

# Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde

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**Global climate change has stimulated efforts to reduce CO<sub>2</sub> emissions. One approach to addressing this problem is to recycle CO<sub>2</sub> directly into fuels or chemicals using photosynthesis. Here we genetically engineered *Synechococcus elongatus* PCC7942 to produce isobutyraldehyde and isobutanol directly from CO<sub>2</sub> 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<sup>1</sup>, hydrogen<sup>2</sup> or lipid<sup>3</sup> production by cyanobacteria or algae. These results underscore the promise of direct bioconversion of CO<sub>2</sub> into fuels and chemicals, which bypasses the need for deconstruction of biomass.**

According to the US Energy Information Administration, world energy-related CO<sub>2</sub> 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<sub>2</sub> have increased by ~25% over the past 150 years<sup>5</sup>. Thus, it has become increasingly important to develop new technologies to reduce CO<sub>2</sub> emissions.

Photosynthetic organisms use solar energy to generate reducing equivalents and incorporate atmospheric CO<sub>2</sub> into organic molecules. Currently, cellulosic biofuels<sup>6</sup> and algal biodiesels<sup>7</sup> are prominent biological approaches to sequester and convert CO<sub>2</sub>. Producing biodiesel from algae has been proposed as one of the most efficient ways of generating biofuels<sup>7</sup> 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<sub>2</sub> 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<sub>2</sub> indirectly<sup>8</sup>. However, the volumetric hydrogen productivity reported is still relatively low<sup>2</sup>. Anaerobic hydrogen production using anaerobic photosynthetic bacteria requires a hydrogen source other than water<sup>9,10</sup>, and the productivity remains to be improved<sup>8</sup>.

Another approach is direct conversion of CO<sub>2</sub> to fuels<sup>11</sup> or chemicals<sup>12</sup>. Previously, the photosynthetic bacteria *Rhodospirillum rubrum* and *Rhodospirillum rubrum* were engineered to produce ethanol from CO<sub>2</sub> and a non-water hydrogen source, with a final titer of ~800 mg/l<sup>11</sup>.

Ethanol has also been produced from CO<sub>2</sub> and water using an engineered *S. elongatus* strain at a rate of 0.18 μg l<sup>-1</sup> hr<sup>-1</sup> (ref. 1). Although these efforts demonstrated the biochemical feasibility of direct ethanol production from CO<sub>2</sub>, 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.

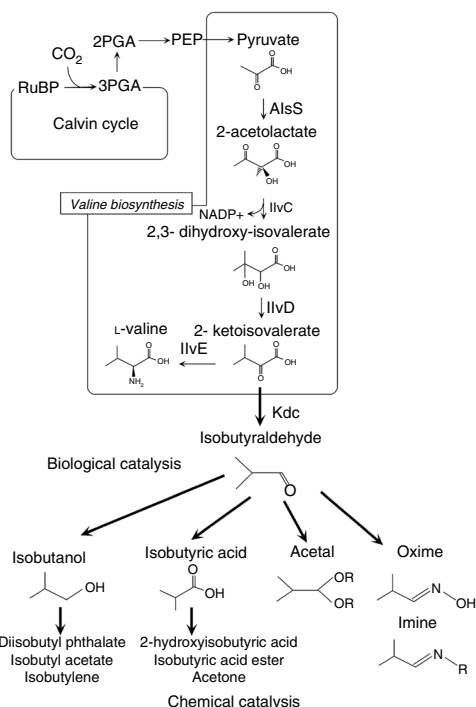
Productivity is a key determinant for the feasibility of direct CO<sub>2</sub> conversion to chemicals or fuels. As biodiesel production from algae is considered one of the most efficient ways of generating biofuels<sup>7</sup>, we estimated the required productivity of isobutyraldehyde by comparison with biodiesel production. The average productivity of microalgal biodiesel in a well-designed production system can be ~1 × 10<sup>5</sup> liter ha<sup>-1</sup> 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 μg l<sup>-1</sup> h<sup>-1</sup> or higher to be competitive with algal biodiesel production. This level of productivity serves as our initial benchmark productivity for direct conversion of CO<sub>2</sub> to isobutyraldehyde.

