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Expression Analysis of β-Ketothiolase and Acetoacetyl-CoA Reductase of *Rhodobacter sphaeroides*

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Abstract By a sequential action of β-ketothiolase and acetoacetyl-CoA reductase, two molecules of acetyl-CoA are converted into D-3-hydroxybutyryl-CoA, a substrate for PHB synthase to form poly-3-hydroxybutyrate (PHB) of *Rhodobacter sphaeroides*. The β-ketothiolase gene, *phbA*, and acetoacetyl-CoA reductase gene, *phbB*, were cloned and analyzed for their expression. Enzyme activities of β-ketothiolase and acetoacetyl-CoA reductase showed constitutive levels during aerobic and photoheterotrophic growth of *R. sphaeroides*. In addition, no difference of each enzyme activity was observed between cells grown aerobically and photoheterotrophically. The constitutive level of the enzyme activities are regulated at the level of *phbAB* transcription. Cellular PHB content, however, was reported as being regulated according to the growth phases along with growth conditions [15]. Thus, *phbAB* expression is not determinative in regulating the PHB content. On the other hand, *phbA*-deleted cell AZ1 accumulated only 10% PHB of the wild-type, and an elevated dosage of *phbAB in trans* in *R. sphaeroides* resulted in a higher content of PHB, indicating that *phbAB* codes for the enzymes responsible for providing the main supply of substrate for PHB synthase. PHB formation by an alternative pathway that does not depend on the *phbA*- and *phbB*-coding enzymes is also proposed.

Key words: *Rhodobacter sphaeroides*, PHB, β-ketothiolase, acetoacetyl-CoA reductase

Polyhydroxyalkanoic acids (PHA) are polyesters of various 3-, 4-, and 5-hydroxyalkanoic acids of C_3-to-C_{14} monomer units. The polymers have received wide attention because they have properties of being biodegradable plastic [1, 2, 3, 8, 17]. Poly-3-hydroxybutyric acid (PHB), a polyester of D-3-hydroxybutyric acid, is produced by a number of bacteria as intracelluar carbon- and energy-storage materials [1, 2, 3, 11, 19, 27, 28]. Genes coding for PHB biosynthetic enzymes have been isolated and analyzed at a molecular level from various bacteria. *Ralstonia eutropha* accumulates PHB by the sequential action of three enzymes, β-ketothiolase (*phbA*), acetoacetyl-CoA reductase (*phbB*), and PHB synthase (*phbC*). The three genes of *R. eutropha* are organized in a single operon as *phbCAB* [27, 28].

Rhodobacter sphaeroides phbC is not linked to the *phbAB* genes [11, 12, 15], and its expression was analyzed to localize the promoter DNA [15]. The cellular PHB content of aerobically grown *R. sphaeroides* is regulated at a level of *phbC* transcription, while the photoheterotrophic control of PHB content is not mediated by PHB synthase activity. Thus, it remains to be determined whether *phbAB* expression is possibly involved in regulating the PHB contents of photoheterotrophically grown cells. In a related bacterium, *Rhodobacter capsulatus*, *phbAB* genes were cloned and the *phbA* translation employing the *lacZ* fusion was shown to be constitutive, irrespective of the carbon sources which were included for cell growth [16].

In this work, we have cloned and analyzed the *phbAB* expression of *R. sphaeroides* in an attempt to understand the cellular physiology regulating the PHB content of *R. sphaeroides*. In particular, *phbAB* transcription and cellular activities of the coding enzymes were examined under photoheterotrophic conditions, to examine any difference from the aerobically grown cell. As a result, the activities of β-ketothiolase and acetoacetyl-CoA reductase were found to be constitutive, regardless of growth phase and growth conditions. In addition, the two enzyme activities were regulated at the level of *phbAB* transcription. Thus, *phbAB* expression is not directly associated with the PHB level, which is regulated according to the growth phases as well as growth conditions [15]. However, *phbA*- and *phbB*-coding enzymes are responsible for providing a main supply of substrate for PHB synthase.

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Table 1. Bacterial strains and plasmids.

