Journal of Microbiology and Biotechnology
Vol. 7. No. 4, 229-236, 1997

Cloning, Nucleotide Sequence and Expression of Gene Coding for Poly-3-hydroxybutyric Acid (PHB) Synthase of *Rhodobacter sphaeroides* 2.4.1

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A gene, $phbC_{2.4.1}$ encoding poly-3-hydroxybutyric acid (PHB) synthase of *Rhodobacter sphaeroides* 2.4.1 was cloned by employing heterologous expression in *Escherichia coli*. *R. sphaeroides* chromosomal DNA partially digested with *Mbo*I was cloned in pUC19 followed by mobilization into *E. coli* harbouring $phbA,B_{AC}$ in pRK415, which code for β-ketothiolase and acetoacetyl CoA reductase of *Alcaligenes eutrophus*, respectively. Two *E. coli* clones carrying *R. sphaeroides* chromosomal fragment of $phbC_{2.4.1}$ in pUC19 were selected from ca. 10,000 colonies. The PHB-producing colonies had an opaque white appearance due to the intracellular accumulation of PHB. The structure of PHB produced by the recombinant *E. coli* as well as from *R. sphaeroides* 2.4.1 was confirmed by [H^+]-nuclear magnetic resonance (NMR) spectroscopy. Restriction analysis of the two pUC19 clones revealed that one insert DNA fragment is contained as a part of the other cloned fragment. An open reading frame of 601 amino acids of $phbC_{2.4.1}$ with approximate M.W. of 66 kDa was found from nucleotide sequence determination of the 2.8-kb *Sal*I-*Pst*I restriction endonuclease fragment which had been narrowed down to support PHB synthesis through heterologous expression in the *E. coli* harbouring $phbA,B_{AC}$. The promoter (s) of the $phbC_{2.4.1}$ were localized within a 340-bp DNA region upstream of the $phbC_{2.4.1}$ start codon according to heterologous expression analysis.

Poly-3-hydroxybutyric acid (PHB), a homopolymer of 3-hydroxybutyric acid accumulates intracelluarly when optimal growth conditions are not met in both gram-positive and gram-negative bacteria (for a review, see ref. 2). The PHB functions as a carbon storage compound or as a sink for reducing equivalents. The PHB belongs to polyhydroxyalkanoic acids (PHAs) which are polyesters of varoius 3-, 4-, and 5-hydroxyalkanoic acids. The thermoplastic properties of some of the PHAs have attracted industrial interest as a biodegradable plastic.

Although many research works have focused on increasing the PHB-production yield using a variety of bacterial strains (a few illustrated with ref. 15, 17, 18, 21, and 38), biosynthesis of PHB has been studied in most detail in *Alcaligenes eutrophus*. In this bacterium three enzymes of the PHB biosynthetic pathway are organized *phbC-A-B* with an operon coding for PHB synthase, β-ketothiolase, and acetoacetyl-CoA reductase, respectively. The β-ketothiolase condenses two acetyl-CoAs to form acetoacetyl-CoA which is subsequently reduced to D-(-)-3-hydroxybutyryl-CoA by a NADPH-dependent acetoacetyl-CoA reductase. Next, the PHB is produced by polymerization of the D-(-)-3-hydroxybutyryl-CoA by PHB synthase (24, 25). In *Rhodospirillum rubrum*, however, L-(+)-3-hydroxybutyryl CoA is formed first and converted to D-(-)-3-hydroxybutyryl-CoA by the action of both L-(+)- and D-(-)- enoyl-CoA hydratases (23).

Genes for PHB biosynthesis have been cloned and characterized from several bacteria (8, 22, 24-26, 30, 33, 35, 36). Recently, Hustede and Steinbüchel isolated and sequenced *phbC* of *R. sphaeroides* ATCC 17023 (9). However, not much is known about *phbC* expression at the level of genetic regulation. As an initial attempt to understand *phbC* expression in photosynthetic bacterium, we cloned and sequenced *phbC* ($phbC_{2.4.1}$) of *R. sphaeroide* 2.4.1 which has been commonly used as a model organism in which to study the molecular genetic regulation of the genes relevant for photosynthetic complexes and photosynthetic membrane development (14, 19). In addition, the DNA region containing promoter(s) of $phbC_{2.4.1}$ were localized by employing heterologous expression in *E. coli*.

