

RESEARCH PAPER



## FabG can function as PhaB for poly-3-hydroxybutyrate biosynthesis in photosynthetic cyanobacteria *Synechocystis* sp. PCC 6803

Haowei Zhang<sup>a,†</sup>, Yinghui Liu<sup>b,†</sup>, Changhong Yao<sup>c</sup>, Xupeng Cao <sup>b</sup>, Jing Tian<sup>a</sup>, and Song Xue<sup>b</sup>

<sup>a</sup>School of Engineering, Dalian Polytechnic University, Liaoning, Dalian, China; <sup>b</sup>Marine Bioengineering Group, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China; <sup>c</sup>Department of Pharmaceutical & Biological Engineering, School of Chemical Engineering, Sichuan University, Chengdu, Sichuan, China

### ABSTRACT

The production of poly-3-hydroxybutyrate (PHB) by photosynthetic cyanobacteria is a potentially sustainable production method for the biodegradable plastics industry.  $\beta$ -Ketoacyl-ACP reductase (FabG), from the photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803 (SpFabG), is the first NADPH-dependent reductase in the fatty acid biosynthesis pathway. Its structure is similar to that of acetoacetyl-CoA reductase (SpPhaB), which is critical for PHB synthesis and can replace SpPhaB for acetoacetyl-CoA reduction *in vitro*. However, the specific function of SpFabG in fatty acid synthesis and whether SpFabG could participate in PHB synthesis *in vivo* were not yet clear. In this study, the role of SpFabG in fatty acid synthesis was first verified *in vivo* by knocking down and overexpressing of *fabG*. It was shown that SpFabG was essential yet not rate-limiting for fatty acid biosynthesis. The biochemical characterization of SpFabG using acetoacetyl-CoA as the substrate showed that the optimum temperature, optimum pH,  $K_m$  and  $k_{cat}$  were 30°C, 7, 2.30 mM, and 19.85 s<sup>-1</sup>, respectively, which exemplified the ability of SpFabG to reduce acetoacetyl-CoA with a relatively low affinity and weak catalytic efficiency. Functional analysis of SpFabG *in vivo* indicated that SpFabG was able to partially complement SpPhaB under nitrogen-deprived conditions, and overexpression of *fabG* led to the diversion of partial carbon flux from fatty acid toward PHB synthesis.

### ARTICLE HISTORY

Received 13 March 2017  
Revised 30 March 2017  
Accepted 3 April 2017

### KEYWORDS


$\beta$ -Ketoacyl-ACP reductase;  
Acetoacetyl-CoA reductase;  
Poly-3-hydroxybutyrate;  
Fatty acid; *Synechocystis*

## Introduction


Poly-3-hydroxybutyrate (PHB), the most common type of polyhydroxyalkanoate (PHA), is a biodegradable polymer of biologic origin that has increasingly wide applications in the materials field.<sup>1</sup> PHB production in photosynthetic cyanobacteria has been regarded as a sustainable way for developing the biodegradable plastics industry.<sup>2</sup> PHB has been found to act mainly as an energy and carbon reserve inside microorganisms and algal cells in response to stressful conditions. In the cyanobacterium *Synechocystis* sp. PCC 6803, PHB biosynthesis can be triggered under N- or P-deprived conditions.<sup>3,4</sup> PHB synthesis in *Synechocystis* sp. PCC 6803 involves 3 steps. First, 2 molecules of acetyl-CoA are converted by acetoacetyl-CoA thiolase (PhaA) to form acetoacetyl-CoA. Next, acetoacetyl-CoA reductase (PhaB) (EC 1.1.1.36) catalyzes

NADPH-dependent reduction of acetoacetyl-CoA to yield D-( $\beta$ )-3-hydroxybutyryl-CoA. Finally, D-( $\beta$ )-3-hydroxybutyryl-CoA is polymerized to form PHB by PHA synthase (PhaC).<sup>5</sup> It was reported that the overexpression of *phaAB* led to a 2.6-fold increase in PHB content in *Synechocystis* sp. PCC 6803.<sup>6</sup>

FabG (EC 1.1.1.100), which is responsible for reducing  $\beta$ -ketoacyl-ACP to  $\beta$ -hydroxyacyl-ACP, is the first NADPH-dependent reductase in the fatty acid (FA) synthesis pathway in bacteria, plants and algae.<sup>7,8</sup> It was found that *fabG* was an essential gene in *Escherichia coli*,<sup>9</sup> and overexpression of *fabG* led to a 2–3-fold increase in fatty acid production.<sup>10</sup> So far, there are no reports on the specific functions of FabG in cyanobacteria *in vivo*. Acetoacetyl-ACP, the substrate of FabG for fatty acid (FA) synthesis, is structurally

**CONTACT** Xupeng Cao  [c\\_x\\_p@dicp.ac.cn](mailto:c_x_p@dicp.ac.cn)  457 Zhongshan Road, Dalian 16023, China; Jing Tian  [tianjing@dlpu.edu.cn](mailto:tianjing@dlpu.edu.cn)  No.1st Qinggongyuan, Ganjingzi, Dalian 116034, China; Song Xue  [xuesong@dicp.ac.cn](mailto:xuesong@dicp.ac.cn)  457 Zhongshan Road, Dalian 16023, China.

Color versions of one or more of the figures in the article can be found online at [www.tandfonline.com/kbie](http://www.tandfonline.com/kbie).

 Supplemental data for this article can be accessed on the publisher's website.

<sup>†</sup>These authors made equal contributions.

