and NSF-DBI #1427621; the latter provided support for the acquisition of the LC-MS/MS used in this project. The authors would like to thank Dr. Himadri Pakrasi, Dr. Justin Ungerer, Dr. Marcus Foston and Virginia Johnson for their assistance in lab equipment and measuring photosynthesis/respiratory parameters.

Abbreviations:

2PG

2-phosphoglycolate

2PGA	2-phosphoglycerate
3PGA	3-phosphoglycerate
6PG	6-phosphogluconate
ACA(or AcCoA)	acetyl-CoA
AKG	α -ketoglutarate
ADPG	adenosine diphosphate glucose
CARB	carbohydrate
CIT	citrate
DHAP	dihydroxyacetone phosphate
E4P	erythrose-4-phosphate
F6P	fructose-6-phosphate
FBP	fructose biphosphate
FUM	fumarate
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G3P	glyceraldehyde-3-phosphate
GA	glycerate
gDCW	gram dry cell weight
GOX	glyoxylate
MAL	malate
OAA	oxaloacetate
PEP	phosphoenolpyruvate
PYR	pyruvate
R5P	ribose-5-phosphate
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
Ru5P	ribulose-5-phosphate
RuBP	ribulose-1,5-bisphosphate
S7P	sedoheptulose-7-phosphate
SBP	sedoheptulose-1,7-bisphosphate

SUC	succinate
UDPG	uridine diphosphate glucose
X5P	xyulose-5-phosphate

References

- Abernathy MH, Yu J, Fa M, Liberton M, Ungerer J, Hollinshead WD, Gopalakrishnan S, He L, Maranas CD, Pakrasi HB, Allen DK, Tang YJ, 2017a Deciphering cyanobacterial phenotypes for fast photoautotrophic growth via isotopically nonstationary metabolic flux analysis. *Biotechnol. Biofuels* 10, 273. [PubMed: 29177008]
- Abernathy MH, He L, Tang Y, 2017b Channeling in native microbial pathways: implications and challenges for metabolic engineering. *Biotechnol. Adv* 35 (6).
- Abernathy MH, Zhang Y, Hollinshead WD, Wang G, Baidoo EEK, Liu T, Tang YJ, 2019 Comparative studies of glycolytic pathways and channeling under *in vitro* and *in vivo* modes. *AIChE J* 65, 483–490.
- Agarwal R, Ortleb S, Sainis JK, Melzer M, 2009 Immunoelectron microscopy for locating Calvin cycle enzymes in the thylakoids of *Synechocystis* 6803. *Mol. Plant* 2(1), 32–42. [PubMed: 19529819]
- Allahverdiyeva Y, Ermakova M, Eisenhut M, Zhange P, Pichaud P, Hagemann M, Cournac L, Aro EM, 2011 Interplay between flavodiiron proteins and photorespiration in *Synechocystis* sp. PCC 6803. *J. Biol. Chem* 286, 24007–24014. [PubMed: 21602273]
- Andrews TJ, Kane HJ, 1991 Pyruvate is a by-product of catalysis by ribulosebiphosphate carboxylase/oxygenase. *J. Biol. Chem* 266 (15), 9447–9452. [PubMed: 1903385]