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Key words: *Rhodobacter sphaeroides*, PHB, $phbC_{2.4.1}$, heterologous expression

MATERIALS AND METHODS

Bacteria, Plasmids and Cell Growth

The bacterial strains and plasmids used in this study are described in Table 1. *Rhodobacter sphaeroides* 2.4.1 was grown at 28°C in Sistrom's minimal medium (31). *Escherichia coli* strains were grown at 37°C in Luria medium (28). For PHB synthesis by recombinant *E. coli* Luria medium containing 2% glucose was used (20). Ampicillin, tetracycline, streptomycin, and spectinomycin (final concentrations, 50, 10, 50, and 50 μg/ml, respectively) were added to the growth medium for *E. coli* carrying plasmids encoding these drug resistance genes.

DNA Manipulation and Sequence Analysis

Large- and small-scale plasmid DNA were prepared as previously described (13, 28). DNA was treated with restriction enzymes and other nucleic acid-modifying enzymes in accordance with the manufacturers' instructions. DNA fragments were analyzed on agarose gels or polyacrylamide gels, and restriction fragments were isolated as described previously (3).

For sequence determination subclones were prepared on pUC19 and pUC18. These clones were used to determine the DNA sequence of *phbC* ($phbC_{2.4.1}$) of *R. sphaeroides* 2.4.1. The dideoxy-nucleotide sequencing reactions (29) were carried out with double-stranded DNA using T7 DNA polymerase of Cy5™ AutoRead™ Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden). The universal primer (5'-CGACGTTGTAAAACGACGGCCAGT-3') labelled with Cy5 at its 5'-end was used. Sequence determination was performed with an ALF express automatic DNA Sequencer (Pharmacia Biotech) at the Molecular Microbiology Center in Seoul National University.

Determined nucleotide sequence was assembled and analyzed using the DNASIS program (Version 7.0) of Promega, WI, U.S.A. Nucleotide sequence comparisons with the GenBank, GenEMBL, DDBJ and PDB databanks were made using the BLAST program of NCBI, U.S.A.

Southern Hybridization

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

Determination of PHB

Determination of PHB was performed by spectro-

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*($r_K^- m_K^+$)*supE44* λ⁻ *thi-1 gyrA relA1 phe*::Tn*10d*Cm	6
R. sphaeroides 241		Wild type	W. R. Sistrom
Plasmids	pUC19	Ap^r	37
	pRK415	Tc^r	12
	pTZ18U-PHB	*A. eutrophus phbC,A,B* in pTZ18U; Ap^r	S. Kaplan
	pSU102	pUC19/*Hin*dIII and *Hin*cII, 2.9-kb *Hin*dIII-*Stu*I fragment of *A. eutrophus phbC* (+)*; Ap^r	this study
	pDH100	pRK415/*Hin*dIII and *Eco*RI, 2.6-kb *Hin*dIII-*Eco*RI fragment of *A. eutrophus phbA,B* (+)*; Tc^r	this study
	pJH1700	pUC19/*Bam*HI, 4.0-kb fragment containing $phbC_{2.4.1}$ of *R. sphaeroides* 2.4.1 (+); Ap^r	this study
	pJH2600	pUC19/*Bam*HI, 6.0-kb fragment containing $phbC_{2.4.1}$ of *R. sphaeroides* 2.4.1 (-); Ap^r	this study
	pJH1701	pUC19/*Eco*RI, 1.8-kb fragment containing carboxy portion of $phbC_{2.4.1}$ (+); Ap^r	this study
	pJH1702	pJH1700 derivative, deletion of 1.5-kb *Sma*I fragment (+); Ap^r	this study
	pJH1703	pJH1700 derivative, deletion of 1.65-kb *Stu*I fragment (+); Ap^r	this study
	pJH1704	pJH1700 derivative, deletion of 1.3-kb *Sal*I fragment (+); Ap^r	this study
	pJH1705	pJH1704 derivative, deletion of 0.8-kb *Bam*HI-*Sma*I fragment (+); Ap^r	this study
	pCP100	pJH1704 derivative, deletion of 283-bp *Sal*I-*Pst*I fragment (+); AP^r	this study
	pOH1704	pJH1704/*Hin*dIII, 2.0-kb Ω cartridge; Ap^r, Sm^r/Sp^r	this study
	pOH1703	pJH1703/*Hin*dIII, 2.0-kb Ω cartridge; Ap^r, Sm^r/Sp^r	this study
	pOH100	pCP100/*Hin*dIII, 2.0-kb Ω cartridge; Ap^r, Sm^r/Sp^r	this study

