Metabolic engineering of *Synechococcus* sp. PCC 7002 to produce poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-4-hydroxybutyrate

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Abstract

Cyanobacteria are an important group of photoautotrophic bacteria that have been engineered and used to produce a wide range of biomaterials and biofuels, which are usually derived from important intermediates of the central metabolic pathways. In this study, the production of poly-3-hydroxybutyrate and poly-3-hydroxybutyrate-co-4-hydroxybutyrate in cyanobacteria was studied, and metabolic engineering strategies to improve the yields were also investigated. The genes involved in the biosynthetic pathway for poly-3-hydroxybutyrate from *Chlorogloeopsis fritschii* PCC 9212 were introduced into *Synechococcus* sp. PCC 7002, and the resulting strain was able to accumulate 2.77% of total cell dry weight as poly-3-hydroxybutyrate. When the *ccmR* gene was deleted in this strain, the yield of poly-3-hydroxybutyrate increased to 3.97% of total cell dry weight. A biosynthetic pathway for the production of 4-hydroxybutyryl-CoA was also constructed and introduced into the poly-3-hydroxybutyrate-producing strain. The resulting strain was able to produce ~4.5% of total cell dry weight as poly-3-hydroxybutyrate-co-4-hydroxybutyrate, in which 4-hydroxybutyrate accounted for ~12% of the co-polymer. These results demonstrate that poly-3-hydroxybutyrate-co-4-hydroxybutyrate can be produced in cyanobacteria and confirm that succinic semialdehyde is an important TCA cycle metabolite in cyanobacteria. This study also demonstrates the potential for future metabolic engineering in cyanobacteria that is based on recently discovered metabolites.

1 Introduction

Due to the ever-increasing demand for sustainable energy and materials, cyanobacteria, one group of photoautotrophic bacteria that are able to assimilate CO₂, are projected to play important roles as resources for the production of biofuels and biomaterials. Cyanobacteria have been engineered and used to produce a wide range of biomaterials and biofuels, which are usually derived from major metabolites of the central metabolic pathways inside cells (Atsumi et al., 2008; Kumaraswamy et al., 2013; Lan and Liao, 2011; Machado and Atsumi, 2012; Oliver et al., 2013). Thus, the discovery of novel metabolic pathways should not only result in a better understanding of metabolic diversity in cyanobacteria, but could also provide new metabolic intermediates that can potentially serve as targets for future metabolic engineering purposes. We recently identified a TCA cycle variant in cyanobacteria, and this not only clarified a very long-term misunderstanding about the nature of the TCA cycle, but it also identified succinic semialdehyde, which might serve as a potential metabolic engineering target, as an important new metabolite in the TCA cycle variant (Zhang and Bryant, 2011). Indeed, many approaches have been explored to use succinic semialdehyde as the starting metabolite to produce important biomaterials, including 1,4-butanediol and polyhydroxyalkanoates (PHA) (Lee et al., 2012; Li et al., 2010; Yim et al., 2011).

A variety of bacteria have been reported to synthesize PHAs as intracellular carbon and energy storage compounds, especially when those organisms are grown under stress conditions produced by limitation for essential nutrients (e.g., nitrogen, phosphate) (Lee, 1996; Saito et al., 1996; Steinbüchel and Valentin, 1995; Zinn et al., 2001). The synthesized polyesters can be degraded by PHA depolymerases, and the decomposition products are used as nutrients for microorganisms in environments as diverse as soils and

oceans (Saito et al., 1996). Thus, due to their biodegradable and biocompatible properties, potential industrial applications for PHAs have been investigated over a long time (Chen, 2009; Martin and Williams, 2003; Park et al., 2005). Depending on the bacterial strain, specific PHA synthases, and the growth substrates, PHAs can be synthesized as homopolymers or copolymers (Gao et al., 2011; Rehm and Steinbüchel, 2005). Among the different types of PHAs synthesized in bacteria, the homopolymer of poly-3-hydroxybutyrate (hereafter P3HB) is the most common and most studied polymer (Gao et al., 2011). However, because P3HB is relatively amorphous, elastic and viscous, potential applications for P3HB are limited. On the other hand, the incorporation of 4-hydroxybutyrate (4HB) into P3HB generates a copolymer, poly-3-hydroxybutyrate-co-4-hydroxybutyrate (hereafter P3HB-co-4HB), which has significantly improved thermal and mechanical properties (Li et al., 2010). Depending on the exact co-polymer composition, the resulting biomaterials exhibit a wide range of physical properties (e.g., crystalline, plastic, elastic rubber) and have many potential industrial applications (Ishida et al., 2001; Saito et al., 1996).

P3HB-co-4HB was first found to be produced in *Ralstonia eutropha* when this bacterium was cultured with 4HB or 4-chlorobutyric acid as carbon sources (Doi et al., 1988), and later on its production was also found in many other bacteria, such as *Alcaligenes latus*, *Comamonas testosteronii* and *Hydrogenophaga pseudoflava* (Choi et al., 1999; Ishida and Inoue, 2004; Saito et al., 1996). However, carbon sources structurally related to 4HB were required in the medium to produce P3HB-co-4HB, and the high cost of using these precursors adds additional costs to the production of P3HB-co-4HB, which constrains further industrial applications of P3HB-co-4HB (Song et al.,

1999; Valentin et al., 2000). Some previous studies have been performed in *E. coli* to produce P3HB-co-4HB from carbon sources not directly linked to 4HB (Li et al., 2010; Valentin and Dennis, 1997). However, additional organic carbon sources (e.g., glucose) are still needed in the medium or the 4HB content in the synthesized co-polymer was relatively low (Li et al., 2010; Valentin and Dennis, 1997; Valentin et al., 2000). All of these disadvantages limit the further production and utilization of P3HB-co-4HB.

