Photoheterotrophic Fluxome in *Synechocystis* sp. Strain PCC 6803 and Its Implications for Cyanobacterial Bioenergetics

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This study investigated metabolic responses in *Synechocystis* sp. strain PCC 6803 to photosynthetic impairment. We used 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; a photosystem II inhibitor) to block O₂ evolution and ATP/NADPH generation by linear electron flow. Based on ¹³C-metabolic flux analysis (¹³C-MFA) and RNA sequencing, we have found that *Synechocystis* sp. PCC 6803 employs a unique photoheterotrophic metabolism. First, glucose catabolism forms a cyclic route that includes the oxidative pentose phosphate (OPP) pathway and the glucose-6-phosphate isomerase (PGI) reaction. Glucose-6-phosphate is extensively degraded by the OPP pathway for NADPH production and is replenished by the reversed PGI reaction. Second, the Calvin cycle is not fully functional, but RubisCO continues to fix CO₂ and synthesize 3-phosphoglycerate. Third, the relative flux through the complete tricarboxylic acid (TCA) cycle and succinate dehydrogenase is small under heterotrophic conditions, indicating that the newly discovered cyanobacterial TCA cycle (via the γ -aminobutyric acid pathway or α -ketoglutarate decarboxylase/succinic semialdehyde dehydrogenase) plays a minimal role in energy metabolism. Fourth, NAD(P)H oxidation and the cyclic electron flow (CEF) around photosystem I are the two main ATP sources, and the CEF accounts for at least 40% of total ATP generation from photoheterotrophic metabolism (without considering maintenance loss). This study not only demonstrates a new topology for carbohydrate oxidation but also provides quantitative insights into metabolic bioenergetics in cyanobacteria.

Cyanobacteria, which first appeared in shallow marine settings as early as 3 billion years ago (1, 2), are now widely distributed in diverse nutrient and light environments (3–5). They can perform oxygenic photosynthesis and respiration simultaneously in the same compartment (6, 7). Cyanobacteria contain two photosystems to harvest light energy. Photosystem II (PSII) splits water and transports electrons sequentially through plastoquinone (PQ), cytochrome *b₆f*, plastocyanin, and photosystem I (PSI), forming a linear electron flow (LEF) to produce ATP and NADPH. Alternatively, a cyclic electron flow (CEF) runs around PSI to generate ATP (8, 9). The cyanobacterial CEF involves respiratory electron transport reactions and PSI enzymes. NAD(P)H dehydrogenase complex (NDH-1) oxidizes NADPH and provides electrons for the PQ pool. Then the electrons from PQ flow to PSI and ferredoxin to regenerate NADPH via ferredoxin-NADP⁺ reductase (FNR). The CEF can regulate ATP and NADPH ratios for CO₂ fixation and respiration in plants (10). Cyanobacteria have a much larger PSI content than plants. For example, the PSI/PSII ratio in *Synechocystis* sp. strain PCC 6803 is about 5, suggesting the significant role of PSI in energy metabolism (6). To decipher photosynthesis mechanisms, researchers often use the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to block the PQ binding site of PSII so that the LEF is inactivated. Under DCMU treatment, cyanobacteria are unable to grow autotrophically but can recover their growth in the presence of glucose (the photoheterotrophic condition). The metabolic responses to DCMU stress can provide insights into the bioenergetics of photosystems. However, it is still difficult to directly quantify ATP generation from the CEF or LEF experimentally.

This study applied ¹³C-metabolic flux analysis (¹³C-MFA) to analyze the carbon and energy metabolisms in *Synechocystis* PCC 6803 after DCMU impaired its photosynthesis (11–13). *Synechocystis* PCC 6803 is a model cyanobacterium for investigation of the mechanisms of CO₂ fixation, photosynthesis, circadian rhythm, ecological effects, and the evolution of eukaryotic algae

and plants. Flux analysis of *Synechocystis* PCC 6803 aimed to reveal how cyanobacteria reorganize their metabolism when only PSI is active. The study also examined whether the recently discovered tricarboxylic acid (TCA) cycle in cyanobacteria (either via the γ -aminobutyric acid pathway [GABA shunt] [14] or via α -ketoglutarate decarboxylase and succinic semialdehyde dehydrogenase [15]) can provide sufficient reducing equivalent if cyanobacterial phototrophic NADPH generation is inhibited. The photoheterotrophic fluxome, together with the transcription analysis, unravels the energy flows of the photosystems and complements the recent ¹³C-MFA studies on cyanobacterial photo-mixotrophic metabolism (12, 13). This study may also broadly improve our understanding of the evolution of photosystems, the effectiveness of herbicides, and cyanobacterium-based photobiorefineries.