- Antoniewicz MR, Kraynie DF, Laffend LA, Gonzalez-Lergier J, Kelleher JK, Stephanopoulos G, 2007 Metabolic flux analysis in a nonstationary system: fed-batch fermentation of a high yielding strain of *E. coli* producing 1,3-propanediol. *Metab. Eng* 9 (3), 277–292. [PubMed: 17400499]
- Benson PJ, Purcell-Meyerink D, Hocart CH, Truong TT, James GO, Rourke L, Djordjevic MA, Blackburn SI, Price GD, 2016 Factors altering pyruvate excretion in a glycogen storage mutant of the cyanobacterium, *Synechococcus* PCC 7942. *Front. Microbiol* 7, 475. [PubMed: 27092129]
- Boyle NR, Sengupta N, Morgan JA, 2017 Metabolic flux analysis of heterotrophic growth of *Chlamydomonas reinhardtii*. *PLoS One* 12 (5), e0177292. [PubMed: 28542252]
- Broddrick JT, Rubin BE, Welkie DG, Du N, Mih N, Diamond S, Lee JJ, Golden SS, Palsson BO, 2016 Unique attributes of cyanobacterial metabolism revealed by improved genome-scale metabolic modeling and essential gene analysis. *Proc. Natl. Acad. Sci. U.S.A* 113 (51), e8344–e8353. [PubMed: 27911809]
- Burnap RL, Hagemann M, Kaplan A, 2015 Regulation of CO₂ concentrating mechanism. *Life* 5 (1), 348–371. [PubMed: 25636131]
- Cameron JC, Wilson SC, Bernstein SL, Kerfeld CA, 2013 Biogenesis of a bacterial organelle: the carboxysome assembly pathway. *Cell* 155 (5), 1131–1140. [PubMed: 24267892]
- Cai F, Menon BB, Cannon GC, Curry KJ, Shively JM, Heinhorst S, 2009 The pentameric vertex proteins are necessary for the icosahedral carboxysome shell to functions as a CO₂ leakage barrier. *PLoS One* 4 (10), e7521. [PubMed: 19844578]
- Clark RL, Gordon GC, Bennett NR, Lyu H, Root TW, Pfleger BF, 2018 High-CO₂ requirement as a mechanism for the containment of genetically modified cyanobacteria. *ACS Synth. Biol* 7 (2), 384–391. [PubMed: 29320853]
- Cano M, Holland SC, Artier J, Burnap RL, Ghirardi M, Morgan JA, Yu J, 2018 Glycogen synthesis and metabolite overflow contribute to energy balancing in cyanobacteria. *Cell Rep* 23, 667–672. [PubMed: 29669272]
- Chwa JW, Kim WJ, Sim SJ, Um Y, Woo HM, 2016 Engineering of a modular and synthetic phosphoketolase pathway for photosynthetic production of acetone from CO₂ in *Synechococcus elongatus* PCC 7942 under light and aerobic condition. *Plant Biotechnol. J* 14 (8), 1768–1776. [PubMed: 26879003]

