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Investigation of Regulatory Mechanism of Flux of Acetyl-CoA in *Alcaligenes eutrophus* Using PHB-negative Mutant and Transformants Harboring Cloned *phb*CAB Genes

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The regulatory mechanism of the flux of acetyl-CoA in *Alcaligenes eutrophus* in unbalanced growth conditions was investigated using a PHB-negative mutant and transformants reintroduced PHB-biosynthesis enzymes through the transformation of cloned *phb*CAB genes. The PHB-negative mutant was defected absolutly in PHB synthase but partially in β-ketothiolase and acetoacetyl-CoA reductase, and excreted substantial amount of pyruvate to culture broth at late growth phase. The excretion was due to the inhibitory effect of acetyl-CoA on the activity of pyruvate dehydrogenase. The cloned *phb*C and *phb*CAB genes were transformed to the PHB-negative mutant strain to reintroduce PHB biosythesis enzymes. Pyruvate excretion could be decreased substantially but not completely by transformation of PHB synthase alone, while pyruvate excretion was ceased by transformation of all three PHB biosynthesis enzymes. To identify the most critical PHB biosynthesis enzyme influencing on the flux of acetyl-CoA, the effect of the variation of PHB biosynthesis enzymes on pyruvate dehydrogenase was investigated. β-Ketothiolase influenced the activity of pyruvate dehydrogenase more sensitively than PHB synthase. β-Ketothiolase, the first step enzyme of PHB biosynthesis that condense acetyl-CoA to acetoacetyl-CoA, seems to be the major enzyme determining the flux of acetyl-CoA to PHB biosynthesis or TCA cycle, and the rate of PHB biosynthesis in *A. eutrophus*.

Poly-β-hydroxybutyrate (PHB) is an energy storage material accumulated inside microorganisms cultivated under abnormal growth conditions (14). The biosynthesis of PHB in *Alcaligenes eutrophus* is accomplished by three enzymes; β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase, the products of *phb*A, *phb*B, and *phb*C genes, respectively (12, 13).

In balanced growth conditions acetyl-CoA generated from glycolysis flows through the TCA cycle for cell growth, meanwhile, in unbalanced growth conditions the acetyl-CoA accumulates inside the cell, and then flows to the PHB biosynthesis pathway. The blockage of the TCA cycle in unbalanced conditions is caused by decrement of activities of enzymes related to TCA cycle including citrate synthase, the first step enzyme of the TCA cycle. The intracellular acetyl-CoA/CoA concentration ratio is controlled by accumulated acetyl-CoA and also effects on the actions of the first two step enzymes related to PHB biosynthesis, β-ketothiolase and acetoacetyl-CoA reductase.

Acetyl-CoA can activate not only the TCA cycle but also PHB biosynthesis depending on its concentration inside of the cell as previously described. Investigation of the flux of acetyl-CoA is essential to our understanding of the regulatory mechanism of *A. eutrophus*, and especially the interaction between glycolysis and the PHB biosynthesis pathways, which will facilitate establishing the strategies for the optimal utilization of fructose and assist in developing proper cutivation conditions for achieving the effective biosynthesis of PHB.

Steinbüchel *et al.* (14) have selected several PHB-negative mutant strains of *A. eutrophus* in order to use them as complementation probes to identify *phb*C gene locus of different PHB-producing strains (13). They found that the mutant strain DSM 541 was absolutly damaged in PHB synthase but only partially damaged in β-ketothiolase and acetoacetyl-CoA reductase. The mutant excreted various intermediary metabolites related to glycolysis, especially pyruvate under unbalanced growth conditions, however, the excretion mechanism has not

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been studied in detail.

In our previous works, the *phb*CAB operon of *A. eutrophus* was isolated, isolated operon was recombined in the plasmid vector, and an *E. coli-A. eutrophus* shuttle vector was constructed. Then, the constructed recombinant plasmids were transformed again into the parent strain *A. eutrophus* (3, 8, 9). The transformants harboring the cloned *phb*CAB genes showed quite different enzyme activities related to PHB biosynthesis (11).

A PHB-negative mutant might be a good source for studying the flux of acetyl-CoA, because of its unique nature for the excretion of various intermediary metabolites related to glycolysis and PHB biosynthesis, such as, pyruvate, acetate, acetoactate, and 3-hydroxybutyrate (16, 18). Through the transformation of cloned *phb*CAB genes to the PHB-negative mutant strain, the activities of each enzyme related to PHB biosynthesis can be controlled. Therefore, they can also be used as model strains for studying the relationship between PHB biosynthesis and glycolysis pathways, and especially the flux of acetyl-CoA inside *A. eutrophus*.