MATERIALS AND METHODS

Cultivation conditions. *Synechocystis* PCC 6803 was cultivated with a modified BG-11 medium at 30°C in 250-ml shake flasks (150 rpm) with a working volume of 50 ml (12). Continuous illumination of 50 μ mol of photons m⁻² s⁻¹ was supplied. For photoheterotrophic cultures, DCMU (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 10

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μM (16). All tracer experiments began with a 0.5% inoculation ratio (the initial optical density at 730 nm [OD_{730}] was around 0.002) from a photoautotrophic *Synechocystis* stock. Cell density was monitored by a Genesys UV-visible (UV-Vis) spectrophotometer (Thermo Scientific) at 730 nm (one OD_{730} unit is ~ 0.45 g [dry biomass]/liter). Glucose consumption was measured by an enzyme kit (R-Biopharm, Darmstadt, Germany). To examine the PSII functions, we performed chlorophyll *a* fluorescence induction analysis of both photomixotrophic and photoheterotrophic cultures (2 g/liter glucose) using a fluorometer (FL3500; Photon Systems Instruments, Czech Republic). The fluorescence yield rose significantly when DCMU was present in *Synechocystis* glucose cultures (see Table S1 in the supplemental material), confirming that PSII was unable to capture sufficient photons to energize electrons.

$[^{13}\text{C}]$ glucose and $[^{13}\text{C}]$ bicarbonate were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Three tracer experiments were carried out to investigate cyanobacterial metabolism under DCMU stress. The first experiment confirmed the inhibition of photoautotrophic protein synthesis by DCMU. We grew *Synechocystis* with 2 g/liter $\text{NaH}^{13}\text{CO}_3$ in sealed serum bottles (with no source of $^{12}\text{CO}_2$). The resulting biomass in the sealed serum bottle was fully labeled. During the early-exponential-growth phase (OD_{730} , ~ 0.3), DCMU and 2 g/liter unlabeled NaHCO_3 were injected into the ^{13}C -labeled cultures. The biomass was sampled after a 1-day incubation with DCMU and unlabeled NaHCO_3 . The abundance of ^{12}C in proteinogenic amino acids was measured in order to determine whether *Synechocystis* could perform autotrophic biosynthesis under DCMU stress. The second experiment examined cyanobacterial CO_2 fixation under DCMU treatment. We cultivated *Synechocystis* PCC 6803 in open air with $[\text{U}-^{13}\text{C}_6]$ glucose and DCMU in shake flasks. The biomass was collected after 3 days of cultivation (OD_{730} , ~ 0.3) for isotopomer analysis. The third experiment delineated the cyanobacterial metabolic flux profile. *Synechocystis* PCC 6803 was grown in open air with $[1,2-^{13}\text{C}_2]$ glucose and DCMU. Labeled biomass in the exponential-growth phase was collected for isotopomer analysis, which provided distinctive isotopic data for ^{13}C -MFA.