Cyanobacteria are photoautotrophic bacteria that utilize sunlight as their energy source and CO₂ as their carbon source. Because they have adapted or can acclimate to diverse growth environments (Gan et al., 2014a; Gan et al., 2014b), they can serve as “photo-bioreactors” for the production of renewable biofuels and green chemicals (Ducat et al., 2011; Parmar et al., 2011). Some cyanobacteria have been reported to produce P3HB, and different metabolic strategies to maximize the accumulation of P3HB under various growth conditions have been explored (Panda and Mallick, 2007; Tsang et al., 2013; Wu et al., 2002). However, P3HB-co-4HB has not yet been produced in cyanobacteria, probably due to the absence of an appropriate precursor for the biosynthesis of 4HB. Succinic semialdehyde was recently discovered to be an important, new intermediate in the TCA cycle variant that occurs in cyanobacteria (Zhang and Bryant, 2011). Succinic semialdehyde can be converted to 4-hydroxybutyryl-CoA (4HB-CoA) by 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA transferase from *Porphyromonas gingivalis* (Yim et al., 2011). Thus, by introducing the 4-hydroxybutyrate dehydrogenase and the 4-hydroxybutyrate-CoA transferase into a cyanobacterium that produces P3HB, the biosynthesis of P3HB-co-4HB should become possible.

The production of P3HB and P3HB-co-4HB using light and CO₂ in cyanobacteria was explored in this study, and some metabolic engineering strategies to improve the product yield were also investigated. *Chlorogloeopsis fritschii* PCC 9212 has a gene cluster that encodes the biosynthetic pathway for P3HB, and P3HB production in this strain was demonstrated in our recent study (Zhang and Bryant, 2015). However, although gene knockouts have been reported (Stucken et al., 2012) in this strain, the absence of a simple and reliable system for genetic manipulation limits further metabolic engineering in this organism. By using the pAQ1Ex expression system (Xu et al., 2011), this entire gene cluster was instead transferred to *Synechococcus* sp. PCC 7002, which has a faster growth rate and well-established genetic modification tools. The resulting strain could accumulate 2.77% percent of total cell dry weight as P3HB, and the yield was improved to 3.97% when the *ccmR* gene was deleted in this strain. Additionally, the complete biosynthetic pathway to synthesize 4HB-CoA from succinic semialdehyde was constructed and introduced into the P3HB production strain of *Synechococcus* sp. PCC 7002. The resulting strain produced ~4.5% total cell dry weight as P3HB-co-4HB, in which the 4HB monomer accounted for ~12% of the co-polymer. Taken together, this work shows for the first time that P3HB-co-4HB can be produced in cyanobacteria and identifies a genetic modification that improved the yield of P3HB. Furthermore, this work nicely illustrates the possibility for future metabolic engineering of cyanobacteria based on the discovery of novel metabolites.

2 Materials and Methods

2.1 Growth conditions

Wild-type and mutant strains of *Synechococcus* sp. PCC 7002 were grown in liquid A⁺ medium under standard conditions as previously described (Ludwig and Bryant, 2011). Standard conditions are defined as 38°C, an irradiance of 250 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ provided by cool white fluorescent lights, and gentle sparging with 1% (vol/vol) CO₂ in air. Antibiotics were used in the following concentrations when appropriate: spectinomycin (50 $\mu\text{g ml}^{-1}$), kanamycin (100 $\mu\text{g ml}^{-1}$), gentamycin (20 $\mu\text{g ml}^{-1}$) and erythromycin (20 $\mu\text{g ml}^{-1}$).

2.2 Over-expression of the PHA operon in *Synechococcus* sp. PCC 7002

The *phaABEC* operon from *C. fritschii* PCC 9212 was amplified by polymerase chain reaction (PCR) and inserted onto the pAQ1-*P_{cpcBA}*-based expression system by using primer set 3HBExF and 3HBExR (Table 1), in which the *phaABEC* operon was expressed from the strong *cpcBA* promoter of *Synechocystis* sp. PCC 6803 and a His₁₀-tag as also added to the N-terminus of the acetyl-CoA acetyltransferase (*phaA*) (Xu et al., 2011). The resulting plasmid was verified by DNA sequencing, and wild-type *Synechococcus* sp. PCC 7002 was transformed with the resulting plasmid (Frigaard et al., 2004). Successful incorporation of the operon was confirmed by PCR using primer set 3HBExF and 3HBExR (Table 1), and the resulting PCR product was also verified by DNA sequencing. In addition, immunoblotting with commercial antibodies (Rockland) to the His₁₀-tag was also performed as previously described (Shen et al., 2002) to detect the recombinant acetyl-CoA acetyltransferase and to confirm that expression from the *phaABEC* operon was occurring. The resulting strain was denoted as WTPHB.

2.3 Generation of deletion mutants

The operon encoding 2-oxoglutarate decarboxylase (SynPCC7002_A2770) and succinic semialdehyde dehydrogenase (SynPCC7002_A2771) was deleted in the WTPHB strain by transformation and homologous recombination and replaced by a DNA fragment encoding an antibiotic resistance gene (*erm*, erythromycin resistance) as previously described (Frigaard et al., 2004). Primer sets A2771D1-A2771D2 and A2770D3-A2770D4 (Table 1) were used to amplify the upstream and downstream regions of the operon encoding SynPCC7002_A2771 and SynPCC7002_A2770. Full segregation of the deletion mutant was verified by PCR by comparing the products of template DNAs from both the wild type and the mutant strain using primer set A2771D1-A2770D4. The generated strain was denoted as $\Delta 7071$ -PHB.

