Running title: Bioplastic Overproduction in Cyanobacteria

Genetic Engineering and Metabolite Profiling for Overproduction of Polyhydroxybutyrate in Cyanobacteria

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Abbreviations: CE-MS, capillary electrophoresis-mass spectrometry; NMR,
nuclear magnetic resonance; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate;
TEM, transmission electron microscope

Abstract

Genetic engineering and metabolite profiling for the overproduction of polyhydroxybutyrate (PHB), which is a hydrocarbon material in biodegradable plastics, were examined in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Transconjugants harboring cyanobacterial expression vectors that carried the *pha* genes for PHB biosynthesis were constructed. The overproduction of PHB by the engineering cells was confirmed through microscopic observations using Nile red, transmission electron microscopy (TEM), or nuclear magnetic resonance (NMR). We successfully recovered PHB from transconjugants prepared from nitrogen-depleted medium without sugar supplementation in which PHB reached approximately 7% (w/w) of the dry cell weight, showing a value of 12-fold higher productivity in the transconjugant than that in the control strain. We also measured the intracellular levels of acetyl-CoA, acetoacetyl-CoA, and 3-hydroxybutyryl-CoA (3HB-CoA), which are intermediate products for PHB. The results obtained indicated that these products were absent or at markedly low levels when cells were subjected to the steady-state growth phase of cultivation under nitrogen depletion for the overproduction of bioplastics. Based on these results, efficient factors were discussed for the overproduction of PHB in recombinant cyanobacteria.

Key words: acetyl-CoA, cyanobacteria, metabolite analysis, nitrogen depletion, PHB

Poly- β -hydroxybutyrate (PHB, Supplementary Fig. S1) is one of the carbon materials in biodegradable plastics that are environmentally friendly (1,2). PHB is generated by PHA (polyhydroxyalkanoate) biosynthesis genes, which consist of *phaA* (β -ketothiolase producing acetoacetyl-CoA), *phaB* (acetoacetyl-CoA reductase producing 3-hydroxybutyryl-CoA (3HB-CoA)), and *phaC/phaE* (PHA synthase

57 producing PHB) (Fig. 1A). Cyanobacteria are photosynthesizing Gram-negative
58 prokaryotes that produce oxygen. The biological history of cyanobacteria is extensive
59 because they first appeared approximately three billion years ago. The overproduction
60 of PHB has been examined for renewable and high-value-added carbon materials
61 made by CO₂-fixing during photosynthesis in cyanobacteria. The cyanobacterium
62 *Synechocystis* sp. strain PCC 6803 is unicellular and considered a good material for
63 the biotechnology industry because it is transformable and its whole genome sequence
64 has been decoded (3). Previous studies demonstrated that the recombinant
65 PHA-negative mutant *Alcaligenes eutrophus* transformed with plasmids carrying *pha*
66 [exogenous *phaEC*_{Syn} or hybrid *phaECAB* (*phaEC*_{Syn} + *phaAB*_{Ae})] genes from
67 *Synechocystis* sp. PCC 6803 and/or *A. eutrophus* accumulated PHB up to 3.5 to 12%
68 (w/w) of the dry cell weight under nitrogen-depleted conditions supplemented with
69 sugar (gluconate, 0.5 to 2%) as the carbon source (4). On the other hand, PCC6803
70 carrying endogenous *phaAB* and *Escherichia coli tesB* (thioesterase), but not
71 endogenous *phaEC* (knockout of the *phaEC* genes) on the genome resulted in
72 approximately 0.53 g/L of 3-hydroxybutyrate (3HB) being produced under phosphate
73 depletion conditions in which the products were readily secreted from cells without the
74 overexpression of transporters (5). The production of PHB (and PHA) has also been
75 examined in cyanobacteria and other bacteria, which are engineered and
76 non-engineered (6–11). Significantly high PHB biomass production in dry cell weight
77 has been observed. However, studies for PHB overproduction using a novel expression
78 vector have not been sufficient. Furthermore, the metabolic pool of intermediate
79 products for PHB overproduction has not yet been examined in detail.

80 The new cyanobacterial expression vectors pAM500 and pAM461c, which
81 possess the origin of replication that functions in a broad range of Gram-negative
82 bacteria, have recently been constructed (12). To examine shuttle vectors, the gene *gfp*
83 (green fluorescence protein) was cloned downstream from the expression control
84 element (ECE) originating from the regulatory region of the *Microcystis aeruginosa*

gene *psbA2* (for photosystem II D1 protein), and the vectors were introduced into three kinds of cyanobacteria (*Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Limnothrix/Pseudanabaena* sp. ABRG5-3) by conjugation. Multiple copy numbers of the expression vectors (in the range of 14 to 25 copies per cell) and the strong expression of GFP at the RNA/protein level were observed in the cyanobacterial transconjugants. These findings indicated that the vectors in combination with the recombinant cells were useful for overproducing and recovering target gene products from cyanobacteria. In the present study, we constructed recombinant cyanobacteria transformed with expression vectors carrying *pha* genes for PHB biosynthesis, and confirmed the usability of these transconjugants in the overproduction of bioplastics. The PHB productivities and levels of intermediates that accumulated under nitrogen-depletion conditions in the steady-state growth phase were also measured to examine the overproduction of PHB.

