Characterization of the highly active PHA synthase of *Chromobacterium* sp. USM2

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ABSTRACT

Synthesis of bacterial polyhydroxyalkanoate (PHA) is very much dependent on the expression and activity of the key enzyme PHA synthase (PhaC). Enhanced gene expression and enzyme evolution techniques have brought forth numerous improved and active synthases. Nevertheless, the search for a natural synthase with such properties is still widespread. In a recent study, the PhaC of a locally isolated Chromobacterium sp. USM2 (PhaC<sub>Cs</sub>) exhibited high level of PHA accumulation when expressed in mutant Cupriavidus necator PHB<sup>−</sup>4. It showed the ability to use the 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) monomers in PHA biosynthesis. In vitro assay of recombinant PhaC<sub>Cs</sub> expressed in Escherichia coli showed that its activity towards the polymerization of 3-hydroxybutyryl-CoA was nearly 8-folds higher (2132 ± 70 U/g) compared to that of the model strain C. necator (253 ± 21 U/g). Specific activity using a Strep2-tagged, purified PhaC<sub>Cs</sub> was 238 ± 98 U/mg, almost 5-folds higher than previous studies using purified PhaC from C. necator. High poly(3-hydroxybutyrate) [P(3HB)] accumulation in E. coli of up to 76 ± 2 wt% was observed within 24 h of cultivation. PhaC<sub>Cs</sub> has proven to be the first naturally occurring highly active PHA synthase with superior polymerizing ability.

Keywords: Polyhydroxyalkanoate (PHA); Chromobacterium sp.; PHA synthase; High synthase activity; Poly(3-hydroxybutyrate)
INTRODUCTION

Unlike petrochemical polymers, biosynthesis of bacterial polymer is very much dependent on the catalytic activities of various enzymes involved. Polymerization rates and yields vary based on the biosynthesis pathway of the organism and the monomer supply. One such biopolymer that has attracted much interest is polyhydroxyalkanoate (PHA). Owing to its desirable properties, PHA makes an excellent candidate as biodegradable replacements for conventional plastics (7). PHA had gained much interest for applications in various industries such as medicine, pharmacology, agriculture, packaging and cosmetics (2, 26, 44).

PHA has been produced using wild-type as well as recombinant microorganisms (16-18). The biosynthesis of this bacterial polymer is controlled by both monomer supplying enzymes and PHA synthase (PhaC) which is the key enzyme involved in polymerization (28, 35, 37).

The dominant role played by PhaC in determining polymer composition provided an impetus to extensive investigations of PHA synthases. So far, the PhaC of Cupriavidus necator (PhaC<sub>Cn</sub>) (Class 1) has been studied in some mechanistic detail and is the benchmark commonly used to evaluate the performance of other synthases (11, 13, 33). Some studies have also been carried out on the synthase of Allochromatium vinosum (Class III) (12, 22).

Nevertheless, PhaC is a complex enzyme and its complete structure and properties are yet to be fully understood. It is known that the activity and affinity towards the polymerization of different hydroxyalkanoate-CoA substrates vary based on the different classes of PHA synthases. Efforts have been taken to alter and improve the properties of natural synthases towards attaining higher activity and wider substrate specificity via enzymatic evolution (37).

Several successful studies reported mutant synthases with up to 1 – 4-fold increased activity (1, 25, 41). Nevertheless, the search for a natural synthase with comparable properties is still widespread.
The property of PHA is dependent on its monomeric composition which is determined by PhaC (28, 35). Recently, Bhubalan et al cloned the PHA synthase gene (phaC<sub>CS</sub>) from a locally isolated Chromobacterium sp. USM2 and heterologously expressed the gene in a PHA negative mutant of C. necator PHB<sup>−</sup>4 (5). Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] copolymer with high 3-hydroxyvalerate (3HV) fraction was synthesized from mixtures of fructose and sodium valerate. Besides, 3-hydroxyhexanoate (3HHx) monomer was successfully incorporated when crude palm kernel oil (CPKO) was fed as the sole carbon source, resulting in the production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)] copolymer. P(3HB-co-3HV) and P(3HB-co-3HHx) copolymers are known to possess improved mechanical and thermal properties compared to P(3HB) homopolymer. When a combination of sodium valerate or sodium propionate with CPKO was fed to a C. necator PHB<sup>−</sup>4 strain containing heterologously-expressed phaC<sub>CS</sub>, high contents of polymer comprising 3HB, 3HV and 3HHx monomers were produced (4). Some of the P(3HB-co-3HV-co-3HHx) terpolymer produced was found to possess elastomeric properties. However, not many microorganisms have the suitable PhaC with the ability to incorporate monomers of both short-chain-length (scl-) and medium-chain-length (mcl-) PHA. The ability of PhaC<sub>CS</sub> to produce scl-mcl-PHA highlighted the potential of this synthase to be further exploited.