The coding sequence of SynPCC7002_A0171 (*ccmR*) was also deleted in the wild-type *Synechococcus* sp. PCC 7002 and the WTPHB strain, and replaced by a DNA fragment encoding an antibiotic resistance gene (*aphAII*, kanamycin resistance) to produce SynPCC7002_A0171 mutant strains. Transformation and selection were performed as previously described (Frigaard et al., 2004). Primer sets A0171D1-A0171D2 and A0171D3-A0171D4 (Table 1) were used to amplify the upstream and downstream regions of SynPCC7002_A0171. The complete segregation of alleles was verified by PCR by comparing the products obtained with template DNAs derived from the wild type and mutant strains using primer set A0171D1-A0171D4. The resulting strain with deletion of SynPCC7002_A0171 in the wild type *Synechococcus* sp. PCC 7002 was denoted as $\Delta A0171$, and the generated strain with SynPCC7002_A0171 deleted in the WTPHB strain was denoted as $\Delta A0171$ -PHB.

2.4 Construction of the 4-hydroxybutyrate CoA biosynthesis pathways

In order to construct the biosynthetic pathway from succinic semialdehyde to 4HB-CoA (Figure 1), the genes encoding 2-oxoglutarate decarboxylase (SynPCC7002_A2770), 4-hydroxybutyrate dehydrogenase (*gbd1*) and 4-hydroxybutyryl-CoA transferase (*cat2*) were first assembled together on plasmid pAQ1Ex under control of the *psaAB* promoter (from *Synechocystis* sp. PCC 6803) (Xu et al., 2011). Primer set 4HBExF-4HBExR (Table 1) was used to amplify the operon encoding 4-hydroxybutyrate dehydrogenase and 4-hydroxybutyryl-CoA transferase from *Porphyromonas gingivalis* W83, and primer set A2770ExF-A2770ExR (Table 1) was used to amplify the gene encoding the 2-oxoglutarate decarboxylase from *Synechococcus* sp. PCC 7002. To avoid homologous recombination with the chromosomal copy of SynPCC7002_A2770, the operon encoding SynPCC7002_A2771 and SynPCC7002_A2770 was first deleted in the strain Δ A0171-PHB. Then, the malate dehydrogenase gene (SYNPCC7002_A2093) in the resulting strain was replaced with the fused construct by homologous recombination as described (Frigaard et al., 2004). Primer sets A2093D1-A2093D2 and A2093D3-A2093D4 (Table 1) were used to amplify the upstream and downstream flanking regions of malate dehydrogenase. Full segregation of the replacement of the malate dehydrogenase by the fused construct containing the *psaAB* promoter, all three genes, and the spectinomycin gene, was verified using primer set A2093D1-A2093D4. The products were sequenced to verify that no inadvertent changes had occurred during strain construction. The resulting strain was denoted as P3HB4HB.

2.5 Total mRNA profiling

Transcriptome profiling was performed as described (Ludwig and Bryant, 2011). The SynPCC7002_A0171 mutant and wild-type strains were grown in medium A⁺ under

standard conditions and harvested at $OD_{730\text{ nm}} = 0.7$. Total RNA was extracted as described (Ludwig and Bryant, 2011). The construction of cDNA libraries and sequencing (Illumina) were performed in the Genomic Core Facility at The Pennsylvania State University. Mapping against the *Synechococcus* sp. PCC 7002 genome was performed using the BWA software package (Li and Durbin, 2009). The resulting alignment files were further analyzed with self-developed scripts to extract expression levels for each gene as described previously (Zhang and Bryant, 2015). The RNA sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP058241.

2.6 PHB detection

PHB quantifications were performed as previously described (Braunegg et al., 1978; Tsang et al., 2013). Aliquots (20 ml) of liquid cultures of *Synechococcus* sp. PCC 7002 were centrifuged for 10 min at $10,000 \times g$. The pellets were washed once with double-deionized water (20 ml). The resulting cell pellets were then lyophilized to obtain dried cells. The dried cells were put into glass tubes with sealed rubber caps. Sodium 3-hydroxybutyrate and gamma-butyrolactone (Sigma-Aldrich) were used as standards for 3HB and 4HB, respectively, and were analyzed separately following same procedures. Chloroform (1 ml) and acidified methanol (1 ml, 15% vol/vol H_2SO_4) were added to each sample as well as the standards. The tubes were heated in a $97^\circ C$ water bath for 3 h to methanolyze sodium 3-hydroxybutyrate or gamma-butyrolactone into methyl 3-hydroxybutyrate or methyl 4-methoxybutyrate, respectively. After methanolysis, double-deionized water (1 mL) was added to each sample. Following phase separation, an aliquot (2 μ l) of the bottom chloroform phase was collected and loaded directly onto a Shimadzu GC-17A gas chromatograph connected to a Shimadzu GCMS-QP500 mass

spectrometer using a HP-5-MS 30 m column (ID: 0.25 narrow bore; film: 0.25 μ m) (Agilent Technologies; Foster City, CA) for analysis as previously described (Tsang et al., 2013). The concentrations and contents of 3HB and 4HB were calculated on the basis of a standard curve generated with known concentrations of sodium 3-hydroxybutyrate and gamma-butyrolactone (Sigma-Aldrich).