© 2017 Taylor & Francis

similar to acetoacetyl-CoA, the substrate of PhaB in the PHB synthesis pathway. The only difference between them is the thioester bond that connects to ACP and CoA.<sup>11</sup> Therefore, the similarities in the substrates of FabG and PhaB could make it possible for FabG to participate in the synthesis of PHB and become the bridge between fatty acid metabolism and the PHB synthesis pathway. *In vitro* characterization showed that FabGs from some bacteria such as *Pseudomonas* and *Escherichia coli* could reversibly convert  $\beta$ -ketoacyl-CoA to D- $\beta$ -hydroxyacyl-CoA.<sup>12,13</sup> An *in vivo* study also demonstrated that the FabG from *Pseudomonas aeruginosa* could act as  $\beta$ -ketoacyl-CoA reductase for PHA production in *E. coli*.<sup>12</sup> However, to date, there are no reports of investigations on the potential role of FabG in PHB synthesis in photosynthetic cyanobacteria *in vivo*.

Our previous study showed that the structures of FabG and PhaB from the photosynthetic cyanobacterium *Synechocystis* sp. PCC 6803 (SpFabG and SpPhaB, respectively) were very similar, and SpFabG could act as a  $\beta$ -ketoacyl-CoA reductase *in vitro*.<sup>11</sup> These results implied that SpFabG might play a role in PHB synthesis and could participate in carbon flux distribution between FA and PHB in *Synechocystis* sp. PCC 6803. In this study, the role of SpFabG in fatty acid synthesis was first verified *in vivo* by knocking down and overexpressing *fabG*. Next, the kinetic characteristics of SpFabG as a  $\beta$ -ketoacyl-CoA reductase were investigated *in vitro*. Furthermore, the role of SpFabG in complementing SpPhaB and in regulating carbon flux was revealed in *phaB* knocked-down and *fabG*-overexpressed *phaB* knocked-down mutant strains.

## Results and discussion

### *SpFabG was essential but not rate-limiting in fatty acid biosynthesis*

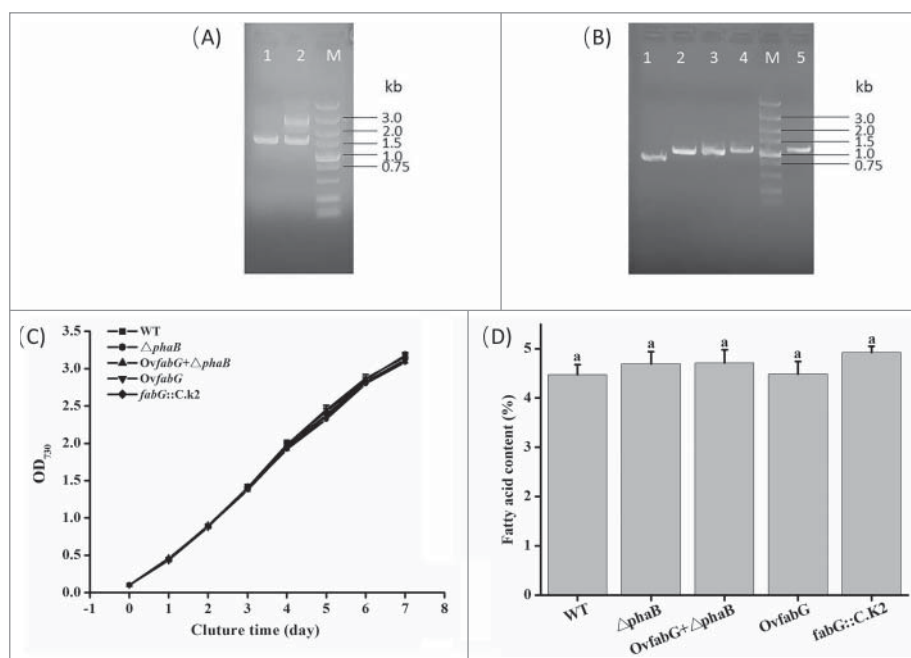
To identify the specific functions of *fabG*, the knock-out of *fabG* was initially attempted. However, the PCR amplification of the *fabG*::C.K2 mutant genome yielded 2 bands, one corresponding to the wild-type *fabG* gene, which was 1426 bp long, and the other band that was 2607 bp long which contained 1426 bp of the WT gene and 1181 bp of the kanamycin fragment (Fig. 1A). These results showed that the *fabG*::C.K2 mutant lacking *fabG* was never completely segregated even after 6 months of culturing of the

transformants in BG11 medium supplemented with kanamycin. The *fabG* could not be completely knocked out in *Synechocystis* sp. PCC 6803 suggesting that it was essential for cell growth. Similarly, EcFabG had been proven to be essential for cell growth in *E. coli*.<sup>9</sup>

To further characterize the role of *fabG* in fatty acid biosynthesis in *Synechocystis* sp. PCC 6803, *fabG* overexpressed (*OvfabG*) and *fabG* knocked-down (*fabG*::C.K2) strains were constructed (Fig. 1B) and evaluated under N-replete conditions (Fig. 1). The results showed that cell growth was almost identical in WT, *OvfabG* and *fabG*::C.K2 strains (Fig. 1C). Furthermore, the fatty acid content in WT, *OvfabG* and *fabG*::C.K2 reached an average of 4.7% of the DW and showed no obvious differences (Fig. 1D). It indicated that FabG was not the rate-limiting enzyme in *Synechocystis* sp. PCC 6803. It was reported that enoyl-ACP reductase (FabI) and  $\beta$ -hydroxyacyl-ACP dehydratase (FabZ) were the rate-limiting enzymes in *E. coli* fatty acid synthesis, while in another cyanobacterium, *Synechococcus* sp. PCC 7002, it was  $\beta$ -ketoacyl-ACP synthase III (FabH).<sup>14,15</sup> This suggested that although the rate-limiting enzyme in different organisms might be different, the *fabG* step did not appear to determine the commitment to fatty acid biosynthesis in bacteria and cyanobacteria despite being essential.