*The transcriptional orientation of the inserted DNA fragment is indicated as being either the same as that of the *lac* promoter (+) or opposite to that of the *lac* promoter (-).

photometric assay using the Law and Slepecky method (16). Cultured cells (2 ml) were centrifuged and resuspended in 2 ml of 5% hypochlorite. After 1 h at 37°C, PHB granules were centrifuged and washed sequentially with water, acetone, and alcohol. The washed polymer was dissolved in chloroform at 60°C and the chloroform was evaporated in a boiling water bath. Next, 2 ml of conc. H_2SO_4 was added and the mixture was heated for 10 min in a boiling water bath to convert the polymer to crotonic acid. The solution was cooled, and the absorbance between 215 and 255 nm measured against conc. H_2SO_4 as a blank. The relative amount of PHB between the culture samples was quantitated from the absorbance of crotonic acid at 235 nm divided by the absorbance of the culture at 600 nm.

Nuclear Magnetic Resonance (NMR) Spectroscopy

[H^+]-NMR analysis of the PHB samples was carried out on a Bruker AMX-500 spectrometer in the pulse Fourier transform (FT) mode. The 500 MHz [H^+]-NMR spectra were recorded at 25°C using $CDCl_3$ solutions of the PHB (5 mg/ml) with 4.0-s pulse repetition, 5000-Hz spectral width, 32 K data points and 16 accumulations (11, 32).

RESULTS AND DISCUSSION

Cloning of Gene for PHB Synthase

Several trials to hybridize the chromosomal DNA of *Rhodobacter sphaeroides* 2.4.1 with *Alcaligenes eutrophus* *phbC* ($phbC_{AC}$) failed to result in any discrete signal (data not shown). As another approach to cloning *phbC* ($phbC_{2.4.1}$) of *R. sphaeroides*, heterologus expression of the *R. sphaeroides* gene(s) in *Escherichia coli* was employed. For this, a genetic background to support the formation of D-(-)-3-hydroxybutyryl-CoA from acetyl CoA was provided in *E. coli* by using $phbA,B_{AC}$ coding for β-ketothiolase and acetoacetyl-CoA reductase of *A. eutrophus*, respectively (24, 25). The idea behind this genetic background was to prvide for easier screening of the recombinant *E. coli* carrying clone (s) of $phbC_{2.4.1}$. Because D-(-)-3-hydroxybutyryl-CoA is the immediate substrate of poly-3-hydroxybutyric acid (PHB) synthase, presence of *phbC* coding for the enzyme in the same *E. coli* cell should result in the accumulation of PHB leading to an opaque white appearance of the colonies (Fig. 1). Two plasmids of compatible groups were stably maintained in *E. coli* using two different antibiotics relevant to antibiotic resistance determinants carried in each of the two plasmids. One plasmid with IncP1 incompatibility was pRK415 (12) or its recombinant derivative, while the other plasmid harboured a ColE1 replicon of pUC19 (37) in which cloning of *R. sphaeroides* chromosomal DNA had been perfrmed. Two plasmids used to test the feasibility of this method were pDH100 and pSU102. The pDH100 contains $phbA,B_{AC}$ in the same orientation as the promoters of *lac* and *tet* of the plasmid pRK415, while pSU102 carries $phbC_{AC}$ in pUC19 in the same orientation as the *lac* promoter. As shown in Table 2, *E. coli* containing both pDH 100 and pSU102 produced PHB sufficiently well to turn the colony appearance opaque white in 2 or 3 days on Luria-glucose plate. Neither *E. coli* (pDH100, pUC19) nor *E. coli* (pRK415, pSU102) accumulated any PHB to form opaque white colonies as expected. No PHB frmation was observed in the *E. coli* (pRK415, pUC19), either.