RNA sequencing and data analysis. In parallel to the ^{13}C -MFA tracer experiments, we examined cyanobacterial gene expression in photoautotrophic, photomixotrophic, and DCMU-treated cultures. Biological triplicates were sampled during the exponential-growth phase. Cell pellets (~ 10 mg) were frozen with liquid nitrogen immediately after centrifugation. Then total RNA was extracted using a miRNeasy minikit (Qiagen, Valencia, CA). DNA was removed with DNase I (Roche Applied Science, Germany). The RNA integrity numbers of all samples used for sequencing were greater than 8.0. RNA profiling (library preparation, sequencing, and data analysis) was performed by the Genome Technology Access Center at Washington University (<https://gtac.wustl.edu/services/>). In brief, total RNA was depleted of rRNA by using a Ribo-Zero Magnetic kit (Epicentre, Madison, WI). Subsequently, mRNA was fragmented and reverse transcribed to prepare cDNA libraries using random primers. End repair was performed by adding an A base to each 3' end. The libraries were then ligated to sequencing adapters and were amplified with index primers. The libraries were quantified at equimolar quantities on a single flow cell lane of a HiSeq 2000 sequencing machine (Illumina, San Diego, CA). Raw image data were converted to base calls using Casava 1.8+. Then the samples were demultiplexed into individual gzipped FASTQ files by using a proprietary demultiplexer algorithm (Illumina, San Diego, CA). The FASTQ files were aligned with the whole-genome transcriptome using TopHat (version 2.0.8.0) (17, 18). To compare gene expression levels, pairwise negative binomial tests in edgeR software were performed (19, 20).

Isotopomer analysis using GC-MS. The labeling of proteinogenic amino acids was measured as reported previously (21). Biomass pellets from 20-ml cultures were harvested by centrifugation and were washed with a 0.9% NaCl solution. The pellets were then hydrolyzed with 6 M HCl at 100°C . The resulting mixtures were subsequently air dried and were derivatized with *N*-tert-butyltrimethylsilyl-*N*-methyltrifluoroacetamide

(TBDMS) prior to gas chromatography–mass spectrometry (GC-MS) analysis. We used a gas chromatograph (Hewlett-Packard model 7890A; Agilent Technologies) equipped with a DB-5ms column (J&W Scientific) and a mass spectrometer (5975C; Agilent Technologies). The GC thermal programs have been described in a previous report (21). A published algorithm was used to calculate the MS data for all derivatized metabolites (22). Due to overlapping peaks or product degradation, several amino acids (proline, arginine, cysteine, and tryptophan) were not used for flux analysis (23). The MS data ($[\text{M}-57]^+$, $[\text{M}-159]^+$ or $[\text{M}-85]^+$, and $[\text{f}302]$) of key amino acids are shown in Table S2 in the supplemental material. Isotopomer labeling fractions designated M0, M1, M2, etc., represent fragments containing unlabeled, singly labeled, and doubly labeled amino acids, and so on.

^{13}C -MFA modeling and flux calculation. ^{13}C -MFA was performed based on proteinogenic amino acids from *Synechocystis* PCC 6803 grown in open air with $[1,2-^{13}\text{C}_2]$ glucose and DCMU. The labeling distribution of key amino acids did not change during the exponential-growth phase (see Table S2 in the supplemental material), indicating the attainment of a pseudo-steady state. Based on previous reports (3, 11, 13, 15), our metabolic network of *Synechocystis* PCC 6803 includes reactions in glycolysis, the Calvin cycle, the TCA cycle, the glyoxylate shunt, photorespiration pathways, and biomass synthesis (see Table S3 in the supplemental material). The model also assumed that internally generated CO_2 from glucose degradation was exchanged with atmospheric CO_2 , resulting in a mixture of CO_2 pools. By normalizing the glucose uptake rate to a value of 100 (unitless), ^{13}C -MFA profiled relative metabolic fluxes through the central metabolism. The relative fluxes were solved by minimizing a quadratic error function that calculated the difference between predicted and measured isotopomer patterns (see Table S3). The biomass composition was based on a previous report (see Table S3) (24). Since DCMU treatment may increase cyanobacterial glycogen content (25), ^{13}C -MFA tested the sensitivity of flux distributions to glycogen composition. This was done by first assuming 10-fold increase of the glycogen content in the biomass composition and then recalculating fluxes by ^{13}C -MFA using the same set of labeling information.

