RNA-Seq Analysis Provides Insights for Understanding Photoautotrophic Polyhydroxyalkanoate Production in Recombinant *Synechocystis* Sp.

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Abstract

The photosynthetic cyanobacterium, *Synechocystis* sp. strain 6803, is a potential platform for the production of various chemicals and biofuels. In this study, direct photosynthetic production of a biopolymer, polyhydroxyalkanoate (PHA), in genetically engineered *Synechocystis* sp. achieved as high as 14 wt%. This is the highest production reported in *Synechocystis* sp. under photoautotrophic cultivation conditions without the addition of a carbon source. The addition of acetate increased PHA accumulation to 41 wt%, and this value is comparable to the highest production obtained with cyanobacteria. Transcriptome analysis by RNA-seq coupled with real-time PCR was performed to understand the global changes in transcript levels of cells subjected to conditions suitable for photoautotrophic PHA biosynthesis. There was lower expression of most PHA synthesis-related genes in recombinant *Synechocystis* sp. with higher PHA accumulation suggesting that the concentration of these enzymes is not the limiting factor to achieving high PHA accumulation. In order to cope with the higher PHA production, cells may utilize enhanced photosynthesis to drive the product formation. Results from this study suggest that the total flux of carbon is the possible driving force for the biosynthesis of PHA and the polymerizing enzyme, PHA synthase, is not the only critical factor affecting PHA-synthesis. Knowledge of the regulation or control points of the biopolymer production pathways will facilitate the further use of cyanobacteria for biotechnological applications.

Introduction

Cyanobacteria are believed to be one of the oldest groups of photosynthetic organisms on Earth and played a significant role in the development of the oxygenic atmosphere we breathe today [1]. In modern day, cyanobacteria continue to play a pivotal role in global carbon recycling, the nitrogen cycle and most importantly, the maintenance of the composition of the atmosphere [2,3]. Cyanobacteria are considered to be ideal producers of various fine chemicals and biofuels because they fix carbon dioxide into biomass using solar energy. Fluctuations of nutrient concentrations constantly occur in natural environments and microorganisms respond to nutrient starvation by accumulating various carbon and energy storage compounds [4]. The study of these storage polymers, particularly polyhydroxyalkanoate (PHA), has gained considerable interest in recent years in an attempt to address the waste disposal problems caused by petrochemical plastics [5].

At present, the major biological processes utilized for industrial production of PHA are fermentations of heterotrophic bacteria. Nevertheless, the economic viability of PHA as a commodity polymer is limited by high production costs due to costly carbon substrates and requirements during the fermentation processes. Substantial effort has been devoted to investigating PHA production processes that are more cost-effective [6]. An interesting and promising approach is the use of photosynthetic cyanobacteria as the host for PHA production. The cyanobacteria, as ‘microbial factories’, can fix carbon dioxide from the atmosphere into high molecular weight PHA directly via photosynthesis. Besides being photoautotrophic, cyanobacteria require minimal nutrients for growth, eliminating the cost of carbon sources and complex growth media [7]. Thus, the application of cyanobacteria offers the potential of a cost-competitive and sustainable approach for the production of this environmentally friendly polymer.

The presence of PHA in cyanobacteria was first described by Carr whom analyzed PHA in *Chlorella fritschi* based on acid hydrolysis of poly(3-hydroxybutyrate), P(3HB), to crotonic acid followed by UV spectroscopic measurement of the hydrolysis product [8]. Since then, much research has demonstrated the presence of PHA in several other cyanobacteria including *Aphanathece* sp. [9], *Oscillatoria limosa* [10], some species of the genus *Spirulina* [11,12] and the thermophilic strain *Synechoccocus* sp. MA19 [13]. So far, cyanobacteria are characterized by their ability to produce PHA containing only 3-hydroxybutyrate (3HB) and/or 3-hydroxyvalerate (3HV) monomers [9,10,14]. Although there are many reports on the occurrence of PHA in cyanobacteria, most of these studies explored the physiology and
fermentation aspects of PHA accumulation in cyanobacteria. The biochemical and molecular basis of PHA synthesis in cyanobacteria are not well understood.

The model cyanobacterium *Synechocystis* sp. strain PCC 6803 is considered as a promising candidate for various biotechnological productions because of the availability of its genome sequence information [15] and the ease of genetic manipulation of this strain due to its naturally transformable feature [16]. In this study, *Synechocystis* sp. was metabolically engineered by increasing the flux of intermediates to PHA biosynthesis and introducing a PHA synthase with higher activity. RNA-seq analysis was carried out to examine the differential expression involved in the global biological processes and metabolic pathways during the improved photoautotrophic production of PHA. This information will facilitate the potential use of cyanobacteria for the sustainable production of this ‘green’ polymer.

### Results

**Enhanced PHA Production in Recombinant Cyanobacteria**

In the well-studied PHA biosynthetic pathway of *Caprioaedium necator*, P(3HB) synthesis occurs in a three-step reaction and starts with the condensation of acetyl-CoA to acetoacetyl-CoA by β-ketothiolase [17]. Under photosynthetic conditions, it was hypothesized that the acetyl-CoA pool in cyanobacteria is insufficient to drive the thermodynamically unfavorable condensation reaction forward [18]. Instead of relying solely on the native β-ketothiolase-mediated condensation to form acetoacetyl-CoA, an acetoacetyl-CoA synthase (nphT7S) from *Streptomyces* sp. CL190 was incorporated in the P(3HB) pathway design. The nphT7S gene catalyzes the irreversible condensation of acetyl-CoA and malonyl-CoA to give acetoacetyl-CoA, driving the reaction toward the formation of PHA. The evolution of carbon dioxide from the condensation reaction effectively pushes the reaction toward the formation of acetoacetyl-CoA [19]. A highly active PHA synthase from *Chromobacterium* sp. USM2 (phaCC) [20] was co-expressed with nphT7S to improve the photosynthetic production of P(3HB) in *Synechocystis* sp. In view of the stimulatory effects of nutrient limitation, carbon supplementation and air-exchange limitation on PHA accumulation [7,21], biosynthesis experiments were designed under these cultivation conditions: N- or P-deficient (nitrogen- or phosphorus-deficient), air-exchange limitation, and/or in the presence of carbon sources (CO2, acetate and/or fructose).

