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Expression Analysis of *phbC* Coding for Poly-3-hydroxybutyrate (PHB) Synthase of *Rhodobacter sphaeroides*

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Abstract Poly-3-hydroxybutyrate (PHB) synthase catalyzes the last enzymic step to synthesize the intracellular PHB of *Rhodobacter sphaeroides*. No PHB was detected when the *phbC* coding for PHB synthase was interrupted, and its expression was regulated at the level of transcription. The cellular PHB content increased about four- to six-fold during the growth transition from the exponential to the early stationary phase under both aerobic and photoheterotrophic conditions. The PHB content during the aerobic growth seemed to be determined by the PHB synthase activity. However, the PHB synthase activity of photoheterotrophically grown cells did not correlate with the PHB content, suggesting a photoheterotrophic regulation different from the aerobic control. Thus, the PHB content of *R. sphaeroides* was regulated at the transcription level only under aerobic conditions.

Key words: *Rhodobacter sphaeroides*, PHB, PHB synthase, *phbC*

Polyhydroxyalkanoic acids (PHA) are polyesters of various 3-, 4-, and 5-hydroxyalkanoic acids, and the thermoplastic properties of some PHAs have attracted industrial interest as a biodegradable plastic [1, 2, 3, 8, 19, 31]. Poly-3-hydroxybutyric acid (PHB) is a PHA [1]. The PHB accumulates intracellularly in *Rhodobacter sphaeroides*, a purple nonsulfur photosynthetic bacterium, by sequential action of three enzymes, β-ketothiolase (*phbA*), acetoacetyl-CoA reductase (*phbB*), and PHB synthase (*phbC*), as observed in *Ralstonia eutropha* [11, 12, 13, 24, 29, 30]. The *phbA* and *phbB* of a related bacterium, *Rhodobacter capsulatus*, form an operon and the operon expression seems to be constitutive regardless of whether the PHB production is induced by a carbon source in the culture medium [18].

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The *phbC* of *R. sphaeroides* has been cloned [11, 17] and its expression was analyzed to localize the promoter DNA of the gene [17]. Although *R. sphaeroides* and *R. capsulatus* have been studied extensively for photosynthesis [6, 9, 15, 16, 21], there have been very little investigation of its PHB metabolism.

An elevated dosage of the *phbC* gene *in trans* in *R. sphaeroides* results in a higher content of PHB, which indicates the importance of PHB synthase activity in determining the PHB level of *R. sphaeroides* [22]. The PHB content per *R. sphaeroides* cell reaches a maximal level during the early stationary phase of growth. Thus, it remains to be determined whether the PHB content during cell growth is regulated by PHB synthase activity. Accordingly, in this study, *phbC* expression and PHB synthase activity were analyzed in an attempt to understand the cellular physiology regulating the PHB content of *R. sphaeroides*. As a result, the PHB synthase activity was found to be regulated at the level of the *phbC* transcription under both aerobic and photoheterotrophic conditions. However, the PHB content appeared to be controlled by PHB synthase activity only during the aerobic growth of the bacterium. Although the photoheterotrophic activity of the PHB synthase was also regulated by the *phbC* transcription, the enzyme did not appear to be solely responsible for determining the PHB level under photosynthetic conditions.

Materials and Methods

Bacteria, Plasmids, and Cell Cultivation

The bacterial strains and plasmids used in this study are described in Table 1. *Rhodobacter sphaeroides* 2.4.1 was used as a wild-type strain and cultured at 28°C in Sistrom's minimal medium [25]. *R. sphaeroides* was grown aerobically with a culture volume of less than one tenth of the culture flask on a shaker with a rotation speed faster than 250 revolutions per minute. Photoheterotrophic conditions were

provided using a screw cap tube filled with culture broth to the top. The light intensity was 10 Watts/m^2. The 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. The early stationary phase corresponded to 165-180 and 260-280 KU during aerobic and photoheterotrophic growth, respectively. One KU of *R. sphaeroides* was approximately 10^7 cells per ml of culture. *Escherichia coli* strains were grown at 37°C in a Luria medium, and ampicillin, tetracycline, streptomycin, and spectinomycin (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.

DNA Manipulation

Plasmid DNA was prepared as previously described [32]. DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes in accordance with the manufacturer's specifications.

