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Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde

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Global climate change has stimulated efforts to reduce CO_2 emissions. One approach to addressing this problem is to recycle CO_2 directly into fuels or chemicals using photosynthesis. Here we genetically engineered *Synechococcus elongatus* PCC7942 to produce isobutyraldehyde and isobutanol directly from CO_2 and increased productivity by overexpression of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). Isobutyraldehyde is a precursor for the synthesis of other chemicals, and isobutanol can be used as a gasoline substitute. The high vapor pressure of isobutyraldehyde allows *in situ* product recovery and reduces product toxicity. The engineered strain remained active for 8 d and produced isobutyraldehyde at a higher rate than those reported for ethanol[1], hydrogen[2] or lipid[3] production by cyanobacteria or algae. These results underscore the promise of direct bioconversion of CO_2 into fuels and chemicals, which bypasses the need for deconstruction of biomass.

According to the US Energy Information Administration, world energy–related CO_2 emissions in 2006 were 29 billion metric tons, an increase of 35% from 1990 (ref. 4). As a result of human activity, atmospheric levels of CO_2 have increased by ~25% over the past 150 years[5]. Thus, it has become increasingly important to develop new technologies to reduce CO_2 emissions.

Photosynthetic organisms use solar energy to generate reducing equivalents and incorporate atmospheric CO_2 into organic molecules. Currently, cellulosic biofuels[6] and algal biodiesels[7] are prominent biological approaches to sequester and convert CO_2. Producing biodiesel from algae has been proposed as one of the most efficient ways of generating biofuels[7] because the lipid productivity of many algae greatly exceeds that of the best cellulosic ethanol production. However, these approaches involve several intermediate stages for recycling CO_2 into usable fuels or chemicals, which increases production costs. Many cyanobacteria and algae have the ability to produce hydrogen, which can be regarded as a way to recycle CO_2 indirectly[8]. However, the volumetric hydrogen productivity reported is still relatively low[2]. Anaerobic hydrogen production using anaerobic photosynthetic bacteria requires a hydrogen source other than water[9,10], and the productivity remains to be improved[8].

Another approach is direct conversion of CO_2 to fuels[11] or chemicals[12]. Previously, the photosynthetic bacteria *Rhodobacrer sphaeroides* and *Rhodobacrer capsulatus* were engineered to produce ethanol from CO_2 and a non-water hydrogen source, with a final titer of ~800 mg/l[11]. Ethanol has also been produced from CO_2 and water using an engineered *S. elongatus* strain at a rate of 0.18 $\mu g\ l^{-1}\ hr^{-1}$ (ref. 1). Although these efforts demonstrated the biochemical feasibility of direct ethanol production from CO_2, the cost of separating ethanol at low concentration remains a formidable problem.

Product-recovery efficiency is an important determinant of the total production cost and a major limitation of biochemical production from photosynthetic organisms. In this work, we chose isobutyraldehyde as a target because it has a low boiling point (63 °C) and a high vapor pressure (66 mm Hg at 4.4 °C), suggesting that it can be readily stripped from microbial cultures during production. Subsequent purification is also relatively easy, and the isobutyraldehyde concentration in the production medium can remain low. Product removal *in situ* can reduce the cytotoxic effects of isobutyraldehyde and enable long-term production. In addition, isobutyraldehyde can be readily converted to various hydrocarbons currently derived from petroleum (such as isobutanol, isobutyric acid, acetal, oxime and imine) using existing chemical catalysis (**Fig. 1**). In particular, it can be converted either biologically or chemically to isobutanol, which is suitable for use as a gasoline substitute, thus making isobutyraldehyde a useful chemical feedstock.

Productivity is a key determinant for the feasibility of direct CO_2 conversion to chemicals or fuels. As biodiesel production from algae is considered one of the most efficient ways of generating biofuels[7], we estimated the required productivity of isobutyraldehyde by comparison with biodiesel production. The average productivity of microalgal biodiesel in a well-designed production system can be $\sim 1 \times 10^5$ liter ha^{-1} per year (ref. 7). Assuming that one-third of the day (8 h) and 80% of the calendar year (292 d) are dedicated to production and a characteristic dimension of 1 m for converting areal to volumetric productivity, the productivity of isobutyraldehyde would need to be 3,420 $\mu g\ l^{-1}\ h^{-1}$ or higher to be competitive with algal biodiesel production. This level of productivity serves as our initial benchmark productivity for direct conversion of CO_2 to isobutyraldehyde.