In this study, the PHA synthase of Chromobacterium sp. USM2 was further characterized via in vitro and in vivo assays using Escherichia coli JM109 to fully understand its PHA synthesizing ability. We also purified a recombinantly-expressed Strep2-tagged version of PhaC<sub>CS</sub> to examine the unique abilities of this enzyme. The results obtained showed that PhaC<sub>CS</sub> is a highly active enzyme in its natural form and is expressed in high concentrations in E. coli. The ability to obtain high concentrations of synthase in vivo might facilitate in overcoming one of the bottlenecks in crystallization of PhaC which is to produce
significant amount of pure protein. Once this is possible, then attempts can be made to
determine the three-dimensional structure of this complex enzyme which to date, still remains
an impenetrable barrier.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

_E. coli_ JM109 was used for all standard genetic engineering and its transformants
were used for PHA biosynthesis. The plasmids used in this study are listed in Table 1. _E. coli_
JM109 was grown at 37 °C in LB broth consisting of the following components: per liter; 10
g casein enzymic hydrolysate, 5 g yeast extract and 10 g NaCl at pH 7.0. In order to
determine the functional expression of the cloned gene _in vivo_, PHA biosynthesis was carried
out by transferring 1.5 mL [3% (v/v)] of inoculum from a preculture grown for 12 h in LB
into 50 mL of fresh LB in 250 mL Erlenmeyer flasks supplemented with 2% (w/v) of
glucose. The cultures were incubated at two different temperatures of 30 °C and 37 °C for 72
h on a reciprocal shaker at 180 rpm. Ampicillin at a final concentration of 100 µg/mL was
added to maintain plasmid stability. For maintenance purposes, bacterial cultures from the
exponential growth phase were stored at −20 °C in 20% (v/v) glycerol.

For extraction of crude protein, _E. coli_ JM109 transformants were grown at 30 °C in 2
mL of LB broth for 14 h. An aliquot of 17.5 µL [1% (v/v)] was inoculated to 1.75 mL of
fresh LB broth and was further grown for 9 h at 30 °C. Ampicillin at a final concentration of
100 µg/mL was added for plasmid maintenance. For Strep2-PhaC<sub>Cs</sub> expression and
purification, _E. coli_ BL21(DE3) was used as a host strain. Expression of Strep2-PhaC<sub>Cs</sub> was
performed as follows. Cells with Strep2-PhaC<sub>Cs</sub> expression plasmid were grown in 1 L LB
broth supplemented with 100 µg/mL ampicillin until an OD<sub>600</sub> = 0.6. Enzyme synthesis was
induced by addition of 0.1 mM (final concentration) of IPTG and allowed to incubate for 2 h at 30°C on a reciprocal shaker at 180 rpm. Cells were then pelleted and protein was purified as described below.

5 DNA manipulation and plasmid construction

Plasmid isolation and DNA manipulation was carried out according to standard procedures (29). All the restriction enzymes were used according to the manufacturers’ protocols such as TaKaRa, Toyobo and Roche respectively. All the other chemicals used were of analytical grade. The plasmid pGEM"AB(phaC Cs) in Figure 1 was constructed to determine the functional expression of PhaC Cs synthase enzyme in *E. coli* JM109 via *in vitro* and *in vivo* experiments. First, the plasmid vector pGEM"C1AB was digested with *Xba*I and *Pst*I to segregate *phaC1* and then ligated with a synthetic linker *Xba*I-*Eco*RI-*Eco*RV-*Asp718-*Hind*III-*Pst*I which was derived by annealing a set of complementary primers (FXbaIPstILK and RXbaIPstIL) (nucleotide sequences are shown in Table 2). The resultant vector was named pGEM"AB(L). Next the *phaC Cs* gene was cloned using the forward primer FEcoRiCs and the reverse primer RAsp718Cs (Table 2) from the plasmid vector pBBR1MCS-C2. The resulting 1.7 kb *Eco*RI—*Asp*718 *phaC Cs* fragment was purified and then digested with the corresponding enzymes and ligated with pGEM"AB(L) which was also digested with the same enzymes. The resultant vector was named pGEM"AB(phaC Cs). DNA sequencing for confirmation of new plasmid constructs was carried out by the dideoxy chain termination method with the Prism 310 Genetic Analyzer DNA sequencer (Applied Biosystems) and the CEQ2000XL DNA Analysis System (Beckman Coulter) using the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter), respectively.
Plasmid pET-phaCCs was constructed using pET51b (Novagen) as the parent plasmid. For construction of a strep2-phaC\textsubscript{Cs}, the phaC\textsubscript{Cs} on the plasmid pBBR1MCS-C2 was amplified by PCR using the forward primer Strep2phaCCsFW and the reverse primer Strep2phaCCsRV (Table 2) to introduce the unique restriction site BamHI 5’ to the phaC\textsubscript{Cs} open reading frame, and the unique restriction site HindIII, 3’ to the phaC\textsubscript{Cs} open reading frame. The amplified gene was subcloned into the pET51b digested with BamHI and HindIII, followed by ligation to produce the plasmid pET-phaCCs. The portion of pET-phaCCs containing the tagged phaC\textsubscript{Cs} gene was sequenced by MIT Biopolymers Laboratory.

