ATP drives direct photosynthetic production of 1-butanol in cyanobacteria

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While conservation of ATP is often a desirable trait for microbial production of chemicals, we demonstrate that additional consumption of ATP may be beneficial to drive product formation in a nonnatural pathway. Although production of 1-butanol by the fermentative coenzyme A (CoA)-dependent pathway using the reversal of β-oxidation exists in nature and has been demonstrated in various organisms, the first step of the pathway, condensation of two molecules of acetyl-CoA to acetoacetyl-CoA, is thermodynamically unfavorable. Here, we show that artificially engineered ATP consumption through a pathway modification can drive this reaction forward and enables for the first time the direct photosynthetic production of 1-butanol from cyanobacteria *Synechococcus elongatus* PCC 7942. We further demonstrated that substitution of bifunctional aldehyde/alcohol dehydrogenase (AdhE2) with separate butyraldehyde dehydrogenase (Bldh) and NADPH-dependent alcohol dehydrogenase (YqhD) increased 1-butanol production by 4-fold. These results demonstrated the importance of ATP and cofactor driving forces as a design principle to alter metabolic flux.

biofuel | malonyl-CoA | metabolic engineering | synthetic biology

Biological production of chemicals and fuel from renewable resources is an attractive approach to a sustainable future. In particular, 1-butanol has received increasing attention because it is a potential fuel substitute and an important chemical feedstock. 1-Butanol can be produced by two distinct routes: the coenzyme A (CoA)-dependent pathway (1, 2) and the keto acid pathway (3–5). The CoA-dependent pathway follows the chemistry of β-oxidation in the reverse direction, in which acetyl-CoA is condensed to form acetoacetyl-CoA and then reduced to 1-butanol. The keto acid pathway utilizes 2-ketobutyrate as an intermediate, which goes through keto acid chain elongation to 2-ketovalarate using the leucine biosynthesis enzymes. 2-Ketovalarate is then decarboxylated and reduced into 1-butanol. In each case, the pathway can be extended to produce 1-hexanol and other higher alcohols (6–8).

The CoA-dependent reverse β-oxidation is a natural fermentation pathway used by *Clostridium* species (9–11) and has been transferred to various recombinant heterotrophs, resulting in 1-butanol titers ranging from 2.5 mg/L to 1.2 g/L with glucose as the substrate (12–16). One of the challenges in transferring this pathway to other organisms lies in the hydrogenation of crotonyl-CoA to butyryl-CoA catalyzed by the butyryl-CoA dehydrogenase/electron transferring flavoprotein (Bcd/EtfAB) complex. Bcd/EtfAB complex is difficult to express in recombinant systems, is presumably oxygen sensitive (12, 17), and possibly requires reduced ferredoxin as the electron donor (18). This difficulty was overcome by expressing trans-2-enoyl-CoA reductase (Ter) (19, 20), which is readily expressed in *Escherichia coli* and directly reduces crotonyl-CoA using NADH. This modified 1-butanol pathway (Fig. 1; outlined in blue) is catalyzed by five enzymes: thiolase (AtoB), 3-hydroxybutyryl-CoA dehydrogenase (Hbd), crotonase (Crt), Ter, and bifunctional aldehyde/alcohol dehydrogenase (AdhE2). Simultaneously expressing these enzymes and engineering NADH and acetyl-CoA accumulation as driving forces, 1-butanol production with a high titer of 15 g/L and 88% of theoretical yield has been achieved using *E. coli* in flasks without product removal (19). This result demonstrates the feasibility of transferring the CoA-dependent pathway to nonnative organisms for high-titer 1-butanol fermentation from glucose.

However, the success of the CoA-dependent pathway in *E. coli* is not directly transferrable to photoautotrophs. By expressing the same enzymes in cyanobacteria *Synechococcus elongatus* PCC 7942, photosynthetic 1-butanol production from CO_2 was barely detectable (21). 1-Butanol production was achieved by this strain only when internal carbon storage made by CO_2 fixation in light conditions was fermented under anoxic conditions (21). We hypothesized that both the acetyl-CoA and NADH pools in this organism under photosynthetic conditions may be insufficient to drive 1-butanol formation. Acetyl-CoA is the precursor for fermentation pathway and the TCA cycle, both of which are not active in light conditions. Furthermore, photosynthesis generates NADPH, but not NADH, and the interconversion between the two may not be efficient enough. Without a significant driving force against the unfavorable thermodynamic gradient, 1-butanol production cannot be achieved. The difficulty of direct photosynthetic production of 1-butanol is in sharp contrast to the production of isobutanol (450 mg/L) and isobutyraldehyde (1,100 mg/L) by *S. elongatus* PCC 7942 (22), which has an irreversible decarboxylation step as the first committed reaction to drive the flux toward the products. This difference suggests the importance of driving forces in altering the direction of metabolic flux.

