Appl Biochem Biotechnol (2010) 162:2355–2364
DOI 10.1007/s12010-010-9008-y

Functional Expression of *pha*CAB Genes from *Cupriavidus taiwanensis* Strain 184 in *Escherichia coli* for Polyhydroxybutyrate Production

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Received: 22 February 2010 / Accepted: 7 June 2010 /
Published online: 16 June 2010

Abstract Polyhydroxyalkanoates are polyesters synthesized by numerous microorganisms. These polyesters are biodegradable and have similar properties to those of conventional plastics. *Cupriavidus taiwanensis* strain 184 is phylogenetically related to the well-known polyhydroxybutyrate (PHB) producer *Ralstonia eutropha* (*Cupriavidus necator*) and is also shown to be able to accumulate significant amounts of PHB. In this study, we cloned the PHB synthesis genes (*phaCAB*) from *C. taiwanensis* 184 into *Escherichia coli* for biosynthesis of PHB. The recombinant *E. coli* strains were able to synthesize significant amounts of PHB. The PHB amounted to about 66~70% of total cell material of these recombinant strains.

Keywords Polyhydroxyalkanoates · PHA · *Cupriavidus taiwanensis* · PHB · Polyhydroxybutyrate

Introduction

Polyhydroxyalkanoates (PHA) are known to be accumulated in many environmental bacteria [1–4]. These biopolymers have attracted considerable interest of scientists and engineers from various areas of discipline not only because of their potential use as an alternative for petro-based plastics but also because of their biodegradability and

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biocompatibility [5–7]. Polyhydroxyalkanoates are classified according to the number of carbons in the repeating units in the polymers as short (three to five C-atoms) chain-length PHA (scl-PHA) and medium (six or more C-atoms) chain-length PHA (mcl-PHA) [2, 8, 9]. Examples of scl-PHA include polyhydroxybutyrate (PHB) and hydroxyvalerate. Accumulation of PHA in microorganisms in their natural niches can vary greatly ranging from less than 1% to as high as 80% of their cellular dry weight [2]. However, only those with the ability to accumulate very high contents of PHA have the potential for further strain improvement for use in industry. Therefore, despite the prevalence of PHA in bacteria in nature able to synthesize PHA, only few species have actually been intensively investigated for their application in industry [10]. Besides indigenous PHA producing microorganisms, genetic engineered microorganisms such as recombinant *Escherichia coli* have also been intensively investigated for industrial PHA production [11–15].

In *Cupriavidus necator* (formerly known as *Ralstonia eutropha*), the amount of PHB accumulation can be as high as 80–90% of its cell dry weight [2, 16, 17]. Since the genetics and enzymes involved in PHB synthesis in this bioplastic-producing "Knallgas" bacterium were well studied decades ago, its PHB syntheses genes (*pha*CAB_{RE}) were most often to be used in recombinant *E. coli* for production of the biopolymer [15, 18, 19]. With the increasing interests on biodegradable polymers generated from PHA as possible alternatives to petro-based plastics, recent endeavors have focused on improvement of PHB productivity by the recombinant microorganisms. In this way, they will serve as a viable and cheap alternative to conventional petro-based plastics in the market. Therefore, applications of *pha*CAB_{RE} on PHB production have been extensively investigated by numerous researchers both in academia and industry [15, 18, 20, 21]. Efficient enzymes for the production of diverse PHA from the microbial world are of interest to the scientists and microbiologists.

Cupriavidus taiwanensis, a symbiont with the *Mimosa* spp., has a strikingly similar genome to that of *C. necator* H16 [17, 22]. Besides its importance in symbiotic nitrogen fixation in the environment, we have demonstrated its recent capability of accumulating PHB recently (Y-H Wei, personal communication). Although the wild-type strains of both *Cupriavidus* (*C. necator* and *C. taiwanensis*) can accumulate high amounts of PHB, their inability to use some common carbohydrates such as glucose has limited and hampered their application as industrial production strains. The success of utilization of PHB synthesis genes of *C. necator* expressed in *E. coli* has led to our attempt to clone and express the PHB synthesis genes of *C. taiwanensis* in *E. coli* in this study.