Conjugation Techniques

pRK415- and pSUP202-derived plasmids were mobilized into *R. sphaeroides* using the procedure described previously [5, 14]. The pSUP202 derivative was used for the construction of the *phbC*-deleted mutant strain, C2. The even-numbered crossovers were isolated, as previously described [7, 34].

Southern Hybridization

R. sphaeroides chromosomal DNA was digested to completion with appropriate restriction enzymes and electrophoresed on 0.8% agarose gels. Southern blots using a Hybond-N membrane (Amersham, U.K.) were performed as previously described [4]. Probes were prepared using a Fluorescein Gene Images labeling and detection kit (Amersham, U.K.). Hybridization with the fluorescein-labeled probes and the washing of the membranes were carried out according to the instructions included in the kit.

β-Galactosidase Assay

R. sphaeroides cultures used for measuring the β-galactosidase activities were grown aerobically or photoheterotrophically as described earlier. The β-galactosidase assays with *o*-nitrophenyl-β-galactoside hydrolysis were performed as described previously. All determinations were conducted in duplicate and repeated at least three times [23].

PHB Determination and PHB Synthase Assay

The amount of PHB was determined spectrophotometrically using the Law and Slepecky method [20], and PHB synthase activity was assayed according to the method of Miyake *et al.* [26]. Cell extracts were prepared from cells cultivated aerobically or photoheterotrophically and harvested by centrifugation for 10 min at 10,000 ×g, followed by washing and resupension in a Tris-HCl buffer (25 mM, pH 7.5). The cells were broken at a pressure of 10,000 psi using a French® press (SLM, IL, U.S.A.), and the unbroken cells and cell debris were removed after centrifugation at 200 ×g for 15 min. The reaction mixture in a final volume of 2 ml contained 0.05 mM DL-3-hydroxybutyryl-CoA, 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and cell extracts in the Tris-HCl buffer. The assay temperature was 30°C and the reaction was initiated by the addition of DL-3-hydroxybutyryl-CoA. The optical density at 412 nm (ε= 29.63 $mM^{-1}cm^{-1}$) of the thiobenzoate anion (TNB^-) resulting from the reaction of CoA and DTNB was measured with a spectrophotometer (model UV-2401PC, Shimadzu, Japan).

Table 1. Bacterial strains and plasmids.

Strains or plasmids	Relevant characteristic(s)	Source or reference
E. coli		
DH5α*phe*	FΦ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(rk^-mk^+) *supE44*λ^- *thi-1 gyrA relA1 phe* ::Tn*10dCm*	9
HB101	*supE44 hisdS20*(rB^-mB^-) *recA13 ara-I4 proA2 lacY1 galk2 rpsL20 Xyl-5 mtl-1*	10
R. sphaeroides		
2.4.1	Wild-type	W. Sistrom
C2	2.4.1 derived, *phbC* deleted, and replaced by km^r gene from Tn*903*	This study
Plasmids		
pRK415	Tc^r	14
pSUP202	pBR325 derivative; Mob^+, Ap^r, Cm^r, Tc^r	34
pRK505	pRK415 derivative containing 2.1-kb *Pst*I-*Xho*I DNA of *phbC*; Tc^r	This study
pSUP2-C2	2.9-kb *Pst*I DNA of *phbC* cloned at the *Pst*I site of pSUP202; 1.1-kb *Stu*I-*Bam*HI DNA internal to the gene was deleted and replaced by km^r gene	This study
pCF200	pLV106 derivative (IncQ), *phbC::lacZYA'*, transcriptional fusion, 992 bp *Sal*I-*Stu*I DNA of *phbC* fused; Sm/Sp^r	This study
pCLZ627	pLV106 derivative (IncQ), *phbC::lacZYA'*, translational fusion, 622 bp *Sal*I-*Puv*II DNA of *phbC* fused; Sm/Sp^r	This study

One unit of enzyme activity was defined as the amount of enzyme, which catalyzed the release of 1 nmol TNB^- per min [26]. All analyses were performed in duplicate at least twice, and the data presented are the averages of the values obtained within standard deviations of 10- 15%.

