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Augmenting the Calvin-Benson-Bassham cycle by a synthetic malyl-CoA-glycerate carbon fixation pathway

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The Calvin-Benson-Bassham (CBB) cycle is presumably evolved for optimal synthesis of C3 sugars, but not for the production of C2 metabolite acetyl-CoA. The carbon loss in producing acetyl-CoA from decarboxylation of C3 sugar limits the maximum carbon yield of photosynthesis. Here we design a synthetic malyl-CoA-glycerate (MCG) pathway to augment the CBB cycle for efficient acetyl-CoA synthesis. This pathway converts a C3 metabolite to two acetyl-CoA by fixation of one additional CO2 equivalent, or assimilates glyoxylate, a photorespiration intermediate, to produce acetyl-CoA without net carbon loss. We first functionally demonstrate the design of the MCG pathway in vitro and in Escherichia coli. We then implement the pathway in a photosynthetic organism Synechococcus elongates PCC7942, and show that it increases the intracellular acetyl-CoA pool and enhances bicarbonate assimilation by roughly 2-fold. This work provides a strategy to improve carbon fixation efficiency in photosynthetic organisms.
The ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)-dependent CBB cycle is the most prevalent CO₂ assimilation mechanism on Earth. The CBB cycle fixes atmospheric CO₂ into a C3 metabolite, which serves as a precursor for all cellular constituents and most of the reduced carbon on Earth. However, the CBB cycle has its limitations. First, the CBB cycle is presumably evolved for optimal synthesis of C3 compound, but not for the production of acetyl-CoA, the C2 building block (Supplementary Table 1). When 3-phosphoglycerate (C3), the product of the CBB cycle, is converted to acetyl-CoA, one fixed carbon is lost as CO₂ (Supplementary Fig. 1a). Second, the oxygenation reaction of Rubisco causes carbon loss during metabolism of its side product, 2-phosphoglycerate. Improvement of Rubisco specificity has been challenging, and no natural pathway is practically feasible to convert the photosynthesis intermediates, such as glycolate or glyoxylate, into acetyl-CoA without carbon loss. Furthermore, the CBB cycle involves significant ATP consumption for CO₂ fixation (Supplementary Table 1). Since acetyl-CoA is one of the central precursor molecules involved in biosynthesis of numerous products, its inefficient synthesis from the CBB cycle limits the maximum carbon yield of photosynthetic products and presents a major challenge for the development of bio-based economy.

Various solutions have been proposed to reduce carbon loss during acetyl-CoA synthesis, including a synthetic non-oxidative glycolytic (NOG) pathway that can bypass the C3 decarboxylation step. The NOG pathway converts two glyceraldehyde 3-phosphate (C3) into three molecules of acetyl-phosphate (C2) without carbon loss. In plants, Rubisco shunt is evolved for carbon-conservational acetyl-CoA synthesis from sugar, which yields 20% more acetyl-CoA with 40% less carbon loss compared to glycolysis.

In contrast to Rubisco, phosphoenolpyruvate (PEP) carboxylase (Ppc) is known to be one of the most active carboxylases with no oxygenase activity. The enzyme catalyzes the carboxylation of PEP (C3) to produce oxaloacetate (OAA) (C4). Ppc is used to replenish intermediates of the tricarboxylic acid (TCA) cycle for amino acid biosynthesis, or to shuttle CO₂ between the mesophyll and bundle sheath cells in C4 plants. In most organisms, however, C4 compounds cannot be metabolized to acetyl-CoA without carbon loss (Supplementary Fig. 1a). Without such a capability, carbon fixation through Ppc is of limited use.

Here we introduce a synthetic malyl-CoA-glycerate (MCG) pathway to complement the deficiency of the CBB cycle for efficient acetyl-CoA synthesis. This designed pathway is capable of converting one C3 sugar to two acetyl-CoA via fixation of one CO₂ equivalent, or assimilating glyoxylate, a downstream product of 2-phosphoglycolate, into acetyl-CoA without net carbon loss. We first investigate the feasibility of the MCG pathway in vitro and in Escherichia coli. Then we demonstrate the effect of coupling the MCG pathway with the CBB cycle for acetyl-CoA synthesis in a photosynthetic organism Synechococcus elongatus.

