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Control of Poly-β-Hydroxybutyrate Synthase Mediated by Acetyl Phosphate in Cyanobacteria

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Poly-β-hydroxybutyrate (PHB) synthesis in a cyanobacterium, *Synechococcus* sp. strain MA19, is controlled at the enzyme level and is dependent on the C/N balance in the culture medium. The control involves at least two enzymes. The first enzyme is PHB synthase. Little PHB synthase activity was detected in crude extracts from cells grown under nitrogen-sufficient conditions ($MA19_{+N}$). The activity was detected exclusively in membrane fractions from nitrogen-deprived cells ($MA19_{-N}$) under light but not dark conditions. The shift in the enzyme activity was insensitive to chloramphenicol, which suggests posttranslational activation. Acetyl phosphate activated PHB synthase in membrane fractions from $MA19_{+N}$. In vitro, the activation level of PHB synthase changed, depending on the concentration of acetyl phosphate. The second enzyme was phosphotransacetylase (EC 2.3.1.8), which catalyzes the conversion of acetyl coenzyme A (acetyl-CoA) to acetyl phosphate. The activity was detected in crude extracts from $MA19_{-N}$ but not in those from $MA19_{+N}$. The results suggested that intracellular acetyl phosphate concentration could be controlled, depending on C/N balance and intracellular acetyl-CoA concentration. Acetyl phosphate probably acts as a signal of C/N balance affecting PHB metabolism in MA19.

In many cyanobacteria, small amounts (<6% of cell weight [dry weight]) of poly-β-hydroxybutyrate (PHB) have been found and chemically identified, as reviewed by Stal (17). Electron microscopy revealed that the shapes of PHB granules are very similar to those in PHB-accumulating bacteria (1, 9, 21). PHB is accumulated under conditions of nutrient deprivation (1, 9, 17) or excess reducing power (4) or in the presence of excess acetate (4, 9, 20). However, the control of PHB accumulation in cyanobacteria is poorly understood.

Recently, we discovered a thermophilic cyanobacterium, *Synechococcus* sp. strain MA19 (14), that rapidly accumulates PHB to 27% (wt/wt) under nitrogen-limited conditions. During nitrogen-sufficient cultivation, PHB does not accumulate at all, despite the presence of intracellular acetyl coenzyme A (acetyl-CoA) flux.

PHB metabolism has been studied in PHB-accumulating bacteria, especially in *Alcaligenes eutrophus*. PHB is synthesized from acetyl-CoA via three enzymatic reactions (18): 3-thiolase (EC 2.3.1.9) converts two acetyl-CoA molecules to an acetoacetyl-CoA molecule, NADPH-dependent acetoacetyl-CoA reductase (EC 1.1.1.36) converts acetoacetyl-CoA to D-3-hydroxybutyryl-CoA with NADPH oxidation, and the last enzyme, PHB synthase (no EC number), catalyzes the linkage of the D-3-hydroxybutyryl moiety to an existing PHB molecule by an ester bond. In *A. eutrophus*, the three enzymes are constitutively synthesized. CoA inhibits 3-thiolase (5, 7). No regulation of the other two enzymes has been reported. Therefore, control of PHB metabolism in *A. eutrophus* appears to be dependent to some extent on the intracellular acetyl-CoA concentration (11). In an *Acinetobacter* sp. which shows regulated PHB accumulation, the PHB biosynthesis gene operon is controlled at the transcriptional level (15).

We examined PHB synthase of *Synechococcus* sp. strain MA19 and found that PHB synthase activity was controlled. In this paper, we describe how the role of PHB synthase in the control of PHB metabolism is dependent on the C/N balance in the culture medium and a key factor of PHB synthase activation in this microorganism.

MATERIALS AND METHODS

Chemicals. Acetyl-CoA, acetoacetyl-CoA, DL-3-hydroxybutyryl-CoA, phosphoenolpyruvate, 3-phosphoglycerate, ATP, and acetyl phosphate were purchased from Sigma, St. Louis, Mo. Other chemicals were purchased from Nakarai tesque, Kyoto, Japan.