Reaction reversibility was characterized by the exchange coefficient *exch* and the net flux v^{net} (12, 26). Optimization for ^{13}C -MFA was achieved by the EMU (elementary metabolite units) method (27). The MATLAB optimization solver fmincon was used to minimize the quadratic error function. To avoid local minima, 100 initial guesses were randomly generated, and the solution set that minimized the objective function was used as the best fit. The 95% confidence intervals were calculated using the Monte Carlo method as described previously (28), in which the measured isotopomer data (M0, M1, M2, etc.) were perturbed with normally distributed noise within measurement errors (± 0.005) 500 times, and the optimization solver was restarted with the optimal solution. The confidence intervals of the fluxes were determined based on 500 simulations. The MATLAB program for ^{13}C -MFA can be downloaded from the Tang laboratory website (<http://tang.eece.wustl.edu/ToolDevelopment.htm>).

RESULTS

Carbon utilization in DCMU-treated *Synechocystis* PCC 6803 cultures. We added 10 μM DCMU to the photoautotrophic cultures right after inoculation (initial OD_{730} , ~ 0.002), and no biomass growth was observed (see Fig. S1 in the supplemental material). We also added DCMU to *Synechocystis* cultures during the exponential-growth phase, and photoautotrophic growth stopped (Fig. 1). If DCMU-treated cultures were supplemented with glucose (Fig. 2), *Synechocystis* growth could be recovered (growth rate $[\mu]$, ~ 0.038 h $^{-1}$). Next, we investigated *Synechocystis* protein synthesis under DCMU stress. Photoautotrophic cultures started with an OD_{730} of ~ 0.002 and were grown with $\text{NaH}^{13}\text{CO}_3$ as the only carbon source. DCMU and unlabeled

FIG 1 CO₂ fixation in photoautotrophic cultures under DCMU stress. The photoautotrophic cultures were first grown on NaH¹³CO₃ to an OD₇₃₀ of ~0.3. Then DCMU and unlabeled NaHCO₃ were added to the cultures, which were incubated for another day. (Inset) Growth of the cultures before and after DCMU treatment. The ¹²C abundance in the biomass of amino acids was measured before and after DCMU treatment. ¹²C abundance was calculated as $\sum_{i=0}^n \frac{i}{n} M_{(n-i)}$, where i is the number of unlabeled carbons, n is the total number of carbons in an amino acid, and M is the relative molar concentration of mass isotopomer $n - i$. Error bars represent standard deviations for biological triplicates.

NaH¹²CO₃ were added during the exponential-growth phase (OD₇₃₀, ~0.3). Biomass samples, collected after 24 h of DCMU-treatment, showed negligible ¹²C incorporation (proteinogenic amino acids remained mostly ¹³C labeled [Fig. 1]). This result further confirmed that DCMU blocks protein synthesis under autotrophic conditions (25). In contrast, both ¹³C and ¹²C were present in the biomass if DCMU-stressed *Synechocystis* PCC 6803 was grown with uniformly labeled [U-¹³C₆]glucose and atmospheric ¹²CO₂ (Fig. 3). Amino acids from glycolysis (e.g., alanine and serine) and the pentose phosphate pathways (e.g., phenylalanine and histidine) had around 10 to 15% ¹²C enrichment, indicating that RubisCO in the cells still actively fixed atmospheric ¹²CO₂ under

FIG 3 CO₂ fixation by heterotrophic cultures of *Synechocystis* PCC 6803. The culture began with an OD₇₃₀ of 0.002 and was grown in open air with [U-¹³C₆]glucose and DCMU. Biomass samples were harvested after a 3-day incubation for isotopomer analysis. Significant ¹²C carbon incorporation was observed in all the key amino acids, indicating an active CO₂ fixation reaction under this condition. Error bars represent standard deviations for biological triplicates.

DCMU stress. In addition, aspartate, synthesized from oxaloacetate (OAA), had even higher ¹²C enrichment (>20%) due to additional ¹²CO₂ fixation (possibly by the anaplerotic reactions).