### Results

#### Design of the MCG pathway for efficient acetyl-CoA synthesis.

In theory, if the NOG pathway is integrated with the CBB cycle (Supplementary Fig. 1c and d), it requires only two CO₂ turnovers by Rubisco and six ATP to synthesize each acetyl-CoA as opposed to the endogenous route (Supplementary Fig. 1b) that needs three CO₂ turnovers and seven ATP (Table 1). Since Rubisco is a major rate-limiting step in photosynthetic organisms, the reduced dependence on Rubisco turnover reaction is expected to improve the overall photosynthesis rate. However, over-expression of xpk (coding for phosphoketolase), the key gene of the NOG pathway, severely inhibited growth of Synechococcus elongatus (Supplementary Fig. 1e and f). Since both pathways compete for the same intermediates (Supplementary Fig. 1b), the integration of the NOG pathway with the CBB cycle was not readily feasible.

We thus designed two other synthetic pathways, termed the reverse glyoxylate shunt-citrate (rGS-citrate) pathway (Supplementary Fig. 2a) and the MCG pathway (Fig. 1a), to couple with the CBB cycle. These two pathways do not share the same intermediates with the CBB cycle, and both of them are more efficient in acetyl-CoA synthesis compared to the NOG. These pathways can convert one PEP (C3) to generate two acetyl-CoA via fixation of one CO₂ equivalent (Table 1). We showed the feasibility of part of the rGS-citrate pathway in an oxaloacetate auxotrophic E. coli strain (Supplementary Fig. 2a). However, we were unable to demonstrate the complete rGS-citrate pathway, possibly due to its non-robustness predicted by computational analysis. The metabolic activities from malate to glyoxylate and to succinate need to be balanced in order to maintain equal flux. Otherwise, imbalanced flux would cause accumulation or depletion of pathway intermediates, and ultimately stop the rGS-citrate pathway.

We then focused on the MCG pathway. In this pathway (Fig. 1a and Supplementary Table 2), an input PEP together with a regenerated PEP are carboxylated to produce two oxaloacetate via assimilation of two bicarbonate. Two oxaloacetate molecules are reduced to malate, which is activated to malyl-CoA, and further split into two acetyl-CoA and two glyoxylate. The two acetyl-CoA are the products of this pathway, and two glyoxylate are recycled to regenerate one PEP through a bacterial glyoxylate assimilation route. To do so, two glyoxylate are condensed to one tartronate semialdehyde (C3), releasing one CO₂, through glyoxylate carboligase (Gcl). Then tartronate semialdehyde is reduced to d-glycerate and phosphorylated to form 2-phosphoglycerate by tartronate semialdehyde reductase (Tsr) and glyceraldehyde kinase (Gk), respectively. Thus, the net reaction of the MCG pathway is to convert one PEP and one bicarbonate to produce two acetyl-CoA with the expense of three ATP and three NADH (Fig. 1a). If the pathway is constructed in a photosynthetic organism (Supplementary Fig. 2c), the cell will need only 1.5 CO₂ assimilation by Rubisco to produce one acetyl-CoA with the expenditure of 5.5 ATP and 4 NADH (Table 1). This is a significant improvement over the native system.

### Table 1 Comparison of different pathway combinations for synthesizing each acetyl-CoA from CO₂ equivalents

| Pathway Combination | ATP consumption | Rubisco turnover | Theoretical Carbon Yield |
|---------------------|-----------------|------------------|-------------------------|
| CBB + PDH³         | 7               | 3                | 66% (1 Ac-CoA/C3)        |
| CBB + NOG          | 6               | 2                | 100% (1.5 Ac-CoA/C3)     |
| CBB + rGS-citrate  | 5               | 1.5              | 100% (2 Ac-CoA/C3 + C1) |
| CBB + MCG          | 5.5             | 1.5              | 100% (2 Ac-CoA/C3 + C1) |

³PDH: pyruvate dehydrogenase complex; CBB + PDH is the native pathway.
We showed that expression of source unless supplemented with acetate (Supplementary Fig. 3a). Such a strain cannot grow in minimal medium with glucose as the sole carbon source (Fig. 2b). The MCG pathway, coupling with glycolate dehydrogenase, can assimilate glycolate to acetyl-CoA without net carbon loss. Gdh, glycolate dehydrogenase. The net reactions are shown in the yellow boxes.

Meanwhile, the MCG pathway can also assimilate C2 metabolites, such as photorespiration intermediates glycolate and glyoxylate (Fig. 1b), to acetyl-CoA with 100% theoretical carbon efficiency. This capability is particularly useful for C3 plants which suffer from severe carbon loss by photorespiration under hot dry conditions. The net reactions of converting glycolate to acetyl-CoA by various pathways are compared in Table 2. To our knowledge, no natural pathway can perform the complete carbon conversion from glycolate to acetyl-CoA, and the synthetic MCG pathway is the only one with such type of activity.