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

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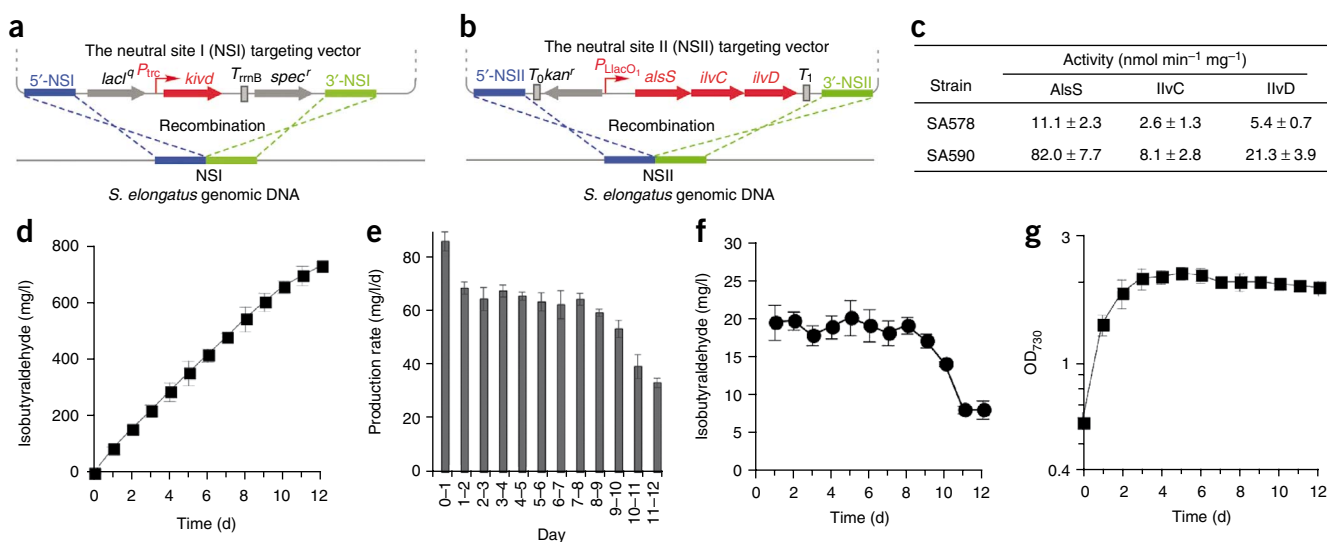


**Figure 1** The pathway for isobutyraldehyde production. The Kdc-dependent synthetic pathway for isobutyraldehyde production. AlsS, acetolactate synthase; IlvC, acetohydroxy acid isomeroreductase; IlvD, dihydroxy-acid dehydratase; Kdc, 2-ketoacid decarboxylase.

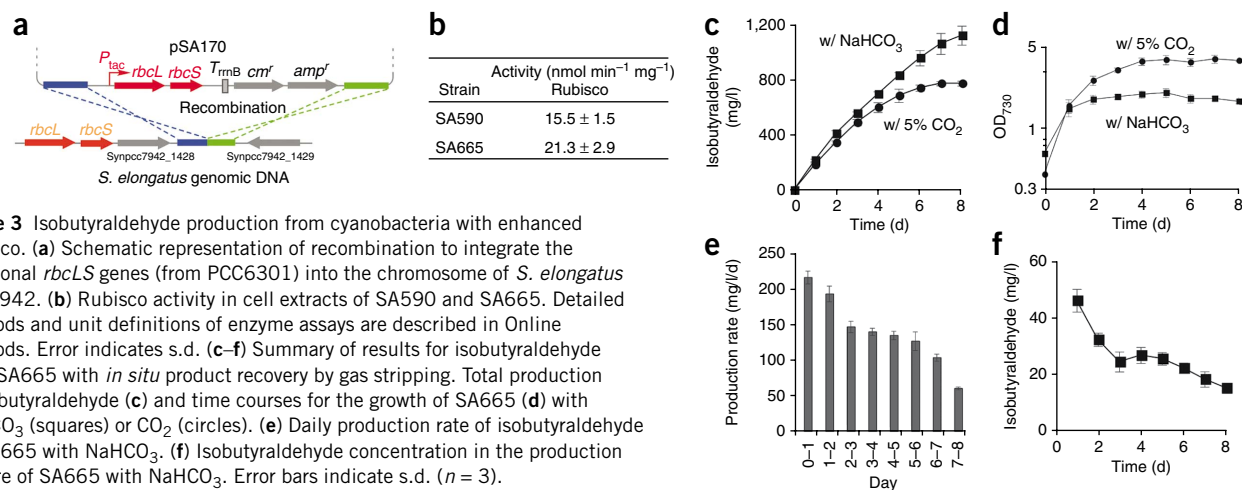
(Fig. 2b). All three enzyme assays of SA590 lysates demonstrated higher activity than those from SA578 (Fig. 2c), indicating that *alsS* (*B. subtilis*), *ilvC* (*E. coli*) and *ilvD* (*E. coli*) are expressed and functional in *S. elongatus*. Because the vapor pressure of isobutyraldehyde is relatively 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  $\mu\text{g l}^{-1} \text{h}^{-1}$  (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 ( $\sim 60 \text{ mg l}^{-1} \text{d}^{-1}$ ), suggesting that some inhibitory metabolites<sup>18</sup> 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<sub>2</sub> fixation reaction in the Calvin-Benson-Bassham (CBB) cycle is Rubisco<sup>19</sup>, which is implicated as the limiting step in CO<sub>2</sub> fixation because of its poor turnover rate and the competition between O<sub>2</sub> and CO<sub>2</sub> at the active site<sup>20</sup>. 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  $\sim 1.4$ -fold higher than that of SA590 (Fig. 3b). The strain was cultured in BG-11 medium with 50 mM NaHCO<sub>3</sub> 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  $\mu\text{g l}^{-1} \text{h}^{-1}$  (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<sub>2</sub> fixation is one of the bottlenecks of the isobutyraldehyde production. Thus, it is possible that the optimization of CO<sub>2</sub> fixation could further improve isobutyraldehyde production. To probe the impact of Rubisco overexpression, photosynthetic O<sub>2</sub> 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<sub>3</sub> (squares) or CO<sub>2</sub> (circles). **(e)** Daily production rate of isobutyraldehyde by SA665 with NaHCO<sub>3</sub>. **(f)** Isobutyraldehyde concentration in the production culture of SA665 with NaHCO<sub>3</sub>. Error bars indicate s.d. ( $n = 3$ ).

