NOTE

Characterization of a Photosynthetic Mutant Selected by Increased Formation of Poly-3-Hydroxybutyrate in *Rhodobacter sphaeroides*

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Abstract Various mutants either lacking or having decreased levels of light-harvesting complexes and reaction center complex were obtained with a high frequency by an increased formation of poly-3-hydroxybutyrate (PHB) in *Rhodobacter sphaeroides*. One of the photosynthesis-defective mutants, PY-17, which was devoid of any of the light-harvesting complexes (B800-850⁻, B875⁻) as well as the reaction center complex, was analyzed further. The mutant showed substantial transcription of the *puhA*, *pufKBALMX*, and *pucBAC* operons coding for the structural proteins of the photosynthetic complexes although each of the activities was lower than that of the wild type. Translation of the *pufKBALMX* and *pucBAC* operons were also active in the mutant although with activities different from the corresponding one of the wild type. From these results the mutation appears to exert its effect at the post-translational level of the photosynthetic complex assembly. Complementation of the photosynthesis-defective phenotype of the mutant was achieved with an about 12-kb DNA region containing the *puhA* gene. The relationship between the formation of PHB and photosynthetic complexes is discussed.

Key words: *Rhodobacter sphaeroides*, PHB, photosynthetic mutant, photosynthetic complex assembly

Poly-3-hydroxybutyrate (PHB) is accumulated intracellularly by both gram-negative and gram-positive bacteria as a carbon storage compound or as a sink for reducing equivalents [2]. The PHB is one of the poly-3-hydroxyalkanoic acids (PHAs) which are composed of 3-hydroxy fatty acids. Some of the PHAs have received much attention as sources for biodegradable plastics because of their thermoplastic properties.

Genes for PHB biosynthesis have been cloned and characterized from several bacteria [9, 16, 18-20, 22, 27-29]. The biosynthesis of PHB from acetyl CoA minimally

requires consecutive action of three enzymes, β-ketothiolase, acetoacetyl CoA reductase, and PHB synthase encoded by *phbA*, *phbB*, and *phbC*, respectively, based on biochemical studies of *Alcaligenes eutrophus*. In this bacterium, the three genes are organized in an operon of *phbCAB* [18, 19].

Recently, we have cloned, sequenced, and characterized the expression of *phbC* gene coding for the PHB synthase of *Rhodobacter sphaeroides* in which the *phbC* is not linked to the *phbA* or the *phbB* genes [13]. During the study of the elevated formation of PHB in *R. sphaeroides*, with acetate as a sole carbon source, various mutants showing altered pigmentation were isolated with a high frequency and found to contain very reduced levels of (or no) photosynthetic complexes. One of the photosynthesis-defective mutants was chosen for further analysis in an attempt to understand the relationship between PHB formation and photosynthetic complex assembly.

The bacterial strains and plasmids used in this work are described in Table 1. *R. sphaeroides* 2.4.1 was grown at 28°C in Sistrom's minimal medium containing succinate (34 mM) or acetate (30 mM) as a sole carbon source [24]. Dark anaerobic culture with dimethyl sulfoxide (DMSO, 75 mM) as a terminal electron acceptor was employed to evaluate the gratuitous formation of photosynthetic complexes. When appropriate, tetracyclin (Tc), kanamycin (Km), streptomycin (Sm), and spectinomycin (Sp) were added to the Sistrom's minimal medium to final concentrations of 1, 25, 50, and 50 μg/ml, respectively. *Escherichia coli* strains were grown at 37°C in Luria medium [21]. Antibiotics were added to the growth medium for *E. coli* strains carrying plasmids encoding the drug resistance genes, as above, except that 20 mg/ml tetracyclin and 50 μg/ml ampicillin (Ap) were used.