Establishing an in vivo platform for Mtk/Mcl activity test. The most important step of the MCG pathway is to split malate to generate acetyl-CoA for growth-supporting. Thus, we used this E. coli system to screen for a suitable Mtk/Mcl combination. The results showed Mtk (originally annotated as SucCD-2) from M. capsulatus was still the most active enzyme to convert malate to malyl-CoA. However, a more active Mcl (MexAM1-M and mcl from Rhodobacter sphaeroides could rescue the growth defect of the acetyl-CoA auxotroph (ΔaceEF ΔpoxB ΔpflB) and allowed the E. coli strain to grow in minimal medium with only glucose addition (Fig. 2a), which suggested Mtk(M.c) together with Mcl(R.s) split malate to generate acetyl-CoA for growth-supporting. Thus, we used this E. coli system to screen for a suitable Mtk/Mcl combination.

**Fig. 1** Design of the MCG pathway for efficient acetyl-CoA synthesis. a The MCG pathway can convert one C3 sugar to two acetyl-CoA via fixation of one CO2 equivalent. PEP, phosphoenolpyruvate; Ac-CoA, acetyl-CoA; Ppc, phosphoenolpyruvate carboxylase; Mdh, malate dehydrogenase; Mtk, malate thiolase; Mcl, malyl-CoA lyase; Gcl, glyoxylate carboligase; Tsr, tartronate semialdehyde reductase; Gk, glycerate kinase; Eno, enolase. b The MCG pathway, coupling with glycolate dehydrogenase, can assimilate glycolate to acetyl-CoA without net carbon loss. Gdh, glycolate dehydrogenase. The net reactions are shown in the yellow boxes.

**Table 2** Comparison of ATP/NADH consumption and carbon yield among different pathways in assimilation of glycolate to produce acetyl-CoA

| Per Ac-CoA synthesis from glycolate | NAD(P)H consumption | ATP consumption | The theoretical carbon yield |
|-----------------------------------|----------------------|------------------|-----------------------------|
| Native photorespiration pathway    | 0                    | 0                | 50% (1 Ac-CoA/2 glycolate)  |
| The bacterial glycolate assimilation route | −2                   | 0                | 50% (1 Ac-CoA/2 glycolate)  |
| The MCG pathway                    | 1                    | 2                | 100% (1 Ac-CoA/1 glycolate) |

The MCG pathway can convert one glycolate to produce one acetyl-CoA without net carbon loss. The bacterial glycolate assimilation route converts two glycolate to only one acetyl-CoA.
Demonstration of the feasibility of the MCG pathway in vitro. Some synthetic pathways, such as the rGS–citrate pathway (Supplementary Fig. 2a), designed based on stoichiometry and thermodynamics, may be difficult or impossible to realize in vivo because of the lack of kinetic robustness. For example, a narrow range of enzyme activity ratio may need to be satisfied in order to distribute the flux precisely for the cycle. To test if the MCG pathway can be readily balanced, we first set up an in vitro system (Supplementary Note 1, Supplementary Fig. 4 and Supplementary Table 4) to investigate the kinetic feasibility of the pathway by using pyruvate (C3) or glyoxylate (C2) as an initial substrate. Pyruvate, which can be phosphorylated to PEP by Pps (PEP synthase), is the direct source for acetyl-CoA synthesis in nature. On the other hand, using glyoxylate as the substrate can evaluate the capability of the MCG pathway to assimilate glycolate to produce acetyl-CoA.

The results showed that after 3 h, the substrate, 2 mM pyruvate (or glyoxylate), was completely consumed. About 3.8 mM acetyl-CoA was produced with the complete pathway enzymes using pyruvate as the initial carbon input (Fig. 2c). While as controls, only 1.2 mM acetyl-CoA was detected in the mixture without Gcl addition through the action of Mtk/Mcl, and no acetyl-CoA was produced in the mixture without Mtk. The acetyl-CoA/pyruvate molar ratio was 1.91 using the complete pathway, reaching 95% of the theoretical value (=2). The lack of complete conversion is presumably due to intermediates accumulating in the system. However, when Gcl was absent, the acetyl-CoA/pyruvate ratio was 0.6, representing only 60% of the theoretical value (=1), which was presumably caused by the inhibited Mcl (M.e) activity resulting from glyoxylate accumulation.