For the design of PHA production pathway in *Synechocystis* sp., the vector plasmid pTKP2031V was used for the insertion of transgenes into the genome via homologous recombination between sites *sly2030* and *sbr2031* [22]. *Synechocystis* sp. was transformed with a plasmid harboring *phaC*<sub>Cn</sub>, *nphT7<sub>Cn</sub>*, and *C. necator* acetoacetyl-CoA reductase (*phaC*<sub>C</sub>) genes under the control of the light-inducible *psbAII* promoter. The successful transformant strain *C*. *n*<sub>C</sub>*NphT7<sub>B</sub>*<sub>Cn</sub> was analyzed for PHA production under a two-stage culture system consisting of sequential cell growth and PHA accumulation phases. The strain C<sub>C</sub>*NphT7<sub>B</sub>*<sub>Cn</sub> achieved an encouraging direct photosynthetic production of PHA from CO₂ with a maximum of 14 wt% P(3HB) content on day 7 of cultivation (Fig. 1A). In comparison, strain C<sub>C</sub>*A*<sub>C</sub>*<sub>B</sub>*<sub>Cn</sub> expressing *phaC*<sub>C</sub>, *C. necator* β-ketothiolase (*phaC*<sub>C</sub>) and *phaB*<sub>Cn</sub> recorded a reduction in P(3HB) content (7 wt%) under the same cultivation conditions. The strain pTKP2031V, with only a kanamycin resistance cassette integrated into the genome, showed the lowest P(3HB) production potential (5 wt%). Prolonged incubation until day 14, however, did not exert any significant impact on the P(3HB) accumulation potential of the cyanobacteria under photoautotrophic conditions. At higher cell densities, P(3HB) accumulation may be limited by competition for carbon dioxide and light.

In order to boost P(3HB) production, an exogenous carbon source [0.4%(w/v) acetate] was provided to the cyanobacterial cultures at the PHA accumulation phase. Strain C<sub>C</sub>*NphT7<sub>B</sub>*<sub>Cn</sub> recorded the highest PHA content of 29 wt% on day 10 of incubation (Fig. 1B). The increase in the P(3HB) pool resulting from the addition of a carbon source affirms earlier findings on the effect of external carbon source supplementation on PHA production [7,21]. In the case of air-exchange limitation effect, a significant increase in P(3HB) was observed (up to 41 wt%) for strain C<sub>C</sub>*NphT7<sub>B</sub>*<sub>Cn</sub> (Fig. 1C). These observations imply that the P(3HB) accumulation potential of *Synechocystis* sp. is affected by the provision of carbon source and air-exchange. Interestingly, the increase in CO₂ supply (5%) to photoautotrophic cultures of *Synechocystis* sp. was found to increase the PHA content up to 16 wt% in strain C<sub>C</sub>*NphT7<sub>B</sub>*<sub>Cn</sub> (Table 1). The simultaneous
addition of acetate and fructose to N- or P-deficient cultures of strain pTKP2031V showed a reduction in P(3HB) accumulation compared to photoautotrophic conditions (5% CO2). There were no significant changes in the P(3HB) content of strains CCsACnBCn and CCsNphT7BCn under the same cultivation conditions. The order of PHA-producing potential of the recombinant Synechocystis sp. on day 7 of incubation and under the cultivation conditions tested in this study is CCsNphT7BCn > CCsACnBCn > pTKP2031V.

**Table 1.** P(3HB) accumulation in recombinant Synechocystis sp. PCC 6803 under various treatment conditions.

| Treatment            | P(3HB) (%) w/w of dry cells |
|----------------------|-----------------------------|
| pTKP2031V            |                             |
| N-deficiency, CO2 (5%) | 10 ± 1                      |
| P-deficiency, Acetate, Fructose | 3 ± 1          |
| N-deficiency, Acetate, Fructose | 6 ± 1          |
| CCsACnBCn            |                             |
| N-deficiency, CO2 (5%) | 10 ± 2                      |
| P-deficiency, Acetate, Fructose | 8 ± 1          |
| N-deficiency, Acetate, Fructose | 12 ± 1         |
| CCsNphT7BCn          |                             |
| N-deficiency, CO2 (5%) | 16 ± 4                     |
| P-deficiency, Acetate, Fructose | 18 ± 3         |
| N-deficiency, Acetate, Fructose | 15 ± 2         |

Comparison of P(3HB) accumulation in Synechocystis sp. PCC 6803 strains pTKP2031V, CCsACnBCn and CCsNphT7BCn. Cells cultivated on modified BG-11 media under the indicated cultivation conditions were harvested after 7 days of incubation. Data shown are the means and standard deviation of triplicates. doi:10.1371/journal.pone.0086368.t001

**Expression Levels of PHA Synthesis-related Genes**

The expression levels of native PHA biosynthetic genes in Synechocystis sp. consisting of *phaCSs*, *phaASs* and *phaBCs* were monitored by real-time PCR analysis (Fig. 2A and 2B). Surprisingly, comparative quantification of *phaCSs* and *phaBCs* expression levels in Synechocystis sp. strain CCsNphT7BCn relative to *phaASs* expression that was approximately 2-fold lower. However, there were no significant differences in the expression level of the native *phaCSs* gene in the recombinant Synechocystis sp. (pTKP2031V, CCsACnBCn and CCsNphT7BCn) investigated. The expression levels of *phaCSs* and *phaBCs* that were introduced into the genome on the same operon showed at least 3-fold lower expression in strain CCsNphT7BCn compared to CCsACnBCn. Despite higher levels of PHA accumulation in CCsNphT7BCn, the expression levels of most PHA synthesis-related genes in this strain were relatively lower compared to CCsACnBCn and pTKP2031V.