Plasmid DNA was prepared as previously described [12, 21], and the DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes in accordance with the manufactures' specifications. Plasmids derived

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Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli		
DH5α*phe*	F⁻ Φ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *recA1 endA1* *hsdR17*(r$_K$⁻m$_K$⁺) *supE44λ⁻thi-1 gyrA relA1 phe*::Tn*10d*Cm	[5]
S17-1	C600::RP-4 2-(Tc::Mu) *thi pro hsdR hsdM⁺ recA*	[23]
HB101	*lacY1 galK2 supE44 ara14 proA2 rpsL20 recA13 xyl-5* *mtl-1 hisdS20*(r$_B$⁻m$_B$⁻)	[7]
R. sphaeroides		
2.4.1	wild type	Laboratory strain
PY-17	2.4.1 derivative: PS⁻ B800-850⁻ B875⁻ RC⁻	This study
Plasmids		
pRK415	Mob⁺ Tc^r *lacZα* IncP	[11]
pRK1704	pRK415 derivatives, 2.7-Kb *Sal*I-*Eco*47III fragment containing *phbC* of *R. sphaeroides* 2.4.1, Tc^r	J. H. Kim and J. Lee, Sogang University
pRK2013	Km^r IncP1 ColE1 Mob	[7]
pWS2	R' R68.45	[24]
pLA2917	Tc^r Km^r IncP cosmid vector	[1]
pUI8484	pLA2917-derived cosmid from *R. sphaeroides* 2.4.1 cosmid library, Tc^r	[4]
pUI8523	pLA2917-derived cosmid from *R. sphaeroides* 2.4.1 cosmid library, Tc^r	[4]
pUI8714	pLA2917-derived cosmid from *R. sphaeroides* 2.4.1 cosmid library, Tc^r	[4]
pCF200	*pucB-lacZ* transcriptional fusion; Sm^r/Sp^r Tc^r IncQ	[15]
pHZ300	*puhA-lacZ* transcriptional fusion; Sm^r/Sp^r Tc^r IncQ	S. K. Lim and J. Lee, Sogang University
pUI1830	*pufB-lacZ* transcriptional fusion; Sm^r/Sp^r Tc^r IncQ	S. Kaplan, University of Texas
pUI1665	*pucB-lacZ* translational fusion; Sm^r/Sp^r Tc^r IncQ	[6]
pUI1851	*pufB-lacZ* translational fusion; Sm^r/Sp^r Tc^r IncQ	[8]

from pRK415 or RSF1010 were mobilized into *R. sphaeroides* as described previously [3].

β-Galactosidase assays were performed at least twice as previously described [26]. *R. sphaeroides* cultures used for the assays were grown anaerobically in the dark with DMSO.

Determination of PHB was performed by spectrophotometric assay using the Law and Slepecky method [14]. The relative amount of PHB between culture samples was compared with each of the cellular PHB content, which is calculated from the PHB amount (μg/ml culture) divided by the culture Klett units (KU) monitored by using a Klett-Summerson colorimeter (No. 66 filter); 1 KU corresponds to approximately 10^7 cells /ml.

Absorption spectra of *R. sphaeroides* cell-free extracts were analyzed with a UV-2041PC spectrophotometer (Shimadzu, Japan). The same concentration of protein was used when the spectral profiles of different strains of *R. sphaeroides* were examined. Protein was determined by a modified Lowry method [17] with bovine serum albumin as a standard.

The PHB accumulation of *R. sphaeroides* during chemoheterotrophic growth was investigated with each of the two organic acids, acetate or succinate, as a sole carbon source in the Sistrom's medium, as shown in Table 2, since the acetate and succinate have been known as

Table 2. PHB accumulation of *R. sphaeroides* in the presence of extra copies of the *phbC in trans* in the medium containing succinate or acetate.[a]

Plasmid	PHB (μg/ml culture · KU)[b]	
	Succinate	Acetate
pRK1704	0.177±0.017[c]	1.343±0.106
pRK415	0.040±0.001	0.594±0.051

[a]Succinate and acetate were used at 34 and 30 mM, respectively.
[b]Cells were grown chemoheterotrophically and harvested for PHB assay between 150 and 250 KU.
[c]Standard deviations of the PHB amount determined are indicated with ±.