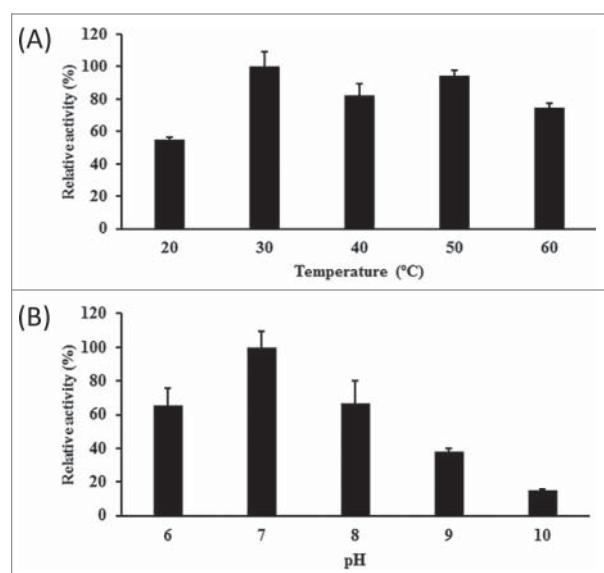
### *Biochemical characterization of SpFabG activity with acetoacetyl-CoA as substrate*

To examine the kinetics of the catalysis of acetoacetyl-CoA by SpFabG, the effects of temperature and pH on SpFabG activity were first explored. As shown in Figure 2A, SpFabG showed the highest activity at 30°C, although the enzyme retained 55, 82, 94 and 75% of its peak activity at 20, 40, 50 and 60°C, respectively. The preferred temperature of 30°C for SpFabG was consistent with the optimum temperature for the growth of *Synechocystis* sp. PCC 6803. The optimum temperature for SpFabG from *Synechocystis* sp. PCC 6803 was consistent with that of FabG from *Streptococcus pneumoniae* (Table 1).<sup>16</sup> As shown in Figure 2B, SpFabG displayed the highest activity at pH 7, which was also the preferred pH for growth, and the activities at pH 6, 8, 9 and 10 were only 66, 67, 38 and 15% of that at pH 7, respectively. The optimum pH for SpFabG of *Synechocystis* sp. PCC 6803



**Figure 1.** Verification of the mutant genotype (A and B) and characterization of their growth (C) and fatty acid synthesis (D). (A) Verification of the genotype of *fabG::C.K2*. Lanes 1, 2: The PCR products amplified with the primers *KfabG-1* and *KfabG-2* using WT and *fabG::C.K2* strain DNA. (B) Verification of the genotypes of  $\Delta phaB$ , *OvfabG* and *OvfabG + \Delta phaB* DNA. Lanes 1, 2, 3: The PCR products amplified with the primers *phaB-1* and *phaB-2* using WT,  $\Delta phaB$  and *OvfabG + \Delta phaB* DNA, and the correct sizes of the amplicons were 765 bp, 1002 bp and 1002 bp, respectively. Lanes 4, 5: The PCR products amplified with the primers *psbAII-1* and *fabG-2* using *OvfabG + \Delta phaB* and *OvfabG* strain DNA; the correct size was 1065 bp. (C) Growth curves and (D) fatty acid content of WT,  $\Delta phaB$ , *OvfabG + \Delta phaB*, *fabG::C.K2* and *OvfabG* mutant strains grown under N repletion for 7 d. The letter (a) implies that no significant differences were observed ( $p > 0.05$ ) using Tukey's HSD test. The data indicate the means  $\pm$  SD ( $n = 4$ ).

was very close to that of FabG from *Plasmodium falciparum*, which is 6.8 (Table 1).<sup>17</sup> The results described above demonstrated that the optimum temperature



**Figure 2.** Biochemical characterization of SpFabG with acetoacetyl-CoA as the substrate. (A) Effects of temperature on SpFabG activity. (B) Effects of pH on SpFabG activity.

and pH for SpFabG activity was in line with the optimum culture conditions for *Synechocystis* sp. PCC 6803.

After confirming the optimum temperature and pH, the kinetics of SpFabG with acetoacetyl-CoA as the substrate was determined. As shown in Table 1, the  $K_m$  and  $k_{cat}$  were 2.3 mM and 19.85 s<sup>-1</sup>, respectively, which resulted in a  $k_{cat}/K_m$  value of 8.63 mM<sup>-1</sup> s<sup>-1</sup>. These kinetic parameters were comparable to those obtained with the enzyme from *Streptococcus pneumonia* under optimal conditions (Table 1).<sup>16</sup> However, the FabG from *Plasmodium falciparum* showed a much lower  $K_m$  and a higher  $k_{cat}$ , as well as a larger  $k_{cat}/K_m$  (Table 1).<sup>17,18</sup> These results indicated that SpFabG was able to utilize acetoacetyl-CoA as its substrate *in vitro*, although its affinity and catalytic efficiency were relatively weak.

### **SpFabG was able to partially complement SpPhaB *in vivo***

To further confirm its role in using acetoacetyl-CoA as a substrate *in vivo*, the  $\Delta phaB$  mutant, in which the