To achieve this goal, we engineered a cyanobacterium, *S. elongatus*. The ketoacid decarboxylase gene *kivd* from *Lactococcus lactis*[13] was expressed using an expression cassette under the control of the isopropyl-β-D-thiogalactoside (IPTG) inducible promoter *P*trc (**Fig. 1**). This DNA fragment was integrated into neutral site I (NSI)[14] by homologous recombination[15], resulting in SA578 (**Fig. 2a**). To increase the flux to the keto acid precursor, 2-ketoisovalerate (KIV)[16], we integrated the *alsS* gene from *Bacillus subtilis* and the *ilvC* and *ilvD* genes from *Escherichia coli* into neutral site II (NSII)[17] of the SA578 genome, resulting in SA590

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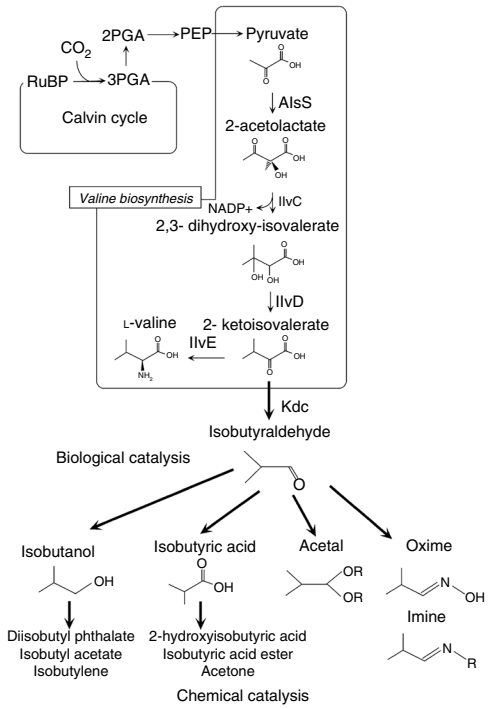

Figure 1 The pathway for isobutyraldehyde production. The Kdc-dependent synthetic pathway for isobutyraldehyde production. AlsS, acetolactate synthase; IlvC, acetohydroxy acid isomeroreductase; IlvD, dihydroxy-acid dehydratase; Kdc, 2-ketoacid decarboxylase.

(**Fig. 2b**). All three enzyme assays of SA590 lysates demonstrated higher activity than those from SA578 (**Fig. 2c**), indicating that *alsS* (*B. subtilis*), *ilvC* (*E. coli*) and *ilvD* (*E. coli*) are expressed and functional in *S. elongatus*. Because the vapor pressure of isobutyraldehyde is relatively high, it can be removed readily from the culture medium during production by the bubbling of air. Evaporated isobutyraldehyde was then condensed with a Graham condenser.

The strain was cultured in a Roux culture bottle at 30 °C. Isobutyraldehyde concentrations in the culture medium and the trap were measured as described in Online Methods (**Fig. 2**). The trap was refreshed daily. The strain produced 723 mg/l isobutyraldehyde in 12 d with an average production rate of 2,500 μg l^{-1} h^{-1} (**Fig. 2d,e**). This number is very encouraging, as it is already close to our benchmark. The isobutyraldehyde production rate remained constant for the first 9 d, but the production rate decreased dramatically after the tenth day (**Fig. 2e**) for unknown reasons. When the culture was resuspended in fresh medium after 10 d, the bacteria regained their productivity (~60 mg l^{-1} d^{-1}), suggesting that some inhibitory metabolites[18] accumulated during the cultivation. As expected, during the production process the isobutyraldehyde concentration in the culture medium remained low, around 20 mg/l (**Fig. 2f**). This low concentration would reduce toxicity to cells and prolong the production phase. This strain did not produce isobutanol, indicating that endogenous alcohol dehydrogenase (ADH) activity toward isobutyraldehyde was not detectable.

