0273-2289/00/84–86/0991/$13.00

Polyhydroxybutyrate Production from Carbon Dioxide by Cyanobacteria

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Abstract

Genetic characterization and enhancement of polyhydroxybutyrate (PHB) accumulation in cyanobacteria were investigated for efficient PHB production from CO_2. The genome DNAs in the PHB-accumulating strains *Synechococcus* sp. MA19 and *Spirulina platensis* NIES46 retained the highly homologous region to *phaC* of *Synechocystis* PCC6803, whereas low homology was detected in the nonaccumulating strains *Synechococcus* sp. PCC7942 and *Anabaena cylindrica* NIES19. *Synechococcus* sp. MA19, which accumulates PHB up to 30% of dry cell weight from CO_2 as the sole carbon source, was mutated by insertion of transposon Tn*5* to enhance the PHB accumulation. Genetic and physiological analysis of the mutant indicated that decreased phosphotransacetylase activity could trigger an increase of acetyl coenzyme A leading to enhancement of PHB accumulation. PHB synthase in *Synechococcus* sp. MA19 was probably attached to thylakoid membrane since PHB granules were associated with pigments. A genetically engineered cyanobacteria retaining soluble PHB synthase from *Ralstonia eutropha* accumulated pigment-free PHB granules, which is an advantage for the purification of PHB.

Index Entries: Polyhydroxyalkanoates; cyanobacteria; carbon dioxide.

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Introduction

Bacterial storage polymers, polyhydroxyalkanoates (PHAs), are increasingly gaining interest in research and in industry. The most common of these PHAs is polyhydroxybutyrate (PHB), which is a raw material for biodegradable plastics and can thus significantly contribute to solving environmental pollution problems generated by petrochemical plastics.

Many photosynthetic bacteria, and several species of cyanobacteria among them, are capable of accumulating PHB (reviewed in ref. *1*). Industrial utilization of cyanobacteria as PHB producers has the advantage of converting waste CO_2, a greenhouse gas, to environmentally friendly plastics using the energy of sunlight *(2–5)*. Various species of cyanobacteria accumulate considerable amounts of PHB: *Spirulina platensis* (6% of cell dry wt; *[2]*), *Gloeothece* sp. (6% of cell dry wt; *[1]*), *Oscillatoria limnosa* (poly-β-hydroxyvalerate, 6% of cell dry wt; *[1]*), and *Synechococcus* sp. MA19 (~30% of cell dry wt; *[4]*). Furthermore, non-PHB accumulating cyanobacteria have recently been genetically engineered by heterologous transformation with genes involved in the PHB pathway of *Ralstonia eutropha*, leading to the accumulation of the polymer *(5,6)*.

PHB is synthesized from acetyl coenzyme A (acetyl-CoA) via three enzymatic reactions *(7)*: 3-ketothiolase converts two acetyl-CoA molecules to one acetoacetyl-CoA molecule, NADPH-dependent acetoacetyl-CoA reductase converts acetoacetyl-CoA to D-3-hydroxybutyryl-CoA and the last enzyme, and PHB synthase catalyzes linking of the D-3-hydroxybutyryl moiety to an existing PHB molecule via an ester bond. PHB synthase of the unicellular cyanobacterium *Synechocystis* PCC6803 consists of two subunits (PhaC and PhaE) *(8)*, similar to purple sulfur bacteria *(9–11)*. The regulation of PHB synthase was investigated in *Synechococcus* sp. MA19 *(12)*. The enzyme isolated from *Synechococcus* sp. MA19 was found to be characterized as membrane bound. The enzyme activity is controlled by nitrogen sources in the culture media and is activated by acetyl phosphate in vitro. *Synechococcus* sp. MA19 accumulates PHB under nitrogen-deprived conditions *(4)* and the regulation system of PHB synthase reported in Miyake et al. *(12)* (Fig. 1).

PHB granules in *Synechococcus* MA19 have recently been shown to be strongly associated with thylakoid membranes, which are probably mediated by the membrane-bound PHB synthase *(13)* since the enzyme attaches to the surface of PHB granule in general *(14)*. This strong association can be broken on extensive ultrasonication of samples, finally yielding pure PHB granules that are not contaminated with pigments, which usually pose problems for the purification of PHB from cyanobacteria.