We reason that instead of the acetyl-CoA pool, ATP may be used to drive the thermodynamically unfavorable condensation of two acetyl-coA molecules under photosynthetic conditions. Thus, we engineered the ATP-driven malonyl-CoA synthesis and decarboxylative carbon chain elongation used in fatty acid synthesis to drive the carbon flux into the formation of acetoacetyl-CoA, which then undergoes the reverse β-oxidation to synthesize 1-butanol. We further replaced the subsequent NADH-dependent enzymes with NADPH-dependent ones and successfully achieved 1-butanol synthesis under photosynthetic conditions. In theory, excess ATP consumption in the cell might cause a decrease in biomass. Thus, with notable exceptions (23–26), most metabolic engineering design do not choose to increase ATP consumption. Although many natural examples of microbes using ATP to drive reactions, most of them are highly regulated. Therefore, it is unpredictable whether it is feasible to use ATP consumption to push flux in a nonnative pathway, for which no regulation exists.

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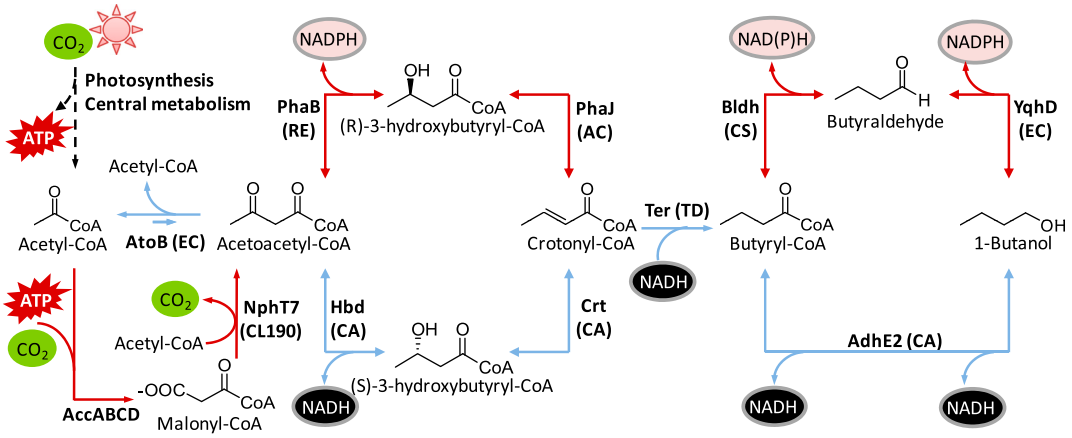


Fig. 1. Variations in the CoA-dependent 1-butanol pathway. The fermentative CoA 1-butanol pathway is in blue. Alternative routes are in red. EC, *E. coli*; RE, *R. eutropha*; CA, *C. acetobutylicum*; AC, *A. caviae*; TD, *T. denticola*; CS, *C. saccharoperbutylacetonicum* N1-4; *CL190*, *Streptomyces* sp. strain CL190.

Results

Incorporating an ATP Driving Force in 1-Butanol Pathway Design. The thiolase-mediated condensation of two acetyl-CoA molecules is reversible but strongly favors the thiolysis of acetoacetyl-CoA. To examine the thermodynamic property of this reaction, we overexpressed and purified *E. coli* thiolase (AtoB) and used an in vitro assay to determine its equilibrium constant and $\Delta G^{\circ\prime}$. The result showed that the condensation reaction is unfavorable (Fig. 2) with equilibrium constant (K_{eq}) of $(1.1 \pm 0.2) \times 10^{-5}$ at pH 8.0, within the optimum pH range for cyanobacteria (27). This experimentally determined K_{eq} approximately corresponds to a $\Delta G^{\circ\prime}$ of 6.8 kcal/mol, consistent with the literature reported K_{eq} using partially purified thiolase from pig heart protein homogenate (28). Therefore, without a sufficiently large acetyl-CoA pool or an efficient product trap, there is no driving force for the formation of acetoacetyl-CoA. Although the irreversible hydrogenation of crotonyl-CoA catalyzed by Ter provides a driving force, it is insufficient to drive the reaction forward without large pools of acetyl-CoA and reducing equivalent (19).