3 Results

3.1 Generation and verification of overexpression strain

The *phaABEC* genes form an apparent operon in *C. fritschii* PCC 9212, and are also co-located with the *aceBA* genes for the enzymes of the glyoxylate cycle (Figure 2). The *phaABEC* operon was cloned onto the pAQ1Ex system and expressed in *Synechococcus* sp. PCC 7002 as described in the Materials and Methods. The incorporation of this operon into *Synechococcus* sp. PCC 7002 was verified by PCR as shown in Figure 2. Immunoblotting was also performed using antibodies against His₁₀-tag, which demonstrated the production and accumulation of the acetyl-CoA acetyltransferase (Figure 2). These results clearly demonstrate that the *phaABEC* operon was successfully incorporated into *Synechococcus* sp. PCC 7002, and verified that at least the first gene of the operon was expressed successfully. The over-expression strain had a somewhat yellowish color compared to wild-type strain. This was probably due to protein over-expression as well as the biosynthesis and accumulation of P3HB, which might shift the overall carbon and nitrogen balance of the cells.

3.2 P3HB production in the overexpression strain

With the successful incorporation of the *phaABEC* operon and demonstration of expression of acetyl-CoA acetyltransferase, the production of P3HB in the WTPHB strain

was further investigated by GC-MS as described in the Materials and Methods. The GC-MS results showed that P3HB production occurred in the WTPHB strain but not in wild-type *Synechococcus* sp. PCC 7002, and the mass fragmentation pattern confirmed that 3HB was the only monomer in the polymer (Figure 3). Our previous results showed that *C. fritschii* PCC 9212 could accumulate P3HB to about 5% (w/w) of cell dry weight under standard growth conditions for this cyanobacterium (Zhang and Bryant, 2015). Cells of the WTPHB strain accumulated a lower content (2.77% of cell dry weight) of P3HB under standard conditions (Figure 4) for *Synechococcus* sp. PCC 7002. The difference presumably is due to metabolic differences in these two organisms (e.g., the glyoxylate cycle is present in *C. fritschii* PCC 9212 but not *Synechococcus* sp. PCC 7002). The glyoxylate cycle was demonstrated to play an important role in the metabolism of acetyl-CoA, which is the building block for P3HB (De Philippis et al., 1992; Ensign, 2006). However, the growth rate of wild type *C. fritschii* PCC 9212 is much slower compared to *Synechococcus* sp. PCC 7002 and *C. fritschii* PCC 9212 is currently not easily amenable to genetic analysis (Stücken et al., 2012). Thus, *Synechococcus* sp. PCC 7002 still has advantages in rapidly accumulating P3HB as well as in allowing metabolic engineering to improve the production of P3HB (see below).

3.3 A carbon concentration mechanism regulator

The biosynthesis of P3HB utilizes acetyl-CoA as the building block, and thus metabolic engineering to increase the intracellular acetyl-CoA pool should potentially improve the production of P3HB. Acetyl-CoA is an important carbon metabolite in both central carbohydrate metabolic and fatty acid biosynthesis and degradation pathways. Thus, it is likely that improved carbon fixation could potentially provide more carbon resources to the central metabolic pathways and thus increase the cellular level of acetyl-CoA. It has

been reported that the LysR-type transcription factor, CcmR, controls the expression of genes encoding the carbon concentration mechanism of cyanobacteria, and it represses its own transcription along with structural genes encoding high affinity C_i transporters in *Synechocystis* sp. PCC 6803 (Daley et al., 2012). This indicates that CcmR is also likely to play an extremely important role in connecting central metabolism to inorganic carbon acquisition for photosynthesis. Thus, deletion of the *ccmR* gene (SYNPCC7002_A0171) in *Synechococcus* sp. PCC 7002 might potentially improve carbon fixation and thus increase the acetyl-CoA pool inside cells. In order to test this idea, a SYNPCC7002_A0171 deletion mutant of *Synechococcus* sp. PCC 7002 was constructed and confirmed by PCR (data not shown).

3.4 RNA profiling of SYNPCC7002_A0171 mutant

Transcription profiling of the SYNPCC7002_A0171 mutant was performed in comparison with the wild-type strain. The profiling results showed that transcripts for genes encoding bicarbonate transport systems as well as the Na^+/H^+ antiporter increased quite substantially, which is consistent with previously reported results (Figure 5) (Woodger et al., 2007). Along with these changes, transcript abundances for the two genes encoding glyceraldehyde-3-phosphate dehydrogenase (*gap*) also changed differently. Transcripts for the *gap1* gene, whose product, glyceraldehyde-3-phosphate dehydrogenase-1, is involved in glycolysis, increased about 5-fold, while transcripts for the *gap2* gene, whose product is thought to be primarily used in the Calvin-Benson-Bassham cycle (Koksharova et al., 1998), decreased to about 20% of the wild-type level. These results suggested that carbon flux might be shifted toward the glycolytic pathway, which might lead to the synthesis of more acetyl-CoA. Additionally, transcripts for the *nifJ* gene, which encodes pyruvate:ferredoxin (flavodoxin) oxidoreductase that catalyzes

the oxidative decarboxylation of pyruvate to acetyl-CoA, increased dramatically (~15-fold), which further suggested that acetyl-CoA might accumulate in this mutant strain. In addition to these observations, mRNA levels for the hydrogenase genes (e.g., *hoxF*, *hoxU*) as well as the transhydrogenase genes (*pntABC*) also increased in the mutant. Collectively, these changes implied that the redox cofactors or redox potential in the mutant might change to balance changes in carbon metabolism.