The enzyme that catalyzes the CO_2 fixation reaction in the Calvin-Benson-Bassham (CBB) cycle is Rubisco[19], which is implicated as the limiting step in CO_2 fixation because of its poor turnover rate and the competition between O_2 and CO_2 at the active site[20]. To compensate for the inherent limitations of Rubisco, the *rbcLS* genes from the related *S. elongatus* strain PCC6301 (ref. 21) were integrated downstream of the endogenous *rbcLS* genes of SA590, resulting in SA665 (**Fig. 3a**). The Rubisco activity of SA665 was found to be ~1.4-fold higher than that of SA590 (**Fig. 3b**). The strain was cultured in BG-11 medium with 50 mM $NaHCO_3$ at 30 °C. With the expression of the additional *rbcLS* genes, SA665 produced 1.1 g/l of isobutyraldehyde over 8 d with a steady state (first 7 d) production rate of 6,230 μg l^{-1} h^{-1}(**Fig. 3c–f**), which is roughly twofold higher than SA590, which lacks the additional *rbcLS* genes. The increase in Rubisco activity of SA665 and the concomitant increase in isobutyraldehyde production (**Fig. 3b,c**) suggest that CO_2 fixation is one of the bottlenecks of the isobutyraldehyde production. Thus, it is possible that the optimization of CO_2 fixation could further improve isobutyraldehyde production. To probe the impact of Rubisco overexpression, photosynthetic O_2 production of SA590 and SA665 were measured under the same conditions as isobutyraldehyde

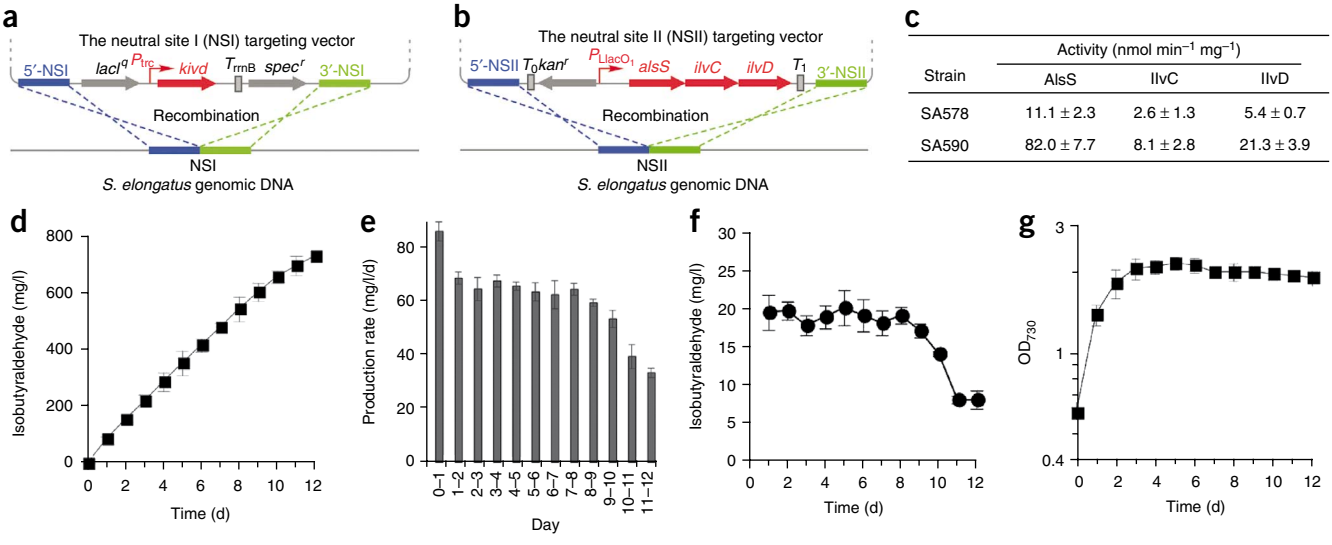

Figure 2 Isobutyraldehyde production from cyanobacteria. (**a**,**b**) Schematic representation of recombination to integrate *kivd* (**a**) *alsS*, *ilvC* and *ilvD* (**b**) genes into the *S. elongatus* chromosome. (**c**) Specific activities of AlsS, IlvC and IlvD in cell extracts of SA578 (with integrated *kivd* only) and SA590 (with integrated *kivd, alsS*, *ilvC* and *ilvD*). Detailed methods and unit definitions of enzyme assays are described in Online Methods. Error indicates s.d. (**d**) Cumulative production of isobutyraldehyde production by SA590. (**e**) Daily production rate of isobutyraldehyde by SA590. (**f**) Isobutyraldehyde concentration in the production culture of SA590. (**g**) Time courses for the growth of SA590. Error bars indicate s.d. (n = 3).

