Engineering Cyanobacteria to Convert Carbon Dioxide to Building Blocks for Renewable Plastics

by

Bo Wang

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Graduate Supervisory Committee:

Deirdre R. Meldrum, Chair
Weiwen Zhang
Todd R. Sandrin
David R. Nielsen

ARIZONA STATE UNIVERSITY

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ABSTRACT

The production of monomer compounds for synthesizing plastics has to date been largely restricted to the petroleum-based chemical industry and sugar-based microbial fermentation, limiting its sustainability and economic feasibility. Cyanobacteria have, however, become attractive microbial factories to produce renewable fuels and chemicals directly from sunlight and CO_2. To explore the feasibility of photosynthetic production of (*S*)- and (*R*)-3-hydroxybutyrate (3HB), building-block monomers for synthesizing the biodegradable plastics polyhydroxyalkanoates and precursors to fine chemicals, synthetic metabolic pathways have been constructed, characterized and optimized in the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803). Both types of 3HB molecules were produced and readily secreted from *Synechocystis* cells without over-expression of transporters. Additional inactivation of the competing PHB biosynthesis pathway further promoted the 3HB production. Analysis of the intracellular acetyl-CoA and anion concentrations in the culture media indicated that the phosphate consumption during the photoautotrophic growth and the concomitant elevated acetyl-CoA pool acted as a key driving force for 3HB biosynthesis in *Synechocystis*. Fine-tuning of the gene expression levels via strategies, including tuning gene copy numbers, promoter engineering and ribosome binding site optimization, proved critical to mitigating metabolic bottlenecks and thus improving the 3HB production. One of the engineered *Synechocystis* strains, namely R168, was able to produce (*R*)-3HB to a cumulative titer of ~1600 mg/L, with a peak daily productivity of ~200 mg/L, using light and CO_2 as the sole energy and carbon sources, respectively. Additionally, in order to establish a high-efficiency transformation protocol in cyanobacterium *Synechocystis* 6803,

methyltransferase-encoding genes were cloned and expressed to pre-methylate the exogenous DNA before *Synechocystis* transformation. Eventually, the transformation efficiency was increased by two orders of magnitude in *Synechocystis*. This research has demonstrated the use of cyanobacteria as cell factories to produce 3HB directly from light and CO_2, and developed new synthetic biology tools for cyanobacteria.

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BACKGROUND LITERATURE

1.1 Introduction

Cyanobacteria and algae are endowed with the complex photosynthesis systems (Mulkidjanian et al., 2006) which can absorb solar radiation with a broad wave length and thereafter channel the absorbed energy to other forms of energy carriers such as chemicals (Chisti, 2007; Takahashi et al., 1998; van de Meene et al., 2006) and electricity (Furukawa et al., 2006; Pisciotta et al., 2010). Solar radiation is a clean, abundant and renewable energy resource and, if being properly and efficiently transferred, would be more than enough to power the entire human society (Rittmann, 2008). In addition, growing cyanobacteria and algae do not require arable land, which would eventually alleviate the increasing food prices due to the growing crop-based microbial industries (Rittmann, 2008). In contrast, they can fix carbon dioxide (CO_2), a type of greenhouse gas, during photosynthesis. Furthermore, cyanobacteria and algae grow faster than plants and bear relatively simple genetic background which is relatively easy to manipulate (Koksharova and Wolk, 2002).

As an emerging discipline that tackles biotechnology from a rational-design approach, synthetic biology aims to redesign existing biological systems or create artificial life (Benner, 2003; Endy, 2005; Mukherji and van Oudenaarden, 2009). In recent years, synthetic biology research has been focused on model species such as *Escherichia coli* and yeast, and has greatly boosted not only the in-depth understanding of the biological mechanisms in these cells, but also the capability and efficiency of these systems in

biological production of various useful products (Atsumi et al., 2008b; Bayer et al., 2009; Benner, 2003; Dwyer et al., 2007; Inui et al., 2008; Isaacs et al., 2004; Keasling, 2008; Lee and Lee, 2003; Ma et al., 2009; Martin et al., 2003; Mukherji and van Oudenaarden, 2009; Prather and Martin, 2008; Ro et al., 2006; Steen et al., 2010; Yim et al., 2011; Zhang et al., 2008; Zhang et al., 2012). However, with over 40 cyanobacterial genome sequencing projects (http://www.genomesonline.org/) and more than 60 algal genome sequencing projects (http://genome.jgi.doe.gov/) being completed or undergoing, application of synthetic biology in cyanobacteria and algae has significantly lagged behind those in *E. coli* and yeast. Considering the aforementioned inherent merits of the photosynthetic microbes, it would be of great scientific and application value to further develop synthetic biology tools and apply them in cyanobacteria and algae. The recent progresses and the challenges in developing and applying synthetic biology for cyanobacteria and algae are reviewed next.

1.2 Tools for synthetic biology in cyanobacteria and algae

1.2.1 Development of "BioBricks" for cyanobacteria and algae

"BioBricks" stands for standardized DNA parts with a common interface and can be assembled in living organisms. They are the basic interchangeable elements for regulating genetics (http://biobricks.org/). The focus here is on the development of the most common BioBricks for cyanobacteria and algae (*i.e.*, promoters, transcriptional terminators, ribosome binding sites and other regulatory factors).

Promoters. Both native and foreign promoters have been evaluated in cyanobacteria, mostly using *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus* 7942) and *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) as model species (**Table 1**). The native promoters used are usually from genes essential to photosynthesis such as carbon dioxide fixation (P_{rbcL}, P_{cmp}, P_{sbt}), photosystem I (PSI) ($P_{psaA,}$ P_{psaD}), PSII ($P_{psbA1,}$ P_{psbA2}), and photosynthesis antenna protein phycocyanin (P_{cpc}). A native nickel-inducible promoter, P_{nrsB}, has also been successfully utilized to express phage lysis genes in *Synechocystis* 6803 (Liu and Curtiss, 2009). Besides the native promoters, a limited number of foreign promoters have also been characterized in cyanobacteria. The chimeric P_{tac}/P_{trc} promoter, a strong promoter in *E. coli*, has been used in *Synechococcus* and *Synechocystis* species to initiate high-level expressions of the interest genes (Atsumi et al., 2009; Geerts et al., 1995; Huang et al., 2010; Lan and Liao, 2011; Ng et al., 2000; Niederholtmeyer et al., 2010). It is noteworthy that the composition of the cyanobacterial holopolymerase is quite different from those in most bacteria (including *E. coli*), so commonly used *E. coli* promoters might perform differently when introduced into cyanobacteria (Heidorn et al., 2011). A recent study on gene expression analysis in *Synechocystis* 6803 showed that the strength of P_{trc1O} (a version of the P_{trc}/P_{tac} promoter) was more than 4-fold higher than all versions of the promoter of native ribulose bisphosphate carboxylase/oxygenase (RuBisCO) large subunit, $P_{rbcL,}$ whereas the common *E. coli* promoters P_{lac}, P_{tet} and λ P_R exhibited very low or no detectable activities in the same system (Huang et al., 2010). Since currently very little is known about the performance of various native and foreign promoters in cyanobacteria, a systematic investigation on behaviors of various promoters in cyanobacteria is important.

In algae, CaMV 35S and SV40 promoters from viruses have been used to express target genes (Benfey et al., 1990; Wang et al., 2010). However, the most effective promoters have been derived from highly expressed algal genes. For example, the widely used promoters for *Chlamydomonas* transformation have been derived from the 5′ untranslated region of the *Chlamydomonas reinhardtii* RuBisCO small subunit gene (*rbcS2*) (Stevens et al., 1996), *Chlamydomonas* heat shock protein 70A gene *hsp70A* (Schroda et al., 2000), marine diatom fucoxanthin-chlorophyll a/c binding protein gene *fcp* (Apt et al., 1996; Miyagawa-Yamaguchi et al., 2011), *Dunaliella* duplicated carbonic anhydrase 1 (DCA1) (Li et al., 2010; Lu et al., 2011), *Porphyra yezoensis* actin1 gene (PyAct1) (Takahashi et al., 2010) and two *Nannochloropsis* unlinked violaxanthin/chlorophyll a-binding protein (VCP) genes, *VCP1* and *VCP2* (Kilian et al., 2011).

Table 1 Selected promoters used in cyanobacteria.

Promoters	**Sources**	**Gene(s)**	**Expression hosts**	**References**
P_{rbc}	*Synechococcus* 6301, *Synechocystis* 6803, *Synechococcus* 7942	firefly luciferase, *pdc, adh, far, accBCDA, accD, accA, fatB2*	*Synechococcus* 6301, *Synechocystis* 6803, *Synechococcus* 7942	Deng and Coleman, 1999; Liu et al., 2011b; Takeshima et al., 1994; Tan et al., 2011
P_{petE}	*Synechocystis* 6803	*far, far1, far2, accBCDA*	*Synechocystis* 6803	Tan et al., 2011
P_{psbA1}	*Synechococcus* 7942	*efe, hydEF, hydG, cvrbcLS*	*Synechococcus* 7942	Ducat et al., 2011a; Iwaki et al., 2006; Sakai et al., 1997; Takahama et al., 2003
P_{psbA2}	*Synechocystis* 6803	*pdc, adh, cvrbcLS, ispS, tesA, fatB1, fatB2*	*Synechocystis* 6803, *Synechococcus* 7942	Dexter and Fu, 2009; Iwaki et al., 2006; Lindberg et al., 2010; Liu et al., 2011b

Promoters	**Sources**	**Gene(s)**	**Expression hosts**	**References**
P_{psaA}	*Synechocystis* 6803	*luxAB*	*Synechocystis* 6803	Muramatsu and Hihara, 2006
P_{psaD}	*Synechocystis* 6803	*luxAB*	*Synechocystis* 6803	Muramatsu and Hihara, 2007
P_{cpc}	*Synechocystis* 6714	*luxAB, accB, accC*	*Synechococcus* 7942	Imashimizu et al., 2003
P_{rnpB}	*Synechocystis* 6803	GFP	*Synechocystis* 6803	Huang et al., 2010
P_{cmp}	*Synechocystis* 6803	*fol, gpl, shl*	*Synechocystis* 6803	Liu et al., 2011a
P_{sbt}	*Synechocystis* 6803	*gpl*	*Synechocystis* 6803	Liu et al., 2011a
P_{nrsB}	*Synechocystis* 6803	holin, endolysin, auxiliary lysis enzyme	*Synechocystis* 6803	Liu and Curtiss, 2009
P_{T7}	Coliphage T7	*luxAB*	Anabaena sp. 7120	Wolk et al., 1993
P_{lac}	*E. coli*	*atoB, adhE2, ter, hbd, crt, hydA, alsS, ilvC, ilvD,*	*Synechococcus* 7942	Atsumi et al., 2009; Ducat et al., 2011a; Lan and Liao, 2011
$P_{A1lacO-1}$	*E. coli*	*efe*	*Synechocystis* 6803	Guerrero et al., 2012
P_{trc}/P_{tac}	*E. coli*	*petE, atoB, adhE2, ter, hbd, crt, kivd, rbcLS, invA, glf, ldhA, lldP, phrA,* GFP, EYFP	*Synechococcus* 7942, *Synechocystis* 6803	Atsumi et al., 2009; Geerts et al., 1995; Huang et al., 2010; Lan and Liao, 2011; Ng et al., 2000; Niederholtmeyer et al., 2010
P_{tet}	*E. coli*	GFP	*Synechocystis* 6803	Huang et al., 2010

Transcriptional terminators. Placing a transcription terminator downstream of the introduced genes will prevent effects on the expression of genes adjacent to the insertion loci; meanwhile, placing a terminator upstream of the promoter of an introduced gene will also prevent any background transcription effect on the upstream genes (Adhya and Gottesman, 1982). So far only a few native and foreign terminators have been utilized in cyanobacteria, including the cyanobacterial RuBisCO terminator (Takeshima et al., 1994) and strong *E. coli* terminators such as *rrnB* terminator (Atsumi et al., 2009; Geerts et al., 1995; Takahashi et al., 1998), bacteriophage T7 terminator (Argueta et al., 2004; Lang and Haselkorn, 1991) and *rrnBT1-T7TE* double terminator (http://partsregistry.org/Part:BBa_B0015) (Huang et al., 2010). Very little work has been conducted to characterize the termination efficiencies in cyanobacteria and algae.

Ribosome binding sites. The ribosome binding sites (RBS) play a crucial role in initiating the translation of downstream target genes. Upon translation initiation, the 3'-terminal sequence of the 16S rRNA interacts with the core Shine-Dalgarno (SD) sequence of RBS by complementary pairing of the nucleic acids. For example, in cyanobacterium *Synechocystis* 6803, the 3'-terminal sequence of the 16S rRNA is AUCACCUCCUUU (Kaneko et al., 1996; Ma et al., 2002) and therefore the optimal complementary Shine-Dalgarno (SD) sequence should be AAAGGAGGUGAU (core SD sequence underlined). Heidorn and colleagues studied the efficiencies of different RBS in expressing GFP in *Synechocystis* 6803 and found that the RBS sequence UAGUGGAGGU gave about 2-fold higher translation efficiency than a RBS sequence AUUAAAGAGGAGAAA and about 4-fold higher than those of sequences UCACACAGGAAAG and AAAGAGGAGAAA (Heidorn et al., 2011). However, it is

found that the efficiency of the same RBS might vary across species, such as *E. coli* vs. *Synechocystis* (Heidorn et al., 2011). The efficiency of a given RBS also depends on the surrounding nucleotide sequence that may result in secondary structures and the spacing between the SD sequence and the translation start codon AUG (Chen et al., 1994; de Smit and van Duin, 1990; Pfleger et al., 2006). In order to predict the translation efficiency of a given RBS in various genetic contexts, Salis and coworkers have established a thermodynamic model that calculates the impact from the SD sequence, the start codon, the spacing between the SD sequence and the start codon, and the mRNA secondary structure; the model can accurately predict protein expression levels within a factor of 2.3 over a range of 100,000-fold in *E. coli* (Salis et al., 2009). A similar model should be employed to optimize the RBS for gene expression in cyanobacteria.

Negative regulation of gene expression. The down-regulation of the target gene expression has been studied at transcriptional, translational and post-translational levels in cyanobacteria. The negative transcriptional factor, LacI, has been utilized as a repressor in regulating the P_{tac}/P_{trc} controlled target gene expression (Atsumi et al., 2009; Ducat et al., 2011a; Geerts et al., 1995; Huang et al., 2010; Lan and Liao, 2011; Niederholtmeyer et al., 2010). However, the lacI-P_{tac}/P_{trc} expression system can result in severe leaky expression of target genes (Huang et al., 2010). By placing dual *lac* operators upstream of the P_{tac}/P_{trc} promoter the leaky expression of downstream target genes was significantly repressed; however, this also resulted in a limited induction of the promoter with presence of the inducer IPTG (Huang et al., 2010). Degradation tags which can be fused to the target proteins through genetic engineering have also been investigated in cyanobacteria. Three *ssrA* protease degradation tags including ASV, AAV

and LVA were fused to EYFP and expressed in *Synechocystis* 6803. The results indicated that LVA is the strongest degradation tag, AAV is the weaker one, and ASV is the weakest (Huang et al., 2010). Recently, it has been discovered that antisense RNAs (asRNAs) play an important role in cyanobacterial gene regulation (Cerutti et al., 2011; Georg et al., 2009; Hernández et al., 2006; Mitschke et al., 2011). Thus, asRNAs provide another approach for gene silencing in cyanobacteria. For example, Mussgnug and coworkers have successfully down-regulated the expression of light-harvesting antenna complexes through RNA interference (Mussgnug et al., 2007).

Endogenous enhancers. The transcription of an interest gene can be positively affected by placing in the gene cluster an enhancer, a short DNA fragment which interacts with certain proteins to enhance the transcription. In cyanobacteria, some light-responsive elements exhibit enhancer activities. For example, the 5'-untranslated regions of the *psbAII* and *psbAIII* genes of *Synechococcus* 7942 have been found to be enhancers which can increase the expression of downstream genes by 4- to 11-fold when combined with an E. coli promoter (conII) in the *Synechococcus* 7942 host strain (Li and Golden, 1993). A recent study by Eichler-Stahlberg et al. (2009) showed that inserting three introns from the native alga *C. reinhardtii* RBCS2 gene into the recombinant luciferase and erythropoietin resulted in up to 4-fold increase of the expression levels. Fusing the recombinant luciferase with the endogenous RuBisCO LSU protein, Muto et al. (2009) has achieved enhanced luciferase expression by 33-fold.

1.2.2 Plasmid vectors

Both integrative and replicative plasmids have been developed for cyanobacteria. In cyanobacteria, integrative plasmids are usually utilized as vectors to integrate foreign genes into the cyanobacterial genomes via homologous recombination (Eaton-Rye, 2004; Golden et al., 1987; Heidorn et al., 2011). Integrative plasmids usually cannot replicate themselves and would eventually be eliminated through cell division. Replicative plasmids are those which can replicate in host cyanobacteria and the replication properties can be descended to daughter cells. Replicative cyanobacterial plasmids can be classified into two types: those with replicons of broad-host-range plasmids (Huang et al., 2010; Mermet-Bouvier et al., 1993; Mermet-Bouvier and Chauvat, 1994; Ng et al., 2000) and those derived from endogenous cryptic plasmids (Argueta et al., 2004; Deng and Coleman, 1999; Iwaki et al., 2006; Lang and Haselkorn, 1991; Reaston et al., 1982; Summers et al., 1995; Wolk et al., 1984). Representative shuttle vectors for cyanobacteria are listed in **Table 2**. The copy numbers of the broad-host-range RSF1010-derived plasmids have been reported as about 10 per chromosome in *E. coli* cells and 10-30 per cell in *Synechocystis* (Huang et al., 2010; Ng et al., 2000) which is slightly higher than the average copy number (approximately 10) of the *Synechocystis* chromosome (Eaton-Rye, 2004). Due to lack of an active partitioning mechanism, RSF1010-derived plasmids tend to be eliminated in cells and thus antibiotic selection pressure is required for the maintenance (Becker and Meyer, 1997; Meyer, 2009).

Plasmid vectors have been developed to transform algae (León-Bañares et al., 2004). Recombinant eukaryotic algal viruses as transformation vectors (Langridge et al., 1986)

and *Agrobacterium tumefaciens*-mediated method (Kumar et al., 2004) were also successfully developed for both marine and freshwater algae (Wang et al., 2010).

Table 2 Representative shuttle vectors for cyanobacteria.

Cyanobacterial replicons	***E. coli* replicons**	**Representative vectors**	**Host cyanobacteria**	**References**
pDU1	pMB1	pRL1, pJL3	*Anabaena* 7120, *Anabaena* 7118, *Anabaena* M-131, *Nostoc* 7524	Lang and Haselkorn, 1991; Reaston et al., 1982; Wolk et al., 1984
pDC1	pMB1	pSCR119/202, pSUN119/202	*Nostoc* sp. MAC 8009, *Nostoc punctiforme*, *Nostoc* ATCC 29133	Argueta et al., 2004; Lambert and Carr, 1983; Summers et al., 1995
pUH24	P15A	pUC303	*Synechococcus* 7942	Iwaki et al., 2006; Kuhlemeier et al., 1983
pUH24	pMB1	pCB4, pSG111	*Synechococcus* 7942	Deng and Coleman, 1999; Golden and Sherman, 1983; Luinenburg and Coleman, 1993
pAQ1	pMB1	pAQE17	*Synechococcus* 7002	Buzby et al., 1985
PBA1	pMB1	pARUB19	*Synechococcus* 6301	Takeshima et al., 1994
RSF1010	RSF1010	pFC1, pSL1211, pPMQAK1,	*Synechosystis* 6803, *Synechosystis* 6714, *Synechococcus* 7942, *Synechococcus* 6301, *Anabaena* 7120, *Nostoc* ATCC 29133	Huang et al., 2010; Mermet-Bouvier et al., 1993; Mermet-Bouvier and Chauvat, 1994; Ng et al., 2000

1.2.3 Codon usage

Since different organisms usually bear particular codon usage patterns, when a gene is cloned from one species and expressed in a second organism, some codons might become rare codons in the new host, leading to poor translation efficiency (Kane, 1995). Genes in cyanobacteria show a bias in use of synonymous codons (Beck et al., 2012; Campbell and Gowri, 1990; Nakamura et al., 2000; Yu et al., 2012); it is thus very important to examine the difference in codon usage of a heterologous gene before it is expressed in cyanobacteria. In a recent study, Lindberg and coworkers (Lindberg et al., 2010) studied the effects of codon usage on heterologous expression of kudzu *IspS* gene (encoding the isoprene synthase) in *Synechocystis* 6803. The results showed that the codon-optimized *IspS* showed remarkable improved expression, 10-fold higher than that of the native *IspS* gene under control of the same promoter. The importance of codon optimization in algal genetic applications is also increasingly acknowledged. For instance, it has been shown that codon bias significantly affects the GFP expression in *C. reinhardtii* (Heitzer et al., 2007). As a result, in recent transgenic research, the codon-optimized luciferase gene was used in a green alga *Gonium pectorale* (Lerche and Hallmann, 2009) and the codon-modified *β*-glucuronidase gene was transformed in a red seaweed *Porphyra yezoensis* (Takahashi et al., 2010).

1.2.4 Transformation of cyanobacteria and algae

Methods to introduce DNA into cyanobacteria include conjugation (Elhai and Wolk, 1988; Thiel and Wolk, 1987), electroporation (Zang et al., 2007) and natural

transformation (Grigorieva and Shestakov, 1982; Kuhlemeier and Vanarkel, 1987; Shestakov and Khyen, 1970). The methods have been well summarized in several recent reviews (Eaton-Rye, 2004; Heidorn et al., 2011; Koksharova and Wolk, 2002). Compared to cyanobacteria, transformation methods for algae are less developed and more complicated. Since the chloroplast and nucleus of alga *C. reinhardtii* were stably transformed more than two decades ago (Boynton et al., 1988; Fernández et al., 1989; Debuchy et al., 1989), different methods have been employed in algal transformation which include, but not limited to, particle bombardment, glass bead agitation, microinjection, electroporation and *Agrobacterium tumefaciens*-mediated transformation (Coll, 2006; León and Fernández, 2007; León-Bañares et al., 2004; Potvin and Zhang, 2010). Specifically, bombardment of target cells with DNA-coated metal particles turns out to be an effective and highly reproducible method to transform algae. This method has been so far applied in the transformation of nuclear and chloroplast of many algal species such as *C. reinhardtii*, *Volvox carteri*, *Chlorella sorokiana*, *Chlorella ellipsoidea*, *Chlorella kessleri*, *Haematococcus pluvialis, Phaeodactylum tricornutum* and *Gonium pectorale* (Boynton and Gillham, 1993; Potvin and Zhang, 2010). In addition, agitation of the cell wall-deficient algal cells with glass beads, polyethylene glycol (PEG) and foreign DNA has been used to transform algae such as *C. reinhardtii*, *Dunaliella salina*, and red alga *Porphyra haitanensis* (Feng et al., 2009; Kindle, 1990; Wang et al., 2010). Microinjection of the viral SV40 DNA or the chimeric construction pSV2neo into the marine unicellular green alga *Acetabularia mediterranea* also resulted in a high yield and stable nuclear transformation (Neuhaus et al., 1986); nevertheless, it is difficult to operate and the transformation throughput is low. *Agrobacterium tumefaciens* has been used to

mediate the transformation of *C. reinhardtii* (Kumar et al., 2004) and *H. pluvialis* (Kathiresan et al., 2009). Recently, it was discovered that the industrially relevant oil-producing alga *Nannochloropsis* sp. is haploid and can be transformed with high efficiency using high electric field electroporation. It has also been found that efficient stable transformation of this species via homologous recombination requires using linear DNA fragments rather than circular plasmid DNA (Kilian et al., 2011). However, the mechanism for the high homologous recombination efficiency is to be elucidated.

1.3 Applications of modified cyanobacteria and algae

The focus in this section is on recent progress in producing biofuels and other useful chemicals using genetically modified cyanobacteria and algae. For other applications, readers can refer to several other excellent reviews published recently (Qin et al., 2012; Radakovits et al., 2010; Ruffing, 2011).

1.3.1 Biofuels

The United States consumed 24.9 quadrillion Btu equivalent of petroleum for transportation purposes in 2013, whereas the renewable biofuel contributed to only 1.2 quadrillion Btu equivalent of energy in the same year (,http://www.eia.gov/totalenergy/data/monthly/pdf/sec2_11.pdf). Many alternatives to current liquid fuels have been proposed, including ethanol, 1-butanol, isobutanol, short chain alcohols, short chain alkanes, biodiesel (FAME, fatty acid methyl esters), fatty alcohols, alkanes, linear and cyclic isoprenoids (Connor and Atsumi, 2010; Lee et al.,

2008). Current routes for biological production of fuels and chemicals are summarized in **Figure 1**. Traditionally people follow a two-step route to firstly collect plant biomass and then convert biomass to fuels by microbial fermentation (Stephanopoulos, 2007); whereas recently interest in harnessing photosynthetic microbes to directly convert CO_2 to fuels has been dramatically increased (Chisti, 2007; Lu, 2010; Mascarelli, 2009). Compared to crops, the per-hectare oil yield of cyanobacteria or microalgae is about two orders of magnitude higher and the cultivation land needed is around two orders of magnitude less (Chisti, 2007). It is anticipated that sustainable production of biofuel may be achieved via developing and applying synthetic and systems biology in cyanobacteria and algae in the coming decades.().

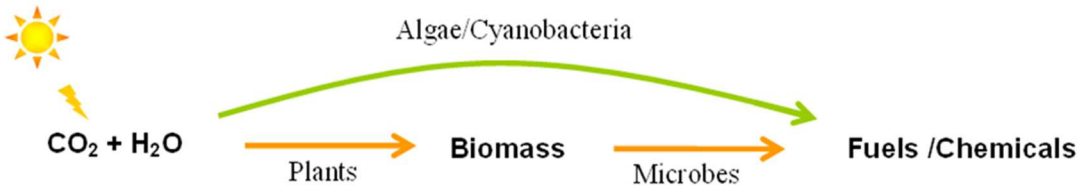

Figure 1 Routes for biological production of fuels and chemicals. Arrows indicate the carbon and energy flow between different carriers.

Biodiesel. Cyanobacteria and algae are rich in energy stock compounds, such as diacylglycerol (DAG), triacylglycerol (TAG) and starch, which can be extracted and used for biodiesel production (Chisti, 2007; Radakovits et al., 2010; Sheng et al., 2011; van de Meene et al., 2006). To further increase the oil contents in the cells, effects have been made to block metabolic pathways as well as to overexpress genes of limiting steps. For example, two different starch-deficient strains of *C. reinhardtii*, the *sta6* and *sta7* mutants

that carries gene knockout in the ADP-glucose pyrophosphorylase and isoamylase genes, respectively, have been isolated (Mouille et al., 1996; Posewitz et al., 2005); and these mutants accumulated increased levels of TAG during nitrogen deprivation (Wang et al., 2009). Another starchless mutant of *Chlorella pyrenoidosa* has also been reported that the lipid content of this mutant has been elevated by nearly 2-fold relative to the wild type under nitrogen limitation culture conditions (Ramazanov and Ramazanov, 2006). It indicated that blocking the starch biosynthesis may be an effective way to increase lipid, and thus potentially biodiesel, production.

Nevertheless, the lipid extraction process is energy-intensive and production of a significant amount of glycerol as a byproduct have been two of the major hurdles for commercial production of biodiesel (Chisti, 2007; Liu and Curtiss, 2009; Fernando et al., 2007). Efforts have been made from both process engineering and genetic engineering approaches to facilitate lipid extraction (Liu and Curtiss, 2009; Liu et al., 2011a; Sheng et al., 2011). Specifically, Liu and coworkers have constructed inducible systems to conditionally express phage lysis genes and lipolytic enzyme genes in *Synechocystis* 6803 to trigger the cell lysis upon harvest and thus facilitate the extraction of lipid from this species (Liu and Curtiss, 2009; Liu et al., 2011a). To produce secretable biofuels from a synthetic biology approach is another way to resolve the issues stated above.

Free fatty acids. Enhanced production of free fatty acids (FFAs) has already been achieved in *E. coli* through a series of genetic engineering methods (Davis et al., 2000; Lu et al., 2008). In a recent study, Liu and coworkers (2011b) engineered cyanobacterium *Synechocystis* strains to produce and secrete FFAs to up to 197 mg/L at a cell density of 1.0×10^9 cells/mL (Liu et al., 2011b). The acetyl-CoA carboxylase (ACC) was

overexpressed to drive the metabolic flux towards FFAs, while the fatty acid activation gene *aas* (*slr1609*) was deleted to inactivate the FFAs degradation. Poly-β-hydroxybutyrate (PHB) synthesis genes (*slr1993* and *slr1994*) and the phosphotransacetylase gene *pta* (*slr2132*) were deleted to block competitive pathways. Particularly, two genetic modifications turned to significantly increase the FFAs production and secretion: overexpression of thioesterases and weakening the polar peptidoglycan layer of the cell wall of *Synechocystis* 6803.