production. The O<sub>2</sub> 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<sub>2</sub> fixation in plants<sup>22</sup>, several attempts have been made to increase Rubisco activity. However, so far no approach has been found to successfully improve photosynthetic CO<sub>2</sub> assimilation<sup>23,24</sup>. The main limitations are precise regulation of the expression and inaccurate protein folding of Rubisco in heterologous organisms<sup>23</sup>. For example, Rubisco in tobacco has been replaced with the enzyme from the  $\alpha$ -proteobacterium, *Rhodospirillum rubrum*, which shows naturally high specificity values<sup>25</sup>. Although the transplastomic plants are fully autotrophic and reproductive with CO<sub>2</sub> supplementation, the CO<sub>2</sub>-assimilation rates of the transplastomic plants are much lower than those of the wild type<sup>25</sup>.

One successful approach to improve photosynthesis by genetic manipulation is overexpression of fructose-1,6/sedoheptulose-1,7-bisphosphatase (*fbp*)<sup>26,27</sup>. Overexpressions of *fbp* from *S. elongatus* PCC7942 in tobacco<sup>26</sup> 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

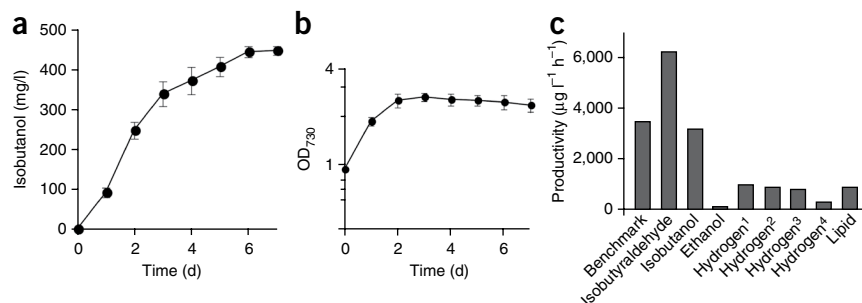
not photosynthetic O<sub>2</sub> production. Continued analysis of these strains and extensions of our approach should provide further insight into the mechanism of the improved production with increased expression of the *rbclS* genes. It also remains to be determined if overexpression of Rubisco will be beneficial in other systems.

For practical applications, utilization of CO<sub>2</sub> instead of NaHCO<sub>3</sub> is more desirable. Thus SA665 was tested for the ability to produce isobutyraldehyde from CO<sub>2</sub> directly by bubbling 5% CO<sub>2</sub> as the sole carbon source. The culture successfully produced isobutyraldehyde directly from CO<sub>2</sub> (Fig. 3c), although the productivity was lower than with NaHCO<sub>3</sub>. Interestingly, the culture with 5% CO<sub>2</sub> reached a higher cell density (OD<sub>730</sub> ~3.6) than that with NaHCO<sub>3</sub> (OD<sub>730</sub> ~2.5) (Fig. 3d).

