Introduction of a Synthetic CO₂-fixing Photorespiratory Bypass into a Cyanobacterium

Received for publication, December 16, 2013, and in revised form, February 19, 2014
Published, JBC Papers in Press, February 20, 2014, DOI 10.1074/jbc.C113.543132

Patrick M. Shih1,†, Jan Zarzycki2,†, Krishna K. Niyogi3,†,‡,§‡¶ and Cheryl A. Kerfeld1,‡,§,‖,‖§1

From the 1Department of Plant and Microbial Biology, 2Howard Hughes Medical Institute, and 3Synthetic Biology Institute, University of California, Berkeley, California 94720, the 4Department of Biochemistry and Molecular Biology, Department of Energy Plant Research Laboratories, Michigan State University, East Lansing, Michigan 48824, and the 5Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720

Oxygenic photosynthesis is the primary source of nearly all biological energy. In this process, light is converted into chemical energy, which is used to fix CO₂ in the CB₁ cycle through the enzyme RuBisCO. The carboxylase activity of RuBisCO results in the addition of one molecule of CO₂ to one molecule of ribulose-1,5-bisphosphate to create two molecules of 3-phosphoglycerate, thus fixing inorganic CO₂ into triose phosphates. However, the competing oxygenase activity of RuBisCO results in the loss of fixed carbon through a process termed photorespiration. One of the “holy grails” of photosynthesis research has been to engineer RuBisCO to improve CO₂ fixation and reduce photorespiration; however, these attempts have been met with limited success. It has been shown that biochemical constraints as well as abiotic factors are crucial considerations in addressing the protein engineering of RuBisCO (1, 2). Given this complexity, a more promising approach may be to accept the inherent “flaws” of RuBisCO and improve net photosynthetic rates through engineered photorespiratory bypasses.

Photorespiration produces the toxic intermediate 2-phosphoglycerate, which is recycled through the photorespiratory C₂ cycle (see Fig. 1A). This pathway is costly, requiring ATP and reducing equivalents in an elaborate reaction sequence involving more than a dozen enzymes and transporters. Furthermore, the reaction catalyzed by glycine decarboxylase, converting two glycine molecules into one serine in the C₂ cycle, releases both NH₃ and CO₂, resulting in a net loss of carbon and nitrogen. To date, only two studies have attempted to experimentally decrease the negative impacts of the photorespiratory C₂ cycle by expression of alternative glycolate metabolic pathways. Kebeish et al. (3) attempted to bypass most of the C₂ cycle by introducing the glycolate catabolic pathway from Escherichia coli (4) into Arabidopsis thaliana chloroplasts. This pathway circumvents the loss of nitrogen, but the glyoxylate carboligase reaction results in the release of one CO₂ per two glyoxylate molecules (see Fig. 1A). Although increased biomass was reported, interestingly, transformants expressing only the first enzyme of that pathway, glycolate dehydrogenase, showed similar results, rendering the approach controversial. In a second study, Maier et al. (5) introduced a glycolate oxidation cycle into Arabidopsis chloroplasts; however, this pathway results in the release of even more CO₂ than the heterologously expressed glycolate catabolism pathway. In both cases, CO₂ release occurs in the chloroplast, where it can potentially be refixed by RuBisCO. The challenges associated with designing experimental approaches to mitigate the losses associated with photorespiration are likewise underscored by results from sys-

Background: Photorespiration limits carbon fixation.
Results: Heterologous expression and functional activity of six enzymes from the 3-hydroxypropionate bi-cycle are demonstrated in cyanobacteria.
Conclusion: A synthetic CO₂-fixing photorespiratory bypass can be introduced into cyanobacteria.
Significance: The results lay the foundation for expressing an alternative CO₂ fixation pathway in cyanobacteria, algae, and plants.

Global photosynthetic productivity is limited by the enzymatic assimilation of CO₂ into organic carbon compounds. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the carboxylating enzyme of the Calvin-Benson cycle, poorly discriminates between CO₂ and O₂, leading to photorespiration and the loss of fixed carbon and nitrogen. With the advent of synthetic biology, it is now feasible to design, synthesize, and introduce biochemical pathways in vivo. We engineered a synthetic photorespiratory bypass based on the 3-hydroxypropionate bi-cycle into the model cyanobacterium, Synechococcus elongatus sp. PCC 7942. The heterologously expressed cycle is designed to function as both a photorespiratory bypass and an additional CO₂-fixing pathway, supplementing the Calvin-Benson cycle. We demonstrate the function of all six introduced enzymes and identify bottlenecks to be targeted in subsequent bioengineering. These results have implications for efforts to improve photosynthesis and for the “green” production of high value products of biotechnological interest.