Metabolic flux analysis of DCMU-treated *Synechocystis* PCC 6803. *Synechocystis* PCC 6803 was grown in the open air with DCMU and [1,2-¹³C₂]glucose. Biomass samples at the mid-exponential-growth phase provided amino acid labeling information for ¹³C-MFA. There were negligible differences between the isotopomer data sets at two different time points, indicating the attainment of a metabolic steady state (see Table S2 in the supplemental material). The model-fitting quality is shown in Fig. S2 in the supplemental material (r^2 , >0.99; the sum of squared residuals [SSR] is 0.0039). The relative flux distributions, the exchange coefficients for reversible reactions, and the 95% confidence intervals are shown in Fig. 4 and Table S3 in the supplemental material. Our previous *Synechocystis* ¹³C-MFA indicated that glucose is used mainly through the glycolysis pathway under photomixotrophic conditions (12). However, ¹³C-MFA of photoheterotrophic cultures revealed that glucose was oxidized to CO₂ by a carbohydrate degradation cycle. Specifically, the oxidizing pentose phosphate (OPP) pathway was highly active in oxidizing glucose-6-phosphate (G6P) to pentose phosphates, which were then used to resynthesize fructose 6-phosphate (F6P). F6P was diverted to G6P through the reverse reaction of glucose-6-phosphate isomerase (PGI), forming a carbohydrate degradation loop (see Fig. S3A in the supplemental material). A similar glucose degradation cycle was observed recently when *Synechocystis* PCC 6803 was under atrazine stress (29). In agreement with the observation in Fig. 3, ¹³C-MFA detected significant CO₂ fixation via RubisCO and phosphoenolpyruvate (PEP) carboxylase. However, 3-phosphoglycerate (3PG), the first metabolite of CO₂ fixation through RubisCO, entered glycolysis instead of the Calvin cycle. Phosphoenolpyruvate carboxylase and malic enzyme, as observed elsewhere under photoautotrophic (11) and photomixotrophic (12, 13) conditions, remained active under the photoheterotrophic condition.

Recently, the GABA shunt was identified as closing the *Syn*-

FIG 2 Representative growth curves of *Synechocystis* PCC 6803. The cultures were grown with glucose with (□) or without (○) DCMU treatment. The ▼ symbol represents glucose consumption in the photomixotrophic cultures with DCMU treatment. Error bars represent standard deviations for biological triplicates.

FIG 4 Flux distribution and energy flows in *Synechocystis* PCC 6803 under DCMU treatment. The biomass samples from *Synechocystis* PCC 6803 cultures grown with $[1,2-^{13}\text{C}_2]$ glucose and DCMU in open air were collected during the exponential phase for isotopomer analysis. The estimated relative flux rates are shown beside the pathways, which are normalized to a glucose uptake flux assumed to be 100. The absolute glucose uptake rate was 0.41 mmol/g (dry weight of cells)/h, and μ was 0.038 h^{-1} (Fig. 2; see also Fig. S7 in the supplemental material). The standard deviations are shown after the flux rates (based on the confidence intervals in Table S3 in the supplemental material). The dashed arrows represent biomass flux, and the shaded arrows represent energy flows. Stars highlight some key bidirectional reactions. Abbreviations: 6PG, 6-phosphogluconate; AceCoA, acetyl coenzyme A; AKG, α -ketoglutarate; CIT, citrate; Cyt, cytochrome; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; FBP, fructose 1,6-bisphosphate; Fd, ferredoxin; FUM, fumarate; GAP, glyceraldehyde 3-phosphate; GLX, glyoxylate; ICIT, isocitrate; PC, plastocyanin; R5P, ribose 5-phosphate; S7P, sedoheptulose-7-phosphate; SUC, succinate; X5P, xylulose-5-phosphate.

echocystis TCA cycle (14). Although cyanobacteria have the potential to use these newly discovered enzymes for running the TCA cycle, ^{13}C -MFA found that TCA cycle flux through the GABA shunt and succinate dehydrogenase was negligible. Thus, *Synechocystis* PCC 6803 cannot activate its TCA cycle to generate significant NAD(P)H and ATP under photoheterotrophic conditions. The low TCA cycle flux also indicated that succinate dehydrogenase plays a small role in quinone reduction.