Isobutyraldehyde can also be converted to isobutanol by cyanobacteria<sup>16</sup>. Increasing attention has been paid to isobutanol as a potential substitute for gasoline or as a chemical feedstock<sup>16</sup>. 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*<sup>16,28</sup>. 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<sub>3</sub> 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 ( $\mu\text{g l}^{-1} \text{h}^{-1}$ ) of isobutyraldehyde production (this work), isobutanol production (this work), ethanol production from *S. elongatus*<sup>1</sup>, hydrogen production from (1) *Anabaena variabilis* PK84 (ref. 2), (2) *Anabaena variabilis* AVM13 (ref. 30), (3) *Chlamydomonas reinhardtii*<sup>31</sup> (4) *Oscillatoria* sp. Miami BG7 (ref. 32), and lipid production from *Haematococcus pluvialis*<sup>3</sup>. The detailed calculation is described in Supplementary Notes.



enzyme<sup>29</sup>, whereas AdhA and ADH2 are NADH-dependent<sup>28</sup>. 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 productivities of cyanobacteria demonstrated for hydrogen or ethanol (Fig. 4c). As producing biodiesel from microalgae has been proposed as one of the most efficient methods<sup>3</sup>, we used the algal diesel productivity ( $1 \times 10^3$  liter ha<sup>-1</sup> per year, which corresponds to about 4,000  $\mu\text{l l}^{-1} \text{h}^{-1}$  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  $\mu\text{g l}^{-1} \text{h}^{-1}$ ) is encouraging (Fig. 4c). This result demonstrates the technical feasibility for direct conversion of CO<sub>2</sub> 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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- Deng, M.D. & Coleman, J.R. Ethanol synthesis by genetic engineering in cyanobacteria. *Appl. Environ. Microbiol.* **65**, 523–528 (1999).
- Tsygankov, A.A., Borodin, V.B., Rao, K.K. & Hall, D.O. H<sub>2</sub> photoproduction by batch culture of *Anabaena variabilis* ATCC 29413 and its mutant PK84 in a photobioreactor. *Biotechnol. Bioeng.* **64**, 709–715 (1999).