MATERIALS AND METHODS

Plasmids and bacterial strains The plasmids and bacterial strains used in this study are listed in Supplementary TABLE S1. Cyanobacterial strains were grown in BG11 or BG11₀ (without NaNO₃ in BG11) (13) under continuous cool white light illumination (100 μmol of photons m⁻² s⁻¹) at 30°C. The *Escherichia coli* strain was used as a host cell for plasmids carrying antibiotic-resistant genes. Transconjugants harboring pAM derivatives as expression vectors were cultivated in BG11 or BG11₀ medium supplemented with chloramphenicol (at a final concentration of 12.5 μg/mL). Details of the respective experimental conditions for cultivation are described in the figure and table legends.

DNA oligonucleotides The oligonucleotides of the DNA primers were as

follows: VZ-F2, 5'-CTGATGTTACATTGCACAAG-3'; VZ-R,
 5'-ATGAAGGAGAAAACCTCACCG-3'; phaAB_FwIF, 5'-
 GAAATTATCCACCATATGATCTTTTCGGACTCGATATTC-3' (underline, *NdeI* site);
 phaABEC_RvIF, 5'-AAGCTTTTACTACCCCGGGTTAAAGCTCTTGCTTTCAAC-3'
 (underlined, the *SmaI* site); phaEC_FwIF
 5'-CAGAAATTATCCACCATATGGATAAACCGACACAA-3' (underlined, the *NdeI*
 site); phaAB_RvIF, 5'- GTCGGTTTATCCCATATGTTTTGCCCTTTTCCTTTAACG
 -3' (underlined, the *NdeI* site); 843phaABEC_Fw3,
 5'-CTTTATCAAGCTAGTATTAAC-3';
 843phaABEC_Fw4, 5'-GATATCCCACCAGAAGCGATC-3'; 843phaABEC_R1,
 5'-CGACGCAATTATCGACATAAC-3'; 843phaABEC_R2,
 5'-AACTTGAATATCAGCCAGCAC-3'; 461c_Fw, 5'-
 CCTTTACATAACTATAAAAACCCG -3'; 843phaE_Rv, 5'-
 CCAGTTTCCGTCCAAAGATTGACG -3'.

PHB expression vector In order to construct the expression vector phaEC461c,
 we initially amplified a 2.2-kbp DNA fragment carrying the *Microcystis aeruginosa*
 NIES-843 *phaEC* genes by PCR, in which LA-taq DNA polymerase (Takara, Tokyo,
 Japan) was used, with the specific primers phaEC_FwIF and phaABEC_RvIF for the
phaEC gene. The resultant PCR fragment was subjected to gel electrophoresis, then
 isolated from an agarose gel. This DNA fragment was inserted into the *NdeI/SmaI* site
 in the expression vector pAM461c (12) in order to create phaEC461c (Fig. 1C), using
 an In-Fusion HD cloning kit (TaKaRa Clontech, Tokyo, Japan). The nucleotide
 sequences of *phaEC* on phaEC461c were verified and the absence of a mutation was
 confirmed by sequencing with the primers of VZ-F2, VZ-R, phaAB_FwIF,
 phaEC_FwIF, and 843phaABEC_Fw3, respectively. We confirmed that the
 phaEC461c plasmid was maintained in 6803KZ (or GT)_EC transconjugants by direct
 colony PCR with a set of specific primers, phaEC_FwIF and phaABEC_RvIF for the

phaEC gene or VZ-F2 and VZ-R for the *phaEC461c* plasmid, respectively. To create *phaABEC461c*, PCR was performed with the primers *phaAB_FwIF* and *phaAB_RvIF* using the NIES-843 genome as template DNA. The 2.0-kbp PCR fragment of *phaAB* was isolated and then inserted into the *NdeI* site in *phaEC461c* using the In-Fusion kit (Fig. 1C). The nucleotide sequences of *phaABEC* on *phaABEC461c* were verified and the absence of a mutation was confirmed by sequencing with the primers *461c_Fw*, *phaAB_FwIF*, *phaEC_FwIF*, *843phaABEC_Fw3* and *Fw4*, *843phaABEC_R1* and *R2*, *phaAB_RvIF*, *843phaE_Rv*, and *phaABEC_RvIF*, respectively. We also confirmed that the *phaABEC461c* plasmid was maintained in 6803GT (or KZ)_{ABEC} transconjugants by direct colony PCR with the specific primers *phaAB_FwIF* and *phaABEC_RvIF* for the *phaABEC* gene or VZ-F2 and VZ-R for the *phaABEC461c* plasmid, respectively.

Transformation of cyanobacteria Conjugations with the expression vectors (IncQ, *mob*⁺) of pAM derivatives and the helper plasmid R751 (IncP, *tra*⁺) were conducted as described previously (12,14,15).

Microscopic observations Cells was morphologically observed using the optical photomicroscope BX53/DP72 (Olympus, Tokyo, Japan) at high resolution with a Nomarski prism (optics, differential interference contrast) or with the fluorescence microscope, BX53/DP72, which was fit with the special cube, BW (Olympus). The fluorescence of cells stained with Nile red (0.36 μ M: Wako Ltd., Tokyo, Japan) was detected using the BW cube with excitation (460 to 495 nm) and emission (510 nm) filters. The exposure time was 0.3 seconds for the fluorescence microscope. Images were processed with the software Viewfinder Lite (Olympus) (12,16,17).