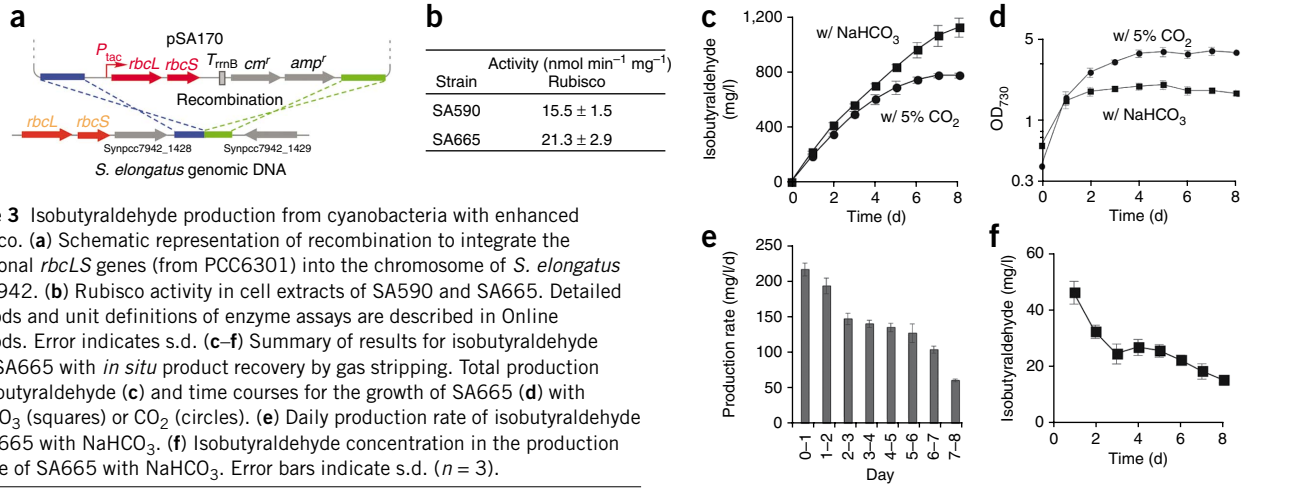

Figure 3 Isobutyraldehyde production from cyanobacteria with enhanced Rubisco. (**a**) Schematic representation of recombination to integrate the additional *rbcLS* genes (from PCC6301) into the chromosome of *S. elongatus* PCC7942. (**b**) Rubisco activity in cell extracts of SA590 and SA665. Detailed methods and unit definitions of enzyme assays are described in Online Methods. Error indicates s.d. (**c**–**f**) Summary of results for isobutyraldehyde from SA665 with *in situ* product recovery by gas stripping. Total production of isobutyraldehyde (**c**) and time courses for the growth of SA665 (**d**) with $NaHCO_3$ (squares) or CO_2 (circles). (**e**) Daily production rate of isobutyraldehyde by SA665 with $NaHCO_3$. (**f**) Isobutyraldehyde concentration in the production culture of SA665 with $NaHCO_3$. Error bars indicate s.d. ($n = 3$).

production. The O_2 production of SA665 was similar to that of SA590 (**Supplementary Fig. 1**), indicating that overexpression of the *rbcLS* genes did not enhance the rate of photosynthesis in this condition. Thus, overexpression of Rubisco either allowed more carbon fixation through more efficient utilization of photosynthesis-generated reducing power or directed more fixed carbon to isobutyraldehyde. As no other by-product was detected (see Online Methods) and the cells were not growing, the former explanation appears more plausible.

Because Rubisco is often implicated as rate-limiting for photosynthetic CO_2 fixation in plants[22], several attempts have been made to increase Rubisco activity. However, so far no approach has been found to successfully improve photosynthetic CO_2 assimilation[23,24]. The main limitations are precise regulation of the expression and inaccurate protein folding of Rubisco in heterologous organisms[23]. For example, Rubisco in tobacco has been replaced with the enzyme from the α-proteobacterium, *Rhodospirillum rubrum*, which shows naturally high specificity values[25]. Although the transplastomic plants are fully autotrophic and reproductive with CO_2 supplementation, the CO_2-assimilation rates of the transplastomic plants are much lower than those of the wild type[25].