†This article contains supplemental Experimental Procedures and Table S1.
1 Supported by National Science Foundation Grant MCB0851054 (to C. A. K.) and by Gordon and Betty Moore Foundation Grant GBMF3070 (to K. K. N.).
2 Supported by United States Department of Energy Contract DE-AC02 05CH11231.
3 An Investigator of the Howard Hughes Medical Institute and the Gordon and Betty Moore Foundation. To whom correspondence may be addressed: 111 Koshland Hall, University of California, Berkeley, CA 94720. Tel.: 510-643-6602; E-mail: niyogi@berkeley.edu.
4 To whom correspondence may be addressed: Michigan State University, Plant Biology, 612 Wilson Rd., East Lansing, MI 48824. Tel.: 517-432-4371; E-mail: ckerfeld@lbl.gov.
5 The abbreviations used are: CB, Calvin-Benson; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; 3OHP, 3-hydroxypropionate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MCH, mesaconyl-CoA hydratase; MCT, mesaconyl-CoA C1:C4 CoA transferase; MCL, malyl-CoA lyase; MEH, mesaconyl-C4-CoA hydratase; MCR, malonyl-CoA reductase; PCS, propionyl-CoA synthase; NS, neutral site; ApMCT, Candidatus Accumulibacter phosphatis mesaconyl-CoA C1:C4 CoA transferase; ACCase, acetyl-CoA carboxylase.
tem-s level genome-scale metabolic modeling that suggests photorespiration is essential for optimal photosynthesis (6).

Introduction of additional, synthetic CO2 fixation pathways provides an approach to increasing photosynthesis that circumvents the complexities associated with manipulating the C2 cycle (7). Of the six known CO2 fixation cycles in nature, only the 3-hydroxypropionate (3OHP) bi-cycle is completely oxygen-insensitive (8, 9), a key consideration when engineering pathways into oxygenic photoautotrophs. The 3OHP bi-cycle from the thermophilic anoxygenic phototroph Chloroflexus aurantiacus offers an attractive starting point for engineering efforts (10) because all of the necessary enzymes have been characterized (9). In this bi-cyclic pathway, bicarbonate is fixed by biotin-dependent acetyl-CoA carboxylase and propionyl-CoA carboxylase. The primary CO2 fixation product resulting from the first cycle is glyoxylate, which is then fed into the second cycle, in which another bicarbonate is fixed and pyruvate is generated as the final product (9).

The concerted function of the MCL, MCH, MCT, and mesaconyl-CoA hydratase (MCH) and mesaconyl-C1-CoA hydratase (MCH) and mesaconyl-CoA hydratase (MEH) was demonstrated in an HPLC-based assay (15) monitoring the malonyl-CoA-dependent oxidation of NADPH at a wavelength of 365 nm (ε365 = 3,400 M⁻¹ cm⁻¹). The assay mixture (400 μl) contained 200 mM MOPS/KOH buffer (pH 7.5), 5 mM MgCl2, 0.4 mM NADPH, 0.4 mM ATP, 0.5 mM CoA, and cell extract. The reaction was started by the addition of 1 mM malonyl-CoA. Notably, two NADPH molecules are oxidized per one malonyl-CoA that is reduced to propionyl-CoA.

Propionyl-CoA synthase activity was monitored either by a previously described and slightly modified spectrophotometric assay (16) or in an HPLC-based assay, as described in the two steps below. (i) The reaction mixture (400 μl) for the photometric assay contained 200 mM MOPS/KOH buffer (pH 7.5), 0.4 mM NADPH, 100 mM KCl, 2 mM ATP, 0.5 mM CoA, and cell extract. The reaction was started by the addition of 5 mM 3OHP. (ii) The same reaction mixture was used for the HPLC-based assay only with 1 mM instead of 0.4 mM NADPH. Samples of 100 μl were withdrawn after different time points and stopped by the addition of 10 μl of 90% formic acid. The supernatants were subjected to HPLC analysis to confirm the formation of acetyl-CoA or other CoA-thioester intermediates.