Instead of using the direct condensation of acetyl-CoA, we contemplated an alternative route through the ATP-driven malonyl-CoA synthesis. Malonyl-CoA is synthesized from acetyl-CoA, HCO_3^- and ATP by acetyl-CoA carboxylase (Acc). The formation of malonyl-CoA is effectively irreversible due to ATP hydrolysis. In fatty acid synthesis, malonyl-CoA is then converted into malonyl-acyl carrier protein (malonyl-ACP) and acts as the carbon addition unit for fatty acid synthesis. For 1-butanol synthesis, malonyl-CoA can react with acetyl-coA in a decarboxylative condensation to form acetoacetyl-CoA, in a reaction analogous to ketoacyl-ACP synthase III (KAS III) that catalyzes the irreversible condensation of malonyl-ACP and acetyl-CoA to synthesize the four carbon intermediate 3-ketobutyryl-ACP.

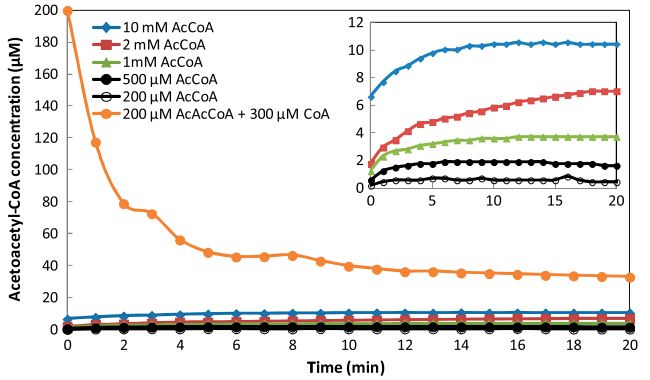


Fig. 2. Determination of equilibrium concentrations for the thiolase (AtoB) mediated reaction. The equilibrium constant (K_{eq}) was determined from the equilibrium concentrations. *E. coli* AtoB was cloned, purified, and used in an in vitro assay. AcCoA, acetyl-CoA; AcAcCoA, acetoacetyl-CoA; CoA, coenzyme A. Detailed conditions and methods are listed in *SI Text*.

We note that the energy release from ATP hydrolysis ($\Delta G^{\circ\prime}$ of −7.3 kcal/mol) would compensate for the energy required for condensation of acetyl-CoA into acetoacetyl-CoA. By combining the reaction catalyzed by thiolase with ATP hydrolysis (Fig. S1), the net reaction is thermodynamically favorable ($\Delta G^{\circ\prime} < 0$), which would reduce the need for high concentration of acetyl-CoA required to push the reaction forward. More importantly, CO_2 released from the second step, decarboxylative chain elongation, shifts the reaction toward the formation of acetoacetyl-CoA. Fatty acid and polyketide syntheses have naturally evolved this mechanism to enable the thermodynamically unfavorable formation of 3-ketoacyl-ACP. By taking advantage of this mechanism, it is possible to push the carbon flux to the 1-butanol pathway without the acetyl-CoA driving force artificially constructed in *E. coli* (19).

Expression of Acetoacetyl-CoA Synthase Enables Photosynthetic Production of 1-Butanol. Therefore, we bioprospected for a KAS III that utilizes malonyl-CoA rather than malonyl-ACP for condensation with acetyl-CoA. Because both ACP and CoA carry phosphopantetheine, which forms thioester bond with the malonyl moiety, KAS III and KAS III-like enzymes may also react with malonyl-CoA. We cloned a variety of KAS III and KAS III-like enzymes from different organisms and examined their expression in *E. coli* (Fig. S2). After His-tag purification, we assayed their activity for condensing malonyl-CoA with acetyl-CoA (Table 1). Among the enzymes tested, NphT7 (29) was the most active. Other enzymes such as Bamb6244, GOX0115, and PAE-FabH2 were also active while the rest showed no detectable activity. The condensation reaction (Fig. S3) catalyzed by NphT7 using malonyl-CoA and acetyl-CoA is irreversible and accumulates acetoacetyl-CoA as the product. At low starting concentrations of malonyl-CoA, conversion yield to acetoacetyl-CoA is higher than high starting substrate concentrations. This result is likely due to the fact that NphT7 also catalyzes malonyl-CoA self condensation and is particularly useful for 1-butanol synthesis, because both malonyl-CoA and acetyl-CoA pools are expected to be low in *S. elongatus* PCC 7942.