Alkanes and alkenes. Although it was known that some cyanobacteria can synthesize alkanes, the molecular mechanism has been mysterious until recently when alkane/alkene biosynthetic pathways were identified in cyanobacteria (Steen et al., 2010; Mendez-Perez et al., 2011). Steen and coworkers identified an alkane/alkene biosynthetic pathway that shows two successive biochemical reactions catalyzed by an acyl-ACP reductase and an aldehyde decarbonylase, respectively, converts acyl-ACP (intermediates of fatty acid metabolism) to alkanes/alkenes (Steen et al., 2010). In order to increase the alkane production in cyanobacteria, heterologous expression of acyl-ACP reductase and aldehyde decarbonylase genes (from *Synechococcus* 7942) has been achieved in *Synechococcus* 7002, which led to a total intracellular accumulation of n-alkane to up to 5% of the dry cell weight (Reppas and Ridley, 2010). In other research, Mendez-Perez and colleagues identified the genes responsible for α-olefin biosynthesis in *Synechococcus* 7002 (Mendez-Perez et al., 2011). In addition, overexpression of the *accBCDA* operon (which encodes ACC) in *Synechocystis* was also reported to enhance alkane/alkene production (Tan et al., 2011), consistent with the aforementioned results of

FFAs production. Although it is believed there are certain alkane/alkene secretion pathways, the specific mechanisms are still under exploration (Radakovits et al., 2010).

Ethanol. Ethanol production via microbial fermentation has undergone a sharp increase in the past decade for its utility as a supplement in transportation fuel (Stephanopoulos, 2007; Energy Information Administration, 2010). In 1999, photosynthetic production of up to 230 mg/L ethanol has been reported using genetically engineered cyanobacterium *Synechococcus* 7942, in which an artificial operon of *pdc-adh* (genes originally from *Zymomonas mobilis*) was expressed under a P_{lac} and P_{rbc} promoters via a shuttle vector pCB4 (Deng and Coleman, 1999). In a recent study, the *pdc-adh* expression cassette was integrated into the chromosome of *Synechocystis* 6803 at the *psbA2* locus. Driven by the light-inducible strong P_{psbA2} promoter, expression of *pdc/adh* resulted in ~550 mg/L ethanol production by the engineered *Synechocystis* under high light (~1000 $\mu E/m^2/s$) conditions (Dexter and Fu, 2009). In algae, although many species have fermentative pathways to produce ethanol, the pathways are only functional under dark and anaerobic conditions (Hirayama et al., 1998). Algal ethanol is currently produced via heterotrophic fermentation of algal biomass using heterotrophs such as yeast and *E. coli* (Harun et al., 2010; Nguyen et al., 2009; Wargacki et al., 2012), which follows the two-step route (**Figure 1**). Direct photosynthetic production of ethanol by algae would be possible using a similar approach: cyanobacteria expressing foreign ethanol biosynthesis pathways, or by tuning the native regulatory pathways in algae.

Isobutanol & 1-butanol. Compared with ethanol, isobutanol and 1-butanol have much higher energy density. The energy density of butanol reaches 29.2 MJ/L, about 90% of that of gasoline, 32.5 MJ/L, and it is also less volatile and less corrosive than ethanol

(Dürre, 2007). Therefore, butanol is regarded as a better gasoline substitute. Recently, significant progress has been achieved for photosynthetic production of butanol. Liao and colleagues introduced an artificial isobutanol biosynthesis pathway into *Synechococcus* 7942 and the engineered strains were able to photosynthetically produce isobutyraldehyde and isobutanol at titers of 1100 mg/L and 450 mg/L, respectively (Atsumi et al., 2009). In contrast, photosynthetic production of 1-butanol in oxygenic cyanobacteria or algae has been difficult because the intrinsic oxygen-sensitivity and NADH-dependence of the 1-butanol biosynthetic pathway are in conflict with the photo-oxygenesis and lack of NADH cofactors in cyanobacteria (Atsumi et al., 2008a; Inui et al., 2008; Lan and Liao, 2011). When a 1-butanol pathway was overexpressed in *Synechococcus* 7942, the 1-butanol was barely detectable (~1 mg/L) after two weeks of cultivation under photosynthetic conditions. Up to 14.5 mg/L 1-butanol has been achieved in *Synechococcus* 7942 under an anoxic condition (Lan and Liao, 2011). Further analysis revealed that the reversible acetyl-CoA condensation reaction catalyzed by thiolase (encoded by *atoB*) strongly favors the thiolysis of acetoacetyl-CoA rather than the condensation of two acetyl-CoA molecules, and thus AtoB may be insufficient to drive the flux from acetyl-CoA pool towards 1-butanol biosynthesis under photosynthetic conditions (Lan and Liao, 2012). To this end, an alternate ATP-driven acetoacetyl-CoA biosynthetic pathway was constructed by overexpressing an acetoacetyl-CoA synthase (NphT7) which instead condenses malonyl-CoA and acetyl-CoA in *Synechococcus*. By co-expressing the downstream NADH-dependent 1-butanol biosynthetic pathway, 6.5 mg/L 1-butanol has been produced under photosynthetic conditions. After the NADH-dependent bi-functional aldehyde/alcohol dehydrogensae (AdhE2) was further replaced

with separate NADPH-dependent butyraldehyde dehydrogenase (Bldh) and alcohol dehydrogenase (YqhD), the 1-butanol production was increased by 4-fold, up to ~30 mg/L, under the same photosynthetic condition (Lan and Liao, 2012).

Longer carbon-chain fatty alcohols. In order to produce long-chain alcohols, Lu and colleagues heterologously expressed fatty acetyl-CoA reductases from different sources in *Synechocystis* and the resultant strains achieved production of fatty alcohols, including hexadecanol (C16) and octadecanol (C18) (Tan et al., 2011). Although the titer was very low (about 0.2 mg/L), it is amenable for further improvement via further enhancing upstream pathways and addressing secretion issues as that in the engineering of *Synechocystis* 6803 for enhanced fatty acid production (Liu et al., 2011b). Production of the intermediate-chain alcohols (C5 to C10) in *E. coli* has been well summarized by Lamsen and Atsumi (2012). Briefly, C5 to C10 alcohols have been successfully biosynthesized via the expanded 1-butanol pathway (Dekishima et al., 2011), the engineered reversal of the β-oxidation pathway (Dellomonaco et al., 2011) and the 2-keto acid metabolic pathways (Atsumi et al., 2008b; Zhang et al., 2008). Since cyanobacteria and algae share with *E. coli* most metabolic pathways required for longer-chain alcohol biosynthesis, it is believed that similar approaches can be used to achieve the biosynthesis of alcohols with carbon chain length >5 in cyanobacteria and algae.

Hydrogen. Besides liquid biofuels, production of hydrogen – a gaseous, carbon-free and high-energy-content fuel – in algae and cyanobacteria has also gained increasing attention in recent years (Ghirardi et al., 2007; Ghirardi et al., 2009; Hankamer et al., 2007; Hemschemeier et al., 2009; Kruse et al., 2005; Lee et al., 2010; Melis et al., 2000; Srirangan et al., 2011). Many cyanobacteria and algae naturally produce hydrogen as a

secondary metabolite to balance the redox energetics. In order to fortify the hydrogen production, endeavors have been made to augment the electron flux, instead of the carbon flux, toward H_2 biosynthesis catalyzed by hydrogenases ($2H^+ + 2e^- \rightarrow H_2$). In alga *C. reinhardtii*, for instance, blocking the cyclic electron transfer around photosystem I eliminated the possible electron competition for electrons with hydrogenase; as a result, the H_2 evolution rate increased 5-13 times under a range of conditions (Kruse et al., 2005). Hydrogenase has been tethered to the photosystem I to obtain a much greater electron throughput and thus H_2 evolution rate (Ihara et al., 2006; Lubner et al., 2011; Schwarze et al., 2010). However, to date, these experiments were all conducted *in vitro* and efforts need to be made from a synthetic biology approach to validate the concept *in vivo*. In another study, expression of an exogenous ferredoxin from *Clostridium acetobutylicum* in addition to the native ferredoxin could fortify the electron flow toward the hydrogenase HydA via siphoning electrons from the fermentation of internal reducing equivalents (such as glycogen). As a result, the hydrogen production was enhanced by approximately 2-fold (Ducat et al., 2011a) under light-dependent anoxic conditions. On the other hand, efforts have been made to block pathways competitive for reductant consumption to facilitate the H_2 production. For example, after the *ldhA* gene (which is responsible for NADH consumption in lactate production) was inactivated in *Synechococcus* 7002, the $NADH/NAD^+$ ratio increased markedly and therefore the hydrogen production by the native bidirectional [NiFe] hydrogenase was increased 5-fold under anoxic dark conditions (McNeely et al., 2010). The oxygen-sensitivity of both two major types of hydrogenases, [NiFe] and [FeFe], is the greatest challenge to date which is discussed in Section 1.4.3.

1.3.2 Other commodity chemicals

Although significant attention has been paid to photosynthetic production of fuels from CO_2, the relative values (in term of USD per photon) of fuels are much lower than those of other commodity chemicals. For example, it is estimated that the relative value of a photon fixed in lactic acid is about 3.5-fold greater than that in octane (Ducat et al., 2011b). Therefore, photosynthetic production of chemicals with higher unit values than fuels is economically more desirable at least in the near term.

Ethylene. Ethylene, the simplest unsaturated alkene, is one of the most important building-blocks in the synthetic chemical industry. However, its production almost exclusively relies on petroleum. To make the production sustainable, biosynthesis of ethylene from renewable resources has been explored. Sakai and colleagues first demonstrated photosynthetic production of gaseous ethylene from CO_2 in genetically engineered *Synechococcus* by heterologously expressing a single *efe* gene of *Pseudomonas syringae* on a pUC303-derived shuttle vector (Sakai et al., 1997). Later, by integrating the *efe* gene into the *psbA1* locus of the *Synechococcus* 7942 genome, the research group achieved higher ethylene production with a titer of ~37 mg/L (Takahama et al., 2003). The engineered *Synechococcus* strains were not genetically stable, resulting in declined ethylene production during successive batch cultivations (Takahama et al., 2003). Since production of every two molecules of ethylenes consumes three molecules of 2-oxoglutarate and one molecule of L-arginine (Fukuda et al., 1992), the genetic instability was speculated to be due to the shortage in the tricarboxylic acid (TCA) cycle intermediates which led to a severe depression on cell growth (Takahama et al., 2003).

However, the genetic instability issue was later resolved by generating several silent mutations at the mutational "hotspots" along the *P. syringae efe* gene (Ungerer, et al., 2012). In order to sustain the ethylene production in cyanobacteria or algae, metabolic flux towards TCA cycle should be enhanced and alternative ethylene biosynthesis pathways might be considered (Fukuda et al., 1989; Kende, 1993; Kosugi et al., 2000; Yang and Hoffman, 1984).

Isoprene. Isoprene is another important feedstock in synthetic chemistry and potentially biofuels. Biosynthesis and emission of isoprene occurs in many plants as a way to cope with heat flecks and reactive oxygen species, and the genetic mechanism has been investigated (Sharkey et al., 2008). Lindberg and colleagues cloned the *IspS* gene (encoding isoprene synthase) from *Pueraria montana* and integrated it into the *psbA2* locus of the *Synechocystis* genome, conferring heterologous expression of the isoprene synthase under the light-dependent P_{psbA2} promoter in *Synechocystis*. Codon usage turned out to be a very important factor for optimal expression of the *IspS* gene. After codon optimization, the *IspS* gene expression was enhanced by about 10-fold. Isoprene was eventually produced at a rate of ~50 mg/g dry cell/day under high light (~500 $\mu E/m^2/s$) culture conditions (Lindberg et al., 2010). It is noteworthy that heterologous expression of *IspS* by replacing the *psbA2* gene did not affect photosynthesis significantly and depress the growth of the transformants (Lindberg et al., 2010), which was different from the aforementioned ethylene-producing cyanobacteria (Sakai et al., 1997; Takahama et al., 2003).

Acetone. Acetone represents the simplest ketone which serves as a solvent and precursor for industrial chemicals (Yurieva et al., 1996). Microbial production of acetone

has been achieved in fermentation of *Clostridia* and recombinant *E. coli* using sugar as feedstocks (Bermejo et al., 1998). However, the maximal yield is merely 50% with the other half carbon being released as CO_2 when hexose is the sole carbon source. Recently, through a combination of co-expression of the acetoacetate decarboxylase (*adc*) and coenzyme A transferase (*ctfAB*) and deletion of the PHB polymerase (PhaEC) in *Synechocystis* 6803, 3-5 mg/L acetone has been produced under nitrogen and phosphate deprived, dark and anaerobic culture conditions. After deleting the phosphotransacetylase encoding gene *pta*, the competitive acetate production was remarkably reduced and the acetone titer has been evidently increased to 36.0 mg/L in the culture (Zhou et al., 2012).

Poly-β-hydroxybutyrates. Cyanobacteria are the natural producers of poly-β-hydroxybutyrate (PHB), a type of polyhydroxyalkanoates (PHAs) that serves as biodegradable plastics (Hein et al., 1998; Taroncher-Oldenberg et al., 2000). However, the yield is very low and nutrient deprivation and acetate addition are usually necessary for accumulation of PHB (Wu et al., 2001). By introducing PHB biosynthesis genes from *Ralstonia eutropha* into *Synechococcus* 7942 coupled with nitrogen starvation and acetate supplementation, the PHB biosynthesis in the recombinant cyanobacteria has reached a maximum of 25.6% of the dry cell weight (Takahashi et al., 1998). Efforts in identifying gene disruptions which might contribute to the increase of PHB accumulation were also made and several gene disruptions with positive effects were discovered (Tyo et al., 2009). Nevertheless, similar with other types of macromolecules, PHB can-not be secreted out of cells; the required extraction process is energy-intensive and remains as one of the major hurdles for commercial applications (Chisti, 2007; Liu and Curtiss, 2009). As a result, 3-hydroxybutyrate (3HB), the monomer of PHB and a building block

molecule for other polyhydroxyalkanoates (PHAs), has been successfully produced and secreted by genetically engineered *E. coli* (Lee and Lee, 2003; Liu et al., 2007; Tseng et al., 2009). Hence, photosynthetic production of 3-hydroxybutyrate in cyanobacteria and algae might be a feasible approach to cope with the secretion problem.

Lactic acid. Lactic acid is another chemical that can serve as a building block for synthesizing biodegradable polyesters with valuable medical properties. It is also used as a preservative and acidulant in food industry, and can serve as an advanced nutrient for neuron cells (Wee et al., 2006). While conventional production of lactic acid relies on microbial fermentation of sugars (Wee et al., 2006), photosynthetic production of lactic acid using CO_2 as the carbon source has been recently demonstrated (Niederholtmeyer et al., 2010). Through heterologously expressing three genes, including *ldhA*, *lldP* and *udhA*, in cyanobacterium *Synechococcus* 7942, Niederholtmeyer et al. (2010) produced lactic acid with a titer of ~56 mg/L under photoautotrophic culture condition. While LdhA catabolizes the conversion of pyruvate to lactate, expression of the lactate transporter gene *lldP* turned out to be essential for lactate secretion from the engineered *Synechococcus* strain (Niederholtmeyer et al., 2010). Repletion of NADH, a cofactor for LdhA, through expression of the NADPH/NADH transhydrogenase (encoded by *udhA*) greatly enhanced the lactate production but reduced the growth rate of *Synechococcus* (Niederholtmeyer et al., 2010).

Sugars. Fresh water cyanobacteria accumulate solutes such as glucosylglycerol and sucrose when they are exposed to salt stress (Hagemann, 2011). By knocking out the *agp* gene (which contributes to the biosynthesis of glucosylglycerol) from the *Synechocystis* 6803 genome, Miao and colleagues achieved sucrose accumulation of up to 44

mg/L/OD_{730} after 0.9 M salt shock for 96 h (Miao et al., 2003). In another study, overexpression of *invA*, *glf* and *galU* genes in *Synechococcus* 7942 resulted in up to 45 mg/L total hexose production (including glucose and fructose) in the culture supplemented with 200 mM NaCl (Niederholtmeyer et al., 2010). While InvA catalyzes the conversion of sucrose to glucose and fructose, expression of the glucose or fructose transporter GLF (encoded by *glf* gene) was essential for glucose or fructose secretion. Additional expression of GalU enhanced the biosynthesis of intracellular precursors and thus further increased the hexose sugar production by over 30% in the culture (Niederholtmeyer et al., 2010).

1.4 Challenges and opportunities of synthetic biology in cyanobacteria and algae

Despite promising progress, there are challenges ahead for synthetic biology to reach its full power in modifying cyanobacteria and algae for biotechnological applications. The challenges and possible strategies are discussed below.

1.4.1 Improving tools for genetic manipulation

Effective "BioBricks". Although a few "BioBricks" have been characterized in cyanobacteria, the limited number of gene expression elements would not fulfill the need of synthetic biology in cyanobacteria. After initial gene expression, a fine-tuning of gene expression is usually the next step in order to further optimize the properties of the genetically engineered strains, which requires a good number of "BioBricks". Currently most of the "BioBricks" were collected from *E. coli*, but the *E. coli* "BioBricks" might behave differently in cyanobacteria. For example, the tightly regulated IPTG-inducible

lacI/P_{tac} gene expression system does not work as well in cyanobacteria as it does in *E. coli* (Huang et al., 2010). Thus, systematic collection and characterization of "BioBricks" in cyanobacteria is necessary. Additionally, in contrast to various commercialized *E. coli* and yeast strains that have been genetically modified to serve as a chassis for different purposes, there are few such cyanobacterial or algal species available nowadays. To design and construct a series of chassis strains is thus an urgent task. Moreover, there has been no study of the performance of a given BioBrick in different cyanobacterial species. A defined BioBrick might behave differently across cyanobacterial species and thus the efficiency of the BioBrick might need to be characterized for each cyanobacterial species.

Improved transformation efficiency. Standardized transformation vectors and protocols have been established for model cyanobacteria, such as *Synechococcus* and *Synechocystis*, although the transformation efficiency still needs further improvement (Eaton-Rye, 2004; Heidorn et al., 2011). However, the transformation methods for model filamentous cyanobacteria, such as *Anabaena* and *Spirulina*, are still under development (Ducat et al., 2011b), and so far no genetic engineering has been conducted in the marine N_2-fixing cyanobacterium *Trichodesmium* despite significant interest on its ability of peaking the fixation of CO_2 and N_2 simultaneously during the day time (Berman-Frank et al., 2001; Chen et al., 1998). *In vivo* restriction activities have been demonstrated as an important barrier for introducing foreign DNA into cyanobacterial cells (Elhai et al., 1997; Koksharova and Wolk, 2002). Hence, it would be helpful to construct methylation-defect cyanobacterium host strains or to establish *in vitro* systems that can methylate the foreign DNA before transformation. Additionally, since the bacteriophage λ recombination system has greatly improved the *E. coli* transformation efficiency (Yu et al., 2000), high-

efficiency homologous recombination in cyanobacterial cells might be achievable by developing a proper cyanophage recombination system.

In order to improve transformation efficiency of other algal species, an endeavor could be made to uncover the mechanism behind the recently discovered truth that highly efficient homologous recombination occurs after electroporation of the industrially relevant oil-producing alga *Nannochloropsis* sp. (Kilian et al., 2011). Recently, through an approach of *ex vivo* assembly of the chloroplast genome before bombarding it into the green alga *C. reinhardtii*, O'Neill and coworkers demonstrated that simultaneous and multi-loci genetic modifications of the chloroplast of the green alga *C. reinhardtii* could occur after one single round of transformation (O'Neill et al., 2012), providing an alternative method to improve the efficiency of multiple-gene transfer.

1.4.2 Improving photosynthesis efficiency

Although the solar energy conversion efficiencies of algae and cyanobacteria are 2-3 fold higher than those of crop plants, the efficiencies are still low with yields around 5-7% during the growing season and around 3% in bioreactors on an annual basis (Blankenship et al., 2011). A recent study on *in silico* modeling of the reconstructed photosynthetic process revealed that regulation of the photosynthesis activity is quite complex and a high degree of cooperation of nine alternative electron flow pathways is responsible for optimized photosynthesis performance in *Synechocystis* 6803 (Nogales et al., 2012).

Light harvesting. Photosynthetic microorganisms in nature have been selected by their abilities to reproduce but not by their ability to produce a maximal amount of biomass or specific products. In order to thrive in the wild environment, cyanobacteria and algae

have maximized their expression of pigments and antenna to compete with competitors for sunlight. However, when a monoculture was employed to produce a high-density of biomass or maximal titers of specific products in photo-bioreactors, excessive photon capture by the cells in the surface layer can block the light availability to the cells underneath (Melis, 2009). To address this issue, studies have been conducted on minimizing the size of the photosystem antenna complex through various strategies, such as by expressing truncated light-harvesting antenna complex (LHC) mutants (Blankenship et al., 2011; Ort et al., 2011; Work et al., 2012), by down-regulating the expression of LHC through RNA interference (RNAi) and expression of LHC translation repressor in both cyanobacteria and algae (Mussgnug et al., 2007; Work et al., 2012). For example, the photosynthetic activity (measured by oxygen evolution) was about 3-fold higher in the alga strain *Stm3LR3* (with LHC being down-regulated via RNAi) than in the parent strain *Stm3* (without RNAi) after 100 min of high-light treatment; the cell growth rate also increased under high-light conditions after the LHC was down-regulated via RNAi (Mussgnug et al., 2007). Another bold proposal was to increase the photosynthesis efficiency by extending the light absorption range of the photosystems in cyanobacteria and algae (Blankenship et al., 2011). As the chlorophyll, carotenoids and other accessory pigments in cyanobacteria and algae capture only the visible region of the spectrum of solar radiation (400 to 700 nm), about 50% of the incident solar energy is dissipated and wasted during photosynthesis. Moreover, since the two photosystems compete for light with the same wavelengths, the overall efficiency is significantly reduced. Thus, it was proposed that one of the two photosystems be engineered to extend the absorption

maxima to ~1100 nm, approximately doubling the solar photon capture, by heterologously expressing bacteriochlorophyll *b* (Blankenship et al., 2011).

CO_2 fixation. RuBisCO is an essential enzyme in photosynthetic carbon fixation in the Calvin-Benson-Bassham (CBB) cycle, catalyzing the combination of ribulose-1,5-bisphosphate with CO_2. However, the reaction is slow. In addition, RuBisCO can also take O_2 as a substrate in addition to CO_2 which further lowers the carbon fixation efficiency. A recent study has revealed that despite slow catalytic turnover and confused CO_2/O_2 substrate specificity, RuBisCOs might have been nearly perfectly optimized (Tcherkez et al., 2006). In nature, cyanobacteria and some algae have evolved certain CO_2-concentrating mechanisms (CCMs) to increase the CO_2 fixation efficiencies. In cyanobacteria, RuBisCOs are sequestered together with carbonic anhydraus in carboxysomes, polyhedral microcompartments (MCPs) with proteinaceous shells. Anhydrase catalyzes the conversion of HCO_3^- to CO_2 which is trapped by (MCPs) for RuBisCOs. Because CCMs can result in much higher CO_2 concentration, and thus higher CO_2 to O_2 ratio, around the RuBisCOs, the carbon fixation efficiency is greatly increased (Espie and Kimber, 2011). It has been found that *Synechococcus* 7942 cells with more carboxysomes exhibited higher CO_2 fixation rates (Savage et al., 2010). Heterologous expression of *Synechococcus* 6301 *rbcLS* (that encodes RuBisCO) in *Synechococcus* 7942 also led to more efficient CO_2 fixation and higher yield of isobutyraldehyde in the genetically modified isobutyraldehyde-producing strain (Atsumi et al., 2009). Besides, overexpression of bicarbonate transporters has also been proposed to improve the photosynthesis efficiency (Price et al., 2011). Alternatively, RuBisCO-independent carbon fixation pathways have been posited. A recent work using *in silico* modeling of

the recombination of existing metabolic building blocks showed that some of the proposed carbon fixation cycles have overall higher kinetic rates (Bar-Even et al., 2010). For example, by coupling the phosphoenolpyruvate caboxylase and the core of the natural C4 carbon fixation cycle, the overall CO_2 fixation rate was predicted as 2-3 fold higher than that of the CBB cycle which employs RuBisCO (Bar-Even et al., 2010).

1.4.3 Overcoming the oxidative stress

Since cyanobacteria and algae are oxygenic microorganisms, the abundant oxygen evolved by splitting water during the photosynthesis process becomes an issue for expressing oxygen-sensitive enzymes. For example, either [NiFe] or [FeFe] hydrogenase required for biological production of H_2 has low oxygen-tolerance (Lee et al., 2010); and the nitrogenases which fix N_2 into NH_4^+ are also extremely oxygen-sensitive (Fay, 1992). From a broader prospect, this oxygen sensitivity issue could be crucial for successful expression of a large number of pathways from anaerobic microorganisms in oxygenic cyanobacteria and algae. To address the issue, efforts have been made to obtain oxygen-resistant enzymes from nature or through mutagenesis. For example, hydrogenases with better oxygen-tolerance have been found from *Ralstonia eutropha* H16 (Saggu et al., 2009) and *Hydrogenovibrio marinus* (Yoon et al., 2011); and elevated oxygen-tolerance has been made for the hydrogenase of *Desulfovibrio fructosovorans* by a single V74M mutation (Dementin et al., 2009). Alternatively, temporal segregation of oxygenic photosynthesis and hydrogen biosynthesis would be another option. In nature, many cyanobacterial species have evolved the mechanism to photosynthetically fix CO_2 during the day time and to fix N_2 by the oxygen-sensitive nitrogenases during the night time

(Fay, 1992). Thereby, the solar energy can be firstly fixed into carbohydrates, such as starch, during oxygenic photosynthesis and then be utilized to power the oxygen-sensitive reactions during dark anoxic conditions. In addition, spatial segregation could be used. Hydrogenases can be localized to certain advantageous space, such as being expressed in heterocysts, to avoid the oxidative stress (Fay, 1992). Recent studies on the assembling of bacterial microcompartments might have provided another opportunity to spatially segregate incompatible oxygenic and oxygen-sensitive processes (Bonacci et al., 2012; Fan et al., 2010; Heinhorst and Cannon, 2010). Moreover, the Mehler reaction can be used to overcome the oxidative stress (Mehler, 1951; Asada, 2006). The Mehler reaction has been evolved to overcome the intracellular oxidative stress by scavenging reactive oxygen species in cyanobacteria and chloroplasts (Kana, 1993; Asada, 2006). For instance, during the N_2-fixation period, the Mehler reaction consumes ~75% of gross O_2 production and therefore maintains the O_2 concentration at a low level (Kana, 1993; Milligan et al., 2007). However, Mehler reaction consumes reductants significantly (Asada, 2006); thus, in the future it will be of great interest and of vital importance to maintain the activity locally around the oxygen-sensitive enzymes rather than in the entire cytoplastic environment.

1.4.4 Systematic approaches

Functional genomics. Functional genomics, *i.e.* transcriptomics, proteomics and metabolomics, would greatly promote the development of synthetic biology in cyanobacteria and algae. Albeit the genomes of some many species of cyanobacteria and algae have been sequenced (http://www.genomesonline.org/ and

http://genome.jgi.doe.gov/), a large portion of the sequenced genomes have not yet been annotated and the regulatory networks are still very poorly understood. The study of cyanobacterial and algal transcriptomes, proteomes and metabolomes would allow for identification of new genes, pathways and regulatory networks which are essential to expand the size and diversity of the pool of genetic tools for synthetic biology. For example, recent transcriptomics studies on *Synechocystis* 6803 has enhanced the understanding of the transcriptional regulation in this photosynthetic microorganism which revealed that approximately two thirds of the transcriptional start sites give rise to antisense RNAs and noncoding RNAs, indicating that asRNAs and ncRNAs play an important role in cyanobacterial genetic regulation (Mitschke et al., 2011). Omics may be the key to collect information about the interactions and regulations to develop a sustainable green chemistry industry.

Metabolic modeling. Although most synthetic biology research in cyanobacteria and algae focus on local pathway optimization, comprehensive synthetic biology summons optimization of the genetic network and metabolic flux at the systems level. Genome-scale metabolic modeling allows theoretically evaluating the impact of genetic and environmental perturbations on the biomass yield and metabolic flux distribution and allows predicting the optimal metabolic flux profile to maximize the value of a given objective function (Knoop et al., 2010; Shastri and Morgan, 2005; Yoshikawa et al., 2011). *In silico* modeling may thus provide a systematic approach to design an optimal metabolic network to maximize the production of the biofuel or chemical of interest. Such genome-scale metabolic network models have been constructed for cyanobacteria and algae, and have been utilized to predict new targets to improve product yields and

new pathways (Dal'Molin et al., 2011; Knoop et al., 2010; Shastri and Morgan, 2005; Yoshikawa et al., 2011). However, reconstruction of the global metabolic networks is still in the infancy stage and the simulation results rely significantly on the included pathways. For instance, with ambiguities in metabolic networks in *Synechocystis* 6803, the estimated metabolic fluxes could be significantly different from the experimental results (Yoshikawa et al., 2011). In order to refine the quality of the reconstructed metabolic networks and thus the simulation of metabolic flux, it is inevitable to couple with experimental characterization of the metabolic networks in cyanobacteria and algae (Yoshikawa et al., 2011). As an example, by firstly investigating the *in vitro* activities of the purified relevant enzyme products (heterologously expressed in *E. coli*) and subsequently verifying their *in vivo* activities in the native host *Synechococcus* 7002, Zhang and Bryant (2011) reported that two enzymes could functionally compensate for the missing 2-oxoglutarate dehydrogenase in the TCA cycle. Further database searches indicated that homologs of these two enzymes occur in all cyanobacteria but *Prochlorococcus* and marine *Synechococcus*, which overturned the previously widely accepted assumption that cyanobacteria possess an incomplete TCA cycle (Zhang and Bryant, 2011). Such discoveries would be of utter importance for reconstructing qualified *in silico* models for simulating metabolic flux in the future.