**TABLE 1**

| Construct | Host       | Description | Insert Length | Reference |
|-----------|------------|-------------|---------------|-----------|
| pAM2991   | S. elongatus| Neutral site 1 genomic integration vector | 12            |          |
| pAM3491PM| S. elongatus| BglBrick-modified pAM2491 vector | This work |          |
| pNS3      | S. elongatus| Neutral site 3 genomic integration vector | 13            |          |
| pNS3:PCS  | S. elongatus| pTrc::PCS | 5492 bp       | This work |
| PMS4032   | S. elongatus| pTrc::rbs.mcl::rbs.mcl::pPsaA1::rbs.mcl::rbs.meh | 5331 bp | This work |
| PMS4570   | S. elongatus| pTrc::rbs.mcl::pPsaA1::rbs.mcl::pPsaA1::rbs.mcl::rbs.meh | 9017 bp | This work |
| PMS4591   | S. elongatus| pTrc::rbs.mcl::rbs.mcl::pPsaA1::rbs.mcl::pPsaA1::rbs.mcl::rbs.meh | 10244 bp | This work |
| PMS4749   | S. elongatus| pTrc::rbs.mcl::rbs.mcl::pPsaA1::rbs.mcl::pPsaA1::rbs.mcl::rbs.meh | 10253 bp | This work |
| pET16b    | E. coli    | IPTG-inducible expression vector |            | Novagen |
| pMct_Ap_JZ33 | E. coli | IPTG-inducible Candidatus Accumulibacter phosphatis mct | 855 bp | This work |

**EXPERIMENTAL PROCEDURES**

**Cloning, Strains, and Growth Conditions**—All constructs were cloned using the BglBrick assembly format (11) in E. coli and subsequently cloned into various neutral site destination vectors, which allow for genomic integration into the S. elongatus genome by previously described transformation protocols (12, 13). Plasmids and strains that were generated and used are summarized in Table 1. All used primers are listed in supplemental Table S1. S. elongatus strains were maintained in BG-11 medium under appropriate selection with constant light at 30 or 37 °C.

**Cloning, Heterologous Expression of Recombinant Enzymes**—The cloning, expression, and purification of the mesaconyl-C1-CoA hydratase (MCH) and mesaconyl-CoA hydratase (MCT) from Chloro- flexus aurantiacus were performed as described previously (9). Cloning, expression, and purification of the malyl-CoA lyase (MCL) from C. aurantiacus were described previously (14). For the expression and purification of the MCT from “Candidatus Accumulibacter phosphatis,” see supplemental Experimental Procedures.
MCT activity was determined in an HPLC-based assay. The reaction mixture (0.4 ml) containing 200 mM MOPS/KOH (pH 7.5), 5 mM MgCl₂, 2 mM propionyl-CoA, 10 mM glyoxylate, 3 units (formation of β-methylmalonyl-CoA) of recombinant MCL, and 25 units of recombinant MCH was preincubated for 10 min at 37 °C to form mesaconyl-C1-CoA as substrate for the CoA transferase. MCT or ApMCT was added to start the reaction. Samples of 100 µl were withdrawn prior to and after MCT addition. Reactions were stopped by the addition of 5 µl of 90% formic acid. Precipitated protein was removed by centrifugation, and the supernatants were analyzed for CoA thioesters by formic acid. Precipitated protein was removed by centrifugation, and the supernatants were analyzed for CoA thioesters by formic acid. Precipitated protein was removed by centrifugation, and the supernatants were analyzed for CoA thioesters by formic acid. Precipitated protein was removed by centrifugation, and the supernatants were analyzed for CoA thioesters by formic acid. Precipitated protein was removed by centrifugation, and the supernatants were analyzed for CoA thioesters by formic acid. Precipitated protein was removed by centrifugation, and the supernatants were analyzed for CoA thioesters by formic acid.

RESULTS

To implement the proposed cycle shown in Fig. 1B, we first tested the constitutive expression and activity of the first four Chloroflexus enzymes required, beginning where glyoxylate enters the cycle (i.e., MCL, MCH, MCT, and MEH). The reactions catalyzed by this sequence of enzymes result in the formation of acetyl-CoA and pyruvate (Fig. 1B) from propionyl-CoA and glyoxylate. Dicistronic operons were assembled to express mcl with mch and mct together with meh (Fig. 2A). Both dicistrons were driven by the previously characterized psbA1 promoter (17). The cassette expressing all four genes (referred to as PMS4032) was integrated into the S. elongatus genome at neutral site 1 (NS1) (18). The resulting transformants were assayed for activity of all four enzymes. Soluble cell extracts from the transformants were incubated with propionyl-CoA and glyoxylate, and the expected disproportionation into acetyl-CoA and pyruvate was confirmed, indicating activity of all four enzymes; the rate of catalysis, however, was low.

The intermediates involved in the last two steps needed to complete the pathway in S. elongatus, MCR and PCS, are toxic to cells. Accumulation of 3OHP, the product of MCR, can lead to organic acid toxicity (19); propionyl-CoA, the product of PCS, inhibits both pyruvate dehydrogenase and citrate synthase (20). The potential toxicity in conjunction with the difficulty of purifying a recombinant His₁₀-tagged version of the ApMCT, which catalyzed the expected intramolecular CoA transfer reaction within mesaconyl-CoA with a specific activity of 37 ± 6 µmol min⁻¹ (mg protein)⁻¹ at 37 °C, corresponding to a turnover number (Kₚ) of 58 s⁻¹ per dimer. Its apparent Kₚ value for mesaconyl-C1-CoA was determined to be 1.49 ± 0.22 mM, which was surprisingly high. In comparison the Kₚ value of the Chloroflexus MCT is only 0.24 mM (9).