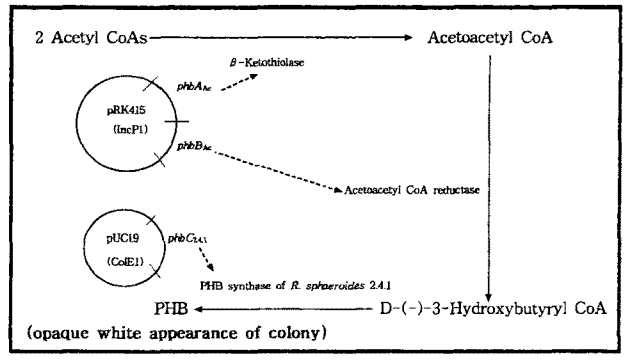

Fig. 1. Strategy for cloning of PHB synthase gene ($phbC_{2.4.1}$) of *R. sphaeroides* 2.4.1.
The $phbA_{AC}$ and $phbB_{AC}$ denote the *phbA* and *phbB* genes of *A. eutrophus*, respectively.

The *R. sphaeroides* chromosome partially digested with *Mbo*I restriction endonuclease was ligated into the *Bam*HI site of pUC19. Next, the ligation mixture was mobilized into *E. coli* containing pDH100. Two *E. coli*

Table 2. The PHB accumulation in *E. coli*.

Plasmids[a]	Relevant markers	Detection of PHB[b]	Colony appearance of the recombinant *E. coli*
pDH100 + pJH1700	$phbA,B_{AC}$ and $phbC_{2.4.1}$	+	opaque white
pDH100 + pJH2600	$phbA,B_{AC}$ and $phbC_{2.4.1}$	+	〃
pDH100 + pSU102	$phbA,B_{AC}$ and $phbC_{AC}$	+	〃
pDH100 + pUC19	$phbA,B_{AC}$	−	clear
pRK415 + pSU102	$phbC_{AC}$	−	〃
pRK415 + pUC19		−	〃

[a] *E. coli* strains harbouring the two plasmids were grown at 37°C in Luria medium containing 2% glucose and appropriate antibiotics. [b] PHB was determined by the Law and Slepecky method (16). +, PHB detected; -, PHB not detected.

colonies were selected according to their opaque white appearance from ca. 10,000 colonies examined. The two pUC19 clones were pJH1700 and pJH2600. PHB formation by the two recombinant *E. coli* strains was also confirmed by the Law and Slepecky method (16) as shown in Table 2.