Strains or plasmids	Relevant characteristic(s)	Source or reference
E. coli		
DH5α*phe*	F*Φ*80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*($r_K^-m_K^+$) *supE44* λ^- *thi-1 gyrA relA1 phe* ::Tn*10dCm*	9
HB101	*supE44 hisdS20*($r_B^-m_B^-$) *recA13 ara-I4 proA2 lacY1 galk2 rpsL20 xyl-5 mtl-1*	10
R. sphaeroides		
2.4.1	Wild-type	W. Sistrom
AZ1	2.4.1 derived, *phbA*-interrupted and transcriptionally fused to *lacZY'*::Ω Sm/Sp^r DNA; Sm/Sp^r	This study
Plasmids		
pRK415	Tc^r	14
pSUP202	pBR325 derivative; Mob$^+$, Ap^r, Cm^r, Tc^r	30
pRuA215	pUC19 derivative with an insert of 4.4-kb *Pst*I DNA containing *phbAB*; Ap^r; +[a]	This study
pUC909	pUC19 derivative with an insert of 2.3-kb *Sal*I-*Xho*I DNA containing *phbC*; Ap^r; +	This study
pRK221	pRK415 derivative with an insert of 2.7-kb *Bam*HI-*Pst*I DNA containing *phbAB*; Tc^r; +	This study
pSuA401	pSUP202 derivative with an insert of 6.0-kb *Pst*I DNA containing transcriptional fusion of *phbA*::*lacZY'*::Ω Sm/Sp^r at the *Eco*RI site of *phbA*; Cm^r, Tc^r, Sm/Sp^r	This study

[a]+ denotes gene orientation in the same direction as the *lac* promoter and/or *tet* promoter of vector DNA.

MATERIALS AND METHODS

Bacteria, Plasmids, and Cell Growth

The bacterial strains and plasmids used in this study are described in Table 1. *R. sphaeroides* 2.4.1 was used as a wild-type strain and cultured at 28°C in the Sistrom's minimal medium [22]. *R. sphaeroides* was grown aerobically or photoheterotrophically as described previously. Light intensity for photoheterotrophic growth was 10 Watts (W)/m^2. Exponential phase was designated as growth between 65 and 85 Klett units (KU) under aerobic conditions, and between 80 and 150 KU under photoheterotrophic conditions. Early stationary phase corresponded to 165–180 and 260–280 KU during aerobic and photoheterotrophic growth, respectively. One KU of *R. sphaeroides* amounted to approximately 10^7 cells per ml of culture. *Escherichia coli* strains were grown at 37°C in a Luria medium. Ampicillin (Ap), tetracycline (Tc), streptomycin (Sm), and spectinomycin (Sp) (final concentrations of 50, 10, 50, and 50 μg/ml, respectively) were added to the growth medium for *E. coli* carrying these drug-resistant genes. The same concentrations of the antibiotics were used for *R. sphaeroides* culture except for Tc at 1 μg/ml.

Conjugation Techniques

pRK415- and pSUP202-derived plasmids were mobilized into *R. sphaeroides* using the procedure previously described [5, 14]. The pSUP202 derivative was used for construction of the *phbA*-deleted mutant strain, *R. sphaeroides* AZ1. Even-numbered crossovers were isolated as previously described [7, 30].

DNA Manipulation and Southern Hybridization

DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes in accordance with the manufacturer's specifications. Southern blots of *R. sphaeroides* chromosomal DNA using a Hybond-N membrane (Amersham, U.K.) were performed as previously described [4]. Probes were prepared by using a Fluorescein Gene Images labeling and detection kit (Amersham, U.K.). Hybridization with the fluorescein-labeled probes and washing of the membranes were carried out according to the instructions included within the kit.

β-Galactosidase Assay

β-Galactosidase assays with hydrolysis of *o*-nitrophenyl-β-galactoside were performed as described previously [20].

PHB Determination

The determination of PHB was performed by using a spectrophotometric assay of the Law and Slepecky method [18].

Assays of β-Ketothiolase and Acetoacetyl-CoA Reductase

β-Ketothiolase activity was assayed according to the method of Nishimura *et al.* [24]. Cell extracts were prepared from cells grown aerobically or photoheterotrophically and harvested by centrifugation for 10 min at 10,000 ×g, followed by washing and resuspension in a Tris-HCl buffer (10 mM, pH 8.0). Cells were broken at a pressure of 10,000 psi by using a French® press (SLM, IL, U.S.A.). The unbroken cells and cell debris were removed after centrifugation at

10,000 ×g for 15 min. Reaction mixture in a final volume of 2 ml contained 0.1 mM acetoacetyl-CoA, 0.057 mM CoASH, 0.5 mM DTT, 40 mM $MgCl_2$, and cell extracts in the Tris-HCl buffer. The assay temperature was 30°C and the reaction was initiated by adding cell extracts. A decrease of the acetoacetyl-CoA-Mg complex at 303 nm (ε=17.26 mM^{-1} cm^{-1}) was measured with a spectrophotometer (model UV-2401PC, Shimadzu, Japan). One unit of enzyme activity was defined as the amount of enzyme, which catalyzed the decrease of 1 μmole acetoacetyl-CoA-Mg complex per min.