In this work, the effect of blockage of the PHB biosynthesis pathway in *A. eutrophus* on cell growth, fructose utilization, and the excretion of various intermediary metabolites were investigated using a PHB-negative mutant strain. The activities of major enzymes related with glycolysis and PHB biosynthesis pathways were measured under nitrogen-limited unbalanced growth condition, and the relationship between major enzyme activities and the excretion of various intermediatory metabolites, especially pyruvate, was investigated. The effect of a supplement of acetyl-CoA and three intermediates of the PHB biosynthesis pathway was examined to investigate the excretion mechanism for pyruvate.

The cloned *phb*C and *phb*CAB genes were reintroduced to PHB-negative mutant through transformation to modify the activities of various PHB biosynthesis enzymes. The effect of variation of PHB biosynthesis enzymes on the pyruvate execretion, PHB accumulation, activity of pyruvate dehydrogenase and PHB biosynthesis enzymes were investigated. The relationships between PHB biosynthesis enzymes and pyruvate dehydrogenase was studied to identify the most critical enzyme influencing the flux of acetyl-CoA in *A. eutrophus*.

MATERIALS AND METHODS

Strains

The parent strain was *Alcaligenes eutrophus* H16 (ATCC 17699). The PHB-negative mutant, *A. eutrophus* DSM 541 as studied by Steinbüchel (15), was also used. Two transformants *A. eutrophus* MAR3 and MAR5, reintroduced the cloned *phb*C and *phb*CAB genes to the PHB-negative mutant by a similar method to that described in our previous works (8, 9).

Medium and Cultivation

The parent, mutant, and transformant strains were cultivated in minimal medium, composed of 3.8 g/l of Na_2HPO_4, 2.65 g/l of KH_2PO_4, 2.0 g/l of NH_4Cl, 0.2 g/l of $MgSO_4$, 20 g/l of fructose, and 1 ml/l of trace minerals. The strains were cultivated in a 250 ml flask shaker at pH 7.0, 30°C, and 200 rpm.

Measurement of Total and Residual Cell Mass

The centrifuged cells collected were dried in a dry oven at 100°C for 24 h to measure the total cell mass. Residual cell mass was determined by subtracting PHB concentration from the total cell mass.

Determination of PHB

PHB was extracted in hot chloroform after treating the cells with 5% sodium hypochlorite solution at 37°C for 1 h (5). PHB concentration was determined by the modified method of Braunegg *et al.* using gas chromatography equipped with flame ionization detector (Young-In Co. Ltd., Seoul, Korea), and a gas column (6 m × 3 mm i.d.) filled with 2% Reoplex 400 on Chromosorb GAW 60 to 80 mesh, using PHB powder of *A. eutrophus* (Sigma Co., MO, U.S.A.) as a standard. The detection conditions were initial temperature at 100°C, final temperature at 150°C, with an increase of the temperature by 5°C/min.

Measurement of Enzyme Activities

Cells were disrupted to obtain cell extract after being suspended in 50 mM phosphate buffer by ultrasonication at 4°C. The enzymatic activities in the cell extract were determined by measurement of the concentration of each detection material at different wavelengths after incubation with appropriate substrates as described in our previous works (4, 6) and summarized in Table 1 (2). Pyruvate dehydrogenase complex was identified by a

Table 1. Various methods used for determination of various enzyme activities (1, 14).

Enzymes	Wavelength employed, nm	Detected material	Reference
β-Ketothiolase	340	NADH	Senior *et al.* (14)
Acetoacetyl-CoA reductase	340	NADPH	Senior *et al.* (14)
PHB synthase	412	CoA	Senior *et al.* (14)
Pyruvate dehydrogenase	340	NADH	Cook *et al.* (1)
Glucose-6-phosphate dehydrogenase	340	NADPH	Senior *et al.* (14)
Citrate synthase	412	CoA	Diagnostic kit
Alanine aminotransferase	340	NADH	(Sigma Co.) Diagnostic kit
Lactate dehydrogenase	340	NADH	(Sigma Co.)

modified method of Sigma Co. using diagnostic kits which determined the concentration of NADH using a spectrophotometer at 340 nm.

Analytical Methods

Pyruvate concentrations were determined by measurement of the reducing concentration of NADH in the UV range at 340 nm after an appropriate enzyme reaction. Acetate and acetoacetate were determined by high-pressure liquid chromatography on a $5C_{18}$ column and a refractive index detector (Waters Co.), using 0.05 M ammonium phosphate buffer (pH 2.0) as the mobile phase. Fructose concentration was determined using sulfuric acid-cystein·HCl-tryptophan (10).