Organisms and cultures. A thermophilic cyanobacterium, *Synechococcus* sp. strain MA19 (14), is in a culture collection of the Molecular Bioenergetics Laboratory of the National Institute of Bioscience and Human-Technology, Tsukuba, Ibaraki, Japan. As reported previously (14), MA19 was grown in an inorganic medium, BG-11, to the concentration of 0.8 mg of cells (dry weight)/ml at 50°C. For nitrogen-sufficient cultivation, 10% (vol/vol) of the culture was inoculated into fresh BG-11 and grown under light (7 W/m^2) with bubbling 2% (vol/vol) CO_2 at 0.4 liter/min. For nitrogen-limited incubation, cells grown in

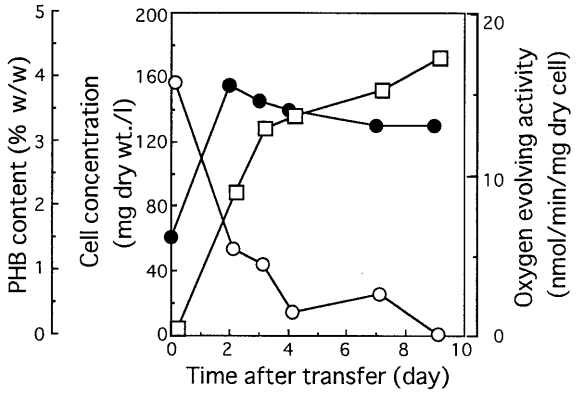

FIG. 1. PHB accumulation by strain MA19 under nitrogen-limited conditions. Open squares, open circles, and closed circles indicate PHB content, oxygen evolving activity, and cell concentration, respectively.

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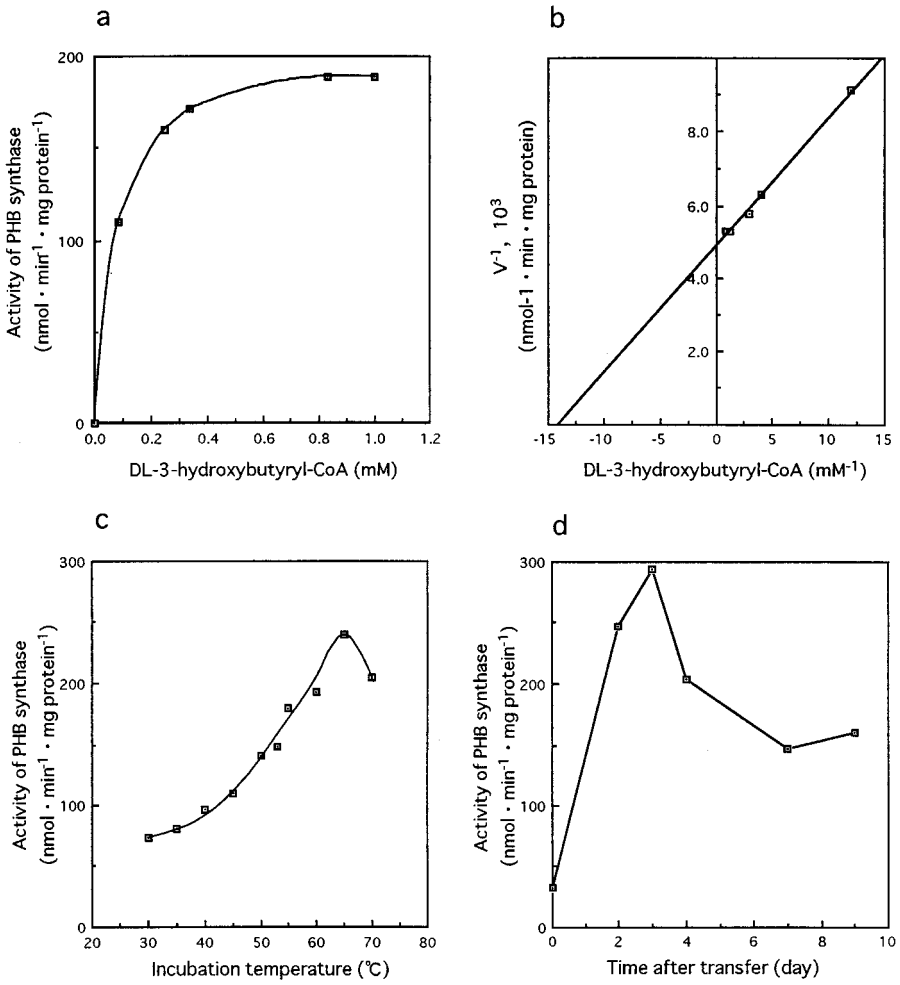

FIG. 2. Characterization of PHB synthase activity in crude extract from $MA19_{-N}$. (a) Dependency on DL-3-hydroxybutyryl-CoA concentration; (b) double-reciprocal plot of the data shown in panel a; (c) dependency on temperature; (d) time course of specific activity after transfer to nitrogen-limited conditions. (a to c) Crude extracts were prepared from $MA19_{-N}$ incubated for 3 days after transfer.