the best-suited and a poor substrate for PHB synthesis in *R. sphaeroides*, respectively [10]. In each case, the PHB amount was measured during the stationary phase of culture since the PHB content per cell reached maximal levels in this phase. In acetate medium, *R. sphaeroides* had about fourteen times more PHB than that found in succinate medium, as expected (Table 2). As a way to increase the PHB accumulation further in the acetate medium, extra copies of *phbC* gene on pRK1704 were put *in trans* in *R. sphaeroides* to show the PHB content of about 1.343 μg/ml culture KU, whose value was about 2.3-fold higher than that of the *R. sphaeroides* containing the vector pRK415. In the succinate medium, extra copies of the *phbC* gene also supported higher accumulation of PHB.
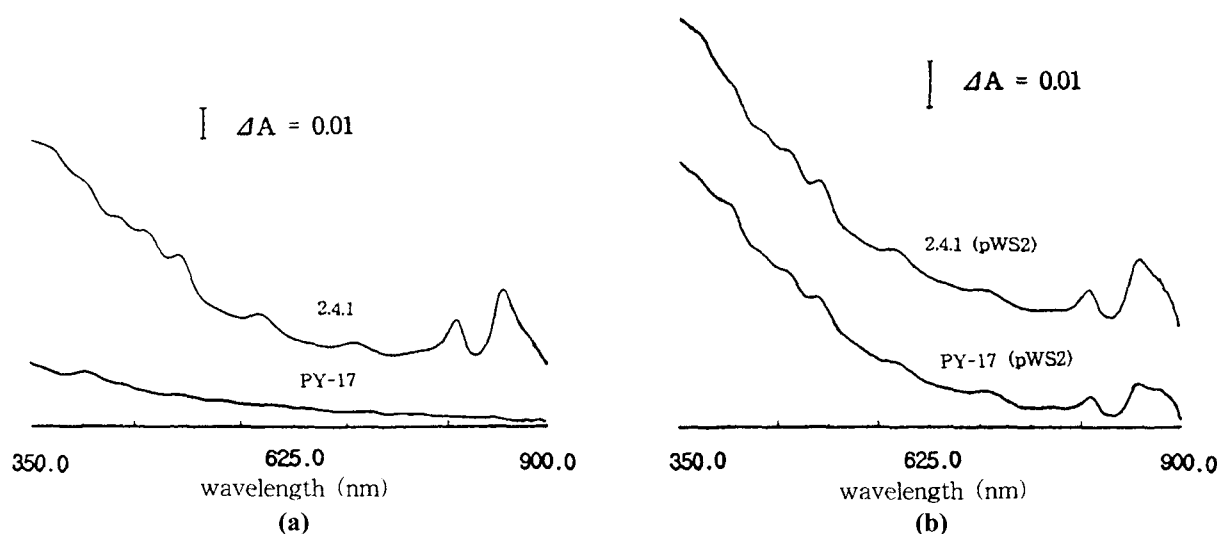
Fig. 1. (A) Absorption spectra of cell-free lysates obtained from *R. sphaeroides* 2.4.1 and PY-17 grown in the dark with DMSO. (B) Absorption spectra of *R. sphaeroides* 2.4.1(pWS2) and PY-17(pWS2) under the same growth condition as in (A). The bar represents an absorbance value of 0.01.

Interestingly, various kinds of mutants showing reduced pigmentation were observed with a high frequency (approximately 3.4×10^{-3}) only after the *R. sphaeroides* containing pRK1704 was grown on the acetate medium. Neither *R. sphaeroides* (pRK1704) grown on the succinate medium nor *R. sphaeroides* (pRK415) grown on any of the two media generated such mutants. The similar mutational event on the acetate medium occurred with *R. sphaeroides* containing *puc::phbC* transcriptional fusion construct, which has the *phbC* structural gene fused to the regulatory DNA region of *pucBAC* operon coding for the structural polypeptides of the B800-850 light-harvesting complex. This result indicates that the high PHB content produced by the extra dosage of the *phbC* gene is responsible for the mutation.

The membranes of the various mutants were analyzed after growth in the dark with DMSO as a terminal electron acceptor to check the gratuitous induction of the spectral complexes. The mutants were classified into two groups; One totally missing the photosynthetic complexes, the other having lower levels of the complexes than that of the wild type. One of the photosynthesis-defective mutants, PY-17, was chosen for further analysis after curing of the pRK1704. As expected, it was devoid of any of the light-harvesting complexes (B800-850⁻, B875⁻) as well as the reaction center complex (Fig. 1A). The PHB amount of PY-17 is comparable with that of the wild type in both the succinate medium and acetate medium (data not shown). This result indicates that the photosynthesis-defective mutation does not significantly affect PHB accumulation, but rather the elevated accumulation of PHB can result in mutations having decreased levels of the photosynthetic complexes, as shown above.