RESULTS AND DISCUSSION

Effect of the Blockage of PHB Biosynthesis of *A. eutrophus* on Cell Growth and Utilization of Fructose

The PHB-negative mutant strain *A. eutrophus* DSM 541 was cultivated in minimal medium containing 20 g/l of fructose and 2 g/l of NH_4Cl for 60 h, and then the effects of the blockage of the PHB biosynthesis pathway on cell growth and fructose utilization were investigated. Fig. 1 illustrates the changes in total cell mass, residual cell mass, PHB concentration, and residual fructose concentration of the parent (Fig. 1A) and the mutant (Fig. 1B) strains during cultivation. The PHB-negative mutant showed somewhat different patterns of cell growth and fructose utilization in the late growh phase. The blockage of PHB biosynthesis substantially decreased the total cell mass, growth rate, and fructose utilization, however, the residual cell mass remained without any significant changes.

Notably, the PHB-negative mutant consumed fructose continuously during the late growth phase after 36 h even though PHB biosynthesis did not occur in contrast with the parent strain that produced PHB continuously. Continuous consumption of fructose suggest that fructose may be either converted to other intermediary metabolites of glycolysis and the PHB biosynthesis pathways or utilized as the energy source for cell maintenance.

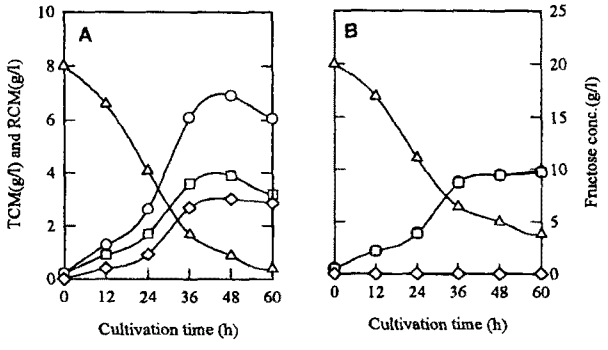

Fig. 1. Comparison of cell growth, PHB concentration, and the amount of fructose used by parent strain *A. eutrophus* H16 (A) and mutant strain *A. eutrophus* DSM 541 (B).
Cultivation: Minimal medium, 20 g/l of frutose, 2 g/l of NH_4Cl, pH 7.0, 30°C, and for 60 h. ○, total cell mass (TCM); □, residual cell mass (RCM); ◇, PHB concentration; △, fructose concentration.

Excretion of Intermediary Metabolites of PHB-negative Mutant *A. eutrophus*

Intermediary metabolites. To investigate the nature of excretion of intermediary metabolites of the PHB-negative mutant, the concentrations of various intermediary metabolites of glycolysis and PHB biosynthesis, such as, pyruvate, acetate, acetoacetate, and 3-hydroxybutyrate excreted extraceullary in the culture broth were measured after cultivation for 48 h when the late growth phase was reached as shown in Table 2. The PHB-negative strain excreted substantial amounts of pyruvate and acetate 1.96 and 0.58 g/l, respectively, meanwhile, small amounts of acetoacetate and β-hydroxybutyrate, less than 0.1 g/l were excreted in the nitrogen-limited late growth phase. Similar excretion of various intermediatory metabolites, such as pyruvate, acetate, and 3-hydroxybutyrate under phosphate-limited growth conditions was also reported by Schlegel *et al.* (1, 15, 16, 18).

The PHB-negative mutant mainly excreted intermediates related to the glycolysis pathway including pyruvate rather than intermediates of the PHB biosynthesis pathway. The excretion of the aforementioned intermediates may be due to the inhibitory effect of the accumulated intermediates of glycolysis and PHB biosynthesis pathways, such as acetyl-CoA, acetoacetyl-CoA, and 3-hydroxybutyryl-CoA, on the activity of pyruvate dehydrogenase.

The following reasons can also postulated. One is that enzymes related to the conversion of pyruvate to alanine or lactate may be damaged, therefore, the consumption of pyruvate does not take place smoothly, and is excreted extracellulary. The other possbility is that the limited supplement of CoA is used as co-substrate of pyruvate dehydrogenase because of the blockage of PHB biosynthesis, so, acetyl-CoA can not be smoothly condensed into acetoacetyl-CoA.

Pattern of pyruvate excretion. The parent and PHB-

Table 2. Comparison of intermediatory metabolites excreted by parent strain *A. eutrophus* H16 and PHB-negative mutant strain DSM 541.