When 2 mM glyoxylate was used as the substrate, 1.7 mM acetyl-CoA was produced from the complete pathway mixture, and no acetyl-CoA was found in the mixture without either Gcl or Mtk (Fig. 2c). The acetyl-CoA/glyoxylate molar ratio was 0.86, achieving 86% of the theoretical yield (=1), which indicated the efficiency of the glyoxylate recycling branch of the pathway. These results demonstrated the in vitro biochemical and kinetic feasibility of using the MCG pathway for acetyl-CoA synthesis.

Construction of the MCG pathway in E. coli. To demonstrate its feasibility in vivo, we first constructed the MCG pathway in E. coli. We deleted the gcl gene in the acetyl-CoA auxotroph ΔaceEF ΔpoxB ΔpflB in order to determine whether the first segment of the MCG pathway could rescue the growth defect of the ΔaceEF ΔpoxB ΔpflB strain without recycling glyoxylate. It showed the expression of mtk(M.c)/mcl(R.s) was indeed able to support the ΔaceEF ΔpoxB ΔpflB strain to grow in minimal medium with glucose as the sole carbon source after 72 h (doubling time of 3.7 h) (Supplementary Fig. 5a). Additional over-expression of gcl(E.c) could accelerate the cell growth (doubling time of 3.3 h), suggesting that glyoxylate recycling was beneficial, even though the steps from tartronate semialdehyde to 2-phosphoglycerate were catalyzed by un-augmented native enzymes (Supplementary Fig. 5b). The reason to use Mcl(R.s), rather than the more active Mcl(M.e) in the experiment, was to enlarge the effect of gcl(E.c) overexpression.

We next constructed a pyruvate auxotroph of E. coli by deleting the enzymes (MaeA<sup>20,21</sup>, MacB, and Pch<sup>22</sup>) that catalyze the C4 decarboxylation to C3 compound (Supplementary Fig. 3c). This strain (ΔmaeAB Δpck) cannot grow in minimal medium with C4 or C2 compound, such as aspartate or acetate, as the sole carbon source, but can grow on pyruvate or its upstream sugars (Supplementary Fig. 3c). Expression of mtk(M.c)/mcl(M.c) could rescue the growth defect of ΔmaeAB Δpck and allowed the strain to grow in minimal medium with aspartate as the sole carbon source (doubling time of 3.8 h) (Supplementary Fig. 5c). However, with an additional gcl knockout in the pyruvate auxotrophic strain, no growth-rescuing was observed by mtk/mcl expression within 6 days. Such results demonstrated the critical role of Gcl for PEP regeneration in the MCG pathway (Supplementary Fig. 5d).

Two glyoxylate assimilation routes can be used to catalyze the conversion of tartronate semialdehyde to glycerate in E. coli (Supplementary Fig. 5f). One uses GlxR (or GarR), functioning as tartronate semialdehyde reductases to directly reduce tartronate semialdehyde to form glycerate. The second route adopts Hyi (hydroxypropiolate isomerase) and GhrA (or GhrB) (hydroxypropiolate reductase). Here tartronate semialdehyde is first converted to hydroxypropiolate and then reduced to glycerate. To investigate which metabolic route works better in E. coli, enzymes in these two routes were overexpressed in the wild-type strain BW25113. The results showed that overexpression of gcl(E.c)/hyi(E.c) led the strain to grow in minimal medium with 50 mM glyoxylate as the sole carbon source (doubling time of 3.1 h), while expressing...
showed Mtk/Mcl were the only heterologous enzymes required to
meditate uptake and phosphorylation of glucose. Its deletion belongs to the PEP-dependent phosphotransferase system, and

Effectiveness of the MCG pathway in E. coli. To demonstrate the effectiveness of the whole pathway, an E. coli strain, ΔaceB ΔglcB ΔfrdB ΔldhA ΔptsG, was created (Fig. 3a). LdhA and FrdABCD are lactate dehydrogenase and fumarate reductase which produce -lactate and succinate, respectively. Their knockouts reduce carbon loss to these products, and channel the metabolic flux towards acetyl-CoA derived C2 compounds, acetate and ethanol, as the main fermentation products. ΔaceB and GlcB were deleted because they function as malate synthases that catalyze the reverse reaction of Mtk/Mcl. PtsG belongs to the PEP-dependent phosphotransferase system, and mediates uptake and phosphorylation of glucose. Its deletion increases the intracellular PEP pool and benefits the carbon flux towards the OAA-forming direction through Ppc.