Nevertheless, the double transformants encoding either a second mct gene from Chloroflexus or the Accumulibacter gene (PCS/PMS4749 or PCS/PMS4591, respectively) were generated (Fig. 2A) and assayed for all enzyme activities. In both cases, the MCT activity was substantially increased, and the activity of all six enzymes engineered into S. elongatus was confirmed (Fig. 2, C, D, E, and G). However, introduction of the additional mct gene upstream of mcr apparently led to a decrease in MCR, MCL, and MCH expression (Fig. 2B) and activity (Fig. 2F).

To estimate whether the resulting enzyme activities were high enough to allow the functioning of the synthetic photorespiratory bypass, we calculated the carbon assimilation rate of a S. elongatus wild-type culture using the equation $dS/dt = (\mu/\lambda) \times X$ (25), which correlates the specific substrate consumption ($dS$) over time ($dt$) with the specific growth rate ($\mu$).
The established growth yield ($Y$) corresponds to a bacterial cell dry mass of 1 g formed per 0.5 g of carbon fixed (~50% of bacterial cell dry mass is carbon). Although $X$ usually refers to the concentration of living cells, in this case it is used to account for the amount of total protein per 1 g of cell dry mass (in bacteria ~50% of cell dry mass is protein). We assumed a typical doubling time of 8 h for a wild-type culture under laboratory conditions with ambient CO$_2$, which corresponds to a $\mu$ of 0.087 h$^{-1}$. This would require a net carbon assimilation rate of 121 nmol min$^{-1}$ (mg protein)$^{-1}$. Taking into account an estimated loss of up to 25% of the fixed carbon due to photorespiration (26) results in 80 nmol min$^{-1}$ (mg protein)$^{-1}$ for the oxygenase activity of RuBisCO and the production of glycolate. To efficiently reassimilate glycolate in the synthetic bypass, the minimal specific activities of the involved enzymes need to be at least as high as the rate of glycolate generation. Based on that estimate, all but one of the introduced enzymes were well above the required threshold (Fig. 2G). Only the specific activity of PCS (~25 nmol min$^{-1}$ (mg protein)$^{-1}$) in the transformant cell extracts was lower than the calculated threshold, despite very high expression (Fig. 2B).

**DISCUSSION**

This study is, to our knowledge, the first successful effort to express a synthetic CO$_2$-fixing photorespiratory bypass in a phototrophic organism, the cyanobacterium *S. elongatus* PCC7942. Unlike previous studies, our pathway differs by directly avoiding the net loss of nitrogen and carbon in the photorespiratory $C_2$ cycle, which actually results in a net gain in carbon fixation through the enzyme acetyl-CoA carboxylase (ACCase).

The unique feature of our pathway is the additional carbon fixation, which must be accounted for in energy balance comparisons of other proposed photorespiratory bypasses. Therefore, we have assumed the stoichiometrically correct values for the formation of two glycolate molecules per CO$_2$ released in the $C_2$ cycle (Table 2). Thus, to reassimilate two glycolate molecules, our cyclic bypass requires 6 ATP equivalents and 4 NAD(P)H, while fixing two additional molecules of bicarbonate, the form of inorganic carbon concentrated in the cytoplasm of cyanobacteria, and circumventing the loss of NH$_3$. Note that if pyruvate, which derives from our bypass, is to be used for replenishing the CB cycle, two more ATP equivalents are required per pyruvate molecule in gluconeogenesis by pyruvate phosphate dikinase because it is AMP-forming. Nevertheless, the synthetic bypass compares favorably over the canonical photorespiratory $C_2$ cycle of cyanobacteria in terms of energy demand; the combined function of the $C_2$ cycle and CB cycle requires 11 ATP equivalents, 4 NAD(P)H, and 2 reduced ferredoxins to first refix the lost CO$_2$ and NH$_3$, as well as additionally fix two more CO$_2$ molecules to arrive at the same level of net carbon fixation as the synthetic bypass (see Fig. 1 and Table 2 for comparison of photorespiratory pathways).

Although the vast majority of metabolic engineering efforts focus on introducing linear pathways for the anabolic production of molecules of interest, our approach introduces a self-sustaining metabolic cycle that fixes CO$_2$ when glycolate/glyoxylate is available. We demonstrate that concomitant expression and activity of all six enzymes necessary to reconstitute the synthetic bypass can be achieved. This required heterologous expression of ~16 kbp of DNA and functional assembly of six multimeric enzymes ranging in molecular mass from 62 to 600 kDa.

