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Complete List of Authors:	Wang, Bo; Arizona State University; National Renewable Energy Laboratory, Biosciences Center Xiong, Wei; National Renewable Energy Laboratory, Biosciences Center Yu, Jianping; DOE National Renewable Energy Laboratory, Maness, Pinching; NREL, Meldrum, Deirdre; Arizona State University, Biodesign Institute, Center for Biosignatures Discovery Automation

Unlocking the photobiological conversion of CO_2 to (*R*)-3-hydroxybutyrate in cyanobacteria

Bo Wang [1,2,‡,*], Wei Xiong [2,‡], Jianping Yu [2], Pin-Ching Maness [2], Deirdre R. Meldrum [1,*]

[1] *Center for Biosignatures Discovery Automation, The Biodesign Institute, Arizona State University, Tempe, AZ 85287-6501, USA.*

[2] *Biosciences Center, National Renewable Energy Laboratory, 15013 Denver West Parkway, Golden, CO 80401, USA.*

[‡] Co-first authors.

* Corresponding authors: bo.wang@nrel.gov (B. Wang); deirdre.meldrum@asu.edu (D.R. Meldrum)

Abstract

Escalating concerns about CO_2 emission from fossil fuels utilization and environmental pollution from fossil-derived plastic waste call for the sustainable production and utilization of renewable biodegradable plastic materials. Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible thermoplastics with thermal and mechanical properties comparable to conventional plastics; thus, they are promising materials to mitigate environmental pollution. (*R*)-3-Hydroxybutyrate (3HB), which is the most common building-block for PHAs, has the potential to be utilized in various medical applications and can also serve as a precursor to a variety of value-added stereospecific chemicals. In addition, it can be produced by microorganisms, such as engineered cyanobacteria, from inexpensive renewable resources such as waste CO_2. However, higher titer and rate of (*R*)-3HBproduction by cyanobacteria beyond that found in the current literature are critical for commercial applications. Herein, we employed a facile strategy to identify the rate-limiting step in photoautotrophic production of (*R*)-3HB by the cyanobacterium *Synechocystis* and found that acetoacetyl-CoA reductase activity is the bottleneck in the process. Optimization of the gene's ribosome binding site led to a 2.2-fold increase in enzyme activity. In the engineered organism, the (*R*)-3HB titer reached 1.84 g L^{-1} within 10 days, with peak productivity of 263 mg L^{-1} day^{-1}, using CO_2 and light as the sole carbon and energy sources. Moreover, dramatic changes in carbon partition were discovered in the (*R*)-3HB-producing cells along the course of cultivation using ^{13}C-metabolic flux analysis; after the rapid growth phase, a majority of carbon flux was redirected from the cell mass formation to the production of (*R*)-3HB in the engineered *Synechocystis* under the

examined experimental conditions.

Introduction

Conventional petroleum-derived plastics have become one of the most popular materials in modern society, with applications across domestic, medical and industrial domains. However, their various "single-use" or "short-life" applications and the concomitant plastic waste generated are causing escalating environmental problems due to their extremely slow degradation rates.[1, 2] Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible thermoplastics that show thermal and mechanical properties comparable to conventional plastics.[1-3] Therefore, PHAs have huge potential market in "short-life" applications, such as medical devices and food packaging.[2, 4-8] In nature, production of PHAs occurs in a variety of microorganisms as a means for carbon and energy storage under nutrient-unbalanced growth conditions.[9] Nevertheless, many efforts towards engineering microorganisms to synthesize PHAs in recent years demonstrate the difficulty in controlling the monomer composition (with several monomer of various carbon chain length), which is critical to the material functionality.[2, 4, 6, 10] Additionally, since PHAs are macromolecules generated intracellularly, harvesting PHAs requires energy-intensive cell lysis.[11] One potential solution is to engineer organisms to produce PHA building-block molecules that can be excreted and then polymerized or co-polymerized at defined ratios to yield PHAs with the desirable thermal and mechanical properties.[12-15] (*R*)-3-Hydroxybutyrate (3HB), which is the most common building-block for PHAs,[2, 4, 6, 8-10, 16, 17] can potentially be used in various medical applications[15] and serve as a precursor for an array of stereo-specific, fine chemicals such as antibiotics, pheromones, and amino acids.[12, 15]

Chemical synthesis of chiral (*R*)-3HB utilizes a complex of 2,2'-bis(diphenylphosphino)-1,1'-

binaphthyl (BINAP)-coordinated Ruthenium (Ru) as a catalyst.[18] Ruthenium is one of the platinum group metals rarely found on earth, with world reserves estimated at only 5,000 tons.[19] BINAP is a complex aromatic molecule and its synthesis and utilization also have negative environmental impact. In recent years, biotechnological synthesis of (*R*)-3HB has been proposed as an alternative to chemical synthesis,[12] and (*R*)-3HB production by genetically engineered *E. coli* has been reported.[20-23] However, biosynthesized (*R*)-3HB or PHAs are expensive owing to many factors, including consumption of glucose by *E. coli*, which contributes to 60% of the PHA cost.[3] The high cost of biologically produced PHAs not only limits their application to medical devices but also prohibits their broader applications in production of other disposable products such as food packaging. To address the cost issue, efforts have been made to convert waste organic carbon or CO_2 (instead of glucose) to PHAs.[3, 24] In particular, cyanobacterial production of PHAs has gained recent attention.[16, 17] Cyanobacteria are capable of using solar energy for CO_2 fixation, and are amenable to genetic manipulation.[25-28] Although photosynthetic production of (*R*)-3HB was demonstrated recently,[29, 30] the reported titers and productivities were relatively low, *e.g.*, 533 mg L^{-1} and 25 mg L^{-1} day^{-1} by engineered *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*)[29] and 1.22 g L^{-1} and 44 mg L^{-1} day^{-1} by engineered *Synechococcus elongatus* PCC 7942 (hereafter *S. elongatus*),[30] and improvements are needed to attain economic feasibility.