Enzymes of the MCG pathway were introduced into the strain ΔaceB ΔglcB ΔfrdB ΔldhA ΔptsG. The cells were grown in Lysogeny Broth (LB) supplemented with 20 mM glucose and 100

either gcl(E).c/glxR(E.c) or gcl(E.c)/garR(E.c) did not display similar positive effect (Supplementary Fig. 5e). It suggested that Gcl/Hyi might be more effective in glyoxylate assimilation in E. coli. The negative results of Gcl/GlxR and Gcl/GarR were not caused by expression problems since the Gcl/GlxR and Gcl/GarR combinations exhibited even higher enzymatic activities than Gcl/Hyi using crude extract assays after IPTG (isopropyl β-D-1-thiogalactopyranoside) pre-induction (Supplementary Fig. 6a and Supplementary Table 5). According to the above results, it showed Mtk/Mcl were the only heterologous enzymes required to achieve the complete pathway activity in E. coli.
mM bicarbonate under oxygen-limited condition. Glucose consumption and C2 compounds production, including acetate and ethanol, were measured after 24 h. The results showed that expression of mtk(M.c) alone was only able to increase the titer of C2 compounds slightly compared to the control containing empty plasmid (Fig. 3b). Overexpression of gcl(E.c), hyi(E.c), garK(E.c), and mdh(E.c) further increased the C2 compound production. Additional expression of ppc from Corynebacterium glutamicum improved the titer of C2 compounds to 70.1 mM. After subtracting the C2 (12.2 mM acetate) consumed in LB medium without glucose, the final corrected C2/C6 molar yield achieved 2.9 (Fig. 3b), approaching the maximum theoretical value of 3 in E. coli (Supplementary Fig. 2b). Ppc(C.g) was used since it displayed much higher carboxylase activity with or without acetyl-CoA compared to the one from E. coli (Supplementary Fig. 6b and Supplementary Table 6).

To determine accurately the carbon fixation ability of the pathway, we grew the strain in LB medium supplemented with uniformly 13C-labeled glucose (M+6) and 13C-bicarbonate (M+1), and measured the production of double labeled C2 compounds (M+2). The M+2 form of the C2 produced and the M+6 form of glucose consumed could evaluate the effect of the MCG pathway. If the MCG pathway is functioning, the (M+2)/C2/(M+6) C6 molar yield should exceed 2, which is the

**Fig. 4** MCG pathway increased the intracellular acetyl-CoA pool and enhanced carbon fixation in S. elongatus. a Expression of the complete pathway genes in the strain McG-140 increased the intracellular acetyl-CoA level compared to wild type and the controls expressing partial pathway genes. Error bars are s.d., n = 3. b GC-MS identification of ketoisocaproate (KIC) production in the McG-140 culture. c The strain McG-SE7 significantly increased the KIC production compared to the controls with expression of the partial pathway genes. The KIC titer in the McG-SE7 achieved the highest amount of 433 mg/L. Error bars are s.d., n = 3. c.f. The strain McG-140 strain assimilated more bicarbonate than wild type and the controls expressing the partial pathway (e), and increased the bicarbonate assimilation rate (f) as well. The bicarbonate assimilation rate (f) was shown between the eighth to tenth hour of e, which displayed the most obvious differences. Strain designations are defined as in a. Error bars are s.d., n = 3. *P < 0.05 (t-test, two tails).
MCG pathway increased acetyl-CoA pool in cyanobacteria. To investigate the effect of MCG coupling with the CBB cycle, we constructed the MCG pathway in cyanobacteria S. elongatus PCC7942. The genes, ppc(E.c.), mdh(E.c.), mtkB(AB)(M.c.), and mcl(M.e.), were integrated into neutral site 1 of the genome, and the remaining genes, gcl(E.c.), mdh(E.c.), and garK(E.c.), were integrated into neutral site 1 of Cupriavidus necator. Gcl(E.c.) displayed higher glyoxylate-condensation activity than the one from E. coli in cyanobacteria (Supplementary Fig. 7a). After verification of chromosomal integration by colony PCR and enzyme assays, the resulting cyanobacterial strains (McG-140, McG-142, and McG-145) were grown under 50 μg/L CO2 continuous light, and cell growth was measured. Unlike the NOG pathway, introduction of the complete MCG pathway enzymes in the strain McG-140 did not negatively affect growth compared to wild type (Supplementary Fig. 7b). Expression of the complete pathway genes also improved cell growth compared with the controls (McG-142 and McG-145) that expressed partial pathway genes.