Next, we constructed a plasmid harboring genes *nphT7*, *hbd*, *crt*, and *adhE2* under an IPTG inducible promoter P_{LlacO1} with Neutral Site II (NSII) recombination sequences flanking the genes and kanamycin resistance marker (Fig. 3). By DNA homologous recombination, we integrated these genes into the genome of *S. elongatus* strain EL9 [expressing *ter* at Neutral Site I (NSI)

Table 1. Specific activities of acetoacetyl-CoA synthases (μmol/ min /mg)

Enzyme	Specific activity	Enzyme	Specific activity
Burkholderia ambifaria BAMB6244	0.0116 ± 0.0002	*Pseudomonas aeruginosa* PAE-FabH2	0.0140 ± 0.0010
Gluconobacter oxydans GOX0115	0.0099 ± 0.0011	*Streptomyces avermitilis* SAV-FabH4	ND
Helicobacter pylori HP0202	ND	*Streptomyces coelicolor* SCO5858	ND
Listeria monocytogenes LMO2202	ND	*Streptomyces sp. strain CL190* NphT7	6.02 ± 0.25

under the control of another IPTG inducible promoter P_{trc}] at NSII and selected for successful transformant on kanamycin containing BG-11 plates. The successful transformant strain EL20 was then analyzed for in vitro enzyme activity and 1-butanol production. As shown in (Fig. 4*A*), crude extract from strain EL20 catalyzed the formation of acetoacetyl-CoA by condensation of malonyl-CoA and acetyl-CoA and did not catalyze the thiolysis of acetoacetyl-CoA (Fig. 4*B*). On the other hand, crude extract from strain EL14 expressing *atoB* along with *hbd*, *crt*, *ter*, and *adhE2* catalyzed thiolysis (Fig. 4*B*) much more efficiently than the condensation reaction (Fig. 4*A*). The two strains EL20 and EL14 exhibited nearly identical growth rate (Fig. 4*C*). However, Strain EL20 produced 6.5 mg/L (Fig. 4*D*) of 1-butanol while Strain EL14 produced only barely detectable amounts (detection limit of about 1 mg/L) of 1-butanol (Fig. 4*D*). This result indicated that ATP-driven acetoacetyl-CoA formation is required for photosynthetic production of 1-butanol using the CoA-dependent pathway.