One successful approach to improve photosynthesis by genetic manipulation is overexpression of fructose-1,6/sedoheptulose-1,7-bisphosphatase (fbp)[26,27]. Overexpressions of fbp from *S. elongatus* PCC7942 in tobacco[26] and fbp from wheat in the *Anabaena* sp. strain PCC 7120 (ref. 27) has enhanced photosynthetic efficiency and growth characteristics. Here, we demonstrated that overexpression of the *rbcLS* genes in *S. elongatus* improved isobutyraldehyde production and *in vitro* enzyme activity, but not photosynthetic O_2 production. Continued analysis of these strains and extensions of our approach should provide further insight into the mechanism of the improved production with increased expression of the *rbcLS* genes. It also remains to be determined if overexpression of Rubisco will be beneficial in other systems.

For practical applications, utilization of CO_2 instead of $NaHCO_3$ is more desirable. Thus SA665 was tested for the ability to produce isobutyraldehyde from CO_2 directly by bubbling 5% CO_2 as the sole carbon source. The culture successfully produced isobutyraldehyde directly from CO_2 (**Fig. 3c**), although the productivity was lower than with $NaHCO_3$. Interestingly, the culture with 5% CO_2 reached a higher cell density (OD_{730} ~3.6) than that with $NaHCO_3$ (OD_{730} ~2.5) (**Fig. 3d**).

Isobutyraldehyde can also be converted to isobutanol by cyanobacteria[16]. Increasing attention has been paid to isobutanol as a potential substitute for gasoline or as a chemical feedstock[16]. Thus, it would be worthwhile demonstrating the biological feasibility of isobutanol production by cyanobacteria. Such a process would be valuable when an efficient product-recovery process is developed. To demonstrate the direct synthesis of isobutanol, we tested three alcohol dehydrogenases (ADH2 from *Saccharomyces cerevisiae*, YqhD from *E. coli*, and AdhA from *L. lactis*) along with Kivd from *L. lactis*[16,28]. Their corresponding genes were integrated downstream of *kivd* (**Fig. 2a**) individually, resulting in strains SA413, SA561 and SA562. After KIV was added to the growth medium the reaction products isobutyraldehyde and isobutanol were detected (**Supplementary Fig. 2a**). Among the three dehydrogenases tested, YqhD was the most active in *S. elongatus* (**Supplementary Fig. 2a**). YqhD is an NADPH-dependent

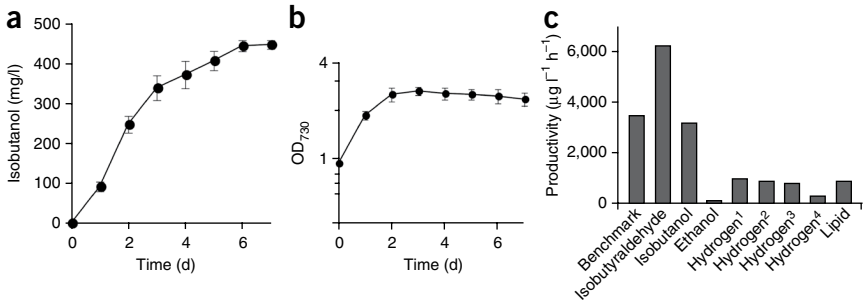

Figure 4 Isobutanol production and comparison of various cyanobacterial and algal productivities. (**a**) Isobutanol production from $NaHCO_3$ using SA579 (with integrated *alsS, ilvCD, kivd* and *yqhD*) in shake flasks without stripping. Only trace amounts (<10 mg/l) of isobutyraldehyde were detected, indicating the dehydrogenase activity of YqhD was sufficient for isobutanol production. (**b**) Time course for the growth of SA579. Error bars indicate s.d. ($n = 3$). (**c**) Productivity comparison of various processes. Productivities (μg l^{-1} h^{-1}) of isobutyraldehyde production (this work), isobutanol production (this work), ethanol production from *S. elongatus*[1], hydrogen production from (1) *Anabaena variabilis* PK84 (ref. 2), (2) *Anabaena variabilis* AVM13 (ref. 30), (3) *Chlamydomonas reinhardtii*[31] (4) *Oscillatoria sp. Miami* BG7 (ref. 32), and lipid production from *Haematococcus pluvialis*[3]. The detailed calculation is described in **Supplementary Notes**.