To evaluate the effect of the MCG pathway, intracellular acetyl-CoA level was determined. The strain (McG-140) expressing the complete pathway genes markedly increased the acetyl-CoA level compared to wild type and the controls (McG-142 and McG-145) expressing partial pathway genes (Fig. 4e). About 25.7 mM 13C-HCO3 was consumed by the McG-140 strain compared to 16.7 mM of wild type after 12 h incubation. The McG-140 strain increased the specific bicarbonate assimilation rate, while McG-142, which expressed only ppc(E.c.)/mdh(E.c.)/mtkB(AB)(M.c.)/mcl(M.e.), or McG-145, which expressed only gcl(E.c.)/glxR(E.c.)/garK(E.c.), did not show any effect (Fig. 4f, Supplementary Table 7 and Supplementary Table 8). This result indicated that the increased bicarbonate consumption could not be attributed to the increased Ppc activity alone. Photosynthetic O2 production was determined under the same conditions except using unlabeled bicarbonate. The O2 evolution of McG-140 was similar to that of wild type under 50 μg/L CO2 light condition (Supplementary Fig. 8d), suggesting that expression of the pathway genes did not affect the ATP production rate by photosystems. Thus, it appeared that coupling the MCG pathway with the CBB cycle increased carbon fixation possibly through more efficient utilization of photosystem-generated energy in cyanobacteria, as predicted in Table 1.

Discussion

Previous work to improve the CBB cycle mainly focused on engineering the cycle enzymes. Here we sought to augment the CBB cycle by constructing a synthetic pathway to complement the deficiency of the CBB cycle. The MCG pathway, coupling with the CBB cycle, allows photosynthetic cells to utilize only 5.5 ATP and 1.5 Rubisco turnovers to produce one acetyl-CoA from CO2 equivalents (Table 1), as opposed to the native pathway that requires seven ATP and three Rubisco turnovers. The MCG pathway has no oxygen sensitivity issue, and does not compete with the major existing metabolic pathways. More importantly, the MCG pathway provides an additional route for CO2 fixation via Ppc, one of the most robust and active carbon fixing enzymes. Although the thaumarchaeal HP/KB cycle, found in Nitrospumulis maritimus, can synthesize one acetyl-CoA from CO2 equivalents with the expense of only four ATP (Table 1), it may be more challenging to implement this cycle in photosynthetic organisms. Since the thaumarchaeal HP/KB cycle requires 16 enzymes to achieve the complete cycle and some of the enzymes have not been characterized yet.
In addition to carbon fixation, the MCG pathway also can reduce carbon loss in photosynthesis by converting glycolate to acetyl-CoA without net carbon loss (Fig. 1b). A previous strategy utilizes a bacterial glycolate assimilation route to save carbon loss from the endogenous photospiration pathway. However, it still assimilates two molecules of glycolate to produce only one acetyl-CoA. The theoretical carbon yield is 50% (Table 2). The MCG pathway, coupling with glycolate dehydrogenase, can convert each glycolate to stoichiometric amount of acetyl-CoA with 100% carbon yield. Thus, coupling the MCG pathway with the CBB cycle in photosynthetic organisms may be a practical approach to improve photosynthetic carbon fixation.

Methods

**Protein synthesis and purification.** Ppc, Eno, and Mdh were purchased from Sigma-Aldrich. Mtk(M2) has two subunits, and the gene coding for each subunit was fused with a 6×His-tag at the C-terminal and cloned into the same operon. The remaining genes, mec(E2c), gcl(Ec), glxR(Ec), garK(Ec), and ppc(Ec), were fused with a His-tag at the N-terminal. All genes were cloned under the T7 promoter and transformed into E. coli BL21(DE3) for expression. Overnight culture was inoculated (2% vol/vol) into fresh LB medium. Cells were grown at 37 °C with agitation at 250 rpm to mid-log phase (OD600 of 0.4–0.6), and induced for gene expression by 0.1 mM IPTG (Zymo Research) for additional 6 h at 30 °C. Cell pellets were lysed with 0.1 mm diameter glass beads at 4 °C. Proteins were purified by His-Spin protein mini-prep columns (Zymo Research). Concentrations of purified proteins were measured using BioRad protein assay kit, and the purity was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis with coomassie staining.