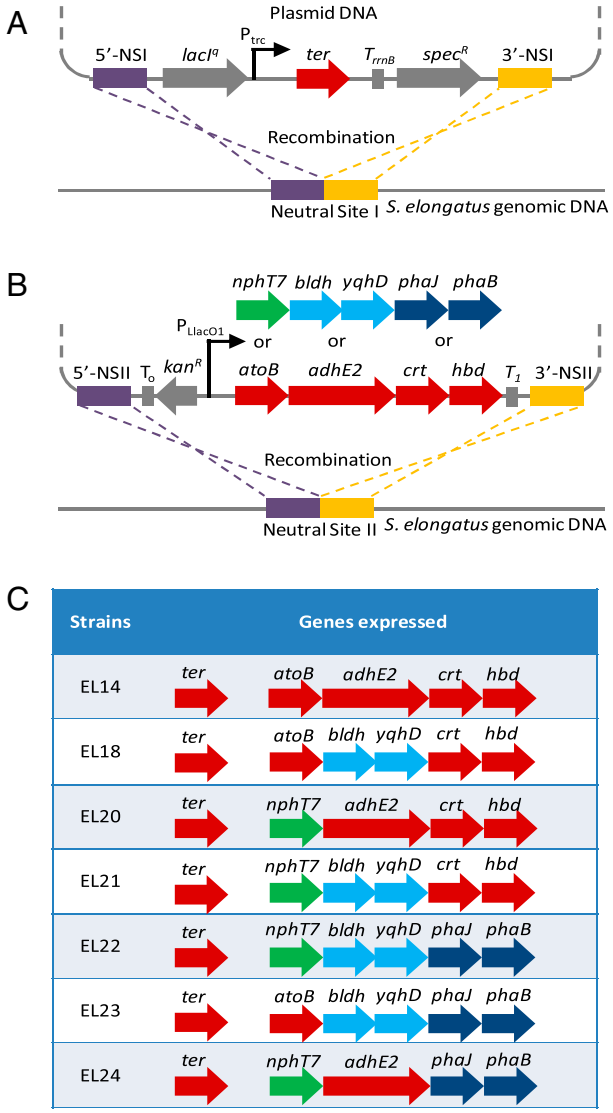


Fig. 3. Schematic representation of recombination to integrate (*A*) *ter* at NSI, (*B*) *atoB*, *adhE2*, *crt*, and *hbd* at NSII in the genome of *S. elongatus*. Different combinations of alternative genes *nphT7*, *bldh*, *yqhD*, *phaJ*, and *phaB* can replace their counterpart enzymes to recombine into NSII. (*C*) List of strains with different combinations of overexpressed genes used in this study.

Substitution of NADPH Utilizing Enzymes Aids 1-Butanol Production. Another useful driving force in 1-butanol synthesis is the reducing equivalent (19). Cyanobacteria produce NADPH as the direct result of photosynthesis. Intracellular NAD^+ and $NADP^+$ levels exist in a ratio of about 1 : 10 (30) in *S. elongatus* PCC 7942. Thus NADH utilizing pathway may be unfavorable in cyanobacteria. To synthesize 1 mol of 1-butanol from acetoacetyl-CoA requires 4 mol of NADH. Therefore, changing the cofactor preference to NADPH may aid the production of 1-butanol.

As depicted in Fig. 1 (outlined in red), we identified enzymes that utilize NADPH or both NADPH and NADH by bioprospecting. Acetoacetyl-CoA reductase (PhaB) (31) was used to replace Hbd. PhaB from *Ralstonia eutropha* is an enzyme found in the poly-hydroxyalkanoate biosynthetic pathway for reducing 3-ketobutyryl-CoA to 3-hydroxybutyryl-CoA using NADPH. However, PhaB produces the (R)-stereoisomer of 3-hydroxybutyryl-CoA instead of the (S)-stereoisomer produced by Hbd. As a result, Crt cannot be used for the subsequent dehydration to produce crotonyl-CoA. Therefore, a different crotonase is necessary for dehydration of (R)-3-hydroxybutyryl-CoA. (R)-specific enoyl-CoA hydratase (PhaJ) (32) is found in *Aeromonas caviae* and dehydrates (R)-3-hydroxybutyryl-CoA into crotonyl-CoA. Together, PhaB and PhaJ can replace Hbd and Crt, respectively,

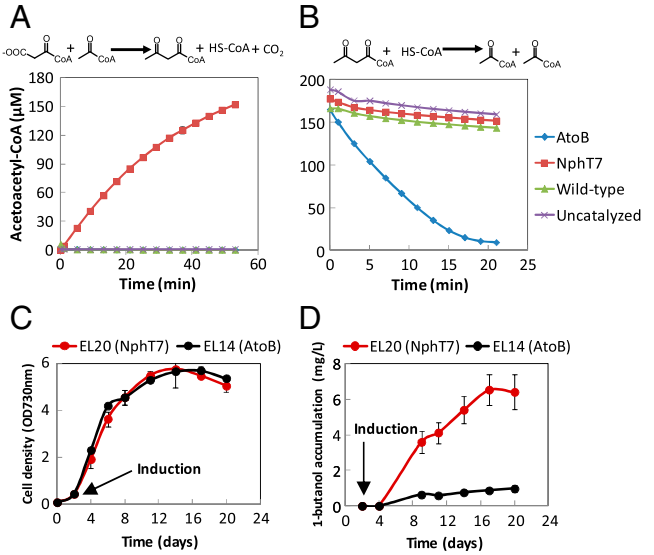


Fig. 4. (*A*) In vitro assay for the synthesis of acetoacetyl-CoA using crude extracts of wild-type *S. elongatus* PCC 7942, strain EL14 and EL20. (*B*) In vitro assay for the thiolysis of acetoacetyl-CoA using crude extracts of wild-type *S. elongatus PCC* 7942, strain EL14 and EL20. (*C*) Cell density and (*D*) 1-butanol accumulation as a function of time of strain EL14 and EL20. 1-Butanol production by strain EL14 was near detection limit of about 1 mg/L.

to change the cofactor preference of 3-ketobutyryl-CoA reduction to NADPH.

