Metabolic Engineering of cyanobacteria for photosynthetic 3-hydroxypropionic acid production
from CO₂ using *Synechococcus elongatus* PCC 7942

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Abstract

Photosynthetic conversion of CO₂ to chemicals using cyanobacteria is an attractive approach for direct recycling of CO₂ to useful products. 3-Hydroxypropionic acid (3HP) is a valuable chemical for the synthesis of polymers and serves as a precursor to many other chemicals such as acrylic acid. 3HP is naturally produced through glycerol metabolism. However, cyanobacteria do not possess pathways for synthesizing glycerol and converting glycerol to 3HP. Furthermore, the latter pathway requires coenzyme B₁₂, or an oxygen sensitive, coenzyme B₁₂-independent enzyme. These characteristics present major challenges for production of 3HP using cyanobacteria. To overcome such difficulties, we constructed two alternative pathways in *Synechococcus elongatus* PCC 7942: a malonyl-CoA dependent pathway and a β-alanine dependent pathway. Expression of the malonyl-CoA dependent pathway genes (malonyl-CoA reductase and malonate semialdehyde reductase) enabled *S. elongatus* to synthesize 3HP to a final titer of 665 mg/L. β-alanine dependent pathway expressing *S. elongatus* produced 3HP to final titer of 186 mg/L. These results demonstrated the feasibility of converting CO₂ into 3HP using cyanobacteria.

Keywords

Cyanobacteria

Biofuel

3-hydroxypropionate

Malonate semialdehyde

Introduction

Currently, the chemical industry is mainly relying on non-renewable fossil resources such as petroleum and natural gas as raw materials, presenting long-term sustainability and environmental problems. To address these issues, both academia and industry have demonstrated increasing interest to develop biological processes for producing chemicals from a variety of renewable substrates (Choi, et al., 2014, Lan and Liao, 2013, Lin, et al., 2014, Wargacki, et al., 2012). In particular, photosynthetic microorganisms such as cyanobacteria are being engineered for the production of chemicals directly from CO₂ (Lan and Liao, 2011, Li and Liao, 2013, Oliver, et al., 2013, Ungerer, et al., 2012, Xue, et al., 2014). Here we report the engineering of *Synechococcus elongatus* PCC 7942 for direct photosynthetic conversion of CO₂ to 3-Hydroxypropionic acid (3HP).

3HP is an important chemical building block with multiple applications. 3HP can be converted to other monomers such as acrylic acid, acrylamide, and 1,3-propanediol. In particular, acrylic acid has an annual market exceeding \$10 billion. Furthermore, having both hydroxyl and carboxyl functional groups, 3HP can be polymerized into poly-3-hydroxypropionate, which is a biodegradable and biocompatible thermoplastic polymer. Microbial 3HP production naturally occurs through the degradation of glycerol or acrylic acid. Acrylic acid is even further removed from renewable substrates than 3HP, and thus is not a good choice as a precursor. The glycerol pathway has been explored in *Escherichia coli* (Jung, et al., 2014, Kim, et al., 2014, Rathnasingh, et al., 2009, Tokuyama, et al., 2014) and *Klebsiella pneumonia* (Ashok, et al., 2013, Luo, et al., 2012, Luo, et al., 2011) for production of 3HP. This pathway involves a coenzyme B12-dependent glycerol dehydratase (Gdh), [requiring excess amounts of coenzyme B12 be externally supplied and thus increasing production cost](#), or a coenzyme B12-independent Gdh, which is oxygen sensitive. Both of these approaches present significant challenges for functional expression in cyanobacteria. Therefore, it is preferable to use alternative pathways for 3HP biosynthesis.

3HP is an intermediate of the carbon fixing 3HP bicycle and 3HP/4-hydroxybutyrate cycle. In these pathways, 3HP is derived from two step of reduction of malonyl-CoA via an

intermediate malonate semialdehyde (MSA), which is hereafter called the malonyl-CoA dependent pathway. Advantage of this pathway is that malonyl-CoA is a central metabolite common to all organisms. This malonyl-CoA dependent pathway has been implemented in heterotrophs such as *Escherichia coli* (Rathnasingh, et al., 2012), *Saccharomyces cerevisiae* (Chen, et al., 2014) and *Pyrococcus furiosus* (Thorgersen, et al., 2014) for the production of 3HP. However, the resulting production titers were generally less than that achieved through the glycerol pathway. This result may be attributed to the highly regulated nature of acetyl-CoA carboxylase (Acc).