3.5 PHB production in mutant strains

Based on the transcription profiling results, it seemed likely that the SYN-PCC7002_ΔA0171 deletion mutant could facilitate carbon assimilation by redirecting more carbon resources to the biosynthesis of acetyl-CoA under standard growth conditions. Thus, the production of P3HB was investigated in the ΔA0171-PHB strain. Complete deletion of SYN-PCC7002_ΔA0171 in the strain ΔA0171-PHB was verified by PCR (data not shown). P3HB accumulated to 3.97% of total cell dry weight in this strain, which represented a 43% increase compared to that of WTPHB strain (Figure 6). To explore further metabolic engineering strategies to improve the production of P3HB in cyanobacteria, P3HB production was also investigated in a mutant strain in which the operon encoding 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase had been deleted (Figure 6). Depletion of 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase results in accumulation of 2-oxoglutarate, and we hypothesized that the acetyl-CoA concentration might also increase because of limited metabolic flux into the TCA cycle due to the accumulation of higher levels of 2-oxoglutarate. However, no significant increase in the accumulation of P3HB was observed in Δ7071-PHB strain compared to WTPHB strain (Figure 6). These results suggested that

the TCA cycle and thus the concentration of acetyl-CoA and 2-oxoglutarate concentrations are regulated at levels that are currently still unknown in cyanobacteria, despite the fact that acetyl-CoA and 2-oxoglutarate are important cellular metabolites.

3.6 Production of P3HB-co-4HB in cyanobacteria

As mentioned in the introduction, P3HB is relatively amorphous, elastic, and viscous, and the incorporation of some 4HB into this polymer greatly improves its physical properties. The co-polymerization of 3-HB-CoA and 4HB-CoA monomer subunits results in P3HB-co-4HB, the synthesis of which is catalyzed by the poly-hydroxybutyrate synthase (*phaEC*). The newly discovered TCA cycle variant of cyanobacteria showed that succinic semialdehyde is a potentially important metabolic intermediate (Zhang and Bryant, 2011), and thus we evaluated the ability of cyanobacteria to produce 4HB-CoA from succinic semialdehyde. To shift the metabolic flux further to the oxidative branch of the TCA cycle and increase the accumulation of 2-oxoglutarate (the precursor for succinic semialdehyde), the fused construct encoding the biosynthetic pathway of 4HB-CoA was used to replace the malate dehydrogenase gene on the chromosome of the $\Delta A0171$ -PHB strain, in which the operon encoding 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase was also deleted to avoid possible problems arising from homologous recombination. Complete segregation of the construct for the replacement of malate dehydrogenase gene by the fused construct was confirmed by PCR as shown in Figure 7. The production of P3HB-co-4HB in the resulting strain P3HB4HB was detected by GC-MS as described in the Materials and Methods. The ratio of 3HB and 4HB was determined by GC analysis, and the identity of the two monomers was verified by mass fragmentation (Figure 8). In total, P3HB-co-4HB accumulated to ~4.5% of total cell dry weight, and 4HB accounted for about 12% of the monomers in the P3HB-co-4HB

copolymer produced in this strain. These results clearly demonstrate that succinic semialdehyde can be converted to 4HB-CoA by the constructed 4HB-CoA biosynthetic pathway. Furthermore, these results establish that succinic semialdehyde is an important metabolic intermediate in the TCA cycle variant that occurs in cyanobacteria.

4 Discussion

Cyanobacteria have been used to produce many useful biomaterials as well as biofuels, which are usually derived from important metabolites inside cells (Ducat et al., 2011; Machado and Atsumi, 2012; Zhao et al., 2015). Thus, the discovery of new metabolic pathways as well as new metabolic intermediates in cyanobacteria should not only enrich our knowledge about cyanobacterial metabolism but can also provide novel targets for potential metabolic engineering. Although the genes involved in the biosynthesis of P3HB have been described in cyanobacteria (Tsang et al., 2013) and the production of P3HB was also reported in cyanobacteria (Miyake et al., 2000; Wu et al., 2002), the production of P3HB-co-4HB had not yet been demonstrated in cyanobacteria. This study explored the production of P3HB-co-4HB, using 4HB-CoA that was derived from succinic semialdehyde, a metabolic intermediate of the recently discovered TCA cycle variant in cyanobacteria (Zhang and Bryant, 2011; Zhang and Bryant, 2014). Overall, our results demonstrated the production of both P3HB as well as P3HB-co-4HB in the cyanobacterium *Synechococcus* sp. PCC 7002, and some metabolic engineering strategies to improve the yield were also investigated.

Although previous flux balance analyses showed that, under photoautotrophic conditions in cyanobacteria, the main carbon flux occurred within the Calvin-Benson-