- Dou Z, Heinhorst S, Williams EB, Murin CD, Shively JM, Cannon GC, 2008 CO₂ fixation kinetics of *Halothiobacillus neapolitanus* mutant carboxysomes lacking carbonic anhydrase suggest the shell acts a diffusional barrier for CO₂. *J. Biol. Chem* 283 (16), 10377–10384. [PubMed: 18258595]
- Emlyn-Jones D, Woodger FJ, Price GD, Whitney SM, 2006 A *Synechococcus* PCC7942 ccmM (cyanophyceae) mutant pseudoreverts to air growth without regaining carboxysomes. *J. Phycol* 42 (4), 769–777.
- Gordan GC, Korosh TC, Cameron JC, Markley AL, Begemann MB, Pfleger BF, 2016 CRISPR interference as titratable, trans-acting regulatory tool for metabolic engineering in the cyanobacterium *Synechococcus* sp. Strain PCC 7002. *Metab. Eng* 38, 170–179. [PubMed: 27481676]
- Graham JWA, Williams TCR, Morgan M, Fernie AR, Ratcliffe G, Sweetlove LJ, 2007 Glycolytic enzymes associate dynamically with mitochondria in response to respiratory demand and support substrate channeling. *Plant Cell* 19, 3723–3738. [PubMed: 17981998]
- Hackenberg C, Huege J, Engelhardt A, Wittink F, Laue M, Matthijs HC, Kopka J, Bauwe H, Hagemann M, 2012 Low-carbon acclimation in carboxysome-less and photorespiratory mutants of the cyanobacterium *Synechocystis* sp. Strain PCC 6803. *Microbiology* 158, 398–413. [PubMed: 22096149]
- Heise R, Fernie AR, Stitt M, Nikoloski Z, 2015 Pool size measurements facilitate the determination of fluxes at branching points in non-stationary metabolic flux analysis: the case of *Arabidopsis thaliana*. *Front. Plant Sci* 6, 386. [PubMed: 26082786]
- Hendry JI, Prasannan C, Ma F, Mollers B, Jaiswal D, Digmurti M, Allen DK, Frigaard NU, Dasgupta S, Wangkar PP, 2017 Rerouting of carbon flux in a glycogen mutant of cyanobacteria assessed via isotopically non-stationary ¹³C metabolic flux analysis. *Biotechnol. Bioeng* 114 (10), 2298–2308. [PubMed: 28600876]
- Ishikawa M, Tsuchiya D, Oyama T, Tsunaka Y, Morikawa K, 2004 Structural basis for channeling mechanism of a fatty acid β -oxidation multienzyme complex. *EMBO J* 23, 2745–2754. [PubMed: 15229654]
- Jackson SA, Eaton-Rye JJ, Bryant DA, Posewitz MC, Davies FK, 2015 Dynamics of photosynthesis in a glycogen-deficient glgC mutant of *Synechococcus* sp. Strain PCC 7002. *Appl. Environ. Microbiol* 81, 6210–6222. [PubMed: 26150450]
- Jazmin LJ, Xu Y, Cheah YE, Adebisi AO, Johnson CH, Young JD, 2017 Isotopically nonstationary ¹³C flux analysis of cyanobacterial isobutyraldehyde production. *Metab. Eng* 42, 9–18. [PubMed: 28479191]
- Kern R, Bauwe H, Hagemann M, 2011 Evolution of enzymes involved in the photo-respiratory 2-phosphoglycolate cycle from cyanobacteria via algae toward plants. *Photosynth. Res* 109, 103–114. [PubMed: 21222161]
- Knoop H, Grundel M, Zilliges Y, Lehmann R, Hoffmann S, Lockau W, Steuer R, 2013 Flux balance analysis of cyanobacterial metabolism: the metabolic network of *Synechocystis* sp. PCC 6803. *PLoS Comput. Biol* 9 (6), e1003081. [PubMed: 23843751]
- Li X, Shen CR, Liao JC, 2014 Isobutanol production as an alternative metabolic sink to rescue the growth deficiency of the glycogen mutant of *Synechococcus elongatus* PCC 7942. *Photosynth. Res* 120 (3), 301–310. [PubMed: 24590366]
- Ludwig M, Bryant DA, 2012 *Synechococcus* sp. Strain PCC 7002 transcriptome: acclimation to temperature, salinity, oxidative stress, and mixotrophic growth conditions. *Front. Microbiol* 3, 354. [PubMed: 23087677]
- Ma F, Jazmin LJ, Young JD, Allen DK, 2014 Isotopically nonstationary ¹³C flux analysis of changes in *Arabidopsis thaliana* leaf metabolism due to high light acclimation. *Proc. Natl. Acad. Sci. U.S.A* 111 (47), 16967–16972. [PubMed: 25368168]
- Mangan NM, Brenner MP, 2014 Systems analysis of the CO₂ concentrating mechanism in cyanobacteria. *eLife* 02043.
- Moriyama T, Tajima N, Sekine K, Sato N, 2014 Characterization of three putative xylulose 5-phosphate/fructose 6-phosphate phosphoketolases in the cyanobacterium *Anabaena* sp. PCC 7120. *Biosci. Biotechnol. Biochem* 79 (5), 767–774. [PubMed: 25530123]