TEM Cells were harvested and fixed with the reagents, 2% formaldehyde (PFA) + 2% glutaraldehyde (GA) in 0.05 M cacodylate buffer (pH 7.2) for prefixation, and

2% osmium tetroxide in 0.1M cacodylate buffer for postfixation. The resulting cells that stained with uranyl acetate and lead solution were observed using transmission electron microscope (TEM) (JEM-1200EX: Jeol, Tokyo, Japan) by an ultrathin sectioning method (17).

NMR analysis The extraction of PHB from a small scale cell culture (50 mL) for an nuclear magnetic resonance (NMR) analysis was described below (Fig. 4C). PCC 6803KZ transconjugants (6803KZ_EC) harboring phaEC461c were prepared as shown in Figure 4C and Supplementary Figure S3. The resultant cell culture of 50 mL was harvested, directly subjected to sonication (output 40, 1 min $\times$ 3 times; UD-100: TOMY, Tokyo, Japan), and then freeze-dried (FreeZone: LABCONCO, Missouri, USA). The resultant pellet (10 mg) was dissolved in 300 μ L of chloroform (CHCl_3), directly subjected to sonication (output 40, 1 min $\times$ 3 times; UD-100: TOMY), and then centrifuged ($2,500 \times g$, 8 min) and the supernatant was collected. This extract of PHB was transferred into a 15-mL egg-plant-shaped flask. The extract was then subjected to vacuum evaporation (V-850: BUCHI, Flawil, Switzerland). The pellet in the flask was again dissolved in 300 μ L of chloroform and this extract was subjected to capillary column filling with cotton wool. The flow-through fraction was collected and subjected to vacuum evaporation. The resultant pellet was dissolved in 600 μ L of deuterated chloroform (CDCl_3) and subjected to an NMR analysis. The ^1H -NMR (400 MHz) spectrum was measured in deuterated chloroform with tetramethylsilane (TMS) as an internal standard using the AVANCE III FT-NMR spectrometer (Bruker BioSpin, Rheinstetten, Deutschland) equipped with a 5-mm BBFO probe.

Metabolite analysis using CE-MS The transconjugants of 6803GT_PAM, EC, or ABEC were cultivated under the conditions shown in Figure 3. Samples for the metabolite analysis were prepared according to a previously reported method (18) with minor modifications. Cell cultures (50 mL) were centrifuged and cells were collected.

They were then dissolved in 10 mL of ammonium hydrogen carbonate (NH_4HCO_3 , 20 mM as a final concentration). Pellets were collected by centrifugation at 15°C (2,500 × g, 8 min) (Suprema 21: TOMY) and dissolved in 1 mL of pre-cooled (-30°C) methanol containing 31 μM L-methionine sulfone and 31 μM piperazine-1,4-bis (2-ethanesulfonic acid) as internal standards for a mass analysis. The resultant sample (1 mL) was transferred into a microtube. A total of 300 μL of chloroform and 100 μL of ultrapure water were then added. This was vortexed at the maximum range for 1 min by a microtube mixer (MT-360: TOMY) and then directly subjected to sonication (UD-100, TOMY) at 4°C for 30 min under a dim light. The sample in the microtube was subjected to microtube mixing (MT-360: TOMY) at 4°C for 30 min in the dark, centrifuged (13,500 × g, 5 min, 4°C), and the supernatant (980 μL) was then collected. Ultrapure water (440 μL, 4°C) was added to the microtube and mixed well with the supernatant. The supernatant was again subjected to centrifugation (13,500 × g, 5 min 4°C) and then collected (900 μL). This supernatant (450 μL x 2 times) was filtrated using a spin column (Ultracel-3K: Amicon/Millipore, Darmstadt, Germany) at 4°C for 5 (to 60) min at 13,500 × g. The flow-through fraction (300 μL) was collected and freeze-dried (FreeZone: LABCONCO). The sample was stored at -80°C until use. After being dissolved in water, the metabolite extract was subjected to capillary electrophoresis-mass spectrometry (CE-MS). The CE-MS analysis was performed as described previously (18). Authentic standards of acetyl-CoA, acetoacetyl-CoA, and 3-hydroxybutyryl-CoA, were purchased from Sigma-Aldrich (St Louis, USA).

Statistical treatment The two-way analysis of variance was conducted using aov function of a computer soft R (R Foundation for Statistical Computing Platform: x86_64-w64-mingw32/x64 (64-bit), Vienna, Austria; <http://www.R-project.org/> (2014)). The bacteria strain and the presence of nitrogen source were used for the factors.