RESULTS

Construction of *phbC*-Deletion Mutant and Complementation of Mutation

In order to examine whether isozymes of PHB synthase were present in *R. sphaeroides*, a *phbC*-deleted mutant strain, *R. sphaeroides* C2, was constructed through homologous recombination using pSUP2-C2 (Table 1), derived from pSUP202 which is a suicide vector. For the construction of C2, the Tc^s and Km^r exconjugants were regarded as recombinants resulting from even-numbered crossovers between the donor and recipient DNA. The net result was an insert DNA on the plasmid pSUP2-C2 incorporated into the host chromosome, replacing the 1.1-kb *Stu*I-*Bam*HI restriction endonuclease fragment of *phbC* on the chromosome with the km^r gene [27] derived from Tn*903* (Fig. 1A). The other class of exconjugants was resistant to both kanamycin and teteracyclin and regarded as recombinants after odd-numbered crossovers between the donor and the recipient, thereby resulting in the entire plasmid pSUP2-C2 being inserted into the host chromosome. The odd-numbered crossovers were checked for their plasmid integration by genomic Southern hybridization (data not shown) and not analyzed any further. Three exconjugants, resistant only to kanamycin, were obtained out of about 60 recombinants. All three Tc^s and Km^r recombinants were PHB^-. One representative strain was chosen for a genomic Southern hybridization analysis.

The genomic Southern hybridization analysis of C2 demonstrated that the chromosomal segment corresponding to the 1.1-kb *Stu*I-*Bam*HI DNA fragment (Fig. 1A, probe *B1*) was missing (Fig. 1, panel B1, lanes 1 to 4) and replaced by the km^r gene through homologous recombination (panel B3, lanes 1 to 4). The restriction of C2 DNA with *Pst*I showed a 2.6-kb *Pst*I DNA fragment hybridized with

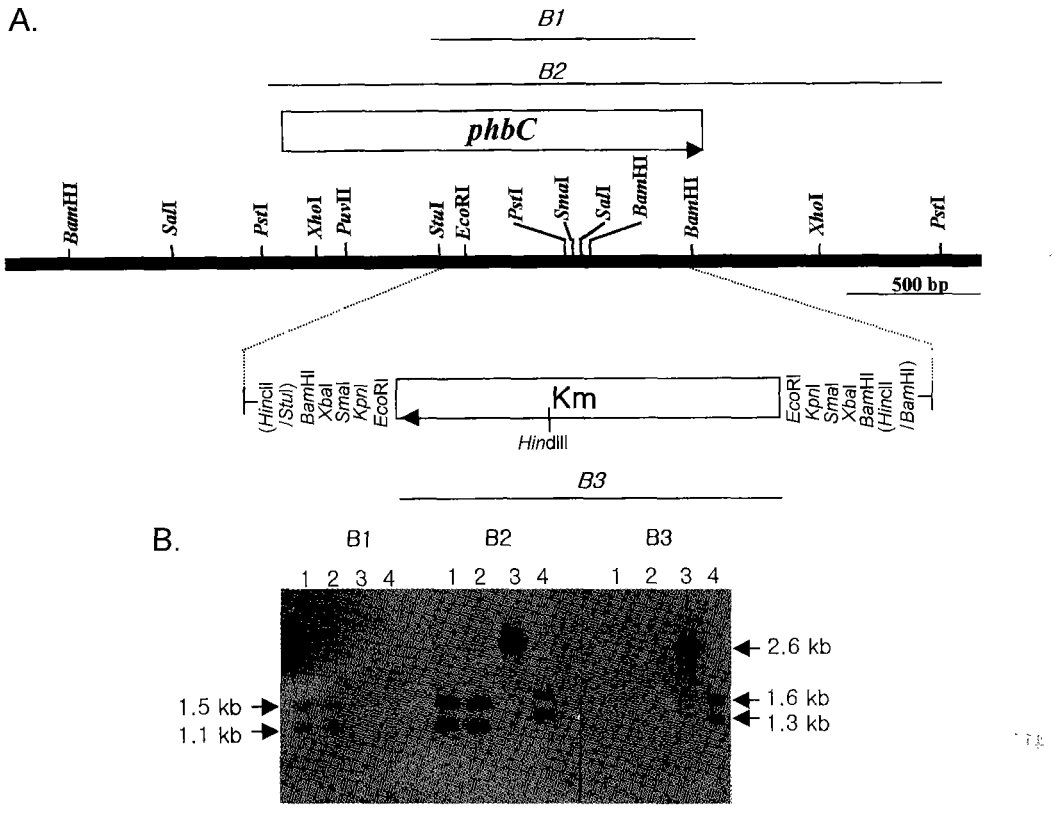

Fig. 1. Restriction map of DNA region containing *phbC* and chromosomal structure of *phbC*-deleted cell, C2, followed by genomic Southern hybridization analysis.

