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

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**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 \textit{in vitro}. However, the specific function of SpFabG in fatty acid synthesis and whether SpFabG could participate in PHB synthesis \textit{in vivo} were not yet clear. In this study, the role of SpFabG in fatty acid synthesis was first verified \textit{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\(^{-1}\), respectively, which exemplified the ability of SpFabG to reduce acetoacetyl-CoA with a relatively low affinity and weak catalytic efficiency. Functional analysis of SpFabG \textit{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.

**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.\(^1\) PHB production in photosynthetic cyanobacteria has been regarded as a sustainable way for developing the biodegradable plastics industry.\(^2\) 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.\(^3,\(^4\) 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).\(^5\) It was reported that the overexpression of phaAB led to a 2.6-fold increase in PHB content in *Synechocystis* sp. PCC 6803.\(^6\)

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

To further characterize the role of *fabG* in fatty acid biosynthesis in *Synechocystis* sp. PCC 6803, *fabG* over-expressed (Ov*fabG*) 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, Ov*fabG* and *fabG*::C.K2 strains (Fig. 1C). Furthermore, the fatty acid content in WT, Ov*fabG* 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). 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). 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...
was very close to that of FabG from *Plasmodium falciparum*, which is 6.8 (Table 1).17 The results described above demonstrated that the optimum temperature 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$^{-1}$, respectively, which resulted in a $k_{cat}/K_m$ value of 8.63 mM$^{-1}$s$^{-1}$. These kinetic parameters were comparable to those obtained with the enzyme from *Streptococcus pneumoniae* under optimal conditions (Table 1).16 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).17,18 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 $\triangle$phaB mutant, in which the
The phaB gene participating in PHB synthesis was knocked out, was constructed. This was followed by the construction of the OvfabG + △phaB mutant, in which fabG was overexpressed in the △phaB mutant (Fig. 1B). Next, PHB biosynthesis and physiologic features of the △phaB and OvfabG +△phaB mutants as well as the WT strain were tracked under conditions of N deprivation (Fig. 3).

The cell growth of △phaB, OvfabG +△phaB and WT as revealed by OD730 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 △phaB strain (Fig. 3C) indicating that △phaB had lost the ability to accumulate PHB. It demonstrated that phaB is an essential gene in the PHB synthesis pathway. Notably, the OvfabG + △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 acetoacetyl-CoA as the substrate was 16% of that of SpPhaB in vitro,11 which could account for the lower levels of PHB in the OvfabG + △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.

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^-1) | k_cat/K_m (mM^-1s^-1) | Optimum conditions | Reference |
|---------------------------|-----------|--------------|------------------------|--------------------|-----------|
| Plasmodium falciparum     | 0.08      | 0.01         | 0.19                   | pH 7.5             | 18        |
| Streptococcus pneumoniae  | 2.20      | 11.00        | 5.00                   | pH 7.6, 30°C       | 16        |
| Plasmodium falciparum     | 0.43      | 259.00       | 602.33                 | pH 6.8, 22°C       | 17        |
| Synechocystis sp. PCC 6803| 2.30      | 19.85        | 8.63                   | pH 7.0, 30°C       | This study|

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 ± 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. 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. 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, 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−PHA biosynthesis. This could offer a new method for the photoautotrophic production of PHA in cyanobacteria.

**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 pJ2A was kindly provided by Dr. Paul Hudson (KTH Royal Institute of Technology of Sweden). 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 pJ2 resulting in the recombinant plasmid pJA-fabG. The \(\Delta\)phaB mutant strain of Synechocystis sp. PCC 6803

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**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 ± SD \((n = 4)\).
with phaB deleted was kindly provided by Dr. Weiw en Zhang (Tianjin University). 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 conformed 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 the ClaI site of that plasmid, resulting in plasmid accession no. EU346690) by EcoRI, was inserted into fragment, excised from pRL446 (NCBI GenBank PCC 6803. The constructed plasmid dicp1 was pMD18-T, and consequn with PBS (pH 6–8) and glycine-NaOH (pH 9–10) buffers object to obtain a final concentration of 0.1 M. The mixtures were preincubated at 30°C before the reaction was initiated.

The kinetic parameters $K_m$, $V_{max}$ and $k_{cat}$ with acetocetyl-CoA as the substrate were determined by varying its concentration from 100 μmol/L to 1600 μmol/L under saturating conditions of NADPH at 400 μmol/L in a volume of 100 μL. Each reaction was initiated by the addition of 0.2 μg 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: ΔphaB (chloramphenicol 17 μg/ml), OvfabG+ΔphaB (chloramphenicol 17 μg/ml, kanamycin 25 μg/ml), OvfabG (kanamycin 25 μg/ml), fabG::C.K2 (kanamycin 25 μg/ml). The cultures with an initial OD$_{730}$ of 0.2 (determined by a Jasco V530 spectrophotometer, Japan) were incubated aerobically at 30°C under continuous illumination of 40–50 μmol/m²/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 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 NaNO$_3$) in which ferric ammonium citrate and Co(NO$_3$)$_2$-6H$_2$O

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

| strain       | Genome modification                                      | Notes                           |
|--------------|----------------------------------------------------------|---------------------------------|
| OvfabG       | fabG was overexpressed by pJA2 in Synechocystis          | This study                      |
| fabG::C.K2   | C.K2 was inserted in ClaI sites of fabG                  | This study                      |
| ΔphaB        | phaB was deleted in Synechocystis                        | Acquired from Dr. Weiw en Zhang |
| OvfabG+ΔphaB | fabG was overexpressed by pJA2 and phaB was deleted      | This study                      |
| WT           | Wild type Synechocystis sp. PCC 6803                     | Wild type                      |

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

SpFabG was expressed and purified as described previously. 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 β-mercaptoethanol), and protein concentrations were determined using the Bradford assay with BSA as the standard. For optimum temperature determination, a mixture containing 200 μM acetocetyl-CoA and 200 μM NADPH in 0.1 M PBS buffer (pH 7.0) was prepared in a volume of 100 μL at different temperatures ranging from 20°C to 60°C. The mixtures were preincubated for 5 min, and the reaction was initiated by the addition of 2 μL of diluted protein solution containing 0.2 μg SpFabG. The relative activity was determined from the rate of conversion of NADPH to 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°C before the reaction was initiated.

The kinetic parameters $K_m$, $V_{max}$ and $k_{cat}$ with acetocetyl-CoA as the substrate were determined by varying its concentration from 100 μmol/L to 1600 μmol/L under saturating conditions of NADPH at 400 μmol/L in a volume of 100 μL. Each reaction was initiated by the addition of 0.2 μg of the enzyme. The data were evaluated by double reciprocal plots.
were replaced with equimolar concentrations of ferric citrate and CoCl₂·6H₂O, respectively. The washed cells, with an initial OD₇₃₀ 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.²⁸ 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% H₂SO₄–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 μL 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,²⁹ equipped with a DB5-column (Agilent, 30 m × 0.25 mm × 0.25 μm) 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% H₂SO₄–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 × 0.32 mm × 0.25 μm) and an FID detector.²⁹

**Carbohydrate analysis**

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

**Statistical analysis**

Statistical calculations were performed using STATISTICA® 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.

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