[H$^+$]-Nuclear Magnetic Resonance (NMR) Analysis

To ensure that the polymer detected by the Law and

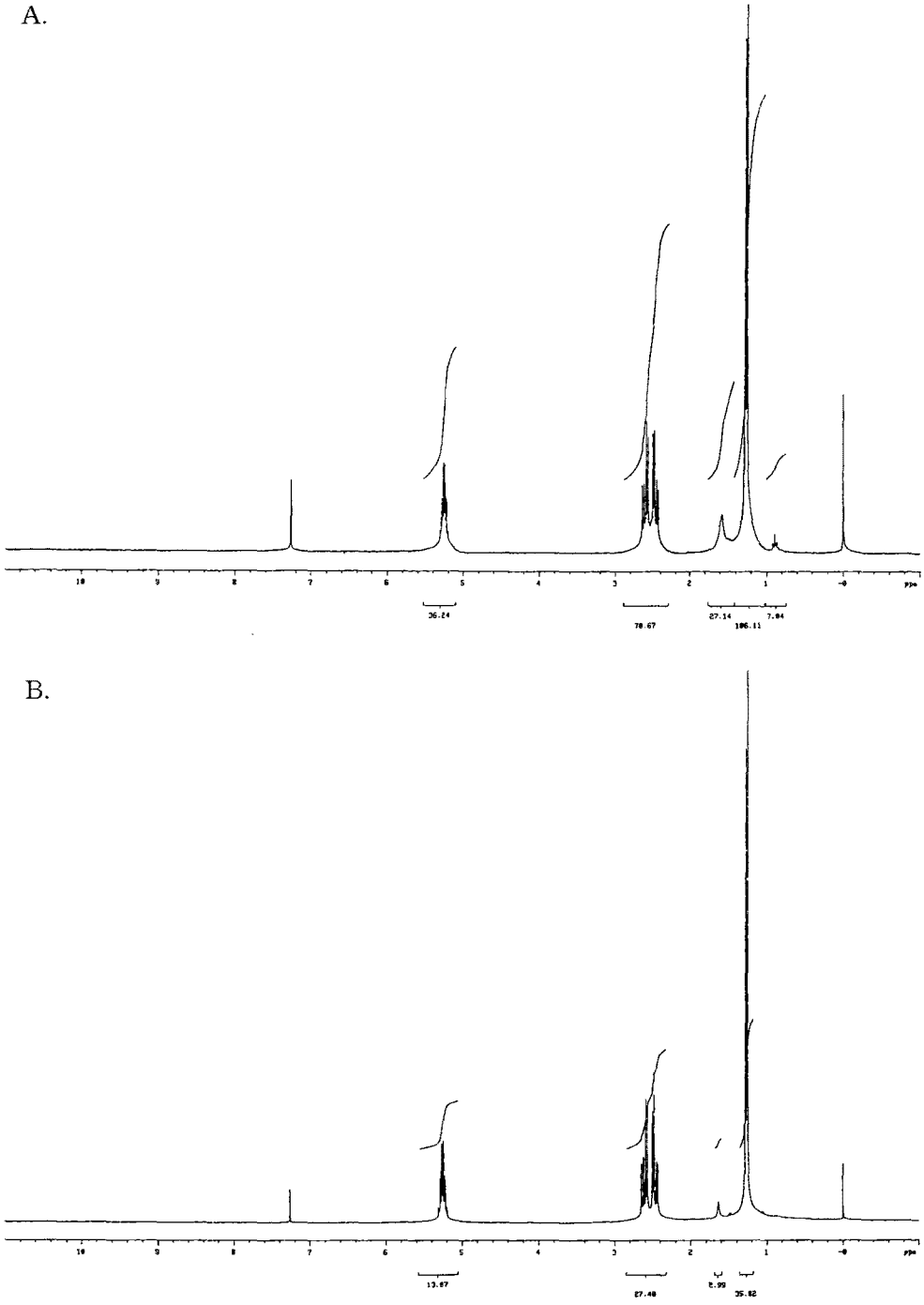

Fig. 2. [H$^+$]-NMR profiles of PHB isolated from *R. sphaeroides* 2.4.1 and *E. coli* (pDH100, pJH1700).
(A) PHB from *R. sphaeroides* 2.4.1, (B) PHB from *E. coli* (pDH100, pJH1700).

Slepecky method was a homopolymer of 3-hydroxybutyric acid, that is PHB, polymers extracted from *R. sphaeroides* 2.4.1 and *E. coli* (pDH100 and pJH1700) were analyzed by [H$^+$]-NMR analysis. As shown in Fig. 2, both [H$^+$]-NMR profiles of the polymers extracted from *R. sphaeroides* 2.4.1 (Fig. 2A) and *E. coli* (pDH100 and pJH1700) (Fig. 2B) showed patterns of peaks typical of PHB (11). Therefore, *R. sphaeroides* 2.4.1 produced PHB under the growth conditions employed in this work, and the clones of pJH1700 should carry the gene(s) of PHB synthase responsible for the formation of PHB in the *E. coli* containing pDH100.

Since the DNA fragment inserted in pJH2600 contains the whole insert fragment of the pJH1700 (see below and Fig. 3), [H$^+$]-NMR analysis was not performed on the *E. coli* (pDH100, pJH2600).