1.5 Conclusion

Owing to the relatively simple genetic contents and the ability to capture solar energy, fix CO_2, grow fast and directly synthesize specific products, cyanobacteria and algae have become excellent candidates for building autotrophic cell factories to produce

renewable surrogate fuels and chemicals. With a large pool of genome sequences and improved genetic tools being available, application of synthetic biology in these photosynthetic microorganisms are highly desirable. In recent years, exciting results have been achieved not only in understanding of the fundamental molecular mechanisms but also in producing various products of interest, such as biofuels and chemicals, utilizing cyanobacteria and algae as the production platforms. Nevertheless, synthetic biology in cyanobacteria and algae is still in its infancy and synthetic biologists are facing great challenges and opportunities in addressing various issues, such as improving the tools for genetic manipulation, enhancing light harvesting, increasing CO_2 fixation efficiency and overcoming intracellular oxidative stress. Systematic approaches, such as functional genomics and metabolic modeling, may also diversify the genetic tools and help the metabolic network design. It is doubtless that synthetic biology would be indispensable for the future success in applying cyanobacteria and algae for various biotechnological purposes.

The contributions of this dissertation include:

1. Cyanobacterium *Synechocystis* sp. PCC6803 was engineered to produce (*S*)- and (*R*)-3-hydroxybutyrate (3HB) directly from light and CO_2.
2. Integration of the thioesterase-encoding gene *tesB* into the *Synechocystis* chromosome as well as screening of the thiolase and acetoacetyl-CoA reductase isoenzymes from different microbial sources to drive the 3HB biosynthesis from central metabolite acetyl-CoA. Isoenzymes from *Ralstonia eutropha* H16 were proven bearing highest enzyme activities when expressed in *Synechocystis*.

3. Analysis of the dynamics of the intermediate metabolite acetyl coenzyme A and the concentrations of essential nutrients, including nitrate and phosphate, in the culture media. It was found that the phosphate consumption during the photoautotrophic growth and the concomitant elevated acetyl-CoA pool acted as a key driving force for 3HB biosynthesis.
4. Characterization of a total of six promoters in *Synechocystis* and discovery that the light-independent P_{tac} promoter was the strongest in expressing 3HB biosynthesis genes in *Synechocystis*.
5. The acetoacetyl-CoA reductase activity was found to be the bottleneck for 3HB biosynthesis in *Synechocystis*, and was mitigated either by introducing a second copy of the *phaB* gene or by optimizing the upstream ribosome binding site.
6. The re-engineered *Synechocystis* strain R168 was able to produce (*R*)-3HB to a cumulative titer of 1600 mg/L, with a peak daily productivity of ~200 mg/L, using light and CO_2 as the sole energy and carbon sources, respectively, which is to date the highest productivity of hydroxyl-fatty acid in cyanobacteria
7. The integrative transformation efficiency in *Synechocystis* 6803 was increased by about two orders of magnitude after pre-methylation of the exogenous DNA. Effective expression of methylase genes, which could be achieved by optimizing the 5'-untranslated region, proved critical to efficient pre-methylation of the donor DNA.

A list of publications:

1. Wang, B., Wang, J., Zhang, W., Meldrum, D. R. (2012). Application of synthetic biology in cyanobacteria and algae. *Front. Microbiol.* 3, 344.

2. Wang, B., Pugh, S., Nielsen, D. R., Zhang, W., Meldrum, D. R. (2013). Engineering cyanobacteria for photosynthetic production of 3-hydroxybutyrate directly from CO_2. *Metab. Eng.* 16, 68-77.

3. Wang, B., Zhang, W., Meldrum, D. R. Pre-methylation of foreign DNA improves integrative transformation efficiency in *Synechocystis* sp. PCC 6803. (submitted)

4. Wang, B., Meldrum, D. R. Enhanced photosynthetic production of 3-hydroxybutyrate via gene expression fine-tuning in *Synechocystis*. (in preparation)

5. Wang, B., Pugh, S., Nielsen, D. R., Zhang, W., Meldrum, D. R. Photosynthetic production of 3-hydroxybutyrate directly from CO_2. 112th ASM General Meeting. San Francisco, California. (Poster | Jun. 2012)

6. Wang, B., Nielsen, D. R., Zhang, W., Meldrum, D. R. Photosynthetic conversion of CO_2 to building-blocks for renewable plastics. BIOT Division, 247th ACS National Meeting. Dallas, Texas. (Poster | Mar. 2014)

7. Wang, B., Zhang, W., Meldrum, D. R., Nielsen, D. R. Photosynthetic production of 3-hydroxybutyrate from carbon dioxide. Patent pending: PCT/US13/29997; WO/2013/172928.

CHAPTER 2

PHOTOSYNTHETIC PRODUCTION OF 3-HYDROXYBUTYRATE FROM LIGHT AND CARBON DIOXIDE

2.1 Introduction

Poly-β-hydroxybutyrate (PHB) represents the most common polyhydroxyalkanoates (PHAs) that can serve as renewable plastics (Madison and Huisman, 1999). It is naturally synthesized as energy and carbon stocks in many types of bacteria. Since decades ago, scientists have been fascinated in microbial production of PHB (Byrom, 1987; Madison and Huisman, 1999). Microbes have also been modified to produce PHAs with improved material properties, such as poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (Doi et al., 1990; Li et al., 2010), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (Aldor et al., 2002; Chen et al., 2011; Slater et al., 1992) and poly(3-hydroxybutyrate-co-lactate) (Jung et al., 2010). However, the monomer composition of the copolymers is hard to control, and consumption of carbohydrates as energy and carbon sources is relatively expensive for PHA production (Byrom, 1987). Additionally, to crack the cells for PHA extraction is energy-expensive (Chisti, 2007; Liu and Curtiss, 2009). These hurdles greatly limited the real world application of PHAs.

As an alternative approach, microorganisms have been engineered to produce hydroxyalkanoates (HAs) (Chen and Wu, 2005; Ren et al., 2010). Different from PHAs which accumulate inside cells as insoluble granules, HAs are the monomers and small molecules that may be secreted into the extracellular environment without damage to the

cells, which would significantly reduce the cost in product recovery. Once recovered, HAs can then not only be chemo-catalytically polymerized to produce PHB, but also be co-polymerized with other monomers to synthesize PHAs with a broader range of chemical and material properties (including adjustable molecular weight and improved purity; Tokiwa and Ugwu, 2007). In addition, (*R*)- or (*S*)-3HA can also serve as a precursor for many stereo-specific fine chemicals such as antibiotics, pheromones and amino acids (Chen and Wu, 2005; Ren et al., 2010; Tokiwa and Ugwu, 2007; Tseng et al., 2009).

3-Hydroxybutyrate (3HB) is a very common and important HA and the microbial production of both types of (*R*)- and (*S*)-3-hydroxybutyrate (3HB) has been demonstrated in the genetically engineered *Escherichia coli* (Gao et al., 2002; Lee and Lee, 2003; Lee et al., 2008; Liu et al., 2007; Tseng et al., 2009). Particularly, Liu et al. (2007) and Tseng et al. (2009) demonstrated that the *E. coli* thioesterase II (encoded by *tesB*) can cleave off the coenzyme A (CoA) from both (*R*)- and (*S*)-3-hydroxybutyryl-CoA, resulting in (*R*)- and (*S*)-3HB, respectively (**Figure 2**). The pathway from acetyl-CoA to (*R*)- or (*S*)-3-hydroxybutyryl-CoA involves two catalytic steps, including the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA by a thiolase and the reduction of acetoacetyl-CoA to (*R*)- or (*S*)-3-hydroxybutyryl-CoA by a acetoacetyl-CoA reductase (Liu et al., 2007; Tseng et al., 2009) (**Figure 2**). Notably, there are two types of acetoacetyl-CoA reductases. Gene *phaB* from *Ralstonia eutropha* H16 reduces acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA which eventually contributes to (*R*)-3HB biosynthesis, while *hbd* from *Clostridium acetobutylicum* ATCC 824 reduces

acetoacetyl-CoA to (*S*)-3-hydroxybutyryl-CoA which eventually leads to (*S*)-3HB production (**Figure 2**).

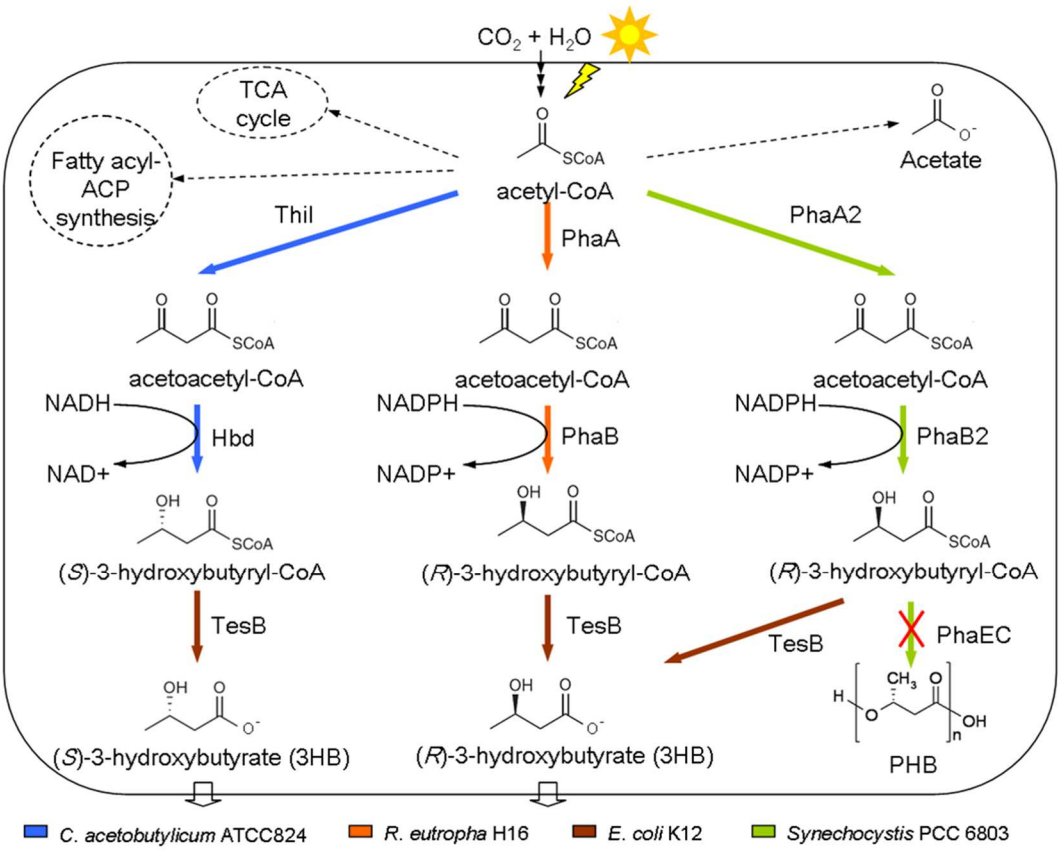

Figure 2 Schematic representation of (*S*)-3HB and (*R*)-3HB biosynthesis from CO_2 in engineered *Synechocystis*.

Cyanobacteria which can utilize sunlight and CO_2 as energy and carbon sources have become an attractive "microbial factory" to produce renewable biofuels and green chemicals (Ducat et al., 2011; Heidorn et al., 2011; Koksharova and Wolk, 2002; Robertson et al., 2011; Wang et al., 2012). In cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803), the native *slr1993* (*phaA2*)-*slr1994* (*phaB2*) operon

(Taroncher-Oldenberg et al., 2000) is responsible for the production of (*R*)-3-hydroxybutyryl-CoA which serves as the building-block for *in vivo* biosynthesis of PHB catalyzed by the PHB polymerase (encoded by *phaEC*; Hein et al., 1998; **Figure 2**). Despite efforts to enhance PHB biosynthesis through both genetic engineering and culture condition optimization strategies (Panda and Mallick, 2007; Takahashi et al., 1998; Tyo et al., 2009; Wu et al., 2001), PHB biosynthesis by cyanobacteria was a multi-stage cultivation process that involved nitrogen or phosphate starvation followed by supplementation with sugar or acetate, approaches that do not capitalize on the photosynthetic potential of cyanobacteria. Further study revealed that biosynthesis of PHB, particularly the expression and activation of the PHB synthase, involves complex regulations (Evaggelos et al., 2012; Schlebusch and Forchhammer, 2010). In this chapter, the 3HB biosynthesis pathways are constructed and optimized in cyanobacterium *Synechocystis* 6803, and the feasibility of photosynthetic production of (*R*)- and (*S*)-3HB from CO_2 in the engineered cyanobacteria is explored.

2.2 Materials and methods

2.2.1 Strains and culture conditions

All strains used in this chapter are listed in **Table 3**. *E. coli* XL1-Blue MRF' (Stratagene, La Jolla, CA) was used as a host to construct and store all recombinant plasmids. *Synechocystis* 6803 and its derivatives were grown in BG11 medium (Rippka et al., 1979) under a light intensity of 35 $\mu E/m^2/s$ unless otherwise specified. For solid agar plates, 10 mM TES (pH 8.2), 3 g/L thiosulfate and 1.5% agar were supplemented to BG11 before autoclaving.

2.2.2 Construction of integration vectors

All plasmids used in this chapter are listed in **Table 3** and all PCR primers are listed in **Table 4**. Plasmid pBS-SPtTeK was constructed by inserting the P_{tac}-*tesB-kan* expression cassette between two homologous fragments *SR*56 and *SL*56 which had been cloned into the SacI and KpnI restriction sites of the plasmid pBluescript II SK(+). Plasmid pBS-GCPU was constructed by inserting the *cat*-P_{tac} fragment between two homologous fragments *GTP* and *PHAU* which had been cloned into the *Sac*I and *Kpn*I restriction sites of the plasmid pBluescript II SK(+). Plasmid pBS-SCPTH was constructed by inserting the *cat*-P_{tac}-*thil-hbd* expression cassette between the two homologous fragments *SR*12 and *SL*12 which had been cloned into the *Sac*I and *Kpn*I restriction sites of the plasmid pBluescript II SK(+) (Stratagene, La Jolla, CA). Plasmid pBS-SCPAB was constructed by replacing the *thil-hbd* of pBS-SCPTH with *phaA-phaB*. Plasmid pBS-PHA was constructed by inserting two homologous fragments *PHA*1 and *PHA*2 between the *Sac*I and *Kpn*I restriction sites of the plasmid pBluescript II SK(+). Plasmid pBS-SPSK3 was constructed by inserting P_{tac}-*sacB-kan* between *PHA*1 and *PHA*2 of the plasmid pBS-PHA.

Synechocystis 6803 genomic DNA was purified by DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) and subsequently used as template for PCR amplification of *SR12* (*slr1495*) and *SL12* (*sll1397*) DNA fragments. *SR12* and *SL12* were recombined together by overlapping PCR and were inserted into the SacI and KpnI restriction sites of the plasmid pBluescript II SK(+) (Stratagene, La Jolla, CA) to construct pBS-SRSL. From the genomic DNA of *Clostridium acetobutylicum* ATCC 824, the *thil* gene was

PCR amplified using primers Th5 and Th8. The purified product was then again PCR amplified by primers Ptac and Th8 to construct P_{tac}-*thil*, wherein *thil* was under the control of the P_{tac} promoter. The gel-purified *Ptac-thil* product was then again PCR amplified using primers TAC5 and Th8, the product of which was purified and restriction digested before being inserted into the *Bam*HI and SalI sites of pBS-SRSL to construct pBS-SPT. Next, *hbd* of *C. acetobutylicum* was PCR amplified with primers HBD3 and HBD6. The resultant fragment was purified and restriction digested before being inserted between the *Nco*I and *Sal*I sites of pBS-SPT to construct pBS-SPTH. Two fragments of the *cat* (Cm^R) gene on pACYC 184 (New England Biolabs, Ipswich, MA) were amplified using primer pairs Cat3 and Cat4, Cat5 and Cat6, and then were recombined by overlapping PCR using primers Cat3 and Cat6 to remove the NcoI restriction site in the open reading frame. The *Nco*I-removed *cat* gene was then inserted between the *Pst*I and *Bam*HI sites of pBS-SRSL to construct pBS-SCat. The P_{tac}-*thil-hbd* fragment of pBS-SPTH was PCR amplified using primers TAC5 and HBD6 and then inserted between the *Bam*HI and SalI sites of pBS-SCat to construct pBS-SCPTH.

The P_{tac}-*thil* fragment from pBS-SCPTH was PCR amplified using primers TAC5 and primer Th10 and was used to replace the original P_{tac}-*thil* fragment of pBS-SCPTH between *Bam*HI and *Nco*I to construct pBS-SCPTH2. The *R. eutropha* H16 gene *phaB* was PCR amplified with primers PHAB11 and PHAB12 using pETphaAphaB (reconstructed based on the methods of Tseng *et al*. in constructing pET-P-P (Tseng et al., 2009)) as a template and was inserted between the *Mlu*I and *Hind*III sites of pBS-SCPTH2 to construct pBS-SCPTB. The gene *phaA* from *R. eutropha* H16 was PCR amplified using primers PHAA11 and PHAA12 with pETphaAphaB as template. The

purified product was then amplified using primers Ptac and primer PHAA12 to construct the P_{tac}-*phaA* fragment. P_{tac}-*phaA* was further PCR amplified using primers TAC5 and PHAA12 before being inserted between the *Bam*HI and *Mlu*I sites of pBS-SCPTB to construct pBS-SCPAB.

The DNA fragment containing *GTP* from *Synechocystis* 6803 was PCR amplified using primers GTP1 and GTP2 and was inserted between the *Sac*I and *Pst*I sites of pBS-SCat to construct pBS-SCG. The DNA fragment *PHAU* from *Synechocystis* 6803 was PCR amplified using primers PHAU1 and PHAU2 before being further PCR amplified using primers Ptac and PHAU2 to construct P_{tac}-*PHAU*. P_{tac}-*PHAU* was then amplified using primers TAC5 and PHAU2 and the product was inserted between the *Bam*HI and *Kpn*I sites of pBS-SCG to construct pBS-GCPU.

The DNA fragments *SR56* and *SL56* were PCR amplified using primer pairs SR5 and SR6 and SL5 and SL6 with *Synechocystis* 6803 genomic DNA as template. Fragments *SR56* and *SL56* were recombined together by overlapping PCR before being inserted into the *Sac*I and *Xho*I restriction sites of the plasmid pBluescript II SK(+) to construct pBS-S2. pBS-S2 was digested with MluI and SalI before being ligated with *kan* (Kan^R) which was amplified from pET-30a(+) (Novagen, Madison, WI) using primers Kan1 and Kan2 to construct pBS-S2K. The *E. coli* gene *tesB* was amplified with primers TESB1 and primer TESB2 using the *E. coli* XL1-Blue MRF' genomic DNA as template. The P_{tac} promoter was PCR amplified with primers TAC11 and TACTESB1 using pBS-SPTH as a template. The P_{tac} and *tesB* containing PCR products were then recombined by overlapping PCR using primers TAC11 and TESB2 to construct the fragment P_{tac}-*tesB*.

P_{tac}-*tesB* was digested with *Bgl*II and *Hind*III before being inserted between the corresponding sites of pBS-S2K to construct pBS-SPtTeK.

The DNA fragment *PpasD56* was PCR amplified from the *Synechocystis* 6803 genomic DNA using primers PpsaD5 and PpsaD6. The *thil* gene was PCR amplified from *C. acetobutylicum* ATCC 824 genomic DNA using primers Th1 and Th2. The PCR product was recombined with *PpsaD56* by overlapping PCR using primers PpsaD5 and Th2 and the resultant P_{psaD}-*thil* product was inserted between the *Bam*HI and MluI sites of pBS-S2K to construct pBS-SPTK. *Ptac* was amplified from pBS-SPTH using primers TAC5 and TAC-PTB3 and then inserted between the *Bam*HI and *Nde*I sites of pBS-SPTK to construct pBS-SPtK. The *sacB* gene was PCR amplified using primers SACB8 and SACB9 using *B. subtillus* genomic DNA as template. The product was restriction digested and inserted between the NdeI and MluI sites of the pBS-SPtK plasmid to construct pBS-SPSK2. DNA fragments *PHA1* and *PHA2* were each PCR amplified from *Synechocystis* 6803 genomic DNA using primer pairs PHA11 and PHA12 and PHA21 and PHA22. Fragments *PHA1* and *PHA2* were then recombined together by overlapping PCR using primers PHA11 and PHA22 to construct the DNA fragment *PHA*. *PHA* was then inserted between the *Xho*I and *Sac*I sites of pBS-S2 to construct pBS-PHA. The P_{tac}-*sacB-kan* fragment was removed from pBS-SPSK2 by digestion with *Bam*HI and *Sal*I and then inserted between the corresponding sites of pBS-PHA to construct pBS-SPSK3.

2.2.3 Modification of *Synechocystis* genome

Synechocystis strains were grown to an OD_{730} of 0.2-0.4, at which time point 0.5 mL culture was pelleted by centrifugation at 2700×g for 10 min at room temperature. The cell

pellet was re-suspended in 50 µl fresh BG11 medium to which approximately 2 µg of the integration vector was added and mixed. The mixture was incubated at 30 ºC under light (~25 µE/m^2/s) for 5 h before being plated on BG11 solid agar plates with appropriate antibiotics supplements, 10 ng/µl kanamycin or 5 ng/µl chloramphenicol. The plates were placed at 30 ºC under light and colonies could be seen within two weeks. Individual colonies were then isolated and re-streaked on BG11 solid agar plates with appropriate antibiotics for additional one to two weeks to achieve full chromosome segregation, as was verified by colony PCR.

Alternatively, markerless modification of the *Synechocystis* genome was conducted using the method described previously (Liu and Curtiss, 2009) with minor modifications. Briefly, fragment *Ptac-sacB-kan* was inserted into the neutral site of *Synechocystis* 6803 using a marker modification method according to the aforementioned method. After confirming that the resultant strain was genotypically pure as verified using colony PCR, the strain were grown in BG11 medium to an OD_{730} of 0.2-0.4, when cells were centrifuged at 2700g for 10 min at room temperature and was resuspended to OD_{730} of 4.0 by 50 µl BG11. About 2 µg of integration vector pBS-PHA was added and mixed well with the cells. The mixture was incubated at 30 ºC under light (25 µE/m^2/s) for 5 h before being transferred into 25 mL BG11 medium in a 50 mL flask. Cells were then further cultivated for 4-5 days after which about 1.3×10^8 cells (assuming OD_{730} of 0.6 equals to 10^8 cells/mL; Liu et al., 2011) were spread onto a BG11 plate containing 4.5% (w/v) sucrose for counter-selection. The plates were incubated at 30 ºC under light for one or two weeks before colonies appeared. Individual colonies were then re-streaked on

fresh BG11 plates with 4.5% sucrose for additional one to two weeks until full chromosome segregation was achieved, as verified by colony PCR.

Synechocystis strain TESB was constructed by transforming *Synechocystis* 6803 with plasmid pBS-SPtTeK to allow the expression cassette to be integrated at the S2 neutral locus of the chromosome via homologous recombination. Similarly, *Synechocystis* strain TPU3 was constructed by transforming *Synechocystis* TESB with plasmid pBS-GCPU, resulting in an extra P_{tac} promoter placed just upstream of the codon region of the native *phaA2-phaB2* operon (Site 4). HB5 and TAB1 were constructed by transforming the strain TESB with plasmids pBS-SCPTH and pBS-SCPAB, respectively, after which the *thil* and *hbd* (as a pair) and *phaA* and *phaB* (as a pair) were integrated into the S1 neutral locus of the *Synechocystis* chromosome, respectively. *Synechocystis* strain SPA:ΔphaEC was constructed using a markerless modification method (Liu and Curtiss, 2009). Firstly, the intermediate *Synechocystis* strain SPA:SPSK3 was constructed by transforming the *Synechocystis* SPA (stored in lab; **Table 3**) using plasmid pBS-SPSK3. Then, the resultant genotypically pure strain *Synechocystis* SPA:SPSK3 was transformed with plasmid pBS-PHA before being subjected to sucrose counter-selection to screen out the strain SPA:ΔphaEC. Strain SPA:ΔphaEC was then transformed with plasmid pBS-SPtTeK to obtain strain TESBd, which was further transformed with plasmids pBS-GCPU, pBS-SCPTH and pBS-SCPAB, respectively, to construct strains TPUd, HBd and TABd.

Table 3 Strains and plasmids used in Chapter 2.

	Genotype*	References
Strains		
E. coli XL1-Blue MRF'	*Δ*(*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA*1 *gyrA96 relA1 lac* [*F´ proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]	Stratagene
Synechocystis		
PCC6803	Wild-type	ATCC
TESB	*Ptac-tesB-Kan*^{R} integrated at S2 site in *Synechocystis* 6803	This study
TPU3	*Ptac-tesB-Kan*^{R} integrated at S2 site and *Cm*^{R}*-Ptac* integrated at S4 site	This study
HB5	*Ptac-tesB-Kan*^{R} integrated at S2 site and *Cm*^{R}*-Ptac-thil-hbd* integrated at S1 site	This study
TAB1	*Ptac-tesB-Kan*^{R} integrated at S2 site and *Cm*^{R}*-Ptac-phaA-phaB* integrated at S1 site	This study
SPA	*Ptac-adhe2* integrated at S2 site	In the lab
SPA:SPSK3	*Ptac-adhe2* integrated at S2 site and *Ptac-sacB-Kan*^{R} integrated at S3 site	This study
SPA:ΔphaEC	*Ptac-adhe2* integrated at S2 site, *phaE* and *phaC* deleted at S3 site	This study
TESBd	*phaE* and *phaC* deleted at S3 site, *Ptac-tesB-Kan*^{R} integrated at S2 site	This study
TPUd	*phaE* and *phaC* deleted at S3 site, *Ptac-tesB-Kan*^{R} integrated at S2 site, *Cm*^{R}*-Ptac* integrated at S4 site	This study
HBd	*phaE* and *phaC* deleted at S3 site, *Ptac-tesB-Kan*^{R} integrated at S2 site, *Cm*^{R}*-Ptac-thil-hbd* integrated at S4 site	This study
TABd	*phaE* and *phaC* deleted at S3 site, *Ptac-tesB-Kan*^{R} integrated at S2 site, *Cm*^{R}*-Ptac-phaA-phaB* integrated at S4 site	This study
Plasmids		
pBluescript II SK(+)	Amp^{R}, pUC ori, f1(+) ori	Stratagene
pBS-SCPTH	*Ptac-thil-hbd* integrated between the *Nco*I and *Sal*I sites of pBS-SPT	This study
pBS-SCPTH2	MluI site added between *thil* and *hbd* of pBS-SCPTH	
pBS-SCPAB	*Ptac-phaA* inserted between the *BamH*I and *Mlu*I sites of pBS-SCPTB	This study
pBS-GCPU	*Ptac-PHAU* inserted between the *BamH*I and *Kpn*I sites of pBS-SCG	This study
pBS-SPtTeK	*Ptac-tesB* integrated between *Bgl*II and *Hind*III site of pBS-S2K	This study
pBS-PHA	*PHA* inserted between the *Xho*I and *Sac*I sites of pBS-S2	This study
pBS-SPSK3	*Ptac-sacB-kan* of pBS-SPSK2 inserted between *BamH*I and *Sal*I of pBS-PHA	This study

*S1, the site on the genome of *Synechocystis* 6803 between *slr1495* and *sll1397*; S2, the site between *slr1362* and *sll1274*; S3, the site between *slr1828* and *sll1736*; and S4, the site between *slr1992* and *phaA2*.

Table 4 Primers used in Chapter 2.