PHB preparation The transconjugant of 6803GT_ABEC harboring phaABEC461c was cultivated for the preparation of PHB under the conditions described in the legend of Figure 4D and TABLE 1. The resultant cell culture as a large scale (2 L for Fig. 4D, 1.35 L for TABLE 1) was harvested and freeze-dried (FreeZone: LABCONCO). The dry cell weight was measured. PHB was prepared as described previously (11) with the following modifications. The dried sample was moved to a glass tube (TE-32: IWAKI, Tokyo, Japan), dissolved in 7 mL of chloroform, and directly subjected to sonication (output 70, 10 min $\times$ 3 times; UD-100: TOMY). The resultant pellet was left to stand for 3 hours at 70°C, again subjected to sonication (output 70, 10 min $\times$ 3 times; UD-100), then heated for 2 days at 70°C. Sonication (output 70, 10 min $\times$ 3 times; UD-100) was performed two more times and the samples were left to stand overnight at 70°C. The respective resultant pellets were dissolved in chloroform (10 mL) and subjected to filtration using a paper filter (ϕ 90 mm: ADVANCE, Tokyo, Japan). This filtrated sample was collected in a glass tube (approximately 7 to 10 mL of the chloroform extraction), subjected to vacuum evaporation (V-850: BUCHI), and then dissolved in 1 mL of chloroform (70°C). Ten milliliters of hexane [$\text{CH}_3(\text{CH}_2)_4\text{CH}_3$] was added and mixed well in a glass tube covered with tin foil, and the samples were left to stand overnight at room temperature. Pellets of PHB were collected by centrifugation ($550 \times g$, 15 min) (M-2420: KUBOTA, Tokyo, Japan). The resultant pellet was dissolved in 1 mL of hexane, centrifuged ($550 \times g$, 15 min), and again subjected to vacuum evaporation. The pellet was dissolved in 1 mL of chloroform, mixed well with 10 mL of methanol (CH_3OH), and left overnight at room temperature. The supernatant was discarded. The resultant solution was transferred into a microtube (1.5 mL) and the pellet was collected by centrifugation ($14,500 \times g$, 15 min). This process was repeated one more. The resultant pellet was again subjected to vacuum evaporation. The weights of the prepared pellets (PHB) were measured.

RESULTS AND DISCUSSION

Construction of the PHB expression vector and transconjugants Many value-added carbon materials are synthesized from acetyl-CoA as a key product in metabolic pathways in cyanobacteria that can photosynthesize (Supplementary Fig. S1). Previous studies reported the construction of new cyanobacterial expression vectors possessing an origin of replication that functioned in a broad range of Gram-negative bacteria, and confirmed their potential utility in overproduction for the green fluorescent protein (GFP) in *Synechocystis* sp. strain PCC 6803 as a trial case (12). In the present study, we attempted to overproduce polyhydroxybutyrate (PHB), a hydrocarbon material in biodegradable plastics, the biosynthesis of which requires *pha* gene products. The *pha* genes were arranged on the genomes as *phaAB* and *phaEC* in *Synechocystis* sp. PCC 6803, or *phaABEC* in *Microcystis aeruginosa* NIES-843, respectively (Fig. 1B). Previous studies revealed that the recombinant PHA-negative mutant *A. eutrophus* transformed with plasmids carrying exogenous *phaEC_{Syn}* from *Synechocystis* sp. PCC 6803 accumulated PHB at up to 12% (w/w) of the dry cell weight when cells were cultivated under nitrogen-depleted conditions in mineral salt medium supplemented with gluconate (2%) as the sole carbon source (4). On the other hand, the recombinant *A. eutrophus* transformed with plasmids carrying hybrid *phaECAB* (*phaEC_{Syn}* + *phaAB_{Ae}*) genes from *Synechocystis* sp. PCC 6803 and *A. eutrophus* accumulated PHB at up to 6.7% (w/w) of the dry cell weight under the same conditions (4). These findings prompted us to construct cyanobacterial expression vectors that carried *phaEC* or *phaABEC* for the overproduction of PHB in cyanobacteria. Since four *pha* genes are known to be distributed as a cluster on the genomic DNA of *Microcystis aeruginosa* NIES-843 (Supplementary TABLE S1) (19), as shown in Figure 1B, we used these genes as a template DNA for gene cloning. Details for the construction of PHB expression vector were described in the

MATERIALS AND METHODS. The constructed expression vectors phaEC461c and, phaABEC461c, and the original vector pAM461c were introduced into the 6803KZ_WT and 6803GT_WT strains, respectively, by conjugation to obtain recombinant 6803KZ (or GT)_EC, ABEC, or PAM harboring phaEC461c, phaABEC, or pAM461c (Supplementary TABLE S1). We confirmed the maintenance of these expression vectors in the transconjugants (MATERIALS AND METHODS). In this study, we used two types of host cyanobacterial strains, 6803KZ and 6803GT. 6803KZ is a representative strain, the whole genome sequence of which has been decoded (3). The 6803GT strain has previously been described as a glucose-tolerant strain that grows under heterotrophic conditions in the dark, in which the carbon source replaces the support of light energy (20). Therefore, we initially used the 6803KZ strain, and then the 6803GT strain because its effects have not yet been tested in different host strains of PCC 6803. We speculated that the transconjugant may be advantageous for the overproduction of PHB in two ways. One was the simple cloning of the *phaEC* or *phaABEC* cluster into the expression vector pAM461c, which is a broad-host range plasmid in not only cyanobacteria, but also Gram-negative bacteria. The *phaABEC* cluster has never been cloned as an endogenous unit. Another advantage is the use of the glucose-tolerant strain 6803GT instead of the glucose-sensitive strain 6803KZ. The utilization of 6803GT expands test engineering using organic waste (instead of glucose) as carbon sources under dark conditions and reduces electricity usage. However, experiments were performed using 6803KZ and 6803GT transconjugants cultivated under nitrogen-depleted conditions without glucose supplementation to examine PHB overproduction as a foundation for a PHB study.