BG-11 were washed with BG-11 without sodium nitrate (−N BG-11) and transferred to −N BG-11 and incubated as described previously (14).

Enzyme assays. Cell extracts were prepared at 4°C or on ice. MA19, harvested from nitrogen-limited or nitrogen-sufficient cultures by centrifugation for 10 min at 10,000 × *g*, was washed with 25 mM Tris-HCl (pH 7.5) (Tris buffer) and resuspended in the same buffer containing 5% (vol/vol) glycerol (Tris-glycerol buffer), which was effective in stabilizing PHB synthase and phosphotransacetylase activities. Cells were disrupted by sonication with a Sonifier 450 (Branson) at 45 W for 10 min for large-scale volumes (20 to 30 ml) and 25 W for 10 min for small-scale volumes (2 to 3 ml). Cell debris was removed by centrifugation at 20,000 × *g* for 10 min. The supernatant (crude extract) was diluted with Tris-glycerol buffer to a 1.5-mg/ml protein concentration and was used for the enzyme assay. Soluble fractions and sediments (membrane fractions) were separated by ultracentrifugation of the crude extracts at 130,000 × *g* for 2 h. Soluble fractions were also diluted to a 1.5-mg/ml protein concentration. Membrane fractions were washed once with cold Tris buffer and resuspended in Tris-glycerol buffer (0.5 mg of protein/ml). Crude extracts, soluble fractions, and membrane fractions were frozen in liquid nitrogen and stored at −80°C until the enzyme assay.

Assay of PHB synthase activity was carried out according to the method of Valentin and Steinbüchel (19). The assay mixtures (600 μl) contained 0.3 mg of protein (except in some cases [see Table 1]), 1.6 mM DL-3-hydroxybutyryl-CoA, and 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in Tris-glycerol buffer. The reaction was started by addition of DL-3-hydroxybutyryl-CoA as the substrate. The temperature for assay was fixed at 50°C, the optimal growth temperature for MA19. The optical density at 412 nm ($\varepsilon = 29.63$ $mM^{-1} \cdot cm^{-1}$) of the thiobenzoate anion resulting from the reaction of CoA and DTNB was measured for 10 min at 50°C with a spectrophotometer (model U-3500; Hitachi, Tokyo, Japan).

Assay of phosphotransacetylase (EC 2.3.1.8) was done according to the method of Mayer et al. (12). The assay mixtures (600 μl) contained 0.15 mg of protein, 0.8 mM acetyl-CoA, 0.5 mM DTNB, and 50 mM potassium phosphate in Tris-glycerol buffer. The reaction was started by addition of acetyl-CoA, unless otherwise stated.

In vitro activation of PHB synthase. The mixtures (300 μl) for in vitro activation contained 0.45 mg of protein and the putative activators in Tris-glycerol buffer. The mixtures were incubated for 10 min at 50°C. Longer incubation resulted in an inactivation of the enzyme. The mixtures were kept on ice until the enzyme assay. Two hundred microliters of the mixtures was used for assay of the PHB synthase.

Dry cell weight and PHB content. Dry cell weight and PHB content were determined as described previously (14).

Measurement of O_2 evolving rate. Cultures (3 ml) were sampled and put into an O_2-electrode system (Biott, Shinjuku, Japan). O_2 evolution rates were measured by monitoring dissolved O_2 concentration for 5 min at 50°C under light-saturated conditions (under a tungsten lamp; 7 W/m^2).

Protein assay. Protein assay was carried out by the Bradford method (2), with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

PHB accumulation. During nitrogen-sufficient cultivation, no PHB was detected from the cells. When the cells were transferred into −N BG-11, photosynthetic O_2 evolving activity of MA19 was reduced and cell growth ceased. PHB accumulation by MA19 was observed under nitrogen-limited con-

TABLE 1. PHB synthase activities in fractionated samples

Cell and protein sample[a]	PHB synthase activity (nmol · min^{-1} · mg of protein^{-1})[b]	
	−Acetyl phosphate	+Acetyl phosphate
$MA19_{-N}$		
Crude extract	58	ND[c]
Membrane fraction	378	ND
Soluble fraction	<15	ND
$MA19_{+N}$		
Crude extract	<15	31
Membrane fraction	<45	245
Soluble fraction	<15	<15

[a] The protein samples were prepared from cells incubated for 7 days after transfer. For membrane fractions, the protein concentration in the assay mixture was adjusted to 0.1 mg per 600 μl.