Strains	Acetate (g/l)	Pyruvate (g/l)	Aceto-acetate (g/l)	β-Hydroxy-butyrate (g/l)
A. eutrophus H16	–	–	–	–
A. eutrophus DSM 541	0.58	1.96	< 0.1	< 0.1

Cultivation: Minimal medium, 20 g/l of fructose, 2 g/l of NH_4Cl, pH 7.0, 30°C, and after 48 h cultivation.

negative mutant *A. eutrophus* were cultivated in minimal medium for 60 h, and then the excretion pattern of pyruvate was measured during cultivation. Fig. 2 compares the changes of NH_4Cl concentrations, pyruvate excreted in culture broth, and PHB accumulated during cultivation. The PHB-negative mutant *A. eutrophus* did not excrete pyruvate during the 30 h of initial cell growth phase, meanwhile, significantly excreted pyruvate after 30 h when NH_4Cl was limited. The pyruvate excretion reached its maximum value of 1.98 g/l after 36 h, and then slightly decreased thereafter. This indicates that pyruvate may flow smoothly to acetyl-CoA during the initial balanced growth phase, however, during the unbalanced growth phase pyruvate starts to accumulate substantially in the inside of cell because of the blockage of PHB biosynthesis, and then is excreted extracellulary into the culture broth.

Meanwhile, the parent strain started to accumulate PHB after 30 h when nitrogen-limited condition were proceeds instead of excreting pyruvate. The excretion pattern of the pyruvate of PHB-negative mutant strain accords with the pattern of the PHB biosynthesis of the parent strain *A. eutrophus*. Steinbüchel *et al.* (15) who compared the rate of pyruvate excretion of PHB-negative mutant and PHB biosynthesis in *A. eutrophus* also observed a similar pattern in both strains.

In order to investigate the detailed mechanism for the excretion of pyruvate in the PHB-negative mutant, the variation of the major enzyme activities related to glycolysis need to be investigated. Pyruvate excretion may also be closely connected with the concentration level of various intermediary meatabolites of glycolysis and PHB biosynthesis, therefore, the effects of a supplement of intermediates at the late growth phase on pyruvate excretion needs to be investigated.

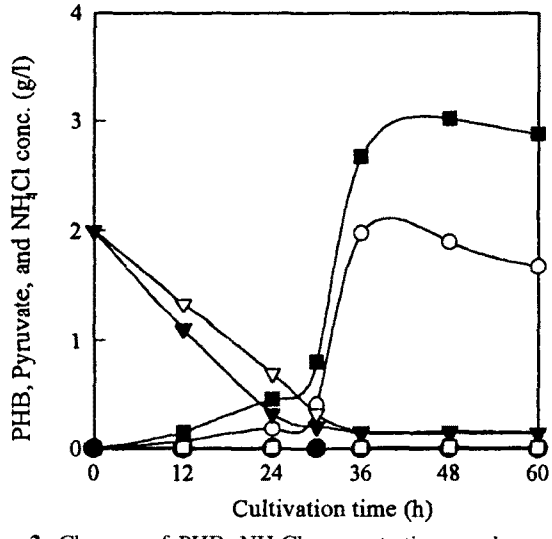

Fig. 2. Changes of PHB, NH_4Cl concentrations, and pyruvate excreted during cultivation of parent strain *A. eutrophus* H16 and PHB-negative mutant strain *A. eutrophus* DSM 541.
Cultivation: Minimal medium, 20 g/l of fructose, 2 g/l of NH_4Cl, flask shaker, 30°C, and for 60 h. Closed symbols, parent strain *A. eutrophus* H16. Open symbols, PHB-negative mutant strain *A. eutrophus* DSM 541. □, PHB concentration; ▽, NH_4Cl concentration; ○, pyruvate concentration.

Mechanism of Pyruvate Excretion of *A. eutrophus*

Enzymes related to pyruvate metabolism. To examine the pyruvate excretion mechanism of the PHB-negative mutant, the activities of five enzymes closely connected with pyruvate metabolism were measured, and compared with those of the parent strain as shown in Table 3, including intracellular glucose-6-phosphate dehydrogenase, pyruvate dehydrogenase, citrate synthase, alanine aminotransferase, and lactate dehydrogenase after cultivation for 24 and 48 h, respectively.

Most of the above mentioned enzymes of both the mutant and parent strains did not differ much after 24 and 48 h, even under balanced and unbalanced growth conditions. The pyruvate dehydrogenase of the PHB-negative mutant strain was not much different at 24 h, however, it was reduced by 75% at 48 h compared to the parent strain. The above observation indicates that the excretion of pyruvate is directly connected with the changes in the activity of pyruvate dehydrogenase rather than for other reasons including the damage to other pathways such as the flow of pyruvate to alanine or lactate. The lower activity levels of pyruvate dehydrogenase under unbalanced growth conditions may be due to either the inhibition of pyruvate dehydrogenase by intermediates accumulated or the decreased supplement of CoA, a co-substrate of pyruvate dehydrogenase, caused by partial damage to the first step reaction of PHB

Table 3. Comparison of specific activities of glucose-6-phosphate dehydrogenase, pyruvate dehydrogenase, citrate synthase, lactate dehydrogenase, and alanine aminotransferase of parent strain *A. eutrophus* H16 and mutant strain DSM 541.