Preparation of crude protein samples

\textit{E. coli} JM109 harboring either pGEM"AB(phaC\textsubscript{Cs}), pGEM'CAB or pGEM"AB(L) was cultured as discussed above. Cells were harvested by centrifugation and whole-cell extract of the transformant was prepared by resuspending the cells in 2 mL of ice cold 40 mM potassium phosphate buffer (pH 7.5) and subsequent disrupting by sonication (5 s, 3 times) on ice using UD-200, TOMY sonicator. A soluble fraction was obtained as resulting supernatant when the disrupted cells were centrifuged at 13,700 × g for 10 min at 4 °C and the insoluble fraction was obtained from the precipitate. Protein concentrations were determined using Bradford assay (6).

Expression and purification of Strep2-Tagged PhaC\textsubscript{Cs}

\textit{E. coli} BL21(DE3)/pET-phaCCs was cultured as discussed above. Cells (6.5 – 7.8 g wet weight) were pelleted by centrifugation at 2988 × g at 4 °C. The cell pellet was resuspended in 25 mL Buffer A (100 mM Tris-HCl, pH 8.0) and lysed using a French pressure cell (2 passes at 12,000 psi). The resulting cell lysate was centrifuged at 100,000 × g to remove cell debris. The clarified lysate was loaded onto a Strep-tactin column (IBA,
GmbH, Göttingen, Germany; 10 mL column volume) preequilibrated with 80 mL buffer B (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). The lysate and column were incubated at 4 °C for 15 – 20 min. The column was eluted and washed with 5 × 10 mL buffer B. Strep2-PhaC<sub>Cs</sub> was eluted from the column with 6 × 5 mL fractions of buffer C (Buffer B + 2.5 mM desthiobiotin). The Strep-tactin column was regenerated according to the manufacturer’s instructions. Protein concentration of each fraction was determined by Bradford assay. The pooled fractions were then concentrated using a Vivaspin 15R concentrator (Sartorius AG, Göttingen, Germany) to 5.5 – 25.5 mg protein/mL, and dialyzed twice versus 100 mM Tris-HCl (pH 8.0), containing 0.5 mM EDTA and 0.5 mM dithiothreitol, for 12 – 16 h using a Slide-a-Lyzer dialysis cassette (Thermo Scientific).

Aliquots of 100 µL of the protein preparation were stored at −80°C. Protein concentrations of pooled, concentrated fractions were determined by Bradford assay and confirmed spectrometrically at A<sub>280</sub>, using the molar absorption coefficient 110,810 M<sup>−1</sup> cm<sup>−1</sup>. Strep2-PhaC<sub>Cs</sub> purification was performed three separate times.

**In vitro enzymatic assay of crude PhaC<sub>Cs</sub>**

The activity of PHA synthase from crude extract was determined by measuring the amount of CoA released from 3HB-CoA during the polymerization of 3HB-CoA (40). The assay mixture contained 2 mM 3HB-CoA, 40 mM potassium phosphate buffer (pH 7.5, 30 °C), 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and 1 mg/mL BSA. The reaction was initiated by adding 35 – 40 µg of protein obtained from the soluble fraction of disrupted cells into the above reaction mixture and the absorbance at 412 nm was measured at 30 °C.

The concentration of CoA was determined spectrometrically (11) using a molar absorption coefficient of 15,600 M<sup>−1</sup> cm<sup>−1</sup> at 412 nm using Hitachi U-3900H Spectrophotometer. One
unit of enzyme activity (U) is defined as the amount of enzyme that catalyzed the release of 1.0 µmol CoA/min. Enzymatic assays were performed in triplicate.

**In vitro enzymatic assay of Strep2-PhaC<sub>c</sub>s**

Assays were carried out as previously described (42). Final enzyme concentrations of 7.5 – 30 nM of Strep2-PhaC<sub>c</sub>s and 600 µM of 3HB-CoA (final concentrations) were used. The concentration of CoA was determined spectrometrically(11) using a molar absorption coefficient of 13,600 M<sup>-1</sup> cm<sup>-1</sup> at 412 nm using an Agilent 8453 Spectrophotometer. Preparations of Strep2-PhaC<sub>c</sub>s were used as a control and purified as described elsewhere (9). One unit of enzyme activity is defined as described above. Enzymatic assays were performed in triplicate.

**Western blot analysis**

A total of 10 µg of proteins prepared from both soluble and insoluble fractions of the disrupted *E. coli* JM109 transformants were separated using 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins from the soluble fraction were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Immun-Blot<sup>TM</sup> PVDF Membrane [Bio-Rad]) using a Criterion Blotter (Bio-Rad). An immunoblot analysis of PHA synthase was carried out using specific rabbit anti-serum raised against the C-terminal peptides of PhaC<sub>c</sub>n as described by Murata *et al* (23). PhaC protein was detected using goat anti-rabbit IgG conjugated with alkaline phosphatase as a secondary antibody.

**Gas chromatography (GC) and polymer isolation**

Methanolation of the lyophilized cells in the presence of 15% (v/v) sulfuric acid and 85% (v/v) methanol was carried out prior to determining P(3HB) content through GC
analysis (8). P(3HB) was extracted by refluxing lyophilized cells with chloroform for 4 h at 60 °C. The polymer solution was then purified by precipitation with chilled methanol. The purified polymer was then air dried in a fume cupboard.