Another potential pathway for 3HP production is through β -alanine, hereafter refer to as the β -alanine dependent pathway. This synthetic pathway bypasses malonyl-CoA and Acc. As depicted in Figure 1, phosphoenolpyruvate (PEP) is carboxylated to oxaloacetate, which is transaminated into aspartate. Aspartate is then decarboxylated to β -alanine using aspartate decarboxylase. β -Alanine is subsequently transaminated to MSA and reduced to 3HP. This strategy has been proposed (Jiang, et al., 2009, Kumar, et al., 2013), and [recently used for production of poly-3-hydroxypropionate \(Wang, et al., 2014\)](#).

In this study, we expressed both the malonyl-CoA dependent and the β -alanine dependent pathway for 3HP production in *S. elongatus* PCC 7942. To express the malonyl-CoA dependent pathway, we introduced malonyl-CoA reductase (Mcr) and malonate semialdehyde dehydrogenase (Msr) into *S. elongatus*, we observed photosynthetic production of 3HP from CO₂. To further increase the carbon flux from central metabolism to 3HP biosynthesis, we introduced the β -alanine dependent pathway, containing PEP carboxylase, aspartate transaminase, aspartate decarboxylase, and β -alanine transaminase, into *S. elongatus* in addition to the malonyl-CoA pathway. The resulting strains expressing both pathways achieved increased 3HP production compared to strain expressing only one of the pathways. These results demonstrate the feasibility of photosynthetic production of 3HP using cyanobacteria, the *in vivo* functioning of the β -alanine dependent pathway, and the effect of carbon flux enhancement by bypassing Acc.

Materials and methods

Chemicals and reagents

All chemicals were purchased either from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified. 3HP was purchased from Tokyo Chemical Industry (Portland, OR) as an approximate 3.6 M solution in water. For quantification of commercial 3HP concentration, please see below section on 3-hydroxypropionate quantification. iProof high-fidelity DNA polymerase was purchased from Bio-Rad (Hercules, CA). Restriction enzymes, Phusion DNA polymerase, and ligases were purchased from New England Biolabs (Ipswich, MA). T5-Exonuclease was purchased from Epicentre Biotechnologies (Madison, WI). KOD and KOD xtreme DNA polymerases were purchased from EMD biosciences (Gibbstown, NJ).

DNA manipulations

All chromosomal manipulations were carried out by homologous recombination of plasmid DNA into *S. elongatus* PCC 7942 genome at neutral site I (NSI) (Bustos and Golden, 1992) and II (NSII) (Andersson, et al., 2000). All plasmids were constructed using Gibson isothermal DNA assembly method (Gibson, et al., 2009). For strains with synthetic operons in both NSI and NSII, operon integrated at NSI was always introduced first. For example, strain EL63 was constructed by transforming strain DC3 with plasmid pEL233. Plasmids were constructed in *E. coli* XL-1 blue for propagation and storage (Table S1). Coding regions of all plasmids were sequenced by Genewiz (San Diego, CA). Gene *skpyd4* was synthesized by DNA 2.0 with codon optimization for *E. coli*. Gene *mcr* from *Sulfolobus tokodaii* was synthesized by Genewiz with codon optimization for *E. coli*.

Culture medium and condition

All *S. elongatus* PCC 7942 strains were grown on modified BG-11 (1.5 g/L NaNO₃, 0.0272 g/L CaCl₂·2H₂O, 0.012 g/L ferric ammonium citrate, 0.001 g/L Na₂EDTA, 0.040 g/L K₂HPO₄, 0.0361 g/L MgSO₄·7H₂O, 0.020 g/L Na₂CO₃, 1000x trace mineral (1.43 g H₃BO₃, 0.905 g/L MnCl₂·4H₂O, 0.111 g/L ZnSO₄·7H₂O, 0.195 g/L Na₂MoO₄·2H₂O, 0.0395 g CuSO₄·5H₂O, 0.0245 g

Co(NO₃)₂·6H₂O), 0.00882 g/L sodium citrate dihydrate (Bustos and Golden, 1991)) agar (1.5% w/v) plates. All *S. elongatus* PCC 7942 strains were cultured in the same BG-11 medium containing 50 mM NaHCO₃ in 250 mL screw cap flasks. Cultures were grown under 50 μE/s/m² light measured by Licor quantum sensor (LI-250A equipped with LI-190 Quantum Sensor), supplied by 3 Lumichrome F30W-1XX 6500K 98CRI light tubes, at 30 °C. Cell growth was monitored by measuring OD₇₃₀ with Beckman Coulter DU800 spectrophotometer.