The goal of this study is to identify and mitigate the rate-limiting steps in (*R*)-3HB production in *Synechocystis*. We first designed and implemented a facile experiment to probe the kinetic bottleneck and the potential for photosynthetic (*R*)-3HB production in *Synechocystis*, and subsequently were able to mitigate the identified rate-limiting step via genetic engineering. The

dramatic changes in carbon partitioning in the engineered (*R*)-3HB-producing cells along the course of cultivation were also quantitatively delineated by ^{13}C-metabolic flux analysis. day-1

Results and Discussion

A facile method to identify the bottleneck in cyanobacterial production of (*R*)-3HB

A variety of chemicals are toxic to cyanobacteria, which limits their production by these organisms.[31-33] Thus, we first assessed the tolerance of *Synechocystis* to 3HB. Wild-type *Synechocystis* was cultivated in BG11 medium supplemented with (±)-3HB at concentrations of up to 50 g L^{-1} inside a growth chamber containing 5% CO_2. As shown in Figure S1, *Synechocystis* was able to grow at 3HB concentrations as high as 50 g L^{-1}, though its growth rate was severely impaired at this level. Supplementation with 25 g L^{-1} 3HB only slightly hampered cell growth, whereas 3HB supplemented at or below 10g L^{-1} had no apparent effect on cell growth. Overall, our results indicate that 3HB at up to 25 g L^{-1} has almost no toxicity to the growth of *Synechocystis*, which suggests that *Synechocystis* is a suitable host for photosynthetic production of these high levels of 3HB.

In vivo, the stereospecific (*R*)-3HB is biosynthesized from the central carbon metabolite, acetyl-CoA, two molecules of which are first condensed to form acetoacetyl-CoA in a reaction catalyzed by a thiolase (PhaA). Thereafter, acetoacetyl-CoA is reduced by acetoacetyl-CoA reductase (PhaB), the subsequently the coenzyme A group is removed, catalyzed by a thioesterase (TesB; Figure 1). Rational design and successful engineering efforts towards enhanced production of a compound, herein (*R*)-3HB, rely heavily on precise identification of

the rate-limiting steps.[26, 34] The traditional trial-and-error genetic engineering approach is time-consuming, given that the doubling time of *Synechocystis* is about 8 to 12 hours, which is much longer than that of *E. coli* (20 – 30 min). Kinetic flux profiling (KFP) is a powerful tool to identify bottlenecks in a biosynthetic pathway. Recently ^{13}C KFP was successfully applied to characterize the metabolic flux profiles of several recombinant cyanobacteria.[35, 36] Nevertheless, it might not have been effective in our case, due to the low abundance of the coenzyme A- associated intermediate compounds in the 3HB biosynthesis pathway and instrument's low sensitivity for detecting them.[36]

[Figure 1 to be inserted here]

To circumvent these issues, a facile method was designed to probe the metabolic bottleneck in cyanobacterial (*R*)-3HB production, the principle of which is depicted in Figure 2. It was demonstrated in previous studies that the TesB enzyme from *E. coli* exhibits relatively high activity towards the medium-chain-length acyl-CoAs over the two-carbon acetyl-CoA.[29, 37] Under photoautotrophic growth conditions, wild-type *Synechocystis* does not produce acetate, whereas heterologous expression of the *E. coli* TesB leads to acetate production and its excretion, likely owing to TesB's activity on acetyl-CoA (Figure 1).[29, 37] Thus, it would be expected that if TesB activity were the rate-limiting step, the (*R*)-3HB productivity would increase upon higher expression of TesB (Figure 2A, B). On the other hand, if PhaAB activities were the bottlenecks in (*R*)-3HB production, increased TesB activity would not affect production of (*R*)-3HB but would rather lead to an elevated ratio of acetate: (*R*)-3HB (Figure 2B, C). To this end, we devised a promoter library to tune the expression level of TesB in a *phaAB*-expressing *Synechocystis* strain. As shown in Figure 3, six natural and synthetic promoters,

including the *E. coli* σ^{70} promoter P_{tac}, the *Synechocystis* native promoter P_{psbA2} and four of their derivatives, were compared in expressing TesB in *Synechocystis*. The *rrnB* T1 terminator was added downstream from the *tesB* gene in all six constructs (Table 1), which apparently did not affect the production of (*R*)-3HB or acetate under the examined condition (Figure S2). The P_{tac} promoter is a strong and near-constitutive promoter in *Synechocystis*.[38] The native *Synechocystis psbA2* promoter (hereafter P_{psbA12}) is light inducible and its 5'-untranslated region (UTR) plays an important role in stabilizing the *psbA2* mRNA.[39] Therefore, the whole 5'-UTR or merely the ribosome binding site (RBS, including the Shine-Dalgarno [SD] sequence and the spacer sequence between the SD region and the start codon) of the P_{psbA12} was placed downstream of the P_{tac}, resulting in promoters P_{tac}-UTR or P_{tac}-SD. Because the AU-box in the 5'-UTR of P_{psbA12} was a negative element for gene expression,[40] it was deleted from the P_{psbA12} promoter, leading to promoter P_{psbA14} (Figure 3). Moreover, promoters P_{psbA12} and P_{tac} were fused together to form the dual promoter, P_{psbA16}-P_{tac}. The TesB expression cassettes were each inserted into the *Synechocystis* strain Abd which expresses PhaAB under the control of P_{tac} promoter (Table 1), resulting in strains TTrK, SDTrK, UTRTrK, PTrK12, PTrK14 and PTrK16, respectively (Figure 3; Table 1).