Localization of $phbC_{2.4.1}$

The DNA fragments inserted in pJH1700 and pJH2600 were approximately 4.0 and 6.0 kb, respectively. Restriction analysis of the two DNA fragments revealed that the 4.0-kb DNA of pJH1700 is in apprximately the same region as two thirds of the 6.0-kb DNA in pJH2600 and shares one of the two ends generated by the partial digestion with the *Mbo*I restriction endonuclease used for cloning (Fig. 3). The relative orientation of the inserted DNA fragments in pJH1700 and pJH2600 to the plasmid *lac* promters, however, was different in each case. But the amount of PHB produced by *E. coli* (pDH100 and pJH 1700) did not differ much from that of *E. coli* (pDH100 and pJH2600). Therefore, the expression of $phbC_{2.4.1}$ seems to be mediated by its own promter(s) in *E. coli*.

Further localization of the $phbC_{2.4.1}$ was performed with the 4.0-kb DNA fragment in pJH1700. Five subclones of the 4.0-kb insert DNA in pJH1700 were constructed followed by mobilization into *E. coli* (pDH100) to check for the formation of PHB (Fig. 3). One subclone, pJH1704 contaning approximately 3.0-kb DNA extending from the SalI restriction endonuclease site to the *Mbo*I site used for cloning showed PHB accumulation in *E. coli* (pDH100), while no PHB was detected with the other subclones. From this result it is evident that $phbC_{2.4.1}$ was located on the 3.0-kb insert DNA in pJH1704.

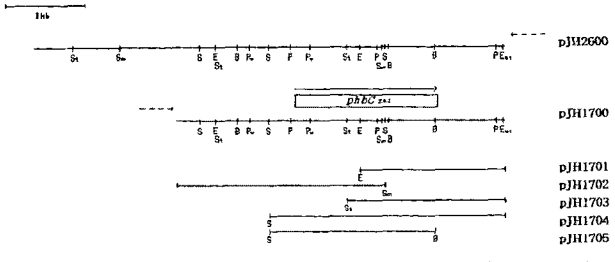

Fig. 3. Restriction map of 6.0- and 4.0-kb DNA fragments inserted in pJH2600 and PJH1700, respectively, and subclones from pJH1700 to localize $phbC_{2.4.1}$ by using heterologous expression in *E. coli*.

The arrows (⋯→) near the restriction maps show transcriptional direction of *lac* promoter of the vector, pUC19. An open reading frame of $phbC_{2.4.1}$ of *R. sphaeroides* 2.4.1 is shown between the restriction maps with the transcriptional direction (—→) of $phbC_{2.4.1}$ (see Fig. 5). Plasmid designations were shown to the right of the each subclone. B, *Bam*HI; E, *Eco*RI; E47, *Eco*47III; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sm, *Sma*I, and St, *Stu*I.

Sequence Analysis of $phbC_{2.4.1}$

Since the $phbC_{2.4.1}$ was further localized to the 2.8-kb *Sal*I-*Pst*I fragment of the 3.0-kb DNA of pJH1704, nucleotide sequence was determined from both strands of the 2.8-kb DNA (Fig. 4). A DNA sequence encoding an open reading frame (ORF) of 601 amino acids was found. This had an ATG start codon preceded by the potential Shine-Dalgarno sequence, AGGGGG (5). The predicted molecular mass of the putative polypeptide of PHB synthase was estimated to be 66,828 Da. The average G + C content, 67%, is similar to that of other genes isolated from *R. sphaeroides* (14). A potential - 10 sequence recognized by the σ^{70} (7) is located at approximately 104 bp upstream of the translation start codon (Fig. 5, the first underline). One inverted repeat was detected at 53 nucleotides downstream of the stop codon, TGA. The free enegy value (ΔG) calculated according to the method of Tinoco *et al.* (34) for the possible stem-loop structure is -22.6 kcal/mol. The proposed structure is followed by five T residues, a feature that resembles a typical rho-independent terminator structure in *E. coli* (1).

The nucleotide sequence of the $phbC_{2.4.1}$ exhibited 99.9% homology to the that of phbC of *R. sphaeroides* ATCC 17023 (9). Only one differece was found at the second nucleotide of the codon for the 144th amino acid. It is C of GCG for alanine in *R. sphaeroides* 2.4.1, while *R. sphaeroides* ATCC17023 has glycine by the codon of GGG.