Cell growth by PCC 6803 transconjugants Cell growth by the transconjugant 6803KZ_EC was examined under normal physiological cultivation conditions using BG11 medium. The results obtained were shown in Figure 2A. The growth curve of 6803KZ_EC revealed that growth was slightly inhibited when the transconjugant was

cultivated in BG11 medium (+N) under an atmosphere containing 0.04% CO₂ gas. The effective induction and accumulation of PHB was previously achieved in microalgae under nitrogen- or phosphate-limiting conditions (4,5). Cell growth by the transconjugant 6803KZ_EC was also examined under nitrogen-depleted (-N) conditions in an atmosphere containing 2% CO₂ gas because the use of medium supplied with a high concentration of CO₂ gas may effectively induce the overproduction of PHB (Fig. 2B). Our results indicated that the inhibition of growth was absent or very slight when 6803KZ_EC was cultivated under such conditions, and this was also independent of the nitrogen resource (+N or -N). These results implied that supplying a high concentration of CO₂ gas for the accumulation of PHB overcame the inhibition of growth. The depletion of nitrogen also resulted in low turbidity in the cell culture. This low turbidity may depend on a biological energy-flow-balance between the accumulation of PHB in the carbon and nitrogen pathways involving amino acid biosynthesis related to cell growth (Supplementary Fig. S1). We also observed cell growth by the transconjugants 6803GT_PAM, EC, and ABEC, respectively, under conditions in which 2% CO₂ gas was supplied in BG11 (+N, Fig. 2C) or BG11₀ (-N, Fig. 2D) medium. When 6803GT_WT (wild type) and 6803GT_PAM (plasmid only) were used as control strains, similar results were observed to those shown in Figure 2B. This result indicated that differences in the genetic backgrounds of PCC 6803KZ and PCC 6803GT may have had no (or almost no) effect on the growth of cells harboring phaEC461c or phaABEC461c. The colors of the 6803KZ_WT and EC cell cultures were shown in Supplementary Figure S2. The colors of the cell cultures changed from green to yellow with both strains of 6803KZ_WT and EC, indicating that nitrogen depletion (-N) caused discoloration even in wild-type (WT) cells. These results were consistent with those shown in the growth curves in Figure 2B.

Microscopic observations of cyanobacteria that accumulated PHB The

overproduction of target gene products in transconjugants has not yet been elucidated in sufficient detail. Therefore, we microscopically observed the accumulation of carbon materials in strains that were stained with Nile red (Fig. 3). Nile red is a lipophilic stain involving hydrocarbons, lipids (21), and other carbon materials. Optical or fluorescent microscopic images were obtained for the PCC 6803 wild type strains (WT), transconjugants of 6803KZ_EC and 6803GT_ABEC. When the 6803KZ (or GT)_WT strain was stained with Nile red, fluorescent microscopic observations showed that its color changed from red to orange (or yellow) under nitrogen depletion (BG11₀ medium) conditions. This result demonstrated the accumulation of carbon materials as PHB even if wild type cells were cultivated under nitrogen depletion conditions. When the transconjugants were examined under the same conditions, significantly strong yellow (or orange) signals, reflecting the accumulation of carbon materials as PHB, were observed under nitrogen depletion (BG11₀ medium) conditions. The accumulation of carbon materials was observed within several days from the start of nitrogen depletion and continued until 8 days (data not shown). These results indicated that PHB accumulated in the transconjugants of 6803KZ_EC and 6803GT_ABEC. The analysis of PHB as accumulated carbon materials was verified and described in a later section. However, we could not determine which transconjugant was the best strain (KZ or GT for strain, EC or ABEC for genes) for the overproduction of PHB at this stage. Nevertheless, the PCC 6803 wild type strain potentially possessed endogenous *phaAB* (*slr1993-1994*) and *phaEC* (*slr1829-1830*) on an independent locus in the genome (Fig. 1B). Therefore, we speculated whether the overexpression of exogenous NIES-843 *phaEC* genes cloned into the expression vector could be an efficient method for reinforcing the accumulation of PHB in the transconjugant of 6803KZ_EC. A previous study reported that the overexpression of exogenous *phaEC*_{Syn} cloned into a plasmid resulted in the accumulation of PHB at up to 12% (w/w) of the dry cell weight from the recombinant PHA-negative mutant *A. eutrophus* cultivated under nitrogen-depleted conditions (4). This finding and Figure 3

led us to perform TEM observations on the 6803KZ_EC strain that accumulated PHB. The results obtained were shown in Figure 4. When 6803KZ_WT cultivated under normal physiological conditions in BG11 medium (+N) was examined, clear cytoplasmic contents from which thylakoid membranes prominently expanded were observed by TEM (Fig. 4A). On the other hand, many vacuoles were observed as inclusion bodies that accumulated with PHB in 6803KZ_EC cells cultivated under nitrogen-depleted conditions, in which most thylakoid membranes no longer disappeared (BG11₀, -N; Fig. 4B). This was consistent with previous findings (4,10).

¹H-NMR spectrum for PHB We prepared a PHB extract from transconjugant 6803KZ_EC cells cultivated in a small scale culture (50 mL) under the conditions shown in Figure 4B and subjected it to a ¹H-NMR analysis. The spectrum obtained was shown in Figure 4C and Supplementary Figure S3. A unit of the polymer structure in PHB was shown as (-O-CHCH₃-CH₂-CO-)*n* and the positions of carbons in the unit structure are shown as an inset in the figure. Four proton signals of PHB were observed at 5.26, 2.61, 2.48, and 1.28 ppm and the area ratio was 1:1:1:3. The signal at 5.26 ppm (dq, *J* = 7.4, 6.3, and 5.8 Hz) was assigned to a methine proton. The signals at 2.61 (dd, *J* = 15.5 and 7.4 Hz) and 2.48 ppm (dd, *J* = 15.5 and 5.8 Hz) were assigned to geminal methylene protons. The signal at 1.28 ppm (d, *J* = 6.3 Hz) was assigned to a methyl proton. These results were consistent with the PHB structures reported previously (22,23). A minor signal was observed at approximately 1.25 ppm (Supplementary Fig. S3). This signal may have been caused by an impurity in the PHB extract. These signal profiles were very similar when powder PHB prepared from large scale cell cultures of 6803GT_ABEC (TABLE 1 and Fig. 4D) was used (data not shown).