Acetoacetyl-CoA reductase activity was assayed according to the method of Ploux *et al.* [25]. Reaction mixture in a final volume of 2 ml contained 0.1 mM acetoacetyl-CoA, 0.125 mM NADPH, and cell extracts in the 100 mM Tris-HCl buffer (pH 8.0). The reaction was initiated by adding the cell extracts and the absorbance at 340 nm was monitored at 30°C. One unit is defined as the amount of enzyme that catalyzes the transformation of 1 nmol NADPH per min.

All analyses in this work were performed in duplicate at least twice, and the data presented are the average of the values obtained within standard deviations of 10–15%.

RESULTS

Cloning of *phbA* and *phbB* of *R. sphaeroides*

β-Ketothiolase and acetoacetyl-CoA reductase mediate the first two sequential enzymic steps to produce D-3-hydroxybutyryl-CoA, a substrate for polymerization by PHB synthase forming PHB [15]. Since the expression of β-ketothiolase and acetoacetyl-CoA reductase of *Rhodobacter* have not been analyzed in terms of regulatory association with cellular contents of PHB, the expression of *phbA* and *phbB* were examined throughout the growth phases under aerobic and photoheterotrophic conditions.

As an approach to clone the *phbA* gene of *R. sphaeroides*, its genomic DNA was digested with *Pst*I and probed with 995-bp *Stu*I DNA containing *R. eutropha phbA'*, which was derived from pTZ18U-PHB [27]. The genomic Southern hybridization analysis under less stringent conditions revealed a signal of 4.4-kb *Pst*I DNA of *R. sphaeroides*. The DNA, pRuA215 (Table 1), was obtained through colony hybridization after cloning of the *Pst*I DNA in pUC19. The 4.4-kb *Pst*I DNA containing *R. sphaeroides phbA* was also hybridized with a 941-bp *Dde*I DNA containing the *R. eutropha phbB* gene (data not shown), in which it was indicative of the genetic linkage of the *phbA* and *phbB* genes of *R. sphaeroides* on the 4.4-kb *Pst*I DNA.

The 4.4-kb *Pst*I DNA fragment of pRuA215 was completely sequenced on both strands with numerous subclones and several synthetic oligonucleotides. Two adjacent open reading frames with properly positioned ribosome-binding sequences were identified, which were regarded to be *phbA* and *phbB*, respectively, based on sequence homology. During the progress of this study, the whole genome of *R. sphaeroides* has been sequenced by DOE Joint Genomic Institute. The nucleotide sequence of *phbA* and *phbB* that we obtained were the same as those obtained from the *R. sphaeroides* genome project site at http://www-mmg.med.uth.tmc.edu/sphaeroids/. The *phbB* start codon is located 99 bp downstream from the *phbA* stop codon. The *R. sphaeroides phbA* is 1,185 bp and the deduced amino acid sequence of the gene showed a significant similarity to the β-ketothiolase of *Paracoccus denitrificans* (74% identity), *Rhizobium meliloti* (69%), and *R. eutropha* (61%). Similarly, the primary structure of the deduced amino acid sequence of *phbB* (723 bp) showed a high similarity to the primary structures of acetoacetyl-CoA reductase of *P. denitrificans* (85% identity), *Rhizobium meliloti* (80%), and *R. eutropha* (50%).

Heterologous Expression of *R. sphaeroides phbA* and *phbB* in *E. coli*

It was determined whether or not *phbA* and *phbB* of *R. sphaeroides* were functionally able to provide substrate for PHB synthase by examining heterologous expression in recombinant *E. coli* that carried *R. sphaeroides phbC*. The *phbC* was cloned in pUC19 to generate pUC909 (Table 1), while the *phbA* and *phbB* were maintained in *E. coli* by using a pRK415 clone, pRK221. As shown in Table 2, *E. coli* containing both pUC909 and pRK221 produced a significant amount of PHB. Thus, *R. sphaeroides phbA* and *phbB* form D-3-hydroxybutyryl-CoA, a substrate for PHB synthase.