**Analysis of Synechocystis sp. Transcriptional Response Under Photoautotrophic PHA Accumulation Conditions**

To gain insight into PHA accumulation in cyanobacteria, transcriptomes of recombinant Synechocystis sp. with different PHA-producing potential were analyzed. RNA-seq libraries were prepared from cells cultivated for 7 days in N-deficient BG-11 under photoautotrophic conditions. Sequencing was performed using the Illumina platform yielding a total of 93-million reads for 6 samples, with an average of 15.5-million reads per sample. Scatter plots between the two biological replicates for each recombinant Synechocystis sp. sample show correlation coefficients between 0.96-0.99, indicating the reproducibility of the sequencing data (Fig. S1). The expression levels for each gene were quantified as reads per kilobase of exon model per million mapped reads (RPKM).

The RNA-seq data provide detailed information on the genes that are regulated in response to photoautotrophic PHA accumulation conditions in recombinant Synechocystis sp. strains pTKP2031V, CCsACnBCn and CCsNphT7BCn. In general, the highly expressed genes in Synechocystis sp. were mainly involved in photosynthesis, the electron transport chain, protein metabolic processes and nucleic acid metabolism (Table S1). In particular, the transcript levels of genes involved in photosystem I (*psaB, psaA, psaF* and *psaD*), photosystem II (*psbA3, psbA2, psbX, psbY, psbU, psbK and psbD2*) activities were among the most abundant. A comparison between gene expression in the recombinant strains that were more efficient in PHA production (CCsACnBCn and CCsNphT7BCn) relative to the reference strain (pTKP2013V) was made (Table 2). The up-regulated genes in strains CCsACnBCn and CCsNphT7BCn significantly enriched photosynthesis, transport and cell communication. In contrast, the down-regulated genes were found to be involved mostly in the metabolism of cofactors and vitamins, protein metabolic processes and DNA-binding. The photosystem I reaction center subunit XII gene (*psaM*) that is detected only in cyanobacteria was strongly up-regulated in both CCsACnBCn and CCsNphT7BCn. Another photosystem I reaction center subunit gene, *psaF* was found to be up-regulated more than 10-fold. Both of these subunits are required to form a functional photosystem I [23]. In addition to genes encoding the photosystem I subunits, photosystem II-associated genes were among the significantly up-regulated genes. PsbX and PsbK, that have been found essential for the stability of photosystem II [24], were induced more than 5-fold in both CCsACnBCn and CCsNphT7BCn. Up-regulation of cytochrome B6-f complex subunits, PetG and PetL that are important for either stability or assembly of the complex, was also observed [25]. Two genes involved in porphyrin and chlorophyll metabolism, magnesium-protoporphyrin IX monomethyl ester cyclase (*dd1874*) and protoheme IX farnesyltransferase (*dd1899*) were up-regulated in both strains. Collectively, genes encoding proteins involved in several aspects of photosynthetic activity, e.g. photosystem I and II, cytochrome and chlorophyll metabolism were up-regulated in recombinant Synechocystis sp. that were actively synthesizing PHA.

On the other hand, transcript levels of genes encoding protein metabolism (transcription, translation, amino acid synthesis, etc.) decreased in the recombinant Synechocystis sp. strains CCsACnBCn and CCsNphT7BCn (Table S2). The decrease in transcript levels of genes encoding these proteins [DNA mismatch protein (MutL), methionine sulfoxide reductase B (*dd1680*), prohibitin (*dd1106*), exoenzyme S synthesis protein B (*ExsB*), 3-dehydroquinate dehydratase (*AroQ*) and hydrogenase (*HypA*)] may be related to the reduced growth of Synechocystis sp. under N-deficient conditions. Cells response to nutrient-limiting conditions by accumulating PHA and at the same time slowing down metabolic activities. Reductions in expression levels of genes related to the metabolism of cofactors and vitamins [lipopeptide antibiotics iturin a biosynthesis protein (*dd0495*), cobalamin synthase (*CobS*), 4-hydroxythreonine-4-phosphate dehydrogenase (*PdxA*), cobalt-precorrin-6x reductase (*CobK*), riboflavin biosynthesis protein (*RibG*), lipoyltransferase (*LipB*) and o-succinylbenzoate synthase (*dd1049*)] were observed.

A comparison between gene expression in the recombinant Synechocystis sp. strains CCsNphT7BCn and CCsACnBCn was made to gain substantial insights into the global responses of cyanobacteria
to accommodate the extensive accumulation of PHA (Table 3). The analysis showed that strain CCsNphT7BCn employed a combination of induced stress response, photosynthesis, energy metabolism and transport during the PHA accumulation phase. Notably, genes encoding proteins involved in several aspects of photosynthetic activity e.g. uroporphyrinogen decarboxylase (HemE), ferredoxin component (slr1205), protochlorophyllide reductase subunit (BchB), protohome IX farnesyltransferase (CtaB), photosystem II reaction center protein N (PsbN) and iron-stress chlorophyll-binding protein (IsiA) were up-regulated in strain CCsNphT7BCn compared to CCsACnBCn. The increased photosynthetic activity suggests that the carbon fixing capacity was enhanced to accommodate the increased diversion of carbon to polymer formation. Polyhydroxyalkanoate are bacterial storage compounds synthesized in response to conditions of physiological stress [26]. In the current study, stress-related genes in cyanobacteria include co-chaperonin (groES), Holliday junction resolvase (nucC), molecular chaperon (groEL), superoxide dismutase (sodB) and heat shock protein 90 (hspG) were modestly up-regulated. As it was proposed that PHA accumulation confers survival and stress tolerance in a changing environment [27], stress conditions may trigger responses that favor PHA production. In addition, the transcript level for the global nitrogen regulator, NtcA was detected at an increased level. NtcA is known to regulate the expression of a large number of genes involved in nitrogen metabolism [28] and induction of the gene encoding this protein can be related to the N-deficient cultivation conditions that were applied to increase PHA biosynthesis in *Synechocystis* sp. Conversely, down-regulation of the genes involved in DNA-binding, transport, translation and DNA repair were observed in strain CCsNphT7BCn (Table S3).