Materials and Methods

Bacterial Strains, Plasmids, and Cultivation of Cells

Bacterial strains and plasmids used in this study were listed in Table 1. *C. taiwanensis* strain 184 was kindly provided by Professor Y-H Wei and was used for cloning the PHB biosynthesis operon (*phaCAB*$_{CT}$). *C. taiwanensis* was routinely cultivated in M9 medium (3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with sodium gluconic acid (2% or 4%, *w*/*v*) as sole carbon source. The medium was adjusted to pH 6.8 before sterilization. *E. coli* was cultivated in Luria–Bertani medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl) or M9 medium with glucose (2% or 4%, *w*/*v*) as sole carbon source. Bacto™ agar (1.5%, *w*/*v*; BD Diagnostics, Spark, MD, USA) was added to broth when solid growth media were needed.

Table 1 Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant characterizations	Source or reference
Strains		
Cupriavidus taiwanensis strain 184	Wild type	[22]
Escherichia coli DH5α	F^- Φ80 *lacZ*Δ M15 *recA* endA1 Δ(*lacZYA-argF*)U169 *deo*R *gyrA*96 *thi*-I *hsdR*17 *supE*44 *relAI*	Invitrogen
CCH15	*E. coli* DH5α harbored pTA15	The present study
CCH30	*E. coli* DH5α harbored pTA30	The present study
CCH38	*E. coli* DH5α harbored pTA38	The present study
CCH06	*E. coli* DH5α harbored pSR06	The present study
Plasmids		
pGEM-T	Cloning and expression vector	Promega
pBluescript II KS^+	Cloning and expression vector	Stratagene
pSR06	pBluescript II KS^+ with $phaCAB_{CT}$	The present study
pTA30, pTA38, pTA15[a]	pGEM-T with $phaCAB_{CT}$	The present study

[a] pTA30 and pTA38: $phaCAB_{CT}$ was cloned in the same orientation to that of *lac* operon and pTA15: $phaCAB_{CT}$ was in the opposite orientation

Amplification of PHB Synthesis Operon ($phaCAB_{CT}$) from *C. taiwanensis* Strain 184 and Expression of $phaCAB_{CT}$ in *E. coli*

Bacterial DNA was extracted from cell pellets of *C. taiwanensis* 184 by Triton-Prep method after cells were harvested and washed twice with 1 ml of sterile phosphate-buffered saline. Primers (PhaCAB-forward: ACTAGTCCATACATCAGGAAGGTG; PhaCAB-reverse: TCTAGAATGTTCTTTTCCAGGTAGGT) specifically for the amplification of $phaCAB_{CT}$ were designed according to the published genome sequence of *C. taiwanensis* LGM19424 [23]. Polymerase chain reaction (PCR) for amplification of $phaCAB_{CT}$ was carried out in Extenor Hi-Fidelity PCR Master Mix (ABgene, Surrey, UK) with 2 ng of DNA template and 200 nM primers. PCR conditions were as follows: 94 °C for 5 min followed by amplification in which the following conditions were used: denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and elongation for 6 min at 68 °C. A total of 30 cycles were performed, and then a final elongation step consisting of 68 °C for 10 min was performed after the amplification program. The results of the PCR amplification were run on 1% agarose gel, and the DNA was stained with ethidium bromide and visualized under short-wavelength UV light. PCR results of the expected size (approximately 5.1 kb) were purified for sequence determination to confirm the identity of the $phaCAB_{CT}$ amplicon.

Cloning vector pGEM-T (Promega Co., Madison, WI, USA) and pBluescript II KS^+ (Stratagene, La Jolla, CA, USA) were used in this study. The purified DNA fragments of $phaCAB_{CT}$ obtained from PCR described above were cloned into pGEM-T. Cloning of $phaCAB_{CT}$ was also performed with pBluescript II KS^+ as a cloning vector by inserting the operon excised from pGEM-T:: $phaCAB_{CT}$ by restriction digestion with *Spe*I and *Xba*I. The vectors containing $phaCAB_{CT}$ were then transformed into competent *E. coli* DH5α. Transformed *E. coli* DH5α were cultivated on medium containing Nile red (1 μg ml^{-1}) [24], and colonies were directly monitored for the fluorescence by exposing to ultraviolet light to detect accumulation of lipid storage compounds presumably PHB [10].

Comparison of the Production of PHB by *C. taiwanensis* Strain 184 and Recombinant *E. coli* Containing $phaCAB_{CT}$

Accumulation of PHB by different strains of recombinant *E. coli* and *C. taiwanensis* strain 184 grown in M9 basal medium containing 4% (*w*/*v*) glucose (for recombinant *E. coli* strains) or 4% sodium gluconate (for *C. taiwanensis* 184) was evaluated. Cultures were incubated in a rotary shaker (200 rpm) at 37 °C for 72 h. Samples of growth cultures were examined for their cell dry weight (CDW), PHB content, and PHB concentration at 24, 48, and 72 h of incubation, respectively. Production of PHB by *E. coli* strain CCH30 was also examined with M9 medium containing glucose or glycerol as sole carbon source.