Construction of *R. sphaeroides* AZ1 Containing *phbA-lacZ* on its Chromosome and Transcriptional Expression of *phbA*

A mutant strain, *R. sphaeroides* AZ1, having *phbA* interruption as well as *phbA::lacZY'* transcriptional fusion structure on its chromosome, was obtained. The mutant strain was constructed through a homologous recombination by using a suicide plasmid, pSuA401, which has *phbA* that was transcriptionally fused to *lacZY'* at the *Eco*RI site in the middle of the gene. The Ω Sm/Sp^r transcription-translation stop DNA was cloned at the *lacY'* DNA as a selection marker. Exconjugants, resistant only to Sm/Sp, were regarded

Table 2. Expression of *R. sphaeroides phbAB* in *E. coli*.

Plasmid (gene) in *E. coli*	PHB content[a]
pUC909 (*phbC*)+pRK415 (vector control)	0
pUC909 (*phbC*)+pRK221 (*phbAB*)	110.1±27.1

[a]μg/mg of dry weight of cells.

Cells were grown aerobically in LB containing 2% glucose, and harvested at A_{600} between 1.5 and 1.8. Standard deviations are shown following ±.

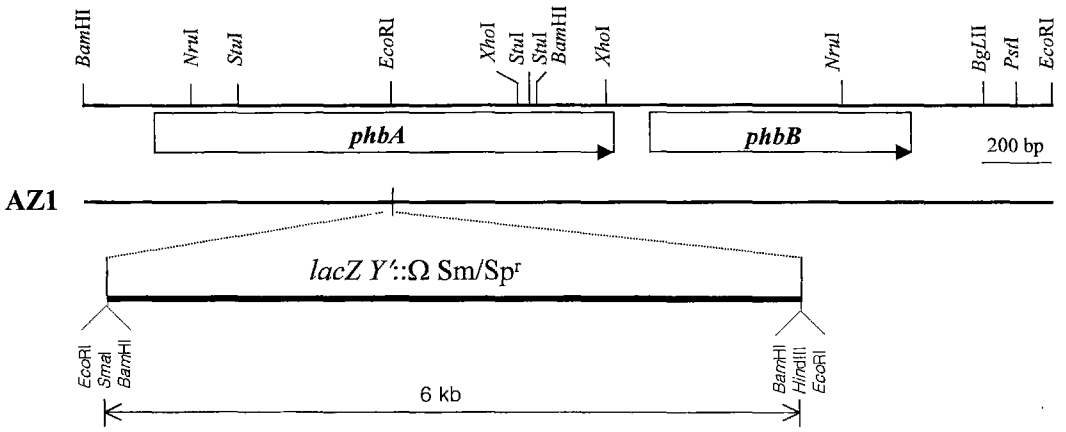

Fig. 1. Restriction map of DNA region containing *phbAB* and chromosomal structure of *phbA-lacZ of R. sphaeroides* AZ1.
Open reading frame of *phbA* and *phbB* comprised of 395 and 240 amino acids, respectively, are shown above the restriction map. A 6.0-kb *lacZY'*::Ω Sm/Sp^r DNA was inserted at the *Eco*RI site of *phbA* through homologous recombination.

as recombinants arising by double crossovers between the donor, pSuA401, and a recipient DNA. Two exconjugants showing Sm/Sp^r and Tc^s were obtained out of approximately 110 recombinants of Sm/Sp^r and Tc^r, which were regarded as recombinants after a single crossover. The two Tc^s exconjugants were confirmed for a correct replacement of genomic DNA by the insert DNA of pSuA401 after Southern hybridization analysis (data not shown). One strain, AZ1, was chosen for further analysis (Fig. 1).

The transcriptional activity of *phbA* was measured under aerobic and photoheterotrophic conditions. As shown in Fig. 2A, the transcriptional activities of *phbA-lacZ* of AZ1 were between 600 and 700 Miller units during the aerobic growth, while the photoheterotrophically grown cell showed a level within the range from 660 to 850 units. Accordingly, the transcriptional expression of *phbA* was constitutive throughout the growth phase that was examined under both aerobic and photoheterotrophic conditions.