^{13}C -MFA could deduce how the cyanobacterium ensures its cofactor and ATP balancing, based on the fluxome and biomass growth (Fig. 4). If the glucose uptake is normalized to 100, 489 NAD(P)H molecules can be synthesized in the central carbon metabolism, of which 342 NAD(P)H molecules were used for biomass synthesis (see Tables S3 and S4 in the supplemental material) (30). A total of 654 ATP molecules were required by the central carbon metabolism (112 ATP molecules) and biomass synthesis

(542 ATP molecules) (Fig. 4; see also Table S4). A maximum of 369 ATP molecules was produced by the oxidative phosphorylation of excess NAD(P)H (see Table S4). This number is still less than the requirement for biomass growth, and thus the CEF around PSI presumably contributes 285 ATP molecules (without considering maintenance loss) (see Table S4).

Transcriptomics analysis of *Synechocystis* photoheterotrophic metabolism. Photoautotrophic (with CO_2), photomixotrophic (with CO_2 + glucose), and photoheterotrophic (with CO_2 + glucose + DCMU) cultures were collected at similar cell densities for transcriptome sequencing (RNA-Seq) analysis. All the samples showed high genome-mapping ratios ($\geq 90\%$). The sequence reads matched more than 3,500 of the 3,561 coding genes in the *Synechocystis* PCC 6803 genome. Multidimensional scaling plots compared the similarities of samples obtained under different conditions (see Fig. S4 in the supplemental material).

FIG 5 Pairwise comparisons of RNA expression levels for genes in the central carbon metabolism. The following $\log_2$ ratios were calculated: $\log_2$ (photomixotrophic data/DCMU data) [$\log_2$ (m/h)], $\log_2$ (DCMU data/photoautotrophic data) [$\log_2$ (h/a)], and $\log_2$ (photomixotrophic data/photoautotrophic data) [$\log_2$ (m/a)]. Abbreviations: GBP, 1,3-bisphosphoglycerate; GLYC, glycolate; SSA, succinic semialdehyde; SucCoA, succinyl-CoA.

The negligible distances among the biological triplicates indicated the overall high quality of RNA sequencing. The RNA-Seq results are shown in Fig. 5 and 6, as well as in Table S5 in the supplemental material. Using a 2-fold change in biological triplicates as the cut-off (31), we observed conserved expression of key genes involved in the central carbon metabolic pathways (e.g., the TCA cycle, anaplerotic pathways, and glycolysis) among all three conditions. In agreement with the ^{13}C -MFA observation, the expression levels of the PGI gene and genes involved in the OPP pathway were highly upregulated after DCMU treatment. The RubisCO genes (GenBank accession no. BAA10190 and BAA10192), involved in

FIG 6 Pairwise comparisons of RNA expression levels for genes in photosystems. The complete RNA-Seq data are shown in the supplemental material. $\log_2$ ratios were calculated as described in the legend to Fig. 5.

CO₂ fixation, also remained highly active in DCMU-treated *Synechocystis* PCC 6803.

As for energy metabolism under DCMU stress, the NDH-1 subunits (GenBank accession no. [BAA17783](#), [BAA17787](#), [BAA18722](#)), participating in NADPH oxidation (6), were downregulated due to the NADPH deficiency after PSII inactivation. The succinate dehydrogenase complex (SDH) supplies electrons to the quinone pool. DCMU stress reduced the expression level of the flavoprotein subunit of SDH ([BAA17519](#)), which converts succinate to fumarate. Moreover, genes encoding the nitrate transport protein (subunits NrtD and NrtC [[BAA10806](#) and [BAA10807](#), respectively]) were downregulated, suggesting a lower rate of nitrogen reduction and assimilation into biomass. On the other hand, most genes associated with CEF around PSI, such as the genes encoding FNR ([BAA18459](#)), cytochrome 553 ([BAA17354](#)), plastocyanin ([BAA10227](#)), and cytochrome *b₆f* complex ([BAA10150](#), [BAA10763](#), and [BAA18236](#)), were upregulated under DCMU stress. The gene encoding ferredoxin ([BAA18248](#)), mediating electron transfer around PSI, had a constant high expression level through all three growth conditions. Finally, we observed correlations between the fluxome and corresponding RNA-Seq data in key pathways. For example, both ¹³C-MFA and RNA-Seq indicated an active OPP pathway and CO₂ fixation through RubisCO. Comparisons between “omics” data may reveal the transcriptional or posttranscriptional regulation of carbon metabolism by cyanobacterial genes (32).