To replace AdhE2, NADP-dependent alcohol dehydrogenase (YqhD) (33) from *E. coli* has been demonstrated to aid the production of higher chain alcohols (22). In addition, we needed a CoA-acylating butyraldehyde dehydrogenase (Bldh) to replace the aldehyde dehydrogenase function of AdhE2. We thus bioprospected for enzymes catalyzing reduction of butyryl-CoA to butyraldehyde. Bldh was found in high butanol producing *Clostridium* species including *C. beijerinckii* NCIMB 8052 (34), *C. saccharobutylicum* ATCC BAA-117, and *C. saccharoperbutylacetonicum* NI-4 (35). In particular, Bldh from *C. beijerinckii* has been purified and demonstrated activity in vitro with both NADH and NADPH as a reducing cofactor.

Using the sequence of *C. beijerinckii* Bldh, we searched by homology and cloned additional Bldh-like enzymes from various organisms including *C. saccharoperbutylacetonicum* NI-4, *C. saccharobutylicum* ATCC BAA-117, *Geobacillus thermoglucosidasius*, *Clostridium kluyveri*, and *E. coli*. We assessed the performance of these Bldh's by 1-butanol production in recombinant *E. coli*. As shown in Fig. 5, the *E. coli* strain expressing *C. saccharoperbutylacetonicum* NI-4 Bldh along with rest of the CoA 1-butanol pathway produced the highest titer of 1-butanol, exceeding the 1-butanol produced by *E. coli* strain expressing AdhE2 by nearly threefold.

To test the effect of cofactor utilization, we constructed various combinations of different routes by overexpressing different genes in *S. elongatus* PCC 7942 (Fig. 3*C*). We constructed plasmids containing different genes and recombined them into the genome of *S. elongatus* strain EL9. We then assayed the activity of overexpressed enzymes to confirm expression (Fig. 6). Of the strains tested, strain EL22 expressing the NADPH utilizing enzymes produced the highest amount of 1-butanol (29.9 mg/L) exceeding that of EL20 (6.4 mg/L) by more than fourfold. This result reinforced the importance of cofactor as driving force.

Discussion

In a metabolic system involving multiple pathways, the direction and rate of each reaction are determined by kinetics, regulated by the enzyme expression levels and metabolite pool sizes. Typically, a reaction with a large positive $\Delta G^{\circ\prime}$ is considered practically unfeasible in the forward direction because it requires high concentrations of the substrate pool to drive the reaction forward. The condensation of two molecules of acetyl-CoA to acetoacetyl-CoA is such an example. On the other hand, the reverse direction, thiolysis of acetoacetyl-CoA, is readily achievable and used as the last step in the β-oxidation. However, some fermentative organisms, such as *Clostridium* species, use direct acetyl-CoA condensation for 1-butanol synthesis. These organisms accomplish this thermodynamically unfavorable reaction presumably through a large pool of acetyl-CoA and high reducing equivalents that drive the subsequent reactions. This situation was recreated in *E. coli* expressing the enzymes for 1-butanol synthesis (19). Unfortunately, this strategy cannot be readily implemented in photosynthetic organisms for multiple reasons: Acetyl-CoA is a precursor for fermentative pathways and the oxidative TCA cycle, which are not active under photosynthetic conditions. Thus, the acetyl-CoA pool size is not expected to be high and is difficult to modulate. Instead of using high acetyl-CoA pool as a driving force, here ATP and the evolution of CO_2 effectively drive the reactions toward 1-butanol synthesis. This strategy is used in fatty acid synthesis in nature, and is used to couple the fatty acid synthesis to the energy status in the cell.