Name		Targets
Primers (5' to 3') used for DNA recombination		
SR1	TCCGAGCTCGACGATTTACCGCTCAAAG	*SR12*
SR2	GTCGACCCGGATCCCGCTGCAGCACAATTGCCTCATTGCCC	*SR12*
SL1	CTGCAGCGGGATCCGGGTCGACTGGAGATTCAGTGCGAATTG	*SL12*
SL2	GGGGTACCCAATCCAGGCTTGTATTTATG	*SL12*
Th5	CACACAAGGAGGATATACATATGAGAGATGTAGTAATAGTAAGTGCTG	*thil*
Th8	ACGGTCGACCATGCCATGGTTAGTCTCTTTCAACTACGAGAGC	*thil*
Ptac	GAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGC GGATAACAATTTCACACAAGGAGGATATACAT	P_{tac}
TAC5	ACGGATCCGAGCTGTTGACAATTAATCATC	P_{tac}
HBD3	CATGCCATGGGGAGGTCTGTTTAATGAAAAAGG	*hbd*
HBD6	CTTGTCGACCCTCTAAAATTATATTATATATAAAGAATCC	*hbd*
Cat3	TTAGGATCCATACCTGTGACGGAAGATCAC	*cat* (Cm^R)
Cat4	CGTATAATATTTGCCAATGGTGAAAACG	*cat* (Cm^R)
Cat5	CGTTTTCACCATTGGCAAATATTATACG	*cat* (Cm^R)
Cat6	CAAGCTGCAGCACACTGCTTCCGGTAGTC	*cat* (Cm^R)
Th10	CATGCCATGGACTGACGCGTTAGTCTCTTTCAACTACGAGAGC	*thil*
PHAA11	CACACAAGGAGGATATACATATGACTGACGTTGTCATCG	*phaA*
PHAA12	GACACGCGTCTTATTTGCGCTCGACTG	*phaA*
PHAB11	GACACGCGTCAAGGAGTGGACATGACTC	*phaB*
PHAB12	ACGAAGCTTTTAGCCCATATGCAGGCCGCCGTTG	*phaB*
GTP1	GTAGAGCTCGGCCCTAATCGTCCATAATG	*GTP*
GTP2	CAAGCTGCAGTAACTCCGCCTCCAGCTC	*GTP*
PHAU1	CACACAAGGAGGATATACATATGGCCGCCCATCCCAAC	*PHAU*
PHAU2	AAGGTACCCTGCGAATACCTTCATCACTG	*PHAU*
SR5	GACCTCGAGGTTTGCTATGAATGCTTCCGC	*SR56*
SR6	GTCGACCCACGCGTCGGGATCCGTATTTATCCCCCTAAATCCC	*SR56*
SL5	ACGGATCCCGACGCGTGGGTCGACCTAGCCTTGCACTTTTCAAACAC	*SL56*
SL6	TGGGAGCTCGGACACTTACCTTTGTAATCGC	*SL56*
Kan1	AAACGCGTTCAAGCTTAGGGGTGTTATGAGCCATATTC	*kan* (Kan^R)
Kan2	ACGGTCGACCAGGTGGCACTTTTCGGGG	*kan* (Kan^R)
TAC11	GAAGATCTGAGCTGTTGACAATTAATCATC	P_{tac}
TACTESB1	GTAAATTTTTTAGCGCCTGACTCATATGTATATCCTCCTTGTGTG	P_{tac}
TESB1	ATATGAGTCAGGCGCTAAAAAATTTACTG	*tesB*

Name		Targets
TESB2	TCAAAGCTTACGGCGAGAATGCCTATAAC	*tesB*
PpsaD5	ACGGATCCGATTACACCAACCTACTGGGC	P_{psaD}
PpsaD6	TACTACATCTCTCATAGGGATGAAAATGGAATT	P_{psaD}
Th1	TTTCATCCCTATGAGAGATGTAGTAATAGTAAGTGCTG	*thil*
Th2	CCTAAGCTTGAACGCGTTTAGTCTCTTTCAACTACGAGAGC	*thil*
TAC-PTB3	CATGATAATTTCATTAAAACTCTTAATCATATGTATATCCTCCTTGTGTG	P_{tac}
SACB8	AAACGCGTTTGCGTTTTTATTTGTTAACTG	*sacB*
SACB9	GGAATTCCATATGAACATCAAAAAGTTTGCAAAAC	*sacB*
PHA11	GACCTCGAGGATAAAAGAGGCAAAGGGAAAC	*PHA1*
PHA12	TCAGTCGACCTAAGCTTTCGGATCCCTGGCCGCTACCTGAATTATG	*PHA1*
PHA21	AGGGATCCGAAAGCTTAGGTCGACTGATGAAAATTTTGCTGGAATACAT TAG	*PHA2*
PHA22	TGGGAGCTCCCTGGGCCCTTTACGGTTTTC	*PHA2*
Primers (5' to 3') used for RT-qPCR		
16S-F2	CCACGCCTAGTATCCATCGT	*Synechocystis* 16S
16S-R2	TGTAGCGGTGAAATGCGTAG	*Synechocystis* 16S
TESB3	ACGCTTCTGATCTTAACTTCCTG	*tesB*
TESB4	TCAAAGCTTTTTAATTGTGATTACGCATCAC	*tesB*
PHAAq3	AAAATATCAACTCAAACCCTTG	*phaA2*
PHAAq4	GTTGTTGCAAGGCATAGAGTAG	*phaA2*
FABGq1	GGGGTAATTGGCATGATG	*fabG*
FABGq2	CGGTGACATAGCTACTGGCTAC	*fabG*

2.2.4 3HB production by the engineered *Synechocystis*

Synechocystis strains were inoculated in 50 mL flasks, each containing 10 mL BG11 (10 mM TES-NaOH), to an initial OD_{730} of 1.5. Then cells were incubated in a shaking bed (150 rpm) at 30 ºC with light intensity of 35 μE/m^2/s. Every 24 h, 0.5 mL 1.0M $NaHCO_3$ was added to each culture and the pH of the culture medium was adjusted to 7.5 by 10 N HCl. Cells were grown for 5 days before cultures were sampled for analysis of

the OD_{730} and the 3HB titers. All culture experiments were conducted in triplicate for each strain.

2.2.5 Photosynthetic production of 3HB from CO_2

Synechocystis was inoculated into a 125 mL flask containing 75 mL autoclaved BG11 (10 mM TES-NaOH) medium to an initial OD_{730} of 0.2. The culture was placed at 30 ºC with continuous illumination of 120 µE/m^2/s and was bubbled with ambient air. The aeration rate was initially set as 75 mL/min. When the culture OD_{730} surpassed about 0.6, the aeration rate was then increased to 250 mL/min. Daily, 1 mL of culture was sampled and 1 mL 5-fold concentrated sterilized BG11 medium was added back into the culture until day 18. After day 18, 1 mL of culture was sampled but no BG11 medium was added back into the culture. The experiments were conducted in duplicates.

2.2.6 Gene expression analysis by RT-qPCR

Cells were grown as described in Section 2.2.4. Approximately 1.67×10^8 *Synechocystis* cells (assuming OD_{730} of 0.6 equals to 10^8 cells/mL; Liu et al., 2011) were collected by centrifugation at 17,000*g*, 4ºC for 1 min. The supernatant was discarded and the cell pellet was used for RNA extraction and RT-qPCR using methods described previously (Gao et al., 2011).

2.2.7 Enzyme activity assay

Cells were grown as described in section 2.2.4. Approximately 3.3×10^9 cells were collected by centrifugation at 5000*g* at 4ºC for 10 min. The crude cell lysate was prepared via sonication (100 cycles of 3-s-on/ 3-s-off) on ice. Cell debris was removed by

centrifugation at 17,000*g* at 4°C for 10 min. Thiolase and acetoacetyl-CoA reductase activities were determined according to previous protocols (Inui et al., 2008). Thioesterase activity was also determined using a previously established method (Zheng et al., 2004).

2.2.8 Measurement of intracellular acetyl-CoA

Synechocystis 6803 was grown to an OD_{730} of 1.0-2.0 as described in Section 2.2.4 before the cells were collected by centrifugation. Cell pellets were re-suspended in 14 mL BG11 containing 10 mM TES-NaOH (pH8.0) and 50 mM $NaHCO_3$ in 50 mL-flasks with an initial cell density of OD_{730} of 2.0. For the phosphate-starvation control culture, *Synechocystis* cells were washed twice with 12 mL of BG11-P (medium deprived of phosphate from the regular BG11) before resuspension with 10 mL of BG11-P containing 10 mM TES-NaOH (pH8.0) and 50 mM $NaHCO_3$ in 50 mL-flasks with an initial cell density of OD_{730} of 2.0. The initial pH of all culture medium was adjusted to 7.5 by 10 N HCl. Once daily, 100 µl of the culture was sampled for cell density analysis, after which 0.5 mL of 1 M $NaHCO_3$ was added into the culture and the pH of the culture medium was adjusted to 7.5 by 10 N HCl. On days 0.5 and day 3.5, 1.67×10^9 cells were sampled from the regular BG11 culture. On day 1.5, 1.67×10^9 cells were sampled from the BG11-P culture. The cells were then collected by centrifuging at 12000*g* at 4 °C for 5 min before the cell pellets were resuspended by 450 µl of ice-bathed 6 N perchloric acid and then processed by sonication (100 cycles of 3-s-on/ 3-s-off) on ice. The acetyl-CoA concentrations in the cell lysates were then analyzed using an Acetyl-CoA Assay Kit (Sigma-Aldrich, St. Louis, MO).

2.2.9 Anion concentration measurement

Synechocystis strain TAB1 was inoculated in 50 mL flasks, each containing 10 mL BG11 (10 mM TES-NaOH), to an initial OD_{730} of 2.0. Then cells were incubated in a shaking bed (150 rpm) at 30 ^{o}C with light intensity of 35 $\mu E/m^2/s$. Every 24 h, 0.5 mL of culture was sampled and replaced with 0.5 mL of 1.0M $NaHCO_3$ after which the pH of the culture medium was adjusted to 7.5 by 10 N HCl. The sampled culture was centrifuged at 17000*g* at for 1 min and the supernatant was used for anion assay. The concentrations of the anions in the sample were measured using an ICS-1000 system (Dionex Corporation, Sunnyvale, CA) equipped with an AS18 column. 32 mM NaOH was used as the eluent and the flow rate was set as 1.0 mL/min for 20 min during the anion assay for each sample.

2.2.10 Product quantification

Standard solutions of 3HB were prepared in water using (±)-3-Hydroxybutyric acid sodium salt. Samples of the culture medium were centrifuged at 17,000*g* for 2 min at room temperature and the supernatant was collected for analysis of products on an 1100 series HPLC equipped with a refractive index detector (Agilent, Santa Clara, CA). Separation of metabolites was achieved using an Aminex HPX-87H anion-exchange column (Bio Rad Laboratories, Hercules, CA). The mobile phase consisted of 5 mM H_2SO_4 at an initial flow rate of 0.55 mL/min before immediately and linearly increasing to a final flow rate of 0.8 mL/min over 12 min, followed by an 8 min hold. The column temperature was maintained at 35°C throughout (Tseng et al., 2009).

2.3 Results

2.3.1 Construction of 3HB-producing strains and comparison of the growth

According to the strategy illustrated in **Figure 2**, four rounds of genetic modification have been made in *Synechocystis* to explore the capability of photosynthetic production of (*S*)- and (*R*)-3HB (**Figure 3**). Promoter P_{tac} was found to be a strong promoter in *Synechocystis* 6803 (Marraccini,et al., 1993), so it was used to express all the 3HB biosynthesis genes in this study. The genotypic purity of each strain was confirmed by colony PCR in all cases. The constructed strains remained genetically stable for at least 6 months (**Figure 4**). No change of the cell shape was found under a microscope. The growth of the engineered strains and the wild-type *Synechocystis* showed marginal difference based on the measurement of OD_{730} (**Figure 5**), indicating that the enrollment of the 3HB biosynthesis pathway does not cause a significant burden to the cell metabolism in *Synechocystis*.

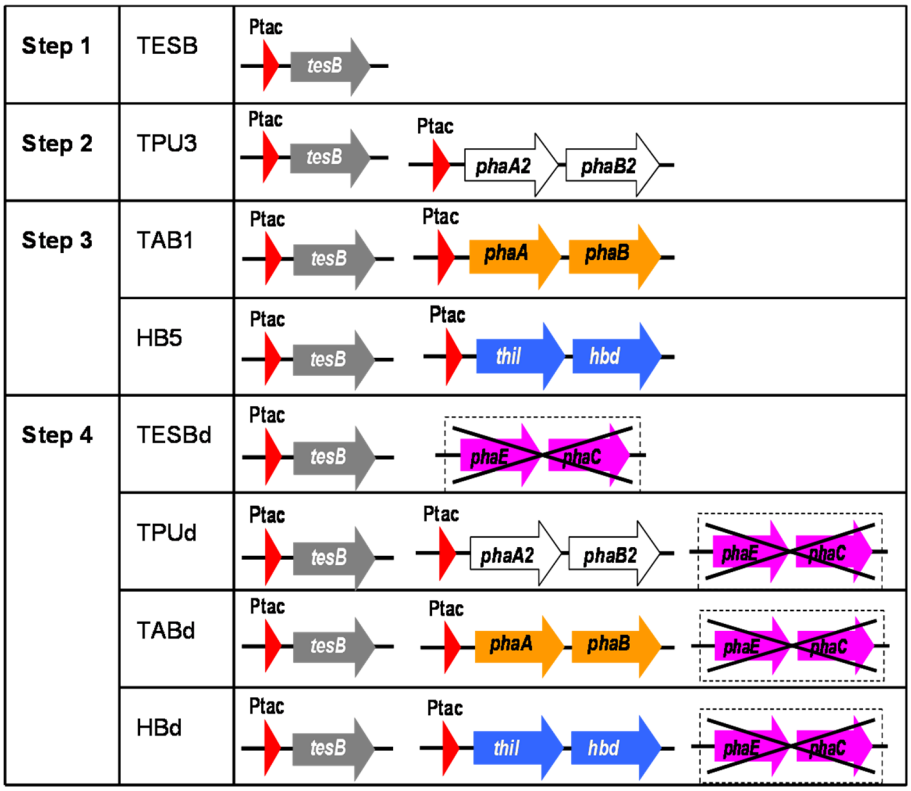

Figure 3 Schematic representation of the modification of *Synechocystis* chromosome for 3HB production.

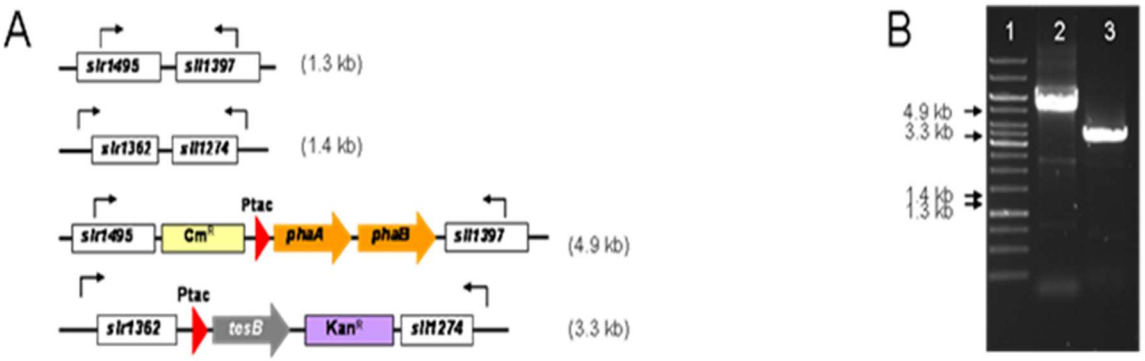

Figure 4 Colony PCR for Synechocystis TABd after 6 months of continuous photoautotrophic cultivation. (A) Schematic representation of the targeted chromosomal regions and the expected PCR products for strain TABd and wild-type *Synechocystis*. (B) Agarose gel electrophoresis result for the colony PCR products of strain TABd that was

continuously cultivated for six months. Lane 1, DNA marker; lane 2, colony PCR product that flanks the region between genes *slr1495* and *sll1397* of the chromosome of *Synechocystis* strain TABd; lane 3, colony PCR product that flanks the region between *slr1495* and *sll1397* of *Synechocystis* TABd.

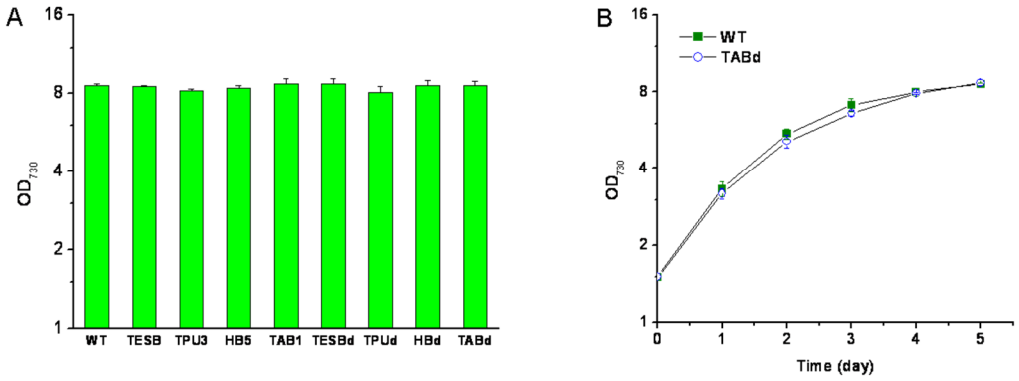

Figure 5 Comparison of the growth of different Synechocystis strains. (A) Cell density of different *Synechocystis* strains after 5 days of photosynthetic cultivation. (B) Growth curves of the wild-type strain *Synechocystis* 6803 (solid squares) and the engineered 3HB-producing strain TABd (open circles).

2.3.2 Production of 3HB by the engineered *Synechocystis*

As wild-type *Synechocystis* 6803 naturally accumulates PHB, the simplest way to realize the production of 3HB in this species is to express a thioesterase responsible in cleaving the coenzyme A off the intermediate product (*R*)-3-hydroxybutyryl-CoA (**Figure 2**). The 3HB in the extracellular medium of *Synechocystis* 6803 was hardly detectable (**Figure 6A**). After the *E. coli* thioesterase II encoded by *tesB* gene has been

expressed under the strong promoter P_{tac} (Marraccini,et al., 1993) in the strain *Synechocystis* TESB, extracellular 3HB was accumulated to up to 20.6 mg/L in the culture under photoautotrophic growth conditions. Interestingly, expression of *E. coli tesB* also resulted in a dramatic increase of acetate production, up to 30.6 mg/L, by the engineered *Synechocystis* relative to the wild-type (**Figure 6A**), consistent with the previous finding that TesB is also able to cleave coenzyme A off acetyl-CoA when expressed in *E. coli* (Liu et al., 2007; Nie et al., 2008). However, TesB prefers medium- to long-chain acyl-CoA as substrates (Nie et al., 2008), and the generated 3HB to acetate ratio in the engineered 3HB-producing *E. coli* strains turned out to be more than 10 (Liu et al., 2007), a sharp contrast to that of the case here. Thus, it can be speculated that the remarkable low 3HB to acetate production ratio in *Synechocystis* TESB was probably due to insufficient conversion of acetyl-CoA to 3-hydroxybutyryl-CoA.

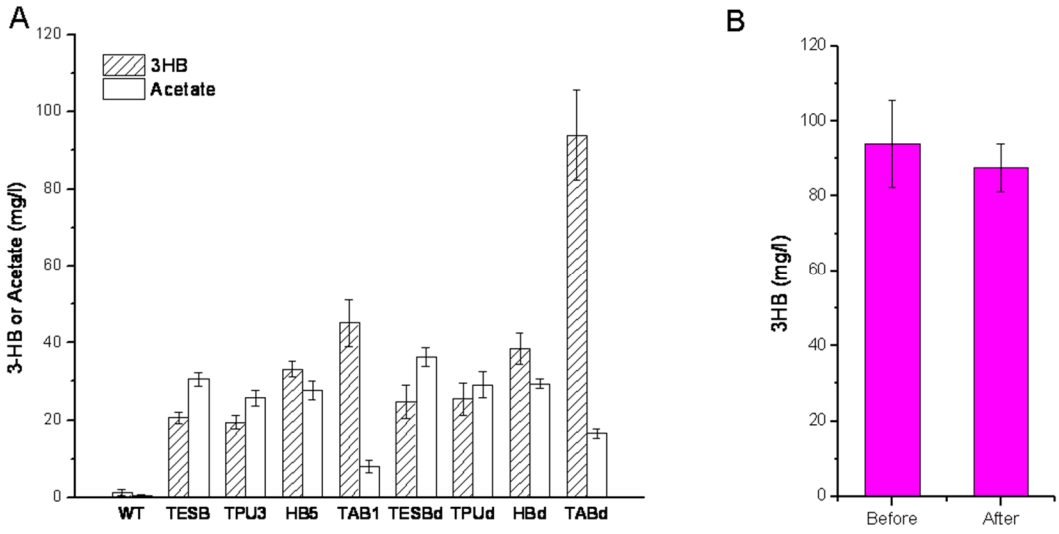

Figure 6 3HB and acetate production by different *Synechocystis* strains. (A) 3HB and acetate accumulation in the extracellular culture medium of different *Synechocystis*

strains. (B) 3HB productivity of *Synechocystis* TABd at the beginning and the end of a period of 6-month photoautotrophic cultivation.

The expression of the native *phaA2-phaB2* operon, which is responsible in driving the metabolic flux from acetyl-CoA to 3-hydroxybutyryl-CoA, was then enhanced by placing an extra promoter P_{tac} just upstream of the codon region of the operon, resulting in the strain TPU3 (**Figure 3**). Although the transcription of *phaA2* and *phaB2* was increased by 6- and 118-fold, respectively (**Table 5**), little difference was observed regarding the production of 3HB and acetate (**Figure 6A**).

Table 5 RT-qPCR for transcription of *tesB, phaA2* and *phaB2*.[a]

Gene	**ΔC_T**	
	WT	**TPU3**
tesB	n.d.	10.87±0.07
phaA2	20.85±0.06	18.17±0.16
phaB2	19.30±1.07	12.42±0.28

[a] The relative abundance of different mRNA molecules could be estimated using $2^{-\Delta\Delta C_T}$; the higher the ΔC_T value is, the less abundant is the corresponding mRNA (Livak and Schmittgen, 2001). For example, the difference of the RNA abundance of the *phaB2* gene is 2^(19.30-12.42), i.e., 118-fold. "n.d." specifies "not detectable".

This led to the next step which was to further express other enzymes that could more efficiently divert the metabolic flux from the acetyl-CoA pool to the 3HB pathway in *Synechocystis*. Genes *phaA* and *phaB* (as a pair) from *R. eutropha* H16 (Pohlmann et al.,

2006) and *thil* and *hbd* (as a pair) from *C. acetobutylicum* ATCC 824 (Inui et al., 2008) were selected (**Figure 2**), as these two sets of genes have shown high efficiency in driving the acetyl-CoA to the intermediate (*R*)- or (*S*)-3-hydroxybutyryl-CoA, respectively. As shown in **Figure 6A**, co-expression of *tesB* with *thil* and *hbd* (strain HB5) resulted in 33.2 mg/L 3HB production, a 1.6-fold increase relative to that of strain TESB. In the culture medium of strain TAB1 which co-expressed *tesB* with *phaA* and *phaB*, the production of 3HB was boosted to 45.1 mg/L (2.2-fold higher) while the acetate accumulation was significantly decreased to 8.0 mg/L (3.8-fold lower; **Figure 6A**). This suggested that *phaA* and *phaB* were able to efficiently increase the metabolic flux from acetyl-CoA to (*R*)-3-hydroxybutyryl-CoA and thus were able to enhance the 3HB production.

PHB biosynthesis is a competing pathway for 3HB production as the PHB polymerase PhaEC consumes (*R*)-3-hydroxybutyryl-CoA (Hein et al., 1998) which directly cripples (*R*)-3HB production, and eventually decreases acetoacetyl-CoA and acetyl-CoA which might also hamper the (*S*)-3HB biosynthesis (**Figure 2**). Next, strains were constructed with the *phaE-phaC* operon being deleted from the *Synechocystis* chromosomes (**Figure 3**; **Table 3**). As shown in **Figure 6A**, all *phaE-phaC*-deleted strains showed increased 3HB production. Particularly, strain TABd exhibited the most significant increase of the 3HB biosynthesis with a production titer of 93.9 mg/L, 2.1-fold higher than that of TAB1. Notably, after *Synechocystis* TABd has been consecutively cultivated for 6 months (during which period one drop of previously-grown culture was re-inoculated into 10 mL of fresh BG11 medium every 2 weeks), the 3HB productivity of *Synechocystis* TABd did not decline (**Figure 6B**). Since little impairment of the cell growth was observed after

Synechocystis was genetically engineered (**Figure 5**), there is probably little pressure for the engineered *Synechocystis* cells to decrease the 3HB productivity.

2.3.3 Production of 3HB from atmospheric CO_2

The ability of *Synechocystis* strain TABd to photosynthetically produce 3HB using CO_2 as the sole carbon source was then investigated by continuously aerating cultures with ambient air. Although the atmospheric CO_2 (400 ppm) is low, the aeration applied in these experiments supplied 5.5 mmol CO_2 into the 75 mL culture daily, which equates to one-time supply of 74 mM (CO_2) day^{-1}. As reported by Summerfield and Sherman (2008) that photoautotrophic growth of *Synechocystis* alkalizes the medium, the pH of the culture medium of *Synechocystis* TABd was 10-11 during the photosynthetic cultivation, simplifying the capture of CO_2 from the ambient air into the medium. The result showed that during the first 7 days the cell growth underwent a dramatic increase, after which the cell growth quickly slowed down. In contrast, the 3HB production came through a lag phase of nearly one week before 3HB production and then quickly accelerated (**Figure 7**). Eventually, 3HB was able to accumulate to a titer of 446.5 mg/L after 18 days and to a titer of 533.4 mg/L after 21 days of continuous cultivation (**Figure 7**). It should be noted that at this point there was no indication that 3HB production would stop; however, the experiment was electively stopped after 21 days. The relationship here between biomass growth and 3HB production rate is consistent with the results of the former experiments in which $NaHCO_3$ was used as the sole carbon source (**Figure 8A**).

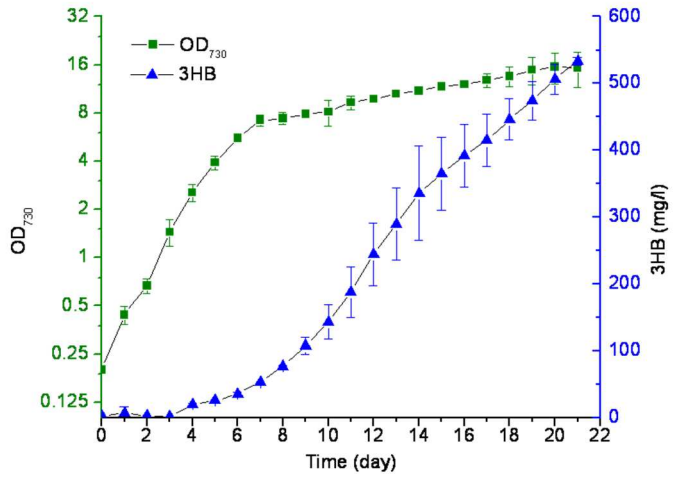

Figure 7 Continuous production of 3HB from atmospheric CO_2.

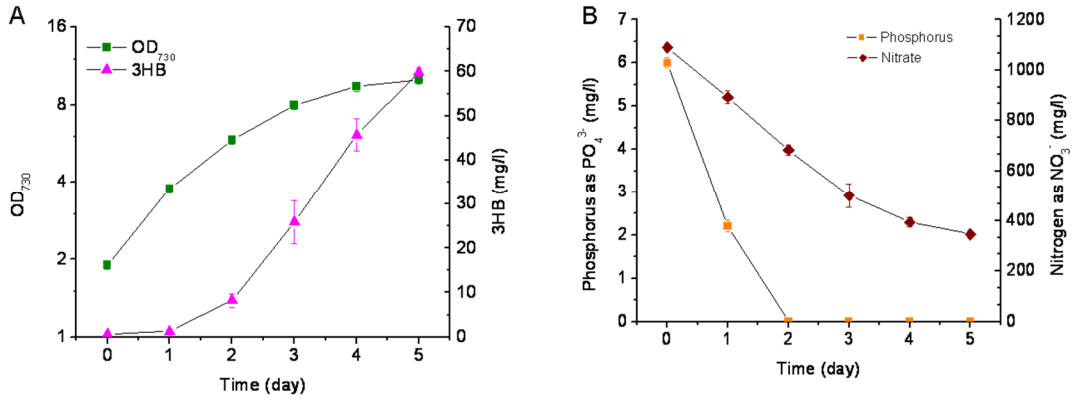

Figure 8 Cell growth, production of 3HB and consumption of nitrate and phosphate in the culture of strain TAB1. (A) Cell growth and production of 3HB. (B) Concentrations of nitrate and phosphate in the culture medium. Starting from day 2, the phosphate concentrations were below the detection limit (2.0 mg/L).

2.3.4 Enzyme activities for 3HB synthesis in the engineered *Synechocystis*

In the wild-type *Synechocystis* 6803, no thiolase or acetoacetyl-CoA reductase enzyme activity was detectable (data not shown), indicating that the expression levels of the corresponding *phaA2* and *phaB2* genes were too low to detect. No thioesterase activity was detectable in the cell lysate of the *Synechocystis* 6803 either. In contrast, in the cell lysate of all engineered *Synechocystis* strains that harbored *tesB*, the thioesterase activity was detected with a value of 0.084±0.021 U when using butyryl-CoA as a substrate (**Table 6**). However, the thioesterase activity increased to 0.484±0.044 U, 6-fold higher, when using decanoyl-CoA as a substrate. This is consistent with the previous report that TesB had higher activities on medium-chain-length acyl-CoA compared to short-chain-length acyl-CoA (Nie et al., 2008). After the transcription of *phaA2* and *phaB2* was enhanced by an extra P_{tac} promoter (**Table 5**), the thiolase activity of PhaA2 became detectable with a value of 1.14±0.14 U (μmol/min/mL cell extract), while the acetoacetyl-CoA reductase activity of PhaB2 was still not detectable, implicating that the expression of PhaB2 might be regulated post-transcriptionally. This may also explain the result that strain TPU3 showed little increase of 3HB production compared to strain TESB (**Figure 6**). When the *R. eutropha phaA* and *phaB* were over-expressed in strain TAB1 (and strain TABd), the thiolase (PhaA) activity was increased by more than 10-fold, up to 13.1±3.4 U; and the acetoacetyl-CoA reductase (PhaB) activity became detectable with a value of 0.067±0.043 U (**Table 6**). In the cell extract of strain HB5, both thiolase (Thil) activity and acetoacetyl-CoA reductase (Hbd) activities were not detectable, indicating low expression of these two enzymes.

Table 6 Enzyme activities for engineered strains.