Electron Microscopy

Cells of strain CCH30 and wild-type *C. taiwanensis* 184 were grown in liquid medium to stationary phase and collected, washed, and suspended in potassium phosphate buffer (pH 7.0) three times. The cells were prefixed with 4% paraformaldehyde and 2.5% glutaraldehyde. Cells were then fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide in phosphate buffer (pH 7.0), stained with 1% uranyl acetate, and then examined under the JEM-1200EXII transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Characterization of PHB Produced by Recombinant *E. coli* Containing $phaCAB_{CT}$

Polyhydroxybutyrate produced by recombinant *E. coli* strain CCH30 and *C. taiwanensis* strain 184 was extracted in an excess of chloroform (1 g of cell dry weight was added in 20 ml of chloroform) at 50 °C for 6 h in a rotary water bath at 200 rpm. The solution was filtered through Whatman® no. 1 filter paper, and the filtrate was collected. Excess of methanol was then added into the filtrate in room temperature for 24 h for the precipitation occurred. The precipitate (PHB) was collected by filtration and dried at 55 °C in an oven. Polyhydroxybutyrate isolated from *C. taiwanensis* 184 and strain CCH30 was subjected to Fourier transform infrared (FTIR) spectroscopy, gas chromatography analysis, and nuclear magnetic resonance (NMR) analysis as described [25–28].

The molecular weight distributions of PHB were determined by gel permeation chromatography (GPC) with columns (PLgel 5-l 10000A, 5-l guard, and 5-l mixed-C columns from Polymer Labs) at 25 °C. An L-2490 refractive-index detector from Hitachi (Taipei, Taiwan) and a PU-980 intelligent high-performance liquid chromatography pump from Jusco (Tokyo, Japan) were used for GPC. Chloroform was used as the eluent at a flow rate of 1 ml/min with PHB concentration of 20.0 mg/ml for GPC analysis. Polystyrene standards (580–841,700 g/mol) were used to generate a calibration curve.

The endotherms and exotherms of PHB produced by *C. taiwanensis* 184 and strain CCH30 were determined by a PerkinElmer differential scanning calorimetry (DSC)-7. Samples (approximately 5–10 mg) were sealed in aluminum pans, and the thermograms were measured at a scanning rate of 10 °C/min from −40 °C to 200 °C (first scan) under a nitrogen flow (with a nitrogen flow rate of 40 ml/min). The specimens were kept at 200 °C for 5 min to erase the thermal history, subsequently cooled to −40 °C at a cooling rate of 10 °C/min, and then reheated to 200 °C at a scanning rate of 10 °C/min (second scan) under the same nitrogen atmosphere. The thermograms were calibrated with the baseline obtained from the empty aluminum pan and an indium standard.

Samples were also further analyzed for the weight loss behavior of the PHB specimens in a dynamic scanning mode with a PerkinElmer Pyris-1 thermogravimetric analyzer

(TGA). Ten to 15 mg of each sample was scanned from 30 °C to about 400 °C at a heating rate of 10 °C/min with a nitrogen flow rate of 40 ml/min. The peak temperatures of derivative weight loss curves and weight loss percentages were obtained from TGA thermograms.

Results

Amplification of PHB Synthesis Operon (*phaCAB*$_{CT}$) of *C. taiwanensis* Strain 184

The *phaCAB*$_{CT}$ of *C. taiwanensis* strain 184 was successfully amplified by PCR, and the approximately 5.1-kb product corresponded with the size of *phaC* (1,781 bp), *phaA* (1,181 bp), and *phaB* (740 bp) consistent with the published sequence [23]. The segment was further confirmed by sequence analysis, and the similarity of the amino acid sequences between PhaC, PhaA, and PhaB of *C. taiwanensis* strain 184 and those of strain LGM19424 was above 96%. The sequences of *phaCAB*$_{CT}$ of strain 184 were deposited in the GenBank under the accession number GQ922052 (*pha*A), GQ922053 (*pha*B), and GQ922054 (*pha*C), respectively.