To measure the transcriptional expression of *phbB*, a plasmid containing the transcriptional fusion structure of *phbAB-lacZ* with a 314-bp *phbA* upstream DNA was constructed and maintained *in trans* in the wild-type. The β-galactosidase activities of the *phbAB-lacZ* showed Miller units between approximately 1,200 and 1,400 throughout the growth phase, under both aerobic and photoheterotrophic conditions (data not shown). Thus, *phbB* is constitutively transcribed as *phbA*, suggesting the organization of the two genes as in an operon of *phbAB*.

Assays of β-Ketothiolase and Acetoacetyl-CoA Reductase

The enzyme activities of β-ketothiolase and acetoacetyl-CoA reductase were examined during the cell growth under aerobic and photoheterotrophic conditions. As shown in Table 3, the specific activity of β-ketothiolase in an exponentially growing cell was 0.963 under aerobic conditions. This activity decreased slightly during the growth transition to the early stationary phase. The β-

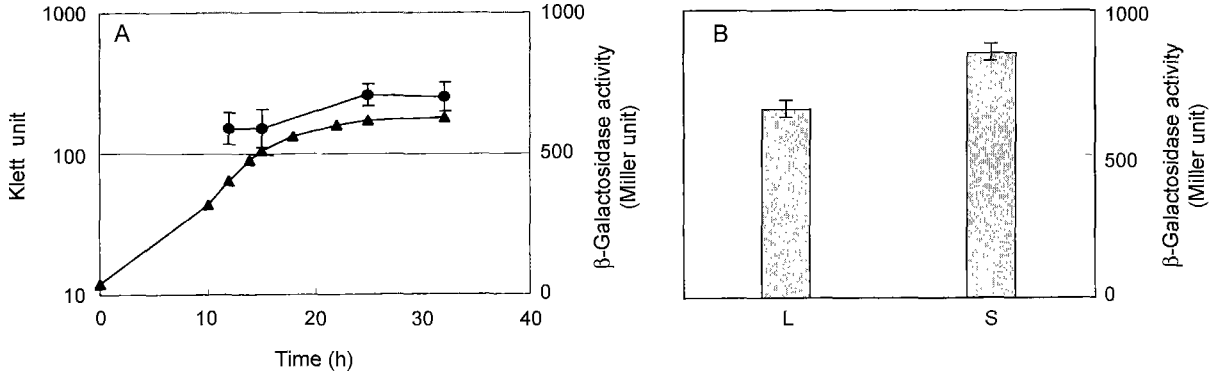

Fig. 2. Transcriptional activities of *phbA* of *R. sphaeroides* AZ1.
Cells were grown aerobically (A) and photoheterotrophically (B) at 10 W/m^2. L, logarithmic growth phase; S, early stationary growth phase. The bars on both the closed circle (A) and the histograms (B) denote the standard deviations.

Table 3. β-Ketothiolase activity of *R. sphaeroides*.

Growth phase	β-Ketothiolase activity[a]	
	O_2	PS
L	0.963±0.2	0.773±0.1
S	0.617±0.2	0.841±0.2

[a]U (μmole/min)/ mg of protein.

L, logarithmic growth phase; S, early stationary growth phase. Cells were grown aerobically (O_2) and photoheterotrophically (PS) at 10 W/m^2. Standard deviations are shown following ±.

ketothiolase activities of photoheterotrophically grown cell were similar to those activities which were measured with the cells grown under aerobic conditions. Thus, the β-ketothiolase activities were constitutive throughout the growth phase, irrespective of the culture conditions. The same is true for the expression of acetoacetyl-CoA reductase (Table 4). Since the *phbAB* transcription also occurred constitutively regardless of the growth phase or growth conditions examined, we conclude that the expression of two enzymes are regulated at the level of *phbAB* transcription.

Previously, it was shown that the PHB content of *R. sphaeroides* increased four- to five-fold during the growth transition from exponential to early stationary phase under aerobic and photoheterotrophic conditions. In addition, the PHB content of the photoheterotrophic cell was about two times as much as that of the aerobically grown cell, when comparison was made with the cells at the corresponding growth phases. Accordingly, the results shown in this work clearly demonstrate that neither β-ketothiolase nor acetoacetyl-CoA reductase controls the PHB content of *R. sphaeroides* under the conditions examined.