- Huntley, M.E. & Redalje, D.G. CO<sub>2</sub> mitigation and renewable oil from photosynthetic microbes: a new appraisal. *Mitig. Adapt. Strategies Glob. Change* **12**, 573–608 (2006).
- Energy Information Administration. *International Energy Outlook 2009*. (US Department of Energy, Washington, DC, 2009) <http://www.eia.doe.gov/oiia/ieo/pdf/0484%282009%29.pdf>.
- Energy Information Administration Greenhouse Gases, Climate Change, and Energy. (US Department of Energy, Washington, DC, 2008) <http://www.eia.doe.gov/bookshelf/brochures/greenhouse/greenhouse.pdf>.
- Ragauskas, A.J. *et al.* The path forward for biofuels and biomaterials. *Science* **311**, 484–489 (2006).
- Chisti, Y. Biodiesel from microalgae beats bioethanol. *Trends Biotechnol.* **26**, 126–131 (2008).
- Dutta, D., De, D., Chaudhuri, S. & Bhattacharya, S.K. Hydrogen production by Cyanobacteria. *Microb. Cell Fact.* **4**, 36 (2005).
- Tavano, C.L. & Donohue, T.J. Development of the bacterial photosynthetic apparatus. *Curr. Opin. Microbiol.* **9**, 625–631 (2006).
- Dubbs, J.M. & Tabita, F.R. Regulators of nonsulfur purple phototrophic bacteria and the interactive control of CO<sub>2</sub> assimilation, nitrogen fixation, hydrogen metabolism and energy generation. *FEMS Microbiol. Rev.* **28**, 353–376 (2004).
- Wahlund, T.M., Conway, T. & Tabita, F.R. Bioconversion of CO<sub>2</sub> to ethanol and other products. *Am. Chem. Soc. Div. Fuel Chem.* **41**, 1403–1406 (1996).
- Tyo, K.E., Zhou, H. & Stephanopoulos, G.N. High-throughput screen for poly-3-hydroxybutyrate in *Escherichia coli* and *Synechocystis* sp. strain PCC6803. *Appl. Environ. Microbiol.* **72**, 3412–3417 (2006).
- de la Plaza, M., Fernandez de Palencia, P., Pelaez, C. & Requena, T. Biochemical and molecular characterization of alpha-ketoglutarate decarboxylase, an enzyme involved in the formation of aldehydes from amino acids by *Lactococcus lactis*. *FEMS Microbiol. Lett.* **238**, 367–374 (2004).
- Bustos, S.A. & Golden, S.S. Light-regulated expression of the *psbD* gene family in *Synechococcus* sp. strain PCC 7942: evidence for the role of duplicated *psbD* genes in cyanobacteria. *Mol. Gen. Genet.* **232**, 221–230 (1992).
- Golden, S.S., Brusslan, J. & Haselkorn, R. Genetic engineering of the cyanobacterial chromosome. *Methods Enzymol.* **153**, 215–231 (1987).
- Atsumi, S., Hanai, T. & Liao, J.C. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* **451**, 86–89 (2008).
- Andersson, C.R. *et al.* Application of bioluminescence to the study of circadian rhythms in cyanobacteria. *Methods Enzymol.* **305**, 527–542 (2000).
- Ikawa, M., Sasner, J.J. & Haney, J.F. Inhibition of *Chlorella* growth by degradation and related products of linoleic and linolenic acids and the possible significance of polyunsaturated fatty acids in phytoplankton ecology. *Hydrobiologia* **356**, 143–148 (1997).
- Watson, G.M. & Tabita, F.R. Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a molecule for phylogenetic and enzymological investigation. *FEMS Microbiol. Lett.* **146**, 13–22 (1997).
- Tcherkez, G.G., Farquhar, G.D. & Andrews, T.J. Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proc. Natl. Acad. Sci. USA* **103**, 7246–7251 (2006).
- Smith, S.A. & Tabita, F.R. Positive and negative selection of mutant forms of prokaryotic (cyanobacterial) ribulose-1,5-bisphosphate carboxylase/oxygenase. *J. Mol. Biol.* **331**, 557–569 (2003).
- Woodrow, I.E. & Berry, J.A. Enzymatic regulation of photosynthetic CO<sub>2</sub> fixation in C3 plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 533–594 (1988).
- John Andrews, T. & Whitney, S.M. Manipulating ribulose bisphosphate carboxylase/oxygenase in the chloroplasts of higher plants. *Arch. Biochem. Biophys.* **414**, 159–169 (2003).
- Suzuki, Y. *et al.* Increased Rubisco content in transgenic rice transformed with the 'Sense' *rbtS* Gene. *Plant Cell Physiol.* **48**, 626–637 (2007).
- Whitney, S.M. & Andrews, T.J. Plastome-encoded bacterial ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) supports photosynthesis and growth in tobacco. *Proc. Natl. Acad. Sci. USA* **98**, 14738–14743 (2001).
- Miyagawa, Y., Tamoi, M. & Shigeoka, S. Overexpression of a cyanobacterial fructose-1,6-bisphosphatase gene enhances photosynthesis and growth. *Nat. Biotechnol.* **19**, 965–969 (2001).
- Ma, W., Shi, D., Wang, D., Wei, L. & Chen, H. Exogenous expression of the wheat chloroplast fructose-1,6-bisphosphatase gene enhances photosynthesis in the transgenic cyanobacterium, *Anabaena* PCC7120. *J. Appl. Phycol.* **17**, 273–280 (2005).
- Atsumi, S. *et al.* Engineering the isobutanol biosynthetic pathway in *Escherichia coli* by comparison of three aldehyde reductase/alcohol dehydrogenase genes. *Appl. Microbiol. Biotechnol.* published online, doi:10.1007/s00253-009-2085-6 (16 July 2009).
- Sulzenbacher, G. *et al.* Crystal structure of *E. coli* alcohol dehydrogenase YqhD: evidence of a covalently modified NADP coenzyme. *J. Mol. Biol.* **342**, 489–502 (2004).
- Happe, T., Schutz, K. & Bohme, H. Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. *J. Bacteriol.* **182**, 1624–1631 (2000).
- Laurinavichene, T., Tolstygina, I. & Tsygankov, A. The effect of light intensity on hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii*. *J. Biotechnol.* **114**, 143–151 (2004).
- Philips, E.J. & Mitsui, A. Role of light intensity and temperature in the regulation of hydrogen photoproduction by the marine cyanobacterium *Oscillatoria* sp. strain Miami BG7. *Appl. Environ. Microbiol.* **45**, 1212–1220 (1983).