(A) An open reading frame of *phbC* comprised of 601 amino acids is shown above the restriction map. The 1.1-kb *Stu*I-*Bam*HI DNA internal to *phbC* was replaced through homologous recombination by 1.5-kb kanamycin-resistant DNA derived from Tn*903* [27]. *B1*, *B2*, and *B3* are the probes used in hybridization. The restriction sites in the parentheses were lost in cloning. (B) Southern hybridization analysis of wild-type and C2 chromosomes. Lanes 1 and 2 contain the DNA of the wild-type, while lanes 3 and 4 show the DNA from C2. *Pst*I restriction in lanes 1 and 3; *Hin*dIII restriction in lanes 2 and 4. The probes used for hybridization are shown above the blots.

Table 2. PHB content of *R. sphaeroides* C2 grown aerobically.

Strain	PHB content[a]
Wild-type	90.0±12
C2	ND[b]
C2 (pRK505)	168.8±20

[a]μg/mg of cell dry weight.
[b]ND, not detected.

the km^r gene (panel B3, lane 3). The 2.6-kb *Pst*I DNA was cleaved by *Hin*dIII into 1.6- and 1.3-kb *Pst*I-*Hin*dIII fragments because the *Hin*dIII site is located in the transcriptional direction of the km^r gene about 915 bp downstream from the *Hin*cII site, which was lost in cloning. The probing of the wild-type and C2 genomic DNA with 2.6-kb *Pst*I DNA (Fig. 1A, probe *B2*) harboring *phb*C revealed the hybridization patterns shown in panel B1 and panel B3, respectively (panel B2).

The PHB content was examined in both the wild-type and mutant strain C2 after aerobic growth. As shown in Table 2, no PHB was detected in C2. The same result was observed in C2 grown photoheterotrophically (data not shown), thus indicating no isozyme of PHB synthase in *R. sphaeroides*. The PHB$^-$ phenotype of C2 was complemented with pRK505, which was a pRK415 derivative containing 2.1-kb *Pst*I-*Xho*I DNA encompassing *phbC*. The 2.1-kb *Pst*I-*Xho*I DNA did not contain the *phbC* promoter (data not shown). However, *phb*C is located in the same transcriptional direction as the tetracyclin-resistant gene as well as the *lacZ'* gene of the plasmid. The higher level of PHB content with pRK505 appeared to result from the copy number of the plasmid. The PHB content of the wild type did not change regardless of whether pRK415 was present *in trans* or not (data not shown).

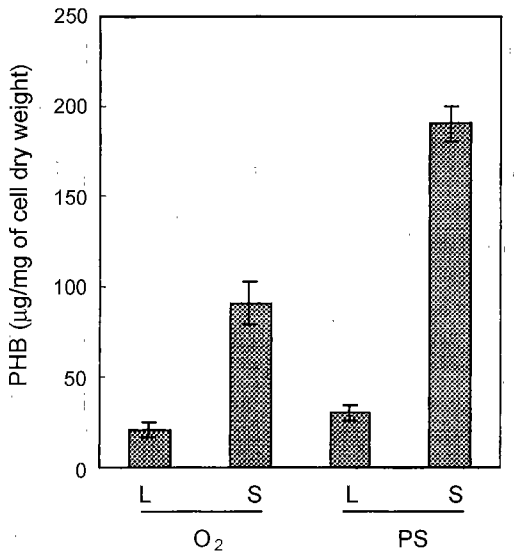

Fig. 2. PHB contents of *R. sphaeroides*.
The cells were grown aerobically (O_2) and photoheterotrophically (PS) at 10 W/m^2. L, logarithmic growth phase; S, early stationary growth phase. The bars on the histograms denote the standard deviations.