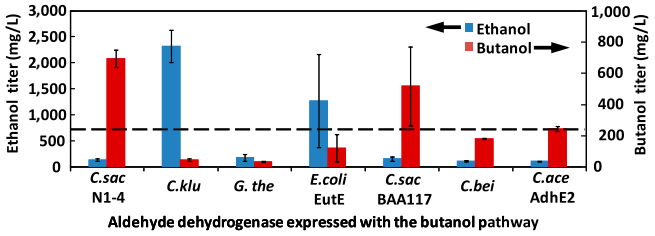


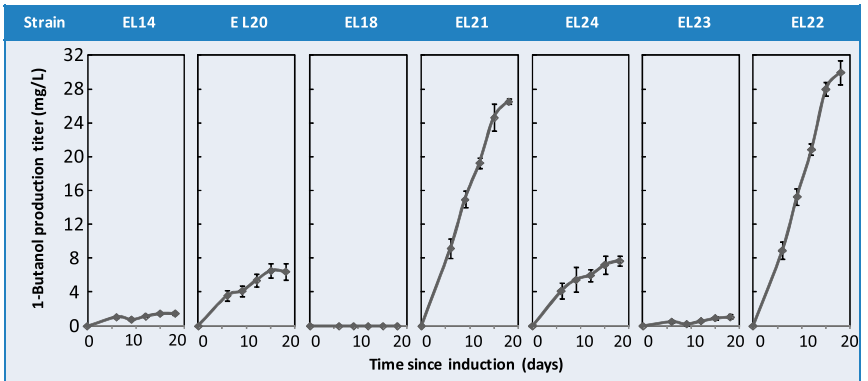
Fig. 5. Production of 1-butanol and ethanol by recombinant *E. coli* strains JCL299 expressing CoA-dependent 1-butanol pathway with YqhD from *E. coli* and Bldh from different organisms. Dashed line represents the baseline production by using AdhE2. Detailed production procedure is listed in *SI Text*.

ATP consumption has been used by cells to drive various thermodynamically unfavorable reactions. In engineered *E. coli*, ATP consumption has been used to stimulate glycolysis by futile cycling (23, 24) or by deletion of membrane-coupling subunits in (F_1F_0)-ATP synthase (25). Increased ATP consumption by overexpressing enzymes that promote malonyl-CoA biosynthesis also increased production yield of compounds downstream of malonyl-CoA (26). However, additional ATP consumption in heterologous pathways may cause adverse effects in the cell and may result in reduced biomass formation. As such, most metabolic engineering design models have primarily focused on maximizing carbon yield and minimizing ATP expenditure. Here we provide a distinct example using ATP to alter the thermodynamics of the CoA-dependent 1-butanol pathway, which naturally does not require additional ATP consumption. By incorporating an ATP-dependent step into the CoA-dependent 1-butanol synthesis pathway, we demonstrated for the first time the direct photosynthetic production of 1-butanol from CO_2.

Direct photosynthetic 1-butanol production from CO_2 is desirable because it reduces the number of processing steps. *S. elongatus* PCC 7942 is naturally competent and therefore is an attractive model organism for engineering. The DNA recombination method used in this study has also been broadly practiced for engineering cyanobacteria for the production of various chemicals. By metabolic engineering, cyanobacteria has also enabled the production of Isobutyraldehyde (1,100 mg/L) (22), isobutanol (450 mg/L) (22), ethanol (550 mg/L) (36), ethylene (451 nl/nl/h/OD_{730}) (37), isoprene (0.05 mg/g dry cell weight) (38), sugars (45 mg/L) (39), lactic acid (56 mg/L) (39), fatty alcohols (0.2 mg/L) (40), and fatty acids (194 mg/L) (41) from CO_2. The pathways for the relatively high production of isobutyraldehyde, isobutanol and ethanol naturally involve a decarboxylation step as the first committed reaction. The loss of CO_2 is considered irreversible and serves as a driving force to the product formation. Our result is also consistent with this phenomenon that decarboxylation aids in directing the carbon flux.

Reducing cofactor preference is another important aspect of pathway design. Depending on the production condition and organisms' natural metabolism, changing cofactor preference is necessary to achieve high flux production. For example, changing NADPH-dependent enzymes into NADH-dependent increases the isobutanol productivity and yield under anaerobic condition in recombinant *E. coli* (42). Replacing the Bcd-EtfAB complex that requires an unknown electron donor with an NADH-dependent Ter is helpful in 1-butanol production (19, 20). In contrast, pathways utilizing NADPH are preferred in cyanobacteria because NADPH is more abundant. By utilizing NADPH-dependent enzymes, our 1-butanol production enhanced from 6.5 mg/L to 29.9 mg/L (Fig. 6).

Current limitation of our 1-butanol production using cyanobacteria may be the synthesis of malonyl-CoA. Compared to the high flux production of isobutanol and isobutyraldehyde in cyanobacteria, the carbon flux through our 1-butanol pathway is suboptimal. Malonyl-CoA biosynthesis is considered as the limiting step in fatty acid synthesis (43). Therefore, increasing carbon flux toward the synthesis of acetyl-CoA and malonyl-CoA may be necessary to increase 1-butanol production. Intracellular acetyl-CoA and malonyl-CoA supply may be increased by increasing CoA biosynthesis (44), overexpression of Acc (45–48), glycolytic