Strain construction and transformation

The strains used and constructed are listed in Table 1 based on homologous recombination using plasmids listed in Table S1. *S. elongatus* PCC 7942 strains were transformed by incubating cells at mid-log phase (OD₇₃₀ of 0.4 to 0.6) with 2 μg of plasmid DNA overnight in the dark. The culture was then spread on BG-11 plates supplemented with appropriate antibiotics for selection of successful recombination. For selection and culture maintenance, 20 μg/ml spectinomycin and 10 μg/ml kanamycin were added into BG-11 agar plates and BG-11 medium where appropriate. Strain Segregation was confirmed by colony PCR (Figure S1). In all cases, three individual colonies were analyzed and propagated for downstream tests.

Production of 3-hydroxypropionic acid

A loopful of *S. elongatus* was used to inoculate fresh 50 mL BG-11 with 50 mM NaHCO₃ and appropriate antibiotics. Initial cell density of culture was typically OD₇₃₀ 0.03 to 0.05. 500 mM IPTG stock solution was used to induce the growing culture at two days after inoculation with cell density typically of OD₇₃₀ 0.4 to 0.6 with 1 mM IPTG as the final concentration. Culture samples (5 mL, 10% of culture volume) were taken every two days for measurement of 3HP concentration in the culture medium and cell optical density. Upon sampling, 5 mL of fresh BG-11 with 500 mM NaHCO₃, appropriate antibiotics, and IPTG were added back to the culture. This procedure ensured the carbon supply for *S. elongatus*.

3-Hydroxypropionic acid quantification

Culture sample (1 mL) was centrifuged for 5 minutes at 15,000 x g. The supernatant was

then directly analyzed by Agilent 1200 HPLC equipped with a BioRad HPX87 column. 3HP was monitored by photodiode array detector at 210 nm absorbance. The mobile phase used was 5 mM H₂SO₄ at a constant flow rate of 0.6 mL/min. The column was maintained at 35°C. Injection volume was 20 µL. Concentration of commercial 3HP was determined biochemically through enzymatic reaction of 3HP with NADP⁺ using purified malonate semialdehyde reductase (Msr). The enzymatic reaction contained approximately 90 µM 3HP, 1.5 mM NADP⁺, 100 mM Tris-HCl pH 8.3, and purified Msr. 3HP standard curve was then constructed by analyzing standard concentrations. Concentration of 3HP present in the culture medium was then calculated based on the standard curve.

Results

The Malonyl-CoA dependent pathway for 3HP biosynthesis

Reduction of malonyl-CoA to 3HP is a natural process in organisms capable of fixing CO₂ via either 3HP bicycle or 3HP/4HB cycle. In particular, Mcr (Hugler, et al., 2002) in *Chloroflexus aurantiacus* (*C. aur*) is a bifunctional alcohol/aldehyde dehydrogenase that catalyzes the two-step reduction of malonyl-CoA to 3HP. As a starting point, we introduced the 3HP biosynthesis pathway into *S. elongatus* by inserting *mcr* (*C. aur*) into the genome of *S. elongatus* PCC 7942 at neutral site I (NSI) via homologous recombination (Figure 2A). The gene *mcr* (*C. aur*) was driven by an IPTG inducible Ptrc promoter. The resulting strain expressing *mcr* (*C. aur*) was unable to synthesize 3HP and suffered growth retardation upon induction with IPTG (Figure S2).

As an alternative, we chose to use mono-functional Mcr coupled with a separate Msr. We used Mcr from *Sulfolobus tokodaii* (*S. tok*), which has been demonstrated to catalyze only the reduction of malonyl-CoA to MSA (Alber, et al., 2006). To identify suitable Msr enzymes, we screened several enzyme candidates using purified enzyme assays (Figure S3). We used Msr from *S. tokodaii* and *Metallosphaera sedula* (*M. sed*), MmsB from *Bacillus subtilis*, and GarR from *E. coli*. In addition to their demonstrated activities, Msr (*S. tok*) and Msr (*M. sed*) were selected because they are naturally present in their native host as part of the 3HP/4HB pathway for CO₂ fixation. MmsB was chosen for its sequence homology to MmsB from *Bacillus cereus*,

which has been previously characterized for its activity for 3HP oxidation to MSA (Yao, et al., 2010). GarR is a tartronate semialdehyde reductase, and it was selected due to the structure similarity between tartronate semialdehyde and MSA. Furthermore, we also chose to clone and express BetA from *Ralstonia eutropha*. BetA was chosen for its sequence homology to DddA, a 3HP dehydrogenase from acrylate degradation pathway of *Halmona* HTNK1 (Todd, et al., 2010).