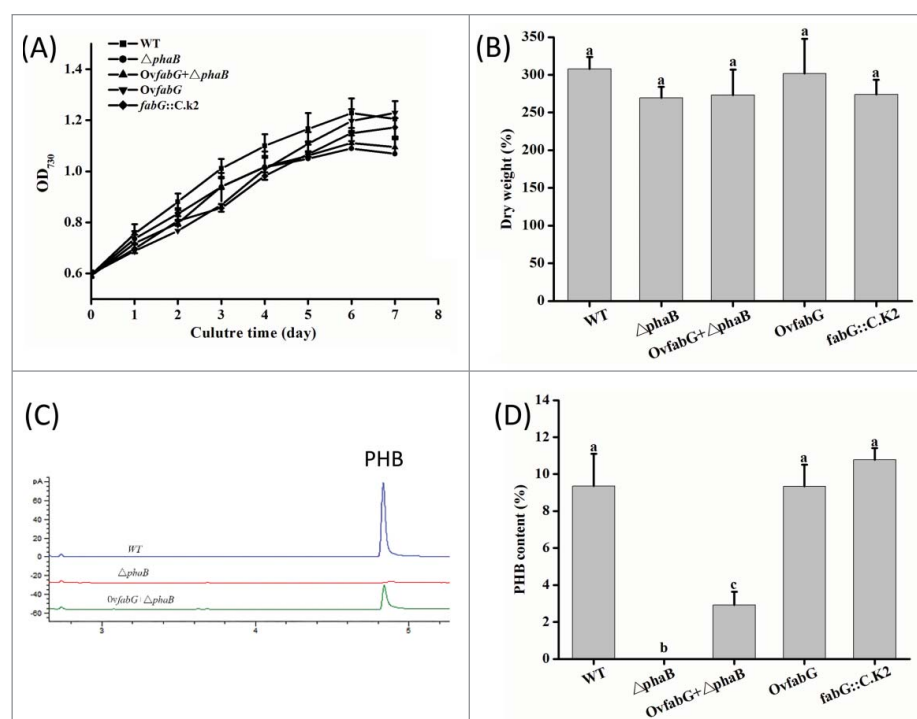
*phaB* gene participating in PHB synthesis was knocked out, was constructed. This was followed by the construction of the *OvfabG* +  $\Delta$ *phaB* mutant, in which *fabG* was overexpressed in the  $\Delta$ *phaB* mutant (Fig. 1B). Next, PHB biosynthesis and physiologic features of the  $\Delta$ *phaB* and *OvfabG* +  $\Delta$ *phaB* mutants as well as the WT strain were tracked under conditions of N deprivation (Fig. 3).

The cell growth of  $\Delta$ *phaB*, *OvfabG* +  $\Delta$ *phaB* and WT as revealed by OD<sub>730</sub> was approximately 1.10, and the DW reached approximately 280 mg/L with no significant differences among the 3 groups (Fig. 3A, B). These results indicated that knocking out *phaB* did not affect biomass accumulation. However, PHB could not be detected in the  $\Delta$ *phaB* strain (Fig. 3C) indicating that  $\Delta$ *phaB* had lost the ability to accumulate PHB. It demonstrated that *phaB* is an essential gene in the PHB synthesis pathway. Notably, the *OvfabG* +  $\Delta$ *phaB* strain regained the ability to accumulate PHB, which reached 31% of the WT levels (Fig. 3D). These results demonstrated that SpFabG could complement PhaB when *phaB* was knocked out, although it could not work as efficiently as PhaB. It was reported that the enzyme activity of SpFabG using

**Table 1.** Kinetic parameters for the catalysis of acetoacetyl-CoA by FabG from *Synechocystis* sp. PCC 6803 and other microorganisms.

Organism	$K_m$ (mM)	$K_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (mM <sup>-1</sup> s <sup>-1</sup> )	Optimum conditions	Reference
<i>Plasmodium falciparum</i>	0.08	0.01	0.19	pH 7.5	18
<i>Streptococcus pneumoniae</i>	2.20	11.00	5.00	pH 7.6, 30°C	16
<i>Plasmodium falciparum</i>	0.43	259.00	602.33	pH 6.8, 22°C	17
<i>Synechocystis</i> sp. PCC 6803	2.30	19.85	8.63	pH 7.0, 30°C	This study

acetoacetyl-CoA as the substrate was 16% of that of SpPhaB *in vitro*,<sup>11</sup> which could account for the lower levels of PHB in the *OvfabG* +  $\Delta$ *phaB* mutant. However, SpFabG did not catalyze acetoacetyl-CoA when *phaB* was present, since overexpressing (*OvfabG*) or knocking down (*fabG::C.K2*) *fabG* had little impact on PHB content (Fig. 3D). PhaB from various organisms had  $K_m$  values ranging from 0.002 to 0.037 mM with acetoacetyl-CoA as the substrate (Supplementary Table S2), which was 100–1000 times lower than those of FabGs, indicating that acetoacetyl-CoA was more likely to be used by PhaB due to its much higher affinity



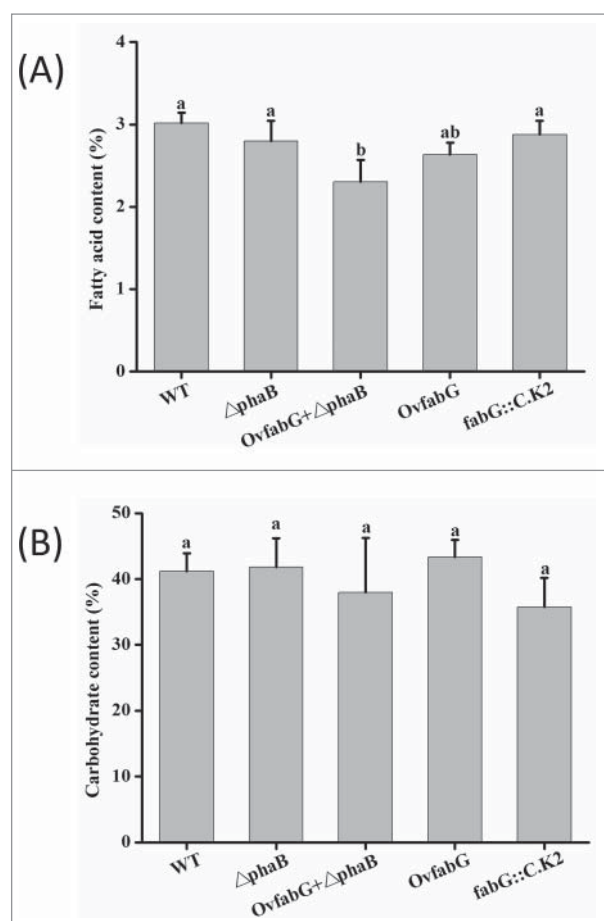
**Figure 3.** The growth curves (A), dry weights (B), PHB peaks detected on GC (C), and PHB content (D) of WT and mutant strains grown under N deprivation conditions for 7 d. The different letters (a-c) indicate the significance of the differences observed ( $p < 0.05$ ) using Tukey's HSD test. The data indicate the means  $\pm$  SD ( $n = 4$ ).