**Discussion**

Current limitation of direct photosynthetic production using cyanobacteria is the relatively low PHA content obtained. In this study, it was encouraging to obtain 14 wt% of P(3HB) from direct photosynthetic fixing of carbon dioxide without the addition of an external carbon source. Although cyanobacteria have simple nutrient requirements, the addition of 0.4%(w/v) acetate was found to increase P(3HB) content up to 41 wt% under air-exchange limiting conditions. Previous studies suggested that enhanced P(3HB) accumulation was the result of direct metabolism of acetate for PHA synthesis by employing an existing pathway operating in cyanobacteria [7,21]. The provision of exogenous carbon was found to have a positive impact on PHA accumulation albeit at concentrations that were 10- to 20-fold lower than those required by heterotrophic bacteria. Recently, the development of new photobioreactors for mass cultivation of cyanobacteria is in progress and these findings will greatly aid the use of cyanobacteria for potential industrial applications [29,30].

Early studies indicate that the PHA biosynthetic genes of *Synechocystis* sp. 6803 do not co-localise together to form an operon...
| Gene ID | Description                                      | Fold change (CCsACnBCn vs pTKP2031V) | Expression levelb (pTKP2031V) | Functional category                                      |
|--------|-------------------------------------------------|-------------------------------------|--------------------------------|----------------------------------------------------------|
| srr1169 | salt-stress induced hydrophobic peptide          | 31.93                              | 22.77                          | cation transport                                         |
| slr1064 | mannosyltransferase                              | 29.17                              | 7.85                           | polysaccharide metabolic process                         |
| smr0005 | photosystem I reaction center subunit XII, PsaM | 22.83                              | 22.21                          | photosynthesis                                            |
| smr0008 | photosystem I reaction center subunit IX, PsaJ  | 17.51                              | 45.43                          | photosynthesis                                            |
| sll1161 | adenylate cyclase                                | 11.6                               | 16.04                          | nucleotide metabolic process                             |
| slr2114 | spore coat polysaccharide biosynthesis protein, SpIC | 10.27                           | 7.83                           | metabolic process                                         |
| smr0002 | photosystem II protein, PsbX                     | 10.71                              | 186.1                          | photosynthesis                                            |
| smr0005 | photosystem II reaction center protein K, PsbK  | 6.98                               | 106.49                         | photosynthesis                                            |
| smr0010 | cytochrome B6-f complex subunit, PetG           | 6.96                               | 175.41                         | photosynthesis                                            |
| sll0247 | iron-stress chlorophyll-binding protein          | 4.49                               | 61.36                          | photosynthesis                                            |
| slr0756 | circadian rhythm protein                         | 3.21                               | 56.8                           | circadian rhythm                                          |
| sll0986 | Transposase                                      | 3.57                               | 84.36                          | DNA-binding                                               |
| slr1318 | iron(III) dictrate ABC transporter permease     | 3.5                                | 19.17                          | transport                                                |
| sll1270 | glutamine ABC transporter                        | 2.52                               | 91.4                           | amino acid transport                                      |
| sll1405 | biopolymer transport protein                     | 2.25                               | 15.07                          | protein transport                                         |
| slr1693 | PatA subfamily protein                           | 3.2                                | 55.32                          | intracellular signal transduction                         |
| sll1994a| cytochrome B6f complex subunit, PetL            | 3.92                              | 17.59                          | energy metabolism                                         |
| sll0778 | ABC transporter                                  | 3.39                               | 14.72                          | lipid transport                                           |
| sll1760 | regulatory components of sensory transduction system | 3.33                          | 23.44                          | signal transduction                                       |
| sll1874 | magnesium-protoporphyrin IX monomethyl ester cyclase | 3.19                        | 21.02                          | porphyrin and chlorophyll metabolism                      |
| sll0312 | NarL subfamily protein                           | 2.54                               | 50.47                          | intracellular signal transduction                         |
| sll0789 | OmpR subfamily protein                           | 1.84                               | 92.86                          | intracellular signal transduction                         |
| sll1755 | NAD(P)H-dependent glycerol-3-phosphate dehydrogenase | 3.02                       | 28.65                          | glycerophospholipid metabolism                            |
| sll1821 | 50S ribosomal protein L13                       | 2.71                               | 86.75                          | translation                                               |
| slr0611 | solanesyl diphosphate synthase                   | 2.52                               | 36.57                          | metabolic process                                         |
| sll0792 | transcriptional repressor, SmtB                  | 1.92                               | 77.57                          | DNA-binding                                               |
| sll0779 | PleD protein                                     | 2.07                               | 36.05                          | signal transduction                                       |
| sll1740 | 50S ribosomal protein L19                       | 1.6                                | 356.8                          | translation                                               |
| sll0984 | CDP-glucose-4,6-dehydratase                     | 2.7                                | 20.43                          | amino sugar and nucleotide sugar metabolism              |
| sll0790 | sensory transduction histidine kinase            | 1.48                               | 98.45                          | signal transduction                                       |
| slr2079 | glutaminase                                      | 2.31                               | 65.16                          | cellular amino acid metabolic process                    |
| slr2123 | D-isomer specific 2-hydroxyacid dehydrogenase   | 2.72                               | 21.83                          | carbohydrate metabolic process                            |
| sll0643 | urease accessory protein G                       | 1.57                               | 44.07                          | GTP catabolic process                                     |
Table 2. Cont.