To identify the mutation nature of the PY-17, transcriptional activities of the *pucBAC*, *pufKBALMX* and *puhA* operons were measured following the introduction of each of the transcriptional *lacZ* fusion constructs *in trans* in the mutant. The three operons code for the structural proteins of the photosynthetic complexes; reaction center complex (by *puhA* and *pufLM*), B875 complex (by *pufBA*), and B800-850 complex (by *pucBA*). As shown in Table 3, substantial transcription of the *puhA*, *pufKBALMX*, and *pucBAC* operons was observed in the mutant although each of the activities was lower than that of the wild type. Translation of the *pufKBALMX* and *pucBAC* operons were also active in the mutant although with activities different from the corresponding one of the wild type. From these results the mutation appears to exert its effect at the post-

Table 3. Expression of photosynthetic genes in *R. sphaeroides* 2.4.1 and PY-17.

Plasmid (fusion structure)	β-Galactosidase activities (Miller units)[a]	
	2.4.1	PY-17
Transcriptional fusion		
pCF200 (*pucB::lacZ*)	353.06±7.19[b]	128.42±8.65
pUI1830 (*pufB::lacZ*)	601.52±14.99	145.17±11.19
pHZ300 (*puhA::lacZ*)	434.69±20.56	395.31±20.92
Translational fusion		
pUI1665 (*pucB::lacZ*)	286.63±10.16	125.82±15.53
pUI1851 (*pufB::lacZ*)	145.68±11.32	284.55±21.99

[a] Cells were grown in the dark with DMSO and were harvested for the assay between 50 and 80 KU.
[b] Standard deviations of the β-galactosidase activities are indicated after ±.

translational level, possibly at the step of the photosynthetic complex assembly.

The photosynthesis-defective mutation of PY-17 was complemented with pWS2 which contains about 109-kb DNA region of photosynthetic gene cluster derived from *R. sphaeroides* WS2 [30]. The spectral profile of PY-17 containing pWS2 after growth in the dark with DMSO is illustrated in Fig. 1B to show the restoration of the photosynthetic complexes to the wild-type level. To localize the mutation site further, the PY-17 was introduced with the cosmid library of *R. sphaeroides* 2.4.1, and two cosmids, pUI8714 and pUI8523, were selected since spectral profiles similar to that with pWS2 were observed with both plasmids. Thus, the mutation site could be localized to about a 12-kb DNA region overlapped between the two plasmids, which contains the *puhA* gene. Previously, it was shown that the disruption of *puhA* resulted in the photosynthesis-defective phenotype, but the mutant was still capable of forming B800-850 complex gratuitously in the dark with DMSO. Since the PY-17, however, did not show any of the photosynthetic complexes under the same conditions, we do not believe the possibility that PY-17 carries a mutation(s) mapped at *puhA*. Thus, gene(s) other than *puhA* in the region are responsible for the complementation of the mutation of PY-17. From the results shown in this work, we deduce that increased flow of reducing power into the formation of more PHB may not be compatible with the assembly of the photosynthetic complexes, and the gene(s) on the 12-kb DNA region may code for gene product(s) which play a key role in regulating formation of photosynthetic complexes responding to the alteration of the intracellular redox balance. The relationship between the PHB accumulation and the photosynthetic complex formation will be explained further after the characterization of the gene(s), of which cloning is in progress from the 12-kb DNA region.

In this work, we showed that the increased formation of PHB is responsible for the mutations leading to a decrease in the amount of photosynthetic complexes. As far as we know, this is the 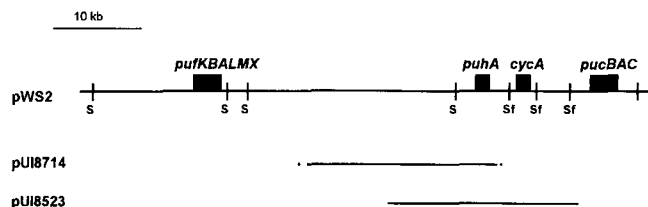first report on the relationship between PHB accumulation and the spectral complex formation in a photosynthetic bacterium.

Acknowledgments

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Fig. 2. Complementation of PY-17.

The *R. sphaeroides* DNA carried by each plasmid was illustrated with relative locations of the *pufKBALMX, puhA, cycA,* and *pucBAC* operons. The S and Sf denote restriction sites of *Ssp*I and *Sfi*I, respectively.

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