Gel permeation chromatography (GPC)

Average molecular weight was estimated using a Shimadzu 10A GPC system and a 10A refractive index detector with Shodex K-806M and K-802 columns. Chloroform was used as the eluent at a flow rate of 0.8 mL/min and analysis was carried out at 40 °C. Sample concentration of 1.0 mg/mL was used. The calibration curve was generated using polystyrene standards with a low polydispersity.

Transmission electron microscopy (TEM)

*E. coli* JM109 harboring pGEM"AB(phaC<sub>Cs</sub>) was cultured for 24 h in LB supplemented with 2% (w/v) glucose, as mentioned above. TEM analysis was carried out to observe the accumulation of PHA granules and the changes in cell morphology under the electron microscope (Philip CM 12/ STEM and JLM-2000FX11). Cells were harvested and fixed in McDowell-Trump fixative at 4 °C for 24 h (21). The cell pellets were then post-fixed with 1% osmium tetroxide (OsO<sub>4</sub>) at room temperature. Cells were dehydrated in an increasing ethanol series (50, 75, 95 and 100%) and then transferred to 100% acetone. Cells were embedded at 60 °C for 24 – 48 h in Spurr’s low viscosity resin (34). Ultra-thin sections were prepared, mounted on copper grids and stained with uranyl acetate and lead citrate for electron microscope examination at an acceleration voltage of 80 kV (Philip CM 12/ STEM and JLM-2000FX11).
RESULTS

In vitro assay of crude PhaC<sub>Cs</sub> in E. coli

The ability of <i>C. necator</i> PHB<sup>−</sup>4 transformant harboring <i>phaC<sub>Cs</sub></i> (GenBank accession no. HM989943) to utilize CPKO and 3HV precursors for the biosynthesis of PHA polymers comprising of 3HB, 3HV and 3HHx monomers served as groundwork to further investigate the interesting properties of this synthase. Hence, in this study PhaC<sub>Cs</sub> was characterized via <i>in vivo</i> and <i>in vitro</i> assays to better understand its PHA synthesizing ability. The <i>phaC<sub>Cs</sub></i> gene was cloned into plasmid pGEM"AB harboring the <i>pha<sub>Ac</sub></i> and <i>pha<sub>Bc</sub></i> genes of <i>C. necator</i> H16 and subsequently expressed in <i>E. coli</i> JM109. <i>E. coli</i> harboring pGEM'CAB plasmid which contains the PHA biosynthetic genes (<i>phaCAB</i>) of <i>C. necator</i> H16 was used as the positive control. The construction of pGEM"AB(<i>phaC<sub>Cs</sub></i>) is shown in Figure 1.

The expression level of PhaC<sub>Cs</sub> was evaluated through a series of <i>in vitro</i> assays. From the SDS-PAGE analysis [Figure 2(A)] of the crude cell lysates, it can be seen that the expression level of PhaC<sub>Cs</sub> appeared to be higher compared to that of PhaC<sub>Cn</sub> using the same background strain. Distinct bands at approximately 66 kDa in size corresponded to the sizes of the synthases. Detection of protein bands in the precipitate (cell pellets) confirmed the presence of some insoluble portions of protein. A more distinctly observed elevation in the concentration of PhaC<sub>Cs</sub> was seen through Western blot analysis. As shown in Figure 2(B), the intensity of PhaC<sub>Cs</sub> band was much greater as compared to that of PhaC<sub>Cn</sub>. This demonstrated the presence of higher concentration of PhaC<sub>Cs</sub> in the bacterial cells. As expected, no protein band was detected with the negative control.

In order to investigate the activity of PhaC<sub>Cs</sub>, 3HB-CoA was used as the substrate and the release of CoA during polymerization was measured to determine the total enzyme activity. The total activity of PhaC<sub>Cs</sub> was measured using the soluble fraction of the crude
extract. PhaC$_{Cs}$ demonstrated superior ability in polymerizing 3HB-CoA as compared to that of PhaC$_{Cn}$. The total activity of PhaC$_{Cs}$ (2132 ± 70 U/g) was nearly 8-folds higher than that of PhaC$_{Cn}$ (253 ± 21 U/g). The high activity of PhaC$_{Cs}$ could be associated with its elevated level of expression in *E. coli*. It was assumed that the availability of higher concentration of PhaC$_{Cs}$ in the cells will ensure efficient and faster accumulation of polymer upon the addition of a carbon substrate. Results obtained from *in vivo* evaluation of PhaC$_{Cs}$ confirmed the above hypothesis.