[b] For activation of PHB synthase, 0.45 mg of protein (crude extract and soluble fraction) and 0.15 mg of protein (membrane fraction) were incubated with 3 mM acetyl phosphate (+Acetyl phosphate) in 300 μl of Tris-glycerol buffer.

[c] ND, not done.

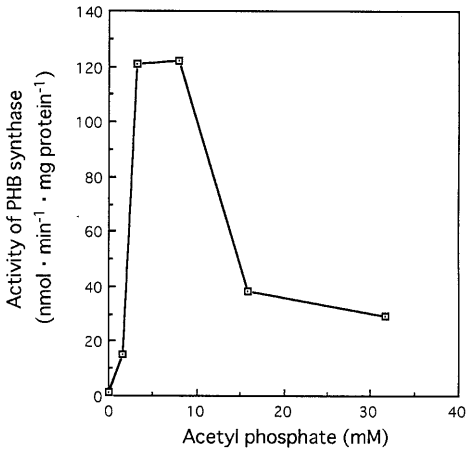

FIG. 4. In vitro activation of PHB synthase in crude extracts from $MA19_{+N}$. Crude extracts were prepared from $MA19_{+N}$ incubated for 7 days after transfer.

ditions (Fig. 1). Cells cultivated under nitrogen-sufficient and nitrogen-limited conditions were designated $MA19_{+N}$ and $MA19_{-N}$, respectively.

PHB synthase activity. Figure 2a shows the DL-3-hydroxybutyryl-CoA saturation curve of the PHB synthase in crude extracts from $MA19_{-N}$. The K_m value, as calculated from the double-reciprocal plots of velocity versus substrate concentration, was 70.7 μM (Fig. 2b). The optimal temperature of PHB synthase activity was about 65°C (Fig. 2c). The specific activity of PHB synthase in crude extracts from $MA19_{-N}$ accumulating PHB was higher than that from $MA19_{+N}$ (Table 1). PHB synthase was increased under nitrogen-limited conditions (Fig. 2d). The results suggest that PHB synthase controlled PHB synthesis by MA19.

Effect of CM and light on PHB synthase activity. The effect of chloramphenicol (CM), an inhibitor of protein synthesis, on the shift of PHB synthase activity in $MA19_{-N}$ was studied. In the absence of CM, the color of the $MA19_{-N}$ culture changed to green-yellow due to degradation of phycobilisomes. Degradation of phycobilisomes is triggered by induction of NblA (3) under conditions of nitrogen deprivation. The MIC of CM for MA19 was 3 μg/ml. The color of the culture, in the presence of 10 μg of CM per ml, remained blue-green, similar to that of the $MA19_{+N}$ culture. Therefore, CM probably inhibited the synthesis of proteins such as NblA in $MA19_{-N}$. Figure 3 shows the shift of PHB synthase activity of $MA19_{-N}$ in the presence versus absence of CM. PHB synthase activity of $MA19_{-N}$ increased when protein synthesis was inhibited by CM. Light was required to increase the PHB synthase activity of $MA19_{-N}$. These results suggest that PHB synthase was activated posttranslationally.

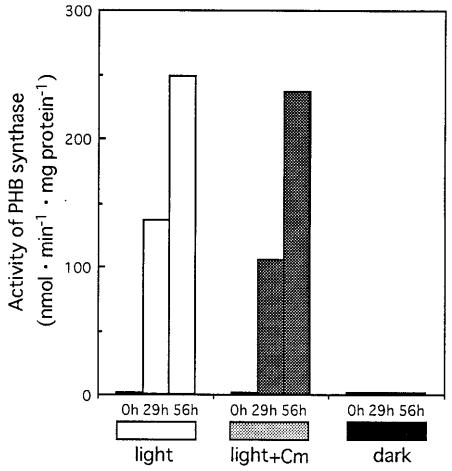

FIG. 3. Effects of CM and light on the shift of specific activity of PHB synthase in $MA19_{-N}$. Light + Cm, MA19 cells grown in BG-11 were transferred into −N BG-11 containing 10 μg of CM per ml and incubated as mentioned in Materials and Methods.