To develop an efficient system for PHB production by cyanobacteria, we studied the genetic characterization of PHB-accumulating cyanobacteria and mutation to enhanced PHB accumulation. In addition, we investigated the production of pigment-free PHB granules by genetically engineered cyanobacteria expressing soluble PHB synthase from *R. eutropha*.

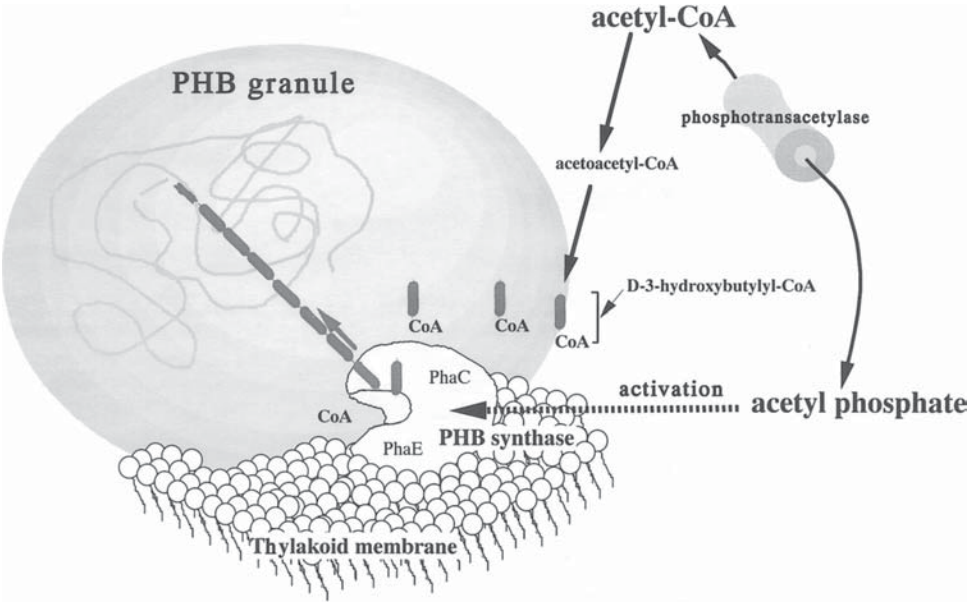

Fig. 1. A model of regulation of PHB accumulation mediated by PHB synthase activation.

Table 1
Cyanobacterial Strains Used

Strain	Phenotype (genotype)	Growth media
Synechococcus sp. MA19	PHB (wild type)	BG11
Synechococystic PCC6803	PHB (wild type)	BG11
S. platensis NIES46	PHB (wild type)	SOT
Synechococcus PCC7942	Nonaccumulation (wild type)	BG11
A. cylindrica NIES19	Nonaccumulation (wild type)	BG11
Synechococcus N1	PHB (PCC7942 transformed with pAEN1)	BG11

Materials and Methods

Bacteria and Culture Conditions

Table 1 gives the cyanobacterial strains and plasmids used in this study. *Synechococcus* sp. MA19 was grown in BG11 medium *(15)* at 50°C according to Miyake et al. *(4)*. *Synechococcus* PCC 7942 and the transformant strain N1 were grown in the BG11 at 30°C as described by Takahashi et al. *(6)*. *Spirulina platensis* NIES46 and *Anabaena cylindrica* NIES19 were maintained at 30°C according to Asada and Kawamura *(16)*. PHB accumulation by cyanobacteria was carried out in the media without nitrogen sources as described previously *(4)*.

Genetic Manipulation

Southern blot analysis was carried out following standard laboratory manuals *(17)* by using an ECL direct nucleic acid labeling and detection system (Amersham Pharmacia, Tokyo, Japan). The probes (*phaC* and *phaE* fragments) for the Southern blot analysis were cloned from *Synechocystis* PCC6803 genome DNA by polymerase chain reaction (PCR) using specific primers: 5'-ATGTTTTTACTATTTTTTATCGTTCA-3' and 5'-AGTGAC AGCAAGGCTATCGG-3' for the *phaC* fragment, and 5'-ATGGAA TCGACAAATAAAAC-3' and 5'-AATCGGACCCAAACGAAGAC-3' for the *phaE* fragment. The PCR fragments were inserted into plasmid pACYC184 and cloned into *Escherichia coli* JM109 to check PHB synthase activity of the gene products (data not shown).