**In vivo evaluation of PhaC$_{Cs}$**

The *E. coli* transformant harboring *phaC*$_{Cs}$ was found to accumulate high amounts of P(3HB) [76 ± 2 wt%] within 24 h of cultivation using glucose as the carbon source (Table 3). The total P(3HB) concentration reached a maximum value of 7.2 ± 0.2 g/L at 48 h of cultivation. *E. coli* strains are known to grow at an optimal temperature of 37 °C. However, the optimal temperature for growth and PHA accumulation by *Chromobacterium* sp. USM2 had been previously identified as 30 °C (5). Therefore, *E. coli* transformant harboring *phaC*$_{Cs}$ was cultivated at a lower temperature of 30 °C to determine the effect of lowered temperature on overall growth and productivity. A significant difference was noticed in the P(3HB) accumulation by this transformant at 30 °C, compared to 37 °C (Figure 3). P(3HB) content up to 88 ± 1 wt% (48 h) was synthesized at 30 °C compared to a maximum of 76 ± 2 wt% (24 h) at 37 °C. The polymerization of P(3HB) by PhaC$_{Cs}$ in *E. coli* appeared to be better at 30 °C. As shown in Figure 4(A), the *E. coli* transformant was packed with granules of various sizes. Some cells contained mainly smaller granules as shown in Figure 4(B). The $M_w$ of P(3HB) produced averaged at $5 \times 10^5$ Da with a high polydispersity of 6.0.
Enzymatic activity of purified Strep2-PhaC<sub>Cs</sub>

To further investigate the polymerization ability of PhaC<sub>Cs</sub>, we constructed a Strep2-tagged PhaC<sub>Cs</sub> for expression in, and purification from, <i>E. coli</i>. Strep2-PhaC<sub>Cs</sub> was cloned into <i>E. coli</i> BL21(DE3) and purified as described in Materials and Methods. The specific activity of this highly purified, Strep2-PhaC<sub>Cs</sub>, was 238 ± 98 U/mg, which is significantly greater than that of purified synthase from <i>C. necator</i> from previously published results (specific activity = 40 U/mg (42)). The Strep2-PhaC<sub>Cs</sub> purified enzyme also exhibited a lag phase in activity (Figure 5), consistent with previous results using purified Class I PhaC proteins (ex. PhaC from <i>C. necator</i>) isolated from <i>E. coli</i> (11, 42, 43). The lag phase in activity of Strep2-PhaC<sub>Cs</sub> is more prevalent when lower concentrations of enzyme are used. Preliminary enzymatic assay experiments using 3HV-CoA also suggested that the specific activity of Strep2-PhaC<sub>Cs</sub> is roughly twice as great as that of PhaC<sub>Cn</sub>, using this substrate (data not shown).

DISCUSSION

<i>In vitro</i> and <i>in vivo</i> characterization of PhaC<sub>Cs</sub> in <i>E. coli</i>, carried out by heterologous expression of phaC<sub>Cs</sub> along with phaA<sub>Cn</sub> and phaB<sub>Cn</sub> under the control of <i>C. necator</i> promoter in a pGEM"AB(phaC<sub>Cs</sub>) expression plasmid, showed increased level of synthase expression and activity. As seen in Figure 2(A) of the crude cell lysates, a distinct band of approximately 66 kDa in size corresponded to the class I PHA synthases (27, 28). A more distinct band exhibited by PhaC<sub>Cs</sub> compared to that of PhaC<sub>Cn</sub> suggested that PhaC<sub>Cs</sub> was expressed at a higher level. This finding was further confirmed by Western blot analysis [Figure 2(B)]. The total activity of PhaC<sub>Cs</sub> towards the polymerization of 3HB-CoA was nearly 8-folds higher compared to that of PhaC<sub>Cn</sub>. This suggested that the total enzymatic activity of PhaC<sub>Cs</sub> can be partially correlated with its elevated level of expression <i>in vivo</i>.
The ability to polymerize 3HB-CoA varies according to the classes of PHA synthases. PHA synthases belonging to class I, III and IV show higher preference towards the polymerization of 3HB-CoA (35, 37). The expression and activity of these genes in *E. coli* are commonly used as benchmarks to compare the performance of other heterologous PHA biosynthesis genes. In this study, the activity of the heterologous PhaC<sub>Cn</sub> expressed in *E. coli* was comparable to that observed in the wild-type *C. necator* whereby its activity in cell extracts is known to range from 180 to 330 U/g during PHA accumulation stages (10, 14, 30).

The activity of several PHA synthases in *E. coli* had been documented. Alterations in their specific activity and expression level were achieved through enzyme evolution studies. The PhaC<sub>Cn</sub> harboring a F420S mutation is known to exhibit 2.4-fold higher specific activity towards the polymerization of 3HB-CoA compared to the wild-type PhaC<sub>Cn</sub> (38). Meanwhile, PhaC<sub>Cn</sub> harboring a double mutation at G4D and F420S exhibited increased synthase concentration *in vivo* and enhanced polymer accumulation (25). In a similar study, the synthase activity in extracts of *A. punctata* cells and its mutants were found to be in the range of 118 – 768 U/g (1). The mutant synthases exhibited 1 – 5-fold increased activity compared to the wild-type synthase. Wild-type and mutant synthases of *Pseudomonas* sp. 61-3 exhibited activity of less than 50 U/g towards the polymerization of 3HB-CoA (41). In a recent study, the enzymatic activities of the PHA synthase of *Aeromonas caviae* (PhaC<sub>Ac</sub>) and some of its mutants when expressed in *C. necator* PHB<sup>−</sup>4 grown on fructose were reported to be in the range of 18 – 249 U/g (40). When compared with the activity levels of these well-known wild-type and mutated PHA synthases, PhaC<sub>Cs</sub> exhibited a clearly much higher 3HB-CoA polymerizing activity.