than that of FabG. Therefore, the probability of SpFabG functioning as an acetoacetyl-CoA reductase in the presence of SpPhaB was low.

Additionally, the fatty acid content in the *OvfabG* +  $\Delta$ *phaB* strain decreased by 18% compared with that in the  $\Delta$ *phaB* strain, whereas the carbohydrate content was similar (Fig. 4A, B). These results indicated that the introduction of SpFabG did not disturb carbohydrate synthesis and facilitated PHB instead of fatty acid biosynthesis. This could be due to the down regulation of fatty acid biosynthesis and the contradicting up regulation of the PHB biosynthesis pathway under nitrogen deprived conditions. It had been demonstrated that when *Synechocystis* sp. PCC 6803 was subjected to nitrogen deprivation, the expression levels of many genes involved in fatty acid biosynthesis, including Acetyl-CoA carboxylase (ACCase) and

Malonyl CoA:ACP transacylase (FabD), were decreased, whereas those involved in PHB biosynthesis, such as PhaA and PhaC, increased compared with those under normal conditions.<sup>19</sup> As a result, the introduction of SpFabG led to the diversion of partial carbon flux from FA toward PHB synthesis.

The present study demonstrated the feasibility of SpFabG functioning as an acetoacetyl-CoA reductase for PHB biosynthesis *in vivo*. This is the first report of a *fabG* from a photosynthetic microorganism that is not only essential for fatty acid biosynthesis but also plays a potential role in PHB accumulation. In fact, PHB in cyanobacteria, like FA in oleaginous microalgae, had been regarded as a redox-sink to store excess NADPH under imbalanced metabolic conditions such as nutrient starvation.<sup>20</sup> The similar physiologic role of PHB and FA might be an indicative of functional multiplicity of FabG. The additional function of SpFabG as a  $\beta$ -ketoacyl-CoA reductase in PHB synthesis might aid cells to acclimate to the stressed conditions more smoothly. This might represent an evolutionary strategy for cyanobacteria to survive complicated situations. Moreover, since FabG is inherently able to reduce  $\beta$ -ketoacyl-ACP with C chain lengths of 4 to 18 in cyanobacteria,<sup>21</sup> it is reasonable to extrapolate from the results of this study that it may also work on  $\beta$ -ketoacyl-CoA with medium C chain lengths (mcl) of 6 to 10 that can be obtained from fatty acid  $\beta$ -oxidation, which could provide the substrate for mcl<sup>-</sup>PHA biosynthesis. This could offer a new method for the photoautotrophic production of PHA in cyanobacteria.



**Figure 4.** Carbohydrate (A) and fatty acid content (B) of WT and mutant strains under conditions of N deprivation for 7 d. The different letters (a-b) show the significance of the differences observed ( $p < 0.05$ ) using Tukey's HSD test. The data indicate the means  $\pm$  SD ( $n = 4$ ).

## Materials and methods

### Construction of plasmids and transformation of *Synechocystis* sp. PCC 6803

All strains used and constructed in this study are listed in Table 2. All primers used are listed in Supplementary Table S1. The replicative vector pJA2 was kindly provided by Dr. Paul Hudson (KTH Royal Institute of Technology of Sweden).<sup>22,23</sup> The *fabG* gene was amplified by PCR using the *Synechocystis* sp. PCC 6803 DNA as the template with *ovfabG1* and *ovfabG2* primers and cloned into the BamHI/XbaI site of pJA2 resulting in the recombinant plasmid pJA-*fabG*. The  $\Delta$ *phaB* mutant strain of *Synechocystis* sp. PCC 6803

**Table 2.** *Synechocystis* strains constructed for this study.

strain	Genome modification	Notes
<i>OvfabG</i>	<i>fabG</i> was overexpressed by pJA2 in <i>Synechocystis</i>	This study
<i>fabG::C.K2</i>	C.K2 was inserted in ClaI sites of <i>fabG</i>	This study
$\Delta phaB$	<i>phaB</i> was deleted in <i>Synechocystis</i>	Acquired from Dr. Weiwen Zhang <sup>24</sup>
<i>OvfabG</i> + $\Delta phaB$	<i>fabG</i> was overexpressed by pJA2 and <i>phaB</i> was deleted	This study
WT	Wild type <i>Synechocystis</i> sp. PCC 6803	Wild type

with *phaB* deleted was kindly provided by Dr. Weiwen Zhang (Tianjin University).<sup>24,25</sup>

The DNA fragment containing the *fabG* gene (*slr0886*) was generated by PCR using the primers *KfabG1* and *KfabG2* with *Synechocystis* sp. PCC 6803 chromosomal DNA as the template, cloned into pMD18-T, and confirmed by sequencing. The C.K2 fragment, excised from pRL446 (NCBI GenBank accession no. EU346690) by EcoRI, was inserted into the ClaI site of that plasmid, resulting in plasmid *dicp1* for the inactivation of *fabG* in *Synechocystis* sp. PCC 6803.<sup>26</sup> The constructed plasmid *dicp1* was introduced into *Synechocystis* sp. PCC 6803 by natural transformation to generate the *Synechocystis* strain *fabG::C.K2* as described by Jiang et al.<sup>27</sup> The constructed plasmid pJA2-*fabG* was introduced into the strain *Synechocystis*  $\Delta phaB$  to generate the *Synechocystis* strain *OvfabG* +  $\Delta phaB$ .