enzyme[29], whereas AdhA and ADH2 are NADH-dependent[28]. These results suggest that the NADH generated in the cell was insufficient for the NADH-dependent ADH. To increase the flux to KIV, the amplified KIV pathway (**Fig. 2b**) was combined with the alcohol-producing pathway (Kivd and YqhD). The strain (SA579) produced 450 mg/l of isobutanol in 6 d (**Fig. 4a**,**b** and **Supplementary Fig. 2b**).

We also measured tolerance of *S. elongatus* to isobutyraldehyde and isobutanol (**Supplementary Fig. 3**). Wild-type *S. elongatus* was able to tolerate concentrations of isobutyraldehyde up to 750 mg/l (**Supplementary Fig. 3a**), but showed growth retardation in the presence of the same concentration of isobutanol. This result shows that isobutyraldehyde is less toxic to the cell than isobutanol. In addition, the isobutyraldehyde tolerance level of *S. elongatus* is much higher than the concentration found in the culture medium during production (**Figs. 2f** and **3f**). These data are consistent with the result that the isobutyraldehyde production strain produced constantly for 9 d in our system. Thus, the *in situ* product removal system effectively avoids toxicity effects.

Although productivity (total product divided by volume and time) is not the only factor that determines the potential of a production system, the productivities of the engineered cyanobacteria for isobutyraldehyde and isobutanol demonstrated here are already higher than the productivites of cyanobacteria demonstrated for hydrogen or ethanol (**Fig. 4c**). As producing biodiesel from microalgae has been proposed as one of the most efficient methods[3], we used the algal diesel productivity (1×10^5 liter ha^{-1} per year, which corresponds to about 4,000 $\mu l\, l^{-1}\, h^{-1}$ assuming 1 m characteristic dimension) as a benchmark for isobutyraldehyde production. Although the productivity of lab-scale experiments cannot be directly translated to industrial-scale production, our productivity of isobutyraldehyde (6,230 $\mu g\, l^{-1}\, h^{-1}$) is encouraging (**Fig. 4c**). This result demonstrates the technical feasibility for direct conversion of CO_2 to fuels or chemicals, which could become an economically feasible option after further improvement. The strategy further expands the utility of photosynthesis and bypasses the need for biomass deconstruction and may therefore provide an alternative path for addressing two of humanity's most pressing problems: energy and climate change.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

S.A. designed and performed research, analyzed data and wrote the manuscript; W.H. designed and performed research and analyzed data; J.C.L. designed and coordinated research, and wrote the manuscript.

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ONLINE METHODS

Reagents. Restriction enzymes and Antarctic phosphatase were from New England Biolabs. Rapid DNA ligation kit was from Roche. KOD DNA polymerase was from EMD Chemicals. Oligonucleotides were from Eurofins MWG Operon. The chemicals, ribulose-1,5-bisphosphate, ribulose-1,5-bisphosphate carboxylase, NADPH, 2,4-dinitrophenylhydrazine, propionic acid, acetoin, 2-keto-isovalerate and cocarboxylase were obtained from Sigma-Aldrich. $NaH^{14}CO_3$ (specific activity 5 mCi/mmol) was purchased from American Radiolabeled Chemicals.

Strains and plasmids construction. Strains and plasmids used in this work are described in **Supplementary Table 1**. *S. elongatus* strain PCC7942 was obtained from Susan S. Golden. The primers used are listed in **Supplementary Table 2**.