Bassham cycle and that the TCA cycle mostly functioned as a branched pathway, it was also suggested that dark consumption of glycogen in cyanobacteria should include some cyclic flux through the TCA cycle, possibly through 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase (Knoop et al., 2013; Steuer et al., 2012). Furthermore, recent ^{13}C -MFA studies reported that after unlabeled glutamate was added to ^{13}C -labeled cultures of *Synechocystis* sp. PCC 6803, substantial fractions of unlabeled 2-OG, succinate, and malate were observed, which may also be produced by the newly identified TCA bypass through succinic semialdehyde (You et al., 2014). Meanwhile, the detection of 4HB monomer in the PHA produced by the P3HB4HB strain under photoautotrophic conditions also establishes that succinic semialdehyde is not only present in *Synechococcus* sp. PCC 7002 but that it can be converted to useful biomaterials (e.g., 4HB-CoA) if appropriate enzymes are introduced into cells. Taken together, these results imply that a complete and functional TCA cycle exists in *Synechococcus* sp. PCC 7002 and probably other cyanobacteria as well, and they indicate that some flux through the TCA cycle occurs even under photoautotrophic growth conditions, which may be enhanced when succinic semialdehyde or other TCA cycle intermediates are consumed by introduced enzymes. Indeed, it was reported that the TCA cycle can be predominantly cyclic, and metabolic flux through the TCA cycle was significantly enhanced, when an engineered ethylene pathway that uses 2-oxoglutarate as substrate was introduced into *Synechocystis* sp. PCC 6803 (Xiong et al., 2015). It should also be noted that the genes responsible for the newly discovered TCA cycle bypass could readily be transferred into other bacteria (e.g., *E. coli*), when succinic semialdehyde is desired as a metabolic intermediate for the production of important biomaterials.

Although the amounts of P3HB produced by cyanobacteria are currently lower than those from *E. coli* (Li et al., 2010), the requirements of substrate glucose or other organic carbon sources in the growth medium certainly increases the costs of producing P3HB or P3HB-co-4HB in *E. coli*. Besides, the results from this study only represent initial attempts, and future metabolic engineering strategies in cyanobacteria may improve P3HB or P3HB-co-4HB yields to levels comparable to those for *E. coli*. The main building block for the production of P3HB is acetyl-CoA, and thus metabolic engineering strategies to increase the cellular acetyl-CoA pool would potentially increase the yield of P3HB. One direct method might be adding acetate to the medium, which could be converted to acetyl-CoA directly once taken up by cyanobacteria. Our previous results have shown that adding acetate to the medium increased the P3HB content from ~5% to ~15% of total cell dry weight in *Chlorogloeopsis fritschii* PCC 9212 (Zhang and Bryant, 2015). However, addition of acetate to the growth medium of the WTPHB strain did not lead to obvious changes in the P3HB content of cells (data not shown). The reason(s) for this are unclear, but could include the absence of an efficient acetate uptake (e.g., acetate transporter) or assimilation (i.e., the glyoxylate cycle) pathway in *Synechococcus* sp. PCC 7002. Additionally, it was demonstrated that adding 2-oxoglutarate to the growth medium could increase the 4HB ratio in P3HB-co-4HB in *E. coli* (Li et al., 2010), and thus adding structurally related carbon sources to the growth medium might further improve the yields of P3HB or P3HB-co-4HB. However, it should be noted that the added carbon source(s) will certainly increase the production cost and should be balanced with the production yield in large-scale production.

Other than direct addition of acetate or 2-oxoglutarate to the medium, metabolic engineering strategies could also be used to increase the cellular concentration of acetyl-CoA. Pyruvate is oxidatively cleaved to form acetyl-CoA and CO₂ with the involvement of coenzyme A to accept the acetyl group (Wolfe and O'Kane, 1953). The conversion of pyruvate to acetyl-CoA can be catalyzed by two distinct enzymes, pyruvate dehydrogenase (PDH) and pyruvate:ferredoxin (flavodoxin) oxidoreductase (PFOR) (Arjunan et al., 2002; Schmitz et al., 1993). Many cyanobacteria possess both PDH and PFOR to accommodate changing O₂ levels, especially in dark environments where O₂ is rapidly consumed due to the high intrinsic respiratory activities of cyanobacteria as well as those of surrounding organisms (Schmitz et al., 2001). Thus, overexpression of one or both of these two enzymes might improve the conversion of pyruvate to acetyl-CoA and thus increase the pool of acetyl-CoA for P3HB biosynthesis.

Other approaches to enhance the beta-oxidation pathway, and thus the degradation of fatty acids, should also increase the acetyl-CoA pool. Overexpression of the long-chain-fatty-acid CoA ligase (*fadD*) may result in a higher concentration of acyl-CoA and thus increase the acetyl-CoA pool (Choi and Lee, 2013). Furthermore, it was also reported in *E. coli* that the pool of acetyl-coA could be increased by overexpression of pantothenate kinase (Lin et al., 2004). The first enzyme in the biosynthesis of P3HB is acetyl-CoA acetyltransferase, which catalyzes a reversible reaction that might limit the carbon flux toward P3HB biosynthesis. Thus, other than improving the intracellular concentration of acetyl-CoA, making the first step in the conversion of acetyl-CoA to acetoacetyl-CoA irreversible could also increase the carbon flux toward P3HB biosynthesis. Recently, an acetoacetyl-CoA synthase of the thiolase superfamily (*nphT7*,

from *Streptomyces* sp. strain CL190) was identified, which catalyzes the reaction that condenses acetyl-CoA and malonyl-CoA to form acetoacetyl-CoA with the release of CO₂, which makes this an essentially irreversible reaction (Okamura et al., 2010). This enzyme has been used to improve the production of 1-butanol in cyanobacteria (Lan and Liao, 2012). All of these results suggest that replacement of acetyl-CoA acetyltransferase with the newly identified acetoacetyl-CoA synthase may further improve the carbon flux toward biosynthesis of P3HB. Attempts to test this possibility are in progress.