| Gene ID | Description                          | Fold change | Expression level | Functional category                  |
|---------|--------------------------------------|-------------|-----------------|--------------------------------------|
| slr1498 | hydrogenase isoenzyme formation protein, HypD | 2.12 2.72   | 33.82 71.78 91.98 | protein metabolic process            |
| slr1041 | ABC transporter                       | 1.95 2.61   | 75.65 147.32 197.09 | phosphate transport                  |
| slr1982 | chemotaxis protein, CheY              | 1.59 2.6    | 298.23 474.89 774.35 | intracellular signal transduction    |
| slr2131 | cation or drug efflux system protein  | 2.29 2.6    | 26.11 59.75 67.83 | transport                            |
| slr1595 | Na/H antiporter                       | 2.45 2.59   | 13.76 33.65 35.64 | cation transport                     |
| slr1912 | anti-sigma factor antagonist           | 1.87 2.59   | 75.93 141.71 196.56 | regulation of transcription          |
| ssl2296 | pterin-4-alpha-carbolamine dehydratase | 1.81 2.51   | 56.88 103.21 142.56 | tetrahydrobipterin biosynthetic process |
| sll428  | P3 protein                            | 2.18 2.49   | 17.28 37.61 43.1 | cation transport                     |
| sll0080 | N-acetyl-gamma-glutamyl-phosphate reductase | 1.6 2.48    | 127.18 204 315.28 | amino acid metabolic process         |
| sll1899 | protoheme IX farnesyltransferase      | 1.43 2.47   | 79.37 113.61 195.82 | porphyrin and chlorophyll metabolism |
| sll1291 | PatA subfamily protein                | 2.03 2.45   | 89.47 181.58 219.06 | signal transduction                 |
| sll0889 | ABCI-like protein                     | 1.82 2.41   | 33.73 61.35 81.44 | energy metabolism                   |
| sll1249 | bifunctional pantoate ligase/cytidylate kinase | 1.5 2.39  | 50.72 75.95 121.45 | pyrimidine base metabolic process    |
| sll1040 | Narl. subfamily protein               | 2.13 2.38   | 35.46 75.5 84.51 | signal transduction                 |
| sll1805 | sensory transduction histidine kinase  | 1.34 2.32   | 80.73 108.44 187.15 | signal transduction                 |
| sll1229 | hybrid sensory kinase                 | 1.78 2.31   | 49.42 88.21 113.98 | signal transduction                 |

*Only the top 50 highest increase in fold-change and genes encoding known proteins are shown. The values shown represent the mean of two independent biological replicates.

[31,32]. Instead, the PHA synthase of *Synechocystis* sp. consisting of *phaC* and *phaE* subunits are linked in the genome and co-expressed. On the other hand, the β-ketothiolase and acetoyl-CoA reductase of *Synechocystis* sp. do not map close to the PHA synthase locus but are probably clustered together and constitute an operon in a different section of the genome. The expression levels of these two genes were surprisingly lower in the recombinant *Synechocystis* sp. strains C**C**N**A**C**B**C** vs pTKP2031V that had higher PHA production potential compared to strain pTKP2031V that accumulated a lower content of PHA. These results suggest that the endogenous PHA biosynthetic pathway operating in *Synechocystis* sp. did not have a significant impact on the PHA-synthesizing abilities of strains C**C**N**A**C**B**C** and C**C**N**P**h**T**B**C**n**.

The *Chromobacterium* sp. PHA synthase and *C. necator* acetoyl-CoA reductase that were introduced into the genome as an operon showed similar lower expression in strain C**C**N**P**h**T**B**C**n**. The observation that the expression levels of most of the PHA biosynthetic genes were lower in strain C**C**N**P**h**T**B**C**n** suggests that the concentration of these enzymes is not the limiting factor in achieving higher PHA accumulation. Based on the results presented here, the transcription of genes encoding enzymes involved in PHA biosynthesis is highly regulated and may be affected by the PHA content in the cells (Fig. 3). When the PHA accumulated by the cells has exceeded a certain threshold level, adequate levels of the enzymes may already be present to meet the biosynthetic demand. Thus, the PHA granule itself or some other sensing factors may exert negative feedback on the expression of these enzymes. However, the expression levels of the enzyme catalyzing the last step of PHA biosynthesis, *Synechocystis* sp. PHA synthase, remained grossly constant in all recombinant *Synechocystis* sp. because negative feedback regulations are likely exerted in the upper part of the pathway.

Previous genetic studies have focused on the engineering of various bacteria or plant hosts for PHA production, but less is known about the global transcriptional changes of the recombinant host under a PHA-synthesizing environment. A comprehensive view of the cyanobacterial transcriptome during cultivation under conditions favorable for PHA synthesis was generated using RNA-seq analysis. One particularly interesting observation is the up-regulation of photosynthetic activity in recombinants *Synechocystis* sp. with higher PHA-synthesizing potential (Fig. 4 A and B). In recent years, there has been tremendous interest in strategies to improve photosynthetic activity in crops [33,34]. It has been suggested that an increase in photosynthetic activity will improve the yield of crops and provide a potential solution to future food shortages [35]. In this context, the increase of photosynthetic activity in cyanobacteria may explain the higher PHA accumulation observed in recombinant *Synechocystis* sp. strains C**C**N**P**h**T**B**C**n** and C**C**N**A**C**B**C** (pTKP2031V).