Strain	Enzyme (gene)	Activity [a]	Strains with same expression cassette
TESB	Thioesterase (*tesB*)	0.084±0.021	TPU3, HB5, TAB1, TESBd, TPUd, HBd, TABd
TPU3	Thiolase (*phaA2*)	1.14±0.14	TPUd
	Acetoacetyl-CoA reductase (*phaB2*)	n.d.	
HB5	Thiolase (*thil*)	n.d.	HBd
	Acetoacetyl-CoA reductase (*hbd*)	n.d.	
TAB1	Thiolase (*phaA*)	13.1±3.4	TABd
	Acetoacetyl-CoA reductase (*phaB*)	0.067±0.043	

[a] Enzyme activities are given in μmol/min/mL cell extract; "n.d." stands for "not detectable".

2.3.5 Increased acetyl-CoA pool drives 3HB biosynthesis

Previous results indicate that the acetyl-CoA pool in cyanobacteria under photosynthetic conditions is not sufficient to drive the formation of downstream products (Asada et al., 1999; Lan and Liao, 2011; Young et al., 2011; Zhou et al., 2012). In order to increase the abundance of acetyl-CoA in cyanobacterial cells to drive the biosynthesis of the products derived from acetyl-CoA, dark and anoxic, salt-stressed or nutrient-starvation growth conditions were usually used (Asada et al., 1999; Lan and Liao, 2011; Zhou et al., 2012). Nevertheless, in the culture of the engineered 3HB-producing *Synechocystis* strains, a decent amount of 3HB was produced in the culture under

photosynthetic growth conditions (**Figures 6, 7**). To resolve the contradiction, the anion concentrations in the culture of *Synechocystis* were monitored. The results showed that the extracellular phosphate was almost completely absorbed in the first couple of days (**Figure 8B**), during which the cell density was dramatically increased (**Figures 5B, 8A**). The nitrate concentration was also decreased 37% compared to the starting concentration, but not as significant as that of phosphate (**Figure 8B**). Similarly, the phosphate in the medium became undetectable while the nitrate concentration was kept around half of the initial value (data not shown) after 8 days of cultivation using the growth condition as shown in **Figure 7**. Based on this discovery, it is thus hypothesized that the *Synechocystis* cells have suffered phosphate starvation before entering the plateau growth phase which would favor the 3HB biosynthesis thereafter. After adding additional K_2HPO_4 of up to 30 mg/L (same level as in the fresh BG11 medium) into the culture of *Synechocystis* TABd at the end of day 3, the 3HB production was dramatically impaired (**Figure 9**). Further measurement of acetyl-CoA in the cell lysate of *Synechocystis* indicated that the intracellular acetyl-CoA concentration of *Synechocystis* cells almost doubled on day 3.5 relative to that of day 0.5 (p-value=0.01; n=4), reaching the same level as that of the phosphate-starved *Synechocystis* culture (**Figure 10**). Thus, it can be concluded that phosphate consumption and the concomitant increased acetyl-CoA pool act as a key driving force for 3HB biosynthesis in *Synechocystis*.

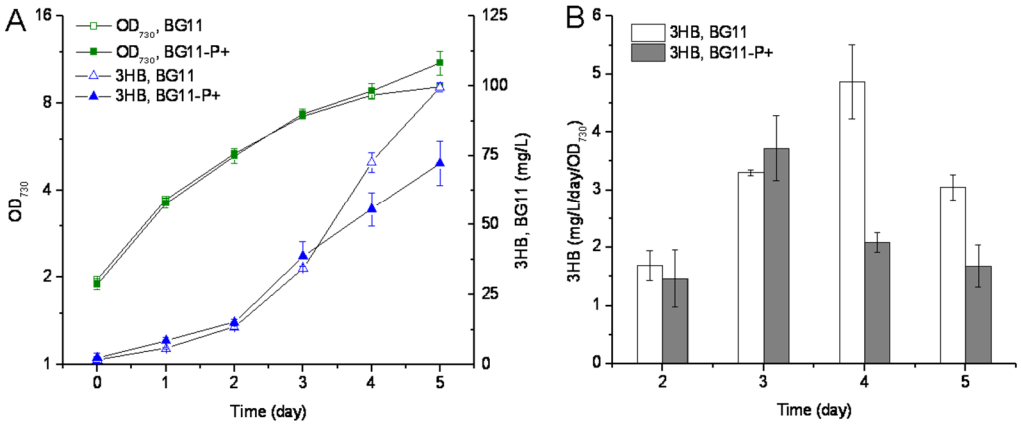

Figure 9 Impaired 3HB production after phosphate spiking. Potassium phosphate dibasic was spiked into the culture medium of *Synechocystis* TABd to 30 mg/L just after day 3. (A) Cell growth and 3HB production curve. (B) 3HB productivity before and after phosphate spiking.

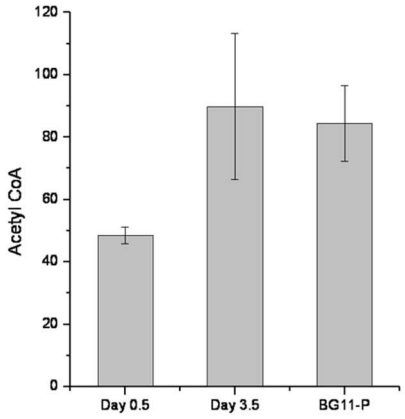

Figure 10 Abundance of acetyl-CoA in *Synechocystis* cells cultivated in normal BG11 medium and under phosphate-starvation conditions. *Synechocystis* cells were harvested from the culture on day 0.5 and day 3.5 when grown in normal BG11 medium. BG11-P

means cells were grown under phosphate-starvation conditions for 1.5 days before being harvested. The y axis shows the total *p* mol of acetyl-CoA in the cell lysate.

2.4 Discussion

(*R*)- and (*S*)-3HB are important bulk chemicals that can serve as monomers in synthesizing renewable plastics PHAs and important building-blocks in producing many chiral fine chemicals (Chen and Wu, 2005; Ren et al., 2010; Tokiwa and Ugwu, 2007; Tseng et al., 2009). Despite the high production yield of 3HB in genetically engineered *E. coli* (Lee and Lee, 2003; Liu et al., 2007; Tseng et al., 2009), the consumption of relatively expensive sugar as energy and carbon sources for production of this type of bulk chemical prevents it from being economically feasible. In the study here 3HB was photosynthetically produced and secreted into the culture medium to up to 93.9 mg/L (after 5 days of cultivation) when using $NaHCO_3$ as the carbon source (**Figure 6A**) and up to 533.4 mg/L (after 21 days of cultivation) when using atmospheric CO_2 as the carbon source (**Figure 7**). Albeit the production titers were not yet comparable to that of the engineered *E. coli* (Lee and Lee, 2003; Liu et al., 2007; Tseng et al., 2009), for the first time, (*R*) and (*S*)-3HB were both produced using renewable sunlight and CO_2 as the sole energy and carbon sources, respectively.

The thioesterase TesB which cleaves the coenzyme A off 3-hydroxybutyryl-CoA (Liu et al., 2007) turned out to be essential in realizing the 3HB biosynthesis in cyanobacterium *Synechocystis* (**Figure 5A**). The performance of TesB was better than that of the combination of the phosphotransbutyrylase (encoded by *ptb*) and butyrate kinase (encoded by *buk*) when expressed in *E. coli* (Tseng et al., 2009). TesB takes a

variety of fatty acyl-CoA as substrates, but has higher specific activities on medium- to long-chain acyl-CoA (Nie et al., 2008). The specific activity of TesB on butyryl-CoA is about 6-fold lower than that on decanoyl-CoA but was around 33-fold higher than that on acetyl-CoA (Wang et al., 2013). This feature determined that efficient driving forces are required to convert acetyl-CoA to acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which means both enzymes need to function efficiently to catalyze these two cascade reactions. Albeit the enhancement of the transcription of *phaA2-phaB2* in *Synechocystis* TPU3 (**Table 5**), only the thiolase activity was increased but not the acetoacetyl-CoA reductase activity (**Table 6**). Genes *phaA* and *phaB* from *R. eutropha* H16 have each been found to exhibit the highest activity among all isoenzymes (**Table 6**), which resulted in relatively high 3HB titers when co-expressed with *tesB* in *Synechocystis* (**Figure 6**). This result is also consistent with the previous studies on 3HB biosynthesis by engineered *E. coli* strains (Tseng et al., 2009). Although the expression of *C. acetobutylicum* genes *thil* and *hbd* in *Synechocystis* strains HB5 and HBd resulted in increased production of 3HB (**Figure 6**), activities of their associated enzymes were undetectable in the cell lysates (**Table 6**). This might be explained when looking at the nucleotide contents of the genes. The G+C contents of *thil* and *hbd* (34.8% for *thil* and 33.1% for *hbd*) are found to be much lower than that of their *Synechocystis* host (G+C content 47.7%; Kaneko et al., 1996). Additionally, there are significant codon usage differences between *Synechocystis* 6803 and *C. acetobutylicum* ATCC 824. For instance, AUA (which encodes for Ile) is a rare codon in *Synechocystis* 6803 (Nakamura et al., 2000) and 16 AUA codons were found in *thil*. In contrast, only one AUA codon was found in the *phaA2* gene which encodes for the native thiolase, and no AUA codons were found in the thiolase-encoding

gene *phaA* of *R. eutropha*. Similarly, up to 18 AUA codons were found in the gene *hbd* from *C. acetobutylicum*. The high abundance of rare codons could decrease the translation efficiency of the heterogeneous genes in *Synechocystis* (Lindberg et al., 2010). In order to improve the performance of the engineered strains, the codons should be optimized before expression.

Although high yields of isobutyraldehyde and isobutanol have been achieved in the engineered cyanobacterium *Synechococcus* via diverting the metabolic flux from pyruvate by a committed decarboxylation reaction (Atsumi et al., 2009), production of chemicals derived from acetyl-CoA turned out to be inefficient because of the thermodynamic property of the thiolase (Lan and Liao, 2012; Masamune et al., 1989) and low concentration of acetyl-CoA accumulated inside cyanobacterial cells under photoautotrophic growth conditions (Asada et al., 1999; Lan and Liao, 2011; Young et al., 2011; Zhou et al., 2012). The condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA catalyzed by the thiolase is a reversible reaction which strongly favors the hydrolysis of acetoacetyl-CoA rather than the reverse reaction (Lan and Liao, 2012; Masamune et al., 1989). This mechanistic feature guarantees the usage of low concentrations of acetyl-CoA in other metabolic routes, such as the TCA cycle and fatty acyl-ACP synthesis (**Figure 2**), which are critical for the cell growth, and the biosynthesis of carbon stocks occurs only when there is excessive acetyl-CoA. As indicated in **Figure 5**, the growth of the TABd strain was almost the same as that of the wild-type strain, probably due to the protection by this mechanistic feature of the thiolase (PhaA). Biosynthesis of acetoacetyl-CoA from acetyl-CoA and malonyl-CoA has been explored in the engineered cyanobacterium *Synechococcus* for 1-butanol production (Lan

and Liao, 2012). Albeit this approach circumvented the issue of low acetyl-CoA abundance, it involves the consumption of ATP, the impact of which is not quite clear yet (Lan and Liao, 2012). Or otherwise, in order to drive the synthesis of acetoacetyl-CoA and thus downstream products using thiolase, increased acetyl-CoA is desirable. Herein, different from the anaerobic fermentation strategy used in other research (Lan and Liao, 2011; Zhou et al., 2012), it is demonstrated that the phosphate in the culture medium was quickly depleted during the biomass growth phase of the photoautotrophic culture of *Synechocystis* (**Figure 8B**), and concomitantly the acetyl-CoA abundance was boosted to around 2-fold higher (**Figure 10**) which provides an important driving force for the formation of acetoacetyl-CoA. Supplementation of extra phosphate into the culture medium greatly impaired the 3HB production but enhanced the cell growth (**Figure 9**).

Other driving forces also play crucial roles in photosynthetic production of 3HB by the engineered *Synechocystis*. NADPH, a cofactor of acetoacetyl-CoA reductase PhaB, is produced abundantly during photosynthesis (Lee et al., 2010) and thus acts as a key driving force in the reduction of acetoacetyl-CoA to form (*R*)-3-hydroxybutyl-CoA (**Figure 2**). The immediate elimination of acetoacetyl-CoA also releases the inhibition of the acetoacetyl-CoA biosynthesis catalyzed by thiolase, drawing in more metabolic flux from acetyl-CoA toward 3HB biosynthesis. In contrast, Hbd utilizes NADH as a cofactor, which is relatively less abundant, resulting in low efficiency in driving the 3HB production. This speculation could be supported by the previous study (Lan and Liao, 2011) in which an *hbd*-harboring and NADH-demanding pathway exhibited low activity in production of 1-butanol in the engineered *Synechococcus*. Additionally, the hydrolysis of 3-hydroxybutyl-CoA by the thioesterase (TesB) is a committed reaction which

provides another driving force for 3HB biosynthesis. Overall, according to the enzyme activity assay results, the acetoacetyl-CoA reductase (PhaB; 0.067±0.043 U) and thioesterase (TesB; 0.084±0.021 U) activities in strain TAB1 were nearly two orders of magnitude lower than that of the thiolase (PhaA; 13.12±3.36 U), suggesting that the driving forces for 3HB biosynthesis in strain TAB1 and TABd (which have the same genetic cassettes for 3HB biosynthesis) could be further enhanced by fine-tuning the expression of the former two enzymes. Blocking the competing pathway of 3HB biosynthesis by deletion of *phaEC* which encodes the PHB synthase is another important driving force for 3HB production. It dramatically increased the 3HB production by about 2-fold in the culture of strain TABd relative to that of strain TAB1 (**Figure 6**).

It has been suggested that the hydrophobic cell membrane became a significant barrier for production and secretion of hydrophilic products, such as hexose and lactic acid, by the genetically engineered cyanobacteria (Niederholtmeyer et al., 2010). Secretion of both products into the culture medium thus relied on the co-expression of the product-specific transporters, namely those encoded by gene *glf* and *lldP* from *Zymomonas mobilis* and *E. coli*, respectively (Niederholtmeyer et al., 2010). However, without co-expressing any transporter genes in *Synechocystis*, hydrophilic 3HB molecules were secreted to the culture medium to up to 533.4 mg/L. While it is possible that *Synechocystis* 6803 naturally expresses unidentified transporters for the 3HB secretion, the genetic system responsible for such a process has not yet been identified.

CHAPTER 3

GENE EXPRESSION FINE-TUNING ENHANCES BIOSYNTHESIS OF (*R*)-3-HYDROXYBUTYRATE IN *SYNECHOCYSTIS*

3.1 Introduction

Cyanobacteria are gaining increasing attention in microbial production of renewable fuels and chemicals due to their capability in harvesting solar energy and recycling carbon dioxide (Wang et al., 2012). However, it remains challenging to engineer cyanobacteria to produce high titers of fuels and chemicals, partially due to limited synthetic biology tools and low level expression of the genes of interest.

To expand the library of genetic tools, endeavors have been made in developing and characterizing a few clusters of promoters for their use in cyanobacteria (Mohamed and Jansson, 1989; Eriksson et al., 2000; Huang et al., 2010; Xu et al., 2011; Qi et al., 2013). Among those characterized promoters, the light-independent P_{trc}/P_{tac} promoter (Marraccini et al., 1993) and the photosynthesis-related promoters are the strongest ones in *Synechocystis* sp. PCC6803 (hereafter *Synechocystis* 6803). It was found that when the repressor protein, LacI, was not expressed, the P_{tac} promoter behaved like a strong constitutive promoter in expressing GFP in *Synechocystis* 6803 (Huang et al., 2010; Guerrero et al., 2012). Angermayr et al. (2012) expressed the *ldh* and *sth* genes in *Synechocystis* 6803 using the P_{trc} promoter without adding inducer (IPTG) and achieved photosynthetic production of 288 mg/L L-lactatic acid in 17 days. Varman et al. (2013) used the P_{trc} promoter to express the *sth* gene and a mutated glycerol dehydrogenase-

coding gene, *gldA101*, and achieved 1.14 g/L photoautotrophic production of D-lactic acid in *Synechocystis* 6803. In the previous study described in Chapter 2 (Wang et al., 2013), P_{tac} was successfully utilized to constitutively express the 3-hydroxybutyrate biosynthesis genes in *Synechocystis* 6803, and the photoautotrophic production of 3HB reached 533.4 mg/L in 21 days. P_{trc} was also used to express heterologous genes for 2,3-butanediol production in *Synechocystis* (Savakis et al., 2013). The light-inducible *psbA* family promoters, which express the Photosystem II reaction center D1 protein, are strong promoters in a broad range of cyanobacterial species under medium to high light conditions (Mohamed et al., 1989, 1993; Elhai et al., 1993; Golden, 1995; Agrawal et al., 2001; Nair et al., 2001; Ludwig and Bryant, 2011; Sakurai et al., 2012). Particularly, the P_{psbA2} promoter in *Synechocystis* 6803 was proven a strong one (Mohamed et al., 1989, 1993). In order to achieve photosynthetic production of ethanol, for example, Dexter and Fu (2009) inserted the *pdc* and *adh* genes into the *psbA2* locus of the *Synechocystis* 6803 chromosome and realized efficient gene expression via the native P_{psbA2} promoter, leading to 552 mg/L ethanol production after 6 days. The pea plant chloroplast P_{psbA} promoter was also proven as an efficient promoter in expressing the *efe* gene in ethylene production in *Synechocystis* (Ungerer et al., 2012). However, it was recently reported that the strength of the P_{psbA2} promoter was only ~10% of that of the P_{trc} promoter in cyanobacterium *Synechocystis* 6803 as indicated by the L-lactic acid productivity and the corresponding enzyme activity (Angermayr and Hellingwerf, 2013).

Insertion of multiple copies of the interest genes into the chromosome of cyanobacterial strains has been proven as an effective way to increase gene expression. For instance, when the genetically engineered cyanobacterium *Synechocystis* 6803 was

engineered by inserting a second copy of the pyruvate decarboxylase and alcohol dehydrogenase genes into the chromosome, the relevant gene expression levels doubled and ethanol production was increased by about 4-fold compared to that of the parent strains which were endowed with only one single copy of those two genes (Gao et al., 2012). Similarly, when a second copy of the *efe* gene was integrated into the chromosome of *Synechocystis*, both the *efe* gene expression level and the ethylene production level doubled under photoautotrophic cultivation conditions (Ungerer et al., 2012).

Modification of the 5'-untranslated region (UTR) is another effective strategy to modulate the gene expression in microbes (Makrides, 1996; Salis et al., 2009). Most recently, it has been found that replacing the 5'-UTR (including the ribosome binding site) upstream of the 2,3-butanediol biosynthesis genes significantly affected the corresponding gene expression in the engineered *Synechococcus elongatus* sp. strain PCC 7942, proven by the enzyme activity assay and the acetoin and 2,3-butanediol analysis (Oliver et al., 2014).

3-Hydroxybutyrate (3HB) is the precursor for synthesizing biodegradable plastics polyhydroxyalkanoates (PHAs), and also serves as a building-block for many fine chemicals. In Chapter 2, it was found that among the isoenzymes of thiolase and acetoacetyl-CoA reductase from different microbial sources, *i.e.*, the native *Synechocystis* 6803, *Ralstonia eutropha* H16 and *Clostridium acetobutilicum* ATCC 824, the isoenzymes from *R. eutropha* (**Figure 11**) showed highest enzyme activities and endowed highest 3HB production rate, on average ~25 mg/L (*R*)-3HB by the engineered *Synechocystis* (Wang et al., 2013). Enzyme activity assay results indicated that the relatively low activities of the acetoacetyl-CoA reductase (encoded by *phaB1*) and the

thioesterase (encoded by *tesB*) might be the bottleneck for the (*R*)-3HB biosynthesis in the engineered *Synechocystis* (Wang et al., 2013).

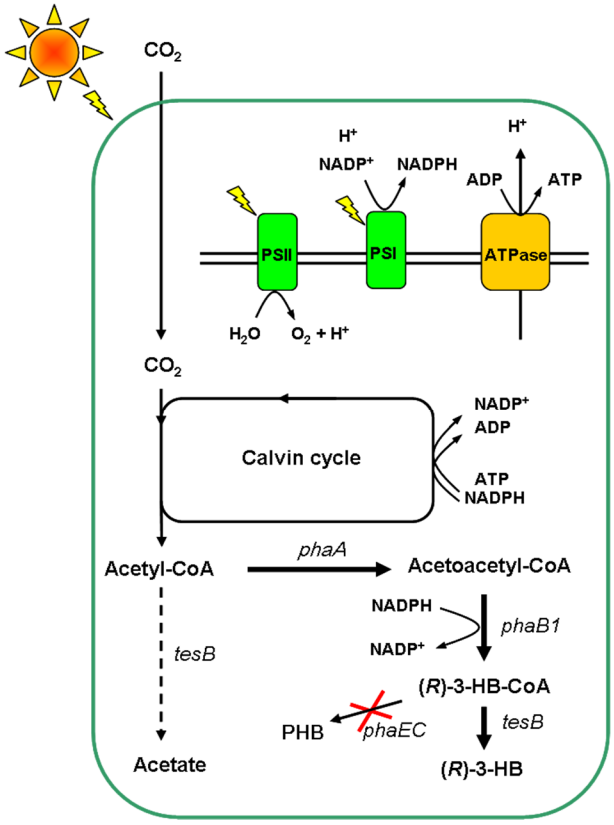

Figure 11 Schematic representation of (R)-3HB biosynthesis from CO_2 in engineered *Synechocystis.*

In this chapter, the goal is to enhance the photosynthetic production of (*R*)-3HB. Different strategies were explored to improve the expression efficiency of the target genes, including tuning the gene copy number, transcription and translation levels. Firstly, the efficiency was compared for five natural and synthetic promoters, *i.e.*, P_{tac} and P_{psbA2} and three of their derivatives, in expressing the *tesB* gene in *Synechocystis*. Since light might play a significant role in regulating the intracellular metabolic flux levels and the light-inducible P_{psbA2} promoter and its derivatives, further studies investigated the (*R*)-

3HB production rate under different light intensities. The effect of inserting a second copy of acetoacetyl-CoA reductase encoding gene and a second copy of the *tesB* gene into the chromosome of the 3HB producing *Synechocystis* strain was investigated. To explore the effectiveness of regulating gene expression at the translation level, the ribosome binding site (RBS) upstream of the *phaB1* gene was optimized. The optimized strains were able to continuously produce and secrete (*R*)-3HB at an average rate of 86 mg/L/day (with a peak rate of ~200 mg/L) under the photoautotrophic condition and finally reached a cumulative titer of 1.6 g/L.

3.2 Materials and methods

3.2.1 Culture conditions

All recombinant plasmids were constructed and stored using *E. coli* XL1-Blue MRF' (Stratagene, La Jolla, CA) as the host strain. *Synechocystis* strains were grown in BG11 medium (Rippka et al., 1979) supplemented with 50 mM $NaHCO_3$ under a light intensity of 60 $\mu E/m^2/s$ unless otherwise specified. For BG11-agar plates, 10 mM TES (pH 8.0), 3 g/L thiosulfate and 1.5% agar was supplemented into BG11 medium before autoclaving.

3.2.2 Modification of *Synechocystis* genome

PCR primers used to construct integrative vectors in this study are listed in **Table 7**. High fidelity DNA polymerase, Phusion™ (New England BioLabs, Ipswich, MA), was used in PCR amplification unless otherwise specified. The kanamycin resistance cassette containing its promoter was PCR amplified using primers Kan9 and Kan2. The PCR product was purified and further amplified by primers rT1KP and Kan2 using LongAmp

Taq DNA polymerase (New England BioLabs, Ipswich, MA) before being digested with MluI and SalI-HF restriction enzymes and inserted into the pBS-S2 plasmid digested with the same restriction endonuclease. The resultant plasmid was denominated as pS2-rT1PK. P_{tac}-*tesB* fragment was PCR amplified from plasmid pSPtTeK using primers TAC21 and TESB2, and was then digested with BglII and MluI and inserted into pS2-rT1PK digested with the same restriction enzymes, resulting in plasmid pSTTrK. Promoters P_{psbA12}, P_{psbA14} were PCR amplified from the genomic DNA of *Synechocystis* sp. PCC 6803 using primers PSBA1 and PSBA2, and primers PSBA1 and PSBA4, respectively. The P_{psbA12} and P_{psbA14} PCR products were then digested with PstI-HF and NdeI, and was subsequently inserted into plasmid pSTTrK cut with the same enzymes, resulting in plasmids pSPTrK12 and pSPTrK14, respectively. Promoters P_{tac}-SD and P_{tac}-UTR were PCR amplified from plasmid pSPtK using primers TAC21 and TAC-SD2, and primers TAC21 and TAC-UTR2, respectively, before being digested with PstI and NdeI and inserted into plasmid pSPTrK14 digested with the same restriction endonucleases. The resultant plasmids were denominated as pSTTrK-SD and pSTTrK-UTR, respectively. To construct plasmid pSPTrK16, promoter P_{psbA12} was PCR amplified using primers PSBA1 and PSBA6, digested with PstI-HF and SphI restriction endonucleases, and inserted between the same restriction sites on plasmid pSTTrK. To construct plasmid pSTTe, plasmid pSTTrK was restriction digested with MluI and SalI, end-blunted by incubating with Phusion DNA polymerase and PCR buffer and dNTP at 72 ^{o}C for 20 min, and eventually self-circulated with T4 DNA ligase. Plasmid pUC-TTB2 with codon optimized for genes *tesB* and *phaB2* for expression in *Synechocystis* sp. PCC 6803 was synthesized by GenScript USA Inc (Piscataway, NJ, USA). The DNA fragment P_{tac}-$tesB_{opt}$-$phaB2_{Re}$-

opt was then cut off from pUC-TTB2 using restriction enzymes BglII and HindIII, and inserted between the BamHI and HindIII sites on plasmid pBS-SPSK3, resulting plasmid pSTTB2K3.

Integrative vectors used in this study were constructed using the same method as described in Chapter 2 (Wang et al., 2013). The features of integrative vectors are indicated in **Table 8**. The chromosome of *Synechocystis* 6803 was modified using the same methods as described previously (Wang et al., 2013). The genotype of each engineered *Synechocystis* strain is described in **Table 8**. The genotypic purity of each strain was achieved by a series of streaking of the colonies on the antibiotic-supplemented BG11 plates and was confirmed by colony PCR.

Table 7 Primers used in Chapter 3.

Name		**Targets**
Primers (5' to 3') used for DNA recombination		
Kan9	GTCAACGCGTCCTAACTACGGCTACACTAGAAG	*kan* (Kan^R)
Kan2	ACGGTCGACCAGGTGGCACTTTTCGGGG	*kan* (Kan^R)
rT1KP	GTCAACGCGTCAAATAAAACGAAAGGCTCAGTCGAAAGACTG GGCCTTTCGTTTTATCTGCCTAACTACGGCTACACTAGAAG	*rrnB T1* and Kan^R
TAC21	GAAGATCTAACTGCAGACATGCATGCGAGCTGTTGACAATTA ATCATC	P_{tac}
TESB2	TCAAAGCTTACGGCGAGAATGCCTATAAC	*tesB*
TAC-SD2	GGAATTCCATATGTTGGTTATAATTCCTTAATGTATATCCTCC TTGTGTG	P_{tac}-SD
TAC-UTR2	GGAATTCCATATGTTGGTTATAATTCCTTATGTATTTGTCGAT GTTCAGATTGGAACTGACTATGTATATCCTCCTTGTGTG	P_{tac}-UTR
PSBA1	AAACTGCAGGCCCTCTGTTTACCCATGGAAAAAACG	P_{psbA12}, P_{psbA14}
PSBA2	GGAATTCCATATGTTGGTTATAATTCCTTATGTATTTGTCG	P_{psbA12}
PSBA4	GGAATTCCATATGTTGGTTATAATTCCTTAGTTCAGATTGGAA CTGACTAAAC	P_{psbA14}

Name		Targets
PSBA6	ACATGCATGCTTGGTTATAATTCCTTATGTATTTGTCG	P_{psbA12}
PHAB15	GACACGCGTACAAGGAGGTAACATGACTCAG	*phaB1*
PHAB14	ACGAAGCTTGCTCTAGATTAGCCCATGTGCAGGCCGCCGTTG	*phaB1*
Primers (5' to 3') used for RT-qPCR		
16S-F2	CCACGCCTAGTATCCATCGT	*Synechocystis* 16S
16S-R2	TGTAGCGGTGAAATGCGTAG	*Synechocystis* 16S
TESB3	ACGCTTCTGATCTTAACTTCCTG	*tesB*
TESB4	TCAAAGCTTTTTAATTGTGATTACGCATCAC	*tesB*

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Table 8 Strains and plasmids used in Chapter 3.