It is interesting to note that PhaC<sub>Cs</sub> revealed a homology of 46% with PhaC<sub>Cn</sub> but only 34% with PhaC<sub>Ac</sub> even though PhaC<sub>Cs</sub> is also known to incorporate 3HHx. As mentioned earlier, evolved PhaC<sub>Cn</sub> synthases are known to exhibit improved levels of synthase activity.
and polymer accumulation. The mutant synthases which harbors mutations at F420S, G4D or G4D/F420S showed improved activities and higher concentration in vivo (24, 25, 38). It was found that the amino acids sequences at the corresponding positions of 4 and 420 in PhaC<sub>Cs</sub> did not show any alterations such as G4D or F420S. The amino acids at these positions were identified as phenylalanine. This indicated that the PHA synthase of Chromobacterium sp. USM2 was highly active in its natural form. Characterization of PhaC<sub>Cs</sub> in vitro showed that this enzyme was produced at high concentrations in the E. coli cells. Such a high level of expression exhibited by PhaC<sub>Cs</sub> in E. coli might be correlated with a high degree of translation capability due to efficient codon usage (32).

Besides the high level of expression, the PhaC<sub>Cs</sub> also showed a very high level of activity, approximately 8-folds higher than that of PhaC<sub>Cn</sub>. Furthermore, the activity of purified PhaC<sub>Cs</sub> was shown to be at least 3 to 5 times greater than the activity of pure PhaC<sub>Cn</sub>. It was reported previously that mutant synthase of A. caviae had an increased specific activity towards 3HB-CoA of approximately 1.6-folds compared to the wild-type (0.016 U/mg) (15). PhaC<sub>Cs</sub> exhibits much higher preference towards 3HB-CoA compared to the other Class I PHA synthases such as PhaC<sub>Cn</sub> and PhaC<sub>Ac</sub>. The characteristics of PhaC<sub>Cs</sub> in its wild-type strain Chromobacterium sp. USM2 or in the C. necator PHB<sup>−4</sup> transformant are yet to be investigated in order to determine if these elevated levels of protein expression and enzymatic activity are strain-dependent. Nevertheless, the findings from this study have given us invaluable insights on the interesting properties of this synthase.

Results of in vivo evaluation on PhaC<sub>Cs</sub> correlated with results obtained from the in vitro experiments. The synthase could efficiently polymerize P(3HB) when glucose was fed as the carbon source. Rapid accumulation was noticed as the cells were able to achieve 76 ± 2 wt% of P(3HB) within 24 h of cultivation. Highest P(3HB) content of 88 ± 1 wt% was accumulated at 48 h. This accounted for a P(3HB) concentration of 7.2 ± 0.2 g/L. Previously,
E. coli transformants harboring phaCn are known to accumulate P(3HB) in the range of 60 – 70 wt% (36). By observing the residual cell biomass (Table 3), it can be clearly seen that the increase in total cell biomass was caused by increasing amounts of polymer accumulation. The average $M_w$ of P(3HB) synthesized by PhaCn$_s$ ($5 \times 10^5$ Da) was found to be lower than that produced by some E. coli transformants harboring different PHA synthases such as PhaCn$_s$ ($9.7 \times 10^5$ Da) and mutant PHA synthase of Pseudomonas sp. 61-3 ($7.2 \times 10^5$ Da) (3, 6, 39). The lower molecular weight of the resulting polymer could be attributed to the high in vivo concentration of the synthase (31). As observed in Figure 4B, many small granules were present in some of the transformants. It is possible that higher concentrations of synthase in vivo could have resulted in the formation of many small granules which leads to the formation of short P(3HB) chains, thus increasing the polydispersity.

The Chromobacterium sp. USM2 synthase produced higher amounts of P(3HB) at 30 °C compared to at 37 °C. Significant difference was noticed in the polymer accumulation whereby a reduction in P(3HB) content of approximately 18% was noticed at the end of cultivation at 37 °C (Figure 3). Nevertheless, residual cell biomass values (data not shown) indicated that cell growth was not affected by the different cultivation temperature. Higher accumulation of P(3HB) at a lower temperature could be correlated with the temperature optimum of PhaCn$_s$. Since the wild-type Chromobacterium sp. USM2 is known to grow and accumulate PHA at an optimum temperature of 30 °C, the synthase is probably more active at this temperature. The performance of PHA synthase is known to be affected by varying temperatures (25).

CONCLUSION

The PHA synthase of a locally isolated Chromobacterium sp. USM2 has been successfully characterized via in vitro and in vivo assays in E. coli. The synthase exhibited
very high levels of expression and specific activity towards the polymerization of 3HB-CoA compared to the PHA synthase of model strain C. necator. The activity of this natural synthase was found to be higher than some of the engineered mutant synthases. This finding questioned the possible existence of other such natural synthases which are yet to be discovered. Rapid accumulation of P(3HB) in vivo within a short period of time further confirmed the in vitro findings. The naturally active PhaC Cs can potentially be developed as a model synthase to compare the activity of other synthases.