Cloning and Expression of *phaCAB*$_{CT}$ in *E. coli*

The amplicons of *phaCAB*$_{CT}$ were cloned into commercially available cloning vectors pGEM-T (pTA30, pTA38, and pTA15) and pBluescript II KS^{+} (pSR06) and then transformed into *E. coli* DH5α. Expression of *phaCAB*$_{CT}$ could be visualized by the additional bands (64.6, 40.5, and 26.3 kDa for PhaC, PhaA, and PhaB, respectively) appeared on the SDS-PAGE (results not shown).

Successful clones of transformed *E. coli* were confirmed by their exhibition of fluorescent Nile red staining on agar plates. Strains CCH30, CCH38, and CCH15 were selected for further examination for their ability to accumulate PHB. Polyhydroxybutyrate produced by strain CCH30 was extracted for analysis of the polymer's properties.

Comparison of PHB Accumulation by Recombinant *E. coli* Harboring *phaCAB*$_{CT}$ and by *C. taiwanensis* 184

A comparison of cell dry weight, PHB content, and accumulated PHB in *C. taiwanensis* 184 grown in M9 medium containing sodium gluconate (2% or 4%, *w*/*v*) to the recombinant *E. coli* (strains CCH30, CCH38, and CCH15) grown in M9 medium containing glucose (2% or 4%, *w*/*v*) was shown in Table 2. There are no significant differences on these results when cells were grown on either 2% (*w*/*v*) or 4% (*w*/*v*) carbon sources in shake flask cultures.

In the expression of p*haCAB*$_{CT}$, these recombinant strains were under the control of the promoter indigenous to p*haCAB*$_{CT}$; therefore, isopropyl β-D-1-thiogalactopyranoside (IPTG) was not added in the above experiments. Since the p*haCAB*$_{CT}$ in the recombinant *E. coli* strains (e.g., strain CCH30) was also controlled under an IPTG inducible promoter, induction of the expression of p*haCAB*$_{CT}$ with and without the addition of IPTG in strains CCH30 was examined. The difference in PHB contents between cells grown under IPTG-induced and IPTG-uninduced conditions was insignificant (61% vs. 66%). However, cell dry weights and PHB concentrations were lower in the IPTG-induced grown cells (1.85 g/l for CDW and 0.96 g/l for PHB concentration) compared to those of uninduced

Table 2 Comparison of accumulation of PHB by *C. taiwanensis* strain 184 and recombinant *E. coli* with *pha*CAB$_{CT}$.

Cultivation time	24 h			48 h			72 h		
Bacteria strain	CDW (g l^{-1})	PHB conc. (g l^{-1})	PHB content (%)	CDW (g l^{-1})	PHB conc. (g l^{-1})	PHB content (%)	CDW (g l^{-1})	PHB conc. (g l^{-1})	PHB content (%)
C. taiwanensis 184[a]	1.5	0.65	44.2	4.8	3.07	64.4	3.4	2.16	63.6
C. taiwanensis 184[b]	1.4	0.44	30.4	3.7	2.05	56.0	4.2	2.50	60.1
Strain CCH30[a]	1.9	0.77	40.3	1.6	0.78	49.6	1.9	1.00	51.8
Strain CCH30[b]	1.6	0.64	40.6	1.8	0.97	53.1	2.2	1.20	54.3
Strain CCH38[a]	1.2	0.25	20.5	1.4	0.48	34.8	1.7	0.67	40.5
Strain CCH38[b]	1.5	0.11	7.5	1.2	0.36	30.8	1.8	0.61	34.5
Strain CCH15[a]	0.8	0.16	19.1	1.2	0.32	25.7	1.5	0.57	38.7
Strain CCH15[b]	0.9	0.15	16.6	1.2	0.37	31.2	1.6	0.54	32.8

PHB content: PHB CDW^{-1} (ww^{-1})

CDW cell dry weight

[a] Cells were grown in M9 medium containing 2% (*w*/*v*) sodium gluconate (*C. taiwanensis* 184) or glucose (strains CCH30, CCH38, and CCH15)

[b] Cells were grown in M9 medium containing 4% (*w*/*v*) sodium gluconate (*C. taiwanensis* 184) or glucose (strains CCH30, CCH38, and CCH15)

growth cells (2.17 and 1.44 g/l, respectively). The amount of PHB accumulation in recombinant *E. coli* using pBluescript II KS^+ as cloning vector (e.g., strain CCH06) was similar to that of strains using pGEM-T as cloning vector (1.77 g/l for CDW, 0.99 g/l for PHB concentration, and 67% for PHB content).

