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Genetic engineering and metabolite profiling for overproduction of polyhydroxybutyrate in cyanobacteria

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Genetic engineering and metabolite profiling for the overproduction of polyhydroxybutyrate (PHB), which is a carbon 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; Polyhydroxybutyrate]

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 polyhydroxyalkanoate (PHA) 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 producing PHB) (Fig. 1A). Cyanobacteria are photosynthesizing gram-negative prokaryotes that produce oxygen. The biological history of cyanobacteria is extensive because they first appeared approximately three billion years ago. The overproduction of PHB has been examined for renewable and high-value-added carbon materials made by CO_2-fixing during photosynthesis in cyanobacteria. The cyanobacterium *Synechocystis* sp. strain PCC 6803 is unicellular and considered a good material for the biotechnology industry because it is transformable and its whole genome sequence has been decoded (3). Previous studies demonstrated that the recombinant PHA-negative mutant *Alcaligenes eutrophus* transformed with plasmids carrying *pha* [exogenous $phaEC_{Syn}$ or hybrid *phaECAB* ($phaEC_{Syn} + phaAB_{Ae}$)] genes from *Synechocystis* sp. PCC 6803 and/or *A. eutrophus* accumulated PHB up to 3.5–12% (w/w) of the dry cell weight under nitrogen-depleted conditions supplemented with sugar (gluconate, 0.5–2%) as the carbon source (4). On the other hand, PCC 6803 carrying endogenous *phaAB* and *Escherichia coli tesB* (thioesterase), but not endogenous *phaEC* (knockout of the *phaEC* genes) on the genome resulted in approximately 0.53 g/L of 3-hydrxybutyrate (3HB) being produced under phosphate depletion conditions in which the products were readily secreted from cells without the overexpression of transporters (5). The production of PHB (and PHA) has also been examined in cyanobacteria and other bacteria, which are engineered and non-engineered (6–11). Significantly high PHB biomass production in dry cell weight has been observed. However, studies for PHB overproduction using a novel expression vector have not been sufficient. Furthermore, the metabolic pool of intermediate products for PHB overproduction has not yet been examined in detail.

The new cyanobacterial expression vectors pAM500 and pAM461c, which possess the origin of replication that functions in a broad range of gram-negative bacteria, have recently been constructed (12). To examine shuttle vectors, the gene *gfp* (green fluorescence protein) was cloned downstream from the expression control 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 14–25

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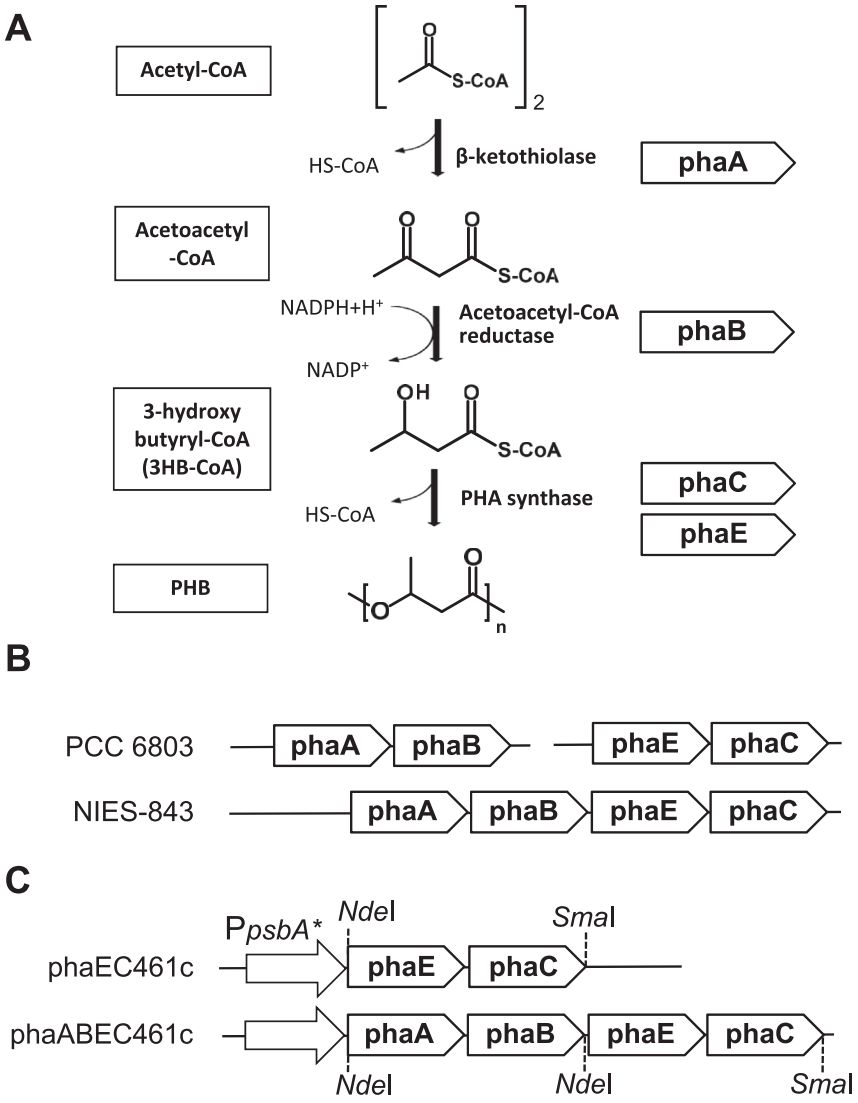

FIG. 1. PHB synthesis genes and cyanobacterial expression vectors. (A) PHB biosynthesis genes. (B) Loci of *pha* genes on the genomes of cyanobacteria *Synechocystis* sp. PCC 6803 and *Microcystis aeruginosa* NIES-843. (C) Regulatory region on the expression vector phaEC461c or phaABEC461c. P*psbA**,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

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 $BG11_0$ (without $NaNO_3$ in BG11) (13) under continuous cool white light illumination (100 μmol of photons m^{-2} s^{-1}) at 30°C. The *E. 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 $BG11_0$ 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′-ATGAAGGAGAAAACTCACCG-3′; phaAB_FwIF, 5′-GAAATTATCCACATATGATCTTTTCGGACTCGATATTC-3′ (underline, *Nde*I site); phaABEC_RvIF, 5′-AAGCTTTTACTACCCGGGTTAAGCTCTTGCTTTCAAC-3′ (underlined, the *Sma*I site); phaEC_FwIF 5′-CAGAAATTATCCACATATGGATAAACCGACACAA-3′ (underlined, the *Nde*I site); phaAB_RvIF, 5′-GTCGGTTTATCCATATGTTTTGCCCTTTTCCTTTAACG-3′ (underlined, the *Nde*I 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 *M. 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 *Nde*I/*Sma*I 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