To introduce *garR*, *mmsB*, *betA*, *msr* (*M. sed*), and *msr* (*S. tok*) individually with *mcr* (*S. tok*) into *S. elongatus* PCC 7942, we constructed shuttle plasmids using the same approach as we did for expressing *mcr* (*C. aur*) (Figure 2A). A synthetic operon containing the two genes were placed under the control of an IPTG inducible P_{trc} promoter. The resulting strains, with the exception of strain EL56 expressing *betA*, enabled photosynthetic production of 3HP at different levels (Figure 2B). Strain DC3 expressing *msr* (*M. sed*) produced the highest amounts of 3HP, reaching to an observed titer of 659 ± 69 mg/L in 16 days with peak productivity of 98 ± 11 mg/L/d occurring between day 8 to 10 (Figure 2B). This result indicated that Msr is likely a limiting step for 3HP production. Slight growth retardation was observed for strain DC3 expressing *msr* (*M. sed*) (Figure 2C), potentially indicating increased cellular stress due to higher flux to 3HP. Notably, strain EL56 expressing *betA* suffered significant growth retardation (Figure 2C), which may be potentially due to the accumulation of toxic intermediate MSA. Together, these results demonstrated the construction of 3HP producing cyanobacteria and showed that the malonyl-CoA dependent pathway for 3HP production functions well in cyanobacteria.

Expression of the synthetic β -alanine dependent pathway increased 3HP productivity

Biosynthesis of malonyl-CoA is a significantly regulated process in the cell. Acc is regulated both transcriptionally and at protein level via product feedback inhibition. Therefore as an alternative, the synthetic β -alanine dependent pathway may be advantageous for 3HP production. To construct this pathway, we identified and cloned the genes necessary to connect PEP to MSA as according to Figure 1. The first step of the pathway requires carboxylation of PEP to oxaloacetate using PEP carboxylase (Ppc). Oxaloacetate then is transaminated to aspartate using aspartate transaminase (AspC). Aspartate is subsequently decarboxylated to β -alanine and

transaminated to MSA through catalysis of aspartate decarboxylase (PanD) and β -alanine aminotransferase (SkPYD4), respectively. We cloned *ppc* and *aspC* from *E. coli*, *panD* from *Corynebacterium glutamicum*, and *Skpyd4* from *Saccharomyces kluyveri* (Andersen, et al., 2007). In particular, *panD* from *C. glutamicum* was chosen for its higher rate of autoproteolysis (Dusch, et al., 1999), which is a required process for PanD activation. While *S. elongatus* naturally has *ppc* and *aspC* genes, we chose to express heterologous genes to avoid homologous recombination between the copy we introduce and the native copy.

To incorporate the β -alanine dependent pathway into *S. elongatus*, we constructed another recombination plasmid harboring *panD*, *Skpyd4*, *ppc*, and *aspC* under the control of an IPTG inducible promoter PLLacO1 (Figure 3A) for inserting these genes into Neutral Site II (NSII). We tested the effect of having this β -alanine pathway on all four of our *S. elongatus* strains already capable of producing 3HP through the malonyl-CoA reduction pathway. As shown in Figure 3B-3E, with exception of strain DC4, co-expressing both the β -alanine dependent and the malonyl-CoA dependent pathways outperformed their parental strains expressing only the latter. We are uncertain why expression of β -alanine dependent pathway was not able to increase 3HP productivity. One potential explanation is the toxicity of 3HP to cyanobacteria. Nonetheless, expression of the β -alanine dependent pathway in the other strains led to increased 3HP productivity by about 25 – 40 %. There was no obvious change in growth phenotype between the strains with and without expression of synthetic β -alanine pathway (Figure S4). These results demonstrated the functional expression of this synthetic pathway for MSA formation and 3HP biosynthesis.