[Figure 2 to be inserted here]

[Figure 3 to be inserted here]

When the engineered strains were grown under photoautotrophic conditions, the cultures reached the same cell densities in five days (Figure 4A). Strains TTrK and PTrK16 exhibited similar growth rate, (*R*)-3HB and acetate productivity, *tesB* transcript level and TesB enzyme

activity (Figure S3). However, the *tesB* transcript level in strain TTrK was much higher compared to all four other strains tested. The *tesB* transcript levels in strains SDTrK, UTRTrK, PTrK12 and PTrK14 were 33%, 56%, 46% and 40% of that in strain TTrK (Figure 4B). Since modification of the 5'-UTR was assumed to have little impact on gene transcription, the much lower *tesB* transcript levels in strains SDTrK and UTRTrK compared to that in strain TTrK might be attributed to the poor stability of the *tesB* mRNA product. The finding is consistent with a previous report that the RBS of the P_{psbA12} promoter might be a target for the RNase E/G in *Synechocystis*.[39, 41] Additionally, removing the AU-box, a possible target for the RNase E,[40] from P_{psbA12} did not lead to a higher level of *tesB* transcript in *Synechocystis* strain PTrK14 (with promoter P_{psbA14}) (Figure 4B).

[Figure 4 to be inserted here]

We analyzed the (*R*)-3HB and acetate production levels in the same strains above and found that strain SDTrK produced the least amounts of (*R*)-3HB and acetate, 70.2 and 2.55 mg L-1, respectively (Figure 4C); the (*R*)-3HB titers for PTrk14, UTRTrK and PTrK12 were 141.3, 147.5, 166.1 mg L-1, respectively, which were 2.0-2.4 times higher than that for SDTrK; the acetate titers reached 1.2-2.7 times that measured in SDTrK (Figure 4C). While the (*R*)-3HB titer for strain TTrK, 174.6 mg L-1, was only slightly higher than those for strains PTrk14, UTRTrK and PTrK12, the acetate titer produced by strain TTrK was over five-fold higher than all other strains (Figure 4C). The well-maintained cell growth rate (Figure 4A) and the excessively produced acetate by strain TTrK (Figure 4C) implied that supply of acetyl-CoA was sufficient and was probably upregulated for biosynthesis of (*R*)-3HB, as evidenced by a previous study. [36] The

scenarios depicted in Figure 4C-D were in line with our models, as illustrated in Figure 2. (*R*)-3HB biosynthesis in SDTrK represented limited TesB activity (Figure 2A); PTrk14, UTRTrK and PTrK12 represented matched PhaAB and TesB activities (Figure 2B); and TTrK stood for excessive TesB but limited PhaAB activities (Figure 2C). From another perspective, the ratio of acetate to (*R*)-3HB may serve as an indicator to probe an excess of the TesB activity: when TesB activity becomes excessive, the acetate to (*R*)-3HB ratio increases significantly (Figure 2, 5D). Overall, we were able to demonstrate that tuning down TesB activity by a factor of at least two had little impact on (*R*)-3HB production, whereas given high enough TesB activity (*i.e.*, in the case of TTrK), the PhaAB activity which converts acetyl-CoA to (*R*)-3HB-CoA became the bottleneck for (*R*)-3HB biosynthesis in *Synechocystis*.

In our previous study, the *in vitro* activity of thiolase PhaA was approximately 200-fold higher than that of the acetoacetyl-CoA reductase PhaB.[29] Therefore, we inferred that the activity of the acetoacetyl-CoA reductase (PhaB) was likely the bottleneck for (*R*)-3HB biosynthesis in strain TTrK.

Optimization of the ribosome binding site for *phaB1*

Optimizing RBS is an efficient strategy for enhancing expression of a target gene in cyanobacteria. [38, 42, 43] Therefore, the RBS upstream of the *phaB1* gene was optimized to enhance the expression of acetoacetyl-CoA reductase for (*R*)-3HB biosynthesis in *Synechocystis*. It has been reported that the SD sequence UAAGGAGG, which is perfectly complementary to the 3'-terminal sequence of the 16S rRNA in the *Escherichia coli* K12 strain, enabled 3- to 6-fold higher translation efficiency than the SD sequence AAGGA, regardless of the spacing between

189 the SD sequence and the translation start codon – ATG.[44] In this study, the RBS upstream of the
190 *phaB1* gene was examined, and it was found that the original SD sequence, 5'-AAGGAGTGG-3',
191 did not perfectly complement the 3'-terminal sequence (5'-ACCUCCUUU-3') of the 16S rRNA in
192 *Synechocystis*. The original RBS sequence, AAGGAGTGGAC, for *phaB1* was therefore replaced by
193 sequence AAGGAGGTAAC (RBS_{opt}) which was fully complementary to the 3'-terminal sequence
194 of *Synechocystis* 16S rRNA (Figure 5A). The resultant strain was denominated strain R154 (Table
195 1).

196 The acetoacetyl-CoA reductase (PhaB) activity in strain R154 was 2.2-fold higher than that in
197 strain TTrK (Table 2; Figure 5B). While the growth of strain R154 was similar to that of strain
198 TTrK under the examined culture condition (Figure 5C), strain R154 was able to produce (*R*)-3HB
199 at a titer of 280.2 mg L-1, 1.6-fold higher than that of strain TTrK, after five days of
200 photoautotrophic growth with $NaHCO_3$ as the sole carbon source (Figure 5D). Our results
201 confirmed that PhaB activity was indeed the bottleneck for (*R*)-3HB production in *Synechocystis*,
202 and they suggest that the new RBS, *i.e.*, RBS_{opt}, is much more efficient in expressing the *phaB1*
203 gene compared to the original RBS in our engineered *Synechocystis* strains.