However, an obvious physiological phenotype was not observed during growth experiments. The transformants exhibited only slight delay in growth when liquid cultures in air were inoculated from agar plates, but they reached the same doubling times and optical densities as the wild type (Fig. 2F). Our results immediately suggest next steps toward improvement. For example, our initial design used enzymes derived from the thermophile *Chlororflexus*, which are evolved to function at higher temperatures than the mesophilic growth conditions of plants and most cyanobacteria. This may underlie the low measured activity of heterologous PCS despite its strong overexpression in our transformants (Fig. 2B). Synthesis and assembly of such a large enzyme (~600 kDa) might impose a considerable stress on the transformant strains. Substitution by a PCS homolog from a mesophile may improve assembly and function of this trimeric enzyme in *S. elongatus*. Mining genome databases for mesophilic homologs of the six enzymes that may exhibit faster enzyme kinetics at lower temperatures could greatly improve flux through the cycle. However, characterization of these mesophilic alternatives is necessary, as our results with the much less efficient ApMCT homolog demonstrate. Nevertheless, mesophilic enzymes may still be advantageous in terms of expression and correct folding at ambient temperatures.

Likewise, an increase in ACCase activity may be required. Our present design relies on the native enzyme to fix bicarbonate. ACCase is required for fatty acid biosynthesis, and endogenous levels of the enzyme may be insufficient to support optimal flux through the heterologously expressed cycle. However, overexpression of up to four separate subunits of the prokaryotic ACCases will significantly complicate DNA assembly and cloning strategies. Suitable alternatives may be eukaryotic ACCases, which have undergone gene fusion events creating one large single multifunctional gene (27).

In addition to the $C_2$ cycle, cyanobacteria can make use of two other strategies, the decarboxylation and glyceraldehyde pathways (28–30) that consume glyoxylate; they potentially com-

---

**FIGURE 1.** A schematic of the conventional photorespiratory $C_2$ cycle (red) in cyanobacteria and the glyceral bypass (turquoise), adapted from Zarzycki et al. (29). Reactions that take part in both pathways are colored purple. (1) ribulose-1,5-bisphosphate carboxylase/oxygenase; (2) phosphoglycerate phosphatase; (3) glycolate dehydrogenase; (4) serine/glyoxylate aminotransferase; (5) glutamate/glyoxylate aminotransferase; (6) serine hydroxymethyl-transferase; (7) glycine decarboxylase; (8) hydroxypropyruvate reductase; (9) glyceraldehyde kinase; (10) glutamine synthetase; (11) glutamine oxoglutarate aminotransferase; (12) glyoxylate carboligase; (13) tartronate-semialdehyde reductase. THF, tetrahydrofolic acid; CH$_2$THF, methylenetetrahydrofolic acid; Fdx$_{ox}$, reduced ferredoxin; Fdx$_{reduced}$ oxidized ferredoxin. *B. subtilis* engineering a synthetic cyclic photoreposory bypass based on part of the 3OHPS bi-cycle, which also fixes bicarbonate. An overview of the design and its interaction with the CB cycle is shown. Enzymes in white boxes are present in cyanobacteria and plants. The six additional enzymes required to establish this CO$_2$ fixing photorespiratory bypass are in colored boxes. One bicarbonate molecule is fixed, while one glyoxylate is consumed to form pyruvate, which can be used for biosynthesis or to replenish the CB cycle. Pgp, 2-phosphoglycerate phosphatase; GlcD, glycolate dehydrogenase.

**REPORT:** *Expression of Synthetic 3OHPS Photorespiratory Bypass*
pete with the synthetic bypass for substrate. In contrast, plants contain only the C₂ cycle, thus simplifying the fate of glyoxylate. With the localization of all six genes of our pathway to the chloroplast, only one additional enzyme, glycolate dehydrogenase, would be necessary to convert glyoxylate and bicarbonate to pyruvate. In fact, glycolate dehydrogenase has already been successfully targeted and expressed in chloroplasts of Arabidopsis (3).

Our results have implications beyond the optimization of photorespiration in plants and cyanobacteria. The successful
introduction of half of the 3OHP bi-cycle into *S. elongatus* provides a platform in which to express the other half to attain the full bi-cyclic CO₂ fixation pathway. Given that CO₂ fixation limits the light-saturated rate of photosynthesis, the presence of two orthogonal CO₂ fixation pathways is expected to significantly enhance the conversion of solar energy into biomass. Although appealing, introducing the whole 3OHP bi-cycle will result in substantial carbon flux toward pyruvate, which could be detrimental to organisms that have evolved carbon metabolism based on sugar phosphates.