PHB Content of *R. sphaeroides* AZ1

Although the enzymic reaction mediated by either β-ketothiolase or acetoacetyl-CoA reductase is not a regulatory step which controls the cellular content of PHB, the role of the enzymes in PHB formation was examined with *R. sphaeroides* AZ1. Firstly, β-ketothiolase activity was measured with AZ1 and compared with that of the wild-type. As shown in Table 5, AZ1 exhibited about 12% β-ketothiolase activity of the wild-type. Acetoacetyl-CoA reductase activity of AZ1 was also reduced to the similar extent (data not shown). As expected, AZ1 showed 10% PHB of the wild-type (Table 6). The results strongly indicate that there is an alternative route(s) for the synthesis of D-3-hydroxybutyryl-CoA, a substrate for PHB synthase. However, it is still valid that *phbA* and *phbB* are coding for the enzymes mediating the main reactions for supplying the substrate for PHB synthase. In addition, elevated gene dosage of *phbAB in trans* in AZ1 increased β-ketothiolase activity and PHB content approximately thirty- to sixty-fold when compared with those of the parental strain, AZ1 (Tables 5 and 6). Thus, the elevated flux of D-3-hydroybutyryl-CoA through the main route resulted in a higher content of PHB.

Table 4. Acetoacetyl-CoA reductase activity of *R. sphaeroides*.

Growth phase	Acetoacetyl-CoA reductase activity[a]	
	O_2	PS
L	0.0936±0.03	0.071±0.016
S	0.0856±0.02	0.090±0.025

[a]U (nmole/min)/ mg of protein.

L, logarithmic growth phase; S, early stationary growth phase. Cells were grown aerobically (O_2) and photoheterotrophically (PS) at 10 W/m^2. Standard deviations are shown following ±.

Table 5. β-Ketothiolase activity of *R. sphaeroides* AZ1.

Strains	β-Ketothiolase activity[a]
Wild-type (pRK415)	0.628±0.094
AZ1 (pRK415)	0.078±0.008
AZ1 (pRK221)	2.512±0.377

[a]U (μmole/min)/mg of protein.

Cells were grown aerobically and harvested at an early stationary phase. Standard deviations are shown following ±.

DISCUSSION

In the previous work, it was shown that the expression of PHB synthase of *R. sphaeroides* is regulated at the level of *phbC* transcription throughout the growth phase, under both aerobic and photoheterotrophic conditions. In addition, it was found that PHB content of the aerobically grown *R. sphaeroides* is regulated at the level of *phbC* transcription, while the photoheterotrophic content of PHB is not controlled by the PHB synthase activity. Thus, it remained to be determined whether *phbAB* expression is possibly involved in regulating the PHB contents of photoheterotrophically grown cells.

In this work, we have cloned *phbAB* and analyzed the transcription of *phbA* and *phbB* by using *lacZ* fusion structures. Both *phbA-lacZ* and *phbAB-lacZ* fusions showed the same patterns of constitutive expressions. Therefore, the *phbA* and *phbB* were regarded as an operon

Table 6. PHB content of *R. sphaeroides* AZ1.

Strains	PHB content[a]
Wild-type (pRK415)	32.2±4.1
AZ1 (pRK415)	3.3±0.8
AZ1 (pRK221)	192.0±16.4

[a]μg/mg of dry weight.

Cells were grown aerobically and harvested at an early stationary phase. Data are shown as ± standard deviations.

that was organized as in *phbAB*, although it remained to be determined whether *phbB* has its own internal promoter(s) or not.

Activities of β-ketothiolase and acetoacetyl-CoA reductase were examined under photoheterotrophic conditions to examine any difference from the aerobically grown cells. The result suggested that both activities were constitutive regardless of growth phase and growth conditions. Comparing the patterns of *phbAB* transcription with those of the enzyme activities, we concluded that the expression of both enzymes was regulated at the level of transcription. Previously, it was shown that the PHB content of *R. sphaeroides* increased four- to five-fold during the growth transition from exponential to early stationary phase under both aerobic and photoheterotrophic conditions. In addition, the PHB content of the photoheterotrophic cell was about two times as much as that of the aerobically grown cell when compared with each other at the corresponding growth phases. Thus, the *phbAB* expression was not directly associated with the PHB level. However, it should be emphasized that the enzymic reactions of both β-ketothiolase and acetoacetyl-CoA reductase provided a main flux of the substrate supply for PHB synthase, because the *phbA*-deleted cell, AZ1, accumulated only 10% PHB of the wild-type. In addition, an elevated dosage of *phbAB in trans* in *R. sphaeroides* significantly increased the PHB content.

