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Quorum Sensing of *Rhodobacter sphaeroides* Negatively Regulates Cellular Poly-β-Hydroxybutyrate Content Under Aerobic Growth Conditions

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Abstract The community escape response of *Rhodobacter sphaeroides* is exerted through the action of CerR and CerI, which code for a LuxR-type regulatory protein and acylhomoserine lactone synthase, respectively. Deletion of chromosomal DNA including *cerR* and *cerI* (mutant RI) or insertional interruption of *cerI* (mutant AP3) resulted in two-fold increase in the cellular poly-β-hydroxybutyrate (PHB) content in comparison with the wild-type under aerobic growth conditions. The PHB synthase (PhbC) activities of the *cer* mutants were doubled, and the enzyme expression was regulated at the level of *phbC* transcription. Thus, CerR, possibly in response to autoinducer (AI), appears to modulate the PHB content of aerobically grown cells by downregulating *phbC* transcription.

Key words: *Rhodobacter sphaeroides*, quorum sensing, *cer* operon, PHB synthase, *phbC*

PHB, a polymer of D-β-hydroxybutyrate [1], is a storage material accumulated during the growth of *Rhodobacter sphaeroides* [7, 10]. PHB synthase mediates the condensation of D-β-hydroxybutyl-CoA to form PHB [5, 7, 16]. The coding gene, *phbC*, is not linked to the *phbAB* which incode β-ketothiolase and acetoacetyl-CoA reductase, which sequentially convert two molecules of acetyl-CoA into D-β-hydroxybutyl-CoA, a substrate for PHB synthase [6].

Recently, it was found that *R. sphaeroides* produces 7,8-*cis*-*N*-(tetradecenoyl)homoserine lactone, which is regarded as an AI that serves as an intercellular signal facilitating quorum sensing [17]. Its synthesis is mediated by acylhomoserine lactone synthase encoded within *cerI*. An open reading frame (ORF), termed CerR, which shows a significant similarity to LuxR family regulators, has been found upstream from *cerI*, and a small ORF of 52 amino acids is located between *cerR* and *cerI*. No known proteins in databases have similarity to the middle ORF. The *cerI*-interrupted mutant reveals cell aggregation phenotype under aerobic conditions, when the growth reaches the stationary phase in broth cultures. The significance of the quorum-sensing system and its associated phenotype in this free-living bacterium is still unclear. However, it has been proposed that light may be a limiting factor in sufficiently large aggregates of cells, and escape from the cell flocks could be advantageous in terms of photosynthesis [17].

Previously, it has been reported that the PHB synthesis of *Vibrio harveyi* is regulated in a cell density-dependent mode, in which luciferase-mediated bioluminescence has also been known to be induced [13, 14]. However, the target gene(s), which is regulated by the quorum sensing of *R. sphaeroides*, is unknown. Interestingly, we found that the cellular PHB contents of *cer*-deleted (from *cerR* to *cerI*) and *cerI*-interrupted mutants are two-fold higher than that in the wild-type throughout aerobic growth. Furthermore, the quorum-sensing system appears to modulate the *phbC* transcription. The physiological significance of PHB synthesis has been implicated in nutrient availability in the cell flocks formed by *cer* mutants.

In the current study, *R. sphaeroides* 2.4.1 [8] was used as the wild-type strain and cultured at 28°C in Sistrom's minimal medium [11]. *R. sphaeroides* AP3 [17], which is a *cerI*-interrupted mutant (Fig. 1A), was kindly provided by Dr. S. Kaplan at the University of Texas-Medical School at Houston. A 0.8-kb *Bam*HI fragment extending from *cerR* to *cerI* (Fig. 1A) was deleted from the 2.3-kb *Hin*dIII-*Not*I DNA including the genes, and replaced by 1.6-kb trimethoprim (Tp)-resistant DNA [18]. The resulting recombinant DNA was then cloned into pSUP202 (tetracycline [Tc]-resistant gene on the plasmid) [19] and mobilized into the wild-type to select a double-crossover recombinant showing Tp^r and Tc^s. Five double-crossover recombinants were obtained from approximately seventy exconjugants