The gene encoding one of the most important enzymes in carbon fixation, the rubulose-1,5-biphosphate carboxylase/oxygenase (RuBiCo) large subunit (*rbcL*), was up-regulated in both C**C**N**P**h**T**B**C**n** and C**C**N**A**C**B**C**. RuBiCo is a biologically important enzyme that catalyzes the first step of the reaction that converts atmospheric carbon dioxide into organic carbon [36]. Besides RuBiCo, genes encoding proteins involved in different
Table 3. Genes up-regulated in recombinant Synechocystis sp. strain C<sub>Cs</sub>NphT7B<sub>Cn</sub> compared with C<sub>Cs</sub>AC<sub>Cn</sub>B<sub>Cn</sub>).

| Gene ID | Description | Fold change | Expression level<sup>b</sup> | Functional category |
|---------|-------------|-------------|-----------------------------|---------------------|
| slr2075 | co-chaperon, GroES | 3.26 | 328.12 1,069.34 | protein folding |
| slr1204 | serine protease, HtrA | 2.73 | 1,046.94 2,860.72 | cell communication |
| slr1316 | iron(III) dicitrate ABC transporter permease | 2.37 | 15.33 36.3 | iron transport |
| ssr2595 | high light inducible protein | 2.19 | 25.55 55.96 | chlorophyll-binding |
| sll0379 | UDP-N-acetylglucosamine acyltransferase | 2.14 | 28.2 60.47 | lipopolysaccharide biosynthetic process |
| sll0789 | OmpR subfamily protein | 2.13 | 108.53 231.24 | intracellular signal transduction |
| sll0790 | sensory transduction histidine kinase | 2.12 | 90.22 191.02 | signal transduction |
| sll0896 | Holliday junction resolvase, RuvC | 2.09 | 17.14 35.89 | DNA repair |
| slr0767 | molecular chaperone, GroEL | 2.05 | 733.79 1,507.39 | protein folding |
| slr1279 | NADH dehydrogenase subunit A | 2.05 | 25.85 52.92 | electron transport chain |
| slr0536 | uroporphyrinogen decarboxylase, HemE | 2.02 | 80.15 162.23 | porphyrin and chlorophyll metabolism |
| slr1675 | hydrogenase expression/formation protein, HypA | 2.01 | 111.92 225.29 | cellular protein modification process |
| slr1202 | lactose ABC transporter permease | 1.96 | 16.49 32.23 | transport |
| sll1740 | 50S ribosomal protein L19 | 1.93 | 427.21 825.93 | translation |
| sll0538 | beta-glucohydrolase | 1.92 | 21.65 41.64 | carbohydrate metabolism |
| slr1205 | ferredoxin component | 1.92 | 214.39 412.13 | photosynthesis |
| ssr2049 | protochlorophyllide reductase 57 kD subunit, BchB | 1.9 | 30.51 57.98 | photosynthesis |
| slr1805 | sensory transduction histidine kinase | 1.89 | 63.65 120.35 | signal transduction |
| sll1899 | protoheme IX farnesyltransferase, CtaB | 1.88 | 67.28 126.68 | porphyrin and chlorophyll metabolism |
| slr1256 | urease subunit gamma | 1.86 | 38.67 72.03 | nitrogen metabolism |
| slr1009 | chorismutemutase | 1.84 | 34.12 62.78 | amino acid biosynthetic process |
| slr1516 | superoxide dismutase, SodB | 1.83 | 1,356.38 2,485.21 | immune system process |
| sll1423 | global nitrogen regulator, NtcA | 1.82 | 67.28 126.68 | photosynthesis |
| slr1204 | sensory transduction histidine kinase | 1.81 | 22.41 40.57 | signal transduction |
| sll1085 | glycerol-3-phosphate dehydrogenase | 1.75 | 93.19 159.74 | transcription |
| slr1843 | glucose-6-phosphate 1-dehydrogenase | 1.73 | 34.66 62.29 | signal transduction |
| ssr1386 | cryptochrome 1 | 1.72 | 578.14 1,156.28 | oxidation reduction process |
| sll1045 | NADH dehydrogenase subunit, NdhL | 1.71 | 376.21 752.42 | photosynthesis |
| sll1869 | 3-chlorobenzoate-3,4-dioxygenase | 1.71 | 27.55 51.28 | carbohydrate metabolism |
| sll0792 | transcriptional repressor, SmrB | 1.71 | 93.19 159.74 | transcription |
| sll1283 | sporulation protein, SpoIID | 1.71 | 19.37 33.61 | transcription |
| sll0247 | iron-stress chlorophyll-binding protein | 1.65 | 186.8 308.95 | photosynthesis |
Table 3. Cont.

| Gene ID | Description                        | Fold change C_{C_{Csp}NphT7BCa} vs C_{C_{Csp}A_{Csp}B_{Csp}} | Expression level C_{C_{Csp}A_{Csp}B_{Csp}} | Functional category |
|---------|------------------------------------|---------------------------------------------------------------|--------------------------------------------|---------------------|
| sll1468 | beta-carotene hydroxylase           | 1.65                                                          | 87.04                                      | 143.75              | carotenoid biosynthetic process |
| sll1476 | aspartate carbamoyltransferase      | 1.65                                                          | 57.68                                      | 95.25               | pyrimidine biosynthetic process |
| sll0648 | lipophilic protein                  | 1.65                                                          | 26.01                                      | 42.85               | lipid transport                 |
| sll0923 | exopolysaccharide export protein    | 1.64                                                          | 181.51                                     | 297.86              | lipopolysaccharide biosynthetic process |
| sll1430 | heat shock protein 90, HtpG         | 1.64                                                          | 186.42                                     | 305.82              | protein folding                 |

*Table shows the mean of two independent biological replicates.