Properties of Polyhydroxybutyrate Produced by Strain CCH30

Inclusions purified from strains of recombinant *E. coli* harboring *pha*CAB of *C. taiwanensis* exhibited intense absorptions at 1,724–1,740 cm^{-1} which corresponds to ester functional groups primarily from lipids, fatty acids, and PHAs when subjected to FTIR analysis. The identities of inclusions produced by these recombinant strains were further confirmed as PHB by ^{1}H NMR and ^{13}C NMR (results not shown).

Microbiological characteristics of PHB produced by recombinant *E. coli* and *C. taiwanensis* 184 were examined by transmission electron microscopy. The appearances of the PHB inclusions showed distinct difference between those in strain CCH30 (Fig. 1a) and in *C. taiwanensis* 184 wild type (Fig. 1b).

The molecular weights of PHB synthesized by recombinant *E. coli*, *C. taiwanensis* 184, and commercially available PHB (Aldrich Co.) were shown in Table 3. The average molecular weight (M_w) of PHB of *C. taiwanensis* 184 was found to be close to that of commercially available PHB from Aldrich Co. but was significantly higher than PHB synthesized by recombinant strains of *E. coli* harboring *pha*CAB$_{CT}$. Comparisons of physical characteristics of above mentioned PHB from different sources analyzed by DSC and TGA were shown in Tables 4 and 5, respectively.

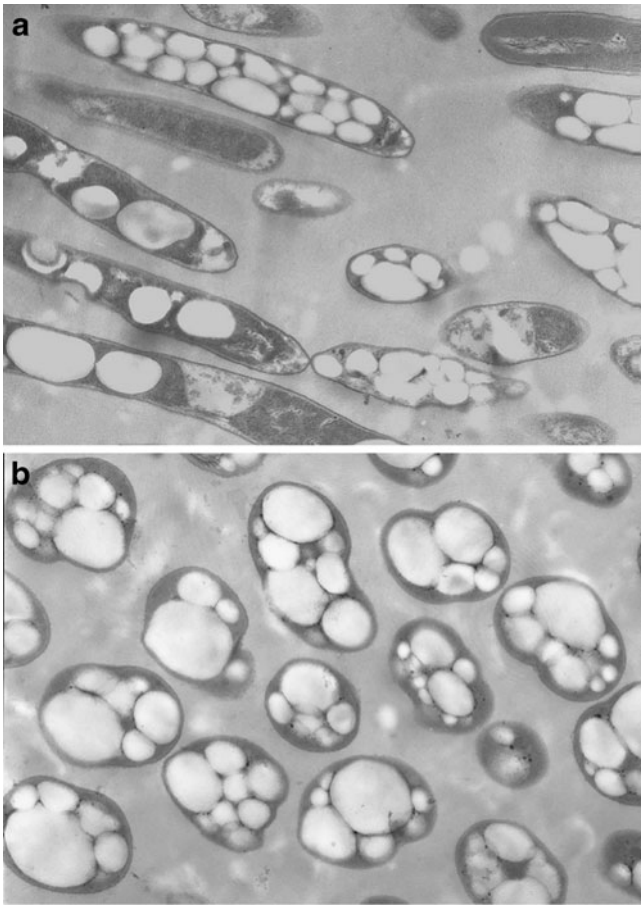

Fig. 1 Electron microscopy of thin section of strain CCH30 (**a**) and *C. taiwanensis* 184 (**b**). Granules of accumulated PHB polymers were observed

Discussion

We have cloned *pha*CAB$_{CT}$ in *E. coli* and the proteins of PhaA, PhaB, and PhaC were successfully expressed in the recombinant strains judging by the correct size of the bands

Table 3 Molecular weights of PHB synthesized by strain CCH30 and *C. taiwanensis* 184.

Source of PHB	Molecular weight		
	M_w	M_n	PDI
Strain CCH30	292,000	90,000	3.23
C. taiwanensis 184	607,000	166,000	3.66
PHB-Aldrich	506,000	343,000	1.47

M_w weight-average molecular weight, M_n number-average molecular weight, *PDI* polydispersity index, *PHB-Aldrich* PHB purchased from Sigma-Aldrich Co., St. Louis, MO, USA

Table 4 Characteristics results of DSC thermograms of PHB examined in this study.