On the other hand, pyruvate or intermediates in the synthetic bypass could be redirected for biotechnological applications, such as biofuels or replacements for chemical feedstocks that are currently petroleum-derived (19). For example, we have shown that 3OHP, a precursor for bioplastics, can be derived from malonyl-CoA by the heterologous expression of MCR in cyanobacteria. Developing cyanobacteria as production strains requires increasing their tolerance to higher concentrations of 3OHP; this has been accomplished in *E. coli* (19). Likewise, the production of propionyl-CoA by the combined function of MCR and PCS in the synthetic bypass could be useful for the production of diverse polyhydroxyalkanoates such as polyhydroxyvalerate, polyhydroxymethylvalerate, or co-polymers.

It is generally assumed that an increase in carbon fixation or decrease in photorespiration will improve the efficiency of photosynthesis (7) and thereby increase growth rates or biomass production; however, the effects on such broad and complex physiological traits are determined by many additional factors (e.g. carbon allocation, translocation, and secretion). Although our growth phenotype is inconclusive (Fig. 2F), future efforts in improving the properties (e.g. finding mesophilic homologs) of the enzymes used in our pathway will be crucial to further investigate the physiological impacts of synthetic carbon fixation pathways and photorespiratory bypasses. However, although several strategies dealing with photorespiration are known to exist in cyanobacteria, its importance is not quite clear, especially in light of the cyanobacterial carbon-concentrating mechanisms that include the carboxysome. Moreover, we still do not fully understand all the effects of the introduced enzymes in *vivo*; thus, future efforts relying on global and systems approaches may shed light on potential avenues for pathway optimization, as utilized in *E. coli* metabolic engineering efforts (31, 32). This study represents an advance toward understanding the effects of altering central carbon metabolism in photosynthetic autotrophs using synthetic biology and metabolic engineering approaches.

In summary, improving photosynthesis holds promise for increasing the sustainable production of food and biofuel crops to meet the challenges of global climate change and population growth, but introducing new pathways and cycles constitutes a daunting challenge. The synthetic photorespiratory bypass

| TABLE 2 | Energy balance comparison of photorespiratory pathways to achieve the same level carbon gain as the 3OHP bypass |
|---------|-----------------------------------------------------------------------------------------------------------|
| 3OHP bypass (this study) | C₂ (glycolate) cycle (30) + Calvin cycle | glycerate bypass (3) + Calvin cycle | glycerate oxidation (5) + Calvin Cycle |
| glycolate dehydrogenase (cyanobacteria) | + 2 NAD(P)H | + 2 NAD(P)H | + 2 NAD(P)H |
| glycine decarboxylase | + 1 NADH | - 1 CO₂ | - 1 NH₃ |
| glutamate synthetase | - 1 ATP | + NH₃ |
| glutamine oxoglutarate aminotransferase | - 2 FdN₃AD | - NADH |
| hydroxypropionate reductase | - 1 ATP | - 1 ATP |
| glycerate kinase | - 1 ATP | - 1 NADH |
| tartronic semialdehyde reductase | - 1 ATP | - 1 NADH |
| glyoxylate carboxylase | - 1 CO₂ | + NADPH | - 1 CO₂ |
| malic enzyme | + NADPH | - 1 CO₂ |
| pyruvate dehydrogenase | - 6 ATP | + NADH | - 4 ATP |
| phosphoglycerate kinase | - 6 ATP | + NADH | - 8 ATP |
| glyceraldehyde 3-phosphate dehydrogenase | - 6 NADPH | - 6 NADPH | - 8 NADPH |
| ribulose phosphate kinase | - 3 ATP | - 3 ATP | - 4 ATP |
| Rubisco | - 3 ATP | - 3 ATP |
| acetyl-CoA carboxylase | - 2 ATP | + 2 HCO₃⁻ |
| malonyl-CoA reductase | - 4 NADPH |
| propionyl-CoA synthase (AMP forming) | - 4 ATP equiv. |
| pyruvate phosphate dikinase (AMP forming) | - 4 ATP equiv. |
| BALANCE | + 2 HCO₃⁻ | - 6 ATP (-10 ATP)* | + 2 ATP | + 2 CO₂ | + 2 CO₂ |
| | - 10 ATP | + 2 NAD(P)H | - 11 ATP | + 10 ATP |
| | + 2 FdN₃AD | - 2 FdN₃AD | - 5 NAD(P)H |
| | - 4 NAD(P)H | - 5 NAD(P)H |

* If pyruvate is used for the regeneration of 3-phosphoglycerate, 2 more ATP equivalents are required per pyruvate molecule by pyruvate phosphate dikinase (AMP-forming). Only 6 ATP are required if pyruvate is channeled into other biosynthesis pathways than gluconeogenesis.
reported in this study provides both a precedent and a platform for future bioengineering efforts.