204

205 [Figure 5 to be inserted here]

206

207 **Enhanced production of (*R*)-3HB from CO_2**

208 Since the bottleneck of (*R*)-3HB biosynthesis in *Synechocystis*, the acetoacetyl-CoA reductase
209 activity, has been mitigated (at least partially) in strain R154 (Figure 5), it would be interesting

to examine if this strain R154 shows higher photosynthetic productivity of (*R*)-3HB directly from CO_2 (rather than daily addition of bicarbonate) than the parental strain.[29] As shown in Figure 6A, strain R154 first showed a relatively fast "growth phase" during the first two days, and then cell growth slowed down in the "production phase". (*R*)-3HB quickly accumulated in the production phase (Figure 6A), consistent with the previous observation that 3HB started to accumulate following the depletion of phosphate and the onset of slowed-down cell growth. [29] During the production phase (starting from day 2 until day 10), strain R154 continuously produced (*R*)-3HB at an average rate of 203 mg L-1 day^{-1} (Figure 6B). Notably, the volumetric productivity peaked on days 6~8, reaching 263 mg L^{-1} day^{-1} on day 7, and the highest specific productivity reached 50 mg L^{-1} day^{-1} OD_{730}^{-1} on day 2 and declined thereafter to 11.5 mg L^{-1} day^{-1} OD_{730}^{-1} on day 10 (Figure 6B). The (*R*)-3HB titer reached 1845 mg L^{-1} at the end of the 10-day photoautotrophic cultivation period (Figure 6A). Compared to the literature,[29] the titer achieved in this study is 3.5-fold higher and the average productivity is 7.3-fold higher than previously reported values.

To our knowledge, this result is, to date, the highest titer and productivity achieved in photoautotrophic production of 3-hydroxyalkanoic acids from CO_2. The productivity is also higher than other compounds branching from the metabolic node of acetyl-CoA (Table S2). Without adding any organic carbon sources into the culture medium, the titer of the excreted (*R*)-3HB achieved in this study, equivalent to ~35% of the dry weight of the cells, has reached the same level as what was reported previously on cyanobacterial mixotrophic production of PHB that was biosynthesized as intracellular granules.[45] The demonstrated (*R*)-3HB productivity has therefore shed light on the potential capacity of engineered cyanobacteria in photoautotrophic production of acetyl-CoA-derived chemicals or biofuels using CO_2 as the only

carbon source.

[Figure 6 to be inserted here]

The dramatic increase of the (*R*)-3HB production rate compared to that in the previous study can probably be attributed to the following reasons. First, the bottleneck in the (*R*)-3HB biosynthesis pathway, *i.e*., the relatively low enzyme activity of acetoacetyl-CoA reductase (encoded by *phaB*), was identified and subsequently alleviated by optimizing the RBS which increased the PhaB activity by 2.2-fold in strain R154 (Figure 5B; Table 2). Second, the culture in current study was aerated with 5% CO_2 instead of air, and the pH of the culture medium was stable at ~8.0 during the entire cultivation process, indicating that the CO_2 supply was sufficient. In contrast, in the previous study when the culture was aerated with ambient air (0.04% CO_2), the pH of the culture medium reached 10~11,[45] which demonstrates that the CO_2 supply was limiting. Third, while in the previous study *Synechocystis* cells were cultivated by simply bubbling air into the flasks without shaking,[45] in this study the flasks containing *Synechocystis* cells were placed on a rotary shaker with a rotation rate of 150 rpm, which can improve CO_2 distribution and light delivery to the *Synechocystis* culture. It is noteworthy that the highest titer, 1.84 g L^{-1}, of (*R*)-3HB achieved in this study is still far below the concentration of 3HB that *Synechocystis* can tolerate without a major decrease in growth rate, *i.e.*, 25 g L^{-1} (Figure S1). Strategies to further increase the titer and productivity may include maximizing the PhaB activity via systematic optimization of the gene expression level, [38] and redirecting the central carbon flux towards (*R*)-3HB biosynthesis by blocking competing pathways, such as using the inhibitor cerulenin to limit the amount of acetyl-CoA channeling to fatty acid synthesis (Figure

1). [46]

Metabolic fluxes redistribution in (*R*)-3HB-producing strain

To better understand the physiology of the (*R*)-3HB-producing strain, we set up a ^{13}C-tracer experiment to profile the fluxome in strain R154 during different growth phases. We used a steady-state ^{13}C-flux strategy which is suitable for quantitative analysis of flux partitioning on metabolic branches.[47] The method can be applied to photomixotrophic growth of cyanobacteria.[48] We grew the R154 strain on fully labeled sodium bicarbonate ($NaH^{13}CO_3$) and unlabeled glucose, a mixture of isotopic carbon substrates that can be proportionally consumed by the cells, hence imprinting unique isotope patterns into the metabolic intermediates. When isotope pseudo-steady state was reached, we collected isotopomer information from proteinogenic amino acids for ^{13}C-based Metabolic Flux Analysis (MFA). Physiological data including cell growth and (*R*)-3HB production were also collected overtime.