Flux sensitivity test and validations. ¹³C-MFA often employs a simplified network and takes advantage of published analytical data (such as biomass composition) for microbial characterizations. ¹³C-MFA results are dependent on model assumptions, flux constraints, and metabolite measurements. To verify our model, we tested the sensitivity of flux topology to modeling constructions. First, we performed *in silico* deletion of RubisCO-based CO₂ fixation (setting its flux as zero). A three-times-greater SSR was observed (see Fig. S5A in the supplemental material). Next, the *in silico* deletion of phosphoribulokinase was tested by blocking the conversion from ribulose-5-phosphate (Ru5P) to ribulose-1,5-diphosphate (RuBP), and the model fit quality decreased (see Fig. S5B in the supplemental material). Both tests, together with the observations shown in Fig. 3, validated CO₂ fixation by RubisCO under DCMU stress.

Second, we forced the reversible reaction F6P → G6P to be unidirectional (G6P → F6P). The SSR increased significantly (see Fig. S5C in the supplemental material), and the overall NAD(P)H production became insufficient for biomass synthesis. Therefore, the carbohydrate degradation cycle, via the reversed PGI reaction and the OPP pathway, is indispensable. Third, DCMU-treated cyanobacteria could accumulate glycogen (25). In the model test, we set a higher glycogen ratio in the biomass equation. With the new biomass composition, the ¹³C-MFA could still fit isotopomer data well (see Fig. S6 in the supplemental material). Compared to the fluxome in Fig. 4, the new flux distribution showed that the flux from G6P toward glycogen increased, and the fluxes to protein synthesis and cell growth decreased (compare Fig. 4 and Fig. S6 in the supplemental material). In spite of these differences in the flux values, the overall metabolic topology—the OPP oxidation cycle, TCA cycle, RubisCO, etc.—remained the same under different assumptions about biomass composition. Fourth, precise measurement of malic enzyme activity is difficult, since the two pathways (PEP + CO₂ → OAA → MAL → CO₂ + PYR and

PEP → PYR) generate identical labeling patterns in pyruvate (PYR) and OAA. However, ¹³C-MFA revealed small fluxes through the glyoxylate shunt and TCA cycle, which fine-tuned the malate (MAL)/OAA/PYR labeling profiles. *In silico* deletion of malic enzyme flux resulted in an increase of the SSR (see Fig. S5D in the supplemental material), suggesting that a measurable malic enzyme flux was favored for model fitting. Fifth, we tested the sensitivity of model fitting to the photorespiration flux (RuBP + O₂ → glycolate + 3PG). The SSR rose if the RuBP oxidation flux was forced to be increased (see Fig. S5E in the supplemental material). This result confirmed that cyanobacterial photorespiration was inhibited by DCMU (33). Comparing to gene expression under the photomixotrophic condition, RNA-Seq analysis also verified decreased expression levels of glycolate oxidase ([BAA10256](#) and [BAA16857](#)) in DCMU-treated *Synechocystis* (see Table S5 in the supplemental material). Finally, recent studies found that the cyanobacterial TCA cycle can be completed via either α-ketoglutarate decarboxylase and succinic semialdehyde dehydrogenase (15) or the GABA shunt (14). In our model test (see Fig. S5F in the supplemental material), the SSR increased slightly after *in silico* deletion of the pathway (α-ketoglutarate → succinate), indicating that the complete TCA cycle could be functional even though its flux is low (~1%).