	Strains	Specific activity (units/mg protein)				
		GPDH[1]	PDH[2]	CS[3]	LDH[4]	AAT[5]
24 h	*A. eutrophus* H16	1.43	1.01	0.84	0.48	0.56
	A. eutrophus DSM 541	1.41	1.00	0.82	0.47	0.55
48 h	*A. eutrophus* H16	1.12	0.87	0.75	0.46	0.42
	A. eutrophus DSM 541	1.12	0.16	0.74	0.46	0.43

Cultivation: Minimal medium, 20 g/l of fructose, 2 g/l of NH_4Cl, pH 7.0, 30°C, and after 24, 48 h cultivation. 1, Glucose-6-phosphate dehydrogenase; 2, Pyruvate dehydrogenase; 3, Citrate synthase; 4, Lactate dehydrogenase; 5, Alanine aminotransferase.

biosynthesis of the PHB-negative mutant.

It can be expected that the supplement of various intermediates of glycolysis and PHB biosynthesis during cultivation will affect their concentrations inside the cells, hence, the influence on the flux of various intermediates related to pyruvate metabolism and PHB biosynthesis pathways. In particular, the effect of suppemented intermediary metabolites on the activity of pyruvate dehydrogenase needs to be examined because pyruvate dehydrogenase is the most critical enzyme determining the flux of pyruvate to other intermediary metabolites including acetyl-CoA.

Effect of supplements of acetyl-CoA and intermediatory metaboltes on pyruvate excretion. To identify the most influential intermediate affects on pyruvate excretion, several intermediates related to pyruvate metabolism and PHB biosynthesis pathways, including acetyl-CoA, acetoacetyl-CoA, β-hydroxybutyryl-CoA, and CoA were added to culture broths after 30 h when pyruvate excretion has been initiated. The concentration levels of the aforementioned intermediates were 3, 5, 5, and 3 mM, respectively, and the intermediate concentration was maintained at sufficient level to overcome the limitation of transport efficiency of cell membrane. The amount of pyruvate excreted was measured at 36 h where its excretion reached the maximum value. Fig. 3 illustrates the effect of supplements of the aforementioned intermediates on pyruvate excretion of the PHB-negative mutant.

Pyruvate excretion was influenced most significantly by supplementing acetyl-CoA indicating that the acetyl-CoA increased by the uptake of acetyl-CoA may not be used effectively for PHB biosynthesis because of the blockage of the PHB biosynthesis pathway, and the accumulated acetyl-CoA strongly inhibits the pyruvate dehydrogenase, as suggested by Voet *et al.* (17) who reported that a high level of acetyl-CoA acts as an inhibitor of pyruvate dehydrogenase.

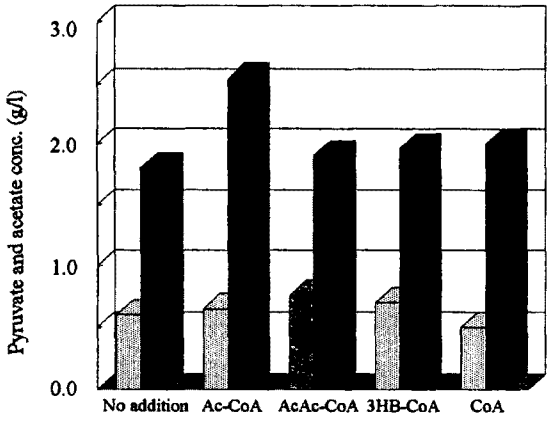

Fig. 3. Effects of supplementation of various metabolites on excretion of pyruvate and acetate of mutant strain *A. eutrophus* DSM 541.

Cultivation: Minimal medium, 20 g/l of fructose, 2 g/l of NH_4Cl, for 48 h, and supplementations of metabolites after 30 h. ▨, acetate concentration; ■, pyruvate concentration. Ac-CoA, Acetyl-CoA; AcAc-CoA, Acetoacetyl-CoA; 3HB-CoA, 3-Hydroxybutyryl-CoA.

On the other hand, pyruvate excretion was not significantly influenced but was slightly increased by the supplemented acetoacetyl-CoA and β-hydrotybutyryl-CoA. It can be hypothesised that the aforementioned two intermediates may not be accumulated at high concentrations inside the cells, because they can flow to acetoacety-CoA or β-hydroxybutyryl-CoA easily due to the reversible characteristics of β-ketothiolase and acetoacetyl-CoA reductase. Also a part of the intermediates uptaken by the cells may be converted to precursor compounds of PHB biosynthesis, acetyl-CoA and inhibits pyruvate dehydrogenase slightly.