Extraction and mass yield of PHB We recovered PHB from the transconjugants of 6803GT_PAM, EC, or ABEC using the method described in

MATERIALS AND METHODS. The productivities of PHB extracted from these cells were summarized in TABLE 1. The mass yield was 0.58, 1.18, or 6.98% (w/w) per dry cell weight in the transconjugants of 6803GT_PAM, EC, or ABEC, respectively. The relative ratio of PHB production was 2.03 of EC/PAM ($= 1.18/0.58$), 5.92 of ABEC/EC ($= 6.98/1.18$), or 12.0 of ABEC/PAM. This result showed that productivity was approximately 2- and 12-fold higher than that of 6803GT_PAM when 6803GT_EC and ABEC were used, respectively. This result clearly indicated that PHB efficiently accumulated in the engineered cyanobacteria of both 6803GT_EC and ABEC. In addition, the combination of *phaAB* and *phaEC* effectively functioned for the overproduction of PHB. Therefore, we used the 6803GT_ABEC strain, and eventually obtained PHB, as shown in Figure 4D. As shown in Figure 4D, we succeeded in preparing powder PHB from a 2-L cell culture in which the cultivation system with a bioreactor may be appropriated to a first step for practical applications. These results demonstrated that the system using the PCC 6803 transconjugants, which harbored expression vectors, had the potential for development within maximum values of approximately 7 kg per 100 kg of dry cell weight obtained from (10 to) 100 tons of cell culture. The approximate 7% mass yield with loss in extraction of PHB recovered from transconjugants will be ameliorated in future. To date, various cultivation conditions have been tested using the PCC 6803 strains engineered for the overproduction of PHB. Osanai's group reported that PHB productivities were 0.6% (w/w) in 6803GT_WT and 1.4% in the transformant, which overexpressed the transcription factor SigE (11). Sudesh's group also demonstrated that PHB productivities were 7% in 6803ATCC27184_WT and 11% in the transconjugants overexpressing the *phaCAB* genes of *Ralstonia* (the previous name was *Alcaligenes*) *eutrophus* (24). Panda's group also showed that PHB productivities were 3.9% in 6803GT_WT and 38% in 6803GT_WT, which was cultivated under the medium supplemented with sugar (gluconate) (25). The value of 12-fold productivity in this time is the best case in PHB studies using expression vectors for PHB overproduction

reported in cyanobacteria, cultivated in medium without sugar supplementation. The results also indicated that the PHB productivities were meaningful and will be further improved by addition of gluconates and phosphate/sulfur-depletion conditions (4,8,10). These improved methods may be advantageous for the biotechnology industry (26,27). We previously reported that a large amount GFP was produced, accounting for 5% (w/w) of all cellular protein in the PCC 6803KZ recombinant harboring the *gfp* gene and that expression vectors carrying *gfp* genes in the transconjugants remained for at least one year (12). In the present study, the expression vectors were stably maintained for at least one year during continuous cultivation using BG11 medium supplemented with chloramphenicol (data not shown).

Metabolite analysis in PHB overproduction Although factors that limit the effective production of PHB need to be identified, they have rarely been examined in the biosynthesis of PHB. Therefore, we measured the intracellular levels of acetyl-CoA, acetoacetyl-CoA, and 3-hydroxybutyryl-CoA (3HB-CoA), which are intermediates in the biosynthesis of PHB. The levels of these intermediates in the transconjugant 6803GT_PAM, EC or ABEC were measured by CE-MS (MATERIALS AND METHODS). We attempted to evaluate the levels of intermediates in transconjugants that were cultivated for 8 days under a steady-state growth phase (a late stage under PHB overproduction) with nitrogen depletion, leading to the accumulation of carbon materials (Fig. 3). Since cells were exposed to conditions in which pools of the intermediates may be consumed during the overproduction of PHB, more detailed information regarding the levels of intermediates remaining at this phase may provide important clues for future strategies for the production of PHB. The results obtained by the CE-MS analysis were shown in Figure 5. The level of acetyl-CoA, which is a key intermediate in the biosynthesis of many carbon materials, was not significantly changed in 6803_PAM, EC, or ABEC when cells were cultivated in BG11 medium (+N). This profile is consistent with the growth curve shown in