- Park JO, Rubin SA, Xu YF, Amador-Nogues D, Fan J, Shlomi T, Rabinowitz JD, 2016 Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. *Nat. Chem. Biol* 12, 482–489. [PubMed: 27159581]
- Porra RJ, Thompson WA, Kriedemann PE, 1989 Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta* 975, 384–394.
- Price GD, Coleman JR, Badger MR, 1992 Association of carbonic anhydrase activity with carboxysomes isolated from cyanobacterium *Synechococcus* PCC7942. *Plant Physiol* 100 (2), 784–793. [PubMed: 16653059]
- Qiao C, Duan Y, Zhang M, Hagemann M, Luo Q, Lu X, 2018 Effects of reduced and enhanced glycogen pools on salt-induced sucrose production in sucrose-secreting strain of *Synechococcus elongatus* PCC 7942. *Appl. Environ. Microbiol* 84 (2) e02023–17.
- Sarnaik A, Abernathy MH, Han X, Ouyang Y, Xia K, Chen Y, Cress B, Zhang F, Lali A, Pandit R, Linhardt RJ, Tang YJ, Koffas MAG, 2019 Metabolic engineering of cyanobacteria for photoautotrophic production of heparosan, a pharmaceutical compound. *Algal Res* 37, 57–63.
- Shearer G, Lee JC, Koo JA, Kohl Dh, 2005 Quantitative estimation of channeling from early glycolytic intermediates to CO in intact *Escherichia coli*. *FEBS J* 272 (13), 3260–3269. [PubMed: 15978033]
- Stevens SE, Patterson COP, Myers J, 1973 The production of hydrogen peroxide by blue-green algae: a survey. *J. Phycol* 9, 427–430.
- Sweetlove LJ, Fernie AR, 2018 The role of dynamic enzyme assemblies and substrate channeling in metabolic regulation. *Nat. Commun* 9, 2136. [PubMed: 29849027]
- Ungerer J, Lin PC, Chen HY, Pakrasi HB, 2018 Adjustments to photosystem stoichiometry and electron transfer proteins are key to the remarkably fast growth of the cyanobacterium *Synechococcus elongatus* UTEX 2973. *mBio* 9 (1) e02327–17. [PubMed: 29437923]
- Wan N, DeLorenzo DM, He L, You L, Immethun CM, Wang G, Baidoo EEK, Hollinshead WD, Keasling JD, Moon TS, Tang YJ, 2017 Cyanobacterial carbon metabolism: fluxome plasticity and oxygen dependence. *Biotechnol. Bioeng* 114 (7).
- Wang X, Liu W, Xin C, Zheng Y, Cheng Y, Sun S, Li R, Zhu XG, Dai SY, Rentzepis PM, Yuan JS, 2016 Enhanced limonene production in cyanobacteria reveals photosynthesis limitations. *Proc. Natl. Acad. Sci. USA* 113 (50), 14225–14230.
- Williams TCR, Sweetlove LJ, Ratcliffe RC, 2011 Capturing metabolite channeling in metabolic flux phenotypes. *Plant Physiol* 157 (3), 981–984. [PubMed: 21896888]
- Wu B, Zhang B, Feng X, Rubens JR, Huang R, Hicks LM, Pakrasi HB, Tang YJ, 2010 Alternative isoleucine synthesis pathway in cyanobacterial species. *Microbiology* 156 (2), 596–602. [PubMed: 19875435]
- Xiong W, Morgan JA, Ungerer J, Wang B, Maness PC, Yu J, 2015a The plasticity of cyanobacterial metabolism supports direct CO₂ conversion to ethylene. *Native Plants* 1, 15053.
- Xiong W, Lee TC, Rommelfanger S, Gjersing E, Cano M, Maness PC, Ghirardi M, Yu J, 2015b Phosphoketolase pathway contributes to carbon metabolism in cyanobacteria. *Native Plants* 2, 15187.
- You L, Page L, Feng X, Berla B, Pakrasi HB, Tang YJ, 2012 Metabolic pathway confirmation and discovery through ¹³C-labeling of proteinogenic amino acids. *J. Vis. Exp* 59, e3583.
- Young JD, Shastri AA, Stephanopoulos G, Morgan JA, 2011 Mapping photoautotrophic metabolism with isotopically nonstationary ¹³C flux analysis. *Metab. Eng* 13 (6), 656–665. [PubMed: 21907300]
- Young JD, 2014 INCA: a computational platform for isotopically nonstationary metabolic flux analysis. *Bioinformatics* 30 (9), 1333–1335. [PubMed: 24413674]
- Yu J, Liberton M, Cliften PF, Head RD, Jacobs JM, Smith RD, Koppenaar DW, Brand JJ, Pakrasi HB, 2015 *Synechococcus elongatus* UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO₂. *Sci. Rep* 5, 8132. [PubMed: 25633131]
- Zhang S, Qian X, Chang S, Dismukes GC, Bryant DA, 2016 Natural and synthetic variants of the tricarboxylic acid cycle in cyanobacteria: introduction of the GABA shunt into *Synechococcus* sp. PCC 7002. *Front. Microbiol* 7, 1972. [PubMed: 28018308]