Determination of PHB Content of Cells

The PHB content of *R. sphaeroides* was determined during growth under aerobic and photoheterotrophic conditions. Interestingly, the PHB content increased more than four-fold during the growth transition from the exponential to the early stationary phase under both conditions (Fig. 2). The PHB content in the photoheterotrophic cell was approximately two-fold higher than that in the aerobically grown cell, when compared for corresponding growth phases. Thus, photosynthesis appeared to be a way to obtain a higher level of PHB content for *R. sphaeroides*.

Relation of PHB Content with PHB Synthase Activities

Since PHB synthase catalyzes the last enzymic step for PHB formation and its expression seems to be important in determining the level of PHB content, the enzyme activities were examined during the growth phases under aerobic and photoheterotrophic conditions. As shown in Table 3, the specific activity of PHB synthase in exponentially growing cell was 4.1 under aerobic conditions, the increase being approximately two-fold during the transition to the early stationary phase. Accordingly, the PHB content of the aerobically grown cell (Fig. 2) was found to be proportionally related with the PHB synthase activity. In contrast, however, the enzyme activities under photoheterotrophic conditions were approximately three- to six-fold lower than the corresponding ones observed with the aerobically grown cell (Table 3). Thus, PHB synthase was not the sole determining factor to regulate the PHB content under photoheterotrophic conditions. Although it remains to be determined how the photoheterotrophic level of the polymer was regulated, its degradation might be a way to control the PHB content, as observed in *Ralstonia eutropha* [33].

Analyses of Translational and Transcriptional Activities of *phbC*

Although the PHB synthase activity did not reflect the level of the PHB content under photoheterotrophic conditions, the regulation of the enzyme expression was examined. A transcriptional fusion of the *phbC-lacZ* of pCF200

Table 3. PHB synthase activities of *R. sphaeroides* 2.4.1 grown aerobically (O_2) and photoheterotrophically (PS) at 10 W/m^2.

PHB synthase activity[a]			
O_2		PS	
L	S	L	S
4.1	7.2	1.3	1.2

[a]U/mg of protein.
L, logarithmic growth phase; S, early stationary growth phase.

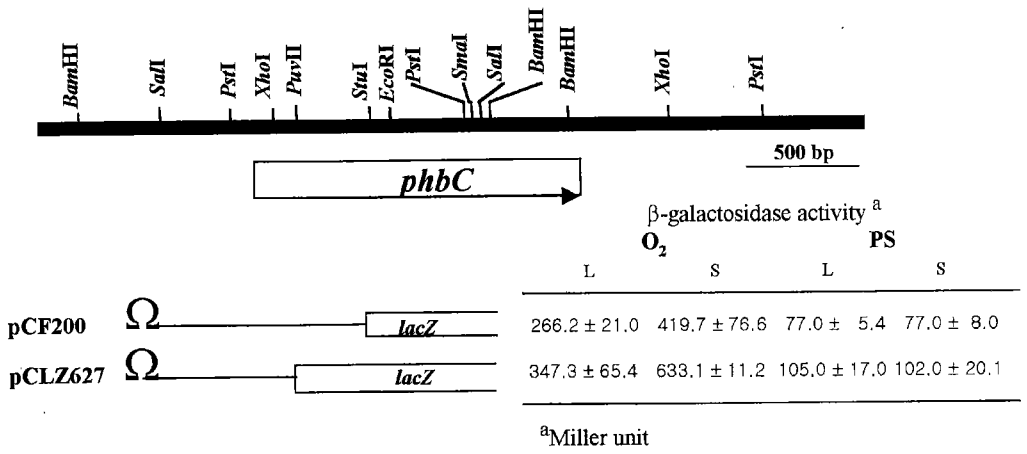

Fig. 3. Transcriptional and translational activities of *phbC*.
The plasmid pCF200 contains the transcriptional fusion of *phbC-lacZ*, while pCLZ627 harbors the translational fusion of the gene Ω is a streptomycin- and spectinomycin-resistant DNA flanked by transcriptional and translational stop signals [28]. The cells were grown aerobically (O_2) and photoheterotrophically (PS) at 10 W/m^2. L, logarithmic growth phase; S, early stationary growth phase. Standard deviations are shown following ±.

contained a *phbC* upstream DNA limited by a *Sal*I site, located 340 bp upstream from the ATG start codon of the *phbC*. Based on the results obtained with several *phbC-lacZ* fusion constructs, in which the upstream DNA of the *phbC* was serially 5' deleted (data not shown), the upstream DNA was found to be sufficient for the maximal transcription of the gene. The plasmid pCF200 was mobilized into the wild-type and maintained *in trans*. As shown in Fig. 3, the transcriptional activity of the *phbC-lacZ* increased about two-fold as the cell growth reached the early stationary phase under aerobic conditions. However, the photoheterotrophic activities of the *phbC* transcription were lower than the aerobic values and remained constitutive throughout the growth.