Meanwhile, pyruvate excretion was not affected at all by the supplement of CoA. This means that CoA may be supplied smoothly by the PHB-negative mutant strain because the β-ketothiolase, generating CoA by condensation of acetyl-CoA to acetoacetyl-CoA, is partially activated. It can also be hypothesised that pyruvate excretion may be closely connected with the flux of acetyl-CoA to PHB biosynthesis and the TCA cycle. Therefore, the effect of variation of the three PHB biosynthesis enzymes on pyruvate excretion and PHB biosynthesis needs to be examined. The activities of the PHB biosynthesis enzyme can be controlled easily by reinforcing cloned *phb*CAB genes with the PHB-negative mutant by means of genetic transformation (3, 8, 9).

Pyruvate Metabolism and PHB Accumulation of Transformants Reintroduced Cloned *phb*C and *phb*CAB Genes to PHB-negative Mutant

Pyruvate excretion and PHB accumulation. Fig. 4 compares the patterns of pyruvate excretion in cuture broth (Fig. 4A), PHB content (Fig. 4B), and PHB concentration (Fig. 4C) in the inside of the cell of the parent, mutant, transformant MAR3 that reintroduced all three enzymes through the transformation of the cloned *phb*CAB gene, and transformant MAR5 that reintroduced PHB synthase only through the transformation of the cloned *phb*C gene to the PHB-negative mutant. As shown in Fig. 4A, pyruvate excretion was completely prevented in transformant MAR3 that reinforced all PHB biosynthsis enzymes to PHB-negative mutant strain. Meanwhile, the MAR5 that reinforced PHB synthase in the PHB-negative mutant strain still excreted a small amount of pyruvate continuously even though the amount is slightly lower than the PHB-negative mutant defecting PHB synthase by around 18.5%. The observed difference between transformants MAR3 and MAR5 could be explained by the variation of activities of the

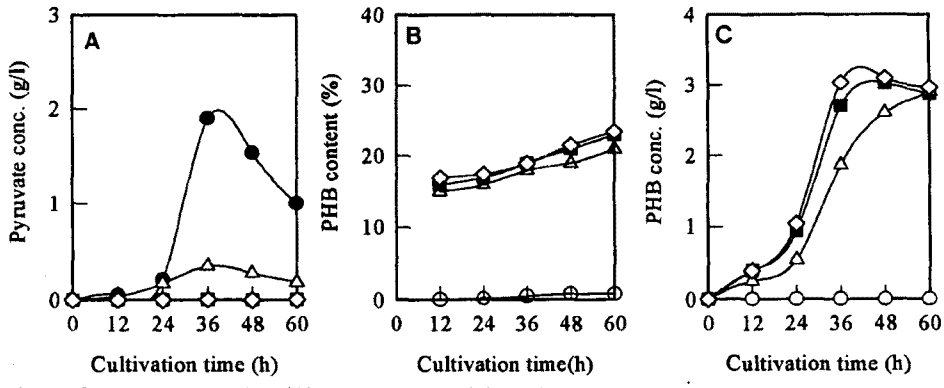

Fig. 4. Comparison of pyruvate excretion (A), PHB content (B), and PHB concentration (C) of parent, mutant, and transformant MAR3 reintroduced cloned *phb*CAB and MAR5 reintroduced cloned *phb*C genes.
Cultivation: Minimal medium, 20 g/l of fructose, 2 g/l of NH_4Cl, 50 μg/ml of kanamycin, pH 7.0, 30°C, and 60 h cultivation. □, parent *A. eutrophus* H16; ○, mutant *A. eutrophus* DSM; ◇, transformant MAR3; △, transformant MAR5.

enzyme related to PHB biosynthesis that was expressed differently according to the types of cloned *phb*CAB genes.

The accumulated PHB contents were also compared. The transformant MAR3 reintroduced all three PHB biosynthesis enzymes and resumed a PHB content of 23% a similar level to the parent strain after 60 h as shown in Fig. 4B. On the other hand, the transformant MAR 5 reinforced PHB synthase alone also asumed a similar PHB content to the parent and transformant MAR3. This indicates that the PHB synthase, involved in the final step of PHB biosynthesis is a polymerizing monomer 3-hydroxybutyrate to poly-3-hydroxybutyrate, and may be the most critical enzyme determining the accumulation of intracellular PHB (11).

The PHB accumulation rates of the above strains were also compared. The transformant MAR 3 was maintained at a similar level compared to the parent strain, meanwhile, the transformant MAR5 was maintained at a lower level of around 20% when compared to the parent strain as shown in Fig. 4C. This decrease of MAR5 may be due to the limited of supply of acetyl-CoA because the PHB-negative mutant has been partially deactivated in β-ketothiolase and acetoacetyl-CoA reductase, as reported in Steinbüchel *et al.* (15). It can be postulated that the above two enzymes may play a key role in determining the rate of PHB biosynthesis, and it will discussed in detaile in the next section.