PLP-dependent aspartate decarboxylase outperformed PanD

To further increase 3HP production, we examined each enzyme of the pathway in more detail. In particular, PanD is an amino acid decarboxylase that is first translated into a pro-protein, which is subsequently proteolyzed into α and β subunits that form the active enzyme. The maturation of PanD is dependent on temperature and the maturation enzyme (Nozaki, et al., 2012). As the maturation of PanD may be difficult to control, we searched for an alternative

enzyme. We noted that most amino acid decarboxylases such as glutamate decarboxylase and lysine decarboxylase utilize PLP as a cofactor to facilitate decarboxylation. Since glutamate is structurally similar to aspartate, we cloned *Ae_Adc*, a glutamate decarboxylase homologue from *Aedes aegypti* with characterized activity towards aspartate, for aspartate decarboxylation (Richardson, et al., 2010).

We replaced the *panD* gene with *Ae_adc* gene in our recombination plasmids. Using the same DNA recombination strategy, we inserted this synthetic operon containing genes *Ae_adc*, *Skpyd4*, *ppc*, and *aspC* into NSII of the same four individual parental strains capable of producing 3HP through malonyl-CoA reduction. The resulting strains EL61, EL62, EL63, and EL64 expressed *garR*, *mmsB*, *msr* (*M. sed*), *msr* (*S. tok*), respectively, with the synthetic β -alanine pathway utilizing *Ae_adc* instead of *panD*. As shown in Figure 3B-3E, all strains expressing *Ae_adc* outperformed the corresponding strains expressing *panD*. Consistent with the observation from expressing *panD* dependent pathway with *Msr* (*M.sed*), additional carbon flux to MSA was not able to increase the overall productivity and titer of 3HP, indicating a bottleneck. Nevertheless, the results here demonstrated that the PLP-dependent enzyme *Ae_Adc* is in general a better choice for increasing carbon flux to MSA.

3HP production using only the β -alanine dependent pathway

To investigate the individual contribution of this β -alanine dependent pathway for 3HP production, we constructed two new strains expressing only the β -alanine dependent pathway instead of co-expressing both the β -alanine and the malonyl-CoA dependent pathways (Figure 4A). To construct these strains, we first transformed the wild type *S. elongatus* PCC7942 with pAM2991 which allowed the integration of *LacIq* into its genome, resulting in strain EL73. Next, we transformed EL73 with individual plasmids to integrate the operon encoding the β -alanine dependent pathway expressing either *Ae_adc* or *panD* (Figure 4A). The resulting strains DC1 and DC2 expressed *Ae_adc* and *panD*, respectively. As shown in Figure 4B, Strain DC2 barely produced 3HP to a titer of around 20 mg/L. On the other hand, strain DC1 produced 3HP to a final observed titer of around 190 mg/L in 16 days, which correlates to the difference between

the production titer of strain EL55, EL57 and EL60 expressing only malonyl-CoA dependent pathway and EL61, EL62 and EL64 expressing both pathways. The results again verified that Ae_adc outperforms panD for more efficient decarboxylation of aspartate, leading to higher carbon flux to MSA, the direct precursor to 3HP.

Discussion

Production of desirable compounds is often limited by the available metabolic pathways and their suitability in the organism of choice. As a result, additional engineering designs are required to achieve higher flux productions (Lan and Liao, 2012, Lan, et al., 2013). Furthermore, redesign of essential primary metabolic pathways for the purpose of increasing efficiency (Bogorad, et al., 2013, Mainguet, et al., 2013) and favorability of production systems becomes crucial. While 3HP can be synthesized from either 3-hydroxypropionaldehyde or MSA, we reasoned that MSA is a preferred precursor over 3-hydroxypropionaldehyde for 3HP biosynthesis from CO₂ in cyanobacteria because MSA can be derived from malonyl-CoA which is a central metabolite present in all organisms. In addition, MSA can also be produced from β -alanine (Jiang, et al., 2009, Kumar, et al., 2013).