	Genotype*	References
Strains		
E. coli XL1-Blue MRF'	*Δ*(*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA*1 *gyrA96 relA1 lac* [*F´ proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]	Stratagene
Synechocystis		
ABd	P_{tac}-*adhe2* integrated at S2 site, *phaE* and *phaC* deleted at S3 site and Cm^{R}-P_{tac}-*phaA-phaB1* integrated at S1 site	This study
TTrK	P_{tac}-*tesB*-T1-Kan^{R} integrated at S2 site, Cm^{R}-P_{tac}-*phaA-phaB1* integrated at S1 site and *phaE* and *phaC* deleted at S3 site	This study
SD-TrK	P_{tac}-SD-*tesB*-T1-Kan^{R} integrated at S2 site, Cm^{R}-P_{tac}-*phaA-phaB1* integrated at S1 site and *phaE* and *phaC* deleted at S3 site	This study
UTR-TrK	P_{tac}-UTR-*tesB*-T1-Kan^{R} integrated at S2 site, Cm^{R}-P_{tac}-*phaA-phaB1* integrated at S1 site and *phaE* and *phaC* deleted at S3 site	This study
PTrK12	P_{psbA12}-*tesB*-T1-Kan^{R} integrated at S2 site, Cm^{R}-P_{tac}-*phaA-phaB1* integrated at S1 site and *phaE* and *phaC* deleted at S3 site	This study
PTrK14	P_{psbA14}-*tesB*-T1-Kan^{R} integrated at S2 site, Cm^{R}-P_{tac}-*phaA-phaB1* integrated at S1 site and *phaE* and *phaC* deleted at S3 site	This study
PTrK16	P_{psbA12}-P_{tac}-*tesB*-T1-Kan^{R} integrated at S2 site, Cm^{R}-P_{tac}-*phaA-phaB1* integrated at S1 site and *phaE* and *phaC* deleted at S3 site	This study
SPA:ΔphaEC	P_{tac}-*adhe2* integrated at S2 site, *phaE* and *phaC* deleted at S3 site	This study
ABd-SPSK2	P_{tac}-*sacB-kan* integrated at S2 site, *phaE* and *phaC* deleted at S3 site	This study

	Genotype*	References
	and Cm^R-P_{tac}-*phaA*-*phaB1* integrated at S1 site	
ABd-TTe	Ptac-*tesB* integrated at S2 site, *phaE* and *phaC* deleted at S3 site and Cm^R-P_{tac}-*phaA*-*phaB1* integrated at S1 site	This study
TTB2K3	*phaE* and *phaC* deleted at S3 site, P_{tac}-*tesB* integrated at S2 site, P_{tac}-$tesB_{opt}$-$phaB2_{eu\text{-}opt}$-*kan* integrated at S3 site and Cm^R-P_{tac}-*phaA*-*phaB1* integrated at S1 site	This study
ABdTB	*phaE* and *phaC* deleted at S3 site, P_{tac}-$tesB_{opt}$-$phaB2_{eu\text{-}opt}$ integrated at S3 site and Cm^R-P_{tac}-*phaA*-*phaB1* integrated at S1 site	This study
PTrK16-Int	P_{psbA12}-P_{tac}-*tesB*-T1-Kan^R integrated at S2 site and *phaE* and *phaC* deleted at S3 site	This study
R154	P_{psbA12}-P_{tac}-*tesB*-T1-Kan^R integrated at S2 site, *phaE* and *phaC* deleted at S3 site and Cm^R-P_{tac}-*phaA*-(RBS_{opt})-*phaB1* integrated at S1 site	This study
R168	P_{psbA12}-P_{tac}-*tesB*-T1-Kan^R integrated at S2 site, *phaE* and *phaC* deleted at S3 site and Cm^R-P_{psbA12}-P_{tac}-*phaA*-(RBS_{opt})-*phaB1* integrated at S1 site	This study
Plasmids		
pBluescript II SK(+)	Amp^R, pUC ori, f1(+) ori	Stratagene
pSCPAB	Cm^R-Ptac-*phaA*-*phaB1* with arms for S1 site	Chapter 2
pSPtTeK	P_{tac}-*tesB*-*kan* with arms for S2 site	Chapter 2
pSTTrK	P_{tac}-*tesB*-T1-Kan^R with arms for S2 site	This study
pSTTrK-SD	P_{tac}-SD-*tesB*-T1-Kan^R with arms for S2 site	This study
pSTTrK-UTR	P_{tac}-UTR-*tesB*-T1-Kan^R with arms for S2 site	This study
pSPTrK12	P_{psbA12}-*tesB*-T1-Kan^R with arms for S2 site	This study
pSPTrK14	P_{psbA14}-*tesB*-T1-Kan^R with arms for S2 site	This study
pSPTrK16	P_{psbA12}-Ptac-*tesB*-T1-Kan^R with arms for S2 site	This study
pSTTe	P_{tac}-*tesB* with arms for S2 site	This study
pBS-SPSK2	P_{tac}-*sacB*-*kan* with arms for S2 site	Chapter 2
pSTTB2K3	P_{tac}-$tesB_{opt}$-$phaB2_{eu\text{-}opt}$-*kan* with arms for S3 site	This study
pSCPAB154	Cm^R-Ptac-*phaA*-(RBS_{opt})-*phaB1* with arms for S1 site	This study
pSCPAB168	Cm^R-P_{psbA12}-P_{tac}-*phaA*-(RBS_{opt})-*phaB1* with arms for S1 site	This study

*S1, the site on the genome of *Synechocystis* 6803 between *slr1495* and *sll1397*; S2, the site between *slr1362* and *sll1274*; S3, the site between *slr1828* and *sll1736*.

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

Synechocystis strains were inoculated in 50 mL flasks containing 10 mL BG11 (10 mM TES-NaOH) to an initial OD_{730} of 2.0. Then, cells were incubated in a shaking bed (150 rpm) at 30 ^{o}C with a light intensity of 60 μE/m^2/s except otherwise specified. Daily, 0.05 mL cell culture was sampled for analysis of the OD_{730} before 0.5 mL 1.0M $NaHCO_3$ was added to each culture and the pH was adjusted to ~8.0 by 10 N HCl. Each cell culture was sampled at the end of day 3 and day 5, respectively, for analysis of the (*R*)-3HB titers. All culture experiments were conducted at least in triplicate for each strain.

3.2.4 Production of 3HB from carbon dioxide

Synechocystis was inoculated into a 125 mL flask containing 50 mL autoclaved BG11 (10 mM TES-NaOH) medium to an initial OD_{730} of 0.2. The culture was placed at 30 ^{o}C with continuous illumination of 100 μE/m^2/s, bubbled with ambient air during the first 24 h and then switched to 1% (v/v) CO_2. The aeration rate was set as 37.5 mL/min. Every day, 1 mL of culture was sampled and 1.25 mL 2-fold concentrated sterilized BG11 medium was added back into the culture until day 21. The experiments were conducted in duplicates.

3.2.5 Gene expression analysis by RT-qPCR

Cells were resuspended to an initial OD_{730} of 2.0 before they were grown in BG11 (10 mM TES-NaOH) medium under continuous illumination of 60 μE/m^2/s. Daily, 0.05 mL of cell culture was sampled for analysis of the OD_{730} before 0.5 mL 1.0M $NaHCO_3$ was added to each culture and the pH was adjusted to ~8.0 by 10 N HCl. At 3.5 days of cultivation, approximately 1.67×10^8 *Synechocystis* cells (assuming OD_{730} of 0.6 equals to

10^8 cells/mL (Liu et al., 2011)) were collected by centrifugation at 17,000g, 4 ^oC for 1 min. The supernatant was discarded and the cell pellet was used for RNA extraction using ZR Fungal/Bacterial RNA MiniPrep^{TM} Kit (ZYMO Research, Irvine, CA). The RNA was then quantified by RT-qPCR using methods described previously (Gao et al., 2011).

3.2.6 Enzyme activity assay

Synechocystis cells were grown as described in Section 3.2.3 for 12 hours. At the end of the cultivation, approximately 1.67×10^9 *Synechocystis* cells were collected by centrifugation at 8000g, 4 ^oC for 5 min. The supernatant was discarded and the cell pellets were frozen on dry ice and stored at -80 ^oC before performing 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 bath. The cell lysate was centrifuged at 17000g, 4 ^oC for 10 min before the supernatant was analyzed for the thioesterase activity following the previous protocols but using Butyryl-CoA as the substrate (Wang et al., 2013). 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] with 0.1 mM PMSF and lysed by sonication (20 cycles of 3-s-on/ 3-s-off) in ice bath. The supernatant was analyzed for the acetoacetyl-CoA reductase activity using the previously established protocol (Wang et al., 2013).

3.2.7 Product quantification

The (*R*)-3HB and acetate concentrations were quantified by an 1100 series HPLC using the method described previously (Wang et al., 2013). Briefly, samples of the *Syenchocystis* 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 equipped with an Aminex HPX-87H anion-exchange column (Bio-Rad Laboratories, Hercules, CA) and a refractive index detector (Agilent, Santa Clara, CA). 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 to 0.8 mL/min over 12 min, followed by an 8 min hold (Tseng et al., 2009).

3.3 Results and Discussion

3.3.1 Construction of a promoter library

Since promoter is the key element to initiate the expression of the interest genes, to screen out a strong promoter is critical to improving the gene expression level in the host strain. Herein, a promoter library was constructed and characterized in expressing the 3HB biosynthesis gene *tesB*. The library included the constitutive P_{tac} promoter, the wild type light-inducible P_{psbA2} promoter from *Synechocystis* 6803, and three derivative promoters (**Figure 12**). *Synechocystis* strain TTrK, with all the 3HB biosynthesis genes expressed via the P_{tac} promoter, was reconstructed from the TABd strain (Section 2.2.3; Wang et al., 2013) by placing a *rrnB* T1 terminator downstream the *tesB* (**Table 8**). The

P_{tac} promoter upstream the *tesB* gene was replaced by the wild type *psbA2* promoter (thereafter P_{psbA12}), resulting in strain PTrK12.

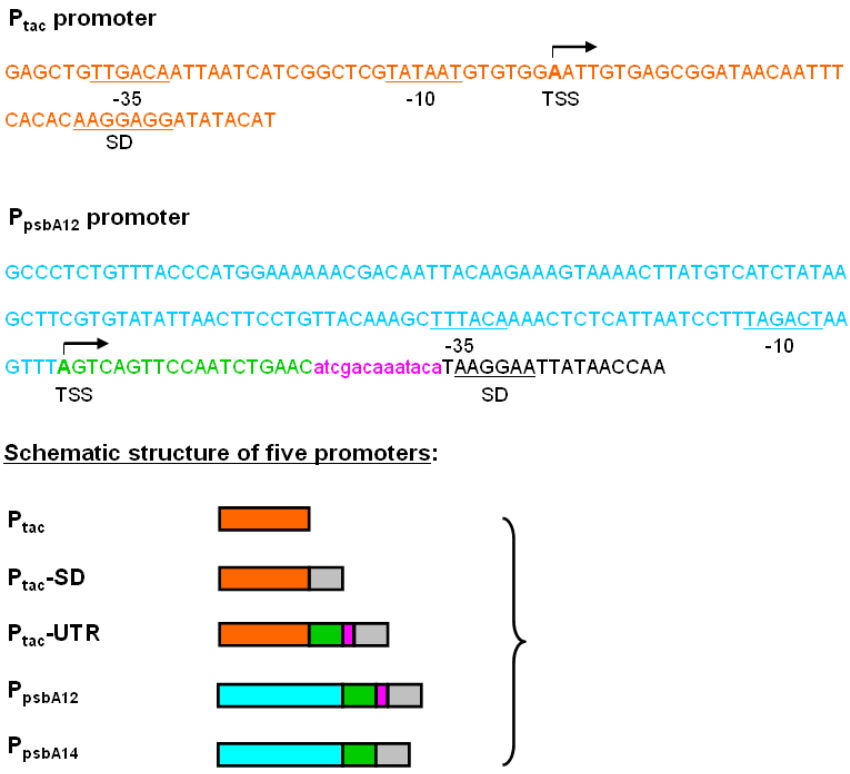

Figure 12 Schematic structure of the five promoters.

It was reported that the 5'-untranslated region (UTR) of the P_{psbA12} promoter from *Synechocystis* 6803 plays an important role in stabilizing the *psbA2* mRNA (Sakurai et al., 2012). It was therefore assumed that the 5'-UTR of P_{psbA12} promoter might be placed upstream of the interest gene to increase the stability of the corresponding mRNA. To this end, the 5'-UTR and the ribosome binding site (RBS, including the SD sequence) of the *psbA2* gene was combined with the P_{tac} promoter, resulting in promoters P_{tac}-UTR and P_{tac}-SD, respectively (**Figure 12**). In addition, since the AU-box in the 5'-UTR of

P_{psbA2} was suggested to be a negative element for gene expression (Sakurai et al., 2012; Agrawal et al., 2001), the AU-box has been deleted from the P_{psbA12} promoter to form promoter P_{psbA14} (**Figure 12**).

3.3.2 The performance of promoters

Since the thioesterase (encoded by *tesB*) and the acetoacetyl-CoA reductase (encoded by *phaB*1) were identified as the bottleneck for (*R*)-3HB biosynthesis in *Synechocsytis* based on the enzyme activity assay (Wang et al., 2013, 2014), the five promoters were used to express the *tesB* gene in the *phaA-phaB*-expressing strains (**Table 8**). When the engineered strains were grown under photoautotrohic conditions, no significant difference was observed regarding the cell growth rates of the strains indicated by the optical density at 730nm (OD_{730}; **Figure 13A**). However, the 3HB production titers exhibited considerable variability among the five strains (**Figure 13B**). Strain TTrK(P_{tac}) exhibited the highest 3HB productivity, reaching 176.9±6.4 mg/L in five days. Strain PTrK12 (P_{psbA12}) produced 166.1±5.2 mg/L of 3HB, slightly lower than that of strain TTrK (P_{tac}). The 3HB productivities of strains UTR-TrK (P_{tac}-UTR) and PTrK14 (P_{psbA14}) were slightly lower than that of strain PTrK12 (P_{psbA12}), reaching 147.5±13.3 mg/L and 141.3±7.3 mg/L. Strain SD-TrK (P_{tac}-SD) produced the least amount of 3HB, reaching only 70.2±7.0 mg/L, less than half of that of any other strain (**Figure 13B**).

RNA analysis of the above five strains indicated that the mRNA level of gene *tesB* in strain TTrK was the highest among all investigated strains (**Figure 13C**) which is consistent with its highest 3HB productivity (**Figure 13B**), suggesting that the P_{tac} promoter might be the strongest promoter under the examined culture condition. In contrast, the *tesB* mRNA level in strain SD-TrK was the lowest (**Figure 13C**). Since

modification of the 5'-UTR was assumed to have little impact on the gene transcription, the finding here indicated that the apparent lower abundance of the *tesB* mRNA in SD-TrK was attributed to the poorer stability of the mRNA product that contained the RBS region of the P_{psbA12} promoter. This result was consistent with the previous report that the RBS of the P_{psbA12} promoter is a target of the RNase E/G in *Synechocystis* 6803 (Horie et al., 2007; Sakurai et al., 2012). Interestingly, the *tesB* mRNA abundance was 57% higher in the strain UTR-TrK compared to that of strain SD-TrK, consistent with the ~2-fold higher 3HB production rate (**Figure 13B**). It suggested that the AU-box and the 19-bp upstream sequence (from the P_{psbA12} promoter) in the P_{tac}-UTR promoter has a positive impact on expression of the downstream gene. While the AU-box is also a target of the RNase E, this region may be bound by the intrinsic *cis*-coded asRNA "PsbA2R" and therefore protect the mRNA from degradation by endoribonuclease RNase E/G in *Synechocystis* (Horie et al., 2007; Sakurai et al., 2012). However, this explanation can-not elucidate why adding the AU-box and the upstream sequence to the 5'-UTR was able to enhance the expression of the *tesB* mRNA. Moreover, after the AU-box was removed, the P_{psbA14} promoter did not show any improved expression of the *tesB* mRNA (**Figure 13C**) and in contrast, the 3HB and acetate production titers decreased compared to those of the strain PTrK12 (**Figures 13B, D**). It can be speculated that the AU-box and/or the upstream sequence of the P_{psbA12} promoter is an enhancer in the *Synechocystis* 6803.

Interestingly, although the *tesB* expression levels in strains SD-TrK, UTR-TrK, PTrK12 and PTrK14 range from 30% to 60% relevant to that of the strain TTrK (**Figure 13C**), the acetate accumulation in these strains reached merely less than 20% of that of strain TTrK, 41.3±4.3 mg/L (**Figure 13D**). TesB has low activity on acetyl-CoA (Wang

et al., 2013), and the acetyl-CoA pool in each strain is presumably the same as indicated by the same cell growth rate (**Figure 13A**). Therefore, the acetate titer is a good indicator of the TesB enzyme activity in each strain. The unmatched TesB enzyme activities (**Figure 13C**) and mRNA abundance (**Figure 13D**) is probably attributed to the different translation efficiency of different promoters, which is discussed thoroughly later on.

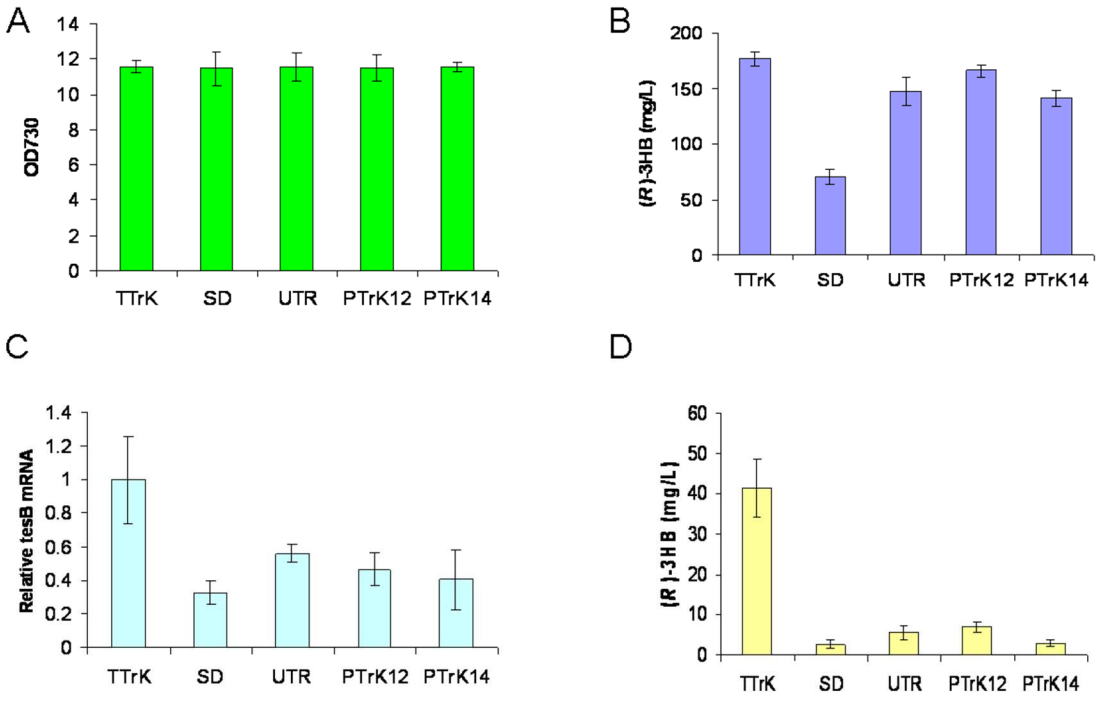

Figure 13 Characterization of the five promoters. (A) Cell density of strains expressing *tesB* using different promoters. (B) Production of (*R*)-3HB by each strain. (C) *tesB* mRNA abundance in each engineered strain. (D) Acetate accumulation in the culture medium of each engineered strain.

3.3.3 Construction and characterization of a dual promoter

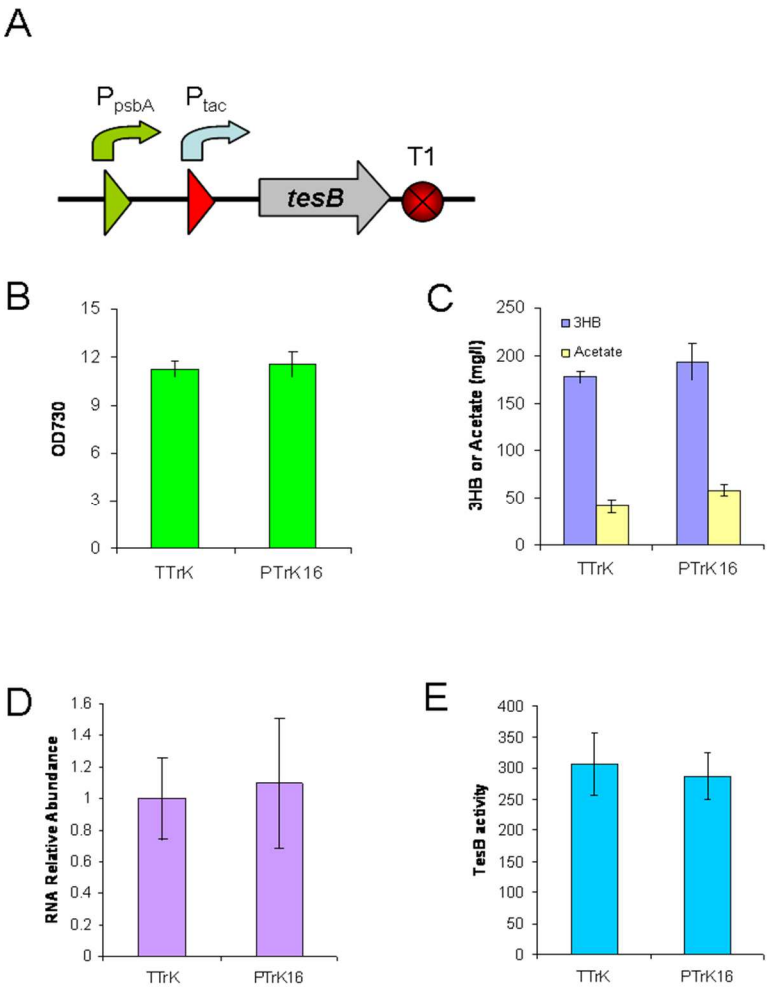

Figure 14 Characterization of the dual promoter system in strain PTrK16. (A) Schematic representation of the dual promoter system. (B) Cell densities for strains TTrK and SPTrK16 after growing under a light intensity of 60 µE/m^2/s for 5 days. (C) Production of (*R*)-3HB and acetate by strains TTrK and PTrK16. (D) *tesB* mRNA relative abundance on day 3.5. (E) Thioesterase activity analysis for TesB.

The P_{tac} and the P_{psbA12} promoter were recombined together to form a cascade structure (**Figure 14A**) in order to investigate if the expression of the downstream gene can be enhanced in such a way. As a result, there were no significant differences between strains PTrK16 and TTrK regarding the cell growth rate (**Figure 14B**), (*R*)-3HB production and acetate accumulation (**Figure 14C**). RT-qPCR analysis results indicated that the abundance of *tesB* mRNA in strain PTrK16 and TTrK was at the same level on day 3.5 (**Figure 14D**), though the *tesB* mRNA only reached approximately 70% of that in strain TTrK on day 0.5 (**Figure 15**). The results were further confirmed by the enzyme activity assay (**Figure 14E**; **Table 9**).

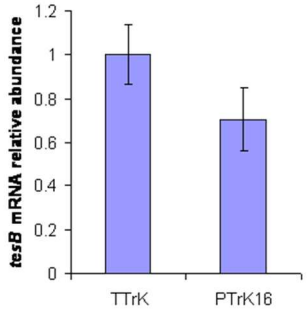

Figure 15 *tesB* mRNA relative abundance on day 0.5.

Table 9 Enzyme activity assay for re-engineered *Synechocystis* strains*.

Strain	Genotype	Acetoacetyl-CoA reductase	Thioesterase
TTrK	P_{tac}-*tesB*, P_{tac}-*phaA-phaB1*, Δ*phaEC*	0.063 ± 0.013	0.345 ± 0.010
PTrK16	P_{psbA12}-P_{tac}-*tesB*, P_{tac}-*phaA-phaB1*, Δ*phaEC*	0.078 ± 0.033	0.243 ± 0.014
TTB2K3	P_{tac}-*tesB*, P_{tac}-*phaA-phaB1*, P_{tac}-	0.093± 0.007	0.386 ± 0.010

Strain	Genotype	Acetoacetyl-CoA reductase	Thioesterase
	tesB$_{opt}$-*phaB2*$_{eu\text{-}opt}$, Δ*phaEC*		
ABdTB	P_{tac}-*phaA*-*phaB1*, P_{tac}-*tesB*$_{opt}$-*phaB2*$_{eu\text{-}opt}$, Δ*phaEC*	n.a.	0.161 ± 0.014
R154	P_{tac}-*tesB*, P_{tac}-*phaA*-(RBS_{opt})-*phaB1*, Δ*phaEC*	0.139 ± 0.020	n.a.

* The cells for enzyme assay were collected after growing in BG11 (10 mM TES-NaOH, pH 8.0) for 12 hours under an irradiation of 60 μE/m^2/s.

3.3.4 The limiting factor for (*R*)-3HB biosynthesis

Though the *tesB* mRNA abundance was two-fold higher (**Figure 13C**) and the thioesterase activity in strain TTrK was more than 5-fold higher than that of strain UTR-TrK (indicated by the acetate titers in **Figure 13D**), it merely led to marginal increase in the production of the (*R*)-3HB in strain TTrK (**Figure 13B**). It implied that the metabolic throughput between the acetyl-CoA and the (*R*)-3HB-CoA might be the limiting factor for (*R*)-3HB biosynthesis. In other words, the thiolase or the acetoacetyl-CoA reductase activity may be the bottleneck. Because the thiolase activity was measured to be more than two orders of magnitude higher than that of the acetoacetyl-CoA reductase (Wang et al., 2013, 2014), it was recognized that the bottleneck was the acetoacetyl-CoA reductase activity.

Increasing the co-factor availability has been proven as an effective approach to improving the enzyme activity (Niederholtmeyer et al., 2010; Shen et al., 2011). NADPH, a product of the photosynthesis process, is not only the driving force for carbon fixation in the Calvin cycle, but also the co-factor for the acetoacetyl-CoA reductase (PhaB1) in

the (*R*)-3HB biosynthesis pathway (**Figure 11**). Since photosynthesis activity is positively associated with the photon flux at lower range of light intensity (< 200 μE/m^2/s) (Williams and Laurensa, 2010; Yang et al., 2011), the next step was to cultivate strains PTrK12 and TTrK under irradiation of 150 μE/m^2/s (vs. previously 60 μE/m^2/s) in hope of stimulating the (*R*)-3HB production. Surprisingly, only a marginal increase of (*R*)-3HB production was observed for both strains TTrK and PTrK12. The acetate titers, however, were elevated to 2-fold and 3-fold higher, reaching 78.6 mg/L and 21.6 mg/L, in strains TTrK and PTrK12, respectively (**Figure 16**), indicating that the intracellular acetyl-CoA pool was significantly elevated. The higher fold change of the acetate production in strain PTrK12 was probably due to the enhanced expression of the TesB by the upregulated P_{psbA12} promoter under higher light condition. Based on the above results, it was speculated that the expression of the acetoacetyl-CoA reductase (PhaB1) rather than the co-factor NADPH supply was the limiting factor for (*R*)-3HB production.

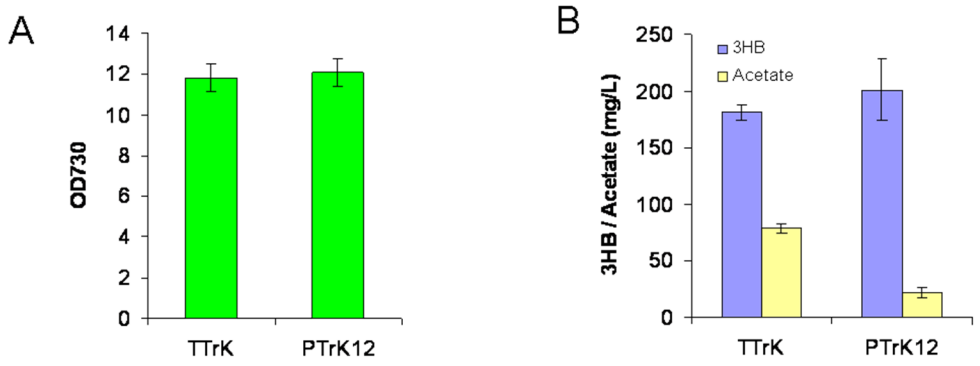

Figure 16 *Synechocystis* cell growth and (*R*)-3HB production under high light condition. (A) Cell density of strains TTrK and PTrK12 after growing for 5 days under a light

intensity of 150 µE/m^2/s. (B) (*R*)-3HB and acetate accumulation in the culture media of strains TTrK and PTrK12.

3.3.5 Multiple copies of *phaB* and *tesB*

In order to increase the acetoacetyl-CoA reductase activity, gene *phaB2* (from *R. eutropha* H16) that encodes an isozyme of PhaB1 was *de novo* synthesized by GenScript USA Inc (Piscataway, NJ, USA) after codon optimization (*phaB2*$_{eu\text{-}opt}$; Appendix A), placed under the control of the P_{tac} promoter and inserted into the chromosome of *Synechocystis* ABd-TTe [P_{tac}-*phaA-phaB1*, P_{tac}-*tesB*, *ΔphaEC*]. During the genetic manipulation, the *tesB* gene was also codon-optimized (*tesB*$_{opt}$; Appendix A), *de novo* synthesized by GenScript USA Inc (Piscataway, NJ, USA) and placed downstream of the *phaB2*$_{eu\text{-}opt}$ gene before being integrated into the chromosome of the *Synechocystis* ABd-TTe. The resultant strain was denominated as *Synechocystis* TTB2K3 [P_{tac}-*phaA-phaB1*, P_{tac}-*tesB*, P_{tac}-*tesB*$_{opt}$-*phaB2*$_{eu\text{-}opt}$, *ΔphaEC*] (**Table 8**). Strain TTB2K3 exhibited the same growth rate under irradiation of 60 µE/m^2/s compared to that of strain TTrK (**Figure 17A**). However, the TTB2K3 strain was able to produce (*R*)-3HB to a titer of 285.1 mg/L after five days, which was 1.6-fold higher than that of strain TTrK (**Figure 17B**). It was also noteworthy that the acetate production by strain TTB2K3 was also increased to 74.4±4.3 mg/L (vs. 41.3±4.3 mg/L for strain TTrK), probably due to the increased thioesterase activity (**Table 9**).