The neutral site I (NSI) targeting vector. Strains that express *kivd* and *adh* were constructed by insertion of an expression cassette into NSI[14]. The genes *kivd* and *adh* were cloned into the NSI targeting vector, pAM2991 (ref. 33) (from Susan S. Golden), under the IPTG-inducible *P*trc promoter. The coding region of *kivd-ADH2, kivd-adhA, kivd-yqhD* and *kivd* were amplified from pSA55 (ref. 16), pSA65 (ref. 28), pSA134 (ref. 28) and pSA129 (ref. 28), respectively, using oligonucleotides A148 and A149, A148-A258, A148-A259 and A148-A262, respectively. The resulting plasmids were named pSA78 (*kivd-ADH2*), pSA149 (*kivd-adhA*), and pSA150 (*kivd-yqhD*) and pSA155 (*kivd*) (**Supplementary Table 1**).

The neutral site II (NSII)[17] targeting vector. Construction of pSA68, which contains *alsS* (*B. subtilis*)-*ilvC-ilvD* (*E. coli*), was as described[16]. To clone the 5′ fragment of NSII, genomic DNA of *S. elongatus* was used as the PCR template with primers A217 and A218. PCR products were digested with SacI and cloned into pSA68 cut with the same enzyme, creating pSA117. A correct orientation of the fragment was confirmed by PCR. To clone the chloramphenicol resistance gene, pACYC184 was used as the PCR template with primers A225 and A226. PCR products were digested with SpeI and cloned into pSA117 cut with the same enzyme, creating pSA122. A correct orientation of the fragment was confirmed by PCR. To clone the 3′ fragment of NSII, genomic DNA of *S. elongatus* was used as the PCR template with primers A219 and A220. PCR products were digested with AvrII and cloned into pSA122 cut with the same enzyme, creating pSA126. A correct orientation of the fragment was confirmed by PCR.

The rbcLS plasmid. To clone *rbcLS*, pETDuet-1 with *rbcLS* of *S. elongatus* PCC6301 (from F. Robert Tabita) was used as the PCR template with A308 and A309 and A310 and A311. These two fragments were then joined by splice overlap extension. The products were digested with MfeI and BamHI and cloned into pMMB66EH (ATCC) cut with EcoRI and BamHI, creating pSA165. To clone the chloramphenicol resistance gene, pACYC184 was used as the PCR template with primers A317 and A318. PCR products were digested with BamHI and PstI and cloned into pSA165 cut with the same enzyme, creating pSA169. The coding region of *rbcLS* with *P*tac and the chloramphenicol resistance gene was amplified from pSA169 with A315 and A316 and cloned into pZA31-luc cut with the same enzyme, creating pSA170.

Transformation of *S. elongatus*. Transformation of *S. elongatus* was carried out as described[15]. Cyanobacterial transformants with the targeting vectors were selected on BG-11 agar plates supplemented with antibiotics as appropriate; 20 μg/ml spectinomycin, 10 μg/ml kanamycin and 5 μg/ml chloramphenicol. Results of the transformation were confirmed by PCR and enzyme assays.

Medium and culture conditions. Wild-type *S. elongatus* and mutant strains were grown in a modified BG-11 medium with the following modifications: 50 mM $NaHCO_3$ and 10 mg/l thiamine were added. For an experiment with 5% CO_2 bubbling, 50 mM $NaHCO_3$ was not added. Cyanobacterial cells were grown at 30 °C under fluorescent light (55 μE $s^{-1}m^{-2}$), which was provided by eight 86-cm 20-W fluorescent tubes placed 15 cm from the cell culture. Cell growth was monitored by measuring OD_{730} of each culture.

Culture conditions for isobutanol and isobutyraldehyde production. For isobutyraldehyde and isobutanol production, cells were grown in 600 ml medium in 1,000-ml Roux culture bottles that were aerated by air or air containing 5% CO_2. The culture was allowed to grow at 30 °C to OD_{730} of 0.4–0.6, at which point 1 mM IPTG was added. Daily, one-tenth the total volume of cell culture was removed from the cell culture. Then the same volume of fresh medium containing 0.5 M $NaHCO_3$ was added to cell culture. pH of cell culture with $NaHCO_3$ was adjusted to 7.5 with 10 N HCl everyday. Utilization of 5% CO_2 stabilized the pH of cell culture around ~7.0, thus the pH was not adjusted. Presumably, the constant pH is due to the balance between CO_2 dissolution and consumption.

Quantification of the products. The alcohol and aldehyde compounds produced were quantified by a gas chromatograph equipped with a flame ionization detector as previously described[16]. Other secreted metabolites were quantified by a high-performance liquid chromatography.