Figure 2C. On the other hand, these levels were significantly reduced under the nitrogen-depleted condition (BG11₀, -N). The amount of acetyl-CoA was higher in 6803GT_ABEC (and 6803GT_EC) than in 6803GT_PAM under the nitrogen-depleted condition. This also agrees with the levels of PHB (TABLE 1), implying that the overexpression of *phaABEC* (and *phaEC*) also had a slight positive feedback effect on the production and/or stability of acetyl-CoA in cells. We also observed similar profiles for coenzyme A (CoA) (Supplementary Fig. S1) under the same conditions (data not shown). Furthermore, the accumulation of acetoacetyl-CoA and 3HB-CoA was absent or markedly low, respectively. We also show an enlarged profiling for the materials in supplementary Figure S4. Because acetoacetyl-CoA was not detected, analysis of variance was not undertaken. In the other substances, effects of strain and interaction between strain and nitrogen were insignificant. The effects of nitrogen were highly significant for acetyl-CoA ($P = 0.000197$) and 3HB-CoA ($P = 0.0214$). Anyway, this is the first case showing levels of the PHB intermediates at the late stage under PHB overproduction. These results demonstrated that the conversion of acetyl-CoA to acetoacetyl-CoA may be a bottleneck in the biosynthesis of PHB. Since the additional introduction of *phaAB* into the cells appeared to affect PHB overproduction (TABLE 1), other possibilities were a quick turnover from acetoacetyl-CoA and 3HB-CoA to PHB and/or the consumption of acetoacetyl-CoA and 3HB-CoA in the overproduction of PHB under steady-state growth conditions. Although we constructed 6803GT_ABEC, the production of PHB was possible when 6803GT (or KZ)_EC was used (Figs. 3, 4, and TABLE 1). This result suggested that the introduction of extragenous NIES-843 *phaEC* (coding PHA synthase for the polymerization of 3HB-CoA to PHB) into PCC 6803 cells was efficient for the overproduction of PHB. In other words, the expression of acetoacetyl-CoA (synthesized by PhaA) and 3HB-CoA (synthesized by PhaB) may have had a synergistic effect on the overproduction of PHB in 6803GT_ABEC. In the genetically-engineered cyanobacterium *Synechococcus elongatus* PCC 7942, which carries *pha* genes from *R.*

eutropha, an acetyl-CoA flux, but not PHB synthase limited the synthesis of PHB (28). To enhance the overproduction of PHB in the future, it may be important to increase the production of acetoacetyl-CoA and 3HB-CoA as metabolites. In the present study, we used BG11₀ (nitrogen-depleted) medium without supplemented gluconate. The addition of gluconates into the medium may also increase the basal level of acetyl-CoA following glycolysis, resulting in the elevated production of PHB. An increase in the production of CoA may also be effective for the overproduction of PHB in the biosynthetic pathway (29). Another approach using synergistic effects, involving increases in the expression of the RNA polymerase sigma factor SigE in the transconjugant 6803GT_ABEC, which may be advantageous for the further overproduction of PHB in future studies (11,30).

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Figure legends

Fig. 1. PHB synthesis genes and cyanobacterial expression vectors. (A) PHB biosynthesis genes. (B) Loci of *pha* genes on the genomes of cyanobacteria *Synechocystis* sp. PCC 6803 and *Microcystis aeruginosa* NIES-843. (C) Regulatory region on the expression vector *phaEC461c* or *phaABEC461c*. *PpsbA**, the *Microcystis aeruginosa* K-81 *psbA2* promoter with a Δ AT sequence; *NdeI* and *SmaI*, sites for restriction enzymes in the expression vectors. Other details were described previously (12).

Fig. 2. Growth curve of PCC 6803 wild type and PHB transconjugants. (A) The *Synechocystis* sp. PCC 6803KZ wild type (6803KZ_WT) or PHB overproduced transconjugant (6803KZ_EC) harboring *phaEC461c* was cultivated in BG11 liquid medium (50-mL culture in a 100-mL Erlenmeyer flask) at 30°C with continuous rotary shaking (110 rpm) and white-light irradiation ($100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Cell turbidities were measured and shown as values of OD₇₃₀ for growth curves. (B) The 6803KZ_WT or EC strain (50 mL) was precultured for 3 weeks, collected by centrifugation, and then suspended in 50 mL (50-mL culture in a 300-mL flask) of new BG11 (+N) or nitrogen-depleted (-N, BG11₀) medium to which sodium dehydroacetate (pH 7, final concentration as 10 mM) was applied. These strains were cultivated at 30°C in flasks with continuous reciprocation shaking (50 rpm) and white-light irradiation ($100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) under 2% CO₂ air, which was supplied to the cultivation chamber (CF-415, TOMY, Tokyo). The relative strains of 6803GT_WT (no plasmid), PAM (pAM461c only), EC (*phaEC461c*), and ABEC (*phaABEC461c*) were cultivated in BG11 (C) or BG11₀ medium (D) under 2% CO₂ air, which were the same conditions as those shown in panel B. The profiles of the respective panels with average values ($n = 3$, error rates are less than 10%) were examined from independent three experiments.

Fig. 3. Accumulation of hydrocarbons in PCC6803 transconjugants. The wild type (WT) or transconjugant (EC or ABEC) cells of PCC 6803KZ or PCC 6803GT harboring phaEC461c or phaABEC461c, respectively, were cultivated for 8 days under the conditions shown in Figure 2B and Supplementary Figure S2. The cell culture was collected and Nile red (final concentration as 10 μ M, Wako, Tokyo) was added. The cells that stained with Nile red were subjected to microscopic observations ($\times$ 1,000) under a fluorescence microscope or photomicroscope. Bars indicate a scale of 10 μ m.