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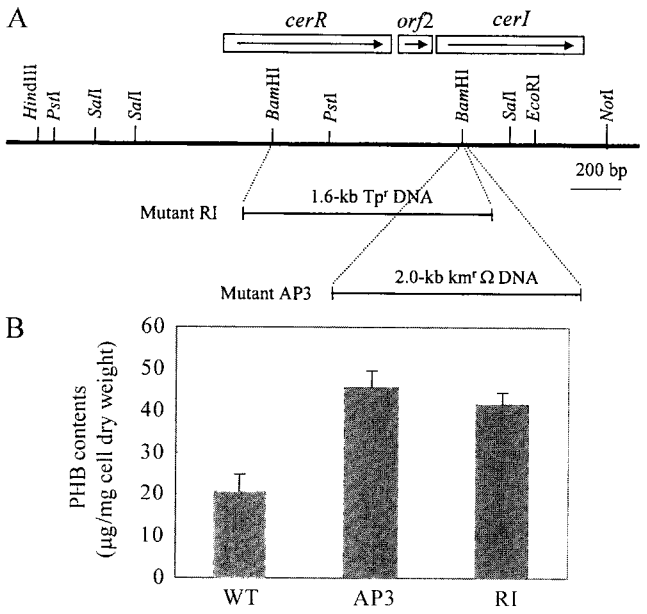


Fig. 1. Restriction map of DNA region including *cerR* and *cerI*. (A) Chromosomal structures of two *cer* mutants, RI and AP3 [17]. (B) Cellular PHB contents of wild-type (WT) and *cer* mutants RI and AP3 during exponential growth (60–100 KU) under aerobic conditions. Standard deviations are shown on each bar.

examined. Their chromosomal arrangement was confirmed by Southern hybridization analysis (data not shown), and one strain, RI (Fig. 1A), was chosen for further analyses.

Interestingly, the cellular PHB content of the *cer*-deleted mutant RI, which was determined spectrophotometrically by the Law and Slepecky method [9], was two times higher than that of the wild-type throughout aerobic growth. The content in RI during the exponential growth phase is shown in Fig. 1B. The other four *cer*-deleted mutants showed similar results. Consistently, the cellular PHB content of AP3 was similar to that of RI (Fig. 1B). However, the PHB content of the photoheterotrophically grown *cer* mutants was not higher than that of the wild-type (data not shown), which was not unexpected, because the PHB content of *R. sphaeroides* under photoheterotrophic conditions is regulated differently from that of the aerobically grown cells [7]. The cellular PHB content is regulated at the level of *phbC* transcription only under aerobic conditions [7]. Therefore, taken together, the results indicated that the lack of quorum sensing resulted only in an increase of cellular PHB content of *R. sphaeroides* during aerobic growth.

The PHB synthase activity was measured according to the method of Miyake *et al.* [12]. Cells were grown aerobically and harvested, when Klett units (KU) of the culture were between 60 and 100. Cells were then broken at 10,000 psi pressure using a French press (SLM, IL, U.S.A.), and the unbroken cells and cell debris were removed by centrifugation at 200 ×g for 15 min. Cell extracts

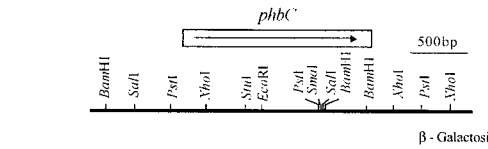
Table 1. PHB synthase activity of *R. sphaeroides* wild-type (WT) and *cer* mutants AP3 and RI under aerobic growth conditions.

Stains	PHB synthase activity[a]
WT	3.9±0.5[b]
AP3	6.9±0.7
RI	7.1±0.3

[a]Unit/mg cell protein.
[b]Standard deviations are shown with ±.

containing intracellular PHB granules were mixed with 0.05 mM DL-β-hydroxybutyryl-CoA and 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in Tris-HCl buffer, and the reaction was carried out at 30°C. Changes in the optical density of the thiobenzoate anion (TNB^-) at 412 nm were measured, as generated from the reaction of DTNB and CoA released from the substrate. One unit of activity was defined as the amount of enzyme to release 1 nmole of TNB^- per min. The PHB synthase activity of the mutants RI and AP3 grown aerobically was found to be approximately two-fold higher than the wild-type value (Table 1), whereas no difference in the PHB depolymerase activity was found between the *cer* mutants and wild-type (data not shown). Thus, the increase in the PHB content of the *cer* mutants appeared to have resulted from a proportional increase of the PHB synthase activity.