In this study, we constructed 14 different strains (Table 2) of *S. elongatus* capable of producing 3HP photosynthetically through either malonyl-CoA dependent pathway and/or the synthetic β -alanine dependent pathway. Both Malonyl-CoA and β -alanine dependent pathways require the same amount of ATP. Malonyl-CoA dependent pathway consumes 2 NADPH while producing 1 NADH. Thus, expression of malonyl-CoA dependent pathway increases NADH/NADPH ratio which is may potentially be less favorable for cyanobacteria. On the other hand, β -alanine dependent pathway only consumes 1 NADPH, leading to less perturbation in NADH/NADPH ratio. Both PEP carboxylase and aspartate decarboxylase catalyze irreversible steps, providing a driving force for 3HP production. Strains expressing the β -alanine dependent pathway with Ae_Adc was the most productive, followed by strains expressing PanD and strains without β -alanine dependent pathway. This result is consistent with our expectation that the β -alanine dependent pathway supplies additional carbon flux towards 3HP synthesis. However,

notably the expression of the β -alanine dependent pathway was not able to increase 3HP productivity for the strains expressing Msr (M.sed). This result indicated the presence of other bottlenecks. One potential bottleneck is the toxicity of 3HP. 3HP has been demonstrated to be toxic to *S. elongatus* PCC 7942 with a minimal inhibitory concentration of 2 mM (Begemann, et al., 2013), equivalent to 180 mg/L, which is 3 to 4 folds lower than the concentration observed in our production cultures. While toxicity and productivity have a complex relationship, it is generally believed that increasing tolerance may aid the production of the desired compound. In the case of 3HP, it has been shown that chromosomal deletion of acetyl-CoA ligase led to several fold increase in tolerance towards 3HP in *Synechococcus* sp. PCC 7002 (Begemann, et al., 2013). As such, photosynthetic 3HP production may be improved further with acetyl-CoA ligase knock out.

Hydroxyacids have both hydroxyl and carboxylic acid groups and are useful compounds for producing plastics. Photosynthetic production of some hydroxyacids including lactic acid (Angermayr, et al., 2012, Varman, et al., 2013) and 3-hydroxybutyric acid (Wang, et al., 2013) have been studied and achieved through metabolic engineering of cyanobacteria. The productivity of 3HP demonstrated in this study exceeds or compares favorably to those of the other hydroxyacids demonstrated. Furthermore, among the various products produced using engineered cyanobacteria, only a few cases were able to achieve production titer over 500 mg/L (Oliver and Atsumi, 2014). The production titers achieved in this study is relatively high with observed titer reaching 665 mg/L from the best producing strain developed in this study. Recent studies have shown that optimization of ribosomal binding sequence (Oliver, et al., 2014) and removal of natural competing pathways (Li, et al., 2014, van der Woude, et al., 2014) in cyanobacteria lead to increase productivity of desired product. Combining these strategies, we may further increase 3HP productivity.

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Figure legends

Figure 1. Schematics for 3-hydroxypropionic acid biosynthesis from CO₂ using cyanobacteria. Photosynthesis generates ATP and reducing equivalents NADPH, which are used for carbon fixation via Calvin cycle and 3HP production. Abbreviations: G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; Ppc, phosphoenolpyruvate carboxylase; AspC, aspartate aminotransferase; PanD, PLP-independent aspartate decarboxylase; Adc, PLP-dependent aspartate decarboxylase; SkPYD4, β -alanine aminotransferase; Mcr, malonyl-CoA reductase; Msr, malonate semialdehyde reductase. Grey line indicates the absence of oxaloacetate α -decarboxylase in nature.

Figure 2. Production of 3HP using engineered cyanobacteria expressing genes coding for malonyl-CoA reductase and malonate semialdehyde reductase. A) Schematics of gene recombination strategy used to incorporate genes *mcr* and *msr* into neutral site I of *S. elongatus* PCC 7942 genome. B) Observed 3HP production profile and C) growth curves of engineered *S. elongatus* expressing *mcr* from *S. tokodaii* with either *garR*, *mmsB*, *betA*, *msr* (*M.sed*), or *msr* (*S.tok*). *garR* was cloned from *E. coli*. *mmsB* was cloned from *B. subtilis*, *betA* was cloned from *R. eutropha*. Fluctuations in observed titers and optical density were due to dilutions made to cyanobacteria cultures during nutrient replenishment. For detailed protocol, see Materials and Methods. Error bars represent standard deviation of triplicate experiments.