Acknowledgments—We thank Tim Hsiau, Josh Kitleson, and the J. Chris Anderson laboratory for sharing expertise with DNA assembly strategies and technical assistance. We also thank David Savage for generously providing the pNS3 vector.

REFERENCES

1. Savir, Y., Noor, E., Milo, R., and Tlusty, T. (2010) Cross-species analysis traces adaptation of Rubisco toward optimality in a low-dimensional landscape. Proc. Natl. Acad. Sci. U.S.A. 107, 3475–3480

2. Tcherkez, G. G., Farquhar, G. D., and Andrews, T. J. (2006) Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. Proc. Natl. Acad. Sci. U.S.A. 103, 7268–7271

3. Kebeish, R., Niessen, M., Thiruvveedhi, K., Bari, R., Hirsch, H. J., Rosenranz, R., Stähler, N., Schönfeld, K., Kreuzaler, F., and Peterhansel, C. (2007) Chloroplastic photosynthetic bypass increases photosynthesis and biomass production in Arabidopsis thaliana. Nat. Biotechnol. 25, 593–599

4. Pellicer, M. T., Badía, J., Aguilar, J., and Baldomá, L. (1996) G6c locus of Escherichia coli: characterization of genes encoding the subunits of glycolate oxidase and the g6c regulator protein. J. Bacteriol. 178, 2051–2059

5. Maier, A., Fahnenstich, H., von Caemmerer, S., Engqvist, M. K. M., Weber, A. P. M., Flügge, U.-I., and Maurino, V. G. (2011) Glycolate oxidation in A. thaliana chloroplasts improves biomass production. Front. Plant Sci. 3, 38

6. Nogales, J., Gudmundsson, S., Knight, E. M., Palsson, B. O., and Thiele, I. (2012) Detailing the optimality of photosynthesis in cyanobacteria through systems biology analysis. Proc. Natl. Acad. Sci. U.S.A. 109, 2678–2683

7. Blankenship, R. E., Tiede, D. M., Barber, J., Brudvig, G. W., Fleming, G., Ghirardi, M., Gunner, M. R., Junge, W., Kramer, D. M., Melis, A., Moore, T. A., Moser, C. C., Nocera, D. G., Nozik, A. J., Ort, D. R., Parson, W. W., Prince, R. C., and Sayre, R. T. (2011) Comparing photosynthetic and photovoltaic efficiencies and recognizing the potential for improvement. Science 332, 805–809

8. Fuchs, G. (2011) Alternative pathways of carbon dioxide fixation: Insights into the early evolution of life? Annu. Rev. Microbiol. 65, 631–658

9. Zarzycki, J., Brecht, V., Müller, M., and Fuchs, G. (2009) Identifying the missing steps of the autotrophic 3-hydroxypropionate CO2 fixation cycle in Chloroflexus aurantiacus. Proc. Natl. Acad. Sci. U.S.A. 106, 21317–21322

10. Mattozozi, M. d., Ziesack, M., Voges, M. J., Silver, P. A., and Way, J. C. (2013) Expression of the sub-pathways of the Chloroflexus aurantiacus 3-hydroxypropionate carbon fixation bicycle in E. coli: Toward horizontal transfer of autotrophic growth. Metab. Eng. 16, 130–139

11. Anderson, J. C., Dueber, J. E., Leguia, M., Wu, C. G., Goler, J. A., Arkin, A. P., and Keasling, J. D. (2010) BglBricks: A flexible standard for biological part assembly. J. Biol. Eng. 4, 1

12. Mackey, S. R., Ditty, J. L., Clerico, E. M., and Golden, S. S. (2007) Detection of rhythmic bioluminescence from luciferase reporters in cyanobacteria. In Circadian Rhythms (Rosato, E. ed), pp. 115–129, Humana Press, New York

13. Niederholmeyer, H., Wolfstätter, B. T., Savage, D. F., Silver, P. A., and Way, J. C. (2010) Engineering cyanobacteria to synthesize and export hydrophilic products. Appl. Environ. Microbiol. 76, 3462–3466

14. Zarzycki, J., and Kerfeld, C. A. (2013) The crystal structures of the tri-functional Chloroflexus aurantiacus and bi-functional Rhodobacter sphaeroides malf-CoA lyases and comparison with CrEt-like superfamily enzymes and malate synthases. BMC Struct. Biol. 13, 28

15. Hügler, M., Menendez, C., Schägger, H., and Fuchs, G. (2002) Malonyl-coenzyme A reductase from Chloroflexus aurantiacus, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO2 fixation. J. Bacteriol. 184, 2404–2410