The insert of plasmid pCLZ627 exhibited the translational fusion construct of *phbC-lacZ*. The plasmid contained the same *phbC* upstream DNA as in pCF200, and the *lacZ* was translationally fused to the 99th amino acid of the *phbC* gene. The plasmid was also maintained *in trans* in the wild-type, and the β-galactosidase activity was measured. The translational activities of pCLZ627 revealed a regulatory pattern similar to that observed with the transcriptional fusion plasmid pCF200.

Interestingly, when the PHB synthase activities of the cells were compared with the corresponding activities of the *phbC* translation determined with pCLZ627, they were found to be proportionately related to each other. The same was true when a comparison was made with the transcriptional activities of pCF200. Therefore, the PHB synthase activities of *R. sphaeroides* would seem to be regulated at the *phbC* transcription level.

From the above results, it was clear that the expression of PHB synthase was regulated by the *phbC* transcription during cell growth under both aerobic and photoheterotrophic conditions. However, the PHB content of the photoheterotrophically grown cell was not reflected by the PHB synthase activities, which were constitutive and lower than those of the aerobically grown cell. Therefore, the PHB content seemed to be controlled by the *phbC* transcription under only aerobic conditions.

DISCUSSION

The PHB is dispensable for the growth of *R. sphaeroides*. When it is not produced, however, other photoheterotrophic activity such as photo-production of molecular hydrogen noticeably increased [13]. This result suggested that the PHB may act as an electron sink for the bacterial photosynthesis. In other bacteria, it has also been regarded as a reserve material of both carbon and reducing power [1].

Previously, extra copies of *phbC* on the plasmid pRK415 were found to increase the PHB content of *R. sphaeroides* by two- to four-fold depending on the carbon source used for cell growth under aerobic conditions [22]. This result suggests that PHB synthase is important in regulating the level of PHB content. In the present study, we analyzed the PHB synthase activity of *R. sphaeroides* cells grown aerobically and photoheterotrophically to determine how the PHB content was related to the enzyme activity. In addition, the expression of the enzyme was analyzed using a *phbC-lacZ* transcriptional fusion as well as a translational fusion construct. The results clearly indicated that the expression of PHB synthase *per se* was regulated at the level of *phbC* transcription under the conditions examined.

The PHB content of the aerobically grown cell varied proportionally with the PHB synthase activity, which increased during the transition to the early stationary phase of the cell growth. The enzyme activities of β-ketothiolase

(*phbA*) and acetoacetyl-CoA reductase (*phbB*) of *R. sphaeroides*, however, remained constitutive during the cell growth (Kho and Lee, unpublished data). The results, taken together, suggested that the aerobic content of the polymer was controlled by the PHB synthase activity, and the aerobic content of PHB seemed to be regulated by *phbC* transcription. In contrast, the photoheterotrophic level of PHB did not exhibit any clear correlation with the synthase activity.

One difference worth mentioning related to the PHB synthase expression of the aerobically and photoheterotrophically grown cells was that the expression and activity of PHB synthase during aerobic growth was regulated depending on the growth phase, whereas the enzyme activity and its expression remained constitutive under photoheterotrophic conditions. This indicates a fundamental difference existing in the PHB metabolism under aerobic and photoheterotrophic conditions, although the PHB content increased during the early stationary phase under both conditions.

In addition, the PHB synthase activities under photoheterotrophic conditions were lower than those found in cells grown aerobically, although the PHB content was about two-fold higher. It remains to be determined how the photoheterotrophic level of PHB was regulated. However, PHB degradation might be another pathway to determine the level of PHB content depending on the culture conditions.

In conclusion, *phbC* transcription determines the expression of PHB synthase, and PHB synthase affects the level of PHB content only under aerobic conditions.

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