Figure 3. Production of 3HP using engineered cyanobacteria expressing *mcr* (*S.tok*) and different genes for Msr in addition to expressing synthetic β -alanine dependent Pathway for 3HP biosynthesis. A) Schematics of gene recombination strategy used to incorporate *mcr* and *msr* into neutral site I and *panD* (or *Ae_adc*), *skpyd4*, *ppc*, and *aspC* into neutral site II of *S. elongatus* PCC 7942 genome. Observed 3HP production profile of strains expressing B) *garR*, C) *mmsB*, D) *msr* (*M. sed*), E) *msr* (*S. tok*) as Msr with either *panD* or *Ae_adc* for aspartate decarboxylase and *ppc*, *aspc*, *skpyd4*. Here, data from Fig. 2 (3HP production from strains expressing *mcr* with either *gar*, *mmsB*, *msr* (*M.sed*), or *msr* (*S. tok*)) were replotted for comparison of the malonyl-CoA pathway only versus addition of the synthetic β -alanine dependent Pathway. Error bars represent standard deviation of triplicate experiments.

Figure 4. 3HP production from strains DC1 and DC2, expressing only synthetic β -alanine pathway with *Ae_Adc* and *PanD*, respectively. A) Schematics of gene recombination strategy. An empty vector was integrated into genome of *S. elongatus* PCC 7942 for expression of LacIq protein. B) Observed production time course of strains DC1 and DC2. C) Growth curves of engineered *S. elongatus* expressing synthetic β -alanine pathway. Error bars represent standard deviation of triplicate experiments.

Table 1. Strain list

Strain	Relevant genotypes	Plasmids used for gene insertion	Reference
PCC 7942	Wild-type <i>Synechococcus elongatus</i> PCC 7942		Lab collection
EL55	P _{Trc} :: <i>garR</i> , <i>mcr</i> (<i>S.tok</i>) integrated at NSI in PCC7942 genome	pCS181	This work
EL56	P _{Trc} :: <i>betA</i> , <i>mcr</i> (<i>S.tok</i>) integrated at NSI in PCC7942 genome	pCS182	This work
EL57	P _{Trc} :: <i>mmsB</i> , <i>mcr</i> (<i>S.tok</i>) integrated at NSI in PCC7942 genome	pCS183	This work
EL58	P _{Trc} :: <i>mcr</i> (<i>C.aur</i>) integrated at NSI in PCC7942 genome	pCS184	This work
DC3	P _{Trc} :: <i>msr</i> (<i>M.sed</i>), <i>mcr</i> (<i>S.tok</i>) integrated at NSI in PCC7942 genome	pCS185	This work
EL60	P _{Trc} :: <i>msr</i> (<i>S.tok</i>), <i>mcr</i> (<i>S.tok</i>) integrated at NSI in PCC7942 genome	pCS186	This work
EL61	P _{Trc} :: <i>garR</i> , <i>mcr</i> (<i>S.tok</i>) integrated at NSI and P _{LlacO1} :: <i>Ae_adc</i> , <i>Skpyd4</i> , <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pCS181 pEL233	This work
EL62	P _{Trc} :: <i>mmsB</i> , <i>mcr</i> (<i>S.tok</i>) integrated at NSI and P _{LlacO1} :: <i>Ae_adc</i> , <i>Skpyd4</i> , <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pCS183 pEL233	This work
EL63	P _{Trc} :: <i>msr</i> (<i>M.sed</i>), <i>mcr</i> (<i>S.tok</i>) integrated at NSI and P _{LlacO1} :: <i>Ae_adc</i> , <i>Skpyd4</i> , <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pCS185 pEL233	This work
EL64	P _{Trc} :: <i>msr</i> (<i>S.tok</i>), <i>mcr</i> (<i>S.tok</i>) integrated at NSI and P _{LlacO1} :: <i>Ae_adc</i> , <i>Skpyd4</i> , <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pCS186 pEL233	This work
EL65	P _{Trc} :: <i>garR</i> , <i>mcr</i> (<i>S.tok</i>) integrated at NSI and P _{LlacO1} :: <i>panD</i> , <i>Skpyd4</i> , <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pCS181 pEL234	This work
EL66	P _{Trc} :: <i>mmsB</i> , <i>mcr</i> (<i>S.tok</i>) integrated at NSI and P _{LlacO1} :: <i>panD</i> , <i>Skpyd4</i> , <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pCS183 pEL234	This work
DC4	P _{Trc} :: <i>msr</i> (<i>M.sed</i>), <i>mcr</i> (<i>S.tok</i>) integrated at NSI and P _{LlacO1} :: <i>panD</i> , <i>Skpyd4</i> , <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pCS185 pEL234	This work
EL68	P _{Trc} :: <i>msr</i> (<i>S.tok</i>), <i>mcr</i> (<i>S.tok</i>) integrated at NSI and P _{LlacO1} :: <i>panD</i> , <i>Skpyd4</i> , <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pCS186 pEL234	This work
EL73	Empty vector with <i>LacI</i> integrated at NSI in PCC7942 genome	pAM2991	This work
DC1	P _{LlacO1} :: <i>Ae_adc</i> , <i>Skpyd4</i> , <i>msr</i> (<i>M.sed</i>), <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pAM2991 pDC207	This work
DC2	P _{LlacO1} :: <i>panD</i> , <i>Skpyd4</i> , <i>msr</i> (<i>M.sed</i>) <i>ppc</i> , <i>aspC</i> integrated at NSII in PCC7942 genome	pAM2991 pDC208	This work