#### Enzyme activity assay and kinetic characterization of SpFabG using the substrate acetoacetyl-CoA

SpFabG was expressed and purified as described previously.<sup>11</sup> The fractions containing SpFabG were concentrated using a 10 kDa Amicon concentrator (Millipore) and stored at  $-80^{\circ}\text{C}$  until further use. The enzyme activity of SpFabG was determined as described previously.<sup>11</sup> Before the reaction, the protein solution was diluted to 0.1 mg/ml using buffer A (50 mM Tris-HCl pH 7.8, 300 mM NaCl, 1 mM EDTA, 5% glycerol (v/v), and 2 mM  $\beta$ -mercaptoethanol), and protein concentrations were determined using the Bradford assay with BSA as the standard. For optimum temperature determination, a mixture containing 200  $\mu\text{M}$  acetoacetyl-CoA and 200  $\mu\text{M}$  NADPH in 0.1 M PBS buffer (pH 7.0) was prepared in a volume of 100  $\mu\text{L}$  at different temperatures ranging from  $20^{\circ}\text{C}$  to  $60^{\circ}\text{C}$ . The mixtures were preincubated for 5 min, and the reaction was initiated by the addition of 2  $\mu\text{L}$  of diluted protein solution containing 0.2  $\mu\text{g}$  SpFabG. The relative activity was determined

from the rate of conversion of NADPH to  $\text{NADP}^{+}$ , which was measured photometrically by the decrease of absorbance at 340 nm (Jasco V630 spectrophotometer, Japan). For determining optimum pH, the same mixture as described above was used except for the use of different pH conditions that were generated with PBS (pH 6–8) and glycine-NaOH (pH 9–10) buffers to obtain a final concentration of 0.1 M. The mixtures were preincubated at  $30^{\circ}\text{C}$  before the reaction was initiated.

The kinetic parameters  $K_m$ ,  $V_{\max}$  and  $k_{\text{cat}}$  with acetoacetyl-CoA as the substrate were determined by varying its concentration from 100  $\mu\text{mol/L}$  to 1600  $\mu\text{mol/L}$  under saturating conditions of NADPH at 400  $\mu\text{mol/L}$  in a volume of 100  $\mu\text{L}$ . Each reaction was initiated by the addition of 0.2  $\mu\text{g}$  of the enzyme. The data were evaluated by double reciprocal plots.

#### Culture conditions and growth measurement

For N-replete cultures, *Synechocystis* sp. PCC 6803 WT strain was grown in BG11 medium buffered with 10 mM HEPES-NaOH (pH 7.5), and the mutant strains were grown in the same medium containing the corresponding antibiotics as follows:  $\Delta phaB$  (chloramphenicol 17  $\mu\text{g/ml}$ ), *OvfabG* +  $\Delta phaB$  (chloramphenicol 17  $\mu\text{g/ml}$ , kanamycin 25  $\mu\text{g/ml}$ ), *OvfabG* (kanamycin 25  $\mu\text{g/ml}$ ), *fabG::C.K2* (kanamycin 25  $\mu\text{g/ml}$ ). The cultures with an initial  $\text{OD}_{730}$  of 0.2 (determined by a Jasco V530 spectrophotometer, Japan) were incubated aerobically at  $30^{\circ}\text{C}$  under continuous illumination of 40–50  $\mu\text{mol/m}^2/\text{s}$  on a rotatory shaker operating at 150 r/min. The growth was tracked every day for 7 d.

For N-depleted cultures, the WT and mutant strain cells were first grown in 5 L flasks under N-replete conditions as described above until the  $\text{OD}_{730}$  reached 2.4. Next, the cultures were centrifuged at 6800 g for 5 min, and the cell pellets were washed twice with BG11-0 medium (BG11 medium lacking  $\text{NaNO}_3$ ) in which ferric ammonium citrate and  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$

were replaced with equimolar concentrations of ferric citrate and  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , respectively. The washed cells, with an initial  $\text{OD}_{730}$  of 0.6, were inoculated in BG11-0 medium supplemented with sodium acetate at a final concentration of 0.4% (w/v) and cultivated for 7 d under the same conditions as the N-replete culture.

The cells were harvested by centrifugation at 6800 g for 5 min before they were vacuum dried. The dry cell weight (DW, g/L) was measured according to the procedure described by Chi et al.<sup>28</sup> Briefly, the cultures (10 mL) were filtered using pre-weighed Whatman GF/C filters (47 mm diameter), washed twice with 2 mL of distilled water, and dried to a constant weight at 60°C. The dry weight of the algal cells was the difference between the final weight and the weight of the clean filter.

### Biochemical composition analysis

#### PHB analysis

Freeze-dried cells (20 mg) were esterified with 1 mL 0.2%  $\text{H}_2\text{SO}_4$ -methanol (v/v) and 1 mL chloroform at 100°C for 5 h during which the PHB was converted to methyl 3-hydrobutyrate. After cooling to room temperature, 0.5 mL of distilled water was added and mixed for 1 min on a vortex mixer. Next, the mixture was centrifuged at 2000 rpm for 2 min and 200  $\mu\text{L}$  of the chloroform layer was used for analysis by gas chromatography (GC). The PHB analysis was performed by Agilent 6890 GC instrument, as described previously,<sup>4</sup> equipped with a DB5-column (Agilent, 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ) and a flame-ionization detector (FID) with the temperatures of the injector and detector set to 270°C and 300°C, respectively. The initial oven temperature was maintained at 60°C for 1 min and increased to 120°C at the rate of 10°C/min. Next, the oven temperature was increased to 200°C at the rate of 45°C/min and maintained for 3 min. A PHB standard (Sigma) was used for calibration.