Cloning of *phaC* from *Synechococcus* sp. MA19 genome DNA was also carried out by PCR using the same primers as just mentioned. The PCR products were directly sequenced by using an automatic DNA sequencer (Perkin-Elmer, Stamford, CT).

The transposon mutagenesis of *Synechococcus* sp. MA19 *(4)* was carried out by mating with *E. coli* S17-1 harboring the Tn*5*-carrying plasmid, pSUP5011 *(18)*, basically according to Elhai and Wolk *(19)*. The recipient strain, *Synechococcus* sp. MA19, obtained from the National Institute of Bioscience and Human-Technology, Ibaraki, Japan, was grown to OD_{600} = 1 to 2 in BG-11 *(15)* with 5% (v/v) of Luria-Bertani (LB) medium (BG-11 + 5% LB) under aeration and continuous light conditions as described previously *(4)* at 50°C. *E. coil* S17-1 with pSUP5011 was transformed by electroporation *(20)*. The transformant of *E. coli* S17-1 as donor strain was grown to OD_{660} = 1.2–1.5 in LB medium supplemented with 30 μg/mL of kanamycin, streptomycin, and ampicillin; and 15 μg/mL of chloramphenicol. The parent strains were harvested by centrifugation (10,000*g*, 4°C for *E. coli*; 37°C for cyanobacteria), and the cells were adjusted to 10^5–10^6 cells/μL with BG-11 + 5% LB medium. The two suspensions were mixed to give an approximate ratio of donor:recipient of 1:1, and 10-μL aliquots were applied to presterilized HATF filters (Millipore, Tokyo, Japan) on the surface of BG-11 + 5% LB plates. Mating was done at 37°C for 8 h. Tn*5* insertion mutants were selected on BG-11 agarose plates at 45°C to allow the cyanobacteria to recover and inhibit growth of *E. coli (21)*. For maintenance of Tn*5* insertion mutants, liquid cultures and BG-11 agarose plates were routinely supplemented with 40 μg/mL of Km.

For enrichment of the high PHB-accumulating mutants, Tn*5* insertion mutants were mixotrophically grown to stationary phase in BG-11 with 1 m*M* acetate and 40 μg/mL of kanamycin and then fractionated by Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation according to Pedros-Alio et al. *(22)*. The buoyant densities of the PHB-accumulating and nonaccumulating cells were approx 1.15 and 1.09 g/mL, respectively. PHB accumulation of each isolate was detected by staining with Nile Blue A *(23)*. A colony of high PHB-accumulating mutant was isolated from a cell

mixture showing a heavy buoyant density (1.12–1.15 g/mL) on BG-11 agarose plates with kanamycin.

Isolation of PHB Granules and Intracellular Membranes

Cell-free extracts were prepared as described previously *(12)*. The duration of cell ultrasonication was either 10 or 30 min. PHB granules were separated from broken cells by centrifugation in a Percoll gradient essentially as described previously *(13)*, with slight modifications. Cells (~1 g wet wt) were harvested by centrifugation (10,000*g*, 10 min, 4°C), washed twice, and resuspended in 25 m*M* Tris-HCl, pH 7.5 (final OD_{660} = 1.3). Cells were disrupted by sonication (20 kHz, 70 W, 4°C), and the resulting homogenate (5 mL) was directly applied onto 90% Percoll in 0.15 *M* NaCl (20 mL) in Oak Ridge–type centrifuge tubes (30 mL, 25.5 mm diameter) and centrifuged (13,000*g*, 55 min, 4°C) in a fixed-angle rotor (RP-50T; Hitachi, Tokyo, Japan). After centrifugation, fractions were collected and analyzed for PHB and chlorophyll content. Density of the Percoll gradient fractions was estimated using color-density marker beads (Pharmacia). To liberate the granules from the residual Percoll, the lowest fraction containing PHB granules was diluted 10 times with buffer and further concentrated by centrifugation (10,000*g*, 20 min, 4°C). Cell membranes were separated by ultracentrifugation (75,000*g*, 60 min, 4°C) of cell-free extracts as described previously *(13)*.