Consistent with our observation of cultures grown under photoautotrophic conditions (Figure 6), R154 batch culture under photomixotrophic conditions showed a typical two-phase metabolism: the growth phase and the production phase (Figure S4). During the growth phase (day 1-2), R154 exhibited a specific growth rate of 0.049 and the excreted (*R*)-3HB was below the detection limit. Upon transition into the production phase (day 3-5), cell growth slowed down significantly and (*R*)-3HB accumulated in the medium (Figure S4). Figure 7 shows a snapshot of quantitative flux distribution during the production phase, and Figure 8 delineates the up- and down-regulation of the metabolic flux during the production phase when compared to that during the growth phase. The relative flux values, the exchange coefficients for

reversible reactions, and the 95% confidence intervals are shown in Supplementary file 2 and fitting results are shown in Figure S5. During the growth phase, ^{13}C-MFA revealed highly active Calvin-Benson-Bassham (CBB) cycle, glycolytic pathway, C4 pathway and relatively moderate fluxes through the TCA cycle in R154. These results were comparable with the previous report on the fluxome in wild type *Synechocystis*,[48] reflecting a relatively stable architecture of *Synechocystis* metabolic network for light-driven growth. However, during the production phase, the fluxes were altered globally for the biosynthesis of (*R*)-3HB. Fluxes in the TCA cycle, photorespiration, and amino acid biosynthesis were 1000-fold lower, whereas the relative flux from 3PGA to (*R*)-3HB and part of the oxidative pentose phosphate (OPP) and CBB pathway was higher (Figure 8). Consequently, during the production phase, fluxes in pathways leading to intermediate compounds, *e.g.*, glyceraldehyde-3-phosphate (GAP) and amino acids (Figure 7), required for biomass formation were down significantly, while the flux from 3-phosphoglycerate (PGA) to acetyl-CoA and (*R*)-3HB production increased. This is consistent with our previous observation that the intracellular acetyl-CoA concentration was about two times higher in the production phase than that in the growth phase.[29]

[Figure 7 to be inserted here]

[Figure 8 to be inserted here]

^{13}C-MFA results showed that 68.8% of the carbon input was redirected to (*R*)-3HB (51.3%) and the by-product acetate (17.5%) on day 4 (when cells were in the production phase) of the cultivation process. To our knowledge, this is the first report detailing that a cyanobacterium redistributes a majority of its carbon flux from biomass formation to the biosynthesis of

298 chemicals and fuels during different metabolic phases, which corroborates the remarkable
299 flexibility of cyanobacterial carbon metabolism. [26, 49]

300 Conclusions

301 We first demonstrated that *Synechocystis* was able to tolerate as high as 50 g L^{-1} of 3HB, with
302 25 g L^{-1} displaying minimal impact on cell growth, which implies that *Synechocystis* is a logical
303 candidate to be engineered for high-level production of 3HB without compromising cell fitness.
304 Subsequently, a facile method was employed to identify the bottleneck in biosynthesis of (*R*)-
305 3HB in *Synechocystis,* which was found to be the enzyme activity of acetoacetyl-CoA reductase
306 (PhaB). Through optimization of its gene's ribosome binding site, the acetoacetyl-CoA reductase
307 activity was increased by 2.2-fold, leading to a dramatic increase in (*R*)-3HB production. The (*R*)-
308 3HB titer reached 1845 mg L^{-1} within 10 days, with a peak productivity of 263 mg L^{-1} day^{-1}, using
309 CO_2 and light as the sole carbon and energy sources. The average (*R*)-3HB productivity of 184
310 mg L^{-1} day^{-1} is four times higher than that of an engineered *S. elongatus* strain reported
311 recently,[29] and it represents to date the highest yield and productivity of any phototrophically
312 produced compound derived from acetyl-CoA (Table S2). ^{13}C-metabolic flux analysis
313 quantitatively delineated the sequential two-phase metabolism profile in the (*R*)-3HB-
314 producing cells. During the early growth phase, carbon fluxes primarily contribute to biomass
315 formation, whereas when cells subsequently enter the production phase, the cyanobacterium
316 redistributed most of carbon fluxes from biomass formation to the heterologous pathway, with
317 68.8% carbon input redirected to (*R*)-3HB (51.3%) and acetate (17.5%) formation under the
318 investigated experimental conditions. The significantly improved titer and rate in production of

(*R*)-3HB directly from CO_2 and sunlight, and the quantitative delineation of metabolic fluxes for cells along the course of cultivation should be seen as an important step in the potential commercialization of this technology for production of renewable (*R*)-3HB and PHAs.

Materials and methods

Culture conditions

All recombinant plasmids were constructed and stored using *E*. *coli* XL1-Blue MRF' (Stratagene, La Jolla, CA, USA) as the host strain. *Synechocystis* strains were grown in BG11 medium supplemented with 50 mM $NaHCO_3$ under a light intensity of 60 μE m^{-2} s^{-1} supplied by cool white fluorescent bulbs unless otherwise specified. For BG11-agar plates, 10 mM TES (pH 8.0), 3 g L^{-1} thiosulfate and 1.5% agar were supplemented into BG11 medium before autoclaving.

Modification of *Synechocystis* genome

The chromosome of *Synechocystis* strains was modified using methods described previously.[38] Basically, recombinant integration plasmids carrying the desired expression cassettes (Table 1) were constructed using pBluescript II SK(+) as the backbone. Each integration plasmid was then used to transform *Synechocystis* using a natural transformation protocol. Premethylation using the cytosine-specific methyltransferase, Slr0214, was used to treat the donor DNA prior to transforming *Synechocystis* whenever necessary.[50] The genotype of each engineered *Synechocystis* strain is described in Table 1. The genotypic purity of each strain was confirmed

by colony PCR.