16. Alber, B. E., and Fuchs, G. (2002) Propionyl-Coenzyme A Synthase from Chloroflexus aurantiacus, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO2 fixation. J. Biol. Chem. 277, 12137–12143

17. Ducat, D. C., Sachdeva, G., and Silver, P. A. (2011) Rewiring hydrogenase-dependent redox circuits in cyanobacteria. Proc. Natl. Acad. Sci. U.S.A. 108, 3941–3946

18. Clerico, E. M., Ditty, J. L., and Golden, S. S. (2007) Specialized techniques for site-directed mutagenesis in cyanobacteria. In Circadian Rhythms (Rosato, E. ed), pp. 155–171, Humana Press, New York

19. Lipscomb, T. W., Lipscomb, M. L., Gill, R. T., and Lynch, M. D. (2012) Metabolic engineering of recombinant E. coli for the production of 3-hydroxypropionate. In Engineering Complex Phenotypes in Industrial Strains, pp. 185–200, John Wiley & Sons, Inc., New York

20. Horwill, A. R., Dudding, A. R., and Escalante-Semerena, J. C. (2001) Studies of propionate toxicity in Salmonella enterica identify 2-methylcitrate as a potent inhibitor of cell growth. J. Biol. Chem. 276, 19094–19101

21. Keasling, J. D. (2010) Manufacturing molecules through metabolic engineering. Science 330, 1355–1358

22. García Martín, H., Ivanova, N., Kunin, V., Warnecke, F., Barry, K. W., McHardy, A. C., Yeates, C., He, S., Salamov, A. V., S. E., Dalin, E., Putnam, N. H., Shapiro, H. I., P. L., Rigoutsos, I., Krypides, N. C., Blackall, L. L., McMahon, K. D., and Hugenholtz, P. (2006) Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. Nat. Biotechnol. 24, 1263–1269

23. Hesselmann, R. P., Werlen, C., Hahn, D., van der Meer, J. R., and Zehnder, A. J. (1999) Enrichment, phylogenetic analysis and detection of a bacterium that performs enhanced biological phosphorus removal in activated sludge. Syst. Appl. Microbiol. 22, 454–465

24. Zarzycki, J., and Fuchs, G. (2011) Coassimilation of organic substrates via the autotrophic 3-hydroxypropionate bi-cycle in Chloroflexus aurantiacus. Appl. Environ. Microbiol. 77, 6181–6188

25. Hu, Z.-H., Yue, Z.-B., Liu, Y.-G., S., and Yu, H.-Q. (2010) Anaerobic digestion of lignocellulosic wastes by rumen microorganisms. Chemical and kinetic analyses. in Environmental Anaerobic Technology: Applications and New Developments (Fang, H. H. P., ed), pp. 259–278, Imperial College Press, London

26. Harper, T. D. (1988) Estimating the rate of photorespiration in leaves. Physiol. Plant. 73, 147–152

27. Sasaki, Y., Konishi, T., and Nagano, Y. (1995) The compartmentation of acetyl-coenzyme A carboxylase in plants. Plant Physiol 108, 445–449

28. Eisenhut, M., Ruth, W., Haimovich, M., Bauwe, H., Kaplan, A., and Hagemann, M. (2008) The photosynthetic glycolic acid metabolism is essential for cyanobacteria and might have been conveyed endosymbiotically to plants. Proc. Natl. Acad. Sci. U.S.A. 105, 17199–17204

29. Zarzycki, J., Axen, S. D., Kinney, J. N., and Kerfeld, C. A. (2013) Cyanobacterial-based approaches to improving photosynthesis in plants. J. Exp. Bot. 64, 787–798

30. Eisenhut, M., Kahlon, S., Hasse, D., Ewald, R., Lienan-Hurwitz, J., Ogawa, T., Ruth, W., Bauwe, H., Kaplan, A., and Hagemann, M. (2006) The plant-like C2 glycolate cycle and the bacterial-like glycater pathway cooperate in phosphoglycolate metabolism in cyanobacteria. Plant Physiol 142, 333–342

31. Kizer, L., Pitera, D. J., Pfleger, B. F., and Keasling, J. D. (2008) Application of functional genomics to pathway optimization for increased isoprenoid production. Appl. Environ. Microbiol. 74, 3229–3241

32. Dahl, R. H., Zhang, F., Alonso-Gutierrez, J., Baidoo, E., Bath, T. S., Redding-Johansen, A. M., Petzold, C. J., Mukhopadhyay, A., Lee, T. S., Adams, P. D., and Keasling, J. D. (2013) Engineering dynamic pathway regulation using stress-response promoters. Nat. Biotechnol. 31, 1039–1046