Preparation of cell-free extracts. Cells were collected 24 h after induction by centrifugation (4,000*g*, 10 min, 25 °C). For the Rubisco assay, the cells were washed once with 50 mM Tris-Cl, pH 8.0, then resuspended in the same buffer. For the Als, IlvC and IlvD assays, the cells were washed once in 1 mM $MgCl_2$ and 100 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 7.0, then resuspended in the same buffer. The cells were broken by passage through a chilled French pressure cell at 20,000 p.s.i. (4 °C) for a total of three times. Total protein measurements were made with the Bradford protein assay kit from Bio-Rad.

Als assay. The Als assay was performed as described previously[34], with the exception that the reaction mixture contained 20 mM sodium pyruvate, 100 mM MOPS buffer, pH 7.0, 1 mM $MgCl_2$ and 100 μM cocarboxylase. The concentration of acetoin produced was determined by a standard curve created using pure acetoin. One specific unit of Als activity corresponds to the formation of 1 nmol of acetoin per mg of soluble protein per min at 37 °C.

IlvC assay. To measure the reduction of 2-acetolactate to 2,3-dihydroxy-isovalerate, the oxidation of NADPH was monitored by a decrease in absorbance at 340 nm. The substrate, 2-acetolactate, was first produced in a separate reaction as described for the Als assay using purified, heterogeneously expressed *B. subtilis* AlsS in *E. coli* strain BL21. From this reaction 180 μl was added to 200 mM potassium phosphate buffer, pH 7.5, 4 mM $MgCl_2$ and 0.1 mM NADPH for a final reaction volume of 1 ml. The samples were incubated at 30 °C for 5 min, then the reaction was initiated with the addition of cell extracts. Absorbance was measured at 340 nm. IlvC activity is expressed as nmol of NADPH oxidized per min per mg of soluble protein at 30 °C.

IlvD assay. The IlvD assay was performed as described previously[35]. The 500 μl reaction mixture contained 5 mM $MgSO_4$, 50 mM Tris-Cl, pH 8.0, cell-free extract and 10 mM 2,3-dihydroxy-isovalerate. The substrate, 2,3-dihydroxy-isovalerate, was synthesized as described previously[36]. After the reaction mixture was preincubated for 5 min at 37 °C, the substrate was added to initiate the reaction. The samples were incubated for 15 min at 37 °C. The reaction was terminated by the addition of 125 μl of 10% trichloroacetic acid, then 250 μl of saturated 2,4-dinitrophenylhydrazine in 2 N HCl was added to the samples. After 20 min at 25 °C, 875 μl of 2.5 N NaOH was added and then the samples were incubated for another 30 min at 25 °C. The samples were then spun down for 1 min to remove coagulated protein. Sample absorbances were measured at 550 nm. Standard curves were created from known amounts of KIV. The specific activity was calculated as 1 nmol of KIV synthesized per mg of soluble protein per min at 37 °C.

Rubisco assay. The assay to measure Rubisco activity was performed as previously described[37]. Briefly, the 250 μl reaction contained 50 mM Tris-Cl, pH 8.0, 20 mM $MgCl_2$, 0.1 mM EDTA, 20 mM $NaH^{14}CO_3$ (specific activity 0.5mCi/mmol), 0.4 mM ribulose bisphosphate, along with cell extracts. All components, except the ribulose bisphosphate, were incubated at 30 °C for 5 min to activate the enzyme. The reaction was started by

the addition of ribulose bisphosphate and the reaction was incubated for another 5 min at 30 °C. To terminate the reaction, 100 µl of 99% propionic acid was added. The unincorporated $^{14}CO_2$ was removed by heating the samples overnight at 65 °C. The acid-stable products were resuspended in 200 µl of 2 N HCl, then added to 3 ml of scintillation cocktail. The samples were counted in a Beckman LS6500 scintillation counter. One enzyme unit catalyzes the fixation of 1 nmol of CO_2 per min per mg of soluble protein at 30 °C.

O_2 production measurements. The *S. elongatus* cultures were similarly cultured and induced as they were for isobutyraldehyde production. Periodically, 2ml culture samples were measured for OD_{730} and O_2 production using the Oxygraph System (Hansatech Instruments). Data points represent triplicate measurements.

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