Enzyme Specific activity (μmol/min/mg)							
AtoB	0.73±0.01	N/A	0.62±0.04	N/A	N/A	0.69±0.01	N/A
NphT7	N/A	0.019± 0.001	N/A	0.022± 0.001	0.024± 0.001	N/A	0.021± 0.001
Hbd (NADH)	0.31±0.01	0.22±0.01	0.16±0.01	0.21±0.01	N/A	N/A	N/A
PhaB (NADPH)	N/A	N/A	N/A	N/A	0.030± 0.005	0.007± 0.003	0.014± 0.005
Crt (PhaJ)	41.2±0.7	41.3±0.3	23.6±2.7	39.8±3.3	30.7±5.1	13.7±0.8	25.0±1.8
Ter	0.71±0.14	1.12±0.31	1.25±0.51	1.25± 0.043	1.10±0.20	0.91±0.70	1.18±0.56

Fig. 6. 1-Butanol production and enzyme activity of strains expressing different enzymes. Strain genotype is listed in Fig. 3C. Expression of *nphT7* enables direct photosynthetic production of 1-butanol under oxygenic condition. Strains EL21 and EL22 expressing *bldh* and *yqhD* achieved the highest production.

enzymes such as phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (26), and inhibition of fatty acid biosynthesis (49). With these approaches, the 1-butanol production in cyanobacteria may be further improved.

Materials and Methods

For details, see *SI Text*.

Culture Medium and Condition. All *S. elongatus* PCC 7942 strains were grown on modified BG-11 (1.5 g/L $NaNO_3$, 0.0272 g/L $CaCl_2{\cdot}2H_2O$, 0.012 g/L ferric ammonium citrate, 0.001 g/L Na_2EDTA, 0.040 g/L K_2HPO_4, 0.0361 g/L $MgSO_4{\cdot}7H_2O$, 0.020 g/L Na_2CO_3, 1,000× trace mineral (1.43 g H_3BO_3, 0.905 g/L $MnCl_2{\cdot}4H_2O$, 0.111 g/L $ZnSO_4{\cdot}7H_2O$, 0.195 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.0395 g $CuSO_4{\cdot}5H_2O$, 0.0245 g $Co(NO_3)2{\cdot}6H_2O$), 0.00882 g/L sodium citrate dihydrate (50)) agar (1.5% w/v) plates. All *S. elongatus* PCC 7942 strains were cultured in BG-11 medium containing 50 mM $NaHCO_3$ in 250 mL screw cap flasks. Cultures were grown under 100 $\mu E/s/m^2$ light, supplied by four Lumichrome F30W-1XX 6500K 98CRI light tubes, at 30 °C. Cell growth was monitored by measuring OD_{730} with Beckman Coulter DU800 spectrophotometer.

Production of 1-Butanol. A loopful of *S. elongatus* PCC 7942 was used to inoculate fresh 50 mL BG-11. 500 mM IPTG was used to induce the growing culture at cell density $OD_{730\,nm}$ of 0.4 to 0.6 with 1 mM IPTG as final concentration. 5 mL of growing culture was sampled for cell density and 1-butanol production measurements every 2 d for up to day 8 after which sampling time was switched to every 3 d. After sampling, 5 mL of fresh BG-11 with 500 mM $NaHCO_3$, appropriate antibiotics, and IPTG were added back to the culture. Method for 1-butanol quantification is listed in *SI Text*.

Enzyme Assays. Thiolase activity was measured via both condensation and thiolysis direction. The enzymatic reaction was monitored by the increase or decrease of absorbance at 303 nm. Acetoacetyl-CoA synthase activity was measured by monitoring the increase of absorbance at 303 nm, which corresponds to appearance of acetoacetyl-CoA. For details, see *SI Text*.

Alcohol Production by *E. coli* Expressing Butyraldehyde Dehydrogenase. Transformed *E. coli* strain JCL299 (Δ*adhE*, Δ*ldhA*, Δ*frd*, Δ*pta*) expressing different Bldh and the CoA-dependent pathway were cultured in Terrific broth (TB; 12 g tryptone, 24 g yeast extract, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, 4 mL glycerol per liter of water) supplemented with 20 g/L glucose. Fermentation was conducted for 2 d, after which samples were taken for measurement of 1-butanol concentration. For details, see *SI Text*.

DNA Manipulations. For details, see *SI Text*.

Plasmid Constructions. For details, see *SI Text*.

Strain Construction and Transformation. For details, see *SI Text*.

Protein Purification and SDS/PAGE. For details, see *SI Text*.

1-Butanol Quantification. For details, see *SI Text*.

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