#### Fatty acid analysis

Freeze-dried cells (5 mg) were transesterified with 5 mL of 0.2%  $\text{H}_2\text{SO}_4$ -methanol (v/v) in a 10 mL round-bottomed flask at 70°C for 1 h during which the FA was converted to fatty acid methyl esters (FAME). Heptadecanoic acid, the internal standard, was transesterified simultaneously. Next, the FAME

content was analyzed by an Agilent 7890 GC instrument equipped with a DB23-column (Agilent, 30 m  $\times$  0.32 mm  $\times$  0.25  $\mu\text{m}$ ) and an FID detector.<sup>29</sup>

### Carbohydrate analysis

Carbohydrate analysis was performed by the sulfuric acid-anthrone method as described by Chi et al.<sup>28</sup>

### Statistical analysis

Statistical calculations were performed using STATISTICA<sup>®</sup> 7.0 (StatSoft Inc., Tulsa, OK, USA). One-way analysis of variance (ANOVA) was used to determine the effects of treatments, and Tukey's honestly significant difference (HSD) test was conducted to test the statistical significance of the differences between the means of various treatments.

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

### Acknowledgments

We appreciate Professor Baosheng Qiu (Central China Normal University) for kindly providing the plasmid PRL446 for the *fabG*::C.K2 mutant and Professor Weiwen Zhang (Tianjin University) for kindly providing the *OvfabG* strain, the  $\Delta$ *phaB* strain and the pJA2-*fabG* plasmid.

### Funding

This work was supported by the National Natural Science Foundation of China (NSFC) under Grant (No. 31500294); and NSFC under Grant (No. 31470432).

### ORCID

Xupeng Cao  <http://orcid.org/0000-0002-7626-7874>

### References

- [1] Chen GQ. A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. *Chem Soc Rev* 2009; 38(8):2434-46; PMID:19623359; <https://doi.org/10.1039/b812677c>
- [2] Balaji S, Gopi K, Muthuvelan B. A review on production of poly  $\beta$  hydroxybutyrate from cyanobacteria for the production of bio plastics. *Algal Res* 2013; 2(3):278-85; <https://doi.org/10.1016/j.algal.2013.03.002>
- [3] Panda B, Jain P, Sharma L, Mallick N. Optimization of cultural and nutritional conditions for accumulation of poly-beta-hydroxybutyrate in *Synechocystis* sp. PCC 6803. *Bioresour Technol* 2006; 97(11):1296-