**Demonstration of the MCG pathway in vitro.** Pyruvate as the initial substrate: The assay was set up at 37 °C in a final volume of 400 μL containing 50 mM Tris-Cl (pH 7.5), 5 mM MgCl2, 0.5 mM TPP, 2 mM pyruvate, 6 mM NaHCO3, 8 mM CoA, 10 mM ATP, 10 mM NADH with enzymes including MtkAB, Mcl, Gcc, GlxR, GarK, Ppc, Eno, and Mdh. Glyoxylate as the initial substrate: The assay was set up at 37 °C in a final volume of 400 μL containing 50 mM Tris-Cl (pH 7.5), 5 mM MgCl2, 0.5 mM TPP, 2 mM glyoxylate, 6 mM NaHCO3, 4 mM CoA, 6 mM ATP, 6 mM NADH with enzymes including MtkAB, Mcl, Gcc, GlxR, GarK, Ppc, Eno, and Mdh. Fifty microliters of the reaction mixture was incubated with 10% formic acid to stop reactions. The detection method of glyoxylate or pyruvate was optimized by sodium dodecyl sulfate polyacrylamide gel electrophoresis with coomassie staining.

**Construction of E. coli strains.** All E. coli strains used in this study are listed in Supplementary Table 10. JC16 was used to create the acetyl-CoA auxotroph ΔaceEFΔpckBΔfl. MC1490 was used to construct the pyruvate auxotroph ΔaceEFΔpckBΔfl. The remaining E. coli strains used BW25113 as the parental strain for construction. Gene deletion was performed by P1 transduction with single knockout strain from the Keio collection.

**Plasmid construction.** Plasmids used in the study are listed in Supplementary Table 10. All plasmids were constructed by using Gibson DNA assembly. The primers used for the cloning are shown in Supplementary Table 11.

**Growth rescue of E. coli strains.** Overnight E. coli culture was inoculated (2% vol/vol) into fresh LB medium. E. coli culture was allowed to grow to 37 °C in a rotary shaker (250 rpm) to an OD600 of 0.4–0.6. About 0.1 mM IPTG was added to induce protein synthesis at 30 °C for 6 h. One milliliter of culture was harvested and washed three times with equal volume of minimal medium. Sixty microliters of culture was added into 3 mL minimal medium for growth rescuing at 37 °C. Minimal medium contains M9 salts (12.8 g/L Na2HPO4·7H2O, 3 g/L KH2PO4, 0.5 g/L NaCl, 1 g/L NH4Cl), 1 mM MgSO4, 0.1 mM CaCl2, 0.1 mg/mL thiamine hydrochloride, 0.1 mM IPTG, appropriate antibiotics (kanamycin 40 μg/mL, ampicillin 100 μg/mL, or tetracycline 50 μg/mL) and carbon sources (all from Sigma-Aldrich) as noted in the study. The growth experiments were performed aerobically.

**Measurement of C2 compounds in E. coli culture.** Overnight E. coli culture was inoculated (2% vol/vol) into fresh 20 mL LB medium. E. coli culture was grown at 37 °C in a rotary shaker (250 rpm) to an OD600 of 0.4–0.6. About 0.2 mM IPTG was used to induce gene expression at 30 °C for 6 h. Six milliliters of culture was harvested and resuspended into 2 mL fresh LB medium supplemented with 20 mM glucose, 100 mM bicarbonate, and 0.1 mM IPTG with appropriate antibiotics. Two milliliters culture (OD600 about 10) was fermented in a BD vacuum glass container capped at 37 °C for 24 h. For isolate labeling experiments, E. coli culture was prepared as stated above except using ΔGlucose-13C6 (from Santa Cruz Bio-technology, Dallas, TX.) and sodium bicarbonate 13C (from Sigma-Aldrich). To measure C2 compounds, culture was centrifuged at 15,000 g for 5 min, and supernatant was diluted for five times and filtered by Amicon 10 kDa protein filters (EMD-Amicon). Twenty microliters of sample was applied to the Agilent 1200 HPLC system with a Bio-Rad Aminex HPX87 column (30 mm 3H2SO4, 0.4 mL/min). Acetate temperature, 30 °C. Acetate detector at 210 nm. Glucose consumption was quantified by a biochemistry analyzer 2300 (YSI). Ethanol was measured by a GC-flame ionization detector (FID) (Agilent Technologies). 1-Propanol was used as the internal standard. 13C-labeled acetate (M = 2) and ethanol (M = 2) were determined by GC-MS (Agilent Technologies) as described in Bogorad et al. (2014)42.

**Measurement of intracellular acetyl-CoA level in cyanobacteria.** For measurement of intracellular acetyl-CoA, cyanobacterial culture was prepared as above, and pellet was lysed with 0.1 mm diameter glass beads at 4 °C. The intracellular acetyl-CoA level was determined by Acetyl-Coenzyme A Assay Kit (from Sigma-Aldrich).