Quantification of PHB and Chlorophyll a

PHB content was determined by gas chromatography as described by Miyake et al. *(4)*. Chlorophyll *a* was extracted and quantified as described by Tandeau de Marsac and Houmard *(24)*.

Enzyme Assays

Samples for assays of PHB synthase and phosphotransacetylase were prepared using a spectroscopic method as described previously *(12)*.

Results and Discussion

Genetic Characterization of PHB- and Nonaccumulating Cyanobacteria

Screening, based on Nile Blue A staining *(23)*, resulted in the observation of PHB-accumulating cyanobacteria, *Synechococcus* sp. MA19 and *S. platensis* NIES46, and nonaccumulating cyanobacteria, *Synechococcus* sp. PCC7942 and *A. cylindrica* NIES 19.

To elucidate the difference between PHB-accumulating and nonaccumulating phenotypes, PHA synthase genes (*phaC* and *phaE*) were detected from the PHA-accumulating and nonaccumulating cyanobacteria by Southern blot analysis using *phaC* and *phaE* from *Synechocystis* sp. PCC6803 as probes. Figure 2 shows the hybridization signals by using *phaC* fragment as the probe. Strong signals were obtained (Fig. 2A,B) in the PHB-accumu-

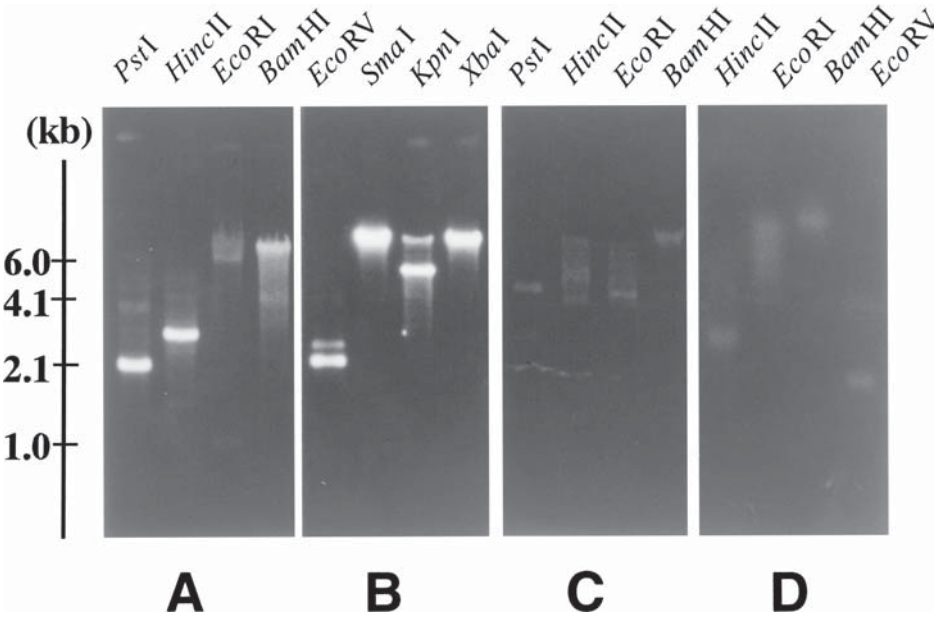

Fig. 2. Detection of *phaC* in the genomes of cyanobacteria. **(A)** *Synechococcus* sp. MA19; **(B)** *S. platensis* NIES46; **(C)** *Synechococcus* PCC7942; **(D)** *A. cylindrica* NIES19. The DNAs of 1 μg were digested by the restriction enzymes and applied onto the 1.0% agarose gels.

lating strains, whereas weak signals, which indicate low homology with the PCC6803 *phaC*, were obtained from the nonaccumulating strains (Fig. 2C,D). The PCC6803 *phaE* homologous region was not detected in any cyanobacterial genome (data not shown).