In vitro activation of PHB synthase. Six putative activators were tested for in vitro activation of PHB synthase in crude extracts from $MA19_{+N}$: the intermediates of PHB synthesis, acetyl-CoA (10 mM) and acetoacetyl-CoA (10 mM); the intermediates of glycolysis, phosphoenolpyruvate (50 mM) and 3-phosphoglycerate (30 mM); and the substrates of phosphorylation, ATP (30 mM) and acetyl phosphate (32 mM), were used at several concentrations up to the values indicated in parentheses. There was no increase in the optical density at 412 nm due to 2-nitro-thiobenzoate anions in the reaction mixture containing the putative activators DL-3-hydroxybutyryl-CoA and DTNB without crude extracts. Activation of the PHB synthase was found only when acetyl phosphate was added to crude extracts and membrane fractions from $MA19_{+N}$ (Table 1). The level of activation was dependent on the concentration of acetyl phosphate added (Fig. 4). These results suggest that PHB synthase activity was subject to posttranslational control. In addition, PHB synthase of MA19 behaved exclusively as a membrane-bound protein under both nitrogen-sufficient (no PHB accumulation) and nitrogen-limited (PHB accumulation) conditions.

Acetyl phosphate synthesis. Under photoautotrophic conditions, acetyl phosphate is exclusively synthesized from acetyl-CoA. Phosphotransacetylase (EC 2.3.1.8) catalyzes this reaction. Figure 5a shows the acetyl-CoA saturation curve for this enzyme in crude extracts from $MA19_{-N}$. The K_m value, as calculated from the double-reciprocal plots of velocity versus substrate concentration, was 82.8 μM (Fig. 5b). Table 2 shows the relationship among phosphotransacetylase, PHB synthase, and PHB accumulation under different culture conditions. Phosphotransacetylase was detected in crude extracts from $MA19_{-N}$ but not from $MA19_{+N}$. The addition of acetate to the nitrogen-sufficient culture was not a trigger for activation

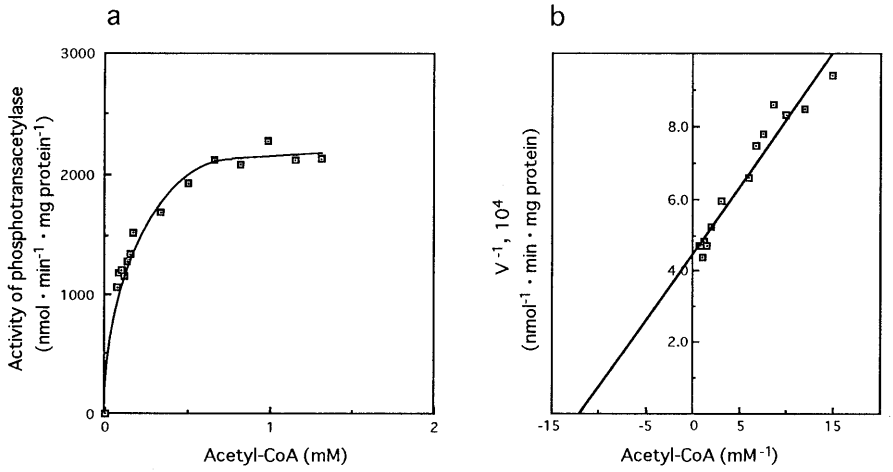

FIG. 5. Effect of acetyl-CoA on phosphotransacetylase activity in crude extracts from MA19$_{-N}$. MA19$_{-N}$ were incubated for 7 days after transfer. (a) Dependency on acetyl-CoA concentration; (b) double-reciprocal plot of the data shown in panel a.

of phosphotransacetylase (Table 2). PHB synthase activity and PHB accumulation also were detected only in MA19$_{-N}$. PHB content in MA19$_{-N}$ grown with acetate was higher than that in MA19$_{-N}$ grown without acetate despite lower PHB synthase activity, which probably means that carbon flux also affects PHB accumulation.