- 301; PMID:16046119; <https://doi.org/10.1016/j.biortech.2005.05.013>
- [4] Panda B, Mallick N. Enhanced poly-beta-hydroxybutyrate accumulation in a unicellular cyanobacterium, *Synechocystis* sp. PCC 6803. *Lett Appl Microbiol* 2007; 44(2):194-8; PMID:17257260; <https://doi.org/10.1111/j.1472-765X.2006.02048.x>
- [5] Taroncher-Oldenberg G, Nishina K, Stephanopoulos G. Identification and analysis of the polyhydroxyalkanoate-specific beta-ketothiolase and acetoacetyl coenzyme A reductase genes in the cyanobacterium *Synechocystis* sp. strain PCC6803. *Appl Environ Microbiol* 2000; 66(10):4440-8; PMID:11010896; <https://doi.org/10.1128/AEM.66.10.4440-4448.2000>
- [6] Khetkorn W, Incharoensakdi A, Lindblad P, Jantaro S. Enhancement of poly-3-hydroxybutyrate production in *Synechocystis* sp. PCC 6803 by overexpression of its native biosynthetic genes. *Bioresour Technol* 2016; 214:761-8; PMID:27213577; <https://doi.org/10.1016/j.biortech.2016.05.014>
- [7] Beld J, Lee DJ, Burkart MD. Fatty acid biosynthesis revisited: structure elucidation and metabolic engineering. *Mol Biosyst* 2015; 11(1):38-59; PMID:25360565; <https://doi.org/10.1039/C4MB00443D>
- [8] Price AC, Zhang YM, Rock CO, White SW. Structure of beta-ketoacyl-[acyl carrier protein] reductase from *Escherichia coli*: Negative cooperativity and its structural basis. *Biochem* 2001; 40(43):12772-81; <https://doi.org/10.1021/bi010737g>
- [9] Zhang Y, Cronan JE. Transcriptional analysis of essential genes of the *Escherichia coli* fatty acid biosynthesis gene cluster by functional replacement with the analogous *Salmonella typhimurium* gene cluster. *J Bacteriol* 1998; 180(13):3295-303; PMID:9642179
- [10] Jeon E, Lee S, Lee S, Han SO, Yoon YJ, Lee J. Improved production of long-chain fatty acid in *Escherichia coli* by an engineering elongation cycle during fatty acid synthesis (FAS) through genetic manipulation. *J Microbiol and Biotechnol* 2012; 22(7):990-9; <https://doi.org/10.4014/jmb.1112.12057>
- [11] Liu YH, Feng YB, Cao XP, Li X, Xue S. Structure-directed construction of a high-performance version of the enzyme FabG from the photosynthetic microorganism *Synechocystis* sp. PCC 6803. *FEBS Lett* 2015; 589(20):3052-7; PMID:26358291; <https://doi.org/10.1016/j.febslet.2015.09.001>
- [12] Nomura CT, Taguchi K, Gan ZH, Kuwabara K, Tanaka T, Takase K, Doi Y. Expression of 3-ketoacyl-acyl carrier protein reductase (*fabG*) genes enhances production of polyhydroxyalkanoate copolymer from glucose in recombinant *Escherichia coli* JM109. *Appl Environ Microbiol* 2005; 71(8):4297-306; PMID:16085817; <https://doi.org/10.1128/AEM.71.8.4297-4306.2005>
- [13] Ren Q, Sierro N, Witholt B, Kessler B. FabG, an NADPH-dependent 3-ketoacyl reductase of *Pseudomonas aeruginosa*, provides precursors for medium-chain-length poly-3-hydroxyalkanoate biosynthesis in *Escherichia coli*. *J Bacteriol* 2000; 182(10):2978-81; PMID:10781572; <https://doi.org/10.1128/JB.182.10.2978-2981.2000>
- [14] Yu XY, Liu TG, Zhu FY, Khosla C. In vitro reconstitution and steady-state analysis of the fatty acid synthase from *Escherichia coli*. *Proc Natl Acad Sci U.S.A.* 2011; 108(46):18643-48; PMID:22042840; <https://doi.org/10.1073/pnas.1110852108>
- [15] Kuo J, Khosla C. The initiation ketosynthase (FabH) is the sole rate-limiting enzyme of the fatty acid synthase of *Synechococcus* sp. PCC 7002. *Metab Eng* 2014; 22:53-9; PMID:24395007; <https://doi.org/10.1016/j.ymben.2013.12.008>
- [16] Patel MP, Liu WS, West J, Tew D, Meek TD, Thrall SH. Kinetic and chemical mechanisms of the *fabG*-encoded *Streptococcus pneumoniae* beta-ketoacyl-ACP reductase. *Biochem* 2005; 44(50):16753-65; <https://doi.org/10.1021/bi050947j>
- [17] Karmodiya K, Surolia N. Analyses of co-operative transitions in *Plasmodium falciparum* beta-ketoacyl acyl carrier protein reductase upon co-factor and acyl carrier protein binding. *FEBS J* 2006; 273(17):4093-103; PMID:16934037; <https://doi.org/10.1111/j.1742-4658.2006.05412.x>
- [18] Pillai S, Rajagopal C, Kapoor M, Kumar G, Gupta A, Surolia N. Functional characterization of beta-ketoacyl-ACP reductase (FabG) from *Plasmodium falciparum*. *Biochem Biophys Res Commun* 2003; 303(1):387-92; PMID:12646215; [https://doi.org/10.1016/S0006-291X\(03\)00321-8](https://doi.org/10.1016/S0006-291X(03)00321-8)
- [19] Huang SQ, Chen L, Te RG, Qiao JJ, Wang JX, Zhang WW. Complementary iTRAQ proteomics and RNA-seq transcriptomics reveal multiple levels of regulation in response to nitrogen starvation in *Synechocystis* sp. PCC 6803. *Mol Biosyst* 2013; 9(10):2565-74; PMID:23942477; <https://doi.org/10.1039/c3mb70188c>
- [20] Hauf W, Schlebusch M, Hüge J, Kopka J, Hagemann M, Forchhammer K. Metabolic changes in *Synechocystis* PCC6803 upon nitrogen-starvation: Excess NADPH sustains polyhydroxybutyrate accumulation. *Metabolites* 2013; 3(1):101-18; PMID:24957892; <https://doi.org/10.3390/metabo3010101>
- [21] Ohlrogge J, Browse J. Lipid Biosynthesis. *Plant Cell* 1995; 7(7):957-70; <https://doi.org/10.1105/Tpc.7.7.957>
- [22] Anfelt J, Hallstrom B, Nielsen J, Uhlen M, Hudson EP. Using transcriptomics to improve butanol tolerance of *Synechocystis* sp. Strain PCC 6803. *Appl Environ Microbiol* 2013; 79(23):7419-27; PMID:24056459; <https://doi.org/10.1128/AEM.02694-13>
- [23] Huang HH, Camsund D, Lindblad P, Heidorn T. Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. *Nucl Acid Res* 2010; 38(8):2577-93; <https://doi.org/10.1093/nar/gkq164>
- [24] Wang HL, Postier BL, Burnap RL. Optimization of fusion PCR for *in vitro* construction of gene knockout fragments. *Biotech* 2002; 33(1):26-28



- [25] Wang YP, Sun T, Gao XY, Shi ML, Wu LN, Chen L, Zhang WW. Biosynthesis of platform chemical 3-hydroxypropionic acid (3-HP) directly from CO<sub>2</sub> in cyanobacterium *Synechocystis* sp. PCC 6803. *Metab Eng* 2016; 34:60-70; PMID:26546088; <https://doi.org/10.1016/j.ymben.2015.10.008>
- [26] Elhai J, Wolk CP. Conjugal transfer of DNA to cyanobacteria. *Method Enzymol* 1988; 167:747-54
- [27] Jiang HB, Lou WJ, Du HY, Price NM, Qiu BS. *Sll1263*, a unique cation diffusion facilitator protein that promotes iron uptake in the cyanobacterium *Synechocystis* sp. Strain PCC 6803. *Plant Cell Physiol* 2012; 53(8):1404-17; PMID:22685083; <https://doi.org/10.1093/pcp/pcs086>
- [28] Chi L, Yao CH, Cao XP, Xue S. Coordinated regulation of nitrogen supply mode and initial cell density for energy storage compounds production with economized nitrogen utilization in a marine microalga *Isochrysis zhangjiangensis*. *Bioresour Technol* 2016; 200:598-605; PMID:26547809; <https://doi.org/10.1016/j.biortech.2015.10.059>
- [29] Liu J, Liu YN, Wang HT, Xue S. Direct transesterification of fresh microalgal cells. *Bioresour Technol* 2015; 176:284-7; <https://doi.org/10.1016/j.biortech.2014.10.094>