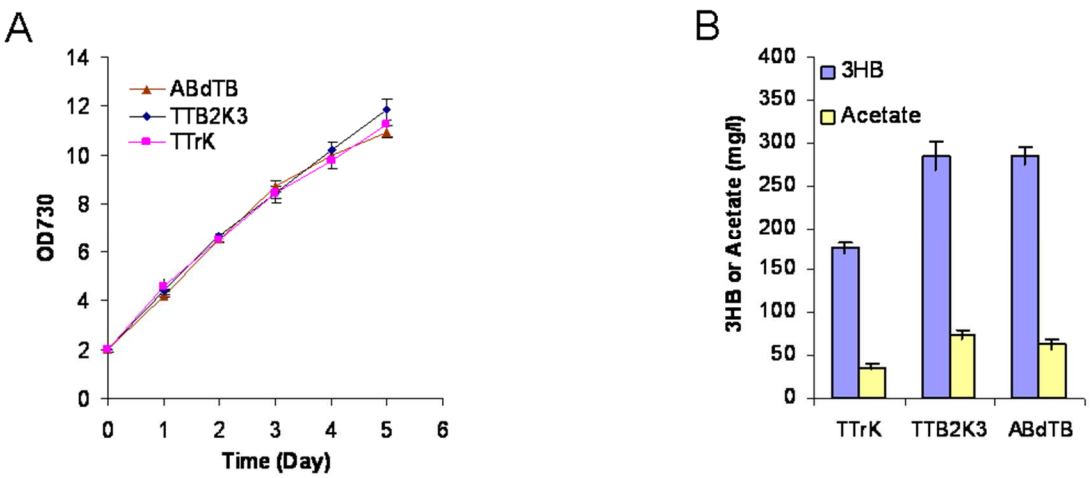

Figure 17 Performance of *Synechocystis* strains with two copies of *tesB* and *phaB*. (A) Cell growth curve of strains TTrK, TTB2K3 and ABdTB. (B) Production of (*R*)-3HB and acetate.

One copy of the *tesB* gene was then removed from the strain TTB2K3 to construct strain ABdTB (**Table 8**) in order to decrease the acetate production as well as to examine if the thioesterase activity was the limiting step for (*R*)-3HB biosynthesis. It was found that strain ABdTB exhibited similar growth rate and (*R*)-3HB productivity compared to that of strain TTB2K3, whereas the acetate production was decreased from 74.4 mg/L to 62.8 mg/L (**Figure 17**) probably due to the decreased thioesterase activity (**Table 9**). It once again provided evidence that the thioesterase activity was not the limiting factor in (*R*)-3HB production in above *Synechocystis* strains.

3.3.6 Optimization of the ribosome binding site for *phaB1*

Since the ribosome binding site plays a crucial role in initiating the translation of the corresponding gene, the ribosome binding site was then optimized to maximize the

expression of the (*R*)-3HB biosynthesis genes. It has been recognized that the Shine-Dalgarno (SD) sequence UAAGGAGG, which is perfectly complementary to the 3'-terminal sequence of 16S rRNA of *Escherichia coli*, could enable 3- to 6-fold higher translation efficiency than AAGGA regardless of the spacing between the SD and the translation start codon – ATG (Makrides, 1996). Next, the ribosome binding site (RBS) was examined upstream of the open reading frame of each 3HB biosynthesis gene, and it was found that the Shine-Dalgarno (SD) sequence upstream of gene *phaB1*, AAGGAGTGG, was not a perfect match to the 3'-terminal sequence (ACCUCCUUU) of the 16S rRNA in *Synechocystis* 6803 (Wang et al., 2012). The original SD sequence for *phaB1* was thus replaced by sequence AAGGAGGT (RBS_{opt}), which is fully complementary with the 3'-terminal sequence of 16S rRNA of *Synechocystis* 6803 (**Figure 18A**). The strain with the new RBS for gene *phaB1* was denominated as R154 (**Table 8**).

The acetoacetyl-CoA reductase (PhaB) activityincreased by about two-fold in strain R154 compared to that of strain TTrK (**Figure 18B**). While the growth of strain R154 exhibited a similar pattern compared to strain TTrK (**Figure 18C**), strain R154 was able to produce (*R*)-3HB at a titer 1.6-fold higher than that of strain TTrK, reaching 280.2 mg/L after five days of cultivation (**Figure 18D**). It suggested that RBS optimization is crucial to enhancing the expression of the downstream gene *phaB1* in *Synechocystis*, and the new RBS_{opt} was much more efficient in initiating the translation of *phaB1* relevant to the original RBS. Further replacing the P_{tac} promoter with the dual promoter P_{psbA12}-P_{tac} for the *phaA-phaB1* operon in *Synechocystis* strain R168 (**Table 8**) did not result in any difference in cell growth rate and the production of (*R*)-3HB and acetate.

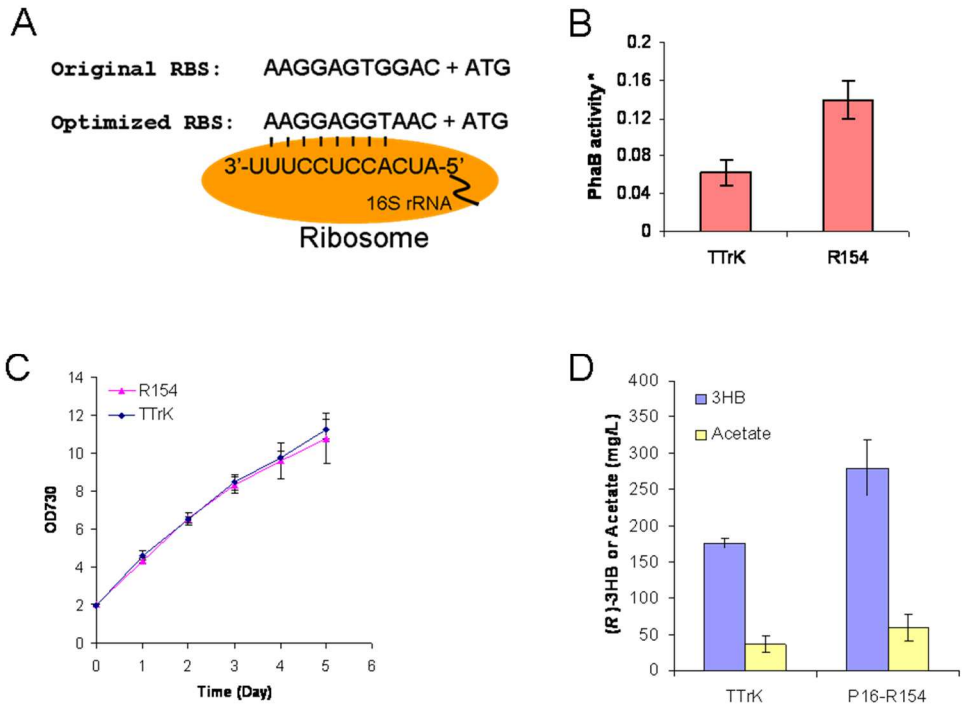

Figure 18 Optimization of the RBS for gene *phaB1* in *Synechocystis* strain R154. (A) The original and optimized ribosome binding site. (B) Acetoacetyl-CoA reductase activity. (C) Cell growth curve of strains TTrK and R54 cultured in BG11 medium under light of 60 μE/m^2/s with daily supplementation of 50 mM $NaHCO_3$. (D) Production of (*R*)-3HB and acetate in the culture.

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

The ability of *Synechocystis* strains to photosynthetically produce (*R*)-3HB directly from CO_2 was then examined by continuously aerating cultures with 1% CO_2. As shown in **Figure 19A**, *Synechocystis* strains R168 and ABdTB underwent relatively fast growth during the first four to five days, and the growth rate slowed down thereafter. In contrast,

the (*R*)-3HB production quickly accelerated after the first three to four days (**Figures 19B, C, D**), consistent with the previous observation described in Chapter 2 (Wang et al., 2013). Starting from day 4 until day 21 (when the experiment was stopped), strains R168 and ABdTB exhibited average (*R*)-3HB production rates of ~86 and ~65 mg/L/day, respectively, with peak productivities of 208 and 139 mg/L/day, respectively. Eventually, (*R*)-3HB was able to accumulate in the culture medium to titers of 1347 and 987 mg/L, respectively, after 21 days of continuous cultivation (**Figure 19B**). It is noteworthy that the cumulative titer of (*R*)-3HB was able to reach 1599 for strains R168 at the end of the cultivation (**Figure 19C**).

The dramatic increase of the (*R*)-3HB production rate compared to the previous result described in Chapter 2 (Wang et al., 2013) can probably be attributed to the following reasons. First, the enzyme activity of acetoaceyl-CoA reductase which is the bottleneck in the (*R*)-3HB biosynthesis (**Figures 17B, 18C**) was increased by 1.5- and 2.2-fold in strains ABdTB and R154, respectively (**Table 9**), leading to dramatic increase of the metabolic flux towards the (*R*)-3HB biosynthesis. Second, as photoautotrophic growth of *Synechocystis* alkalizes the medium (Summerfield and Sherman, 2008), the pH of the culture medium increased to 10~11 when atmospheric air (with 0.04% CO_2) was aerated into the culture in the previous experiment described in Chapter 2 (Wang et al., 2013), indicating that CO_2 supply was not able to meet the demand of *Synechocystis* cells. Herein, with aerating 1% CO_2 into the culture, the pH of the culture medium was able to be maintained at ~8.0 during the whole cultivation process, indicating that CO_2 supply was sufficient in this experiment. Third, in contrast to cultivation of *Synechocystis* cells by simply bubbling air into the flasks in the previous study described in Chapter 2, in this

study the flasks were placed in a shaker with a rate of 150 rpm during aeration, leading to more even distribution of the supplied CO_2 and photons in the cell population.

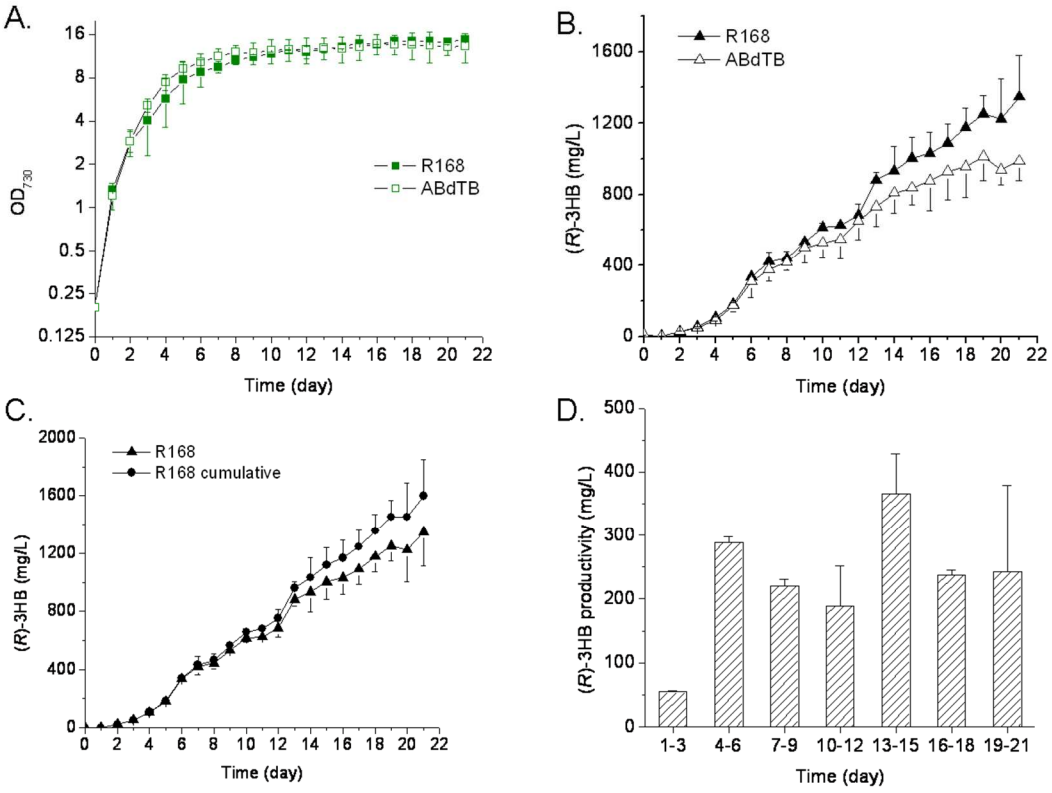

Figure 19 Photosynthetic production of (*R*)-3HB from CO_2 by *Synechocystis* strain R168. (A) Cell growth curve of strain R168. (B) Time course of in-flask and cumulative (*R*)-3HB generated by strain R168. (C) In-flask and cumulative titers of (*R*)-3HB generated by strain R168. (D) (*R*)-3HB generated in every 3 days.

3.4 Conclusion

In order for cyanoabcterial biotechnology to be economically feasible, chemicals of interest need to be produced at desirable high titers so that the expense in cultivation

maintenance and product recovery can be significantly reduced. To date, however, it remains a challenge to construct such types of cyanobacterial strains. Typically, the titers of chemicals that are photosynthetically produced by engineered cyanobacteria are below 1.0 g/L (Wang et al., 2012). The situation is partially due to limited well-characterized genetic tools and low level expression of the interest genes. In this chapter, six promoters in *Synechocystis* were completely characterized and it was found that the constitutive P_{tac} promoter was the strongest under the examined experimental condition (**Figure 13C**). P_{tac} and the light-inducible P_{psbA12} promoter exhibited the best performance regarding to the photosynthetic production of (*R*)-3HB. Recombination of the P_{psbA12} and P_{tac} to form a dual promoter did not improve the gene expression efficiency (**Figure 14**). The acetoacetyl-CoA reductase activity was found to be the limiting factor for biosynthesis of (*R*)-3HB in the engineered *Synechocystis*. Expressing an additional isozyme-encoding gene $phaB2_{eu\text{-}opt}$ or enhancing the *phaB1* gene expression by optimizing the RBS are both effective strategies to enhance the acetoacetyl-CoA reductase enzyme activity (**Table 9**), and both methods increased the (*R*)-3HB production by nearly 1.6-fold (**Figures 17B, 18D**). One of the engineered *Synechocystis* strain, R168, was able to produce and secrete (*R*)-3HB to the extracellular environment at an average rate of ~86 mg/L/day, with a peak productivity of 208 mg/L/day. The eventually achieved cumulative titer of ~1.6 g/L is to date the highest titer reported in cyanoabcterial production of the hydroxyalkanoates, precursors for biodegradable plastics and fine chemicals. In this chapter, it was demonstrated that fine-tuning the expression of the 3HB biosynthesis genes at gene copy number, transcriptional and translational levels is critical to improving the photosynthetic production of (*R*)-3HB in the engineered *Synechocystis*.

CHAPTER 4

PRE-METHYLATION OF FOREIGN DNA IMPROVES INTEGRATIVE TRANSFORMATION EFFICIENCY IN *SYNECHOCYSTIS* SP. PCC 6803

4. 1 Introduction

Despite increasing endeavors in developing and applying synthetic biology tools in cyanobacteria in recent years (Heidorn et al., 2011; Ruffing, 2011; Wang et al., 2012), one of the key elements to assure success of such efforts is to establish genetic transformation methodologies of high efficiency in cyanobacteria. During this research, for a set of fixed-length homologous arms the transformation of *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) with relevant short DNA constructs of interest usually resulted in a decent number of colonies on the selection BG11 plates. However, it was noticed that the integrative transformation efficiency varied between different DNA constructs of the same size. In other words, it is harder to get some genes of interest integrated into the *Synechocystis* chromosome compared to other DNA constructs with the same size. The situation became worse when the size of the DNA constructs of interest increased. Sometimes, only several or zero colonies formed on the selection BG11 plates, impeding the application of synthetic biology and genome engineering in cyanobacterium *Synechocystis*. To this end, it became an urgent need to develop high-efficiency transformation protocols in *Synechocystis*.

Since its first demonstration in cyanobacterium *Anacystis nidulans* 602 in 1970 (Shestakov and Khyen, 1970), genetic transformation protocols for a variety of

cyanobacterial species have been developed and optimized (Stevens and Porter, 1980; Grigorieva and Shestakov, 1982; Golden and Sherman, 1984; Golden et al., 1987; Matsunaga and Takeyama, 1995; Brahamsha, 1996; Koksharova and Wolk, 2002; Eaton-Rye, 2004; Frigaard et al., 2004; Onai et al., 2004; Stucken et al., 2012). However, despite the exciting achievement made in the past decades, the transformation efficiency in cyanobacteria is still typically lower than other model systems, such as *E. coli* and yeast, mainly due to the various biological barriers against invaded DNA among which is the restriction degradation of foreign DNA in the host cyanobacterium strains (Koksharova and Wolk, 2002).

Most bacteria carry specific restriction-modification (RM) systems that are able to recognize and degrade foreign DNA from the self DNA. Each RM system typically consists of one methylase (also called methyltransferase) and one restriction endonuclease; the methylase protects the self DNA from restriction digestion by methylating the nucleotides at specific DNA sequences (*i.e.*, restriction sites), while the foreign DNA which usually bears a different methylation pattern would be recognized and degraded by the endonucleases (Tock and Dryden, 2005). It has been reported that RM systems could dramatically reduce the DNA transformation efficiency in a variety of bacteria (Bian and Li, 2011; Dong et al., 2010; Flett et al., 1997; Koksharova and Wolk, 2002; Monk and Foster, 2012; Purdy et al., 2002; Yasui et al., 2009), including cyanobacteria (Cobley et al., 1993; Elhai et al., 1997; Lyra et al., 2001; Moser et al., 1993; Thiel and Poo, 1989). For instance, Elhai and co-workers reported that the conjugal transfer efficiency of cyanobacterium *Anabaena* sp. PCC 7120 was reduced by three

restriction activities as an exponential function of the number of unprotected sites when the number of the sites was low (Elhai et al., 1997).

In order to overcome the restriction barrier and thus enhance the transformation efficiency in cyanobacteria, two possible approaches may be considered: *i*) temporary or permanent inactivation of the RM systems (Edwards et al., 1999; Iwai et al., 2004); *ii*) pre-methylation of the donor DNA using the methylases from the acceptor cyanobacterium (Aune and Aachmann, 2010; Koksharova and Wolk, 2002; O'Connell Motherway et al., 2009; Yasui et al., 2009; Zhang et al., 2012). For instance, Iwai and colleagues reported that after a putative type I restriction endonuclease of the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 was disrupted, the transformation efficiency in the disruptant was increased by 4- to 6-fold for some constructs and some previously non-transferable constructs were successfully integrated into the genome (Iwai et al., 2004). Although it has been suggested that genetic transformation of cyanobacteria could be improved through pre-methylation of the constructs before transformation (Aune and Aachmann, 2010; Koksharova and Wolk, 2002; Yasui et al., 2009), to date no experimental evidence has been provided.

Cyanobacterium *Synechocystis* 6803 is naturally transformable and the transformation procedure was optimized in the past several decades (Grigorieva and Shestakov, 1982; Kufryk et al., 2002; Zang et al., 2007). It was reported that the transformation efficiency of *Synechocystis* 6803 was increased by two orders of magnitude when the *sll1354* gene, encoding the exonuclease RecJ, was knocked out (Kufryk et al., 2002). However, the physiological effect of inactivation of the exonuclease RecJ was unclear. In contrast, using pre-methylation of foreign DNA to increase the integrative transformation

efficiency does not pose any change to the host genetic background, and therefore is more desirable. In this study, two cytosine-specific methylase genes, *sll0729* and *slr0214*, have been cloned from the chromosome of *Synechocystis* 6803 (Kaneko et al., 1996). Specifically, gene *slr0214* from *Synechocystis* 6803 encodes a cytosine-specific methyltransferase that probably targets the first cytosine of the *Pvu*I site (5'-CGATCG-3') (Scharnagl et al., 1998); and gene *sll0729* has been predicted to encode a cytosine-specific methyltransferase that recognizes and functions on the cytosine base(s) of the sequence 5'-GGCC-3' (Kaneko et al., 1996; Roberts et al., 2007). These two genes were cloned and co-expressed in the integration-plasmid-harboring *E. coli*, and the effects of pre-methylation of foreign DNAs on the integrative transformation efficiency in *Synechocystis* 6803 was investigated..

4. 2 Materials and methods

4.2.1 Strains and culture conditions

All strains used in this chapter are listed in **Table 10**. *E. coli* strain XL1-Blue MRF' (Stratagene, La Jolla, CA, USA) was used as the host for all plasmids. All recombinant *E. coli* strains were cultivated in LB medium under 37^{o}C, 175 rpm. Solid LB plates were prepared by adding agar to a final concentration of 1.5% (*w*/*v*). Antibiotics were supplemented into the LB medium to final concentrations of 100 µg/mL for ampicillin and 100 µg/mL for chloramphenicol when necessary to maintain the plasmids. *Synechocystis* 6803 was cultivated in BG11 medium under light with intensity of 35 µE m^{-2} s^{-1}. 10 mM TES (pH 8.2), 3 g/L thiosulfate and 1.5% (*w*/*v*) agar was supplemented to

BG11 before autoclaving to prepare solid agar plates. A final concentration of 10 µg/mL kanamycin was supplemented into the BG11 plates to select successful transformants.

Table 10 Strains and plasmids used in Chapter 4.

	Genotype	References
Strains		
E. coli XL1-Blue MRF'	*Δ*(*mcrA*)*183 Δ*(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA*1 *gyrA96 relA1 lac* [*F´ proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]	Stratagene
Synechocystis sp. PCC 6803	Wild-type	ATCC
Plasmids		
pBS-SPtK	Amp^{R}, pUC ori, f1(+) ori, P_{tac}-Kam^{R}	This study
pACYC184	Cm^{R}, Tet^{R}, p15A ori	New England BioLabs
pAC-M	*sll0729* inserted between BamHI and SalI sites of pACYC184	This study
pAC-C	*slr0214* inserted between BamHI and SalI sites of pACYC184	This study
pAC-MC	*sll0729* and *slr0214* inserted between BamHI and SalI sites of pACYC184	This study
pAC-Mv	pAC-M but 5'-untranslated region (UTR) optimized for *sll0729*	This study
pAC-Cv	pAC-C but 5'-UTR optimized for *slr0214*	This study
pAC-MCv	pAC-MC but 5'-UTR optimized for *sll0729*	This study

4.2.2 Construction of plasmids

The integration plasmid pBS-SPtK targeting the *Synechocystis* chromosome was constructed as described in Section 2.2.2. The schematic structure of plasmid pBS-SPtK is illustrated in **Figure 20**.

Help Plasmids used to express *Synechocystis* methylases were constructed as follows. Gene *sll0729* (M) which encodes a modification methylase was cloned from the genome

of *Synechocystis* 6803, digested with *Bgl*II and *Sal*I, and inserted between the *Bam*HI and *Sal*I sites on plasmid pACYC184 to construct plasmid pAC-M. Similarly, gene *slr0214* (C; encoding a cytosine-specific methyltransferase) was cloned from the genome of *Synechocystis* 6803, digested with *Bam*HI and *Sal*I, and inserted between the *Bam*HI and *Sal*I sites on and inserted into pACYC184 to constructed plasmid pAC-C. Gene C was digested with *Bam*HI and *Sal*I and placed downstream of the gene M on plasmid pAC-M to construct plasmid pAC-MC. When each gene was cloned by PCR, the native RBS was included. Particularly, a point mutation was introduced by the primer in PCR amplification of gene M to result in a stronger RBS (**Table 11**).

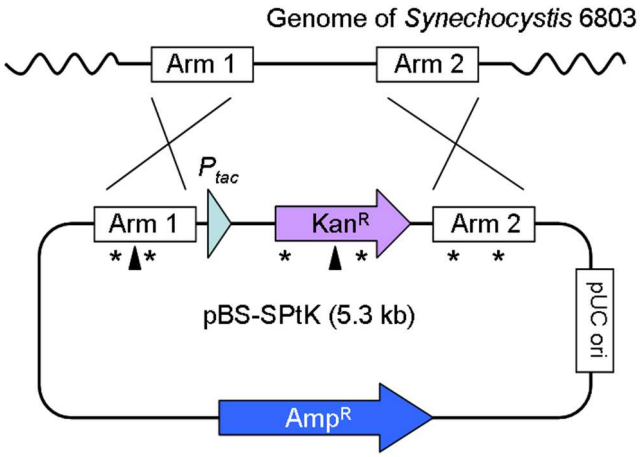

Figure 20 Integrative plasmid pBS-SPtK and schematic representation of the homologous recombination. Arm 1, the left homologous DNA fragment, part of which is *slr1362*. Arm 2, the right homologous DNA fragment, part of which is *sll1274*. Asterisks indicate the sites with DNA sequence 5'-GGCC-3'; black arrow heads represent the sites with DNA sequence 5'-GCGATCGC-3' (*Pvu*I site underlined).

To optimize the 5'-untranslated region for gene M, primers Ptet2 and MMS5 were used in PCR with plasmid pAC-M or pAC-MC as the template. The PCR products were

digested with *Eco*RV before being ligated to form plasmid pAC-Mv and pAC-MCv, respectively. To optimize the 5'-untranslated region for gene C, primers CSM3 and CSM4 were used in PCR with plasmid pAC-C as the template. The PCR products were digested with *Hind*III and *Bam*HI before being inserted between these two sites on plasmid pACYC184 to construct plasmid pAC-Cv. High fidelity *Phusion* DNA polymerase was utilized in all PCR amplifications. All the plasmid constructs were confirmed by DNA sequencing. All the PCR primers are listed in **Table 11**.

Table 11 Primers used in Chapter 4.

Name		**Usage**
Primers (5' to 3') used for DNA recombination		
MMS1	GAAGATCTGAGGAATAGAACTATGGAGGAAAC	pAC-M
MMS2	ATGGTCGACTAGGATCCGTTATAACCTTCAGGATTACTCATG	pAC-M
MMS5	GACGATATCAGGAGGAATAGAACTATGGAGGAAAC	pAC-Mv, pAC-MCv
Ptet2	GACGATATCAGCAATTTAACTGTGATAAACTAC	pAC-Mv, pAC-MCv
CSM1	TAGGATCCAGGAAAAACCATGGCCAGAC	pAC-C
CSM2	ATGGTCGACTTGGAGTGGTAATTCTAACTGC	pAC-C
CSM3	GATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATTGCTAGG AGGAAAAACCATGGCCAGAC	pAC-Cv
CSM4	CATGGATCCTAATTCTAACTGCTTTAGGAATG	pAC-Cv
Primers (5' to 3') used for RT-qPCR		**Targets**
16S-F2	CCACGCCTAGTATCCATCGT	*Synechocystis* 16S
16S-R2	TGTAGCGGTGAAATGCGTAG	*Synechocystis* 16S
MMS1	TTACCGATTCTTCCATTGATAG	*sll0729*
MMS2	TCCTCGGAATCATCATAGG	*sll0729*
CSMq1	CCAATACACTACGCCTTACCTAG	*slr0214*
CSMq2	CCGGCAAATCCTCAACAG	*slr0214*

4.2.3 Preparation of DNA for transformation

Each Help Plasmid was co-transferred into *E. coli* strain XL1-Blue MRF'. As a control, plasmid pACYC184 was co-transferred into *E. coli* XL1-Blue MRF'. Ampicillin and chlorhamphenicol were each supplemented into the LB medium to a final concentration of 100 µg/mL to maintain the plasmids.

The co-transformed *E. coli* strains were cultivated in antibiotics-supplemented LB medium for 12 hours before plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). The plasmid mixture purified from each strain was digested with restriction enzymes *Xho*I, *Sac*I and *Xba*I to confirm the coexistence of the integration plasmid and each Help Plasmid or the control plasmid pACYC184. Each digested sample was further diluted and analyzed using theAgilent DNA 12000 Kit on the Agilent 2100 Bioanalyzer system (Agilent Technologies, Inc., Wilmington, DE, USA) to quantify the concentrations of DNA fragments and thus to eventually calculate the concentration of the integration plasmid. Each plasmid mixture sample was then properly diluted so that the concentration of the integration plasmid pBS-SPtK was 100 µg/mL.

4.2.4 Transformation of *Synechocystis*

Synechocystis 6803 was grown until OD_{730} = ~0.4. Then, 50 µl of the culture was taken into each 1.5 mL Eppendorf tube and mixed with 5.5 µl of above plasmid mixture. The final concentration of the integration plasmid was 10 µg/mL in each transformation mixture. The Eppendorf tubes were then incubated at 30 ^{o}C under light with an intensity of ~15 µE m^{-2} s^{-1} for 5 h, shaken once at 2.5 h. The transformation mixture was

transferred onto BG11 plates amended with 10 μg/mL kanamycin. Colonies were counted after 1~2 weeks.

4.3 Results and discussion

4.3.1 Preparation of DNA for *Synechocystis* transformation

The integration plasmid pBS-SPtK, designated to target the genome of *Synechocystis* 6803, is schematically represented in **Figure 20**. The kanamycin resistance gene was placed under a strong promoter P_{tac}. The two DNA fragments, Arm 1 and Arm 2, designed to target the genome of *Synechocystis* 6803 via homologous recombination were about 650 bp each (**Figure 20**).