Production of (*R*)-3HB from bicarbonate

Synechocystis strains were inoculated in 50-mL flasks containing 20 mL BG11 (10 mM TES-NaOH) to an initial OD_{730} (optical density at 730 nm) of 0.1 and were grown in a shaking bed (150 rpm) at 30 ^{o}C with a light intensity of 60 µE m^{-2} s^{-1} until an OD_{730} of ~1.5. Cells were then harvested by centrifugation and resuspended with 10 mL BG11 (10 mM TES-NaOH) contained in 50-mL flasks to an initial OD_{730} of 2.0. Then cells were incubated under the same culture condition for the following experiments unless otherwise specified. Every day, 0.05 mL cell culture was sampled for OD_{730} measurements, before 0.5 mL 1.0 M $NaHCO_3$ was added to each culture. At that point, the pH was adjusted to ~8.0 with 10 N HCl. After 5 days of cultivation, the culture supernatant was collected after centrifugation and the (*R*)-3HB titers were analyzed by HPLC. All cultivation experiments were conducted at least in triplicate for each strain.

Production of 3HB from carbon dioxide

Synechocystis was inoculated into 50 mL autoclaved BG11 (10 mM TES-NaOH) medium contained in a 250-mL baffled flask with an initial OD_{730} of 0.4. The culture was grown at 30 ^{o}C with continuous illumination of 100 µE m^{-2} s^{-1} for the first day and 300 µE m^{-2} s^{-1} for the rest of the experiment. The headspace of the culture flask was aerated with 5% (v/v) CO_2 (balanced with ambient air) at an aeration rate of 60 mL min^{-1}. At the end of each day, 1 mL of culture was sampled and 1 mL 5x (5-fold concentrated) BG11 medium was added back into the culture until the end of day 3. After day 3, 1 mL of culture was sampled and 1 mL of 10x BG11 medium was

added back into the culture each day until the end of the experiment. Additionally, 0.33 mL sterilized deionized water was supplemented into the culture every day to compensate the water loss due to evaporation. The experiments were conducted in duplicate.

Gene expression analysis by RT-qPCR

Cells were resuspended to an initial OD_{730} of 2.0 in BG11 (10 mM TES-NaOH) medium under continuous illumination of 60 µE m^{-2} s^{-1}. Daily, 0.05 mL cell culture was sampled for analysis of the OD_{730} before 0.5 mL 1.0 M $NaHCO_3$ was added to each culture and the pH was adjusted to ~8.0 with 10 N HCl. After 3.5 days of cultivation, approximately 1.67×10^8 *Synechocystis* cells (assuming OD_{730} of 0.6 equals to 10^8 cells mL^{-1})[51] were collected by centrifugation at 17,000g at 4 °C for 1 min. The supernatant was discarded, and the cell pellet was directly used for RNA extraction using ZR Fungal/Bacterial RNA MiniPrep™ Kit (ZYMO Research, Irvine, CA, USA). The RNA was then quantified by RT-qPCR using methods described previously.[29] The primers used for RT-qPCR analysis are listed in Table S1.

Enzyme activity assay

Synechocystis cells were resuspended with an initial OD_{730} of 2.0 in BG11 (10 mM TES-NaOH) supplemented with 50 mM $NaHCO_3$ and were grown under light of 60 µE m^{-2} s^{-1} for 12 hours. Then, 1.67×10^9 *Synechocystis* cells were collected by centrifugation at 8000g at 4 °C for 5 min. The supernatant was discarded, and the cell pellets were frozen on dry ice and stored at -80 °C before the assay. For the thioesterase enzyme activity assay, the cell pellet was resuspended with 500 µL ice-cold 0.1 M Tris-HCl (pH 7.5) and lysed by sonication (100 cycles of 3-s-on/ 3-s-

off) in an ice-water bath. The cell lysate was centrifuged at 17000g at 4 ^{o}C for 10 min before the supernatant was analyzed for the thioesterase activity, following the previously established protocols but using butyryl-CoA as the substrate.[29] For the acetoacetyl-CoA reductase enzyme activity assay, the cell pellet was resuspended in 500 µL ice-cold Buffer A [50 mM K_2HPO_4-HCl (pH 7.5), 10% glycerol, 1 mM EDTA, 1 mM DTT] supplemented with 0.1 mM PMSF, and was lysed by sonication (20 cycles of 3-s-on/ 3-s-off) in ice-water bath. The supernatant was analyzed for acetoacetyl-CoA reductase activity using the protocol established previously.[29]

Product quantification

The (*R*)-3HB and acetate concentrations were quantified by 1100 series HPLC (Agilent, Santa Clara, CA, USA) using the method described previously.[29] Briefly, samples of the *Synechocystis* culture were centrifuged at 17,000*g* for 1–2 min at room temperature and the supernatant was properly diluted before being analyzed on HPLC that was equipped with an Aminex HPX-87H anion-exchange column (Bio-Rad Laboratories, Hercules, CA) and a refractive index detector (Agilent, Santa Clara, CA, USA). The column temperature was maintained at 35 °C during operation. The mobile phase was 5 mM H_2SO_4 and the flow rate was set as a linear gradient from 0.55 mL min^{-1} to 0.8 mL min^{-1} over 12 min, followed by an 8-min hold.