ACKNOWLEDGEMENTS

This study was supported by Techno Fund provided by The Ministry of Science, Technology and Innovation, Malaysia (MOSTI). K.B and J.C acknowledge National Science Fellowship awarded by MOSTI and USM’s Fellowship Scheme respectively for financial support. We are grateful for the guidance and suggestions provided by Dr. Ken’ichiro Matsumoto and Dr. Miwa Yamada. We also thank Prof. JoAnne Stubbe and Dr. Ping Li for the generous gift of some of the 3HB-CoA used in this study and Ms. Jingnan Lu for helpful assistance with protein purification.

REFERENCES


**FIGURE CAPTIONS**

Figure 1: Construction of the pGEM"AB(phaC Cs) expression plasmid harboring the PHA synthase gene of *Chromobacterium* sp. USM2 with promoter (P Cn), terminator (T Cn) and monomer supplying genes phaA Cn and phaB Cn of *C. necator*. pGEM"C1AB plasmid harbors the PHA synthase gene of *Pseudomonas* sp. 61-3 with promoter (P Cn), terminator (T Cn) and monomer supplying genes phaA Cn and phaB Cn of *C. necator*. pGEM"AB(L) harbors the synthetic XbaI-EcoRI-EcoRV-Asp718-HindIII-PstI linker with promoter (P Cn), terminator (T Cn) and monomer supplying genes phaA Cn and phaB Cn of *C. necator*.

Figure 2: (A) SDS-PAGE analysis of crude extracts of PhaC Cn and PhaC Cs. *E. coli* cells harboring plasmid pGEM"AB(L) was used as negative control. A total of 10 µg of sample was loaded into each well. SM = Size marker, W = Whole-cell extract, S = Supernatant (soluble fraction), P = Precipitate (cell pellets). (B) Comparison of the expression levels of PhaC Cn and PhaC Cs in *E. coli* transformants using Western blot analysis. *E. coli* cells harboring plasmid pGEM"AB(L) was used as negative control. A total of 10 µg of protein from the supernatant was used for the analysis.

Figure 3: Comparison of P(3HB) production at 30 °C and 37 °C by *E. coli* JM109 transformant harboring phaC Cs. Cells were incubated for 72 h at 180 rpm in LB medium supplemented with 2 % (w/v) of glucose and 100 µg/mL of ampicillin. Data shown are means of triplicates. Means with different letters are significantly different (Tukey’s HSD, p<0.05).

Figure 4: TEM showing P(3HB) granules in *E. coli* JM109 transformant harboring pGEM"AB(phaC Cs). Cells were cultured for 24 h at 30 °C, 180 rpm in LB supplemented with 2% (w/v) glucose and 100 µg/mL ampicillin. It could be observed that some cells contained granules of various sizes (A), while others contained a high number of smaller granules (B).

Figure 5: Time course of CoA release from 3HB-CoA catalyzed by purified Strep2-PhaC Cs (black boxes) and purified Strep2-PhaC Cn (black triangles). In the experiment represented by this figure, 30 nM of Strep2-PhaC Cs and 30 nM of Strep2-PhaC Cn were used. The dashed line indicates when the 3HB-CoA substrate was completely used up.
<table>
<thead>
<tr>
<th>Bacterial strains and plasmids</th>
<th>Relevant phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> JM109</td>
<td>E14–(<em>mcrA</em>), recA1, gryA96, <em>thi</em>-1, hsdR17(rk-, mk+), supE44, relA1, D(<em>lac-proAB</em>), [F' <em>tra</em>D36, <em>pro</em>AB, lacI*ZΔM15]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F', <em>ompT</em>, gal, dcm, lon, hsdS&lt;sub&gt;B&lt;/sub&gt;(r&lt;sup&gt;-&lt;/sup&gt;m&lt;sup&gt;-&lt;/sup&gt;), λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmids:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBBR1MCS-C2</td>
<td>pBBR1MCS-2 derivative harboring approximately 2.0 kb fragment of <em>phaC&lt;sub&gt;Cs&lt;/sub&gt;</em> from <em>Chromobacterium</em> sp. USM2 with putative promoter</td>
<td>(5)</td>
</tr>
<tr>
<td>pGEM'CAB</td>
<td>pGEM-T derivative; with <em>C. necator</em> promoter and terminator harboring *phaC&lt;sub&gt;Cn&lt;/sub&gt;, phaA&lt;sub&gt;Cn&lt;/sub&gt;, phaB&lt;sub&gt;Cn&lt;/sub&gt;</td>
<td>(20)</td>
</tr>
<tr>
<td>pGEM'C1AB</td>
<td>pGEM-T derivative; with <em>C. necator</em> promoter and terminator harboring *phaC&lt;sub&gt;1P&lt;/sub&gt;, phaA&lt;sub&gt;Cn&lt;/sub&gt;, phaB&lt;sub&gt;Cn&lt;/sub&gt;</td>
<td>(19)</td>
</tr>
<tr>
<td>pGEM''AB</td>
<td>pGEM-T derivative; with <em>C. necator</em> promoter and terminator harboring *phaA&lt;sub&gt;Cn&lt;/sub&gt;, phaB&lt;sub&gt;Cn&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM''AB(L)</td>
<td>pGEM-T derivative; with <em>C. necator</em> promoter and terminator harboring *phaA&lt;sub&gt;Cn&lt;/sub&gt;, phaB&lt;sub&gt;Cn&lt;/sub&gt;, synthetic XbaI-EcoRI-EcoRV-Asp718-HindIII-PstI linker</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM''AB(*phaC&lt;sub&gt;Cs&lt;/sub&gt;)</td>
<td>pGEM-T derivative; with <em>C. necator</em> promoter and terminator harboring *phaC&lt;sub&gt;Cs&lt;/sub&gt;, phaA&lt;sub&gt;Cn&lt;/sub&gt;, phaB&lt;sub&gt;Cn&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pET51b</td>
<td>Protein expression vector for <em>N</em>-terminally Strep2-tagged proteins.</td>
<td>Novagen</td>
</tr>
</tbody>
</table>
pET-phCCs pET51b derivative; with $phaC_s$ open reading frame inserted into $BamHI/HindIII$ restriction site.