The flux of acetyl-CoA inside the cell is essential for our understanding of the requlatory mechanism of PHB biosynthesis of *A. eutrophus*. The acetyl-CoA is a high energy compound and does not itself remain inside the cell at high concentration but changes other intermediary metabolites including CoA, acetate, and citrate *et al.* (17). The direct measurement of acetyl-CoA concentration is troublesome, hence, the flux of intracellular acetyl-CoA can be profiled indirectly by investigating the change in pyruvate dehydrogenase activity. In order to identify the most critical PHB biosynthesis enzyme affecting the flux of acetyl-CoA, transformants showing different activities of PHB biosynthesis enzymes were used, and the effect of the variation of enzyme activities on pyruvate dehydrogenase was investigated.

Effect of variations of PHB biosynthesis enzymes on pyruvate dehydrogenase. Fig. 5 compares intracellular PHB biosynthesis enzyme activities; the β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase of the parent, mutant, and the two transformant strains was measured after 36 h, respectively, along with

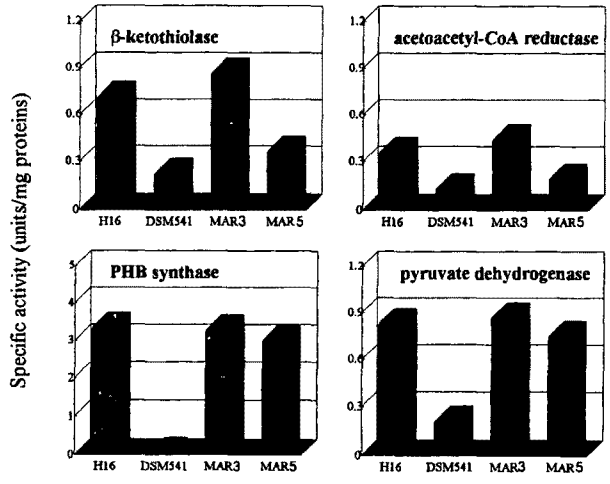

Fig. 5. Comparisons of specific activity of three PHB biosynthesis enzymes and pyruvate dehydrogenase of parent, mutant, and transformant MAR3 reintroduced cloned *phb*CAB and MAR5 reintroduced cloned *phb*C genes.
Cultivation: Minimal medium, 20 g/l of fructose, 2 g/l of NH_4Cl, 50 μg/ml of kanamycin, 30°C, and after 36 h.

the pyruvate dehydrogenase of the above strains.

Two transformants reintroduced PHB biosynthesis enzymes through transformation recovered pyruvate dehydrogenase activity sinificantly comparable to that of the PHB-negative mutant, however, two transformants showed slight differences in pyruvate dehydrogenase. The pyruvate dehydrogenase of transformant MAR5 showing similar PHB synthase activity to parent strain but lower activities of β-ketothiolase and acetoacetyl-CoA reductase. The β-ketothiolase activity of MAR5 increased at around 1.64 folds compared to PHB-negative mutant, and the strain showed significantly improved pyruvate dehydrogenase activity around 3.75 folds compared to PHB-negative mutant strain. The transformant MAR3 reintroduced with all three PHB biosynthesis enzymes fully recovered PHB synthase activity to similar level with parent strain and 1.2 folds higher activities of β-ketothiolase and acetoacetyl-CoA reductase. The MAR3 showed significantly improved pyruvate dehydrogenase activity (4.1 fold) compared to PHB-negative mutant, but similar level with parent strain.

The above results indicate that pyruvate dehydrogenase is influenced in part by all three PHB biosynthesis enzymes. However, β-ketothiolase seems to be the most critical enzyme influencing the activity of pyruvate dehydrogenase even though PHB synthase influences the activity of pyruvate dehydrogenase. This conclusion can be justified by the facts that the activity of pyruvate dehydrogenase was sensitively affected even though the variation of β-ketothiolase is relatively small from 0. 22 to 0.86 units/mg proteins. The activity of pyruvate dehydrogenase did not sensitively respond to the variation of PHB synthase even though it varied significantly from 0.05 to 3.32 units/mg proteins. It is worthy of note that β-ketothiolase is the first step enzyme involved in PHB biosynthesis that condenses acetyl-CoA to acetoacetyl-CoA (7).