Synechococcus sp. MA19 accumulated PHB under nitrogen-deprived conditions and was the highest PHB accumulator (27.5% of cell dry wt) *(4)* among the cyanobacteria previously reported. Figure 3 indicates the sequence of the *phaC* isolated from *Synechococcus* sp. MA19. The DNA sequence was 99.8% identical to the gene of *Synechocystis* PCC6803 *(8)*, which accumulates PHB up to 2% cell dry wt from CO_2. These results suggest that *phaC* could be a suitable probe to survey PHB-accumulating cyanobacteria.

Transposon Tn5 Insertion Mutant Showing Higher PHB Accumulation

To compare the phenotype of the kanamycin-resistant mutant to that of *Synechococcus* sp. MA19, PHB contents of cells under nitrogen-sufficient and nitrogen-deprived conditions were measured (Fig. 4A,B). While *Synechococcus* sp. MA19 showed no PHB accumulation during the mixotrophic growth, the mutant accumulated PHB under the same conditions (Fig. 4A). Under nitrogen-deprived conditions, the PHB accumulation rate

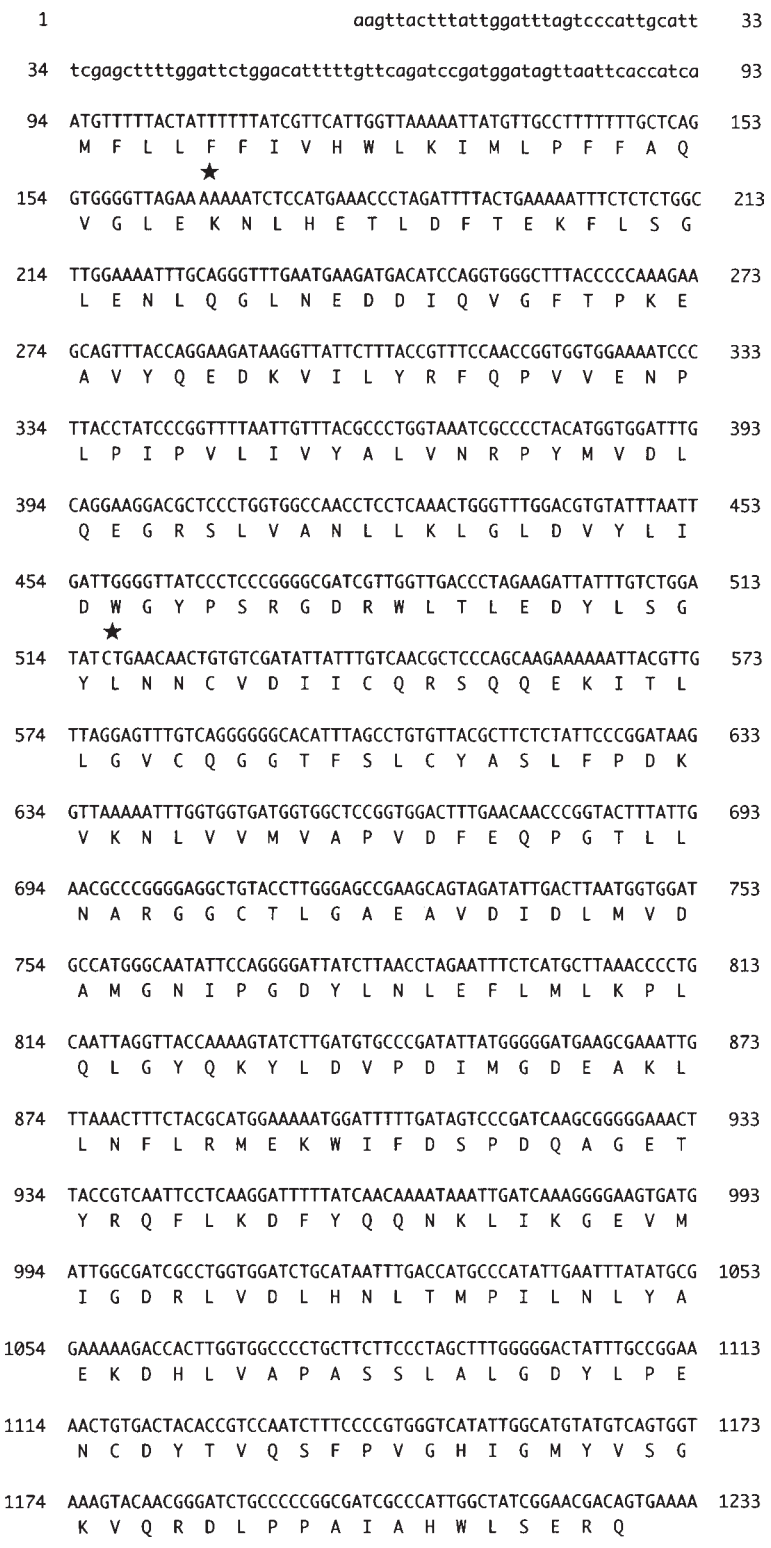

Fig. 3. DNA sequence of *phaC* region in *Synechococcus* sp. MA19. The sequence is identical to that in *Synechocystis* PCC6803 except the points indicated by ★. The open reading frame is from 94 to 1227 bases. The amino acid sequence is indicated under the DNA sequence.

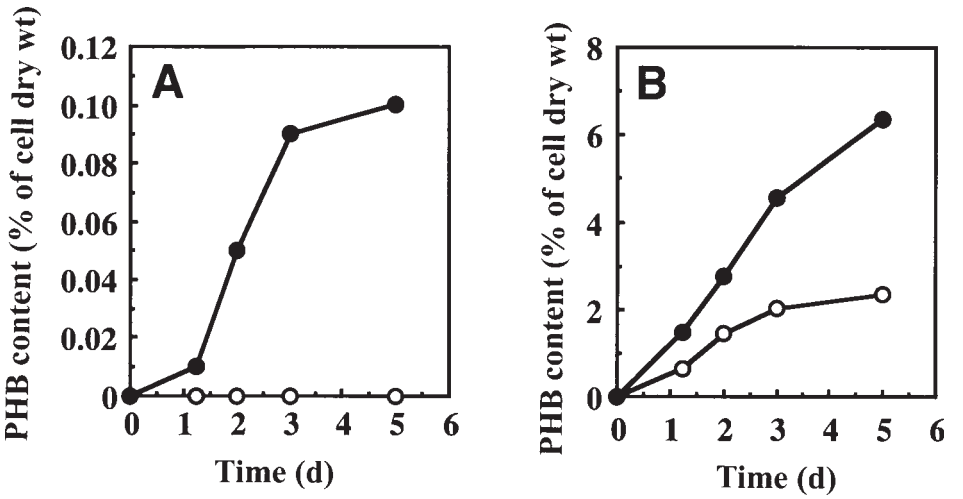

Fig. 4. PHB accumulation in *Synechococcus* sp. MA19 and the mutant. s, *Synechococcus* sp. MA19; d, mutant. **(A)** PHB accumulation in BG-11 with 1 m*M* acetate; **(B)** PHB accumulation in BG-11 without sodium nitrate. Culture conditions are described in detail in ref. *12*.