It is interesting to recognize that AZ1 still showed β-ketothiolase and acetoacetyl-CoA reductase activities, which amount to approximately 10% of the wild-type. These results indicate that *R. sphaeroides* has an alternative metabolic pathway(s) for D-3-hydroybutyryl-CoA formation that does not depend on the *phbA*- and *phbB*-coding enzymes. The 3-hydroybutyryl-CoA should be in D configuration to be a substrate for PHB synthase. *Rhodospirillum rubrum* has been proposed to have alternative routes for D-3-hydroybutyryl-CoA formation from L-3-hydroybutyryl-CoA, which is a normal hydroxyacyl intermediate synthesized during the fatty acid degradation [23]. However, the gene(s) responsible for the conversion has not been identified in *R. rubrum*. Recently, isozymes (Bkt) of β-ketothiolase have been identified in *R. eutropha* and *Azotobacter vinelandii* [31]. These isozymes are not associated with fatty acid degradation and they have been shown to be involved in the formation of C_5- or C_6-β-ketoacyl-CoA, which is used to form PHA copolymers such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate). Therefore, *R. sphaeroides* AZ1 generated in this work will provide a genetic background that will be useful for the elucidation of the PhbA isozyme(s) along with finding any possible metabolic link between the PHB formation and fatty acid degradation.

In conclusion, the results presented above suggest that *phbAB* transcription determines the expression of β-ketothiolase and acetoacetyl-CoA reductase. However, the enzyme activities are not directly associated with the PHB level, although the *phbAB* are coding for the enzymes responsible for the main supply of D-3-hydroxybutyryl-CoA, a substrate for PHB synthase.

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REFERENCES

1. Anderson, J. A. and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.* **54:** 450–472.
2. Choi, J. I. and S. Y. Lee. 1999. Production of poly(3-hydroxybutyrate) [P(3HB)] with high P(3HB) content by recombinant *Escherichia coli* harboring the *Alcaligenes latus* P(3HB) biosynthesis genes and the *E. coli ftsZ* gene. *J. Microbiol. Biotechnol.* **9:** 722–725.
3. Chung, C. W., Y. S. Kim, Y. B. Kim, K. S. Bae, and Y. H. Rhee. 1999. Isolation of a *Pseudomonas* sp. strain exhibiting unusual behavior of poly(3-hydroxybutyrates) biosynthesis and characterization of synthesized polyesters. *J. Microbiol. Biotechnol.* **9:** 847–853.
4. DeHoff, B. S., J. K. Lee, T. J. Donohue, R. I. Gumport, and S. Kaplan. 1988. *In vivo* analysis of *puf* operon expression in *Rhodobacter sphaeroides* after deletion of a putative intercistronic transcription terminator. *J. Bacteriol.* **170:** 4681–4692.
5. Davis, T., T. J. Donohue, and S. Kaplan. 1988. Construction, characterization, and complementation of a *puf* mutant of *Rhodobacter sphaeroides. J. Bacterol.* **170:** 320–329.
6. Donohue, T. J., A. G. McEwan, and S. Kaplan. 1986. Cloning, DNA sequence, and expression of the *Rhodobacter sphaeroides* cytochrome c_2 gene. *J. Bacteriol.* **168:** 962–972.
7. Donohue, T. J., A. G. McEwan, and S. Kaplan. 1988. Phenotypic and genetic characterization of cytochrome C_2-deficient mutant of *Rhodobacter sphaeroides. Biochemistry* **27:** 1918–1925.
8. Doi, Y., A. Tamaki, M. Kunioka, and K. Sopa. 1987. Biosynthesis of terpolyesters of 3-hydroxybutyrate, and 5-hydroxyvalerate in *Acaligenes eutrophus* from 5-chloropentanoic and pentanoic acids. *Makromol. Chem. Rapid Commum.* **8:** 631–635.
9. Eraso, J. M. and S. Kaplan. 1994. *prrA*, a putative response regulator involved in oxygen regulation of photosynthesis gene expression in *Rhodobacter sphaeroides. J. Bacteriol.* **176:** 32–43.
10. Figurski, D. H. and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans. Proc. Natl. Acad. Sci. USA* **76:** 1648–1652.