Cyanobacteria also use glycogen as a carbon and energy source, and PHAs can serve the same two functions. Thus, metabolic engineering to reduce the metabolic flux toward biosynthesis of glycogen might potentially shift more carbon resources toward P3HB biosynthesis (Xu et al., 2013). It was reported previously that a *glgC* deletion mutant was not able to synthesize glycogen under any circumstances and had an increased content of soluble sugars (Guerra et al., 2013). Thus, deletion of the *glgC* gene in the P3HB producing strain might also improve the production of P3HB, because glycogen biosynthesis is not essential during photoautotrophic growth (Xu et al., 2013). Moreover, growing cyanobacteria under nitrogen limitation conditions could rebalance the metabolic flux between nitrogen and carbon and may favor the biosynthesis of PHB. Indeed, it was demonstrated that PHB accumulation increased substantially under nitrogen limitation conditions in *Synechococcus* sp. MA19 (Miyake et al., 2000). Surprisingly, however, no significant changes in the accumulation of PHB contents were found when the WTPHB strain was grown under nitrogen limitation condition (data not shown). Considering that the PHB biosynthetic pathway is not present in wild-type *Synechococcus* sp. PCC 7002 but occurs in *Synechococcus* sp. MA19, this could be due

to differences in the regulatory mechanisms in these two cyanobacteria. However, one should keep in mind that the growth rate would be affected under such limitation conditions and this could significantly alter the total yields of PHB.

Overall, it should be noted that the combination of all the mentioned methods might eventually improve the PHA production in cyanobacteria to the level that is comparable with *E. coli*. During the past several decades, metabolic engineering strategies in *E. coli* have become well established and studied, and *E. coli* has been used to produce many different biomaterials, ranging from small molecules to complicated natural products and related pharmaceutical analogs (Keasling, 2010; Peralta-Yahya et al., 2012; Stephanopoulos, 2007). However, because of the advantage of the low cost to grow cyanobacteria, and recently well-established genetic tools for mutagenesis and protein overexpression, one can envisage a new era in metabolic engineering of cyanobacteria to produce a wide range of biomaterials. The preliminary work toward this goal in this study provides a good example of how newly discovered metabolic pathways in cyanobacteria could lead to new applications of these bacteria. Future investigations of the metabolic diversity in cyanobacteria certainly would not only enrich our knowledge of cyanobacterial metabolism but might also make cyanobacteria a more powerful tool for providing sustainable green energy by providing new feedstocks for the production of biofuels and biomaterials in these organisms.

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Table 1. Primers used in this study.

Name	Sequences (5'-3')
3HBExF	GGTCGTCATATGCGTGACGTGTACATTGTT
3HBExR	GAAGTAGGATCCTTAATCAGCGCGTACCTT
A2771D1	AATCGAAGTCGTCTTTTTACCCGCCGAGCA
A2771D2	AATGATGGATCCAACTCTCTCCTTTGCCGC
A2770D3	CCGGTGAAGCTTTCCCAAAAATCAGGGGAT
A2770D4	ACCTTGGTCAGTGAGCCAATTTTGGCGATG
A2093D1	TCGTCTGCGCCTGTTTCACATCAAACCCGT
A2093D2	GGCCGCAAGCTTGGGACTATTCCAAATTTA
A2093D3	CTGAGTGGATCCCAGATCGATCCATGGCAG
A2093D4	AAGGCTGTTGCTCTGGCGTCAGGAGTTGTG
A0171D1	CCAAAGACTGTTGCCCCACCAGGGGTTCAA
A0171D2	TGCAGGGGATCCGGGTTTACTCCTGAACTT
A0171D3	CTGCAAAAGCTTAAGCTCAAAATTGTTGGG
A0171D4	CAGCCCCAATCCATTTCTAAGTAGGCCACA
A2770ExF	CGATCGCTGCAGATGAATACAGCTGAATTATTGA
A2770ExR	GCCGACGGATCCTTACATACGGCAGATTAA
4HBExF	AACCCTCCATGGCTAAAGACGTATTAGCGG
4HBExR	ACCTTTGGATCCTTAGTAGAGTCTTCTGTA

Figures

Figure 1. Scheme showing the biosynthesis pathways of P3HB and P3HB-co-4HB.

Abbreviations: *cat2*, 4-hydroxybutyryl-CoA transferase; *gbd1*, 4-hydroxybutyrate dehydrogenase; *ogdA*, 2-oxoglutarate decarboxylase; *phaA*, acetyl-CoA acetyltransferase; *phaB*, acetoacetyl-CoA reductase; *phaEC*, poly(R)-hydroxyalkanoic acid synthase, class III, PhaE and PhaC subunits; *ssaD*, succinic semialdehyde dehydrogenase.

Figure 2. Verification of the presence of genes for the PHB biosynthesis pathway in WTPHB. **A.** Scheme showing that *phaABEC* form an apparent operon and co-localized with the glyoxylate cycle genes in *C. fritschii* PCC 9212. *aceA*, isocitrate lyase; *aceB*, malate synthase; *phaA*, acetyl-CoA acetyltransferase; *phaB*, acetoacetyl-CoA reductase; *phaE*, poly(R)-hydroxyalkanoic acid synthase, class III, PhaE subunit; *phaC*, poly(R)-hydroxyalkanoic acid synthase, class III, PhaC subunit. **B.** PCR verification of the presence of *phaABEC* in the WTPHB strain. The template DNA was derived from wild-type *C. fritschii* PCC 9212 (lane 9212), and from the recombinant strain WTPHB (lane WTPHB), which has the *phaABEC* genes from *C. fritschii* PCC 9212 inserted in plasmid pAQ1-Ex as described in the Materials and Methods. **C.** Immunoblotting of whole cell extracts of *Synechococcus* sp. PCC 7002 wild type (WT7002) as well as WTPHB strain with antibodies to His₁₀-tag.