Expression of $phbC_{2.4.1}$ in *E. coil*

To localize the cis-acting regulatory region responsible for expression of $phbC_{2.4.1}$ in *E. coli*, three subclones

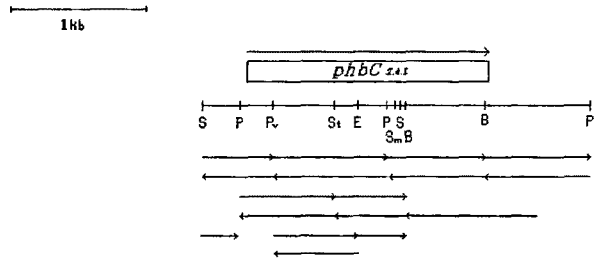

Fig. 4. Sequencing directions of the 2.8-kb *Sal*I-*Pst*I DNA with subclones derived from pJH1704.

Each subclone was constructed in pUC19 (DNA fragments shown with ←) and in pUC18 (DNA fragments shown with →). The arrows also indicate the direction of DNA sequencing. The same abbreviations are used for restriction enzymes as in Fig. 3.

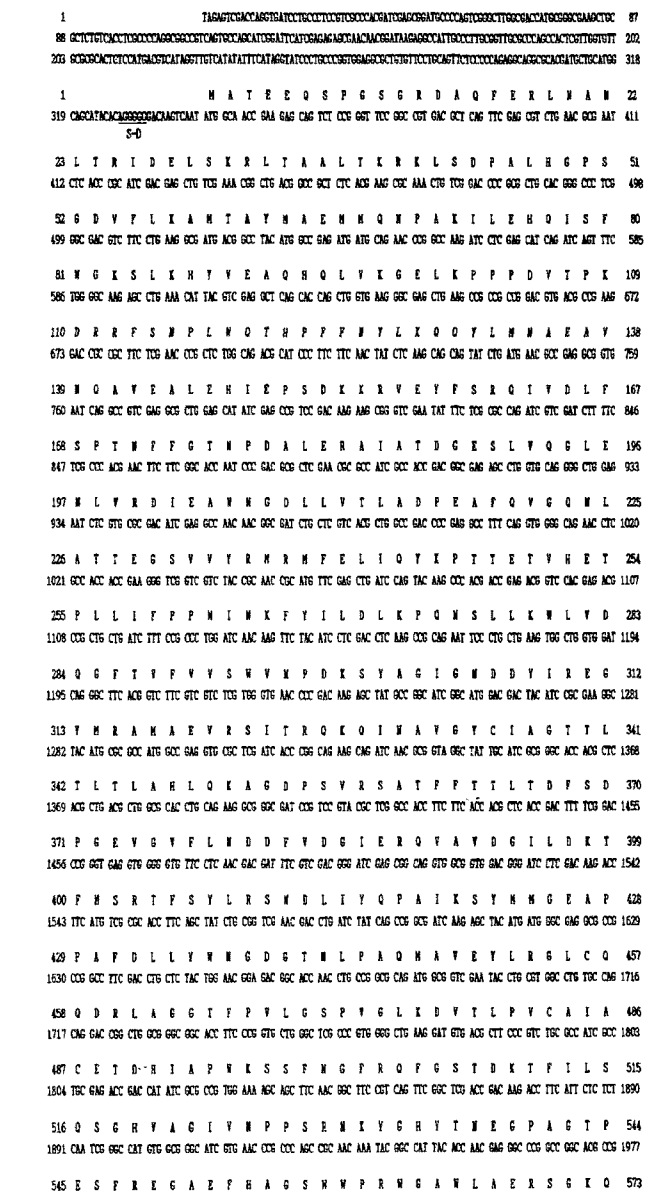

Fig. 5. DNA sequence of the $phbC_{2.4.1}$ region.
The deduced amino acid sequence for PHB synthase encoded by $phbC_{2.4.1}$ is shown above the DNA sequence. A putative -10 region recognized by σ^{70} (7) is underlined at 104 bp upstream of the start codon. The position of the possible ribosme-binding sequence (5) is also underlined and marked with S-D. An inverted repeat sequence for putative transcription terminator structure (1) located at the immediate downstream of the stop codon is underlined with the facing arrows.