11. Hustede, E., A. Steinbüchel, and H. G. Schlegel. 1992. Cloning of poly(3-hydroxybutyric acid) synthase genes of *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* and heterologous expression in *Alcaligenes eutrophus*. *FEMS Microbiol. Lett.* **93:** 285–290.
12. Hustede, E. and A. Steinbüchel. 1993. Characterization of the polyhydroxyalkanoate synthase gene locus of *Rhodobacter sphaeroides*. *Biotechnol. Lett.* **15:** 709–714.
13. Hustede, E., A. Steinbüchel, and H. G. Schlegel. 1992. Relationship between the photoproduction of hydrogen and the accumulation of PHB in non-sulphur purple bacteria. *Appl. Microbol. Biothechnol.* **39:** 87–93.
14. Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger. 1988. Improved broad-host range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70:** 191–197.
15. Kho, D. H., J. M. Yang, K. S. Kim, and J. K. Lee. 2001. Expression analysis of *phbC* coding for poly-3-hydroxybutyrate (PHB) synthase of *Rhodobacter sphaeroides*. *J. Microbiol. Biotechnol.* **11:** 310–316.
16. Kranz, R. G., K. K. Gabbert, T. A. Locke, and M. T. Madigan. 1997. Polyhydroxyalkanoate production in *Rhodobacter capsulatus*; genes, mutants, expression, and physiology. *Appl. Environ. Microbiol.* **63:** 3003–3009.
17. Kunioka, M., Y. Nakamura, and Y. Doi. 1988. New bacterial polyesters produced in *Alcaligenes eutrophus* from organic acids. *Polymer Commun.* **29:** 174–176.
18. Law, J. H. and R. A. Slepecky. 1961. Assay of poly-β-hydroxybutyric acid. *J. Bacteriol.* **82:** 33–36.
19. Lee, J. K. and S. Kaplan. 1996. Molecular genetics of purple bacteria, pp. 225–246. *In* B. Anderson, A. H. Salter, and J. Barber (eds.), *Molecular Genetics of Photosynthesis; Frontiers in Molecular Biology.* Oxford University Press, Oxford, U.K.
20. Lee, J. K. and S. Kaplan. 1992. Isolation and characterization of trans-acting mutation involved in oxygen regulation of *puc* operon transcription in *Rhodobacter sphaeroides*. *J. Bacteriol.* **174:** 1158–1171.
21. Liebergesell, M., E. Hustede, A. Timm, A. Steinbüchel, R. C. Fuller, and R. W. Lenz. 1991. Formation of poly(3-hydroxyalkanoates) by phototrophic and chemolithotrophic bacteria. *Arch Microbiol.* **155:** 415–421.
22. Lueking, D. R., R. T. Fralely, and S. Kaplan. 1978. Intracytoplasmic membrane synthesis in synchronous cell populations of *Rhodobacter sphaeroides*. *J. Biol. Chem.* **253:** 451–457.
23. Moskowitz, G. J. and J. M. Merrick. 1969. Metabolism of poly-β-hydroxybutyrate. II. Enzymatic synthesis of D-(-)-β-hydroxybutyryl coenzyme A by an enoyl hydratase from *Rhodospirillum rubrum*. *Biochemisty* **3:** 2748–2755.
24. Nishimura, T., T. Saito, and K. Tomita. 1978. Purification and properties of β-ketothiolase from *Zoogleoa ramigera*. *Arch. Microbiol.* **116:** 21–27.
25. Ploux, O., S. Masamune, and C. T. Wasish. 1988. The NADPH-linked acetoacetyl-CoA reductase from *Zoogleoa ramigera*: Characterization and mechanistic studies of cloned enzyme over-produced in *Escherichia coli*. *Eur. J. Biochem.* **174:** 177–182.
26. Prentki, P. and H. M. Krisch. 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29:** 303–313.
27. Peoples, O. P. and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase (*phbC*) *J. Biol. Chem.* **264:** 15298–15303.
28. Peoples, O. P. and A. J. Sinskey. 1990. Poly-β-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. *J. Biol. Chem.* **264:** 15293–15297.
29. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.
30. Simon, R., V. Priefer, and A. Puhler. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Bio/Technology* **1:** 784–791.
31. Steven, S., K. Houmiel, T. Minhtien, T. A. Mitsky, N. B. Taylor, S. R. Padgette, and K. J. Gruys. 1998. Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in *Rastonia eutropha*. *J. Bacteriol.* **180:** 1979–1987.