DISCUSSION

In this report, we characterized PHB synthase of a thermophilic cyanobacterium, *Synechococcus* sp. strain MA19. Previously, PHB synthases have been detected in soluble rather than in membrane fractions (5, 8, 10). However, the PHB synthase of MA19 was exclusively detected in the membrane fractions from both MA19$_{+N}$ (PHB-nonaccumulating cells) and MA19$_{-N}$ (PHB-accumulating cells) (Table 1). Our preliminary experiments indicated that MA19 synthase is not easily solubilized from membrane fractions (data not shown). Moreover, the optimal temperature for the PHB synthase activity of MA19 was 65°C (Fig. 2c). The properties of PHB synthase of MA19 may differ from those of PHB synthases of other bacteria. The K_m value of the PHB synthase in the crude extracts from MA19$_{-N}$ for DL-3-hydroxybutyryl-CoA was 70.7 μM. Since the substrate used was the DL mixture, the K_m value for the D form might be as low as or actually lower than those for *Chromatium vinosum* (63 μM) (10) and *Zoogloea ramigera* (53 μM) (5) and much lower than that for *A. eutrophus* (720 μM) (8). The low K_m value may aid the accumulation of PHB under conditions of low carbon flux through the photoautotrophic metabolism of cyanobacteria (16).

The insensitivity of the shift of PHB synthase activity to CM and the demonstration of in vitro activation of the enzyme strongly suggested the existence of posttranslational control of the enzyme. PHB synthase of MA19 was activated by acetyl phosphate. The extent of activation changed in the range of physiologically probable concentrations (0 to 3 mM) of acetyl phosphate. The acetyl phosphate concentration was reported to vary with respect to the intracellular acetyl-CoA flux and/or the catalytic activity of acetyl phosphate synthesis (13). The intracellular concentration of acetyl phosphate in MA19$_{-N}$ was probably higher than that in MA19$_{+N}$, because phosphotransacetylase activity was detected exclusively in MA19$_{-N}$ (Table 2). Since PHB synthase activity was detected only in MA19$_{-N}$, it may be controlled by acetyl phosphate in vivo. *A. eutrophus* PHB synthase requires a posttranslational modifica-

TABLE 2. Activities of phosphotransacetylase and PHB synthase in crude MA19 extracts

Sample[a]	Sp act (nmol · min^{-1} · mg of protein^{-1})		% (wt/wt) PHB content
	Phospho-transacetylase	PHB synthase	
MA19$_{+N}$	<30	<15	<0.1
MA19$_{+N}$ + acetate (1 mM)	<30	<15	<0.1
MA19$_{-N}$	1,230	121	10
MA19$_{-N}$ + acetate (1 mM)	5,120	38	18

[a] The protein samples were prepared from cells incubated for 7 days after transfer.

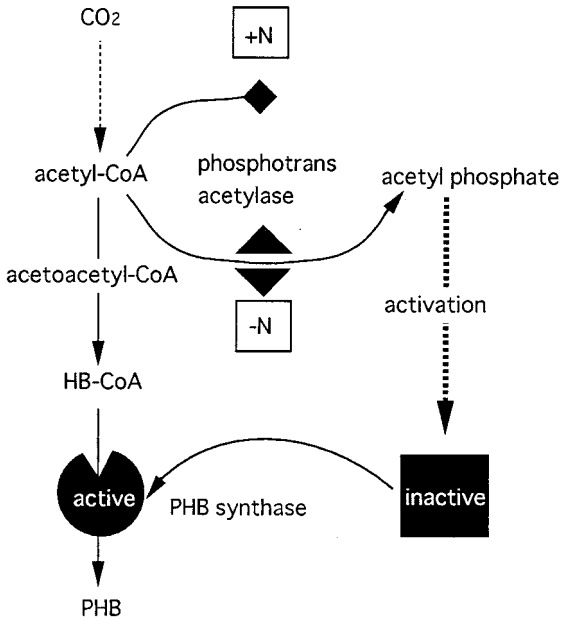

FIG. 6. Model for system of PHB synthesis control in strain MA19.

tion at a serine residue with phosphopantetheine for its activity (6). It is still unclear whether the activation of MA19 synthase by acetyl phosphate is related to the phosphopantetheine modification.

It has been reported that an imbalance in the C/N ratio and the NADPH/ATP ratio triggers PHB accumulation in chemolithotrophic bacteria (18) and cyanobacteria (4, 17). In *Synechococcus* sp. strain MA19, it was found that an imbalance in the C/N ratio results in the accumulation of PHB, since photoautotrophic PHB accumulation occurred under nitrogen-limited conditions (14).

This is the first study to report that posttranslational control by acetyl phosphate regulates PHB synthase. We propose a regulation model (Fig. 6) based on the results described above. In this model, MA19 has two regulatory systems. The PHB synthase acts as a valve for PHB synthesis, and phosphotransacetylase acts as a switch to control the valve. Acetyl phosphate acts as a signal to indicate acetyl-CoA flux. Further studies to confirm this model and to cover the generality of this model for cyanobacteria and other bacteria will be published elsewhere.

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