Figure 3. The scheme shows the regulation of PHA synthesis-related gene expression in recombinant Synechocystis sp.

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aspects of photosynthesis and electron transport chain were significantly induced in both C_{C_{Csp}NphT7BCa} and C_{C_{Csp}A_{Csp}B_{Csp}}. In particular, the induction of photosynthesis and electron transport chain-related genes was most prominent in strain C_{C_{Csp}NphT7BCa} with the highest PHA accumulation, suggesting the possible correlation of photosynthetic activity with PHA content. The Synechocystis sp. cells may utilize enhanced photosynthesis, carbon fixation and electron transport chain activities as a means to provide precursors that are necessary to drive the production of PHA. The increased photosynthetic production of PHA reveals that similar metabolic engineering approaches can be applied to the production of biofuels or chemicals using this versatile organism. As cyanobacteria and plants share similar photosynthetic machinery, it is likely that the strategy can be extended in future efforts to improve PHA production in higher plants.

In living cells, catabolic reactions that produce energy and anabolic biosynthetic reactions are regulated to maintain a balance of supply and demand. To cope with the higher PHA production demand, carbon dioxide fixing was enhanced to replenish the pool of carbon that was lost to PHA formation. Concomitant with the increase in photosynthetic activity, the flow of newly fixed carbon dioxide into biosynthetic reactions other than PHA was reduced. Genes encoding metabolism of cofactors and vitamins as well as protein metabolic process were found to be down-regulated in strains C_{C_{Csp}NphT7BCa} and C_{C_{Csp}A_{Csp}B_{Csp}}. The reduced growth of recombinant Synechocystis sp. under nutrient-deficient cultivation conditions may account for the depression of these metabolic processes. These cellular anabolic reactions were regulated to maintain the balance of resources in cells. The expression levels of genes involved in the tricarboxylic acid cycle (TCA) were shown to be down-regulated in strains C_{C_{Csp}NphT7BCa} and C_{C_{Csp}A_{Csp}B_{Csp}}. These observations agree well with previous finding that reported on the repressed of the TCA cycle genes in C. necator H16 during PHA production [37].

RNA-seq transcriptome analysis reveals that the heterologous expression of PHA synthesis-related genes in Synechocystis sp. affect not only the regulation of PHA biosynthesis but also the preceding pathways that are involved in the provision of precursors for this biosynthesis. The direct photosynthetic production of 14 wt% of P(3HB) from strain C_{C_{Csp}NphT7BCa} is the highest value achieved for Synechocystis sp. 6803 so far. This work suggests the use of carbon flux as a possible driving force for the biosynthesis of intracellular inclusions e.g. PHA. Future work can be done to confirm this finding by enhancing carbon fixation in cyanobacteria through engineering or overexpressing the enzymes involved in the process.

Materials and Methods

Chemicals and Reagents

All chemicals were purchased from Nacalai Tesque (Tokyo, Japan) or Wako Pure Chemical (Tokyo, Japan) unless otherwise specified. KOD Plus high-fidelity DNA polymerase was purchased from Toyobo (Tokyo, Japan). Restriction enzymes and the DNA ligation kits used were from Takara (Shiga, Japan).

Organism and Culture Conditions

All Synechocystis sp. PC6803 strains (Table S4) were cultivated at 30°C in BG-11 medium [38] buffered with 20 mM HEPES-KOH, pH 8.0, under continuous illumination of 100 μmol photons m^{-2} s^{-1}. Liquid cultures were incubated with shaking (100 r.p.m.) or bubbled with air enriched with 2-3% (v/v) CO₂. Escherichia coli DH5α used for plasmid cloning was grown with shaking (180 r.p.m) at 37°C in Lysogeny broth. For the selection and maintenance of plasmids, kanamycin (50 μg/mL) or ampicillin (100 μg/mL) were added. To promote PHA biosynthesis in cyanobacteria, a two-stage cultivation was performed. The cultures were first grown in BG-11 medium until the late exponential phase and then harvested, washed and transferred to BG-11 medium devoid of sodium nitrate. P-deficiency was achieved by cultivating cells in BG-11 medium without K₂HPO₄. Different carbon sources [0.2% (w/v) and 0.4% (w/v) of fructose and/or acetate] were added to study the effects of carbon supplementation on PHA accumulation. Air-exchange limiting conditions on cultures were imposed by sealing the mouth of culture vessels with cotton plugs and covering with aluminium foil [7]. The cyanobacterial cultures were cultivated in the above conditions.
culture conditions for 7, 10 or 14 days, harvested by centrifugation (8000 g, 10 min) and then lyophilized.

Plasmid Construction and Transformation of *Synechocystis* sp

The constructs used for transformation of *Synechocystis* sp. were derived from pTKP2031V (Table S4); pTKP2031V was designed for insertion into the genome via homologous recombination between sites *slb2030* and *slb2031* together with a kanamycin resistance cassette [22]. The expression of all cyanobacterial constructs was under the *psbAII* promoter. The gene cluster containing β-ketothiolase (*phaACn*) and acetocetate-CoA reductase (*phaACn*) were amplified from chromosomal DNA of *C. necator* H16 using primers *phaABc* (F; *NdeI*) and *phaACbc* (R; *HpaI*) (Table S5). The resulting PCR product was digested with *NdeI* and *HpaI* and inserted into *NdeI* and *HpaI*-digested pTKP2031V to obtain pTKP2031V-phaABc. The PHA synthase (phaACn) was prepared from chromosomal DNA of *Chromobacterium* sp. USM2 using primers *phaACn* (F; *SfiI*) and *phaABc* (R; *AatI*). This PCR fragment was digested with *SfiI* and *AatI* and subcloned into the appropriate restriction sites of pTKP2031V-phaABc by ligation to yield pTKP2031V-phaCAG2Bc. The gene cluster containing plastocyanin (*pc*), plastocyanin/NADPH dehydrogenase (*plastoquinone; FNR, ferredoxin-NADP(H) reductase; Pc, plastocyanin; PSI, photosystem I; PSII, photosystem II; Ndh, NADH dehydrogenase; Glc-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-bp, fructose-1,6-biphosphate; Glycerate-1,3-P2, 1,3-biphosphoglycerate; 3-P-Glycerate, 3-phosphoglycerate; Ru-5,1-bisP, ribulose-1,5-biphosphate; PEP, phosphoenolpyruvate. doi:10.1371/journal.pone.0086368.g004