It was also examined whether the increased activities of PHB synthase of the *cer* mutants was controlled at the transcriptional level of *phbC*. 1.3-kb *Bam*HI-*Stu*I DNA containing *phbC* and its upstream DNA was transcriptionally and translationally fused to *lacZ* and *lacZ'* DNA to generate the IncQ plasmid derivatives pCF100 and pPLZ627, respectively (Fig. 2). The plasmids were maintained in *trans* in *R. sphaeroides*, and the β-galactosidase activities of the aerobically grown cells were assayed with *o*-nitrophenyl-β-D-galactoside hydrolysis, as described previously [7]. All the determinations were performed in duplicate and repeated at least three times independently. The data presented are the averages of the values obtained within 10–15% deviations. As shown in Fig. 2, the β-galactosidase activities of the *cer* mutants, RI and AP3,



			β-Galactosidase activity[a]		
			WT	AP3	RI
pCF100	Ω	*phbC* *lacZ*	215.2 ± 5.3	396.4 ± 6.7	408.0 ± 10.2
pPLZ627	Ω	*phbC* *lacZ*	340.7 ± 7.5	612.5 ± 10.4	581.1 ± 6.4

[a]Miller unit

Fig. 2. β-Galactosidase activities of *lacZ* transcriptionally (pCF100) and translationally (pPLZ627) fused to *phbC*.
Cells containing the plasmids were grown aerobically. Standard deviations are shown with ±.

harboring pCF100 and pPLZ627 were approximately two-folds higher than the corresponding values of the wild-type. Accordingly, the results indicate that the increased activities of PHB synthase of the *cer* mutants, RI and AP3, were regulated at the level of *phbC* transcription.

As mentioned above, the cellular PHB contents of the photoheterotrophically grown *cer* mutants were not different from the level of wild-type. However, the PHB synthase activities and β-galactosidase activities of pCF100 and pPLZ627 of the photoheterotrophically grown *cer* mutants were approximately two-folds higher than the corresponding values of the wild-type (data not shown). Therefore, no increase of the PHB content, inspite of higher PHB synthase activity, suggests that the photoheterotrophically grown *cer* mutants controlled the PHB content by another PHB metabolic enzyme(s) other than PHB synthase. Therefore, the mechanisms involved in regulating the cellular PHB content during photoheterotrophic growth remain to be elucidated. However, the PHB synthase activities of the photoheterotrophically grown *cer* mutants were clearly elevated and regulated at the transcription level of *phbC*.

We also examined whether there was any difference in the transcriptional regulation of *phbC* between RI and AP3 in response to treatment with an *R. sphaeroides* AI, which was extracted from the culture supernatant with acidified ethyl acetate, as previously described [15]. Cells were grown aerobically in LB, removed by centrifugation, and the supernatants were extracted twice with equal volume of ethylacetate. The extracts were then combined, dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residues from a 50-ml culture, grown to the early stationary phase, were dissolved in 1 ml of methanol, and used as the crude AI of *R. sphaeroides*. The relative activity of the AI between independent extractions was confirmed using a TraR indicator system of *Agrobacterium tumefaciens* [3]. The mutants RI and AP3 harboring pCF100 were grown aerobically to the mid-exponential phase, and then treated with varying amounts of the AI. The β-galactosidase activities were measured 3 h after the treatment (Table 2). 1× represents the addition of the crude AI at the same concentration as in the original culture from which the molecules were extracted, while 10× represents the addition of a ten-fold higher concentration of the AI. The β-galactosidase activities of AP3 harboring pCF100 decreased inversely to the AI added, and showed approximately the same value as the wild type, which did not change with the AI treatment. In contrast, the β-galactosidase activities of pCF100 *in trans* in RI did not decrease in response to the AI. Thus, the results suggest that CerR, possibly interacting with the AI, was involved in the downregulation of *phbC* transcription. The increased β-galactosidase activities of RI and AP3 harboring pCF100 were lowered to the wild-type level, when the mutants were complemented with cosmid pUI8166 containing the *cerR* and *cerI* genes of *R. sphaeroides* (data not shown).

The 5' end of the *phbC* transcript was determined by a primer-extension analysis (Fig. 3). Total RNA was isolated from cells grown aerobically, as described previously [2]. The primer extension was performed using a primer phb1

Table 2. Effect of extracted AI on β-galactosidase activity of pCF100 *in trans* in *R. sphaeroides* wild-type (WT) and *cer* mutants, RI and AP3, which were grown under aerobic conditions.

Strains	β-Galactosidase activity[a]		
	-AI	+AI	
		1×[b]	10×
WT	225.3±3.3	222.3±1.4[c]	254.0±5.5
AP3	408.0±5.4	275.7±0.7	229.9±6.8
RI	417.1±4.3	418.1±2.1	431.1±8.3

[a]Miller units.

[b]1× represents the same AI concentration as in the original culture from which the molecule was extracted, whereas 10× denotes a ten-fold higher AI concentration.

[c]Standard deviations are shown with ±.