DISCUSSION

Synechocystis PCC 6803 reorganizes its metabolism after PSII inactivation. First, the activities of the Calvin cycle and the OPP pathway in cyanobacteria are sensitive to the intracellular NAD(P)H abundance (7). Several scenarios of the pentose phosphate (PP) pathway in phototrophs are shown in Fig. S3 in the supplemental material. Under photomixotrophic conditions, the allosteric glucose-6-phosphate dehydrogenase in *Synechocystis* is suppressed. Glucose is metabolized mainly through glycolysis (see Fig. S3B) (12, 13). The inactive OPP pathway is also observed in *Rhodospseudomonas palustris* when reducing equivalents are supplied via acetate oxidation (see Fig. S3C) (34). In the photoautotrophic metabolism of *Synechocystis* PCC 6803, OPP could be employed together with the Calvin cycle (reversed pentose phosphate pathway) to regulate the cofactor balance and CO₂ fixation (see Fig. S3D). Under DCMU stress, *Synechocystis* metabolism contains a highly active cycle via the OPP pathway to degrade glucose and produce NADPH (see Fig. S3A), while its RubisCO remains for CO₂ fixation to compensate for carbon loss. This metabolic topology does not rely on a fully functional Calvin cycle. A counterpart metabolic feature is also observed in the embryos of plant seeds (35). On the other hand, the TCA cycle and the PEP-glyoxylate cycle are two common metabolite oxidation routes by which microbes generate reducing equivalents (36). Although *Synechocystis* contains all functional enzymes in the TCA cycle, the flux through the TCA cycle remains minimal after inactivation of its major NADPH generation route (i.e., photosynthesis).

Second, malic enzyme is the main route for supplying pyruvate in *Synechocystis* PCC 6803 under continuous light illumination, because pyruvate kinase is inhibited by ATP from photosynthesis (37). The malic enzyme activity has been revealed under photoautotrophic and photomixotrophic conditions (11–13) and with DCMU treatment. RNA-Seq analyses detected the high expression level of malic enzyme ([BAA16663](#)) under all three conditions. On the other hand, DCMU can alleviate pyruvate kinase inhibition by blocking LEF-based ATP synthesis, rendering malic en-

zyme less important for pyruvate synthesis. The adverse effect of eliminating malic enzyme ($\Delta slr0721$) on the growth of DCMU-treated *Synechocystis* PCC 6803 was shown to be much less significant than that in photoautotrophic or photomixotrophic cultures (37).

Third, regulation of the ATP/NADPH ratio is important for efficient photosynthesis (8). In DCMU-treated *Synechocystis* PCC 6803, the oxidative phosphorylation of NAD(P)H cannot produce sufficient ATP to support cell growth. To fulfill the ATP requirement, *Synechocystis* PCC 6803 upregulates the CEF (Fig. 4). Inside the CEF, NDH-1 oxidizes NAD(P)H and supplies electrons to the PQ pool (38–41). SDH in the TCA cycle could also pump electrons to the CEF (6, 42). However, the TCA cycle flux in *Synechocystis* is small under DCMU stress (Fig. 4), and thus, succinate contributes a minimum number of electrons to the PQ pool. RNA-Seq detected highly upregulated expression of genes associated with PSI (FNR, cytochrome 553, cytochrome *b₆f* complex, and plastocyanin) in DCMU-treated *Synechocystis* PCC 6803 (Fig. 6). Both ^{13}C -MFA and transcriptional analysis indicate that the CEF pumped a significant amount of ATP to sustain cell metabolism after PSII inactivation (8).

Finally, this study offers evolutionary insight into photosynthesis and potential applications of photofermentation. Cyanobacterial photosystems originated from anoxygenic phototrophs, which employed either PSI or PSII (43). It is still unclear how these two photosystems were brought together to form cyanobacterial photosynthesis (44). The metabolism of primitive cyanobacterial ancestors might have employed the photoheterotrophic fluxome (as shown in Fig. 4) before the complete development of oxygenic photosynthesis.

Conclusions. *Synechocystis* PCC 6803 can simultaneously metabolize organic carbons and CO_2 . This study presents a preliminary picture of cyanobacterial carbon and energy metabolisms under DCMU stress. When photosynthesis is sufficient, the Calvin cycle of *Synechocystis* PCC 6803 actively fixes CO_2 . When photosynthesis is limited or inactivated, its OPP pathway can be upregulated to degrade sugar for NADPH generation. *Synechocystis* PCC 6803 shows a high degree of metabolic flexibility for carbon utilization, making it a robust workhorse for synthesizing value-added products via photofermentation. The knowledge of cyanobacterial fluxomics has broad implications for many research fields, such as herbicide technology, plant physiology, evolution, and bioenergy.

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