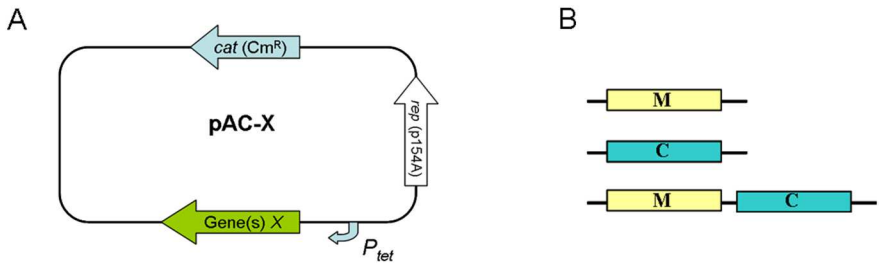

Figure 21 Help Plasmids used to express DNA methylases of *Synechocystis 6803* in *E. coli*. (A) Scheme of the Help Plasmids. Gene(s) *X* represents *Synechocystis* methylase genes. (B) Genetic structures of the region of Gene(s) *X* on Help Plasmids. M, *sll0729*; C, *slr0214*.

A total of three Help Plasmids were constructed to express *Synechocystis* DNA methylase(s) in *E. coli* (**Figure 21**). Each Help Plasmid has an origin of plasmid

pACYC184 which is compatible with the pUC origin of the integration plasmid pBS-SPtK. Therefore each Help Plasmid could coexist with the integration plasmid in the *E. coli* host. After co-transformation of *E. coli*, the coexistence of the integration plasmid pBS-SPtK and the Help Plasmid was confirmed by restriction digestion followed by agarose gel electrophoresis (**Figure 22**). The integration plasmid was digested to 2.5 kb and 2.8 kb; the Help Plasmids were linearized to DNA fragments bigger than 4.0 kb (**Figure 22**).

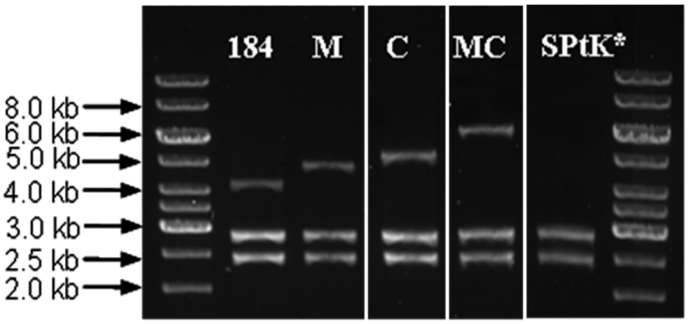

Figure 22 Confirmation of the coexistence of integrative plasmid pBS-SPtK and each Help Plasmid. The leftmost and rightmost lanes are standard DNA ladders. Lanes 184, M, C, MC indicate coexistence of the integrative plasmid pBS-SPtK with pACYC184 (Control 1), pAC-M, pAC-C or pAC-MC, respectively. Lane SPtK*, existence of pBS-SPtK only (Control 2).

As the DNA concentration is critical to the transformation efficiency of *Synechocystis* (Grigorieva and Shestakov, 1982; Kufryk et al., 2002; Zang et al., 2007), each plasmid mixture sample was further analyzed with the Bioanalyzer to quantify the DNA concentration for the integration plasmid pBS-SPtK. After three replicates of DNA

analysis via Bioanalyzer, each DNA mixture sample was then diluted by dH_2O to a final concentration of 100 µg/mL and used for transformation of *Synechocystis* 6803.

4.3.2 Pre-methylation of DNA improves integrative transformation efficiency

The integrative plasmid pBS-SPtK cannot replicate and acts as an integrative plasmid when transferred into cyanobacterium *Synechocystis* 6803 (Heidorn et al., 2011; Wang et al., 2012); thus, *Synechocystis* cells would grow on the kanamycin-amended BG11 plates only when the kanamycin resistance marker has been integrated into the genome via homologous recombination. As a result, the number of colonies shown on the kanamycin-amended BG11 plates can be used as an indicator of the integrative transformation efficiency. As shown in **Figure 23**, expression of individual *Synechocystis* methylase M or C in the pBS-SPtK harboring *E. coli* host has exerted marginal effects on the transformation efficiency in *Synechocystis* 6803 (**Figure 23**). However, after the integration plasmid pBS-SPtK was co-transferred with the Help Plasmid pAC-MC and propagated in *E. coli*, the integrative transformation of *Synechocystis* 6803 by pBS-SPtK dramatically increased, about 7.5-fold higher than the control (**Figure 23**). The results suggested that either there was cooperation of M (Sll0729) and C (Slr0214) on methylation of the integrative plasmid pBS-SPtK or the expression of methylase C (Slr0214) was very poor via plasmid pAC-C but was significantly improved when expressed via plasmid pAC-MC. To date, no study has reported on the cooperation of two or multiple methylase enzymes (especially those targeting different feature sequences) during DNA methylation.

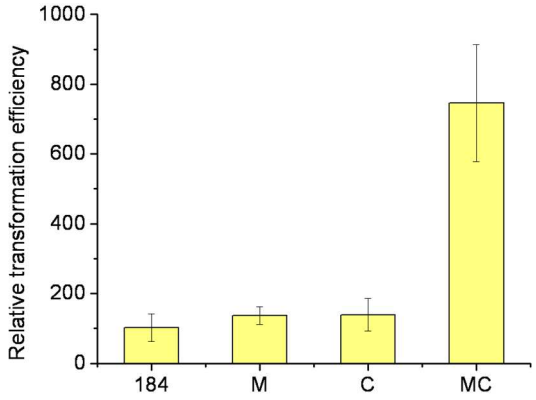

Figure 23 Transformation efficiency of the integrative plasmid pBS-SPtK into *Synechocystis* 6803. Lanes 184, M, C and MC indicate the effect of coexistence with plasmid pACYC184 (Control 1), pAC-M, pAC-C or pAC-MC on the transformation efficiency of plasmid pBS-SPtK.

4.3.3 Improved expression of Slr0214 facilitates integrative transformation

Expression of methylase genes in the pBS-SPtK-harboring *E. coli* cells was confirmed first by analyzing the mRNA using RT-qPCR (Wang et al., 2013). In this study, expression of methylase genes M (*sll0729*) and C (*slr0214*) was driven by the P_{tet} promoter on the pACYC derived plasmids. In order to find out if adding inducer tetracycline would impact the transcription level, 10 µg/mL of tetracycline was added into the LB culture medium when the OD_{600} reached ~0.5. Flasks were then wrapped by aluminum foil to avoid any degradation of tetracycline caused by light. As shown in **Figure 24**, the mRNA abundance of the target methylase genes in the uninduced *E. coli* cells was at the same level as that of the induced cells, indicating that the P_{tet} promoter

was efficient enough in expressing the methylase genes M and C without using any tetracycline.

Next, the Gibbs free energy related to the translation initiation rate (TIR) of each methylase gene was calculated using a previously established method (Salis et al., 2009). The results showed that the ΔG_{total} was 5.13 kcal/mol for translation initiation of gene M when expressed on pAC-M and pAC-MC, indicating expression of gene M was very poor. The ΔG_{total} for gene C translation initiation on plasmid pAC-MC was smaller than that of pAC-C which resulted in 3.4-fold higher TIR on pAC-MC compared to that on pAC-C (**Table 12**). This is consistent with the aforementioned improved integrative transformation efficiency using pAC-MC as the Help Plasmid (**Figure 23**).

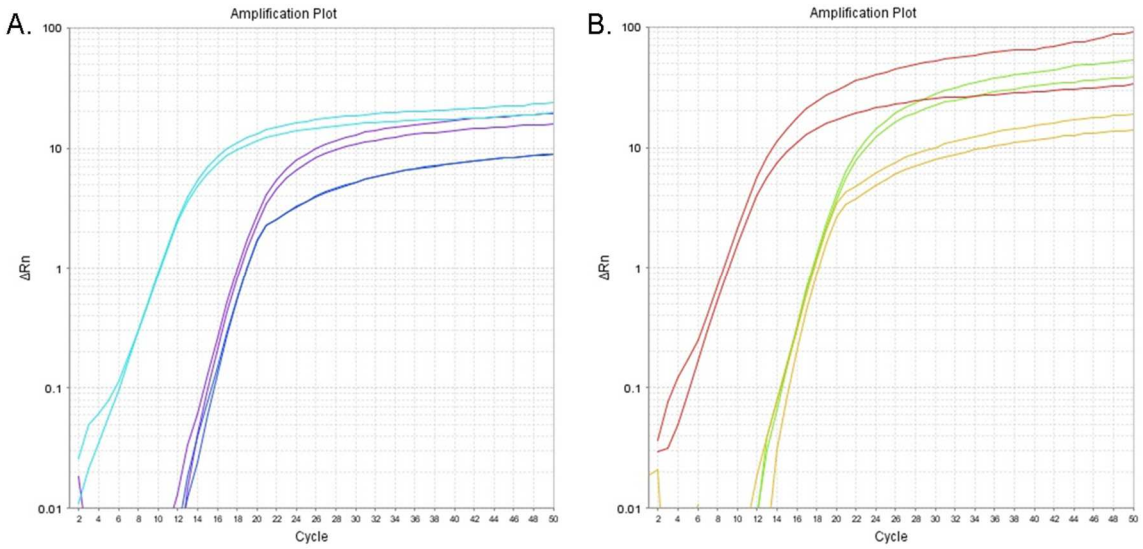

Figure 24 RT-qPCR analysis for the mRNA of methylase genes M and C in *E. coli* strain XL1-BlueMRF'/pAC-MC with (A) and without (B) tetracycline induction.

The 5'-untranslated regions (UTR) of genes M and C were modified by constructing plasmids pAC-Mv, pAC-Cv and pAC-MCv. In the new constructs, the ΔG_{total} value for genes M and C were much smaller, and the TIRs for genes M and C on plasmids pAC-Mv and pAC-Cv were increased by 25- and 151-fold, respectively, compared to the parent plasmids pAC-M and pAC-C (**Table 12**). Each of Help Plasmid pAC-Mv, pAC-Cv and pAC-MCv was used to transform *E. coli* XL1-Blue MRF'/pBS-SPtK. The co-existence of each 5'-UTR modified Help Plasmid and the integrative plasmid pBS-SPtK was confirmed (**Figure 25**). The DNA concentrations of the pBS-SPtK were measured via Bioanalyzer (**Figures 26, 27**), and subsequently carefully diluted to 100 µg/mL before being used to transform *Synechocystis* 6803.

Table 12 Gibbs free energy and translation initiation rates [a]

	ΔG_{total} for M	ΔG_{total} for C
pAC-M	5.13 (249)	-
pAC-C	-	6.35 (143)
pAC-MC	5.13 (249)	3.65 (484)
pAC-Mv	-2.02 (6214)	-
pAC-Cv	-	-4.79 (21556)
pAC-MCv	-2.02 (6214)	3.65 (484)

[a] Values are shown in a unit of kcal/mol; values in bracket indicate the relative translation initiation rate in each case.

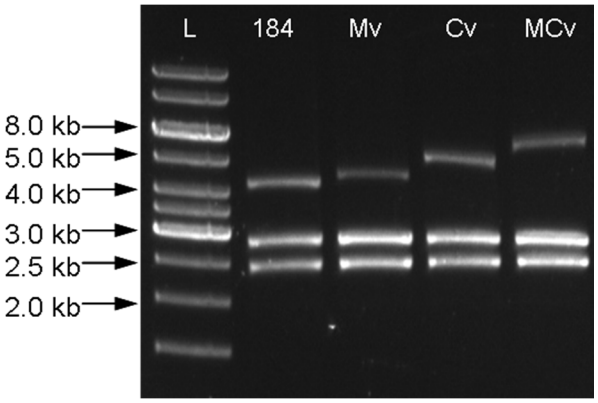

Figure 25 Confirmation of the coexistence of integrative plasmid pBS-SPtK and the optimized Help Plasmids pAC-Mv, pAC-Cv, pAC-MCv and the control plasmid pACYC184. Lane L, standard DNA ladders. Lanes 184, Mv, Cv, MCv indicate coexistence of the integration plasmid pBS-SPtK with pACYC184 (Control 1), pAC-Mv, pAC-Cv or pAC-MCv, respectively.

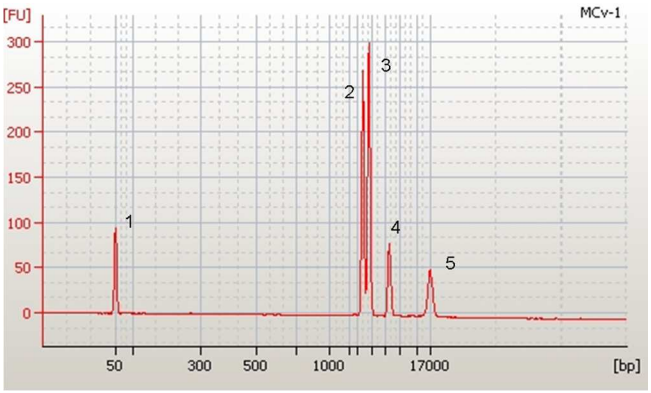

Figure 26 Measurement of DNA concentrations of pBS-SPtK and Help Plasmid pAC-MCv by Bioanalyzer. Peak 1, standard, 50 bp, 8.3 ng/µl. Peak 2 and 3, the 2.5 kb and 2.8 kb fragments of the digested pBS-SPtK, respectively. Peak 4, the linearized Help Plasmid pAC-MCv. Peak 5, standard B, 17000 bp, 4.2 ng/µl.

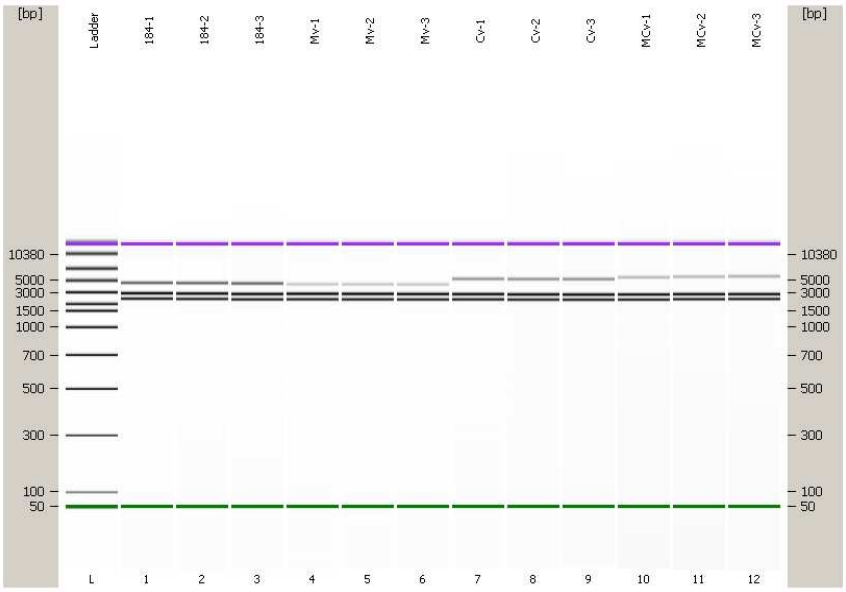

Figure 27 Gel image created by Bioanalyzer for restriction digested plasmids pACYC184, pAC-Mv, pAC-Cv and pAC-MCv.

When these redesigned and subsequently constructed Help Plasmids were co-transferred with the integration plasmid pBS-SPtK into *E. coli* hosts before transforming *Synechocystis* 6803, it resulted in up to 161-fold higher efficiency in the later integrative transformation of *Synechocystis* 6803 (**Figure 28**). The results indicated that optimization of the expression of cytosine-specific methylase C (Slr0214) played an essential role in improving the transformation efficiency, and the improvement of the transformation efficiency was proportional to the TIR of methylase C (**Figures 23, 28**; **Table 12**). However, improved expression of cytosine-specific methylase M (Sll0729) showed little impact on the transformation efficiency in *Synechocystis* 6803 (**Figure 28**), suggesting that methylase M is dispensable for the pre-methylation of foreign DNA.

It was reported that methylase C (Slr0214) specifically methylates the first cytosine of the sequence 5'-CGATCG-3' which blocks restriction digestion from the *Pvu*I and *Sgf*I endonucleases (which recognizes 5'-GCGATCGC-3') (Scharnagl et al., 1998), while modification methylase M(Sll0729) methylates the cytosine base(s) of the sequence 5'-GGCC-3' (Kaneko et al., 1996; Roberts et al., 2007). In the study here, the sequence of the integration plasmid pBS-SPtK was screened and it was found two sites of 5'-CGATCG-3' and six sites of 5'-GGCC-3' along the DNA sequence of the integration fragment (**Figure 20**). It is speculated that the significantly increased transformation efficiency using Help Plasmid pAC-Cv was probably due to the protection of the integration fragment from digestion by the endogenous restriction enzymes in *Synechocystis* 6803. It also suggested that restriction digestion of foreign DNA posed a significant barrier in transformation of cyanobacterium *Synechocystis* 6803.

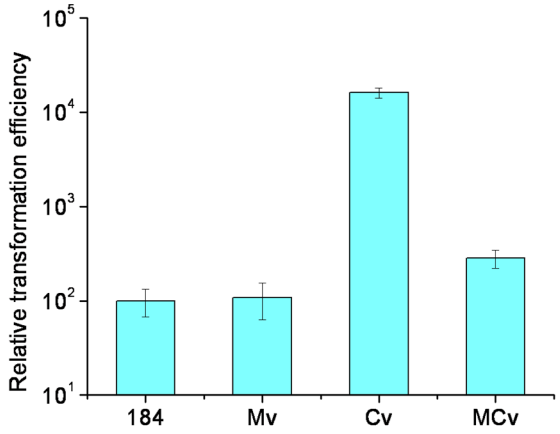

Figure 28 Transformation efficiency after optimizing the translation of methylase genes.

4.4 Conclusion

As synthetic biology applications in cyanobacteria dramatically increased in recent years, strategies to enhance the genetic transformation efficiency in cyanobacterial species have become an urgent need. Albeit pre-methylation of exogenous DNA was proven effective in increasing the genetic transformation efficiency in several bacterial species (O'Connell Motherway et al., 2009; Yasui et al., 2009; Zhang et al., 2012), experimental evidence in cyanobacteria was still missing. In this study, two cytosine-specific methylase genes M (*sll0729*) and C (*slr0214*) were cloned from the chromosome of *Synechocystis* 6803 and expressed via Help Plasmids in the integrative-plasmid-harboring recombinant *E. coli*. Transformation results indicated that while expression of the methylase gene M (*sll0729*) had little effect on the integrative efficiency in *Synechocystis* 6803, expression of the methylase gene C (*slr0214*) was able to dramatically increase the transformation efficiency in *Synechocystis* 6803. Optimization of the C (*slr0214*) expression via redesign of the 5'-UTR to increase the translation initiation rate eventually led to approximately two orders of magnitude higher transformation efficiency in *Synechocystis* (**Figure 28**).

CHAPTER 5

DISSERTATION CONTRIBUTIONS AND FUTURE WORK

5.1 Summary of research contributions

1. Successfully engineered cyanobacterium *Synechocystis* sp. PCC6803 to produce (*S*)- and (*R*)-3-hydroxybutyrate (3HB) directly from light and CO_2, which may contribute to a more sustainable chemical industry (**Figure 29**).
2. Integration of the thioesterase-encoding gene *tesB* into the *Synechocystis* chromosome as well as screening of the thiolase and acetoacetyl-CoA reductase isoenzymes from different microbial sources to drive the 3HB biosynthesis from central metabolite acetyl-CoA. Isoenzymes from *Ralstonia eutropha* H16 were proven bearing highest enzyme activities when expressed in *Synechocystis*.
3. Analysis of the dynamics of the intermediate metabolite acetyl coenzyme A and the concentrations of essential nutrients, including nitrate and phosphate, in the culture media. It was found that the phosphate consumption during the photoautotrophic growth and the concomitant elevated acetyl-CoA pool acted as a key driving force for 3HB biosynthesis.
4. Characterization of a total of six promoters in *Synechocystis* and discovery that the light-independent P_{tac} promoter was the strongest in expressing 3HB biosynthesis genes in *Synechocystis*.
5. The acetoacetyl-CoA reductase activity was found to be the bottleneck for 3HB biosynthesis in *Synechocystis*, and was mitigated either by introducing a second copy of the *phaB* gene or by optimizing the upstream ribosome binding site.

6. The re-engineered *Synechocystis* strain R168 was able to produce (*R*)-3HB to a cumulative titer of 1600 mg/L, with a peak daily productivity of ~200 mg/L, using light and CO_2 as the sole energy and carbon sources, respectively, which is to date the highest productivity of hydroxyl-fatty acid in cyanobacteria
7. The integrative transformation efficiency in *Synechocystis* 6803 was increased by about two orders of magnitude after pre-methylation of the exogenous DNA. Effective expression of methylase genes, which could be achieved by optimizing the 5'-untranslated region, proved critical to efficient pre-methylation of the donor DNA.

A list of publications:

1. Wang, B., Wang, J., Zhang, W., Meldrum, D. R. (2012). Application of synthetic biology in cyanobacteria and algae. *Front. Microbiol.* 3, 344.

2. Wang, B., Pugh, S., Nielsen, D. R., Zhang, W., Meldrum, D. R. (2013). Engineering cyanobacteria for photosynthetic production of 3-hydroxybutyrate directly from CO_2. *Metab. Eng.* 16, 68-77.

3. Wang, B., Zhang, W., Meldrum, D. R. Pre-methylation of foreign DNA improves integrative transformation efficiency in *Synechocystis* sp. PCC 6803. (submitted)

4. Wang, B., Meldrum, D. R. Enhanced photosynthetic production of 3-hydroxybutyrate via gene expression fine-tuning in *Synechocystis*. (in preparation)

5. Wang, B., Pugh, S., Nielsen, D. R., Zhang, W., Meldrum, D. R. Photosynthetic production of 3-hydroxybutyrate directly from CO_2. 112th ASM General Meeting. San Francisco, California. (Poster | Jun. 2012)

6. Wang, B., Nielsen, D. R., Zhang, W., Meldrum, D. R. Photosynthetic conversion of CO_2 to building-blocks for renewable plastics. BIOT Division, 247th ACS National Meeting. Dallas, Texas. (Poster | Mar. 2014)

7. Wang, B., Zhang, W., Meldrum, D. R., Nielsen, D. R. Photosynthetic production of 3-hydroxybutyrate from carbon dioxide. Patent pending: PCT/US13/29997; WO/2013/172928.

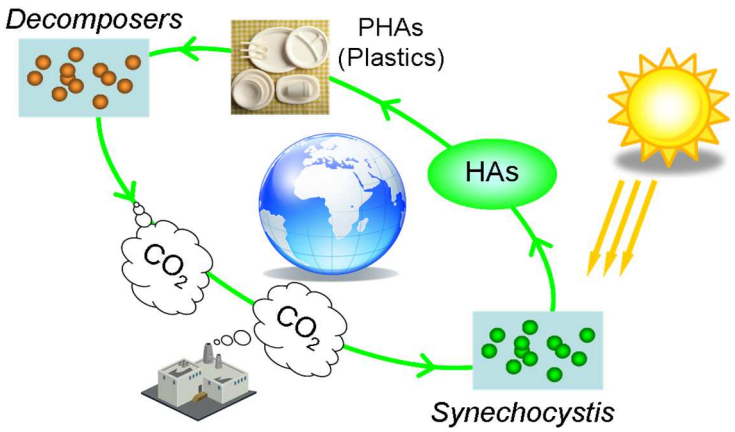

Figure 29 Sustainable production of building blocks for plastics.

5.2 Future work

The metabolic flux towards 3HB biosynthesis can be further enhanced by optimizing the gene network at a global level. Transcriptomics, proteomics and metabolomics followed by flux balance analysis may shed light on engineering the next generation of cyanobacteria for enhanced production of 3HB.

The upstream metabolic pathway, i.e., light harvesting and conversion of CO_2 to acetyl-CoA, may be enhanced in order to further increase the 3HB biosynthesis. One effective approach that was demonstrated was to enhance the expression of the RuBisCO genes in cyanobacteria so that the carbon fixation can be enhanced via the Calvin cycle (Atsumi et al., 2009). Alternative carbon fixation pathways, such as the 3-hydroxypropionate bicycle (Shih et al., 2014), may also be considered.

It was suggested that the hydrophobic cell membrane became a significant barrier for production and secretion of hydrophilic products, such as hexose and lactic acid, by the

genetically engineered cyanobacteria (Niederholtmeyer et al., 2010). Secretion of both products into the culture medium thus relied on the co-expression of the product-specific transporters, namely those encoded by gene *glf* and *lldP* from *Zymomonas mobilis* and *E. coli*, respectively (Niederholtmeyer et al., 2010). However, without co-expressing any transporter genes in *Synechocystis*, hydrophilic 3HB molecules were secreted to the culture medium in this study. While it is possible that *Synechocystis* 6803 naturally expresses unidentified transporters for the 3HB secretion, the genetic system responsible for such a process is yet to be identified.

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APPENDIX A

DNA SEQUENCE OF CODON-OPTIMIZED *$TESB_{OPT}$-$PHAB2_{EU\text{-}OPT}$* CONSTRUCT

The DNA sequenced below includes P_{tac} promoter, *tesB*$_{opt}$, and *phaB2*$_{eu\text{-}opt}$. The start codons and the stop codons are boxed. The ribosome binding sites are underlined. Restriction sites for cloning are also included.

DNA sequence:

AGATCTAACTGCAGACATGCATGCGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTG
G**A**ATTGTGAGCGGATAACAATTTCACACAAGGAGGATATACAT
ATGAGTCAAG CTCTGAAAAA CTTACTGACT TTACTGAATT TGGAAAAAAT TGAAGAAGGA
TTATTTCGTG GTCAGAGTGA AGATTTAGGC TTACGTCAAG TGTTTGGCGG TCAAGTGGTT
GGTCAGGCCT TGTATGCTGC CAAAGAAACC GTGCCCGAAG AACGTTTGGT TCATAGCTTT
CACTCCTATT TTTTGCGTCC CGGTGATAGT AAAAAACCCA TTATTTACGA TGTGGAAACC
CTGCGGGATG GTAATAGCTT TAGTGCCCGT CGTGTGGCCG CTATTCAAAA CGGCAAACCC
ATTTTTTATA TGACTGCCAG CTTTCAAGCC CCCGAAGCTG GGTTTGAACA TCAGAAAACC
ATGCCCAGTG CCCCCGCTCC CGATGGTTTA CCCTCCGAAA CTCAAATTGC CCAAAGTCTG
GCTCATTTGT TACCCCCCGT GTTGAAAGAT AAATTTATTT GTGATCGCCC CCTGGAAGTG
CGTCCCGTGG AATTTCATAA TCCCTTGAAA GGTCATGTGG CCGAACCCCA TCGTCAAGTG
TGGATTCGCG CTAATGGCTC CGTGCCCGAT GACTTGCGCG TTCATCAGTA TCTGTTGGGT
TACGCCAGTG ATTTGAATTT TCTGCCCGTG GCTTTACAAC CCCACGGCAT TGGTTTTCTG
GAACCCGGGA TTCAGATTGC CACCATTGAC CATAGTATGT GGTTTCATCG TCCCTTTAAT
TTGAATGAAT GGTTACTGTA TTCCGTGGAA TCCACCAGTG CCTCCAGTGC TCGTGGCTTT
GTGCGGGGTG AATTTTATAC CCAAGATGGC GTGTTAGTTG CCAGTACCGT GCAAGAAGGT
GTGATGCGTA ATCATAATTA A
TAAGGAGGTAATGAG
ATGGCTGGTC AACGTATTGC TCTGGTGACT GGTGGTATGG GGGGTTTAGG TGAAGCTATT
GCTGTGCGGC TGCTGGCTGA CGGTGCCCGT GTGGTTGTGA CCCATAGCGT TCACAATGAT
CATGTGGCCC AATGGTTAGG CACCCAACGT AGTGCTGGTC GGGAATTTAC CGCCTTTCCC
GTTGATGTGA CTGACTTTGC CAGCTGTCAA CGGTGCGTGA GTCAAGTGCG CAGTGAATTG
GGTGATGTTG ATATTCTGAT TAACAATGCC GGTGTGACCC GTGATCGCAC CTTACGCAAA
ATGGATAAAG CCGATTGGGA CTTTGTGTTA CGCACCGATT TGGACTCCTT ATTTCACATG
ACTCGTCCCT TGGTGGAACC CATGTTAGCT CGTGGTTGGG GTCGTATTGT GAATATTTCC
AGTGTGAATG CCAGTCGTGG TGCCTTTGGT CAAACCAATT ATGCCGCTGC CAAAGCTGGT
GTGCATGGCT TTACCAAAGC CTTAGCTTTA GAATTGGCCC GCAAAGGGAT TACCGTTAAC
ACCGTGTCCC CCGGTTACCT GGATACCCAC ATGGTGACTG ACATGCCCGC CGAAATTTTG
GAACGTGATG TGTTGCCCAC CATTCCCGTT GGGCGCTTGG GTAAACCCGC CGAAGTTGCT
GCCTTAATTT CCTATTTGTG TAGTGATGAC GGGGCCTTTG TGACCGGTGC TAATTTTGCC
ATTAACGGCG GTCAACATTT ACAATAA
AAGCTT