^{13}C-Metabolic flux analysis (MFA)

Steady-state ^{13}C-MFA was adopted to quantify carbon fluxes through the central metabolic network in R154 strain. Cultures were illuminated at 60 µE m^{-2} s^{-1} and grown on unlabeled glucose (10 mM) and $NaH^{13}CO_3$ (60 mM). Cells were collected during the exponential growth

phase (day 2) and (*R*)-3HB production phase (day 4), respectively, and proteinogenic amino acids were analyzed by a GC-MS system including a 7890A GC system and a 5975C inert XL MSD with Triple-Axis Detector (Agilent, Santa Clara, CA, USA) using the same method as previously reported.[52] The central carbon network of *Synechocystis* was constructed based on collected genome knowledge, which has been supported by biochemical and isotope tracer experiments.[48, 53-55] The network includes the Calvin Benson Cycle, the EMP pathway, the C4 pathway, the TCA Cycle, and photorespiration pathways (Figure 8; Supplementary File 3). The cell mass composition (Figure 8; Supplementary File 3) was based on a previous report.[48, 56] The (*R*)-3HB production pathway and the acetate byproduct pathway (owing to the low TesB activity towards acetyl-CoA) were lumped and included into the metabolic model. INCA, a recently developed ^{13}C-flux software based on the MATLAB platform[57] was utilized for flux estimation. The calculation of ^{13}C-metabolic flux was performed by minimizing the sum-of-squared residuals (SSR) between computationally simulated and experimentally determined measurements.

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Competing interests

Part of this work has contributed to a patent application.

Authors' Contributions:

B.W. and D.R.M. conceived the study. B.W. and W.X. designed and carried out the experiments, analyzed the data, and drafted the manuscript. B.W., W.X., J.Y., P.C.M. and D.R.M. revised the manuscript. All authors read and approved the final manuscript.

Table 1 Strains used in this study.

Strains	Genotype*	References
E. coli	*Δ*(*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA*1 *gyrA96 relA1*	Stratagene
XL1-Blue MRF'	*lac* [*F´ proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]	
Synechocystis		
SPA:ΔphaEC	P_{tac}-*adhE2* (S2), Δ*phaEC* (S3)	[29]
ABd	Cm^{R}-P_{tac}-*phaA-phaB1* (S1), P_{tac}-*adhE2* (S2), Δ*phaEC* (S3)	This study
TABd	Cm^{R}-P_{tac}-*phaA-phaB1* (S1), P_{tac}-*tesB*-Kan^{R} (S2), Δ*phaEC* (S3)	[29]
TTrK	Cm^{R}-P_{tac}-*phaA-phaB1* (S1), P_{tac}-*tesB*-T1-Kan^{R} (S2), Δ*phaEC* (S3)	This study
SDTrK	Cm^{R}-P_{tac}-*phaA-phaB1* (S1), P_{tac}-SD-*tesB*-T1-Kan^{R} (S2), Δ*phaEC* (S3)	This study
UTRTrK	Cm^{R}-P_{tac}-*phaA-phaB1* (S1), P_{tac}-UTR-*tesB*-T1-Kan^{R} (S2), Δ*phaEC* (S3)	This study
PTrK12	Cm^{R}-P_{tac}-*phaA-phaB1* (S1), P_{psbA12}-*tesB*-T1-Kan^{R} (S2), Δ*phaEC* (S3)	This study
PTrK14	Cm^{R}-P_{tac}-*phaA-phaB1* (S1), P_{psbA14}-*tesB*-T1-Kan^{R} (S2), Δ*phaEC* (S3)	This study
PTrK16	Cm^{R}-P_{tac}-*phaA-phaB1* (S1), P_{psbA12}-P_{tac}-*tesB*-T1-Kan^{R} (S2), Δ*phaEC* (S3)	This study
R154	Cm^{R}-P_{tac}-*phaA*-(RBS_{opt})-*phaB1* (S1), P_{psbA12}-P_{tac}-*tesB*-T1-Kan^{R} (S2), Δ*phaEC* (S3)	This study

*S1, the insertion site on the chromosome of *Synechocystis* between *slr1495* and *sll1397*; S2, the insertion site between *slr1362* and *sll1274*; S3, the insertion site between *slr1828* and *sll1736*.

Table 2 Acetoacetyl-CoA reductase enzyme activities. *

Strain	**Expression cassette for** acetoacetyl-CoA reductase	**Enzyme activity**
TTrK	P_{tac}-*phaA-phaB1*	0.063 ± 0.013
R154	P_{tac}-*phaA*-(RBS_{opt})-*phaB1*	0.139 ± 0.020

* Enzyme activities were given in μmol min^{-1} L^{-1} cell extract.

Figure captions:

Fig. 1 Photoautotrophic production of (*R*)-3HB and the byproduct acetate in *Synechocystis*. Abbreviations: PSI, Photosystem I; PSII, Photosystem II; Fatty acyl-ACP, fatty acyl-acyl carrier protein; PHB, poly-3-hydroxybutyrate; 3HB, 3-hydroxybutyrate; TCA, tricarboxylic acid; *phaA*, the gene encoding thiolase; *phaB*, the gene encoding acetoacetyl-CoA reductase; *tesB*, the gene encoding thioesterase.

Fig. 2 Schematic illustration of the method for identifying the bottleneck in (*R*)-3HB biosynthesis. Acetyl-CoA and (*R*)-3HB-CoA are competitive substrates for thioesterase TesB, so when the enzyme activity of TesB is excessive (*i.e.*, the activities of thiolase and acetoacetyl-CoA reductase are limiting) it would react extravagantly on acetyl-CoA, leading to higher amount of acetate relative to (*R*)-3HB. Abbreviations: Acetyl-CoA, acetyl-coenzyme A; 3HB, 3-hydroxybutyrate; PhaAB, thiolase and acetoacetyl-CoA reductase; TesB, thioesterase.

Fig. 3 Schematic structures of the constructs used to characterize a library of six promoters in *Synechocystis*. Each construct was integrated into the neutral site S2 (between *slr1362* and *sll1274*) of the chromosome of *Synechocystis* ABd [Cm^R-P_{tac}-*phaA*-*phaB1*, P_{tac}-*adhE2*, Δ*phaEC*], resulting in strains expressing *tesB* under the control of various promoters. Abbreviations: TSS, transcription start site; SD, Shine-Dalgarno sequence; *rrnB* T1, the T1 terminator downstream from the *rrnB* gene.