Zhang Y, Beard KFM, Swart C, Bergmann S, Krahnert I, Nikoloski Z, Graf A, Ratcliffe RG, Sweetlove LJ, Fernie AR, Obata T, 2017 Protein-protein interactions and metabolite channeling plant tricarboxylic acid cycle. Nat. Commun 16(8), 15212.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. Strain physiology under high and low CO_2 and light conditions.

(A) Representative fluorescence images of the *Synechococcus* PCC 7002 control and $\Delta ccmKLMN$. Chlorophyll fluorescence is red, and GFP-labeled RuBisCO is cyan. Scale bars are 1 μm . (B) Growth under 0.2% CO_2 and $25 \mu mol m^{-2} s^{-1}$. (C) Growth under 1% CO_2 and $125 \mu mol m^{-2} s^{-1}$. Glycerol at a concentration of 0.2% (w/w) or sodium bicarbonate ($NaHCO_3$) at a concentration of 50 mM were added to cultures to investigate photo-mixotrophic growth rates.

Fig. 2. Biomass compositional analysis of *Synechococcus* PCC 7002 control and *ccmKLMN* mutant.

(A) Fatty acid profile found in each strain as a relative abundance of the total fatty acid content. (B) Amino acid profile found in each strain as the relative mole percentage. asx: aspartate and asparagine; glx: glutamate and glutamine. (C) Macromolecule biomass composition as a percentage of dry cell weight. * indicates a significant difference (p value < 0.05). Both control and mutant strains were cultivated in high CO_2 /light conditions.

Green arrows represent biomass synthesis reactions. Percentages of total ^{13}C enrichment at steady state of key metabolites are represented by bar graphs. Total ^{13}C enrichment was calculated at isotopic steady state. Standard deviations are based off biological replicates ($n = 2$). The complete metabolic network and full flux results can be found in Tables S2–S4.

Fig. 4. Comparison of photosynthetic efficiency in PCC 7002 control and *ccmKLMN* mutants. (A): PSII activities (F_V/F_M). (B): O₂ evolution and uptake. F_0 : initial fluorescence; F_V : variable fluorescence; F_M : maximum fluorescence. Technical ($n = 2$) and biological replicates ($n = 3$) were used for standard deviations. $*p < 0.001$. Percentages represent the respiratory O₂ uptake (as measured in the dark) as a fraction of the total net O₂ evolution.

Fig. 5. Comparison of metabolite pool size between *Synechococcus* PCC 7002 control and *ccmKLMN*.

(A) Relative pool size comparisons between the two strains. A y-axis of 1 represents equal metabolite concentrations while a y-axis > 1 indicates the factor of pool size in *ccmKLMN* greater than PCC 7002 control. The relative pool size was normalized using a ^{13}C -labeled internal standard and cell density. (B) Succinate and malate in the culture supernatant were detected by LC-MS, and the peak intensity (counts per second, cps) was proportional to their concentrations. Exact succinate concentration was further quantified using an enzyme kit (Table S1).

Fig. 6. Steady-state proteinogenic amino acid labeling from labeled glycerol, acetate and pyruvate to monitor photomixotrophic growth of PCC 7002 control (ctrl) and *ccmKLMN*. GC-MS data for proteinogenic amino acids under photomixotrophic conditions with 25 mM labeled carbon. Standard deviations are estimated based on two biological replicates (n = 2). Percent ¹³C increase is graphed (i.e., evidence of organic carbon utilization). (A) Labeling of amino acids and (B) Ratio of biomass accumulation under mixotrophic and low light/CO₂ conditions. (C) Labeling of amino acids and (D) Ratio of biomass accumulation under mixotrophic and high light/CO₂ conditions. Note: “ratio of biomass accumulation per day” is defined as:
$$\frac{\text{Maximal OD}_{730} \text{ increase over time (with organic carbon)}}{\text{Maximal OD}_{730} \text{ increase over time (photoautotrophic)}}$$
.