of the mutant was higher than that of *Synechococcus* sp. MA19 (Fig. 4B). Since PHB accumulation of *Synechococcus* sp. MA19 was controlled by posttranslational regulation of PHB synthase *(12)*, the results suggest that regulation of PHB synthase by nitrogen sources could be changed in the mutant.

To elucidate physiological differences between *Synechococcus* sp. MA19 and the mutant, PHB synthase and phosphotransacetylase activities were measured according to Miyake et al. *(12)*. Table 1 gives the enzyme activities of the mutant and *Synechococcus* sp. MA19 in the presence and absence of sodium nitrate in the media. Weak activity of phosphotransacetylase was detected from the mutant under nitrogen-sufficient conditions, whereas the enzyme activity in *Synechococcus* sp. MA19 was below detection level. As correlated with the enzyme activity, PHB synthase activity and PHB accumulation were observed in the mutant grown under nitrogen-sufficient conditions in the presence of acetate (Table 2, Fig. 4A). Phosphotransacetylase activity in the mutant was not increased even under nitrogen-deprived conditions, whereas considerable enzyme activity was detected in *Synechococcus* sp. MA19 under the same conditions. A lower activity of PHB synthase in the mutant compared to *Synechococcus* sp. MA19 under nitrogen-deprived conditions could be explained by a possible correlation between phosphotransacetylase and PHB synthase activity as previously reported *(12)*. It is supposed that the increase of the PHB accumulation rate in the mutant (Fig. 4B) resulted from some increase of intracellular acetyl-CoA, owing to low activity of phosphotransacetylase, since acetyl-CoA is the substrate for PHB synthesis.