garR, *ppc*, and *aspC* are from *Escherchia coli*. *mmsB* is from *Bascillus subtilis*, *betA* is from *Ralstonia eutropha*. *Skpyd4* is from *Saccharomyces kluyveri*. *panD* is from *Corynebacterium glutamicum*. *mcr* (*S. tok*) and *msr* (*S. tok*) are from *Sulfolobus tokodaii*. *msr* (*M. sed*) is from *Metallosphaera sedula*. *mcr* (*C. aur*) is from *Chloroflexus aurantiacus*.

Table 2. Summary of 3HP productivity and observed titer from strains used in this study

Strain name	Additional genes	Asparate decarboxylase	Malonyl-CoA reductase	Malonate semialdehyde reductase	Peak 3HP productivity (mg/L/d)	Highest observed Titer (mg/L)
EL58	---	---	<i>mcr (C.aur)</i>	<i>mcr (C.aur)</i>	<i>n.d.</i>	<i>n.d.</i>
EL56	---	---	<i>mcr (S.tok)</i>	<i>betA</i>	<i>n.d.</i>	<i>n.d.</i>
EL60	---	---	<i>mcr (S.tok)</i>	<i>msr (S.tok)</i>	17 ± 42	124 ± 9
EL57	---	---	<i>mcr (S.tok)</i>	<i>mmsB</i>	23 ± 5	202 ± 27
EL55	---	---	<i>mcr (S.tok)</i>	<i>garR</i>	27 ± 4	222 ± 4
DC3	---	---	<i>mcr (S.tok)</i>	<i>msr (M.sed)</i>	98 ± 11	659 ± 69
EL68	<i>ppc, aspC, Skpyd4</i>	<i>panD</i>	<i>mcr (S.tok)</i>	<i>msr (S.tok)</i>	26 ± 4	180 ± 17
EL66	<i>ppc, aspC, Skpyd4</i>	<i>panD</i>	<i>mcr (S.tok)</i>	<i>mmsB</i>	44 ± 7	250 ± 55
EL65	<i>ppc, aspC, Skpyd4</i>	<i>panD</i>	<i>mcr (S.tok)</i>	<i>garR</i>	40 ± 6	286 ± 30
DC4	<i>ppc, aspC, Skpyd4</i>	<i>panD</i>	<i>mcr (S.tok)</i>	<i>msr (M.sed)</i>	99 ± 13	662 ± 26
EL64	<i>ppc, aspC, Skpyd4</i>	<i>Ae_adc</i>	<i>mcr (S.tok)</i>	<i>msr (S.tok)</i>	51 ± 6	350 ± 61
EL62	<i>ppc, aspC, Skpyd4</i>	<i>Ae_adc</i>	<i>mcr (S.tok)</i>	<i>mmsB</i>	70 ± 10	388 ± 89
EL61	<i>ppc, aspC, Skpyd4</i>	<i>Ae_adc</i>	<i>mcr (S.tok)</i>	<i>garR</i>	51 ± 6	409 ± 30
EL63	<i>ppc, aspC, Skpyd4</i>	<i>Ae_adc</i>	<i>mcr (S.tok)</i>	<i>msr (M.sed)</i>	102 ± 9	665 ± 91
DC2	<i>ppc, aspC, Skpyd4</i>	<i>panD</i>	---	<i>msr (M.sed)</i>	5 ± 3	20 ± 2
DC1	<i>ppc, aspC, Skpyd4</i>	<i>Ae_adc</i>	---	<i>msr (M.sed)</i>	25 ± 4	186 ± 24

Figure 1

Figure 2

Figure 3

Figure 4