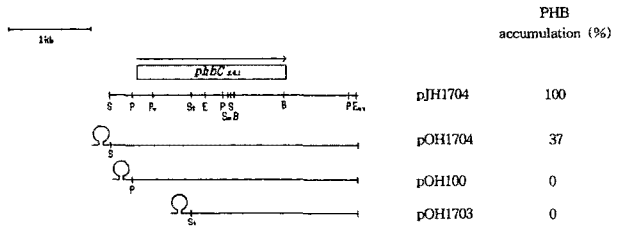

Fig. 6. Subclones to localze cis-acting DNA region responsible for expression of $phbC_{2.4.1}$ in *E. coli*.
The Ω is a transcription and translation stop cartridge (27) to prevent any transcriptional readthrough from *lac* promoter of the vector. The relative amount of the PHB accumulation was quantitated by comparison of A_{235}/A_{600} after PHB determination by the Law and Slepecky method (16).

were constructed as shown in Fig. 6. The pOH1704 has the same 3.0-kb DNA as in pJH1704, while in pOH100 the 2.7-kb insert DNA contains the whole $phbC_{2.4.1}$ as well as its upstream DNA limited by the *Pst*I site at 64 bp upstream of the $phbC_{2.4.1}$ start codon. The pOH1703 was used as a negative control in which the $phbC_{2.4.1}$ DNA region encoding 218 amino acids from its N-terminal was deleted. Although all of the three subclones have the same transcriptional direction of $phbC_{2.4.1}$ as the lac promoter of the vector, the presence of transcription and translation stop cartridge, Ω Sm^r/Sp^r (27) prevents any transcriptional readthrough of the *lac* gene.

After each subclone was transformed into *E. coli* (pDH100), only pOH1704 turned the colony opaque white appearance by accumulating PHB, while the pOH 100 in *E. coli* (pDH100) maintained a clear colony as did pOH1703. Quantitation of PHB by the Law and Slepecky method revealed that pOH1704 in *E. coli* (pDH100) produced about 37% of the PHB accumulated in *E. coli* (pDH100, pJH1704). The reason for the higher yield of PHB production with the pJH1704 is probably due to the additive effect of the transcriptional readthrough from the *lac* promoter. This result strongly suggests that $phbC_{2.4.1}$ is expressed in *E. coli* by its own promoter(s) as shown earlier with pJH1700 and pJH2600. Thus, the promoter(s) responsible for the expression of $phbC_{2.4.1}$ in *E. coli* were localized to the 276-bp DNA region between the *Sal*I and the *Pst*I sites at 340 and 64 bp upstream of the start codon, respectively. It remains to be determined whether the potential -10 of the σ^{70} recognition sequence (Fig. 5, the first underline) at 104 bp upstream of the start codon acts as the promoter.

Previously, Hustede *et al.* (10) reported that PHB synthase activity was not expressed in recombinant *E. coli* containing only the *phbC* of *R. sphaeroides* ATCC17023. That experiment was performed in the absence of both *phbA* and *phbB* genes. However, our results clearly show that $phbC_{2.4.1}$ is expressed in *E. coli* (pDH100) to produce PHB, suggestive of active PHB synthase ex-

pressed in *E. coli*. Therefore, we propose that PHB synthase encoded by $phbC_{2.4.1}$ in *E. coli* is degraded rapidly in the absence of its substrate, 3-hydroxybutyryl CoA.

Acknowledgement

We thank Dr. S. Kaplan, Univ. of Texas, Houston, USA, for providing pTZ18U-PHB and Dr. Yong-Hyun Lee, Kyungpook National University, Taegu, Korea, for his help with the [H^+]-NMR analysis experiment. This research was supported by a Sogang University Research Grant in 1995-1996, and partly supported by the academic research fund (Genetic Engineering) of the Ministry of education, Republic of Korea.

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(Received June 9, 1997)