## 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.  $\text{NaH}^{14}\text{CO}_3$  (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<sup>14</sup>. The genes *kivd* and *adh* were cloned into the NSI targeting vector, pAM2991 (ref. 33) (from Susan S. Golden), under the IPTG-inducible *P*<sub>trc</sub> 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)<sup>17</sup> targeting vector.** Construction of pSA68, which contains *alsS* (*B. subtilis*)-*ilvC-ilvD* (*E. coli*), was as described<sup>16</sup>. 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 *Sac*I 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 *Spe*I 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 *Avr*II 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 *Mfe*I and *Bam*HI and cloned into pMMB66EH (ATCC) cut with *Eco*RI and *Bam*HI, 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 *Bam*HI and *Pst*I and cloned into pSA165 cut with the same enzyme, creating pSA169. The coding region of *rbclS* with *P*<sub>tac</sub> 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<sup>15</sup>. 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  $\text{NaHCO}_3$  and 10 mg/l thiamine were added. For an experiment with 5%  $\text{CO}_2$  bubbling, 50 mM  $\text{NaHCO}_3$  was not added. Cyanobacterial cells were grown at 30 °C under fluorescent light ( $55 \mu\text{E s}^{-1}\text{m}^{-2}$ ), which was provided by eight 86-cm 20-W fluorescent tubes placed 15 cm from the cell culture. Cell growth was monitored by measuring  $\text{OD}_{730}$  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%  $\text{CO}_2$ . The culture was allowed to grow at 30 °C to  $\text{OD}_{730}$  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  $\text{NaHCO}_3$  was added to cell culture. pH of cell culture with  $\text{NaHCO}_3$  was adjusted to 7.5 with 10 N HCl everyday. Utilization of 5%  $\text{CO}_2$  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  $\text{CO}_2$  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<sup>16</sup>. 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 (4,000g, 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  $\text{MgCl}_2$  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<sup>34</sup>, with the exception that the reaction mixture contained 20 mM sodium pyruvate, 100 mM MOPS buffer, pH 7.0, 1 mM  $\text{MgCl}_2$  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-dihydroxyisovalerate, 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  $\text{MgCl}_2$  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<sup>35</sup>. The 500 µl reaction mixture contained 5 mM  $\text{MgSO}_4$ , 50 mM Tris-Cl, pH 8.0, cell-free extract and 10 mM 2,3-dihydroxyisovalerate. The substrate, 2,3-dihydroxyisovalerate, was synthesized as described previously<sup>36</sup>. 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<sup>37</sup>. Briefly, the 250 µl reaction contained 50 mM Tris-Cl, pH 8.0, 20 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 20 mM  $\text{NaH}^{14}\text{CO}_3$  (specific activity 0.5 mCi/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 biphosphate 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  $^{14}\text{CO}_2$  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  $\text{CO}_2$  per min per mg of soluble protein at 30 °C.

**O<sub>2</sub> 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<sub>730</sub> and O<sub>2</sub>

production using the Oxygraph System (Hansatech Instruments). Data points represent triplicate measurements.

33. Ivleva, N.B., Bramlett, M.R., Lindahl, P.A. & Golden, S.S. LdpA: a component of the circadian clock senses redox state of the cell. *EMBO J.* **24**, 1202–1210 (2005).
34. Yang, Y.T., Peredelchuk, M., Bennett, G.N. & San, K.Y. Effect of variation of *Klebsiella pneumoniae* acetolactate synthase expression on metabolic flux redistribution in *Escherichia coli*. *Biotechnol. Bioeng.* **69**, 150–159 (2000).
35. Kiritani, K., Narise, S. & Wagner, R.P. The dihydroxy acid dehydratase of *Neurospora crassa*. *J. Biol. Chem.* **241**, 2042–2046 (1966).
36. Cioffi, E.A., Shaw, K.J., Bailey, W.F. & Berg, C.M. Improved synthesis of the sodium salt of DL-alpha, beta-dihydroxyisovaleric acid. *Anal. Biochem.* **104**, 485–488 (1980).
37. Whitman, W. & Tabita, F.R. Inhibition of D-ribulose 1,5-bisphosphate carboxylase by pyridoxal 5'-phosphate. *Biochem. Biophys. Res. Commun.* **71**, 1034–1039 (1976).