The aforementioned observation indirectly proves that β-ketothiolase is also the most critical enzyme determining the flux of acetyl-CoA to PHB biosynthesis and TCA cycle in *A. eutrophus*. Consequently, it can be concluded that β-ketothiolase may be also be a key enzyme which determines the rate of PHB biosynthesis in *A. eutrophus*. The fortification of β-ketothiolase through the transformation of cloned *phb*A gene seems to be an effective method that can be used for the improvement of the strain for effective accumulation of PHB because it can accelerate the flux of acetyl-CoA into the PHB biosynthesis pathway.

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REFERENCES

1. Cook, A. M. and H. G. Schlegel. 1978. Metabolite concentrations in *Alcaligenes eutrophus* H16 and a mutant defective in polyhydroxybutyrate synthesis. *Arch. Microbiol.* **119**: 231-235.
2. Dixon, G. H. and H. L. Kornberg. 1959. Assay methods for key enzymes of biochemical society. *Biochem. J.* **72**: 3-5.
3. Kim, G. T., J. S. Park, H. C. Park, Y. H. Lee, and T. L. Huh. 1993. Construction of the recombinat *phb*CAB operon of *Alcaligenes eutrophus* for accumulation of poly-β-hydroxybutyric acid in *Escherichia coli. Kor. J. Appl. Microbiol. Biotechnol.* **21**: 221-228.
4. Kim, T. W., J. S. Park, and Y. H. Lee. 1996. Enzymatic characteristics of biosynthesis and degradation of poly-β-Hydroxybutyrate of *Alcaligenes latus, J. Microbiol. Biotechnol.* **6**: 425-431.
5. Law, J. H. and R. A. Slepecky. 1960. Assay of poly-β-hydroxybutyric acid. *J. Bacteriol.* **82**: 33-36.
6. Lee, Y. H., T. W. Kim, J. S. Park, and T. L. Huh, 1996. Effect of the supplement of metabolites on cell growth and poly-β-hydroxybutyrate biosynthesis of *Alcaligenes latus, J. Microbiol. Biotechnol.* **6**: 120-127.
7. Oeding, V. and Schlegel, H. G. 1973. β-Keththiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-β-hydroxybutyrate metabolism. *Biochem. J.* **134**: 239-248.
8. Park, H. C., J. S. Park, Y. H. Lee, and T. L. Huh. 1994. Manipulation of the genes for poly-β-hydroxybutyric acid synthesis in *Alcaligenes eutrophus, Proceedings of IUMS Congress '94*, p. 276, International Union of Microbiological Societies, Prague, Czech Republic, July 3-8.
9. Park, H. C., K. J. Lim, J. S. Park, Y. H. Lee, and T. L. Huh. 1995. High frequency transformation of *Alcaligenes eutrophus* producing PHB by electroporation. *Biotechnol. Tech.* **9**: 31-34.
10. Park, J. S. and Y. H. Lee. 1996. Metabolic characteristics of ioscitrate dehydrogenase leaky mutant of *Alcaligenes eutrophus* and its utilization for poly-β-hydroxybutyrate production, *J. Ferment. Bioeng.* **81**: 197-205.
11. Park, J. S., H. C. Park, T. L. Huh, and Y. H. Lee. 1995. Production of poly-β-hydroxybutyrate by *Alcaligenes eutrophus* transformant harbouring cloned *phb*CAB genes. *Biotechnol. Lett.* **17**: 735-740.
12. Peoples, O. P. and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16, characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. *J. Biol. Chem.* **264**: 15293-15297.
13. Peoples, O. P. and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16, identification and characterization of the PHB polymerase gene (*phbC*). *J. Biol. Chem.* **264**: 15298-15303.
14. Senior, P. J., G. A. Beech, G. A. F. Ritchie, and E. A.

Dawes. 1972. The role of oxygen limitation in the formation of poly-β-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerrinckii*. *Biochem. J.* **128**: 1193-1201.
15. Steinbüchel, A. and H. G. Schlegel, 1989. Excretion of pyruvate of *Alcaligenes eutrophus*, which are impared in the accumulation of poly(β-hydroxybutyric acid)(PHB), under conditions permitting synthesis of PHB. *Appl. Microbiol. Biotechnol.* **31**: 168-175.
16. Vollbrecht, D., E. I., M. A. Nawawy, and H. G. Schlegel. 1978. Excretion of metabolits by hydrogen bacteria, I. autotrophic and heterotrophic fermentations. *European J. Appl. Microbiol.* **6**: 145-155.
17. Voet D. and J. G. Voet. 1994. *Biochemistry*, p. 470-475. 2nd ed. Wiley, New York.
18. Vollbrecht, D. and H. G. Schlegel, 1978. Excretion of metabolits by hydrogen bacteria, II. influences of aeration, pH, temperature, and age of cells. *European J. Appl. Microbiol.* **6**: 157-166.

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