Table 2
Effect of Culture Conditions on PHB Synthase and Phosphotransacetylase Activity in PHB Accumulating Mutant and Wild-Type Strains of *Synechococcus* sp. MA19

Strain	Growth substrate	PHB synthase (nmol/min [mg/protein])	Phosphotransacetylase (nmol/min [mg/protein])
Wild type[a]	Nitrate[b] + CO_2[c]	<15	<30
	Nitrate + CO_2 + acetate[d]	<15	<30
	CO_2 (without nitrate)	700.7	6700
Mutant	Nitrate + CO_2	<15	172
	Nitrate + CO_2 + acetate	17	671
	CO_2 (without nitrate)	316	569

[a]*Synechococcus* sp. MA19.
[b]Sodium nitrate (1.5 g/L).
[c]Bubbling air into the culture.
[d]Sodium acetate (10 m*M*).

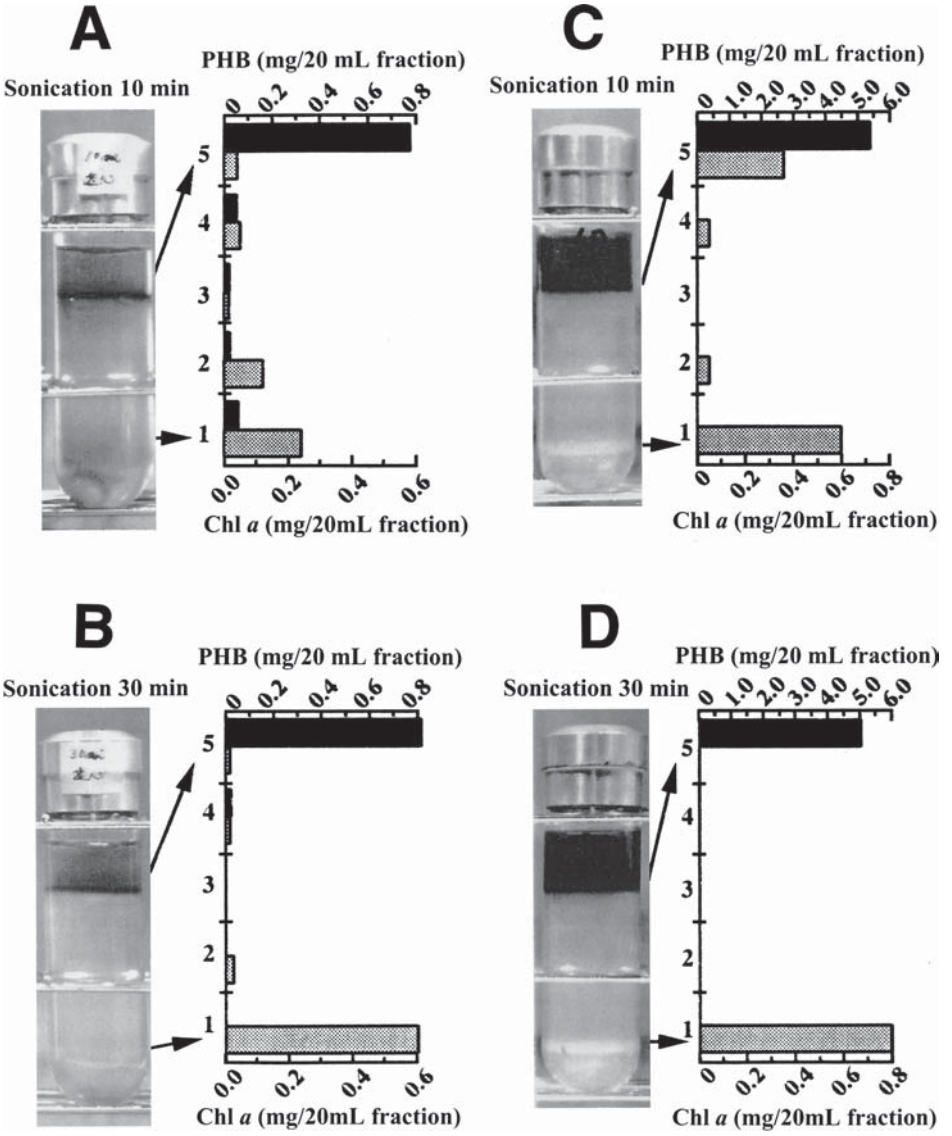

Fig. 5. Fractionation of cell homogenates of **(A,B)** *Synechococcus* sp. MA19 and **(C,D)** strain N1 in Percoll gradients. The volume in each fraction was 5 mL. Buoyant density ranges were >1.15 g/cm^3 in fraction 1, 1.15–1.10 g/cm^3 in fraction 2, 1.10–1.09 g/cm^3 in fraction 3, 1.09–1.06 g/cm^3 in fraction 4, and <1.06 g/cm^3 in fraction 5. Shaded bars, PHB; solid bars, chlorophyll *a* (Chl *a*).

Isolation of PHB Granules

PHB granules isolated from the genetically engineered *Synechococcus* sp. N1 *(6)*, which retained and expressed the soluble PHB synthase of *R. eutropha*, were compared with those from *Synechococcus* sp. MA19, which retained endogenous membrane-bound PHB synthase. Separation of PHB granules from MA19 and N1 resulted in the formation of two bands (Fig. 5): a lower

Table 3
Summary of Separation of PHB Granules from Cyanobacteria

	Sonication (min)	*Synechococcus* MA19	Strain N1
Yield (% of total PHB)	10	52	56
Chlorophyll *a* (mg/mg PHB)	10	0.24	0
Yield (% of total PHB)	30	93	100
Chlorophyll *a* (mg/mg PHB)	30	0	0

distinct band (>1.15 g/cm^3), mostly containing PHB granules; and an upper band (<1.06 g/cm^3), containing intact cells, cell fragments, and membranes. Shorter ultrasonication (10 min) resulted in about 50% recovery of PHB (Fig. 5A,C) in both the MA19 and N1 strains (Fig. 5A,C), whereas longer ultrasonication (30 min) resulted in about 100% recovery (Fig. 5B,D). These results indicate that 10 min of ultrasonication was insufficient for a complete disruption of the cells, and about half of the total PHB either remained in unbroken cells or bound to cell fragments, and, therefore, longer ultrasonication was required to liberate all PHB granules.