Real-time PCR Analysis
cDNA synthesis was performed with 250 ng of RNA using the QuantiTect Reverse Transcription Kit (Qiagen, USA). Real-time PCR quantification was performed using Thunderbird SYBR qPCR Mix (Toyobo, Japan) and gene-specific primers with the Mx3000P QPCR system (Agilent, USA). The cycling conditions were as follows: 95°C for 10 min, 40 cycles: 95°C for 15 s and 60°C for 1 min. A melting curve analysis (60°C-95°C) was performed after each amplification to ensure specificity of the reaction. Transcript levels were quantified based on determination of the quantification cycle (Ct). The transcript levels of genes of interest were normalized to the level of the housekeeping gene (16S rRNA) used in this study. Comparative quantification was used to compare the expression levels of genes of interest in *Synechocystis* sp. PCC 6803 strains *C. necator* H16 and *Synechocystis* sp. CL190 with phaCCn. The phaCCn strain was prepared from chromosomal DNA of *Synechocystis* sp. USM2 using primers *phaCCn* (F; *SfiI*) and *phaABc* (R; *AatI*). Transformation of *Synechocystis* sp. was performed as described previously [39]. Briefly, 100-200 μL of an exponentially growing culture were mixed with a plasmid solution to a final concentration of 1-2.5 μg/mL. The mixture was then spread onto a nitrocellulose membrane filter placed on a BG-11 plate and incubated overnight (12 h) at 30°C. The membrane filter was transferred onto a new BG-11 plate and incubated overnight (12 h) at 30°C. The mixture was then spread onto a nitrocellulose membrane filter placed on a BG-11 plate and incubated overnight (12 h) at 30°C.

RNA-seq Library Preparation, Illumina Sequencing and Data Analysis

For each sample, 2 μg total RNA was subjected to ribosomal RNA depletion using the Ribo-zero rRNA removal kit (Epicentre, USA). The cDNA libraries for RNA-seq were constructed from total RNA depleted of rRNA using the Illumina TruSeq Stranded mRNA Sample Preparation Kit (Illumina, USA) according to the manufacturer’s specifications. In brief, preparation of the cDNA libraries included the following steps: RNA fragmentation, cDNA synthesis, 3’ ends adenylation, adapter ligation and cDNA template enrichment. Quantification of the libraries was carried out using a Bioanalyzer 2100 (Agilent, USA) and 4 to 6 pM of the template was used for cluster generation. Libraries were sequenced on a Miseq (Illumina, USA) instrument using the 2×250 paired end protocol. The sequence data has been submitted to the NCBI Gene Expression Omnibus (GEO) under accession number GSE50688. The RNA-seq data analysis was performed using CLC Genomics Workbench 6 software (CLC bio, Denmark). Sequence reads were pre-processed to trim low-quality reads and filter reads shorter than 20 bp. The qualified sequence reads were mapped to the *Synechocystis* sp. PCC 6803 genome (NC_000911), allowing a maximum of two mismatches. The reference genome sequences and annotations were downloaded from NCBI (downloaded on May 23, 2013). Sequence reads that mapped to non-coding RNA and reads that did not map to unique positions were excluded from further analysis. The transcript levels were expressed as reads per kilobase of exon model per million mapped reads in which the read count for a gene was normalized by the length of the gene and the total number of reads mapped in the sample [41]. Statistical analysis was performed and genes with a False Discovery Rate (FDR) *p*-value correction <0.05 were determined as differentially regulated genes [42].

**Figure 4.** The scheme shows the cellular changes in recombinant *Synechocystis* sp. strains (a) *C. necator* Bc and *C. necator* NphT7BcN (compared with pTKP2031V) (b) *C. necator* NphT7BcN (compared with C. necator BcC) under photoautotrophic PHA biosynthesis conditions. Only a selection of cellular changes is shown. The genes or pathways that are up-regulated are in red; the downregulated ones are in green. Black dashed lines indicate the engineered route. AP, allophycocyanin; PC/PEC, phycoerythrocyanin; Cytb6/f, cytochrome b6/f complex; PQ, plastoquinone; FNR, ferredoxin-NADP(+) reductase; Pc, plastocyanin; PSI, photosystem I; PSII, photosystem II; Ndh, NADH dehydrogenase; Glc-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-bp, fructose-1,6-biphosphate; Glycerate-1,3-P2, 1,3-biphosphoglycerate; 3-P-Glycerate, 3-phosphoglycerate; Ru-5,1-bisP, ribulose-1,5-biphosphate; PEP, phosphoenolpyruvate.
Table S4 Strains and plasmids used in this study.

| Strain | Description |
|--------|-------------|
| pTKP203IV | Polyhydroxyalkanoate (PHA) production plasmid |
| pTKP20B93 | PHA production plasmid |

Table S5 Primers used in this study.

| Primer Name | Sequence |
|-------------|----------|
| Forward | Reverse |
| primerA | primerB |

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Author Contributions

Conceived and designed the experiments: N-SL, KK MM. Performed the experiments: N-SL, YK CPF. Analyzed the data: N-SL MM. Contributed reagents/materials/analysis tools: MM. Wrote the paper: N-SL.