Fig. 4 Characterization of a library of five promoters in *tesB*-expressing *Synechocystis* strains. (A) Cell density of strains achieved after 5 days of photoautotrophic cultivation in BG11 medium with bicarbonate as the carbon source. (B) Abundance of *tesB* mRNA. (C) Titer of (*R*)-3HB (wide bar) and acetate (thin bar). (D) Acetate to (*R*)-3HB ratio in the culture medium.

Fig. 5 Optimization of the RBS for gene *phaB1* in *Synechocystis*. (A) The original and optimized ribosome binding sites. (B) Acetoacetyl-CoA reductase activity before (strain TTrK) and after (strain R154) RBS optimization; *, given in μmol min^{-1} L^{-1} cell extract. (C) Cell growth curves for strains TTrK and R154 under the examined experimental conditions. (D) (*R*)-3HB titers from strains TTrK and R154.

Fig. 6 Photosynthetic production of (*R*)-3HB from CO_2 by engineered *Synechocystis* strain R154. (A) Time courses of cell growth and (*R*)-3HB production in the culture of *Synechocystis* strain R154. (B) Daily volumetric and specific (*R*)-3HB productivity of strain R154.

Fig. 7 Relatively quantitative flux distributions for (*R*)-3HB production in strain R154 on Day 4 (in the production phase). Arrow thickness is scaled proportionally to the flux values which are

relative to the CO_2 uptake rate. Abbreviations: G6P, Glucose 6-phosphate; Ru5P, Ribulose 5-phosphate; RuBP, Ribulose bisphosphate; F6P, fructose 6-phosphate; R5P, Ribose 5-phosphate; FBP, Fructose bisphosphate; X5P, Xylulose 5-phosphate; E4P, Erythrose 4-phosphate; DHAP, Dihydroxyacetone phosphate; GAP, Glyceraldehyde-3-phosphate; SBP, Sedoheptulose bisphosphate; S7P, Sedoheptulose 7-phosphate; PGA, Phosphoglycerate; PEP, Phosphoenolpyruvate; PYR, pyruvate; AcCoA, Acetyl Coenzyme A; CIT, Citrate; ICT, Isocitrate; 2OG, 2-oxoglutarate; SSA, Succinic semialdehyde; SUC, Succinate; FUM, Fumarate; MAL, Malate; OAA, Oxaloacetate. Abbreviations for reactions: G6PD, Glucose 6-phosphate dehydrogenase; PRK, Phosphoribulokinase; PGI, Phosphoglucose isomerase; TKT, Transketolase; PPI, Pentose phosphate isomerase; PFK, Phosphofructosekinase; PPE, Phosphopentose epimerase; FBA, Fructose bisphosphate aldolase; TAL,transaldolase; SBA, Sedoheptulose bisphosphate aldolase; TPI, Triosephosphate isomerase; SBPS, Sedoheptulose bisphosphatase; RBC, Ribulose bisphosphate carboxylase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ENO, Enolase; PEPC, Phosphoenolpyruvate carboxylase; PK, pyruvate kinase; PDH, pyruvate dehydrogenase; CS, citrate synthase; ME, malic enzyme; ACO, Aconitase; ICTDH, Isocitrate dehydrogenase; OGDC, 2-oxoglutarate decarboxylase; SSADH, Succinic semialdehyde dehydrogenase; SDH, Succinate dehydrogenase; FUS, Fumarase; MDH, Malate dehydrogenase.

Fig. 8 Heatmap of relative flux fold changes between Growth Phase (Day 2) and Production Phase (Day 4). Red color indicates up-regulation; green color indicates down-regulation; white color indicates either no change of the reaction rate or the reaction rate was too low to be considered and compared. Abbreviations: 2PGA, 2-phosphoglyceric acid; 2PG, 2-phosphoglycolate; 3HB, 3-hydroxybutyrate; 3PGA, 3-phosphoglyceric acid; AC, acetate; ACA, acetyl-CoA; AKG, α-ketoglutarate; ALA, alanine; ARG, arginine; ASN, asparagine; CIT, citrate; CYS, cysteine; DHAP, dihydroxyacetone phosphate; E4P,erythrose 4-phosphate; F6P, fructose 6-phosphate; FBP, fructose-1,6-bisphosphate; FUM, fumarate; G6P, glucose 6-phosphate; GA, glycerate; GAP, glyceraldehyde 3-phosphate; GLC, glycolate; GLN, glutamine; GLX, glyoxylate; GLY, glycine; GLU, glutamate; HIS, histidine; ICI, isocitrate; ILE, isoleucine; LEU, leucine; MAL, malate; MTHF, 5,10-Methylenetetrahydrofolate (5,10-CH2-THF); OAA, oxaloacetate; PEP, phosphoenolpyruvate; PHE, phenylalanine; PRO, proline; PYR, pyruvate; R5P, ribose 5-phosphate; Ru5P, ribulose-5-phosphate; RuBP, ribulose-1,5-diphosphate; S7P, sedoheptulose-7-phosphate; SBP, sedoheptulose-1,7-bisphosphate; SER, serine; SUC, succinate; SucCoA, succinyl-CoA; THR, threonine; TRP, tryptophan; VAL, valine; X5P, xylulose-5-phosphate.

Table of contents entry (20 words)

Mitigation of a bottleneck significantly improved (*R*)-3HB productivity, and metabolic flux analysis delineated dramatic metabolic flux changes in cyanobacterium *Synechocystis.*

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