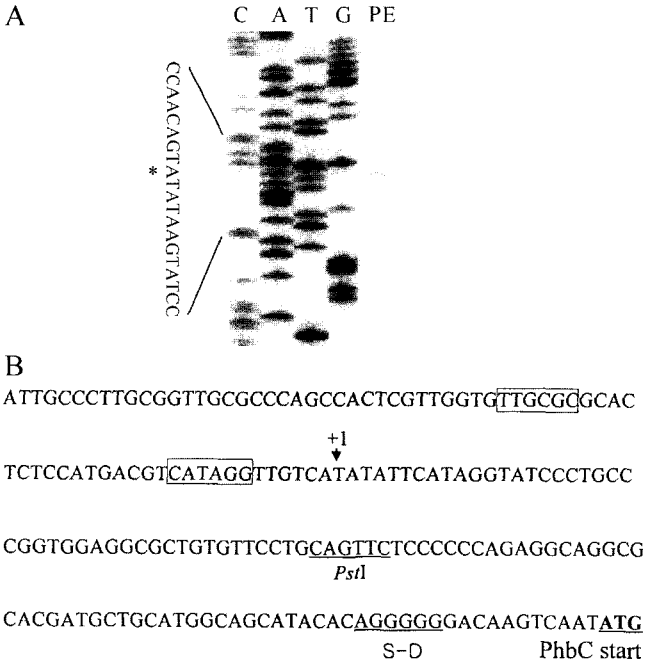


Fig. 3. 5'-End mapping of *phbC* transcript by primer extension analysis.

(A) Primer extension with RNA from *R. sphaeroides* is shown in lane PE. The same ^{32}P-labeled primer was used to generate a sequence ladder (lanes C, A, T, G). DNA sequence of the noncoding strand is illustrated on the left with the 5' end marked with a star. (B) DNA sequence extending from -70 to +113 nucleotides of *phbC* DNA (+1: 5' end of the *phbC* transcript). The putative -35 and -10 elements of a σ^{70}-type promoter are indicated, and a putative ribosome-binding site (S-D) is shown 12 nucleotides upstream from an ATG initiation codon of PhbC, illustrated in boldface letters.

(5'-GCGGCGTGGTGCTTGTC-3'), which approximately corresponded to codons 2-8. Total RNA from *R. sphaeroides* cells during exponential aerobic growth was used for the extension, and the reaction products were analyzed on an 8.3 M urea-8% polyacrylamide sequencing gel. Only one major primer-extended product of approximately 130 nucleotides was observed (Fig. 3A). To determine the 5' endpoint of the transcript, sequencing reactions with the same ^{32}P-labeled primer were performed in parallel with a template DNA containing *phbC* DNA. Sequence shown in Fig. 3A represents the noncoding strand. Accordingly, the 5' end (T) was localized precisely to 105 nucleotides upstream from an ATG initiation codon of PhbC. Putative sequences resembling a σ^{70}-type promoter were found in the DNA at around -35 and -10 (Fig. 3B). A comparison of the *phbC* regulatory DNA with the consensus sequence of the LuxR-binding sequence [4] did not reveal any region of a significant homology. Thus, the action site for CerR may be quite different from that for LuxR, and remains to be determined.

Interestingly, it has been found that the PHB synthesis of *V. harveyi* is controlled in a manner analogous to the induction of cell luminescence [13, 14]. However, unlike the negative regulation of the PHB synthesis of *R. sphaeroides*, the PHB synthesis of *V. harveyi* is under positive regulation by a quorum-sensing system. *N*-(3-hydroxybutanoyl)homoserine lactone, the AI of *V. harveyi*, provides a signal to control PHB production as well as luminescence. Although the molecular basis and physiological implication of PHB synthesis in relation to the light emission of the bacteria have not yet been clearly elucidated, it was proposed that the PHB in *V. harveyi* serves as an energy source for maintaining cell viability in the stationary growth phase.

The physiological implication of the increased PHB formation in *cer* mutants remains unclear. However, *cer* mutants form many cell flocks during aerobic growth, therefore, they may need more PHB than the wild-type, because nutrients may be limited in cell aggregates. No cell aggregation was observed during photoheterotrophic growth. Nonetheless, the *phbC* transcription of *cer* mutants during photoheterotrophic growth was regulated similarly to that during aerobic growth. Therefore, the regulation of *phbC* transcription by the quorum sensing of *R. sphaeroides* was not a pleiotrophic result due to cell flock formation. Thus, the transcription of *phbC* appeared to be directly controlled by the specific interaction of CerR in response to the AI. Accordingly, *phbC* is proposed as the target gene that is controlled by the quorum sensing of *R. sphaeroides*.

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