Figure 3. Detection and verification of the production of P3HB.

A. GC-MS elution profiles showing the detection of methyl-3-hydroxybutyrate from the methanolized 3-hydroxybutyrate standard (3HB standard, dashed line) as well as the methanolized dry cell pellets of WTPHB strain (solid black line), but not the dry cell pellets of wild type strain *Synechococcus* sp. PCC 7002 (WT7002, solid gray line). **B.** Mass fragmentation patterns for the peaks detected in the GC-MS elution profiles, confirming that the compound detected in the WTPHB strain (panel 2) had nearly identical pattern as that for the 3-hydroxybutyrate standard (panel 1).

Figure 4. Accumulation of P3HB in WTPHB strain.

P3HB contents of WTPHB strain were monitored as a function of batch growth under standard conditions. The bars indicate the total cell dry weight (CDW), and the black portions of the bars show the P3HB content at different growth stages. This information is also plotted to emphasize the kinetics and more accurately indicate the amount of P3HB production. The data shown are averages of three biological replicates, and the error bars show the standard deviation.

Figure 5. Relative transcripts abundance for mRNAs in wild type (WT) and SynPCC7002_A0171 mutant (Δ A0171) of *Synechococcus* sp. PCC 7002.

Grey lines indicate a two-fold increase or 50% decrease in mRNA level. *bicA*, bicarbonate transporter, BicA; *cupA*, CO₂ hydration protein; *gap1*, glyceraldehyde-3-phosphate dehydrogenase, type I; *gap2*, glyceraldehyde-3-phosphate dehydrogenase, type II; *hoxF*, hydrogenase large diaphorase subunit F; *hoxU*, hydrogenase small diaphorase subunit U; *hoxY*, hydrogenase small subunit Y; *hyp3*, CBS domain-containing protein; possibly associated with hydrogenase assembly; *mnhB*, multisubunit Na⁺/H⁺ antiporter, B

subunit; *mnhC*, multisubunit Na⁺/H⁺ antiporter, subunit C; *mnhD1*, multisubunit Na⁺/H⁺ antiporter, subunit D1; *mnhD2*, multisubunit Na⁺/H⁺ antiporter, subunit D2; *napA*, Sodium/hydrogen exchanger family protein (Na⁺/H⁺ antiporter); *ndhD-III*, proton-translocating NADH-quinone oxidoreductase (NADH dehydrogenase subunit 4); *ndhF-III*, NADH2 dehydrogenase (plastoquinone) chain 5; *nifJ*, pyruvate:ferredoxin (flavodoxin) oxidoreductase; *pntA*, nicotinamide nucleotide transhydrogenase, chain alpha, part 1; *pntB*, nicotinamide nucleotide transhydrogenase, subunit beta; *pntC*, nicotinamide nucleotide transhydrogenase, chain alpha, part 2; *ppsA*, phosphoenolpyruvate synthase; *sbtA*, sodium-dependent bicarbonate transporter. Red-labeled genes are involved in the CCM mechanism; the hydrogenase genes are labeled in green; and the blue-labeled genes are involved in other metabolic pathways, such as fermentation.

Figure 6. P3HB productions in mutant strains of WTPHB.

P3HB contents in mutant strains of WTPHB, lacking the SynPCC7002_A2771 and SynPCC7002_A2770 operon (Δ 7071-PHB), or lacking SynPCC7002_A0171 (Δ A0171-PHB). The bars indicate the total cell dry weight (CDW), and the black portions of the bars show the P3HB contents. This information is also plotted to emphasize the differences between these strains. The data shown are averages of three biological replicates, and the error bars show the standard deviation.

Figure 7. Construction and verification of the 4-hydroxybutyryl-CoA biosynthesis pathway in the WTPHB strain.

A. Scheme showing the replacement of open reading frame SynPCC7002_A2093 (encoding malate dehydrogenase) by the fused construct containing the 4-hydroxybutyryl-CoA biosynthesis pathway genes by homologous recombination and selection with spectinomycin (*aadA* gene cassette confers resistance to spectinomycin), using the promoter regions (*P_{psaAB}*) for *psaAB* from *Synechocystis* sp. PCC 6803. *cat2*, 4-hydroxybutyryl-CoA transferase; *gbd1*, 4-hydroxybutyrate dehydrogenase; *ogdA*, 2-oxoglutarate decarboxylase. **B.** Agarose gel electrophoresis of PCR amplicons using primer set A2093D1 and A2093D4 to verify the introduction of the fused construct into the respective strains. Template DNAs came from the Δ A0171-PHB strain and the fully segregated mutant strain (P3HB4HB) with SynPCC7002_A2093 replaced with the fused construct.

Figure 8. Detection and verification of the production of poly-3-hydroxybutyrate-co-4-hydroxybutyrate in the P3HB4HB strain.

A. GC-MS elution profiles demonstrate the detection of both methyl-3-hydroxybutyrate and methyl-4-methoxybutyrate from methanolized dry cell pellets of the P3HB4HB strain. **B.** Mass fragmentation patterns for the 4HB peak detected in the GC-MS elution profiles, confirming that the 4HB peak detected in the P3HB4HB strain (panel 2) has a nearly identical fragmentation pattern as that of the γ -butyrolactone standard (panel 1). The other peaks in the elution profiles for P3HB4HB are derived from unrelated contaminating compounds that could not be positively identified.