Source of PHB	DSC scan							
	First heating scan			Cooling scan		Second heating scan		
	T_m (°C)	ΔH_m (J/g)	X_c (%)	T_c (°C)	ΔH_c (J/g)	T_m (°C)	ΔH_m (J/g)	X_c (%)
Strain CCH30	171	93	64	73	−56	170	84	58
C. taiwanensis 184	179	104	71	77	−69	177	103	71
PHB-Aldrich	176	84	58	68	−55	175	83	57

T_m melting temperature, ΔH_m melting enthalpy, ΔH_c crystallization enthalpy, X_c percentage crystallinity based on PHB with 146 J/g of endotherm at 100% crystallinity

visualized on SDS-PAGE. The extent of PHB accumulation was significant in several recombinant strains regardless of the type of cloning vectors and the insertion direction of *pha*CAB_{CT} into the cloning vectors (i.e., pGEM-T or pBluescript II KS^+) used. This suggest that the expression of *pha*CAB_{CT} in the recombinant *E. coli* was under the control of the indigenous promoters of *C. taiwanensis* 184 included in our genetic constructs. Production of PHB in the laboratory scale as well as industrial scale by cloning of *pha*CAB of *C. necator* in *E. coli* host has been extensively studied and discussed [18, 19, 29]. Modification of recombinant *E. coli* strains harboring *pha*CAB of *C. necator* such as co-expressed with a *vgb* gene has also been evaluated in the scale-up production of PHB [15]. These promising results demonstrated that it is feasible to utilize exogenous PHB synthesis genes to synthesize PHB in genetically easily manipulating microorganisms. Successful cloning and expression of *pha*CAB_{CT} will help develop industrial production of PHB and further promote their applications.

The sequences of *pha*CAB of *C. taiwanensis* 184 is highly homolog to that of *C. necator*; therefore, it is not surprised that the ability for accumulation of PHB by recombinant strains of *E. coli* constructed in this research is compatible to those recombinant *E. coli* containing *pha*CAB of *C. necator*. However, the characteristics of PHB produced by recombinant strains containing *pha*CAB_{CT} are still somewhat unique from those obtained from wild-type *C. taiwanensis*, *C. necator*, and that obtained from commercial vendors. For example, the molecular weights of PHB produced by all of the strains of recombinant *E. coli* with *pha*CAB_{CT} in this research are much smaller than those obtained from other sources mentioned above. This, maybe due to the activity of the enzyme(s) related to PHB biosynthesis of *C. taiwanensis*, when expressed in *E. coli*, is not as high as the indigenous origins. The activity of PhaC (polymerase) is known to be directly related to the molecular weight of the polymer produced and therefore also affected the polydispersity index of the polymers synthesized. Characteristics of thermal stability of

Table 5 Degradation temperatures of PHB in this study determined by TGA thermograms.

Source of PHB	T_i (°C)	T_p (°C)
Strain CCH30	240	292
C. taiwanensis 184	245	290
PHB-Aldrich	231	276

T_i the onset temperatures of weight loss, T_p the peak temperatures of weight loss

PHB biosynthesized by strains of this research were also slightly different from those of other sources, although not very significantly and how these differences would affect the applications of the polymers produced by these recombinant strains remained to be studied.

As biodegradable and biocompatible polyester, PHB has drawn much attention of scientists as a potential replacement for petroleum-based plastics [7, 30, 31]. Some of the microorganisms in natural environment such as *C. necator* and *C. taiwanensis* showed to be potent PHB producers. However, due to the limited range of substrates as starting material (e.g., inability to metabolize cheap carbon sources such as glucose), the high cost associated with production of PHB by these bacteria has hampered the development of this industry using these strains. With this construction, we now are able to utilize glucose as sole carbon source for the synthesis of PHB under the act of enzymes encoded by genes of *C. taiwanensis* ($phaCAB_{CT}$). Cloning of $phaCAB_{CT}$ and successfully expressed in *E. coli* offered one more options when selecting industrial PHB-producing strains. In conclusion, we have demonstrated that heterologous expression of PhaCAB of *C. taiwanensis* strain 184 in *E. coli* was useful in the production of PHB. Further endeavors will be focused on selecting other genes that are related to PHA metabolism such as *prpE* (known to be involved in PHBV synthesis) from *C. taiwanensis* strain 184 and co-expression with $phaCAB_{CT}$ in suitable host (e.g., *E. coli*) in order to develop diverse PHA copolymers.

Acknowledgment We thank Dr. Thomas J Lie, University of Washington, Seattle, WA, USA, for his critical review and editing assistance of this manuscript. The present study was supported partly by grants (contract number NSC98-2622-E-155-003-CC2 and NSC97-2221-E-155-006-MY3) from National Science Council.

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