Shorter ultrasonication of MA19 cells resulted in greenish PHB granules owing to bound chlorophyll *a* (Fig. 5A,C; Table 3). Longer ultrasonication of the cells of this strain resulted in white PHB granules, containing no detectable chlorophyll *a*. However, for the transformant strain N1, no chlorophyll *a* was detectable in the lower PHB granule band under any ultrasonication condition studied. Thus, strain N1 showed improvement in recovery and purity of PHB at the PHB separation step.

The presence of chlorophyll *a* in association with PHB granules indicates the binding of the polymer to photosynthetic membranes. There is a correlation between the characteristics of PHB synthase and this pigment-binding phenomenon: only strains having a membrane-bound PHB synthase produce PHB granules bound to photosynthetic membranes, as evidenced by the presence of chlorophyll *a* in the granule fraction after ultracentrifugation in a Percoll gradient. This association may play a role in the process of PHB synthesis. Understanding the role of the binding of PHB synthase to photosynthetic membranes in cyanobacteria remains a future research topic. Our data show that genetic transformation of cyanobacteria with soluble PHB synthase may simplify the process of isolating the PHB granules by eliminating the need for extensive ultrasonication.

Conclusion

The PHB-accumulating cyanobacteria investigated retained the *phaC* homologous region in their genome. This implies that *phaC* is likely to be used as the probe to discover new PHB-accumulating cyanobacteria, although a number of strains should be tested to establish the screening

method. We focused on the strong PHB accumulator *Synechococcus* sp. MA19 and succeeded in isolating the Tn*5* insertion mutant showing higher PHB accumulation. Characterization of the mutant gave the positive effect of decreased phosphotransacetylase activity on the PHB accumulation in cyanobacteria. Additionally, we proved that expression of soluble PHB synthase in cyanobacteria resulted in accumulation of the pigment-free PHB granules.

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