Fig. 4. Microscopic observation and ^1H -NMR spectrum for PHB. The wild type (A) or transconjugant (B) cells of PCC 6803KZ harboring phaEC461c were prepared as shown in Figure 3 and fixed with reagents (MATERIALS AND METHODS). The resulting cells were subjected to transmission electron microscopy (TEM) ($\times$ 18,400) by the ultrathin sectioning method (17). The acceleration voltage was 80 kV. The bar indicates 1 μ m. (C) ^1H -NMR spectrum. PCC 6803KZ transconjugants harboring phaEC461c were prepared as shown in Figure 2B. A 50-mL cell culture was harvested and this culture was subjected to the preparation of samples for an NMR analysis (MATERIALS AND METHODS). The resultant pellet was dissolved in deuterated chloroform (CDCl_3) and then subjected to a ^1H -NMR analysis (400 MHz). The regions of the peaks corresponding to the respective structures on position 2 ($-\text{CH}_2$), 3 ($-\text{CH}$), and 4 ($-\text{CH}_3$) in PHB are shown. (D) PHB prepared from PCC 6803GT transconjugants. The transconjugant of 6803GT_ABEC harboring phaABEC461c was precultivated in BG11 medium (+N) for 1 month in a glass tank, 2 L of the cell culture was then collected by centrifugation, and the pellets were dissolved in 2 L of fresh BG11₀ (-N, $-\text{NaNO}_3$) medium, which was supplemented with sodium dehydroacetate (pH 7, final concentration of 10 mM). These strains were then cultivated for 20 days at 30°C in a rectangular parallelepiped plastic tank (width 10 cm $\times$ depth 30 cm $\times$ height

40 cm) with aeration (3 L/min) with 0.5% CO₂ gas. The cells that accumulated PHB were collected and bioplastics were prepared (section of PHB preparation in MATERIALS AND METHODS). A portion of the PHB powder is shown.

Fig. 5. Metabolite profiling of intermediate materials for PHB. Transconjugants of PCC 6803GT harboring pAM461c (6803GT_PAM, vector only), phaEC461c (6803GT_EC), or phaABEC461c (6803GT_ABEC), respectively, were cultivated for 8 days under the conditions shown in Figure 3. The cells that accumulated PHB were collected and samples for the metabolite analysis were prepared as described previously (18). Details for sample preparation and the analysis using CE-MS were described in MATERIALS AND METHODS. Relative amounts of the respective materials prepared from cells in 50 mL of the cell cultures (per unit cell-culture-volume, UCV) are shown with error bars in three independent experiments ($n = 3$) for acetyl-CoA, acetoacetyl-CoA, and 3-hydroxybutyryl-CoA, referring to the value set as 1 for 6803GT_PAM N+.

Supplementary Fig. S1. Metabolic pathway and carbon materials in cyanobacteria. An outline of photosynthesis, glycolysis, and the TCA pathway linking the carbon and nitrogen pathways with the respective materials is shown. 2-OG, 2-oxysoglutamate; AA Syn/Deg, synthesis/degradation of amino acids.

Supplementary Fig. S2. Cultures of PCC 6803 wild type and PHB transconjugants. The strains (50 mL) of 6803KZ_WT and EC were precultured for 3 weeks, collected by centrifugation, then suspended into 50 mL (30-mL culture in a 300-mL Erlenmeyer flask) of new BG11 (+N) or nitrogen-depleted (-N, BG11₀) medium to which sodium dehydroacetate had been applied (pH 7, final concentration of 10 mM). These strains were cultivated for 7 days at 30°C in flasks with continuous reciprocation shaking (50 rpm) and white-light irradiation (100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)

under 2% CO₂ air, which was supplied to the cultivation chamber (CF-415, TOMY, Tokyo). One milliliter of the cell culture was collected after 7 days. These samples were subjected to photography (Fig. S2) and Nile red staining for microscopic observations (Fig. 3).

Supplementary Fig. S3. ¹H-NMR spectrum for PHB. The regions of peaks shown in Figure 4C corresponding to the respective structures on position 2 (-CH₂), 3 (-CH), and 4 (-CH₃) in PHB are enlarged. Other details are described in the text.

Supplementary Fig. S4. Metabolite profiling of intermediate materials for PHB. The ranges of amount for the materials shown in Figure 5 are enlarged. Other details are described in the text.

TABLE 1. Productivity of PHB from recombinant cyanobacteria.

Type of strain ^a	PCC 6803GT		
	PAM	EC	ABEC
BG11 medium (mL) ^a	1,350	1,350	1,350
Dry cell weight (mg) ^b	1,263±10.2	727±0.35	206±0.02
PHB recovered (mg) ^b	7.33±0.09	11.0±0.62	14.3±0.02
PHB recovered per dry cell weight (%) ^b	0.58±0.04	1.18±0.43	6.98±0.98

^a *Synechocystis* sp. PCC 6803GT transconjugants harboring pAM461c (6803GT_PAM), phaEC461c (6803GT_EC), or phaABEC461c (6803GT_ABEC) were respectively precultivated in 5 L of BG11 medium for 1 month under normal conditions. Each cell culture of 450 mL was centrifuged and the resultant pellets of the cells were transferred into a new 30-mL cell culture of BG11₀ medium in a 500-mL flask. These cell cultures were cultivated under the conditions shown in Figure 2D, with continuous reciprocation shaking (40 rpm) and white-light irradiation under 2% CO₂ air for 8 days. A 90-mL cell culture (from 30 mL x three flasks, according to 1,350 mL of the cell culture = 450 mL × three samples) was collected and the cell pellets were subjected to the preparation of PHB.

^b The dry cell weight (DCW) of the pellets and PHB recovered per DCW were shown with error values from three independent experiments ($n = 3$).

Fig. 3., Hondo *et al.*

Fig. 4., Hondo *et al.*