**Measurement of bicarbonate consumption in cyanobacteria.** For measurement of bicarbonate consumption, the cyanobacterial culture (OD730 about 3) was grown under 50 μE/s/m2 light intensity with continuous shaking at 30 °C. The cyanobacterial culture was fed with 50 mM NaHCO3, and bicarbonate was measured as: bicarbonate consumption (mM)/time interval (2 h)/average OD730 with an Oxygraph System (from Hansatech Instruments)47. Oxygen production was measured by the Oxygraph System (from Hansatech Instruments) as described in Bogorad et al. (2014)45. Glucose consumption was quantified by a biuret assay (from Sigma-Aldrich), was used for NMR spectroscopy. The bicarbonate fixation rate was calculated as: bicarbonate consumption (mM/time interval) (2 h)/average OD730.

**Measurement oxygen production in cyanobacteria.** For measurement of oxygen production in cyanobacteria, the cyanobacterial culture (OD730 about 3) was used to induce gene expression at 30 °C for 6 h. One milliliter of culture was added into 3 mL minimal medium for growth rescuing at 37 °C. Minimal medium contains M9 salts (12.8 g/L Na2HPO4·7H2O, 3 g/L KH2PO4, 0.5 g/L NaCl, 1 g/L NH4Cl), 1 mM MgSO4, 0.1 mM CaCl2, 0.1 mg/mL thiamine hydrochloride, 0.1 mM IPTG, appropriate antibiotics (kanamycin 40 μg/mL, tetracycline 50 μg/mL, and ampicillin 100 μg/mL), and carbon sources (all from Sigma-Aldrich) as noted in the study. The growth experiments were performed aerobically.

**Measurement of C2 compounds in cyanobacteria.** For measurement of C2 compounds in cyanobacteria, cyanobacterial culture was prepared as above, and culture was allowed to grow at 37 °C in a rotary shaker (300 rpm) to an OD730 of 0.4–0.6. About 0.5 mM IPTG was added to induce gene expression at 30 °C for 6 h. Six milliliters of culture was harvested and resuspended into 2 mL fresh LB medium supplemented with 20 mM glucose, 100 mM bicarbonate, and 0.1 mM IPTG with appropriate antibiotics. Two milliliters culture (OD600 about 10) was fermented in a BD vacuum glass container capped at 37 °C for 24 h. For isolate labeling experiments, E. coli culture was prepared as stated above except using ΔGlucose-13C6 (from Santa Cruz Bio-technology, Dallas, TX.) and sodium bicarbonate 13C (from Sigma-Aldrich). To measure C2 compounds, culture was centrifuged at 15,000 g for 5 min, and supernatant was diluted for five times and filtered by Amicon 10 kDa protein filters (EMD-Amicon). Twenty microliters of sample was applied to the Agilent 1200 HPLC system with a Bio-Rad Aminex HPX87 column (30 mm 3H2SO4, 0.4 mL/min). Acetate temperature, 30 °C. Acetate detector at 210 nm. Glucose consumption was quantified by a biochemistry analyzer 2300 (YSI). Ethanol was measured by a GC-flame ionization detector (FID) (Agilent Technologies). 1-Propanol was used as the internal standard. 13C-labeled acetate (M = 2) and ethanol (M = 2) were determined by GC-MS (Agilent Technologies) as described in Bogorad et al. (2014)42.
ketoisocaprate production, the cyanobacterial culture was centrifuged at 15,000 g for 5 min. Supernatant was analyzed by the Agilent 1200 HPLC system equipped with a BioRad HPX87 column (30 mM H2SO4; 0.6 mL/min; column temperature, 30°C). Ketoisocaprate concentration was monitored by a photodiode array detector at 210 nm.

**Data analysis.** Data are presented as mean ± s.d. (standard deviation) unless otherwise indicated in figure legends. For strain growth and production assays, three biological replicates of each strain were measured.

**Data availability.** All the genes used this study are listed in Supplementary Table 9. Their sequences can be obtained by searching accession ID and the associated organism in Biocyc (https://biocyc.org/). All other relevant data are available from the authors upon request.

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Author contributions
J.C.L. designed the rGS–citrate and MCG pathways; H.Y. refined the glyoxylate recycling pathway; H.Y. and J.C.L. wrote the manuscript; H.Y. performed the experiments of the pathway in E. coli; X.L. and H.Y. performed the experiments in cyanobacteria; D.S.C. contributed experiments of phosphoketolase in cyanobacteria and repeated the bicarbonate assimilation experiment; H.Y., X.L., and F.D. contributed the in vitro experiments.

Additional information
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