This study
Table 2: Primer sequences used in this study. Restriction sites are underlined.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’−3’)</th>
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<tbody>
<tr>
<td>FEcoRICs</td>
<td>GCGGCCAACCAAGGAATTCATGC</td>
</tr>
<tr>
<td>RAsp718Cs</td>
<td>GGGACGGTACCTTCGGTTCAG</td>
</tr>
<tr>
<td>FXbaI PstILK</td>
<td>CTAGATAAGAAGGAGATGAATCGATATCGGTACCA</td>
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<td></td>
<td>AGCTTCTGCA</td>
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<tr>
<td>RXbaI PstIL</td>
<td>GAAGCTTGGTACCGATATCGAATTCATCTCCTTCTTAT</td>
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<tr>
<td>Strep2phaCCsFW</td>
<td>CAAGGATCCGATGCAGCAGTGTGCTGCAATTCCCCT</td>
</tr>
<tr>
<td>Strep2phaCCsRV</td>
<td>CTTAAGCTTTCAAGGCGGCCGA</td>
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Table 3: Biosynthesis of P(3HB) by *E. coli* JM109 transformant harboring *phaC*<sub>c</sub>.<sup>a</sup>

<table>
<thead>
<tr>
<th>Cultivation time (h)</th>
<th>CDW (g/L)</th>
<th>P(3HB) content (wt%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>P(3HB) concentration (g/L)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Residual biomass (g/L)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>2.1 ± 0.1</td>
<td>52 ± 3</td>
<td>1.1 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>24</td>
<td>5.9 ± 0.1</td>
<td>76 ± 2</td>
<td>4.5 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>36</td>
<td>7.5 ± 0.3</td>
<td>86 ± 1</td>
<td>6.4 ± 0.3</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>48</td>
<td>8.3 ± 0.1</td>
<td>88 ± 1</td>
<td>7.2 ± 0.2</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>60</td>
<td>8.2 ± 0.2</td>
<td>85 ± 3</td>
<td>6.9 ± 0.2</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>72</td>
<td>7.9 ± 0.2</td>
<td>83 ± 1</td>
<td>6.5 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Incubated for 72 h at 30 °C at 180 rpm in LB medium supplemented with 2 % (wt/v) of glucose and 100 µg/mL of ampicillin.

<sup>b</sup> P(3HB) content in freeze-dried cells were determined via gas chromatography (GC).

<sup>c</sup> P(3HB) concentration = P(3HB) content x CDW

<sup>d</sup> Residual biomass = CDW – P(3HB) concentration

Data shown are means of triplicates.
Figure 1

**pGEM™ C1AB**

- **BamHI**
- **Xhol**
- **PstI**

**pGEM™ C1AB**

- **BamHI**
- **PstI**

**Digested with Xhol and PstI**

**Ligation**

**pGEM™ AB(L)**

- **BamHI**
- **Asp718**
- **EcoRI**
- **Xhol**
- **EcoRI**

**Digested with EcoRI and Asp718**

**Ligation**

**pGEM™ AB(phac_{C_{D}})**

- **BamHI**
- **Xhol**

**Amplified from pBBR1MC3-C2**

- **~1700 bp**

**3D region**

- **EcoRI**
- **Asp718**
- **HindIII**

**Xhol-EcoRI-EcoRV-Asp718-HindIII-PstI**

**linker**
Figure 2

A

Negative control

PhaC\textsubscript{Cn}

PhaC\textsubscript{Cs}

SM W S P

W S P

W S P

75 kDa

50 kDa

66 kDa

B

Negative control

PhaC\textsubscript{Cn}

PhaC\textsubscript{Cs}

...
Figure 3

![Bar chart showing P(3HB) content (wt%) over time of cultivation (h). The chart compares results at 30°C and 57°C.](image)

- Time of cultivation (h): 24, 36, 48, 60, 72
- P(3HB) content (wt%): 0 to 80

Legend:
- • 30°C
- □ 57°C

Legend for bars:
- bc, be
- d
- ab
- a
- cd
- ab
Figure 4

A

B

1 μm

1 μm
Figure 5

[Graph showing the release of CoA over time (in minutes). The y-axis represents [CoA] released (µmol) ranging from 0 to 0.03, and the x-axis represents time (in minutes) ranging from 0 to 4. The graph includes data points for different conditions, indicated by different symbols.]